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New strategies for reversing cancer therapy resistance

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New strategies for reversing cancer therapy resistance

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Editorial: New strategies for reversing cancer therapy resistance

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Editorial on the Research Topic
New strategies for reversing cancer therapy resistance

Cancer stands as one of the leading causes of mortality worldwide. In the past decade, remarkable achievements in anticancer therapy have been made due to tremendous innovations. However, the emergence of drug resistance remains a major challenge in cancer treatment. Drug resistance can be caused by complex molecular mechanisms such as gene mutations, epigenetic dysregulation, microenvironment alterations, etc., which limits the effectiveness of anticancer therapies, and causes cancer recurrence and metastasis, thus being a major cause of cancer-related death (Housman et al., 2014). Various clinical strategies, including combination therapies and the utilization of epigenetic drugs, have been employed to mitigate or reverse drug resistance with some success (Morel et al., 2020). Nevertheless, the continued progression of cancers in treated patients and the resistance observed in some individuals indicate that current approaches to overcome resistance are far from sufficient, thus further research and innovation are required.

To overcome the challenge, identifying the drug resistance-associated genes is important. Zhang et al. reviewed a genome-wide CRISPR/Cas9 screening method for identifying potential drug resistance-associated genes (Zhang et al. *Genome-wide CRISPR/Cas9 screening for drug resistance in tumors*). This screening approach holds substantial promise for advancing the treatment of malignancies that have developed drug resistance. This article provides an overview of drug resistance pathways such as the KEAP1/Nrf2 pathway, MAPK pathway, NF-κB pathway, etc.

In addition to the gene mutation, numerous studies have discovered the close relationship between lipid metabolism and cancer drug resistance. Wang et al. reviewed the alterations in lipid metabolism associated with drug resistance and elucidate how lipid metabolism influences this resistance (Wang et al. *Lipid metabolism as a target for cancer drug resistance: progress and prospects*). It has been indicated that combination therapy could induce changes in lipid-related metabolic pathways, potentially reversing the progression of cancer drug resistance and augmenting or restoring sensitivity to therapeutic drugs. Thus, this review explores the intersection of medication combination with lipid metabolism and drug resistance, offering novel insights and strategies for future tumor treatment.

Endoplasmic reticulum stress (ERS) represents a cellular response mechanism to counter hypoxia and various stresses. Accumulating evidence suggests that prolonged

stress can foster the onset, progression, and drug resistance in tumors through the unfolded protein response. Qing et al. provide an overview of ERS mechanisms and tumor multidrug resistance (MDR), elucidate their interrelationship, and outline the potential of targeting ERS to enhance the therapeutic outcomes (Qing et al. *Crosstalk between endoplasmic reticulum stress and multidrugresistant cancers: hope or frustration*).

Furthermore, two cellular protein biomarkers have been reported in this collection. Wang et al. reported that the nucleolar protein 58 (NOP58) was overexpressed in two 5-fluorouracil (5-FU)-resistant advanced colorectal cancer cell lines, HCT116-5FuR and Lovo-5FuR, coinciding with an increase in aerobic glycolysis rate (Wang et al. *NOP58 induction potentiates chemoresistance of colorectal cancer cells through aerobic glycolysis as evidenced by proteomics analysis*). Knockdown of NOP58 resulted in decreased glycolysis and increased sensitivity of HCT116-5FuR and Lovo-5FuR cells to 5-FU. These findings from proteomic analysis shed light on a novel target implicated in cellular adaptation to 5-FU, offering a potential new therapeutic avenue to combat resistance. The multifunctional molecule ceruloplasmin (CP), involved in iron metabolism, has not been thoroughly investigated regarding its expression pattern, prognostic significance, and association with immune cells in gliomas. Jia et al. revealed a significant association between CP expression and immune pathways in gliomas by analysis of various databases (Jia et al. *Ceruloplasmin is associated with the infiltration of immune cells and acts as a prognostic biomarker in patients suffering from glioma*). This study indicates CP as a potential therapeutic target for gliomas and a predictor of immunotherapy effectiveness.

Breast cancer currently ranks as the most prevalent malignancy with a high mortality rate. A number of target-therapy drugs have been approved by the FDA to treat HER2-positive breast cancer (Cai et al., 2022; Cai et al., 2023). However, prolonged treatment using these drugs often results in drug resistance, complicating subsequent treatment decisions. In a case report, Li et al. used disitamab vedotin (RC48) to treat a female patient with HER2-positive breast cancer who developed drug resistance and disease progression after receiving multiple lines of anti-HER2 targeted therapy (Li et al. *Disitamab Vedotin (RC48) for HER2-positive advanced breast cancer: a case report and literature review*). The tumor exhibited both size reduction and stabilization following treatment with RC48. This case study highlights the potential clinical efficacy of RC48 as a promising therapeutic option for patients resistant to HER2-targeted therapies. Huang et al. reviewed the role of short peptides in breast cancer to overcome the drug resistance (Huang et al. *The Role of Peptides in Reversing Chemoresistance of Breast Cancer: Current Facts and Future Prospects*). In this review, diverse mechanisms through which various peptides reverse drug resistance in breast cancer were described, encompassing the promotion of cancer cell apoptosis, facilitation of non-apoptotic regulatory cell death, inhibition of cancer cell DNA repair mechanisms, alteration of the tumor microenvironment, suppression of drug efflux mechanisms, and enhancement of drug uptake. Deng et al. reviewed a natural product, Ginsenosides as the candidates to treat breast cancer patients (Deng et al. *Updating the therapeutic role of ginsenosides in breast cancer: a bibliometrics study to an in-depth review*). In this review, the various mechanisms of ginsenosides against breast cancer were summarized including apoptosis induction, autophagy stimulation, inhibition of epithelial-mesenchymal transition and metastasis, and regulation of miRNA and lncRNA.

Chronic myeloid leukemia (CML) is a type of myeloproliferative neoplasm triggered by a BCR-ABL fusion gene (Askmyr et al., 2014). The T315I mutation of BCR-ABL is the major cause of resistance to imatinib. Gao et al. investigated the impact of I13, a potent histone deacetylase (HDAC) inhibitor, on differentiation blockage in CML cells (Gao et al. *I13 overrides resistance mediated by the T315I mutation in chronic myeloid leukemia by direct BCR-ABL inhibition*). The results revealed that I13 efficiently depleted BCR-ABL in CML cells expressing the T315I mutation, impeding its function as a scaffold protein that regulates the chronic myeloid leukemia signaling pathway and mediating cell differentiation.

Kanner et al. explored a combination therapy to overcome multidrug resistance (MDR) in cancer cells (Kanner et al. *Cytotoxicity and Reversal Effect of Sertraline, Fluoxetine, and Citalopram on MRP1-and MRP7-mediated MDR*). Three selective serotonin reuptake inhibitor (SSRI) drugs—sertraline, fluoxetine, and citalopram, exhibited inhibitory or reversal effects in conjunction with chemotherapy on both MRP1- and MRP7-overexpressing cells, suggesting their repurposing potential in combating MDR in cancer cells. These findings offer a promising avenue for leveraging FDA-approved medications in combination with therapy protocols to address highly resistant malignancies.

Taken together, this special collection overviews the current development to study the mechanisms of multidrug resistance including the identification of gene mutation, lipid metabolism, endoplasmic reticulum stress involvement, and novel protein markers; and highlights the interesting insights into new and complementary treatments to overcome the drug resistance for cancer treatment.

Author contributions

YD: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing–review and editing. XL: Conceptualization, Investigation, Methodology, Supervision, Writing–review and editing. LW: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing–review and editing. SC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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I13 overrides resistance mediated by the T315I mutation in chronic myeloid leukemia by direct BCR-ABL inhibition

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Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm caused by a BCR-ABL fusion gene. Imatinib has significantly improved the treatment of CML as a first-generation tyrosine kinase inhibitor (TKIs). The T315I mutant form of BCR-ABL is the most common mutation that confers resistance to imatinib or the second-generation TKIs, resulting in poor clinical prognosis. In this work, we assessed the effect of a potent histone deacetylase (HDAC) inhibitor, I13, on the differentiation blockade in CML cells harboring T315I-mutated and wild-type BCR-ABL by MTT assay, flow cytometery, cell colony formation assay, mRNA Sequencing, Quantitative real-time PCR and Western blotting analysis. We found that I13 possessed highly potent activity against T315I-mutated BCR-ABL mutant-expressing cells and wild-type BCR-ABL-expressing cells. I13 induced cell differentiation and significantly suppressed the proliferation of these CML cells *via* the cell cycle G0/G1-phase accumulation. Moreover, it was revealed that I13 triggered the differentiation of BaF3-T315I cells, which was attributed to the block of the chronic myeloid leukemia signaling pathway *via* the depletion of BCR-ABL that was mediated by the inhibition of HDAC activity presented by the acetylation of histones H3 and H4. Taken together, I13 efficiently depleted BCR-ABL in CML cells expressing the BCR-ABL-T315I mutation, which blocked its function, serving as a scaffold protein that modulated the chronic myeloid leukemia signaling pathway mediating cell differentiation. The present findings demonstrate that I13 is a BCR-ABL modulator for the development of CML therapy that can override resistance caused by T315I-mutated BCR-ABL.

KEYWORDS

chronic myeloid leukemia, BCR-ABL-T315I mutation, imatinib resistance, HDAC inhibitor, acetylation of histones

Introduction

Chronic myeloid leukemia (CML) is a clonal proliferation disease representing 15%–20% of newly diagnosed cases of leukemia (O'Hare et al., 2012). CML is caused by the presence of the BCR-ABL fusion gene, which is formed by a translocation between chromosomes 9 and 22 (Liu et al., 2018; Hochhaus et al., 2020). BCR-ABL has a tyrosine kinase activity and triggers several cellular signaling pathways, such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), and phosphatidylinositol 3-kinase (PI3K)/AKT, to regulate cell proliferation,

differentiation, apoptosis, survival, migration, and DNA repair. Hence, BCR-ABL is the molecular target for CML treatment, which protects leukemic cells from the normal programmed cell death that leads to the development of CML (Cortes et al., 2021; Minciacchi et al., 2021). Moreover, CML cells are characterized by high proliferative capacity with a block in mature myeloid cell differentiation (Faderl et al., 1999).

The first-generation tyrosine kinase inhibitor (TKI), imatinib, was approved by the US Food and Drug Administration (FDA) in 2001 for the management of CML. It was shown to significantly improve 5-year relative survival rates for patients with chronic-phase CML due to the inhibition of the activation of BCR-ABL by reducing the phosphorylation of the BCR-ABL oncoprotein (Redner, 2010). Hence, imatinib became the first and best example of a successful targeted therapy against cancer (Jabbour and Kantarjian, 2020). However, clinical studies have shown that approximately 20%–30% of CML patients develop primary or secondary resistance to imatinib. The known mechanisms of resistance to imatinib include mutations in the kinase domain of BCR-ABL, BCR-ABL amplification, BCR-ABL overexpression, and the persistence of quiescent CML leukemic stem cells (Braun et al., 2020; Ozgur Yurttas and Eskazan, 2020). Mutations in the BCR-ABL kinase domain have been the most common mechanism of acquired imatinib resistance (Zhou et al., 2018). More than 70 point BCR-ABL mutations have been found in CML patients. Among them, the T315I mutation was responsible for approximately 20% of the acquired resistance to TKIs, resulting in a poor prognosis (Lee et al., 2021). The second-generation TKIs, such as dasatinib, nilotinib, and bosutinib, have been developed and are effective on patients with all but the T315I mutation (Kantarjian et al., 2009; Osman and Deininger, 2021). Ponatinib, a third-generation TKI, was developed to target BCR-ABL-T315I mutations (Cortes et al., 2012); however, they were shown to induce very serious toxic reactions, which meant they had to be withdrawn from the market (Anagnostou and Litzow, 2018; Ozgur Yurttas and Eskazan, 2020). To the delight of CML patients, asciminib was approved by the US FDA in 2021 to treat patients with CML who suffer resistance to or unacceptable adverse effects from TKIs or with the BCR-ABL-T315I mutation. Therefore, drug resistance mediated by the T315I mutant form of BCR-ABL remains a challenge.

Histone deacetylases (HDACs) are a family of enzymes that play pivotal roles in the modulation of gene expression by deacetylating lysine residues on histones (H2A, H2B, H3, and H4) or other proteins in chromatin. Histone acetylation/deacetylation is an epigenetic process mediated by HDACs and histone acetyltransferases (HATs). HDACs, together with HATs, regulate the dynamic balance between acetylation and deacetylation and play a central role in cell proliferation, apoptosis, and differentiation (Abujamra et al., 2010; Sarkar et al., 2020). Once histone acetylation levels are dysregulated in a normal cell, it leads to the imbalance of the original gene expression levels, which in turn leads to tumorigenesis (Wang et al., 2020). HDAC inhibitors (HDACi) are novel drugs for tumor-targeted therapy that inhibit tumor cell proliferation and induce cell differentiation or apoptosis by increasing the acetylation level of intracellular histones (Manal et al., 2016; Eckschlager et al., 2017). Some representative HDACi, such as SAHA, LBH58925, PDX10124, and FK228, have been approved to treat cutaneous T-cell lymphoma by the US FDA (Pojani and Barlocco, 2021).

I13, an indole-3-butyric acid derivative and a potent HDACi (Chen et al., 2021), was found to reduce the proliferation of acute myeloid leukemic cells by inducing cell differentiation in our previous study (Ma et al., 2022). This current study investigated the inhibitory activity of I13 against CML cells with T315I-mutated and wild-type BCR-ABL and sought to understand the underlying mechanism of I13 action.

Materials and methods

Reagents and instrument

The structures of I13 prepared in our laboratory and the imatinib purchased from Selleckchem (Houston, United States) are presented in Figure 1A. Stock solutions of I13 with 95% purity and imatinib dissolved in DMSO were stored at −20°C. RPMI-1640 medium, streptomycin/penicillin (S/P), and fetal bovine serum were acquired from GIBCO in Carlsbad, United States. Propidium iodide (PI) staining buffer (#550825) and an annexin V-fluorescein isothiocyanate (FITC)/PI kit were acquired from BD Biosciences in San Diego, United States. The MTT reagent, Wright–Giemsa stain solution, and DMSO (dimethyl sulfoxide) were purchased from Sigma-Aldrich in St. Louis, United States. The FITC anti-human/mouse CD11b (#101205), PE anti-human CD13 (#301704), FITC anti-human CD14 (#301804), and PE anti-mouse/human CD15 (#15125605) for the detection of cell surface antigen expression were acquired from Biolegend Inc., San Diego, United States. The FITC anti-mouse CD14 (ab307635) and PE anti-mouse CD13 (ab33490) were acquired from Abcam in MA, United States. MethoCult™ M3134 (#03134) and H4100 (#04100) were acquired from the Technologies Inc. of STEMCELL in Vancouver, Canada. GAPDH mAb (#5174), histone H3 mAb (#4499s), acetyl-histone H3 mAb (Ac-H3, #8173), histone H4 mAb (#2935), acetyl-histone H4 (Ac-H4, #2594), BCR-ABL (#3902), and p-BCR-ABL (phospho-BCR-ABL, #3901) were acquired from Cell Signaling Technology in Beverly, United States. The SPARKscript RT Plus Kit (#AG0304) and SYBR Green qPCR Mix (#AH0104) were purchased from SparkJade (Shandong, China). The flow cytometry analysis was performed on a flow cytometer BD Accuri C6 (San Jose, United States).

Cell culture

Murine BaF3-T315I cells harboring T315I-mutated BCR-ABL, BaF3-WT cells with wild-type BCR-ABL (BaF3-WT), and K562 (human CML cells) were used. The BaF3 cells provided by Dr. Brian J. Druker were stably transfected with either wild-type or T315I-mutated BCR-ABL. The cells were maintained in a RPMI-1640 complete medium with 10% fetal bovine serum and 1% S/P with 5% CO_2 at 37°C.

Measurement of cell proliferation

The inhibitory effects of I13 and imatinib on the proliferation of CML cells were measured by the colorimetric MTT assay. The cells were grown for 24 h in 96-well glass bottom plates. After incubation, I13 or imatinib was added to each well for 72 h, then 20 μL of MTT solution (4 mg/mL) was added, and the mixture was maintained for 4 h. Then, 150 μL DMSO was added to each well to dissolve the obtained formazan crystals. Finally, the absorbance of the formazan

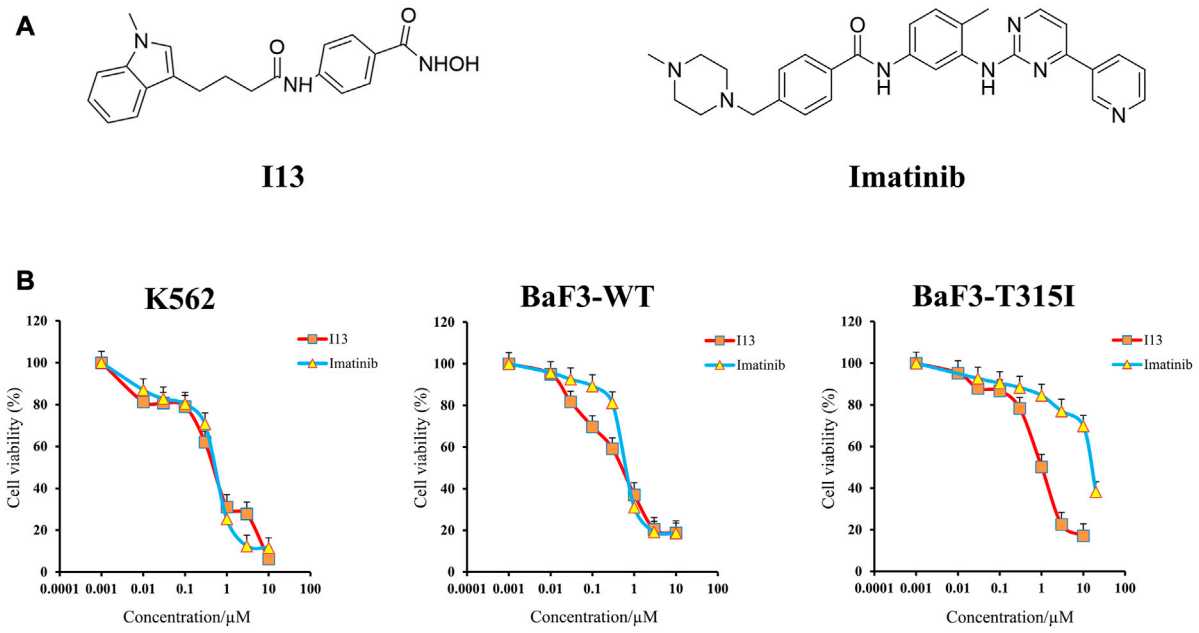

FIGURE 1
Anticancer efficacy of I13 on CML cells. **(A)** Structure of I13 and imatinib. **(B)** The effect of I13 or imatinib on the proliferation of K562, BaF3-WT, and BaF3-T315I cells stimulated with I13 or imatinib (0–20 μM) for 72 h. Error bars represent the SD of the mean.

was determined at 570 nm by Multiskan FC Microplate Photometer (Thermo Scientific Inc., MA, United States).

Analysis of cell cycle distribution

The cells were stimulated with I13 for different periods, then collected, washed, and fixed with ice-cold 70% ethanol solution, and they remained stable at −20°C for 24 h. The cells were washed with PBS and incubated with PI (50 mg/mL) with 100 mg/mL of RNase A for 30 min at room temperature in the dark. The DNA content of sub G1, G0/G1, S, and G2/M, was determined using the flow cytometer. Finally, the cell cycle distribution was analyzed using ModFit software.

Analysis of cell apoptosis rate

The indicated concentration of I13 or imatinib was used to stimulate the cells. After incubation, the cells were harvested and washed twice with a cold PBS. They were collected by centrifugation and resuspended in 100 μL of fresh 1× binding buffer, followed by staining with 5 μL of the annexin V-FITC buffer and 5 μL of PI solution for 30 min at room temperature in the dark. The cell apoptosis rate was detected using the flow cytometer.

Analysis of cell morphology

The indicated concentration of I13 was used to stimulate the cells for 72 h. After incubation, the cells were harvested and washed twice with a PBS buffer. The slides were prepared and air-dried. The adherent cells were stained using Wright–Giemsa dye solution for about 10 min. Finally, a light microscope was used to observe and photograph the cell morphology.

Measurement of expression of cell surface antigens

The cells were exposed to the same concentration of I13 used in the cell morphology analysis for 72 h. After treatment, the cells were harvested, washed, and cultured with a specific antibody for 30 min at room temperature in the dark. The expression level of cell surface markers was detected by the flow cytometer.

Measurement of cell colony formation ability

The cells were incubated with I13 in 24-well plates in a 2.6% methylcellulose medium (MethoCult H4100 and M3134 were used for K562 and BaF3 cells, respectively) containing 10% FBS for 14 days. Colonies composed of ≥50 cells were observed and scored under an inverted microscope.

mRNA sequencing analysis

To quantify the genome-wide distribution of BaF3-T315I cells incubated with the indicated I13, mRNA sequencing was performed. As described previously, Ma et al., 2022 the cells were collected, and

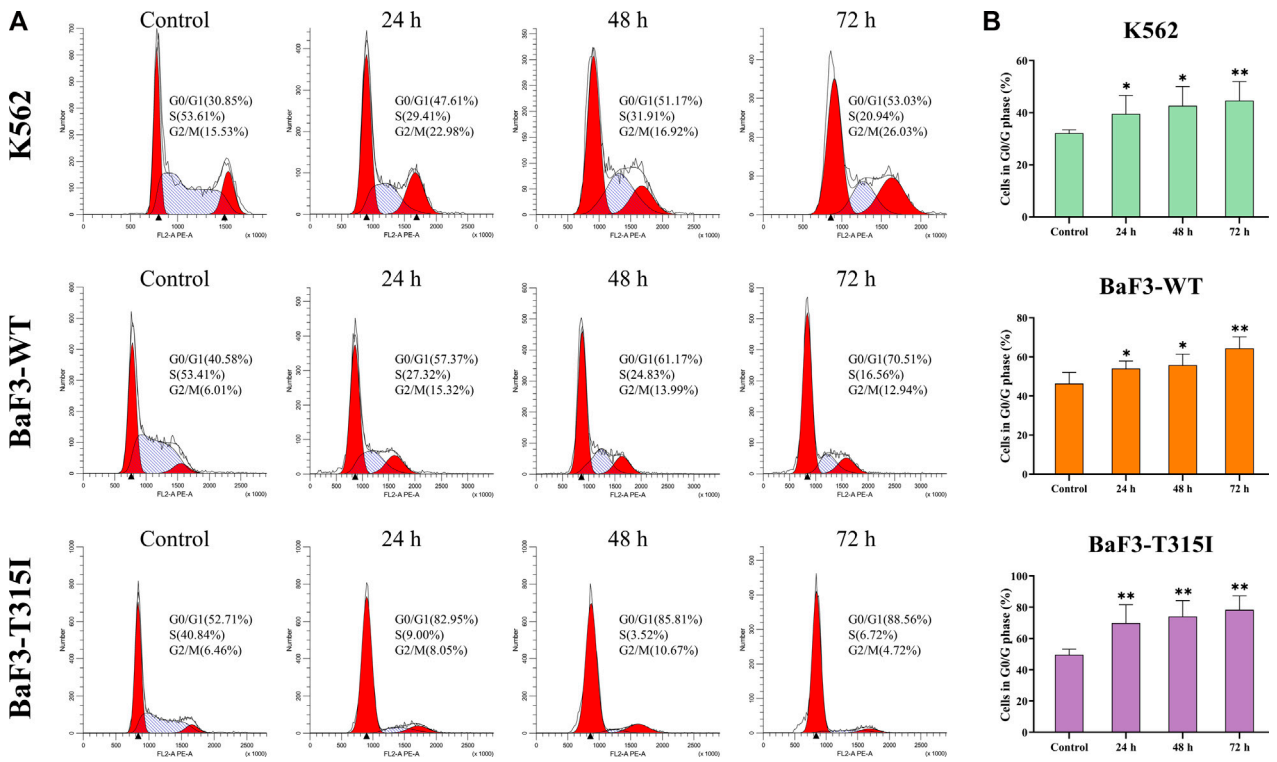

FIGURE 2
I13 blocks cell cycle progression in CML cells. **(A)** K562, BaF3-WT, and BaF3-T315I cells were treated with I13 at 0.5, 0.6, or 1.1 μM, respectively, for 24 h, 48 h, or 72 h. **(B)** A bar graph presenting the population of G0/G1 phase cells (*$p < 0.05$, **$p < 0.01$).

RNA was isolated from the cells. The Illumina genome analyzer was used to sequence the library of cDNA, and the expression levels of individual genes were normalized to the fragments per kilobase of transcript per million of the mapped data. Identification of the differentially expressed genes (DEGs) analysis was performed with an adjusted p value less than 0.05 and $|\log_2(\text{fold change})| \geq 0.58$). KEGG enrichment analysis of these DEGs was carried out using the R language clusterProfiler package, and an adjusted p-value of less that 0.05 was used as the cut-off criteria.

Analysis of relative gene expression using quantitative real-time PCR

The BaF3-T315I cells were incubated with I13 (1.1 μM) for 24, 48, or 72 h and then collected and lysed using the TRIzol reagent (Invitrogen Life Technologies) to obtain the total RNA. The SPARKscript II RT Plus Kit was used to synthesize the first strand cDNA. The Ct value of each gene in triplicate reactions was detected using the SYBR Green method on an Applied Biosystems 7,500 Fast System. The mRNA expression levels of the target genes were normalized to GAPDH expression levels using the $2^{-\Delta\Delta Ct}$ method. The primers were presented as follows: GAPDH (mouse) forward 5′- CAAGGTCATCCATGACAACTT TG-3′, reverse 5′-GTCCACCACCCTGTTGCTGTAG-3′ (Alhasan et al., 2016); BCR-ABL (mouse) forward 5′-AAGCGCAACAAG CCCACTGTCTAT-3′, reverse 5′-CTTCGTCTGAGATACTGG ATTCCT-3′ (Lan et al., 2017).

Western blotting analysis

The BaF3-T315I cells were exposed to I13 for 72 h, and then they were collected, washed, and lysed with a radioimmunoprecipitation assay (RIPA) buffer. The denatured protein lysates were isolated by electrophoresis on sodium dodecyl sulfate (SDS)–polyacrylamide gel (PAGE), and the desired protein lysates were transferred onto a polyvinylidene difluoride (PVDF) membrane. The 5% skim milk was used as a blocking solution, and the membrane was placed in it and blocked at room temperature for 2 h. Then, the membrane was exposed to the desired primary antibody overnight at 4°C. Finally, the secondary antibody was used to incubate the membrane at room temperature for 1 h, and an enhanced chemiluminescence detection reagent (Fluor Chem Q, United States) was used to visualize the protein bands.

Statistical analysis

The results are representative of the data obtained from three independent experiments performed in triplicate. They are

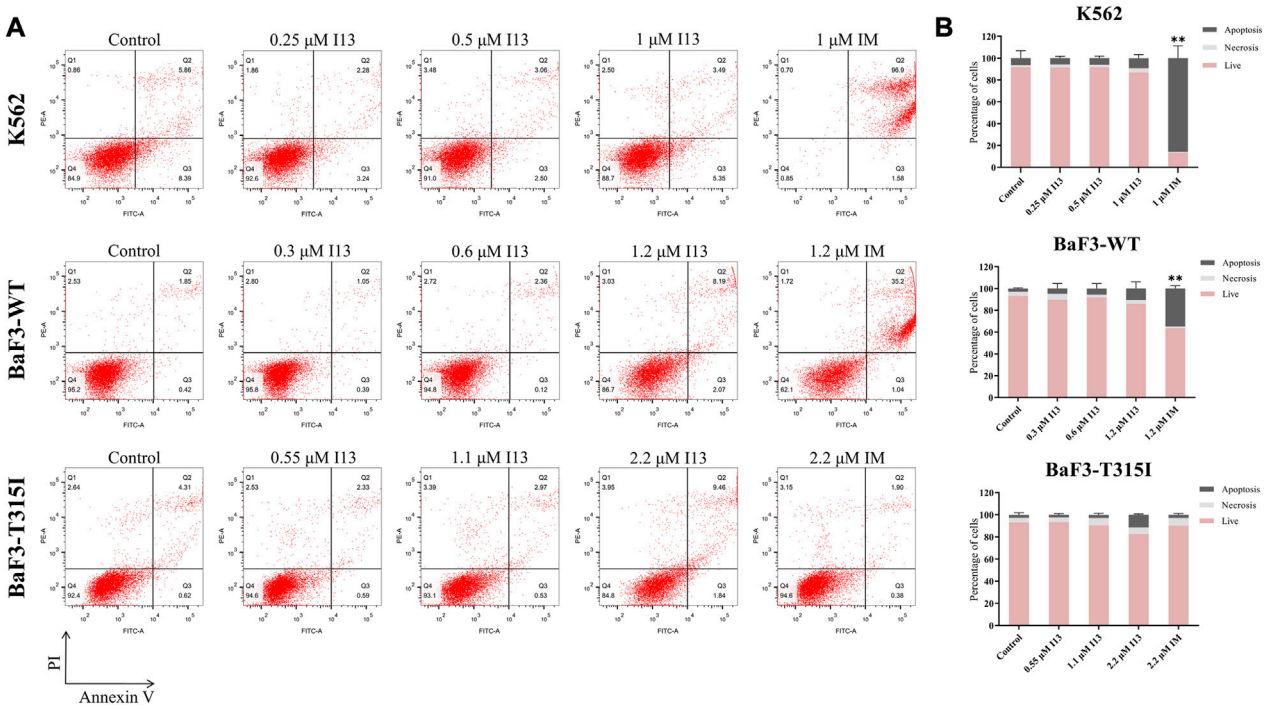

FIGURE 3
I13 had no significant effects on cell apoptosis in CML cells. **(A)** The effect of I13 on the cell apoptosis in cells incubated with I13 or imatinib for 72 h. **(B)** The bar graph showing the cell apoptosis rate. The K562 cell was incubated with 0.25, 0.5, and 1 μM of I13 or imatinib at 1 μM. The BaF3-WT cell was cultured with 0.3, 0.6, and 1.2 μM of I13 or 1.2 μM of imatinib. The BaF3-T315I cell was stimulated with 0.55, 1.1, and 2.2 μM of I13 or 2.2 μM of imatinib for 72 h (**$p < 0.01$).

shown as the mean along with standard deviation denoted by error bars. The difference between the experiment and control group was determined using SPSS 13.0 one-way ANOVA, followed by Dunnett's test. The difference was considered statistically significant when the p-value was less than 0.05 (*$p < 0.05$ and **$p < 0.001$).

Results

I13 shows significant anti-proliferation activity against CML cells expressing T315I-mutated or wild-type BCR-ABL

It can be seen from Figure 1B that the proliferation of BaF3-T315I, BaF3-WT, and K562 cells was significantly inhibited after the treatment of I13 with the IC_{50} value of 1.00 ± 0.02 μM, 0.59 ± 0.01 μM, and 0.57 ± 0.01 μM, respectively. The concern is that I13 was much more efficient (16-fold) than imatinib with the IC_{50} of 16.26 μM in reducing the proliferation of BaF3-T315I. In addition, I13 also suppressed the proliferation of the K562 and BaF3-WT cells with a comparable potency of imatinib with the IC_{50} value of 0.62 ± 0.01 μM and 0.74 ± 0.02 μM, respectively. These results suggest that I13 has strong inhibitory activity against imatinib-resistant CML cells carrying T315I-mutated BCR-ABL.

I13 induces G0/G1 arrest in both BCR-ABL T315I mutation and wild-type CML cells

To better potentially understand the mechanism of the proliferation inhibition induced by I13, the cell cycle progression of cells affected by I13 was evaluated. It can be seen from Figures 2A, B that I13 exposure resulted in a significant increase in the proportion of the cells in the G0/G1 phase, with increasing treatment time in the BaF3-T315I, K562, and BaF3-WT cells, which demonstrated the G0/G1 arrest induced by I13. The data suggest that the inhibitory effect of I13 on the proliferation of CML cells is due to the induction of the G0/G1 cell cycle arrest.

I13 treatment did not promote the appearance of signs of apoptosis in both BCR-ABL T315I mutation and wild-type CML cells

In order to investigate whether the observed inhibition of cell proliferation was caused by cell apoptosis, BaF3-T315I, K562, and BaF3-WT cells were stimulated with the indicated concentrations of I13 or imatinib. Less apoptosis was observed when BaF3-T315I, K562, and BaF3-WT cells were exposed to I13 at less than 2.2, 1, and 1.2 μM, respectively (Figures 3A, B). In contrast, K562 and BaF3-

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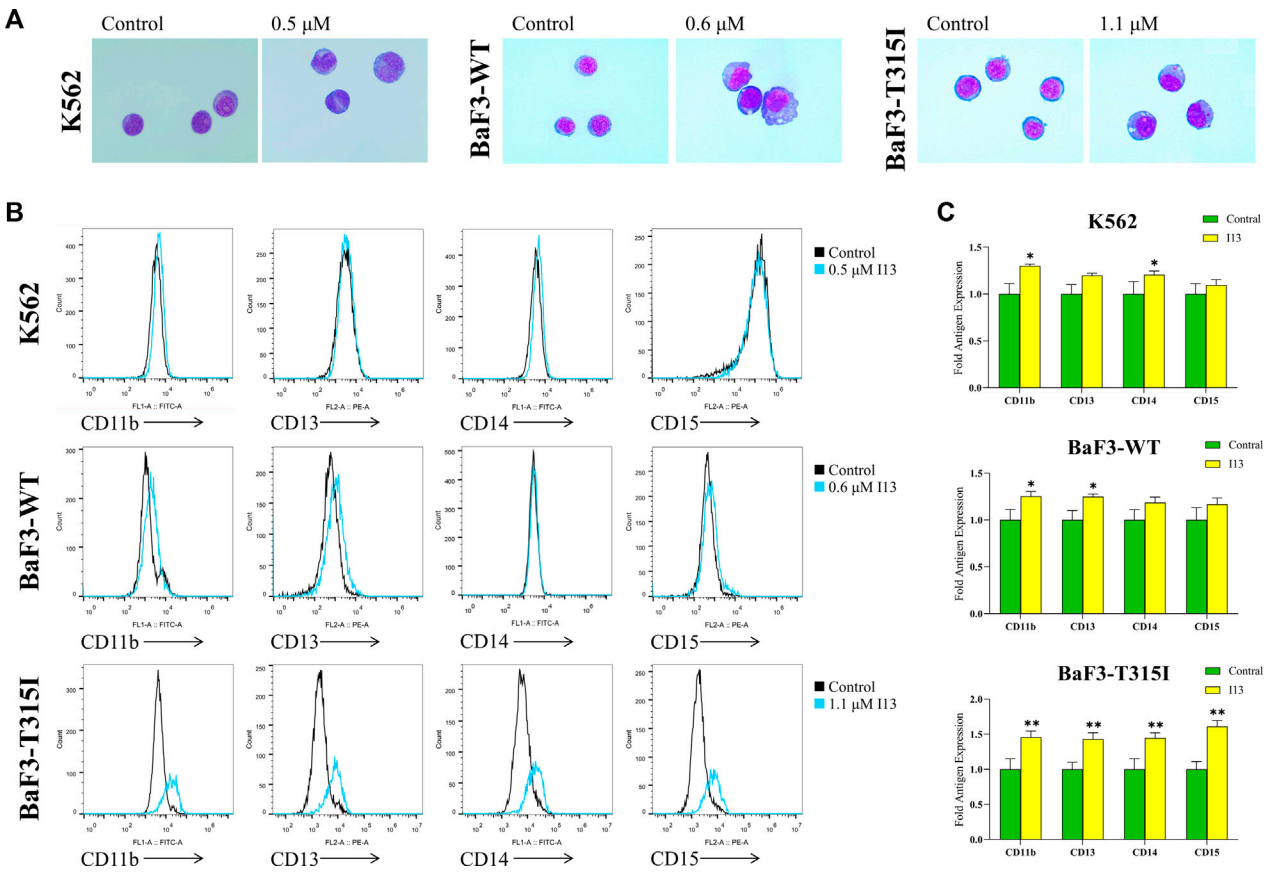

FIGURE 4
I13 promoted the differentiation of K562, BaF3-WT, and BaF3-T315I cells characterized by the change of morphology and cell surface markers. **(A)** Morphological changes of cells stained with the Wright–Giemsa dye solution and examined with light microscopy (×1,000). **(B)** Cell surface antigen expression was detected by flow cytometry. **(C)** The mean intensity of fluorescence is shown in a bar graph. The BaF3-T315I, K562, and BaF3-WT cells were stimulated with 1.1, 0.5, or 0.6 μM of I13, respectively, for 72 h (*$p < 0.05$, **$p < 0.01$).

WT cells treated with 1 and 1.2 μM of imatinib, respectively, showed substantial apoptosis. These data indicate that the proliferation inhibition of CML cells harboring T315I-mutated or wild-type BCR-ABL is not related to apoptosis after the treatment of BaF3-WT, k562, and BaF3-T315I cells with I13 at less than 1.2, 1, and 2.2 μM, respectively.

I13 promotes cell differentiation in both BCR-ABL T315I mutation and wild-type CML cells

Since the proliferation inhibitory effect of I13 on BaF3-T315I, K562, and BaF3-WT cells was not related to induction of cell apoptosis, a cell morphological analysis and cell surface differentiation antigen analysis were performed on these cells. As shown in Figure 4A, BaF3-T315I, K562, and BaF3-WT cells demonstrated significant morphological changes, including a decreased nuclear-to-cytoplasmic ratio and increased cell size, which indicates that the cell differentiation block could be overcome when these cells are stimulated with 1.1, 0.5, and 0.6 μM of I13, respectively. Additionally, the treatment of I13 markedly up-regulated the expression levels of CD11b (a differentiation marker of granulocyte/monocyte), CD13 (a differentiation marker of granulocyte/monocyte), CD14 (a differentiation marker of monocyte/macrophage), and CD15 (a differentiation marker of granulocyte/monocyte) in BaF3-T315I cells. Similarly, I13 treatment elevated the CD11b and CD14 expression levels in the K562 and BaF3-WT cells. In addition, I13 exposure elevated the expression of CD13 in BaF3-WT cells (Figures 4B, C). The data reveal that the inhibition activity of I13 against CML cells harboring T315I-mutated or wild-type BCR-ABL may be associated with the induction of cell differentiation. Hence, these concentrations of I13 were used in the experiments, including the cell cycle, colony formation assay, and mechanism research.

I13 significantly inhibits colony formation capacity in both BCR-ABL T315I mutation and wild-type CML cells

We further assessed how I13 affects the colony formation ability of these CML cells. The cell colony formation ability was significantly decreased in a concentration-dependent manner

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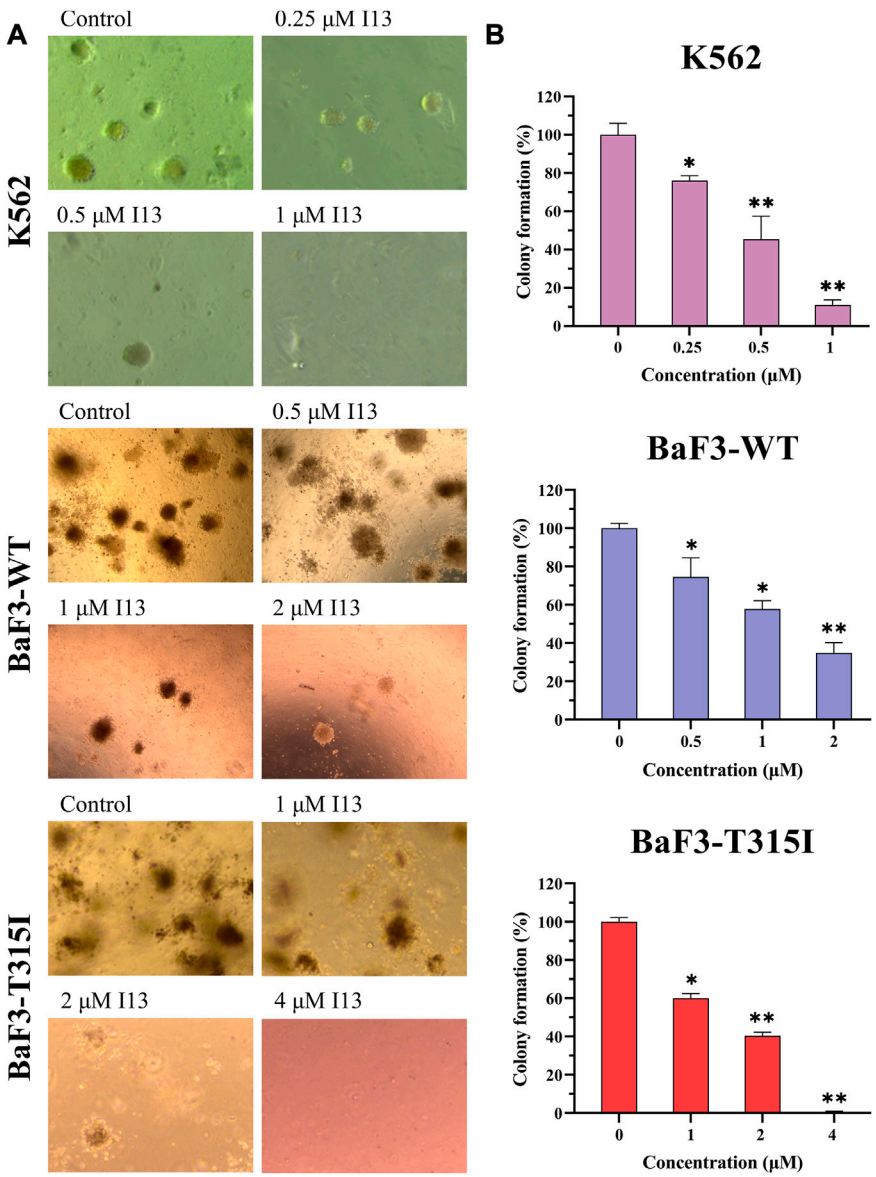

FIGURE 5
The effect of I13 on the colony-forming efficiency of K562, BaF3-WT, and BaF3-T315I cells. **(A)** Colony formation assay of cells exposed to I13 in a methylcellulose medium. **(B)** The relationship of the number of colony formation assays to the concentration of I13 is shown in the bar graph (*$p < 0.05$, **$p < 0.01$).

(Figures 5A, B). These data reveal that I13 significantly depresses the colony-forming ability of both the BCR-ABL T315I mutation and wild-type CML cells.

I13 promotes cell differentiation by HDAC inhibition coupled with block of chronic myeloid leukemia signaling pathway in BCR-ABL T315I mutation CML cells

mRNA sequencing was used to understand the molecular control involved in the differentiation of BaF3-T315I cells mediated by I13. In Figure 6A, it can be seen that the expression of 81 genes declined, and 234 genes were elevated, which was presented in the volcanic diagram, indicating that I13 was not a global transcriptional regulator (Figure 6B). KEGG analysis in the BaF3-T315 cells showed that the chronic myeloid leukemia signaling pathway (genes such as Cdk4, Tgfbr1, Gadd45b, Mdm2, Gadd45g, Polk, Rb1, Ctbp1, and Cdkn2a were enriched) was involved in the I13 treatment (Figure 6C). Since CML is caused by the BCR-ABL, we explored whether I13 affects the BCR-ABL expression at the mRNA and protein level in BaF3-T315I cells. As shown in Figure 7A, treatment with I13 at 1.1 μM significantly down-regulated the BCR-ABL mRNA expression level in a time-dependent manner. Furthermore, the expression level of the BCR-ABL protein was depleted after exposure to I13 at 1.1 μM in the BaF3-T315I cells

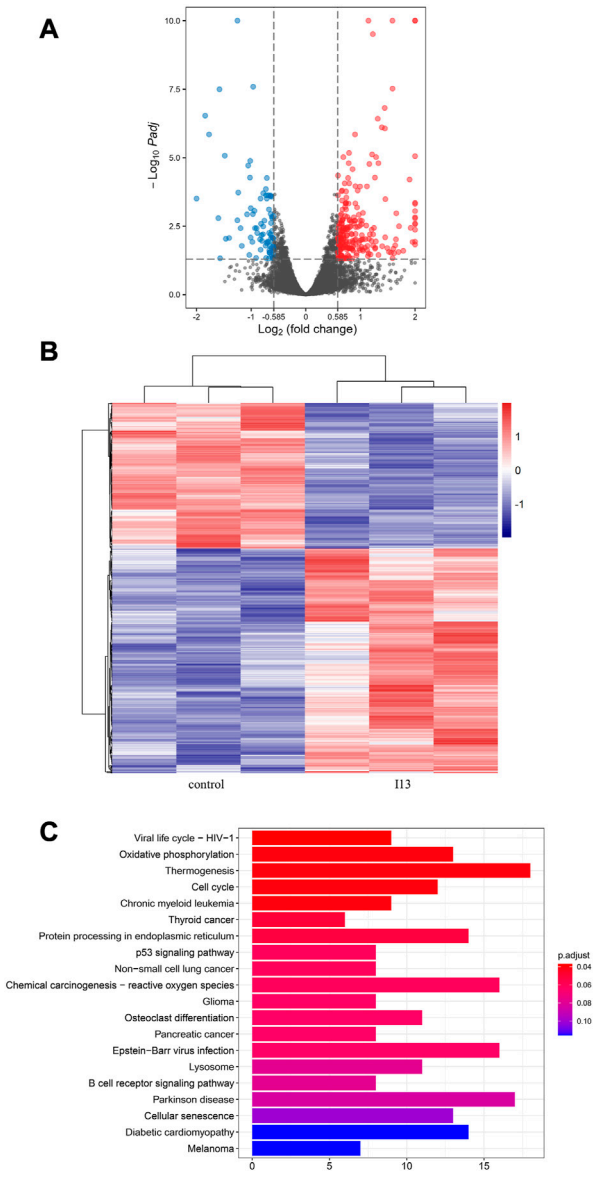

FIGURE 6
mRNA sequencing analysis in BaF3-T315I cells exposed to I13. **(A)** Volcano plots of differentially expressed genes. **(B)** The cluster heatmap of differentially expressed genes. **(C)** KEGG pathway analysis of DEGs. BaF3-T315I cells were stimulated with I13 at 1.1 μM for 48 h.

(Figures 7B, C). These data exhibited that BCR-ABL was markedly down-regulated at both the mRNA and protein level after I13 treatment; however, imatinib could not alter the expression. Hence, the inhibitive activity of I13 against the cellular proliferation of cells carrying the BCR-ABL T315I mutation was attributed to the depleting of the BCR-ABL oncoprotein resulting in the decrease of p-BCR-ABL.

Considering that I13 is a promising HDAC inhibitor with significant inhibitory activity against the HDAC1 and HDAC3 enzymes (Chen et al., 2021), we determined the HDAC inhibition effect of I13 based on the protein level of the acetylation of histones H3 and H4 (Ac-H3 and Ac-H4) in BaF3-T315I cells. It can be seen from Figures 7B, C that the I13 treatment significantly elevated the expression of Ac-H3 and Ac-H4 in the BaF3-T315I cells; however, the imatinib treatment did not demonstrate significant HDAC inhibition activity.

Discussion

CML is a myeloproliferative malignancy caused by the BCR-ABL fusion gene, which is the product of the translocation of the ABL gene located on 9q34 to the BCR gene on 22q11 (Mughal et al., 2007). CML occurs most frequently in middle-aged and older adults and represents 15%–20% of leukemias. In 2001, imatinib, the first generation of TKIs, became a great boon to CML patients. However, it was found that most patients developed resistance to imatinib, predominantly because of the mutation of the BCR-ABL kinase domain. Amongst these mutations, the T315I mutation is the most frequent one, and patients with this mutation have a poor prognosis (Jabbour et al., 2008; Wei et al., 2020). Accordingly, second-generation TKIs, such as dasatinib and nilotinib, and third-generation TKIs such as, ponatinib, have been developed (Miura, 2015; Saussele et al., 2020; Cortes and Lang, 2021); however, the T315I mutation also confers resistance to the second-generation TKIs. Ponatinib was approved by the US FDA in 2012 for the treatment of patients with the T315I mutation, but it was withdrawn from the US market for 7 weeks because of its severe toxic reactions (Moslehi and Deininger, 2015). With this background, asciminib was approved and used to treat patients who had the T315I mutation or failed the two prior TKIs in 2021. Though asciminib is a potent, orally bioavailable drug, it is inevitable that it will bring adverse reactions. In our previous study (Ma et al., 2022), it was found that I13, a HDAC inhibitor, induced the differentiation of acute myeloid leukemia cells and inhibited cell proliferation. Herein, we present that I13 could overcome the differentiation block in CML cells harboring T315I-mutated BCR-ABL and wild-type BCR-ABL, characterized by the changed morphology and increased expression of CD11b, CD13, CD14, or CD15. Therefore, I13 markedly inhibits the proliferation activity and colony-forming capacity of BaF3-T315I, K562, and BaF3-WT cells through G0/G1 exit. In brief, I13 possesses a significant inhibitive effect on the cell proliferation of CML cells harboring T315I-mutated BCR-ABL and wild-type BCR-ABL by inducing cell differentiation.

As shown in Figure 6C, the cell differentiation of BaF3-T315I induced by I13 was attributed to the inhibition of the chronic myeloid leukemia signaling pathway, which was involved in BCR-ABL regulation (https://www.kegg.jp/pathway/map05220). Moreover, I13 treatment markedly decreased both the mRNA and protein expression level of BCR-ABL (Figure 7). Additionally, it was reported that transient silencing of BCR-ABL or BCR-ABL deficiency can induce the differentiation of CML cells, resulting in cell cycle arrest and the inhibition of cell proliferation (Brozik et al., 2006; Rangatia and Bonnet, 2006; Morceau et al., 2008). Hence, the suppression of the proliferation of BaF3-T315I cells by cell differentiation induced by I13 may be originated from the repression of the chronic myeloid leukemia signaling pathway *via* the modulation of BCR-ABL.

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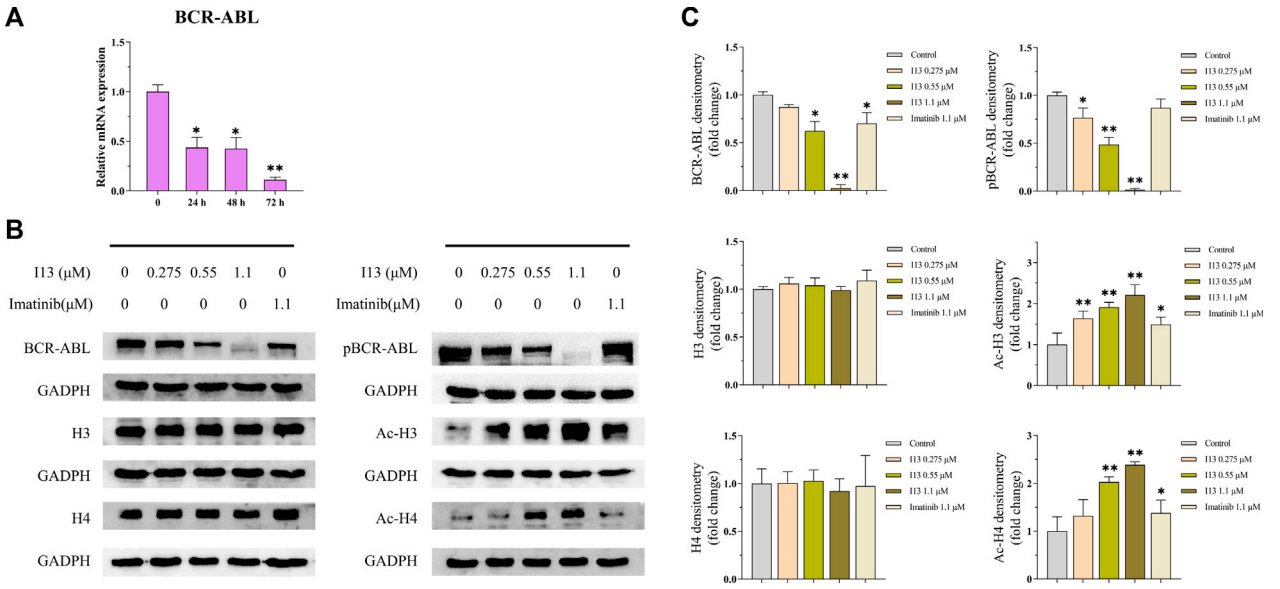

FIGURE 7
The effects of I13 on the mRNA and protein expression levels of differentiation-related genes in BaF3-T315I cells. **(A)** Quantitative real-time PCR analysis of mRNA expression level of BCR-ABL. The cells were exposed to 1.1 μM of I13 for 24, 48, or 72 h. **(B)** Immunoblotting analysis of BCR-ABL, p-BCR-ABL, H3, Ac-H3, H4, and Ac-H4. **(C)** The bar graph shows the intensity of the protein band quantified by AI600 images. The cells were exposed to 0.275, 0.55, and 1.1 μM of I13 or 1.1 μM of imatinib for 72 h (*$p < 0.05$, **$p < 0.01$).

It has been demonstrated that the increased acetylation of histones represses transcription (Marks et al., 2000). We demonstrated that exposure to I13 increased the Ac-H3 and Ac-H4 levels and depleted the BCR-ABL mRNA and protein expression levels in BaF3-T315I cells. This result was in accordance with the report that HDACi, such as SAHA and LAQ824, were shown to inhibit BCR-ABL expression at both the mRNA and protein level in CML cells, paralleled by the increased acetylation of histone H3 levels (Nimmanapalli et al., 2003a; Nimmanapalli et al., 2003b). Moreover, it has been reported that BCR-ABL expression is regulated by global hyperacetylation (Brusa et al., 2006). Therefore, we suggest that the I13-mediated decline in the BCR-ABL protein levels of BCR-ABL may be due to its HDAC inhibitory activity. To conclude, the cell differentiation induced by I13 is likely due to the block of the chronic myeloid leukemia signaling pathway *via* the modulation of BCR-ABL, which is mediated by the inhibition of HDAC activity and presented by the increase of the acetylation of histones H3 and H4 in CML cells harboring BCR-ABL-T315I. These findings indicate that I13 could be a potential epigenetic drug that is worth further investigation through *in vivo* studies. However, the current BCR-ABL-targeted therapies do not target BCR-ABL leukemic stem cells (LSCs). Hence, for future perspectives for CML management, it is necessary to focus on the development of an effective therapeutic approach to target persistent CML leukemic stem cells, which drive the CML and are insensitive to current drugs such as TKIs, thus leading to a long-term treatment for CML patients.

Collectively, I13 possesses an interesting property that makes it a promising compound for further investigation in order to potentially overcome the current limitations of CML therapy caused by the BCR-ABL-T315I mutation. First, I13 produces a marked effect in cells that are resistant to imatinib or other TKIs caused by the BCR-ABL-T315 mechanism. Second, I13 efficiently depletes BCR-ABL, which blocks its function as a scaffold protein that modulates the chronic myeloid leukemia signaling pathway mediating cell differentiation. Third, to be clinically relevant, I13 exerts an antitumoral effect on imatinib-resistant CML cells, demonstrating that it could be a potential epigenetic drug for the development of a CML therapy that can overcome resistance mediated by the BCR-ABL-T315 mutation.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number is GSE 225777.

Author contributions

CG: Performed the experiments and wrote the manuscript. LZ, YX, XM, and PC: Methodology. LW: Designed the research, writing–editing, and supervision. Z-SC: Conceptualization and supervision. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1183052/full#supplementary-material

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The role of peptides in reversing chemoresistance of breast cancer: current facts and future prospects

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Breast cancer is the first malignant tumor in women, and its incidence is also increasing year by year. Chemotherapy is one of the standard therapies for breast cancer, but the resistance of breast cancer cells to chemotherapy drugs is a huge challenge for the effective treatment of breast cancer. At present, in the study of reversing the drug resistance of solid tumors such as breast cancer, peptides have the advantages of high selectivity, high tissue penetration, and good biocompatibility. Some of the peptides that have been studied can overcome the resistance of tumor cells to chemotherapeutic drugs in the experiment, and effectively control the growth and metastasis of breast cancer cells. Here, we describe the mechanism of different peptides in reversing breast cancer resistance, including promoting cancer cell apoptosis; promoting non-apoptotic regulatory cell death of cancer cells; inhibiting the DNA repair mechanism of cancer cells; improving the tumor microenvironment; inhibiting drug efflux mechanism; and enhancing drug uptake. This review focuses on the different mechanisms of peptides in reversing breast cancer drug resistance, and these peptides are also expected to create clinical breakthroughs in promoting the therapeutic effect of chemotherapy drugs in breast cancer patients and improving the survival rate of patients.

KEYWORDS

breast cancer, drug resistance, peptide, apoptosis, chemotherapy

1 Introduction

Breast cancer is one of the global public health problems (Oo et al., 2021), and it is also the most common cancer among women in the world (Wang et al., 2017). By October 2022, breast cancer had surpassed lung cancer to become the most common malignant tumor in the world. Its occurrence is related to many factors, involving a variety of genetic and epigenetic changes (Li et al., 2022). Breast cancer can be divided into different subtypes. From the purpose of treatment, breast cancer can be divided into three subtypes: human epidermal growth factor receptor 2 (HER2) positive, androgen and progesterone receptor (ER, PR) positive, and triple-negative (Weigelt and Reis-Filho, 2009). Triple-negative breast cancer (TNBC), also known as triple-negative breast cancer (TNBC), is named for the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Yeo and Guan, 2017). Breast cancer treatment methods include surgical resection, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy, and chemotherapy is one of the standard therapy (Wang et al., 2017), especially nowadays breast-conserving surgery is performed with neoadjuvant chemotherapy without

resection (Li et al., 2014). Cytotoxic drugs are often used as first-line drugs, such as doxorubicin (DOX), methotrexate (MTX), cisplatin (CDDP), paclitaxel (PTX), etoposide, and so on (Wang et al., 2017). The mechanisms of action of these chemotherapeutic drugs are different. Doxorubicin is an anthracycline drug, and its mechanism of action mainly involves the insertion of chromosome and mitochondrial DNA and the inhibition of topoisomerase II. Methotrexate is a folate antagonist that inhibits dihydrofolate reductase, so the lack of reduced folate substrate impairs the synthesis of purine nucleotides, thereby inhibiting DNA synthesis and ultimately reducing cancer cell proliferation (Lindgren et al., 2006). Paclitaxel is a common microtubule inhibitor, mainly by destroying the mitotic spindle to activate the mitotic checkpoint to prevent cell mitosis, resulting in cell stagnation during mitosis (Singh et al., 2007). These chemotherapy drugs appear early, cancer cells are particularly sensitive to them, effectively inhibit tumor growth and metastasis, and prolong the life of patients in clinically achieved good results. However, with the widespread use of chemotherapy drugs, the joy brought by good therapeutic effects gradually disappeared (Vasan et al., 2019), followed by cancer recurrence, tumor cell metastasis, and uncontrollable conditions. This is the resistance of tumor cells to chemotherapeutic drugs.

Because of the resistance to chemotherapeutic drugs, although the treatment strategy of combined use of multiple chemotherapeutic drugs was later adopted, the effect was still poor (Vasan et al., 2019). Drug resistance can be divided into intrinsic resistance, acquired resistance, and multidrug resistance, and multidrug resistance was later considered to be the most common. Multidrug resistance often leads to poor treatment and poor prognosis of these chemotherapeutic drugs for solid tumors such as breast cancer through various molecular mechanisms (Haggag et al., 2020). According to the study, the response rate of metastatic breast cancer to first-line chemotherapy drugs is usually 30%–70%, but it is not persistent. Generally, drug resistance occurs in 6–10 months, resulting in treatment failure. The 5-year survival rate of patients with metastatic breast cancer is only 27% (Wang H. et al., 2014). Therefore, exploring the molecular mechanism of multidrug resistance of chemotherapeutic drugs has also become a research hotspot. At present, several common drug resistance mechanisms have been studied, including the disorder of the apoptosis mechanism, the activation of the DNA repair mechanism, the mutation of the drug target, and the regulation and disorder of drug inflow (Haggag et al., 2020). For example, cancer cells transport P53 to the cytoplasm through the CRM1-mediated output mechanism to escape the apoptotic effect of P53 (Haggag et al., 2020), resulting in decreased cytotoxicity of drugs such as doxorubicin. For another example, proliferating cell nuclear antigen is involved in DNA replication, DNA repair, DNA methylation, chromatin remodeling, cell cycle regulation, and protein degradation (Lingeman et al., 2014), making cisplatin-induced cell death ineffective. And the effectiveness of drugs like paclitaxel is limited by resistance mechanisms partly mediated by the upregulation of anti-apoptotic BCL-2 and P-glycoprotein (Gupta et al., 2018). In addition, the current chemotherapy is non-specific, regardless of the subtypes of breast cancer, but in terms of drug resistance, for specific types of TNBC, the mechanism of resistance to chemotherapy drugs due to heterogeneity is very complicated (Bakrania et al., 2016). Therefore, the current research is more in the exploration of specific targeted signal transduction pathway therapy, such as a specific β-interferon inducer DEAE-glucan can be targeted to deliver doxorubicin to tumor tissue (Bakrania et al., 2018).

In recent studies, peptides, especially bioactive peptides, are a hot spot in reversing the resistance of breast cancer cells to chemotherapeutic drugs. Peptide is an amino acid with a molecular weight between protein and small molecule (Zhang et al., 2023). It is generally a short chain with more than 50 amino acids, usually containing a disulfide bond. Its sequence and structure are adjustable to increase its interaction with specific molecules. Peptides related to cancer treatment can be divided into three groups: cell-penetrating peptide (CPP), tumor-targeting peptide, and pore-forming peptide (Beheshtirouy et al., 2021). Bioactive peptides are peptides that can bind to molecular targets and affect cells or organisms. They have many favorable properties such as high tissue penetration, good biocompatibility, and good binding affinity with target molecules (Oo et al., 2021). Of course, this has to mention the bioactive cationic peptide (BCP). BCP is widely distributed in nature. The positively charged residues on BCP can interact with the negatively charged groups on the tumor membrane, and then the acyl chain of the lipid membrane. The hydrophobic interaction between non-polar residues induces the instability of the lipid bilayer and structural and physicochemical changes, which in turn leads to cell death (Manrique-Moreno et al., 2021). There are also bioactive peptides from natural aquatic products, including marine peptides, which have therapeutic potential in breast cancer (Ahmed K. S. et al., 2021). Given the different mechanisms of drug resistance and the different roles played by peptides, we will elaborate on the mechanism of different peptides reversing the resistance of breast cancer to first-line chemotherapy drugs in the six aspects of promoting cancer cell apoptosis, promoting non-apoptotic regulatory cell death of tumor cells, hindering DNA damage repair, affecting tumor microenvironment, inhibiting the efflux mechanism of tumor cell chemotherapy drugs and increasing the uptake of chemotherapy drugs Table 1. To study the reversal of drug resistance of breast cancer by peptides, improve the therapeutic effect of chemotherapy and control the metastasis of tumor cells. Scholars who improve the survival rate of breast cancer patients provide references.

2 Peptides reverse drug resistance: promote apoptosis in breast cancer cells

The imbalance of apoptosis is the main reason for the accumulation of immortalized cells and the promotion of cancer development. Of course, it is also related to chemotherapy resistance (Cotter, 2009). Therefore Promoting apoptosis of cancer cells is a good way to improve the sensitivity of tumors to chemotherapeutic drugs (Wang H. et al., 2014), and there are several ways to induce apoptosis. Here, five main apoptotic pathways are mentioned, namely: regulation of apoptotic genes, lysosomal peptide apoptotic pathway, mitochondrial apoptotic pathway, nuclear transcription factor-involved apoptosis, and cell cycle arrest apoptotic pathway. Some peptides induce apoptosis and reverse drug resistance by regulating these apoptotic pathways.

TABLE 1 Mechanism of peptide reversing chemotherapy resistance of breast cancer.

Catalog		Peptide name	Source	Peptide sequence	Reversed drugs	Reversing resistance mechanisms	References
Apoptosis pathways	Apoptosis gene	anti-HSP70 peptide	chemical synthesis	YCAYYSPRHKTTF	DOX	Heat shock protein 70 (HSP70)	Tan et al. (2021)
		NuBCP-9 peptide	Nur77	_	DOX	Exposing the BCL-2 BH3 domain	Gupta et al. (2018)
		AVPIR8	chemical synthesis	FMOC-Arg (pbf)-OH, FMOC-Ala-OH, FMOC-Val-OH, FMOC-Pro-OH, FMOC-Ile-OH	DOX	p53 ↑, a high binding affinity to the X-linked IAPs	Wang et al. (2014a)
	Soluble membrane peptide	Membrane-Lytic Peptide	chemical synthesis	GLLxLLxLLLxAAGW	DOX	ACPs, anionic residues	Chen et al. (2022b)
		HNPs-1	neutrophils, specific subsets of T cells, monocytes, and NK cells	_	DOX	↑ plasma membrane permeability	Li et al. (2014)
		Heterochiral β-Peptide Polymers	chemical synthesis	_	DOX	Destroying the cell membrane	Bakrania et al. (2017)
	Mitochondrial apoptosis pathway	HNPs-1	neutrophils, specific subsets of T cells, monocytes, and NK cells	_	DOX	Destruction of mitochondrial transmembrane potential (△ Ψm)	Li et al. (2014)
		HER-2 peptide	chemical synthesis	YCDGFYACYMDV	DOX	The generation of ROS and damage to mitochondrial respiratory chain components	Shi et al. (2018)
		R 8 H 3 peptide	chemical synthesis	MLRAALSTARRGPRLSRLLHHHRRRRRRRR	DOX	Achieved effective reactive oxygen species (ROS)	Ahmed et al. (2021a)
	Nuclear transcription factor	The peptide aptamer AII-7	chemical synthesis	LNFYRHGFLPNAVMASMLEVGPWFELLGLCGLAGHPLSSL RI	PTX	Interference with AKT activation; ↓ FOXO-1 transcription factor, nuclear factor nB	Kunz et al. (2006)
		T10-ERK	chemical synthesis	HAIYPRHGGCGMPKKKPTPIQLNP	DOX	↓ extracellular signal-regulated kinases (ERK)	Sheng et al. (2016)
		EN1-iPep	the EN1 transcription factor	N-KKKRKVPLVWPAWVYCTRYSDR-C	DTX	↓ homeobox transcription factor Engrailed 1 (EN1)	Sorolla et al. (2016)
	Cell cycle arrest	ANK peptide	chemical synthesis	KGNSALHVASQHGHLGCIQTLVRYGANVTMQNHG	PTX	SNCG with BubR1 interaction	Singh et al. (2007)
		NT21MP	chemical synthesis	H-D-leu-D-Gly-D-Ala-D-Ser-D-Trp-Dhis-D-Arg-D-Pro-D-Asp-D-Lys-Cys-Cys-Leu-Gly-Tyr GlnLys-Arg-Pro-Leu-Pro-OH	PTX	miR-335 ↑; SETD8 ↑, Wnt/β-catenin signaling, genes cyclin D1 and G0/G1	Wang et al. (2017)
		ALT	ALT cell lines	_	palbociclib	↓ the tyrosine phosphorylation of p27Kip1(CDKN1B), CDK4, CDK2, G1	Jilishitz et al. (2021)
Non-apoptotic regulatory cell death		ALT	ALT cell lines	_	palbociclib	↑ RIPK1 phosphorylation; ↓ Ca2+ ion channels and Na + ion channels	Jilishitz et al. (2021)

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TABLE 1 (*Continued*) Mechanism of peptide reversing chemotherapy resistance of breast cancer.

Catalog		Peptide name	Source	Peptide sequence	Reversed drugs	Reversing resistance mechanisms	References
		Acid-Sensitive Peptide	The sequence of cell surface proteins	HAIYPRHGGC, THRPPMWSPVWPGGC	DOX	cathepsin D ↑; caspase-3 ↑	Sheng et al. (2015)
DNA repairing mechanism		caPeptide	proliferating cell nuclear antigen (PCNA)	RRRRRRRRRCCLGIPEQEY, RRRRRRRRRCCEPGLIYEQ	CDDP	↓ proliferating cell nuclear antigen (PCNA)	Lingeman et al. (2014)
		RanGTP inhibitory peptide (RAN-IP)	Ran protein sequence	CAQPEGQVQFK	DOX	↓ Ras-related nuclear protein (Ran-GTP)	Haggag et al. (2020)
Tumor microenvironment		anti-PN peptides	chemical synthesis	TFATHGKHWAAP	DOX	↑ phosphorylation of AKT, ↑ expression of survivin	Oo et al. (2021)
		Abstract. Human neutrophil peptides-1 (HNPs-1)	chemical synthesis	_	DOX	↓ vivo tumor angiogenesis	Li et al. (2014)
		D-CAN	bariatric surgery patients	CSWKYWFGEC	CDDP, PTX	↓ Cancer-associated fibroblasts (CAFs); Extracellular matrix (ECM) remodeling, vascularization; Immunosuppression	Su et al. (2020)
Efflux mechanism		T10-ERK	chemical synthesis	HAIYPRHGGCGMPKKKPTPIQLNP	DOX	↓ ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp)	Sheng et al. (2016)
		TAT	chemical synthesis	CGGGYGRKKRRQRRR	DOX	bypassing the efflux pro- tein (e.g., P-glycoprotein)	Mozaffari et al. (2021)
		NuBCP-9	Nur77	_	PTX	Decomposition of Pgp1 receptor	Gupta et al. (2018)
Enhanced drug uptake	Tumor imaging and drug-delivery	CendR peptide	chemical synthesis	Cys-Arg-Gly-Asp-Lys	CDDP	Binding to the neuropilin-1 receptors (Nrp-1)	Bai et al. (2019)
		Novel peptide	chemical synthesis	NH2-WxEYAAQkFL-CONH2	DOX	Two D-amino acid substitutions at the enzymatic labile sites	Soudy et al. (2013)
		Targeting Peptide	chemical synthesis	Gly–Phe–Leu–Gly, GFLG	DOX	Cathepsin B ↑	Zhi et al. (2019)
		Cancer-selective tetra-branched peptides	chemical synthesis	pyroELYENKPRRPYIL-NH	MTX	Binding to the receptor-related proteins (LRP) receptors and heparan sulfate chains on membrane proteoglycans	Depau et al. (2017)
		bombesin peptide Bombesin (Bn)	chemical synthesis	_	DOX	Binding to the GRPR receptors	Wang et al. (2016)
		T10-EKP	chemical synthesis	HAIYPRHGGCGMPKKKPTPIQLNP	DOX	Binding to the transferrin receptor (TfR)	Sheng et al. (2016)
		YTA2 and YTA4	chemical synthesis	Acetyl-YTAIAWVKAFIRKLRK-amide Acetyl-IAWVKAFIRKLRKGPLG-amide	MTX	Cell-penetrating peptides CPP, across the plasma membrane	Lindgren et al. (2006)

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TABLE 1 (*Continued*) Mechanism of peptide reversing chemotherapy resistance of breast cancer.

Catalog		Peptide name	Source	Peptide sequence	Reversed drugs	Reversing resistance mechanisms	References
		HER-2 peptide	chemical synthesis	YCDGFYACYMDV	DOX	Binding to the human epidermal growth factor receptor-2 (HER-2)	Shi et al. (2018)
		Novel TIMP3 Peptide p700	tissue inhibitor of metalloproteinase 3 (TIMP3)	Azido-PEG12-KIKSCYYLPCFVTSKN-Lys (5-FITC)-amide	DOX	Binding to the VEGFR1	Aldughaim et al. (2020)
		Nap-GFFpYK peptide	chemical synthesis	_	etoposide	↑ solubility, phosphoric acid group	Zhang et al. (2020)
	Only as a carrier	cyclic peptides (CPs)	chemical synthesis	-L-Gln-D-Ala-L-Glu-D-Ala-LGln-D-Ala-L-Cys-D-Ala-	DOX	High drug encapsulation ratio	Wang et al. (2014b)
		amphiphilic peptide dendrimers (AmPDs)	chemical synthesis	_	DOX	Efficient encapsulation	Zhu et al. (2021)

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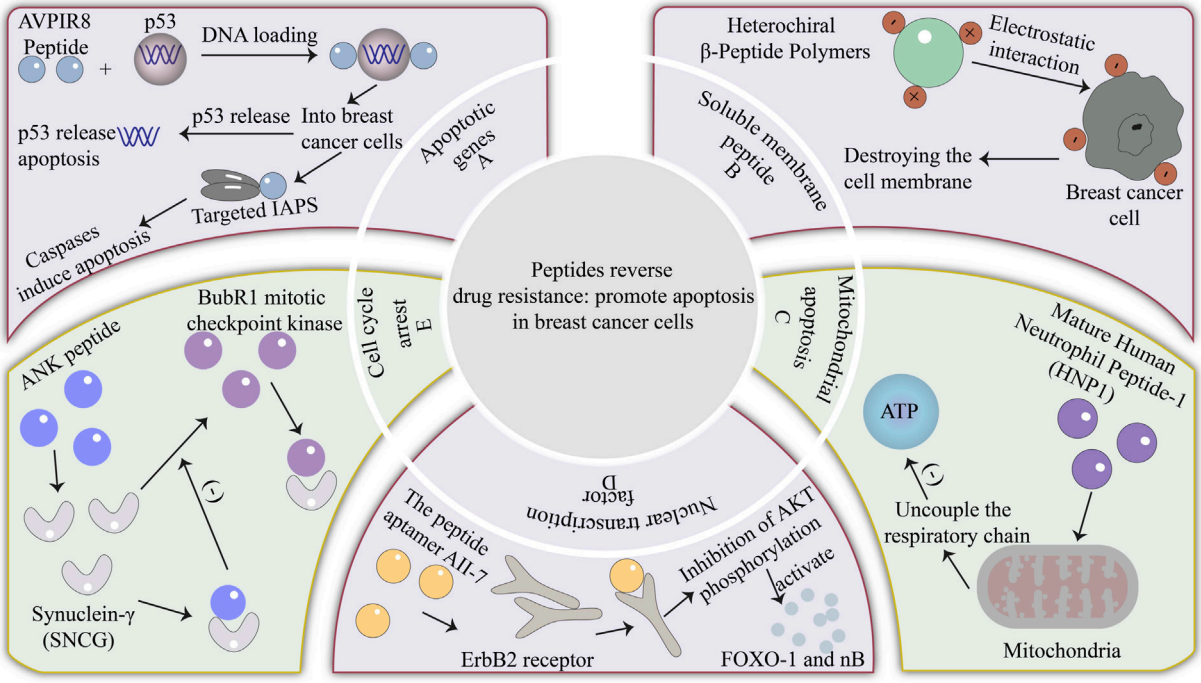

FIGURE 1
The mechanism of reversing drug resistance by promoting apoptosis of cancer cells. **(A)** Smac peptides are apoptosis peptides, the most widely studied is the Smac N-terminal tetrapeptide (AVPI), which has a strong binding force with the chain IAP, and the AVPI peptide and p53 DNA are co-delivered to induce apoptosis. **(B)** The synthesized amphiphilic β-peptide polymer is an ACP, which is composed of cationic and anionic residues. It can bind to the negative charge on the surface of cancer cells through electrostatic interaction, and then the hydrophobic part interacts with the phospholipid bilayer to destroy the cell membrane. **(C)** Mature human neutrophil peptide-1 first mediates the collapse of mitochondrial transmembrane potential in cells, decoupling the respiratory chain from oxidative phosphorylation, damaging the energy source of tumor cells, and leading to apoptosis of cancer cells. **(D)** The peptide aptamer All-7 can specifically bind to the ErbB2 receptor, interfere with the activation of the AKT pathway so it cannot regulate the nuclear transcription factor κB and FOXO-1 through the phosphorylation of AKT, and restore the sensitivity of tumor cells to paclitaxel. **(E)** As an inhibitor of the mechanism of action of SNCG protein, ANK peptide can bind to Synuclein-γ (SNCG) protein, prevent the interaction between SNCG protein and BubR1 mitotic checkpoint kinase, and the role of the mitotic checkpoint will return to normal.

2.1 Peptides regulating apoptotic genes

Anti-apoptosis is the main mechanism of drug resistance in tumor cells (Igney and Krammer, 2002), and the production of anti-apoptosis is closely related to the imbalance of apoptotic genes. Apoptotic genes are mainly divided into two categories: genes promoting apoptosis and genes inhibiting apoptosis according to their functions. There are many kinds of Apoptotic-related genes, such as the Bcl-2 family, p53, Fas, APO-1, and caspase protein family. The main cause of drug resistance is to promote the downregulation of apoptotic genes such as p53 gene expression and inhibit the upregulation of apoptotic genes such as Bcl-2 gene expression. So regulating apoptotic genes to promote cancer cell apoptosis is to enhance its sensitivity to chemotherapy drugs (Wang H. et al., 2014), a feasible way to reverse drug resistance.

The efficacy of paclitaxel, one of the first-line chemotherapy drugs for breast cancer, is limited because of the upregulation of the anti-apoptotic gene Bcl-2 (Kutuk and Letai, 2008). The corresponding countermeasure is to use some Bcl-2 inhibitors, such as navitoclax (ABT-263), but the effect of this method is limited. Therefore, the use of peptides such as NuBCP-9 peptide to combine Bcl-2 protein exposed Bcl-2 BH3 domain, making the Bcl-2 protein conformational changes, bax inhibition (Gupta et al., 2018), hinders the Bcl-2 gene production. In addition to the Bcl-2 gene plays an important role in tumor drug resistance, the decrease of p53 gene expression is another important reason for drug resistance. P53 mainly releases Smac by enhancing mitochondria (Yang et al., 2006; Carter et al., 2010), and Smac peptides are apoptotic peptides. The most widely studied is Smac N-terminal tetrapeptide (AVPI) (Wang, 2011), which has a strong binding force with the linked IAP. Therefore, AVPI peptide and p53 DNA can be synergistically delivered Figure 1. Not only increased the expression of p53 genes but also p53 can promote the release of Smac into the cytoplasm and induce apoptosis.

Caspase-dependent apoptosis also plays an important role in tumor drug resistance. Cells can activate caspase apoptotic genes with the participation of cytochrome C to cause cell death, but heat shock protein (HSP) inhibits the role of caspase apoptotic genes. For example, HSP70 makes breast cancer insensitive to cisplatin through this mechanism. Because HSP70 is expressed in both tumor cells and normal cells, many existing HSP70 inhibitors lack specificity and often lead to side effects (Leu et al., 2017). Therefore, a specific anti-HSP70 peptide was further developed. The anti-HSP70 peptide binds the HSP70 protein to prevent the inhibition of HSP70 on caspase and promote the apoptosis of cancer cells.

Therefore, the use of peptides to regulate apoptotic genes to induce breast cancer cell death is a new means of anti-drug resistance (Brown and Attardi, 2005). Most of these peptides upregulate the expression of the P53 gene or block the expression of the Bcl-2 gene, and enhance the production of apoptotic proteins such as caspase-3 to cope with the escape of cancer cells from apoptosis.

2.2 Soluble membrane peptides

As the name suggests, lysosomal peptides destroy the cell membrane. As a part of the innate immune defense barrier, the cell membrane will not be able to maintain cell survival after being destroyed, and the cells will also undergo apoptosis. It has been actively studied for the treatment of antibacterial, antiviral, and even anticancer (Guha et al., 2019). In anti-cancer treatment, a major advantage of lysosomal peptides is that they also have strong cytotoxicity to drug-resistant cancer cells (McPhee and Hancock, 2005), and can resist the resistance of tumor cells to chemotherapeutic drugs. However, at the same time, lysosomal peptides have certain limitations for anticancer therapy. First, the targeting effect of lysosomal peptides on tumor cells is not strong, and it is not able to accurately identify the subtle differences between tumor cells and normal cells (Leite et al., 2015; Kim et al., 2021). Secondly, the lysosomal peptide is not stable, and there are still difficulties in the way of administration. These are the reasons lysosomal peptides are not yet widely used in cancer treatment.

To kill tumor cells in a non-toxic manner, the first problem to be solved is the targeting of lysosomal peptides. Studies have designed a new type of lysosomal anticancer peptide (ACP) (Chen C. H. et al., 2022). The peptide changes the charge distribution of the peptide by purposefully introducing anionic residues (Andreev et al., 2010; Wiedman et al., 2017; Westerfield et al., 2019), then it can target the anionic lipids on the surface of cancer cells (Gaspar et al., 2012), thereby avoiding affecting healthy cells. The synthesized amphiphilic β-peptide polymer is also an ACP, which is composed of cationic and anionic residues Figure 1. It can bind to the negative charge on the surface of cancer cells through electrostatic interaction, and then the hydrophobic part interacts with the phospholipid bilayer, destroying the cell membrane and effectively resisting multiple drug resistance (Shao et al., 2022). Moreover, the novel membrane-soluble anticancer peptide is a neutral peptide that better targets cancer cells with subtle changes in cell surface pH. In addition to artificially designed and synthesized lysosomal peptides, natural lysosomal peptides such as HNP1-4, expressed primarily by neutrophils (Agerberth et al., 2000), can be found to enhance the plasma membrane permeability of tumor cells (Xu et al., 2008), selectively cytotoxic to cancer cells (Lichtenstein et al., 1986), and equally cytotoxic to drug-resistant cancer cells (Starr and Wimley, 2017).

In the face of the problem of lysing peptide administration route, nano-preparations are now used to assist the intravenous delivery of lysing peptides (Kong et al., 2021), reducing the hemolytic activity of lysing peptides and enhancing the stability of lysing peptides in plasma (Pino-Angeles and Lazaridis, 2018). Moreover, computer modeling is now combined with the research and development of lysosomal peptides, which promotes the speed of programming and design of new lysosomal peptides (Ulmschneider and Ulmschneider, 2018). It is believed that shortly, lysosomal peptides will be increasingly used in the treatment of malignant tumors such as breast cancer to improve the resistance of cancer cells to chemotherapeutic drugs.

2.3 Peptides promoting mitochondrial apoptosis pathway

Targeting the mitochondrial pathway is now also a strategy to reverse tumor drug resistance by inducing changes in mitochondrial function and promoting apoptosis of tumor cells. As for the mechanism of mitochondrial function changes, there are mainly several categories: changes in transmembrane potential, production of reactive oxygen species ROS, and release of apoptotic factors (Zhao et al., 2018; Soukupova and Rudolf, 2019).

Reducing mitochondrial transmembrane potential can block energy supply. The normal transmembrane potential is the premise to maintain the normal function of cells and even mitochondria. The reduction of mitochondrial transmembrane potential can uncouple the respiratory chain (Li et al., 2014), inhibit the production of ATP, block the energy supply of tumor cells and lead to cell death. Mature human neutrophil peptide-1 (HNP1) can induce a decrease in mitochondrial transmembrane potential (Li et al., 2014). Because the outer membrane of tumor cells contains more acidic phospholipids, and the cytoskeleton system of tumor cells is defective, the extracellular matrix components also change, and HNP1 is more likely to act on tumor cells and less affect normal cells (Muller et al., 2002). Mature human neutrophil peptide-1 first mediates the collapse of mitochondrial transmembrane potential in cells, then the respiratory chain is uncoupled with oxidative phosphorylation, damaging the energy source of tumor cells (Xu et al., 2005), leading to apoptosis of cancer cells and achieving the purpose of destroying drug resistance Figure 1.

Mitochondria produces and releases a large amount of reactive oxygen species ROS, which can induce cell death including cancer cells. Targeting mitochondria by designing peptides can destroy the mitochondrial membrane, increase its permeability, and release ROS (Ahmed S. et al., 2021). After a large amount of ROS enters the cytoplasm of breast cancer, it will destroy the cell structure and function by seizing electrons, leading to the death of cancer cells. At the same time, ROS can also lead to oxidative damage of mitochondrial components. Damage to mitochondrial DNA will further destroy mitochondrial oxidative phosphorylation, increase mitochondrial membrane potential (MMP) and release promotes cytochrome c (CytoC), and ultimately promote cell death (Yuan et al., 2021).

The release of apoptotic factors in mitochondria induces an intrinsic apoptosis pathway. The inducing factors of the initiation of the mitochondrial apoptosis pathway mainly include direct caspase activator, indirect caspase activator, and caspase-independent cell death effector (Martinou et al., 2000). The common apoptotic factor cytochrome C released by mitochondria is a direct caspase activator. Studies have shown that HER-2 peptide can mediate the increase of mitochondrial permeability, then release cytochrome C, and activate the caspase apoptotic gene family through a series of events. As a

result, the cells move toward the apoptotic process of self-digestion (Shi et al., 2018). In addition to cytochrome C, reverse drug resistance and promote the death of tumor cells can also start from other apoptotic factors, such as caspase activator Smac, which is derived from the second mitochondria, and apoptosis-inducing factor AIF (Zimmermann et al., 2001). The mechanism is to induce the activation of apoptotic genes by increasing the release of mitochondrial apoptotic factors, and then induce a series of cascade reactions to induce apoptosis of cancer cells and overcome the multidrug resistance of malignant tumors such as breast cancer to many chemotherapeutic drugs such as doxorubicin.

2.4 Peptides regulating nuclear transcription factors

Studies have shown that nuclear transcription factors have a certain relationship with the generation of drug resistance in some malignant tumors. For example, the overexpression of homologous nuclear transcription factor Engrailed 1 (EN1) can lead to the resistance of basal-like breast cancer to the chemotherapeutic drug docetaxel (Sorolla et al., 2016). Some breast cancer cells activate the AKT pathway induced by the heterodimer of ErbB2. After AKT phosphorylation, the FOXO-1 transcription factor is inactivated, thereby inhibiting the expression of apoptotic proteins (Brunet et al., 1999). At the same time, AKT phosphorylation will also induce nuclear factor κB-mediated anti-apoptotic response and inhibit the expression of tumor suppressor (Romashkova and Makarov, 1999), resulting in paclitaxel resistance. Therefore, regulating the expression of nuclear transcription factors is also an idea to reverse drug resistance, including the use of peptides to control transcription factors.

Studies have used the peptide aptamer All-7, All-7 can specifically bind to the ErbB2 receptor, interfere with the activation of the AKT pathway Figure 1, and it cannot regulate the nuclear transcription factor κB (κB was first found in B lymphocytes because it binds to the B site of the immunoglobulin κ light chain gene enhancer and regulates the transcription of the immunoglobulin κ light chain, it is named nuclear transcription factor κB) and FOXO-1 through the phosphorylation of AKT, and restore the sensitivity of tumor cells to paclitaxel (Kunz et al., 2006). For the problem that some nuclear transcription factors are highly expressed in cancer cells, nuclear transcription factor interference peptides are a good solution. Because normal cells hardly express these nuclear transcription factors, they are less affected by nuclear transcription factor interference peptides. For example, the interference peptide (EN1-iPep) designed to block EN1 mainly inhibits the expression of EN1 by binding to the nuclear transcription factor EN1 through a dominant negative-like mechanism (Sorolla et al., 2016). Reduced expression of EN1 induces caspase-3-dependent apoptosis (Beltran et al., 2014), which can reverse the drug resistance of basal-like breast cancer cells. In addition, there is a dual-target hybrid peptide T10-ERK, which mainly interferes with the binding of MEK and ERK, hinders the activation of ERK (Sheng et al., 2016), inhibits the expression of related nuclear transcription factors to regulate the apoptosis of cancer cells, and achieves the purpose of promoting cancer cell death and overcoming drug resistance. In conclusion, it is a feasible scheme to improve the resistance of breast cancer cells to chemotherapeutic drugs by controlling the expression of nuclear transcription factors.

2.5 Cell cycle arrest peptides

Preventing tumor cell mitosis to arrest it in the division phase, that is, blocking the cell cycle is the mechanism of some chemotherapeutic drugs in the treatment of tumors. The commonly used anti-microtubule drugs such as paclitaxel and nocodazole block the cell cycle by causing spindle damage (Blajeski et al., 2002). At the same time based on this mechanism, it also produced resistance. Drug-resistant breast cancer cells can control or inhibit the expression of genes that hinder cell division by avoiding the examination of mitotic checkpoints, thereby successfully avoiding the results of mitotic arrest after chemotherapy.

Now some peptides can be successfully reversed inhibited cell cycle arrest, with the NT21MP peptide can enhance the expression of microRNA-335, and then through the Wnt/β-catenin signaling pathway to inhibit the expression of SETD8 target genes, so cells stagnate in G0/G1 phase. The ANK peptide, as an inhibitor of the mechanism of action of the Synuclein-γ (SNCG), can bind to the SNCG Figure 1, preventing the interaction of the SNCG with the BubR1 mitotic checkpoint kinase, and the role of the mitotic checkpoint will return to normal (Singh et al., 2007). Studies have also found that ALT peptides can also promote the cell cycle arrest of cancer cells. ALT peptides mainly block the tyrosine phosphorylation of p27, and the cyclin D-CDK4 dimer cannot be associated with p27Kip1 (Blain, 2008), thereby inhibiting CDK4 and CDK2. The cell cycle is blocked in the G1 phase, and ALT peptides can also prevent it from re-entering the cell cycle, which can enhance the effect of paclitaxel on cell cycle CDK4/6. After these peptides inhibit the mitosis of cancer cells and block the cell cycle, the body detects these arrested cells, which will induce them to gradually apoptosis and restore the toxicity of chemotherapeutic drugs such as paclitaxel to tumor cells.

3 Peptides reverse drug resistance: promote non-apoptotic cell death in breast cancer

In addition to reversing drug resistance through the apoptotic pathway, peptides can also be used to induce non-apoptotic regulatory cell death leading to tumor cell death (Yu et al., 2022), including autotropic cell death, and necroptosis. Here we mainly introduce two peptides that induce the lysosomal peptide apoptosis pathway and RIPK1-dependent necroptosis, respectively.

The lysosomal apoptosis pathway refers to the release of hydrolases in lysosomes to promote cell death, which is an autophagic cell death that can be applied to kill cancer cells and reverse the resistance of tumor cells to chemotherapeutic drugs. To induce the lysosomal apoptosis pathway, it is necessary to make the cell lysosomal membrane unstable and release hydrolases. There are proteases in these hydrolases, such as the common lysosomal

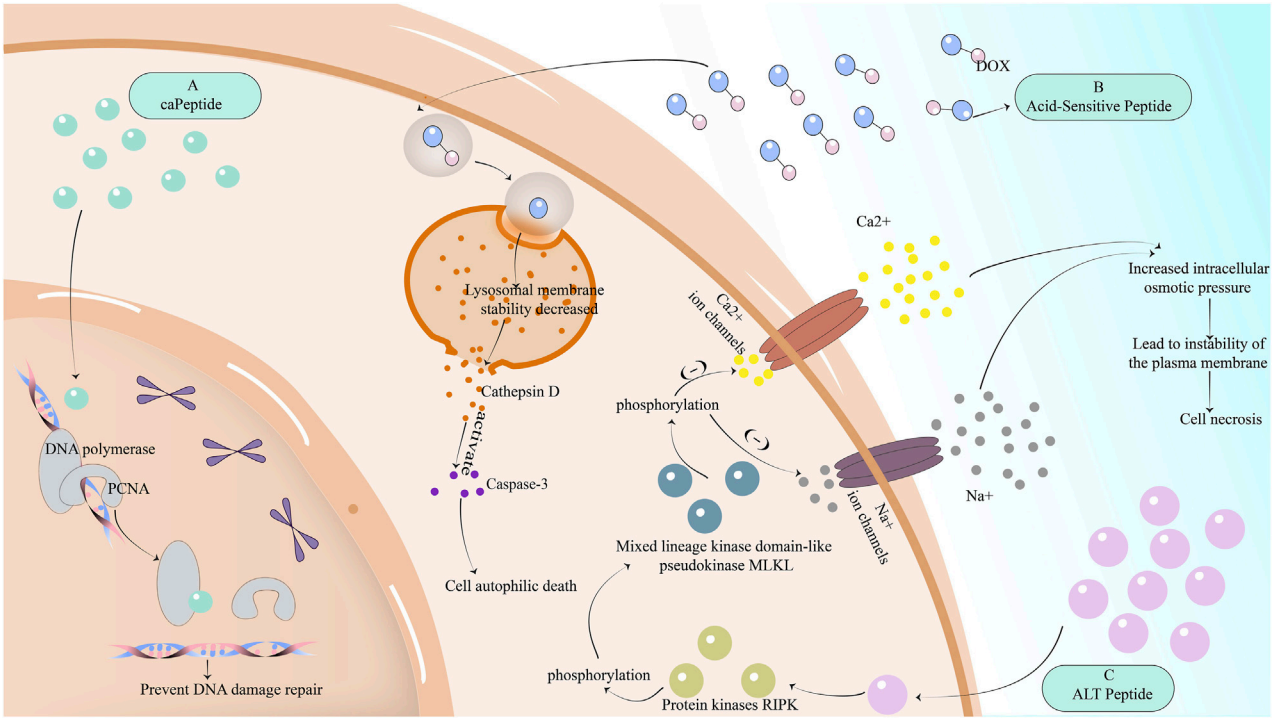

FIGURE 2
The mechanism of reversing drug resistance by partially inhibiting the DNA repair mechanism of cancer cells, or by promoting non-apoptotic regulatory cell death of cancer cells. **(A)** The peptide caPeptide that inhibits DNA repair in breast cancer cells can potentially bind to PCNA-interacting proteins such as POLD3 and increase genomic DNA damage. **(B)** Acid-sensitive peptides are internalized into lysosomes and other organelles through TfR-mediated endocytosis, resulting in decreased stability of lysosome membrane, enhanced permeability, the release of cathepsin D, activation of caspase-3, and induction of cell autophagic death. **(C)** ALT peptide first induces RIPK phosphorylation and then MLKL phosphorylation. MLKL phosphorylation will downregulate Ca2+ and Na + ion channels, resulting in increased intracellular osmotic pressure, instability of plasma membrane, and cell necrosis.

marker cathepsin D (Sheng et al., 2015). These proteases are released into the cytoplasm and activate apoptotic effectors, such as caspase-3, to achieve the purpose of cell death. Therefore, the lysosomal pathway to induce apoptosis must first destroy the organelle membrane of lysosomes.

It has been proved that the accumulation of chloroquine in acidic vesicles can lead to the instability of endosomes and lysosomal membranes (Lubgan et al., 2009). Therefore, acid-sensitive peptides have been designed (Sheng et al., 2015), and acid-sensitive peptides are coupled with chemotherapy drugs. Acid-sensitive peptides can be internalized into lysosomes and other organelles through TfR-mediated endocytosis (Berczi et al., 1993), resulting in decreased lysosomal membrane stability, enhanced permeability, the release of cathepsin D, activation of caspase-3, and induction of cell autophagic death Figure 2.

The ALT peptide has been found to induce necrotic apoptosis of breast cancer cells through the mechanism of RIPK1 (Cekan et al., 2016; Jilishitz et al., 2021). The molecular mechanism of necroptosis is composed of protein kinases RIPK1 and RIPK3 and mixed lineage kinase domain-like pseudokinase MLKL. ALT peptide first induces RIPK phosphorylation and then MLKL phosphorylation. MLKL phosphorylation will downregulate Ca2+ and Na + ion channels, resulting in increased intracellular osmotic pressure, instability of plasma membrane, and cell necrosis Figure 2.

Non-apoptotic regulatory cell death such as cell autophagic death, and necroptosis has become a new method to induce tumor cell death, which is different from the apoptotic pathway and reverse drug resistance. With the deepening of research on non-apoptotic regulatory cell death, it will be more used in the treatment of cancer.

4 Peptides reverse drug resistance: inhibition of DNA repair mechanism in breast cancer cells

The mechanism of action represented by cisplatin in chemotherapeutic drugs for tumors is to damage the DNA of cancer cells, causing DNA damage response (DDR), which cannot be further replicated to cause cell death (Cekan et al., 2016). However, tumor cells later enhanced the repair of damaged DNA and developed resistance to chemotherapy drugs based on damaged DNA. Therefore, inhibiting DNA repair in breast cancer cells can reverse the drug resistance caused by the enhanced repair mechanism after DNA damage.

Ras-related nuclear protein (Ran-GTP) can mediate the repair of DNA damage, while Ran-RCC1 inhibitory peptide (RAN-IP) derived from Ran protein sequence Ran-RCC1 inhibits the

formation of Ran-GTP (Haggag et al., 2017; Haggag et al., 2019) by competitively binding to RCC1 (Haggag et al., 2020), which not only reduces the level of Ran-GTP but also makes the nuclear input and output pathways regulated by Ran-GTP defective, inhibiting its role in enhancing DNA repair in cancer cells. Although RCC1 is also expressed in normal cells and can prevent the aging and death of normal cells due to DNA damage, the use of RAN-IP may have an impact on normal cells, but tumor cells are more affected after reducing Ran levels (Yuen et al., 2013; Yuen et al., 2016). On the other hand, proliferating cell nuclear antigen (PCNA) is also an important mechanism involved in DNA repair (Rassool and Tomkinson, 2010). PCNA can bind to POLD3, one of the dates subunits of DNA polymerase, causing DNA damage repair. To reverse drug resistance, another substance that can bind to POLD3 can be used to interfere with the role of PCNA. Scientists later found caPeptide, a peptide derived from caPCNA, which can potentially bind to PCNA-interacting proteins such as POLD3 (Lingeman et al., 2014), increase genomic DNA damage, and achieve the purpose of reversing the resistance of breast cancer to cisplatin-based chemotherapy drugs Figure 2.

Chemotherapy drugs with the mechanism of damaging cancer cell DNA, such as doxorubicin, mainly inhibit the effect of topoisomerase II (Mozaffari et al., 2021), but after the tumor cells strengthen the repair of DNA, the survival rate of these drugs is increased (Haggag et al., 2020). These peptides make the DNA damage of cancer cells irreparable by inhibiting the expression of Ran-GTP or PCNA. After the body detects DNA damage, the replication will be terminated, and the cells will then be cleared, successfully removing the resistance of cancer cells to doxorubicin and cisplatin.

5 Peptides reverse drug resistance: regulating the tumor microenvironment of breast cancer

Tumor microenvironment (TME) refers to the space around the tumor, which is composed of immune cells, matrix, and vascular system (Vasan et al., 2019). The tumor matrix is composed of non-malignant cell types such as cancer-associated fibroblasts (CAFs) and tumor mesenchymal stromal cells (MSCs) (Sahai et al., 2020). The tumor microenvironment often interferes with the immune clearance of tumor cells by producing some substances, promotes the growth of cancer cells, hinders the absorption of anticancer drugs, and promotes the progress of cancer. It is also the focus of cancer drug resistance research.

Cancer-associated fibroblasts (CAFs) can affect tumor cells through a variety of roles, such as leukocyte recruitment, immunosuppression, tumor microvascular formation, and extracellular matrix remodeling (Gascard and Tlsty, 2016). Due to the strong heterogeneity of CAFs, there has been a lack of therapy targeting CAFs. However, studies have found that many CAFs are related to adipose stromal cells (ASC) (Kolonin and DiGiovanni, 2019; Laurent et al., 2019). Therefore, a pro-apoptotic peptide D-CAN targeting ASC was found. D-CAN peptide indirectly inhibits the formation of CAFs by promoting ASC apoptosis and hindering the formation of CAFs by ASC and reverses the resistance of breast cancer to cisplatin and paclitaxel caused by ASC (Su et al., 2020).

In addition, a matrix protein periostin (PN) has been found, which is secreted by cancer-associated fibroblasts in the tumor microenvironment and can promote tumor growth, proliferation, metastasis, and angiogenesis through various mechanisms (Gonzalez-Gonzalez and Alonso, 2018; Liu et al., 2019). Moreover, PN is also related to the tolerance of a series of chemotherapeutic drugs such as doxorubicin, methotrexate, paclitaxel, cisplatin, and anti-angiogenesis therapy in breast cancer. The mechanism is mainly to enhance cell activity through the P13K/Akt signaling pathway. Because PN is mainly overexpressed in the tumor microenvironment of tumor cells such as breast cancer cells and is rarely expressed in normal cells. Therefore, inhibiting the production of PN is also a major strategy to reverse drug resistance. Researchers have screened a bioactive peptide anti-PN peptide from a phage library (Oo et al., 2021), which has a good affinity with PN by binding to integrin sites Figure 3. Anti-PN peptide binds to PN to inhibit the proliferation and metastasis of cancer cells induced by PN but has little effect on normal cells. Anti-PN peptide also reduces the activity of cancer cells by associating with AKT phosphorylation and surviving expression and reverses the resistance of breast cancer to chemotherapeutic drugs.

The tumor vascular system in the tumor microenvironment has a great influence on the growth of the tumor as a nutritional source of the tumor. Therefore, promoting the normalization of the tumor vascular system is also a method for treating cancer, especially cancer cells that are not sensitive to chemotherapy drugs (Dickson et al., 2007; Shen et al., 2012). For example, DEAE-glucan has an anti-angiogenesis effect by inducing β-interferon production (Bakrania et al., 2017). In addition, human neutrophil peptide-1 (HNP1) (Li et al., 2014), through the formation of a ternary complex with fibronectin and α5β1 integrin, further inhibits VEGF-induced endothelial cell proliferation, thereby making VEGF-mediated angiogenesis impossible, and ultimately promoting the normalization of tumor microvascular.

6 Peptides reverse drug resistance: inhibiting chemotherapeutic drug efflux proteins in breast cancer cells

The resistance of tumors to chemotherapeutic drugs, especially multidrug resistance, is closely related to the increased expression of efflux proteins in cancer cells. The common efflux proteins related to drug resistance are P-glycoprotein (pg) and multidrug resistance protein (MRP) (Sadava et al., 2002). Efflux proteins have a positive significance in normal cells, which can discharge toxic and harmful wastes from cells and ensure normal physiological functions of cells. However, the efflux protein in cancer cells can expel chemotherapeutic drugs from cells and reduce the content of drugs in cells (Seelig and Gatlik-Landwojtowicz, 2005), thereby reducing the biological efficacy and toxicity of anticancer drugs. Therefore, efflux proteins have also become a research direction for reversing drug resistance, including reducing the expression of efflux proteins in cancer cells or avoiding efflux proteins.

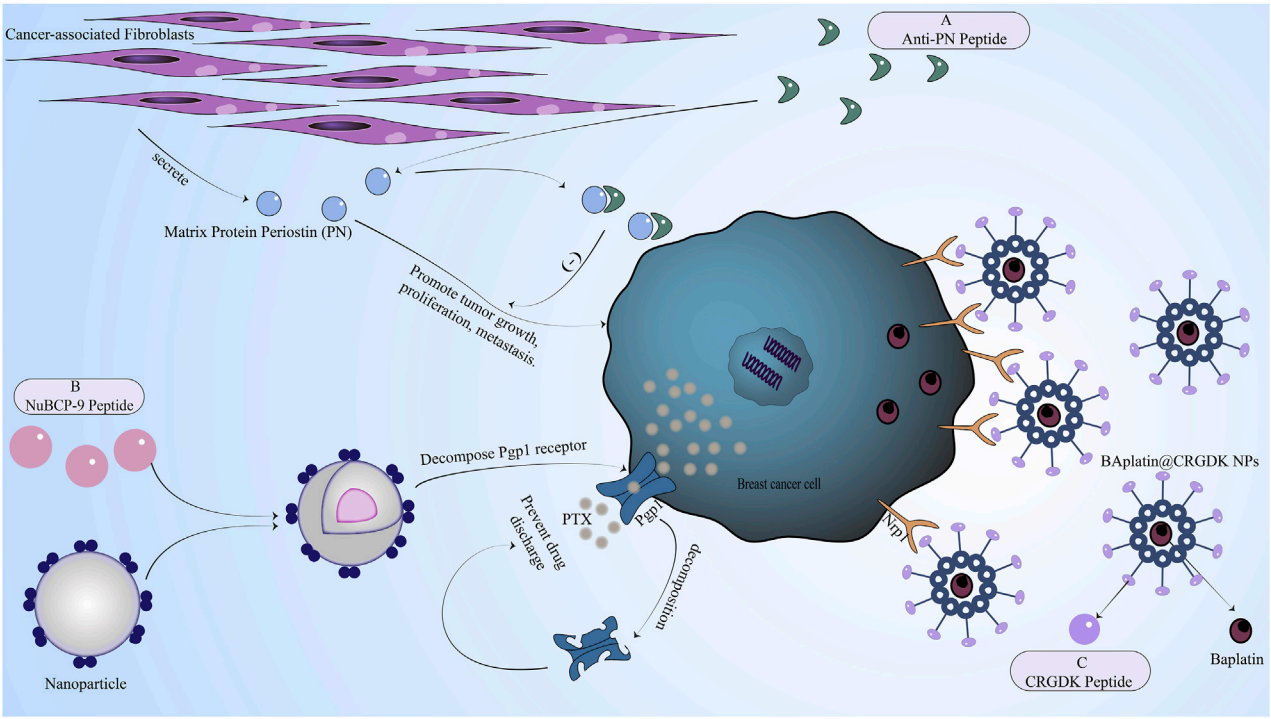

FIGURE 3
The mechanism of reversing drug resistance by regulating the tumor microenvironment of breast cancer, inhibiting chemotherapeutic drug efflux proteins in breast cancer cells, or enhancing breast cancer cell uptake of chemotherapy drugs. **(A)** Anti-PN peptide has a good affinity with PN secreted by cancer-associated fibroblasts in the tumor microenvironment. After binding to PN, the anti-PN peptide can inhibit the proliferation and metastasis of cancer cells induced by PN. **(B)** NuBCP-9 peptide increased drug concentration by inhibiting drug efflux mediated by pg efflux protein. **(C)** The CRGDK peptide binds to the overexpressed Nrp1 receptor of breast cancer cells through the CendR sequence to promote drug absorption.

In breast cancer based on efflux protein reversal resistance strategy, some use drug delivery systems (Yang et al., 2008), then peptides and chemotherapy drugs such as doxorubicin, paclitaxel, and others couple into a common system, the system is to bypass the peptide efflux protein role. The most commonly used peptide in drug delivery systems is cell-penetrating peptide. For example, the small molecule synthetic peptide TAT is a cell-penetrating peptide (Mozaffari et al., 2021), which can successfully avoid efflux proteins such as P-glycoprotein (pg) and can significantly increase the drug concentration in cancer cells. Of course, the use of peptides and the recognition of efflux proteins on the drug surface can also increase the accumulation of intracellular drugs (Lindgren et al., 2006). Another important reason why the drug delivery system can avoid being excreted by the efflux protein is that the molecular weight of the drug delivery system is too large to exceed the size of the efflux protein and naturally cannot be excreted by the efflux protein. In addition, the use of T10-ERK peptide can also interfere with drug efflux mediated by efflux proteins such as P-glycoprotein (pg) to increase drug concentration (Sheng et al., 2016). There are also methods for using Pgp1 inhibitors, such as the inclusion of NuBCP-9 peptide in PLA-PEG-PPG-PEG nanoparticles Figure 3, which can decompose Pgp1 receptors in the acidic environment of the tumor to ensure that the drug is not expelled from the cell (Gupta et al., 2018).

In addition to the drug efflux mechanism related to efflux proteins, there are some other mechanisms. The common one is the overexpression of ATP-binding cassette (ABC) transporters in cancer cells. Therefore, the use of peptides to inhibit the expression of ABC transporters can be considered. ABC transporters cannot convert ATP-binding and hydrolyzed energy into mechanical energy (Dong et al., 2005), and their mediated drug transmembrane transport outflow will also be inhibited. Whether it is inhibiting efflux proteins or inhibiting transporters, it is to increase the drug concentration in cancer cells, enhance drug toxicity, and reverse drug resistance.

7 Peptides reverse drug resistance: enhance breast cancer cell uptake of chemotherapy drugs

A drug delivery system is a good method to enhance drug absorption. It is mainly composed of polymer materials, inorganic materials, stabilizers, accelerators (promoting drug dissolution and absorption), and blockers (controlling drug release). Nowadays, more and more peptides are used in drug delivery systems. Some of them play a role in surface modification to mediate targeted delivery and enhance endocytosis, while some peptides are only used as polymer carriers to deliver drugs (Ahmed K. S. et al., 2021). These effects are intended to enhance the absorption of drugs for tumor cells and can fight drug resistance, because drug absorption disorder is also one of the reasons for cancer cells are not sensitive to chemotherapy drugs.

7.1 Peptides modify chemotherapeutic drugs to target delivery to cancer cells

Peptides have the characteristics of small molecular weight, easy chemical synthesis, and antibodies. Based on these advantages, peptides can be used as a cancer-selective targeting agent (Falciani et al., 2009). Peptides are conjugated to the carrier of the drug delivery system, some for targeted delivery and others to mediate enhanced drug endocytosis. The first is targeted delivery, which is based on the overexpression of tumor cells or specific substances. With these substances as receptors, peptides in the drug delivery system can specifically bind these receptors to achieve targeted drug delivery. In breast cancer cells, neuropilin-1 receptor (Nrp1) (Bai et al., 2019), membrane protein receptor-related protein (LRP) (Depau et al., 2017), GRPR (Wang et al., 2016), human epidermal growth factor receptor 2 (HER-2) (Shi et al., 2018), VEGFR1 (Aldughaim et al., 2020), etc. Are frequently overexpressed.

Many peptides have been found to specifically bind these overexpressed receptors in breast cancer cells. For example, CRGDK peptide (CysArg-Gly-Asp-Lys) can bind to Nrp1 through the CendR sequence (Bai et al., 2019) Figure 3, while cancer-selective tetrapeptide (NT4) can bind to membrane protein receptor-related protein (LRP) and heparan sulfate chain on its glycan (Depau et al., 2017). NT4 peptide can also inhibit the adsorption and migration of matrix proteins and affect cell movement after binding to these specific targets (Brunetti et al., 2016). Bombesin (Bn) isolated from amphibian tissues can specifically bind to GRPR receptors and has been used in experiments to deliver doxorubicin, successfully reversing drug resistance (Wang et al., 2016). HER-2 peptide targeting the HER-2 receptor is an analog of trastuzumab and a good surface modifier for a common drug delivery system targeting the HER-2 receptor (Shi et al., 2018). There is also a p700 peptide, which is an amino acid fragment at the C-terminus of TIMP3 and can specifically bind to receptors such as VEGFR1 and FGFR1-4 (Soudy et al., 2013). These peptides as the surface modification of the drug delivery system can be well-targeted delivery of doxorubicin, cisplatin, and other chemotherapy drugs, thereby more drugs into tumor cells.

There is also a method to enhance the release of chemotherapeutic drugs, because cathepsin B is overexpressed in cancer cells, and cathepsin B can catalyze the hydrolysis of GFLG peptide (Zhi et al., 2019). Therefore, the modification of GFLG on the drug delivery system can achieve the targeted release of chemotherapeutic drugs.

Enhancing endocytosis is also a means of increasing drug concentration in cancer cells. Among them, the transferrin receptor (TfR) -mediated endocytosis pathway has attracted much attention (Sheng et al., 2016). Tf is conjugated to the drug delivery system, and the combination of Tf and TfR can increase the number of chemotherapeutic drugs entering tumor cells (Sheng et al., 2015). The method of enhancing endocytosis also uses cell-penetrating peptides, which can carry macromolecules through the cell membrane. For example, the new cell-penetrating peptide YTA2 successfully solved the problem of MTX lack of transporters during transport (Rousselle et al., 2000), and MTX was delivered to tumor cells (Lindgren et al., 2006). And adjusting the hydrophilic side and hydrophobic side of the peptide affixed to the surface of the drug delivery system to facilitate the endocytosis of the chemotherapy drug into the cancer cell (Bai et al., 2019).

7.2 Peptides are only used as carriers to deliver chemotherapeutic drugs

Polymer materials, an important part of drug delivery systems, can be divided into natural polymer materials, semi-synthetic polymer materials, and synthetic polymer materials. Nanocarriers are very common in drug delivery systems. Commonly used nanocarriers include liposomes, dendritic polymers, and metal nanoparticles (Markman et al., 2013). Nanocarriers can encapsulate the drug well and release the drug slowly, ensuring the local concentration of the drug and prolonging the residence time of the drug in the cell.

Peptides can be used as a natural polymer material carrier to encapsulate drugs. Although peptides are limited by proteolytic enzymes in drug delivery systems, many studies have ensured peptide stability by substituting for unstable sites in enzymatic reactions, such as a protein-stabilized decapeptide WxEYAAQrFL. In addition, a cyclic peptide nanotube made of cyclic octapeptide to load doxorubicin not only has high encapsulation efficiency but also has good dispersion (Wang H. et al., 2014), which can increase the uptake of doxorubicin by cancer cells. There is also the use of binding peptide dendrimers as carriers to deliver drugs because their good biocompatibility can enhance the permeability of drugs (Zhu et al., 2021). The molecular weight of AmPD KK2 peptide in the synthesized peptide dendrites is smaller than that of AmPD KK2K4, and the balance between hydrophobicity and hydrophilicity of AmPD KK2 peptide is better maintained, so it can be better taken up by cells. In summary, peptides have great potential as a natural polymer material that can encapsulate chemotherapy drugs.

8 Peptides as a drug moiety: current challenges and possible solutions

As an anticancer peptide with the effect of reversing the resistance of chemotherapeutic drugs and treating malignant tumors such as breast cancer, it has the characteristics of easy synthesis and modification, various modes of administration, and difficulty in producing multidrug resistance (Chen G. et al., 2022). Moreover, the peptide itself has strong targeting, small adverse reactions, and easy separation and transformation (Qin et al., 2022). Although many peptides have been found to have good anti-tumor activity, the development of peptide drugs faces many challenges. For example, the yield of natural peptides is low, and the later artificial synthesis is expensive (Akbarian and Chen, 2022); at the same time, the peptide will produce immunogenicity in the host, and the presence of peptidase in the serum makes the half-life of the peptide shorter (Duran-Lobato et al., 2021). Secondly, *in vivo*, digestive enzymes will decompose peptides (Muttenthaler et al., 2021). In addition, the anti-tumor mechanism of peptide compounds is not fully understood, and the related clinical and pharmacological studies are still less, which still needs further study.

TABLE 2 Natural peptides reversing chemotherapy resistance and its mechanism.

Peptide name	Source	Active derivative	Reversed drugs	Reversing resistance mechanisms	References
Hemiasterlin and Hemiasterlin C	Sponge (*Hemiasterella minor*, *Auletta* sp., *Cymbastela* sp., and *Siphonochalina* sp.)	Linear tripeptide		Antiproliferative effect; Microtubules Depolymerization; G2/M phase arrest	Gamble et al. (1999)
HTI-286	Sponge (*Hemiasterella minor*, *Auletta* sp., *Cymbastela* sp., and *Siphonochalina* sp.)		Paclitaxel, vincristine, vinblastine, colchicine, and doxorubicin Docetaxel and vinorelbine	Antiproliferative effect	Loganzo et al. (2003)
Milnamide A-D	*Auletta* sp.			Microtubules depolymerization	Sonnenschein et al. (2004)
Symplostatin 1	cyanobacteria of the genus Symploca	Linear Pentapeptide	Taxol and vinblastine	Bcl2 ↓; Microtubules depolymerization	Mooberry et al. (2003)
Cryptophycin	*Nostoc* sp.	Cyclic depsipeptide	paclitaxel and colcemid	Microtubules Depolymerization; P-gp↓	Smith et al. (1994)
Geodiamolide D-E	Sponge (*Auletta* sp. And *Geodia corticostylifera*)			Actin filament disruption	Sonnenschein et al. (2004)
Kulokekahilide-2	Mollusk (Philinopsis speciosa)			↓ cell viability	Nakao et al. (2004)
Stylopeptide 2	Sponge (*Stylotella* sp.)	Cyclic decapeptide		Antiproliferative effect	Brennan et al. (2008)
Ohmyungsamycin A-B	Bacteria (*Streptomyces strain SNJ042*)	Cyclic depsipeptides		↓ cell viability	Um et al. (2013)
Largazole	Cyanobacteria (*Symploca* sp.)	Cyclic depsipeptide		↓ cancer cell growth	Taori et al. (2008)
C-phycocyanin	Cyanobacteria (*Limnothrix* sp. *NS01* and *Spirulina platensis*)	Peptide		DNA fragmentation; Caspase-9 ↑; cyt c ↑; Bcl2↓; Bax↑; PARP ↑; Stat3 ↓ G1 - G2 phase arrest (cyclin D1↓, cyclin E↓; p21↑) Antiangiogenic (VEGFR2 ↓ and MMP- 9 ↓) AKT inhibition; γ-H2AX ↑; Production of ROS and singlet oxygen radicals	Jiang et al. (2018)
Pipecolidepsin A-B	Sponge (*Homophymia lamellosa*)			↓ cell viability	Coello et al. (2014)
Pembamide	Sponge (*Cribrochalina* sp.)	N-methylated linear peptide		↓ cell viability	Coello et al. (2014)
Callyptide A	Sponge (*Callyspongia* sp.)	Cyclic peptide		↓ cell viability	Shaala et al. (2016)
DZ-2384	Ascidia (*Diazona angulate*)	Macrocyclic peptide	Taxane and vinca alkaloid	Microtubule depolymerization	Wieczorek et al. (2016)
Hymenochirin-1B	*Hymenochirus boettgeri* (Pipidae)			↓ cell viability	Attoub et al. (2013)
Alyteserin-2a	the midwife toad *Alytes obstetricans*			Antiproliferative Effect; TGF-β↓	Conlon et al. (2013)
Micro cionamides A-B	the Philippine marine sponge *Clathria (Thalysias) abietina*			↓ cell viability	Davis et al. (2004)
Kahalalide F	Mollusk (*Elysia rufescens*)	Cyclic depsipeptide		PI3K-AKT inhibition; ErbB3 depletion	Suarez et al. (2003)
Elisidepsin	Mollusk (*Elysia rufescens*)		oxaliplatin, cisplatin, 5-FU, gemcitabine and lapatinib	↑ Ca2+ influx; perturbations of membrane conductivity; the formation of giant membranous vesicles; ErbB3 depletion; Bcl2 ↓	Serova et al. (2013)

At present, the targeting, stability, low toxicity, solubility, and half-life of peptides can be improved by using different types of modification, such as peptide engineering, new preparations, and peptide coupling (Mitra et al., 2020). It has been found that many peptide drugs have entered clinical trials or have been approved for marketing (Anand et al., 2023). These peptides can be used as

peptide hormones, radionuclide carriers, peptide vaccines, cytotoxic drug carriers, and anticancer drugs for cancer treatment in different ways (Gu et al., 2021). For example, gonadotropin-releasing hormone receptor (LHRH) agonists such as leuprolide acetate for the treatment of prostate cancer inhibit the proliferation of prostate cancer cells by down-regulating the expression of LHRH in the pituitary, inhibiting the release of follicle-stimulating hormone (FSH) and reducing the production of testosterone (Luo et al., 2023). Octreotide is a radionuclide carrier. After radioactive labeling with indium 111, it can be attached to tumor cells with somatostatin receptors, and the location of tumor cells can be determined by detecting radioactive octreotide (Hijazi, 2021). The drug carrier AEZS-108 exerts its anticancer effect by directly targeting cancer cells expressing luteinizing hormone-releasing hormone receptors by coupling the peptide with the chemotherapeutic drug doxorubicin (Yang et al., 2023). Although peptide drugs have good clinical value in cancer treatment, it is still necessary to overcome the limitations of peptides. It is of great significance to further discover new anticancer peptides or overcome their shortcomings by a modification to reduce cancer mortality.

9 Conclusion and future prospect

With the increasing incidence of breast cancer in recent years (Liu et al., 2022), the problem of breast cancer resistance to chemotherapeutic drugs has also received more attention. Drug resistance has become a huge obstacle to the effective treatment of breast cancer with chemotherapeutic drugs (Wang H. et al., 2014). First-line anticancer drugs with good efficacy, such as doxorubicin, paclitaxel, and are difficult to control tumor growth and metastasis, which shortens the survival of breast cancer patients. There are many reasons for drug resistance, including defects in cancer cell apoptosis, enhanced DNA repair function of tumor cells, reduced drug uptake, enhanced efflux mechanisms, and changes in targets (Bai et al., 2019). To reverse drug resistance, it is necessary to start with the mechanism of these drug resistances. Many studies are exploring ways to solve the problem of drug resistance, and the use of peptides to overcome drug resistance is a feasible solution. Here, we summarize the strategies of different peptides for reversing breast cancer drug resistance and elaborate on the mechanism of these peptides.

We divided the mechanism of action of the peptides currently studied on reversing breast cancer resistance into six categories, including promoting cancer cell apoptosis, promoting non-apoptotic regulatory cell death of cancer cells, inhibiting DNA repair mechanisms of cancer cells, improving tumor microenvironment, inhibiting drug efflux mechanisms and enhancing drug uptake. Among them, promoting apoptosis is divided into several mechanisms, including regulating apoptotic genes (up-regulating pro-apoptotic genes and down-regulating anti-apoptotic genes), using lysosomal peptides, inducing mitochondrial apoptotic pathways, regulating nuclear transcription factors, and arresting cell cycle. Promoting non-apoptotic regulatory cell death includes promoting RIPK1-dependent necrosis and inducing lysosomal-dependent cell death. Finally, the enhancement of drug uptake can be achieved by peptide modified drug delivery system to achieve targeted drug delivery, enhance endocytosis and enhance drug release, or the peptide is only used as a carrier to improve the encapsulation efficiency and dispersion of the drug and increase the drug uptake.

Most of the peptides mentioned in this article are chemically synthesized, and only a few are derived from animals and plants. However, studies have shown that some natural peptides, especially marine peptides, have anti-drug-resistant tumors and can significantly reduce the activity of drug-resistant tumor cells (Ahmed S. et al., 2021) Table 2. However, whether these natural peptides can specifically target breast cancer and reverse its chemotherapy resistance needs further study. Because the peptide molecular weight is small and easy to synthesize (Falciani et al., 2009), it has great advantages in the study of reversing breast cancer resistance. Many peptides that effectively reverse breast cancer resistance have been explored, such as The 8-mer peptide derived from β-fetoprotein inhibits the growth of tamoxifen-resistant estrogen receptor-positive breast cancer cells (Bennett et al., 2002), and Membrane-Lytic Peptide (Chen C. H. et al., 2022), Acid-Sensitive Peptide (Sheng et al., 2015), RanGTP inhibitory peptide (RAN-IP) (Haggag et al., 2020). We hope to use these peptides to overcome the resistance of breast cancer to chemotherapeutic drugs, enhance the therapeutic effect of anticancer drugs, improve the survival rate of breast cancer patients, especially metastatic breast cancer patients, and prolong the life of patients. Although these peptides have shown the effectiveness of overcoming breast cancer resistance in experiments, we need to pay attention to the fact that many experiments are still *in vitro* experiments, and almost no *in vivo* experiments are carried out. Therefore, there may be a long way to go to truly apply them in clinical practice.

Author contributions

YH wrote the manuscript and created the figures. LS supervised the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

HER2	Human epidermal growth factor receptor 2
ER	Estrogen receptor
PR	Progesterone receptor
TNBC	Triple-negative breast cancer
DOX	Doxorubicin
MTX	Methotrexate
CDDP	Cisplatin
PTX	Paclitaxel
CPP	Cell penetrating peptide
BCP	Bioactive cationic peptide
ABT-263	Navitoclax
AVPI	Smac N-terminal tetrapeptide
HSP	Heat shock protein
ACP	lysosomal anticancer peptide
HNP1	Human neutrophil peptide-1
MMP	Mitochondrial membrane potential
CytoC	Promotes cytochrome c
EN1	Homologous nuclear transcription factor Engrailed 1
EN1-iPep	EN1 interference peptide
SNCG	Synuclein-γ
DDR	DNA damage response
Ran-GTP	Ras-related nuclear protein
RAN-IP	Ran-RCC1 inhibitory peptide
PCNA	Proliferating cell nuclear antigen
TME	Tumor microenvironment
CAFs	Cancer-associated fibroblasts
MSCs	Tumor mesenchymal stromal cells
ASC	Adipose stromal cells
PN	Periostin
Pg	P-glycoprotein
MRP	Multidrug resistance protein
ABC	ATP-binding cassette
Nrp1	Neuropilin-1 receptor
LRP	Membrane protein receptor-related protein
NT4	Cancer selective tetrapeptide
GRPR	Gastrin Releasing Peptide Receptor
VEGFR1	Vascular endothelial growth factor
Bn	Bombesin
TfR	Transferrin receptor

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Ceruloplasmin is associated with the infiltration of immune cells and acts as a prognostic biomarker in patients suffering from glioma

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Glioma is regarded as a prevalent form of cancer that affects the Central Nervous System (CNS), with an aggressive growth pattern and a low clinical cure rate. Despite the advancement of the treatment strategy of surgical resection, chemoradiotherapy and immunotherapy in the last decade, the clinical outcome is still grim, which is ascribed to the low immunogenicity and tumor microenvironment (TME) of glioma. The multifunctional molecule, called ceruloplasmin (CP) is involved in iron metabolism. Its expression pattern, prognostic significance, and association with the immune cells in gliomas have not been thoroughly investigated. Studies using a variety of databases, including Chinese Glioma Genome Atlas (CGGA), The Cancer Genome Atlas (TCGA), and Gliovis, showed that the mRNA and protein expression levels of CP in patients suffering from glioma increased significantly with an increasing glioma grade. Kaplan-Meier (KM) curves and statistical tests highlighted a significant reduction in survival time of patients with elevated CP expression levels. According to Cox regression analysis, CP can be utilized as a stand-alone predictive biomarker in patients suffering from glioma. A significant association between CP expression and numerous immune-related pathways was found after analyzing the data using the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA). Tumor Immune Estimation Resource (TIMER) and CIBERSORT analyses indicated a substantial correlation between the CP expression and infiltration of immunocytes in the TME. Additionally, immune checkpoints and CP expression in gliomas showed a favorable correlation. According to these results, patients with glioma have better prognoses and levels of tumor immune cell infiltration when their CP expression is low. As a result, CP could be used as a probable therapeutic target for gliomas and potentially anticipate the effectiveness of immunotherapy.

KEYWORDS

gliomas, ceruloplasmin (CP), The Cancer Genome Atlas (TCGA), Chinese Glioma Genome Atlas (CGGA), tumor immune microenvironment (TIME)

1 Introduction

Glioma is the most commonly occurring and malignant adult brain tumor (Xu et al., 2023), and divided into ependymoma, astrocytoma [including glioblastomas (GBM)], oligodendroglioma, mixed glioma, and a few other tumors that vary widely in histology (from benign ependymoma to the most aggressive and lethal grade-IV GBM) (Antin et al., 2020; Walsh et al., 2023). Despite multiple conventional therapy, including surgical resection, chemoradiotherapy and immunotherapy, glioma still possess a high recurrence and fatality rate (Dong et al., 2023). The impact of immunotherapy on glioma has become one of the hottest areas of research owing to its effectiveness in treating various solid tumors and hematological cancers in recent years (Chang et al., 2023; Hovhannisyan et al., 2023). The advancement of genomics has led to the identification of some molecular markers associated with specific gliomas phenotypes such as isocitrate dehydrogenase (IDH) (Nuechterlein et al., 2021), phosphoinositide 3-kinase (PI3K) (Yan et al., 2020), telomerase reverse transcriptase (TERT) (Park et al., 2022), and phosphatase and tensin homolog (PTEN) (Cheng et al., 2021). However, there is a shortage of reliable biomarkers that can effectively predict the prognosis of glioma patients. Therefore, the search for new immune-related molecular biomarkers affecting glioma is of great importance for its treatment.

Iron, an essential component of hemoglobin, plays a key role in the cell proliferation and differentiation pathways and participates in numerous important physiological processes such as electron transfer, cellular respiration, energy metabolism, and detoxification by catalyzing redox reactions (Meheust et al., 2021; Dugan et al., 2022). Dysregulated iron metabolism can cause accumulation of intracellular ferrous iron (Fe^{2+}), and the overloaded free Fe^{2+} in the cell can generate a large amount of ROS such as hydroxyl radicals through Fenton reaction (Huang et al., 2023a). Excessive ROS can cause oxidative stress, adversely affect the stability of the genome, possibly induce malignant transformation, and also trigger apoptosis signal cascade reaction and ferroptosis. In addition, some studies have proved that the innate immune cells resident in the tumor microenvironment, such as macrophages and neutrophils, are the source of iron and iron related proteins, and can activate the signal pathway controlling iron metabolism and promoting the imbalance of iron metabolism in tumor cells (Weston et al., 2016; Su et al., 2023). Therefore, changes in iron metabolism are one of the important characteristics of tumors and are related to their occurrence and development (Wang et al., 2023). Jaksch-Bogensperger et al. observed higher serum ferritin levels in patients with high-grade glioma (Jaksch-Bogensperger et al., 2020). By increasing the expression of their transferrin Receptor 1 and ferritin genes, GBM stem-like cells are better able to absorb iron from the microenvironment (Pandya Shesh et al., 2023). Additionally, in high-grade gliomas, hypoxia-induced expression of the ferritin light chains is also related to the epithelial-mesenchymal transition (EMT) and chemoresistance (Liu et al., 2020). Current research and drug discovery in iron metabolism have provided effective therapeutic target and a potential prognostic biomarker for glioma (Tan et al., 2023; Zhang et al., 2023).

Ceruloplasmin (CP), an abundant serum α2-glycoprotein and a key iron oxidase in the human body can catalyze divalent iron to trivalent iron, thus promoting iron binding to transferrin (Pal et al., 2022). Since only trivalent iron can bind to transferrin, and CP is essential in the formation of trivalent iron, CP plays a crucial role in iron transport and iron homeostasis in the body (Thepsuwan et al., 2023). Studies have shown that CP in the brain promotes both iron release and iron uptake in brain cells (Squitti et al., 2023). Besides, CP plays a more prominent role in iron uptake than release (Mukhopadhyay et al., 2000; Mietto et al., 2021). Some recent studies stated that the abnormal CP expression occurring in the brain could be attributed to a few neurological diseases (Klomp and Gitlin, 1996; Qian and Ke, 2019; Villadsen et al., 2023). At present, the existing literature studies have reported that in the iron-deficient and iron-saturated cells, CP, regardless of concentration, promotes the uptake of transferrin-bound iron and ferric citrate by glioma cells (Attieh et al., 1999; Roy et al., 2022). Moreover, high expression of CP in tumor cells, such as lung cancer (Chang et al., 2022), liver cancer (Shang et al., 2020), melanoma (Liu et al., 2022a), and breast cancer (Chen et al., 2021), can inhibit ferroptosis, thus reduces tumor cell death and promotes tumor progression. High expression CP in tumor cells transfers electrons to oxygen and oxidizes Fe^{2+} to Fe^{3+} via regulating copper, thus reducing intracellular Fe^{2+} and inhibiting ferroptosis (Li et al., 2020). CP, which is a potential oncogenic factor, is aberrantly expressed in various malignancies like lung cancer (Tsai et al., 2020), ovarian cancer (Dai et al., 2020), and hepatic cancer (Shang et al., 2020), and can be involved in tumor growth and metastasis by regulating ferroptosis, angiogenesis, and tumor microenvironment (TME).

CP expression and carcinogenesis are known to be closely related, although there is currently no information on how CP contributes to the pathophysiology, clinical relevance of glioma or its prognosis. This research used a variety of bioinformatics software and tools to analyze the relationship between CP expression with the clinical feature, patient survival and infiltration of immune cells. Besides, the role of CP in glioma behavior was also presented. CP expression was upregulated significantly in glioma tissues in comparison to non-tumor tissues and increased with glioma grades. The prognosis of the patients suffering from glioma was inversely linked to the high CP expression. Additionally, there was a direct correlation between CP expression and antitumor immune responses, such as immunosuppressive targets and the infiltration of immune cells (including neutrophils, macrophages, dendritic cells, $CD4^+$ T cells, $CD8^+$ T cells and B cells). These findings emphasize the critical function of CP in carcinogenesis and suggest that CP may be essential in the immune landscapes of gliomas TME.

2 Materials and methods

2.1 Raw data acquisition

Two separate databases, the Chinese Glioma Genome Atlas (CGGA) (dataset 1, n = 325) and the CGGA (dataset 2, n = 693),

provided the RNA sequencing information for patients with diffuse glioma (Zhao et al., 2021). The Cancer Genome Atlas (TCGA) was used to retrieve the glioma patient dataset (n = 698), which included the clinical data and gene expression profiles (Blum et al., 2018). Then, from TCGA, CP expression data (normalized into Fragments Per Kilobase per Million [FPKM]) and all clinical information were also retrieved. The clinicopathological data that was acquired, including grade, age, sex, survival status, and overall survival (OS), were combined. Finally, default value samples were removed according to the statistical needs of the follow-up study.

2.2 Relationship between CP expression and glioma grade

Expression data of CP in 31 cancers were obtained from TCGA, analyzed by the HiPlot open-source web platform UCSCXenaShiny module (https://hiplot.com.cn/advance/ucscxena), and grouped into paraneoplastic tissues and tumor tissues. Expression and clinical characteristics of CP genes in TCGA, CGGA, Gravendeel, and Rembrandt glioma databases were obtained through the Gliovis web platform (http://gliovis. Bioinfo.cnio.es/). In addition, the association between the glioma grade, CP expression, and patient prognosis were analyzed. Finally, the CP expression in the normal, low-grade, and high-grade glioma tissues were studied based on the HPA website.

2.3 Correlation between the CP expression and OS of the patients with glioma

FIn order to investigate the correlation between the CP expression and OS of patients with glioma, samples obtained from different glioma-related databases were categorized into the high-CP and low-CP expression categories, according to the median of CP expression. The Kaplan-Meier (KM) survival curves were plotted based on the survival data of the Gliovis patients, based on the information derived from the TCGA, CGGA, Gravendeel, and Rembrandt databases, followed by log-rank and Wilcoxon tests. Events in the high-CP and low-CP expression categories in every dataset were pooled. Subsequently, the relative survival risk ratio (RR) of every group in these databases were calculated by using the binary data meta-analysis tool on the HiPlot web platform. Finally, forest plots of risk factors affecting patient survival were drawn for every database.

2.4 Association between the CP expression and clinical feature

First, the association between the CP expression in glioma and clinical features such as sex, age, glioma subtype, IDH mutation and 1P19q deletion status, was investigated. The CP expression heat map was mapped by the R package "ComplexHeatmap". Afterward, the variations in the clinical feature between the high-CP and low-CP expression groups were investigated, based on the CGGA and TCGA datasets, and subjected to COX regression-based independent prognostic analysis to observe the correlation between the CP expression and the prognosis of glioma patients. Finally, a novel nomogram model incorporating clinical information and the CP expression data was developed, using the R language. Overall, the nomogram model performed better than the conventional staging system in predicting prognosis.

2.5 Gene-gene and protein-protein interaction networks of CP

The GeneMANIA cohort (http://www.genemania.org) was utilized to construct the CP interaction network. In addition, a Protein-Protein Interaction (PPI) network for CP was designed with the help of the STRING online database (https://string-db.org/). The correlation between the CP and iron metabolism-linked genes in glioma was studied using the TCGA, low-grade glioma (LGG), and GBM databases, based on the Timer (https://cistrome.shinyapps.io/timer/) network platform.

2.6 Functional analysis of the differentially expressed genes in the High-CP and Low-CP expression groups in the CGGA and TCGA datasets

Relevant genes co-expressed with CP in the CGGA database were identified by co-expression analysis. Representative genes significantly associated with CP were visualized in circles using the R package "corplot" and the package "Circlize". The samples from CGGA and TCGA were classified into 2 groups based on the CP expression. The LIMMA program package, using the R language, was employed for screening the Differentially Expressed Genes (DEGs) in the two groups, and the heat map was drawn with the pheatmap program package. KEGG and GO enrichment analyses of the DEGs in CGGA and TCGA databases were performed using R language to elucidate the biological functions and pathways involving CP. Subsequently, GSEA enrichment analysis of CP was performed using the c5.go.v7.4.symbols and c2.cp.kegg.v7.4symbents datasets. The groups with the Nominal (NOM) p-values and the False Discovery Rate (FDR) q-values $\leq$0.05 were defined as significantly enriched groups.

2.7 Correlation between the CP and antitumor immunity

Depending on the median level of CP expression, the samples were sorted into the high-CP and low-CP expression groups. R language tools were used to examine the Tumor Mutation Burden (TMB) and the TME scores of both groups. In addition, the Timer web platform (https://cistrome.shinyapps.io/timer/) was used to thoroughly assess the landscape of immune cell infiltration. Using the TCGA database, the "Gene" module of the web platform was utilized to examine the correlation between the CP

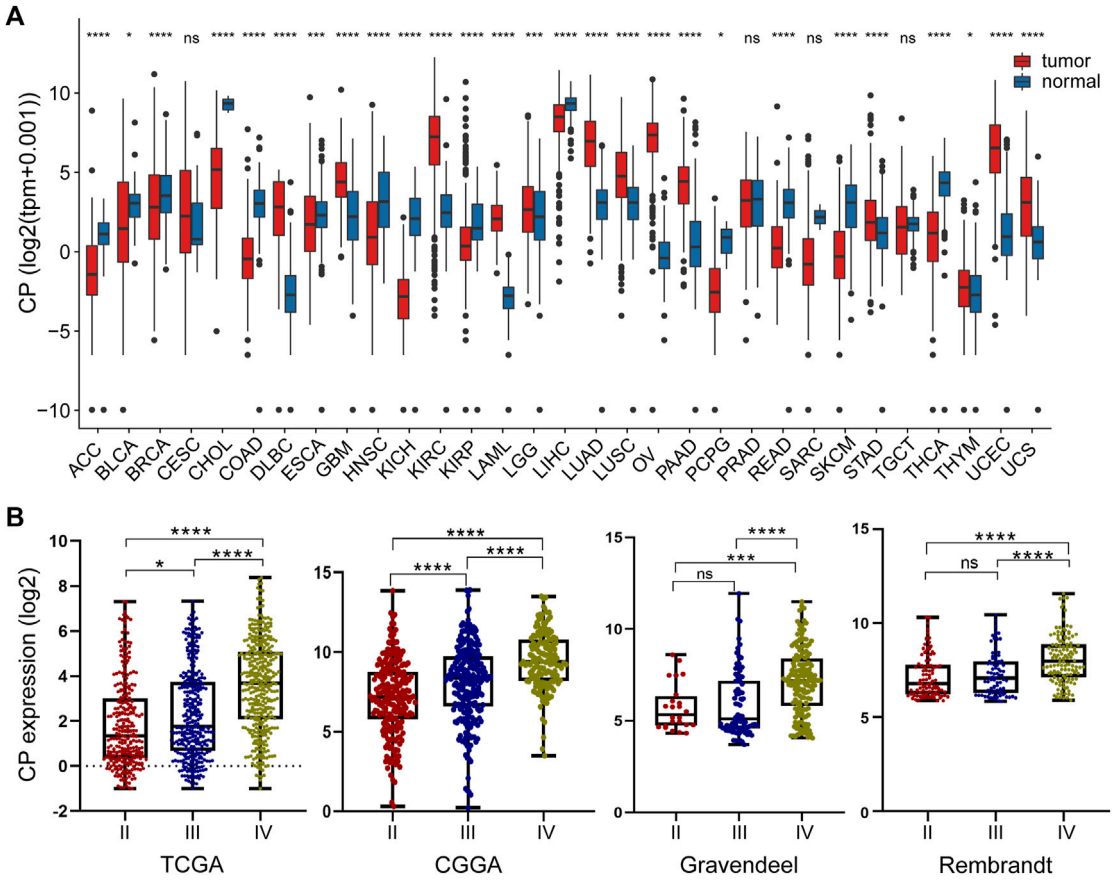

FIGURE 1
Analysis of the relationship between CP expression and glioma grade. **(A)** Pan-cancer analysis of CP expression in the TCGA and GTEx databases. **(B)** Relationship between the expression of CP and grade of glioma. (Note: ***$p < 0.001$ and *$p < 0.05$.).

expression and the immune cell infiltration (including macrophages, dendritic cells, neutrophils, $CD4^+$ T cells, $CD8^+$ T cells and B cells). Immune checkpoint medications that target PD-1, PD-L1, and CTLA-4 are currently showing proving to be effective while treating cancer patients. The GEPIA web platform was used to ascertain the association between CP and PD-L1, PD-1, and CTLA-4 in the GBM and LGG of TCGA. In addition, the GBM dataset derived from the TCIA web portal (https://tcia.at/home) was used to examine the effectiveness of anti-PD-1 and CTLA-4 therapy in the patients categorized into the two groups.

2.8 Relative abundance of the infiltrating immune cells in the TME

The CiberSort web platform, which uses a deconvolution algorithm based on the gene expression that was based on the gene annotation matrix of 22 types of immune cell, was used to examine the relationship between the CP expression and the Tumor-Infiltrating Immune Cells (TIICs) in glioma. *p*-values were computed for each sample in the CGGA dataset. Additionally, the R software was used to display the association between the immune cells in every dataset. The composition of the infiltrating immune cells of each sample can be identified using CiberSort. As a result, the relative quantities of various immune cells in every group can be compared effectively, and the outcomes were plotted as a box plot. The association between immune cells and CP expression was then used to construct a lollipop plot showing the correlation between the immune cells.

2.9 Statistical analysis

Data were presented as the mean ± standard deviation (SD). The Student's *t*-test was used to evaluate whether there were any significant variations between the two groups. To compare more than two groups, a one-way Analysis of Variance (ANOVA) was used. Correlation analysis was conducted using Spearman's technique. In accordance with the 50% cut-off for gene expression, patients were sorted into high-CP and low-CP expression subtypes. The KM survival analysis and the log-rank significance tests were employed to compare the OS rates between the 3 groups. Additionally, COX regression models were utilized to examine the prognosis-influencing components. A software called GraphPad Prism 9.0 was used to create the graphs (GraphPad Inc., San Diego, United States).

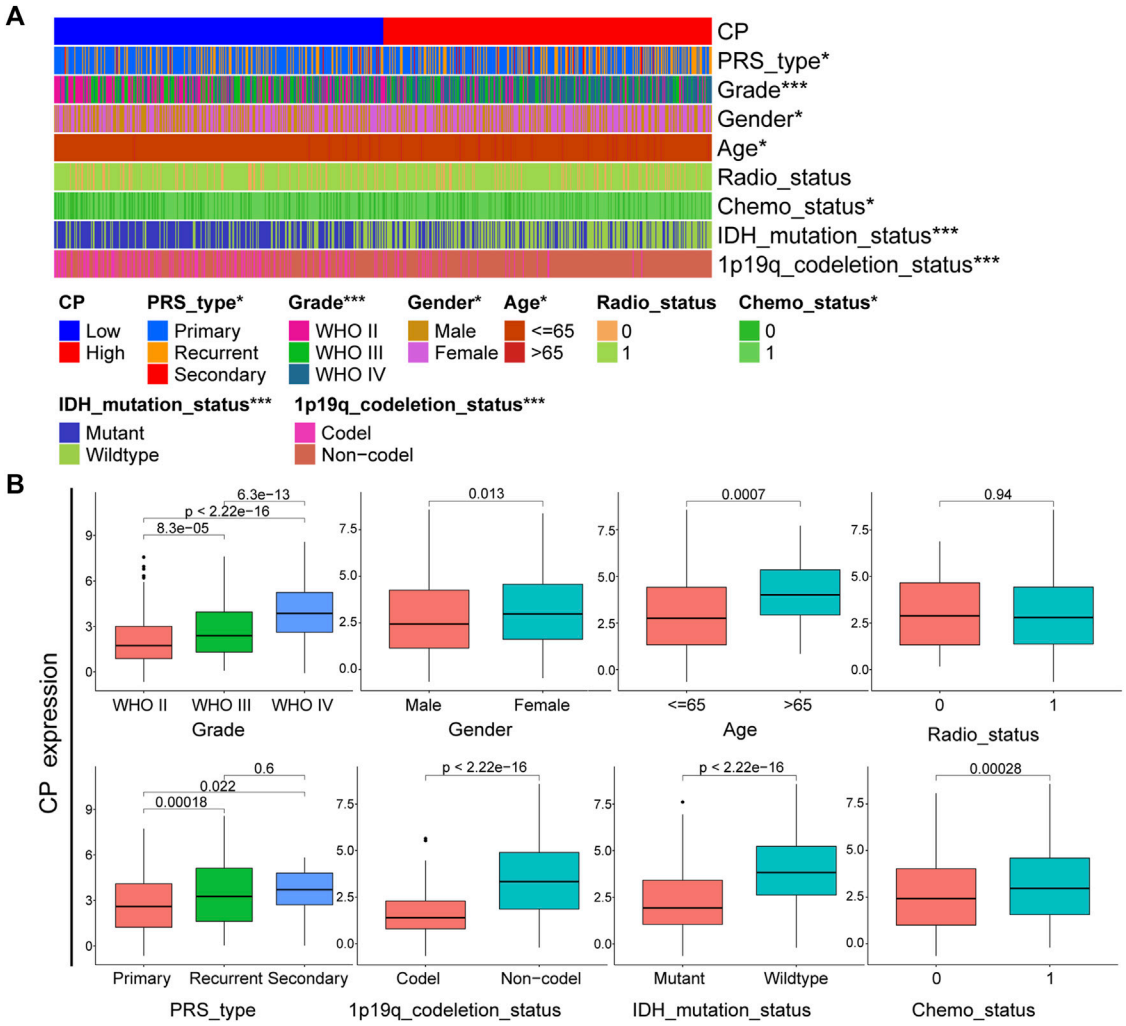

FIGURE 2
Analysis of the relationship between CP and clinical features in CGGA glioma dataset. **(A)** The relationship between CP expression and clinical features of CGGA dataset. **(B)** Each clinical feature was analyzed for differences in the CP high/low expression groups. (Note: ***$p < 0.001$ and *$p < 0.05$.).

3 Results

3.1 Elevated CP expression level with the increase of glioma grade

Pan-cancer analysis suggested significant differences in CP expression between a variety of tumor tissues and paraneoplastic tissues. CP expression was higher in GBM, LGG, ovarian cancer (OV), kidney renal clear cell carcinoma (KIRC), and other tumor tissue samples compared to paraneoplastic tissues (Figure 1A). The link between the CP expression and World Health Organization (WHO) grade in glioma was assessed using the TCGA, CGGA, Gravendeel, and Rembrandt glioma databases. The results revealed a significant increase in CP expression with relatively advanced glioma grade (Figure 1B).

3.2 Relationship between CP expression and clinical feature of patients suffering from glioma

According to the CGGA dataset, CP exhibits significant differences in PRS type, tumor grade, gender, age, chemotherapy status, IDH mutations and 1p19q deletion status ($p < 0.001$) (Figures 2A, B). Consistently, the CP expression was seen to be significantly differences ($p < 0.001$) in the glioma grade, gender and age, validated in the TCGA cohort (Supplementary Figures S1A, B). There is a lower expression of CP in low-grade, IDH-mutant, and chemotherapeutic sensitivity glioma. Elderly (aged over 65 years) and male patients was significantly higher. Besides, no matter male or female, those with high CP expression are more likely to be recurrent and secondary. CP might represent a new biomarker of prognosis and classification-based treatment in patients with gliomas.

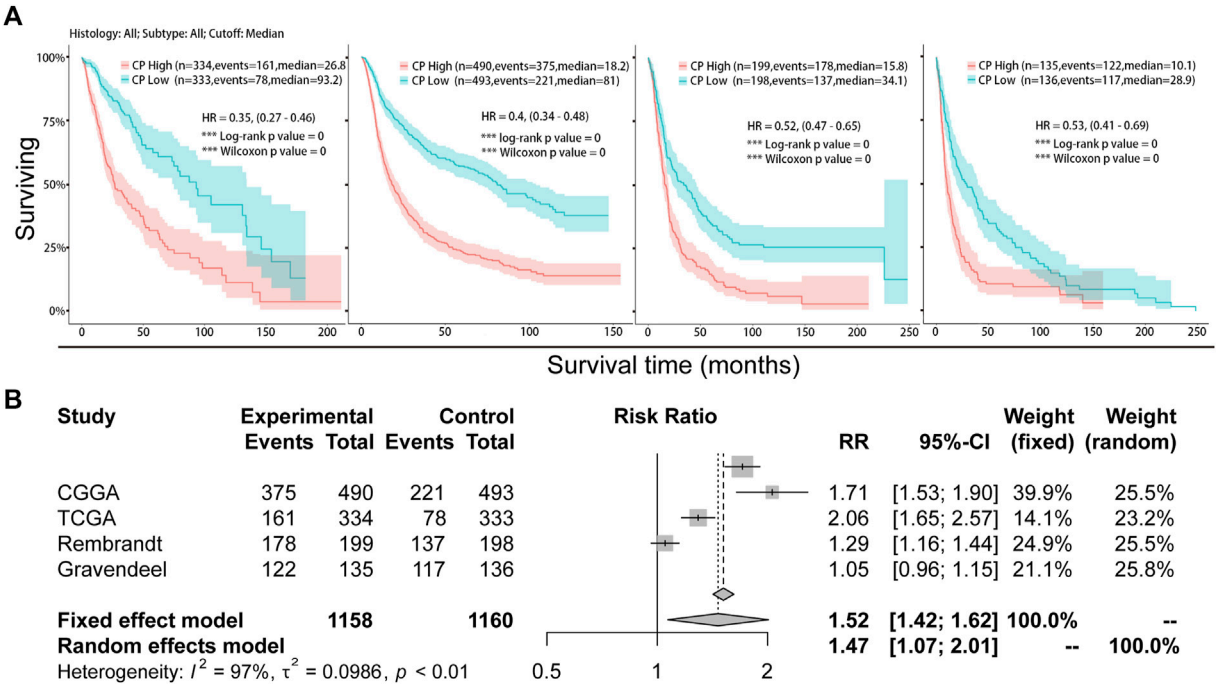

FIGURE 3
Analysis of the relationship between CP expression and survival in glioma. **(A)** Kaplan-Meier plots of CP in a variety of glioma datasets. The 95% confidence interval (CI) is shown. The patients were divided into high and low expression groups by the median expression level. **(B)** Forest plot of the risk ratio for patients with high CP expression levels compared to patients with low CP expression levels.

3.3 Relationship between CP expression and glioma patient survival

Samples from CGGA, TCGA, Rembrandt, and Gravendeel were sorted into the Low-CP and High-CP expression groups, depending on the median CP expression levels. KM curves and statistical tests revealed that the OS rates were reduced significantly in the High-CP expression category compared to the Low-CP expression category in all 4 datasets ($p < 0.001$) (Figure 3A). Considering the significant difference in sample size among the four datasets, RRs (Risk Rates) were calculated for Meta-analysis of the four datasets using a fixed-effects model to improve the reliability of the results. The Low-CP expression patients showed a significantly long OS duration compared to the High-CP expression patients (fixed-effects model: RR: 1.52; 95% Confidence Interval [CI]: 1.42/1.62) (Figure 3B). In the TCGA and CGGA databases, COX regression analysis revealed that CP was an independent factor that affected the prognosis of the patients suffering from glioma ($p < 0.001$) (Figures 4A, B and Supplementary Figures S2A, B). Overall, the nomogram model constructed based on CP expression achieved better prediction of prognosis (Figures 4C, D and Supplementary Figures S2A, B).

3.4 Gene-gene and PPI networks of CP

The CP gene-gene interaction network was developed using GeneMania. Iron metabolism-related genes such as SLC40A1 were closely related to CP (Figure 5A). The PPI network for CP was designed using the STRING database. The most relevant genes primarily included iron metabolism-related genes such as SLC40A1, haptoglobin (Hp), and frataxin (FXN) (Figure 5B). The chord diagram displays the top 11 genes that were co-expressed with CP in the CGGA database (Figure 5C). CP had a positive correlation with CH3L2, FCGR2A, C1S, C1R, SERPINA3, and CF1, and a negative correlation with AMER3, ST6GAL2, PTPRT, AC062021.1, and CHRNB2 (Figure 5C). Furthermore, the correlation between CP expression and the iron metabolism-linked genes was investigated by TCGA database. CP showed a significantly positive link to the HAMP, FTH1, FTL, SLC40A1, TFRC, a negative correlation with RFR2 of LGG, and a significantly positive relationship with the HAMP, FTH1, and FTL of GBM (Figure 5D).

3.5 Functional evaluation of the DEGs in the High-CP and Low-CP expression categories

The heat map shows the Differentially Expressed Genes (DEGs) in the High-CP and Low-CP expression categories in the CGGA dataset (Figure 6A) and the TCGA dataset (Supplementary Figure S3A). The relevant pathways and biological functions of CP were investigated by KEGG and GO enrichment analyses, and the top 10 Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF) terms were listed. In the CGGA dataset, CP was seen to be enriched in the immune response-linked BP pathways, like T cell activation, lymphocyte mediated immunity, and the regulation of the

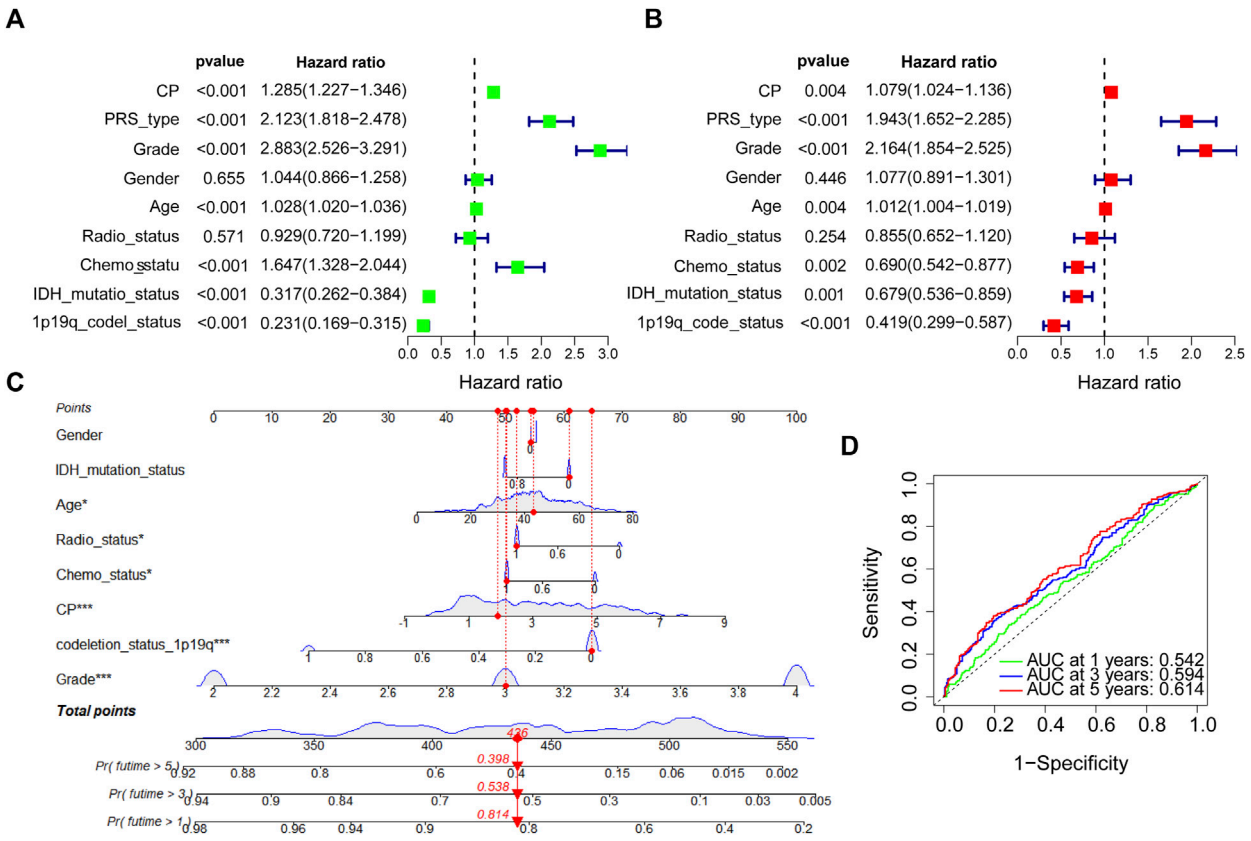

FIGURE 4
COX regression analysis and establishment of prognostic model in CGGA dataset. **(A)** Univariate analysis of CP in the CGGA dataset. **(B)** Multivariate analysis of CP in the CGGA dataset. **(C)** The nomogram was constructed based on four factors for predicting 1, 3 or 5 years survival in CGGA glioma patients. **(D)** The calibration plots of internal validation in CGGA showed well consistency in predicting 1, 3 or 5 years survival. (Note: ***$p < 0.001$ and *$p < 0.05$).

T cell activation (Figure 6B). In addition, the top 10 KEGG pathways related to DEGs are presented in Figure 6C. Out of these pathways, many immune-linked pathways were related to CP, including PD-L1 expression, PD-1 checkpoint and T cell receptor signaling pathway in cancer (Figure 6C). Furthermore, GSEA-GO and KEGG analyses of the CGGA dataset implied that CP was associated with the primary immunodeficiency and adaptive immune-associated pathways, such as T cell and B cell activation, antigen receptor-mediated signaling pathway, T cell receptor signaling pathways (Figure 6D).

CP was primarily enriched in the immune response-linked BP pathways in the TCGA glioma database, including positive regulation of leukocyte activation, B cell-mediated immunity, and adaptive immune response (Supplementary Figure S3B). In addition, Supplementary Figure S3C displays important KEGG pathways associated with DEGs. Among these, many immune-related pathways, including the B cell/lymphocyte-mediated immunity and the B cell receptor signaling pathway, were significantly linked to CP. Additionally, GSEA-GO and KEGG analyses in the TCGA dataset revealed that CP was associated with the immune-linked or T cell- and B cell-linked pathways, such as B cell activation, B cell and T cell receptor signaling pathway (Supplementary Figure S3D).

3.6 Relationship between CP expression and immune microenvironment

The immune and mesenchymal scores were calculated for each glioma sample in CGGA using the “Limma” and “Estimate” software packages in the R language to assess the proportion of immune and mesenchymal components in TME. The violin plot showed that the scores of TME were significantly elevated in the High-CP expression category compared to the Low-CP expression category (Figure 7A). The TMB scores were seen to be significantly increased in the High-CP expression category compared to the Low-CP expression category in the CGGA database, and the CP expression was positively correlated with TMB (Figures 7B, C). The correlation of well-known T cell checkpoints in the GEPIA database, such as PD-L1, PD-1, and CTLA-4 with the CP expression was further investigated. The expression of CP in LGG and GBM was significantly linked to the PD-L1, PD-1, and CTLA-4 expression (Figure 7D). In addition, the correlation of CP expression in LGG and GBM with 6 immune cells, including macrophages, neutrophils, dendritic cells, $CD4^+$ T cells, and B cells, was analyzed by the Timer network platform. CP expression were significantly and positively correlated to the infiltration of $CD4^+$ T cells, B cells, neutrophils, macrophages, and dendritic cells in GBM and negatively correlated

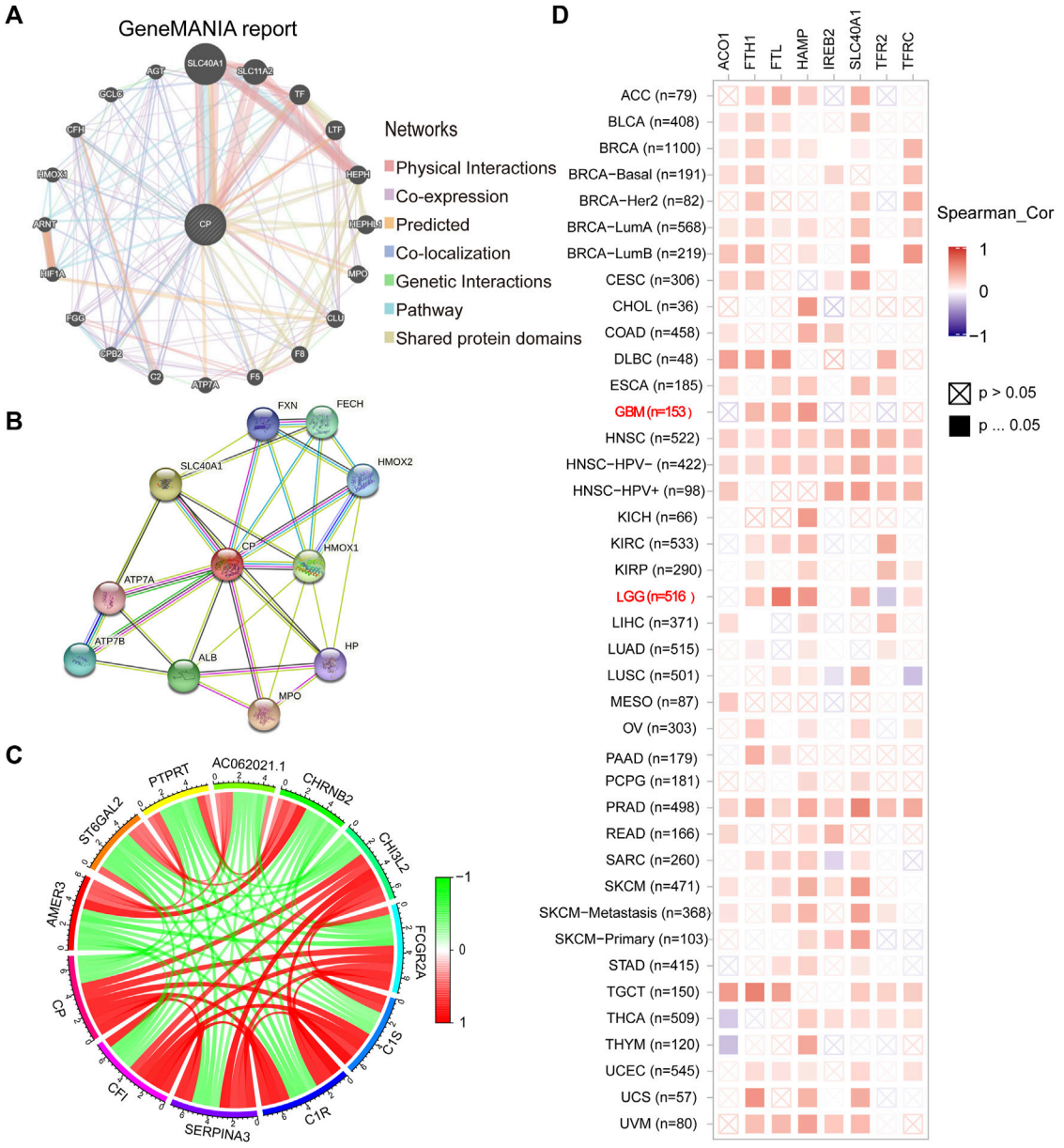

FIGURE 5
Analysis of CP-interacting genes and proteins. **(A)** The gene-gene interaction network of CP was constructed using GeneMANIA. **(B)** The PPI network of CP was generated using STRING. **(C)** The gene chord diagram of CP from co-expression analysis. Red lines represent positive correlations with CP, and green lines represent negative correlations with CP. **(D)** A heatmap shows the correlations between CP and iron metabolism-related genes in LGG and GBM.

to the CD8$^+$ T cells (Figure 7E). Based on the CiberSort resource, the abundance ratios and relationship between each other of 22 types of immune cells in CGGA glioma samples were calculated (Figure 8A). The difference in the concentrations of 22 types of immune cells between the High-CP and Low-CP expression categories suggested that eosinophils, plasma cells, macrophages M0, T-cell CD4 memory resting, and neutrophils were positively and significantly related to the CP expression, whereas macrophages M2, NK-activated cells, B-memory cells, NK-resting cells, and other immune cells showed a significant and negative relation with CP expression (Figures 8B, C). In the GBM dataset of the TCIA web platform (https://tcia.at/home), patients in the high-CP expression group exhibited significant immune escape in CTLA-4 and PD-1 positive GBM tissues compared to the low-CP expression group after anti-PD-1 and CTLA-4 treatments (Figure 8D).

4 Discussion

Glioma is a serious disease that affects human health (Brown, 2023). For decades, a large number of studies on molecular markers and molecular targeted drugs only had a little effect in prolonging life expectancy of patients with glioma (Huang et al., 2023b). Therefore, searching for glioma-related molecular

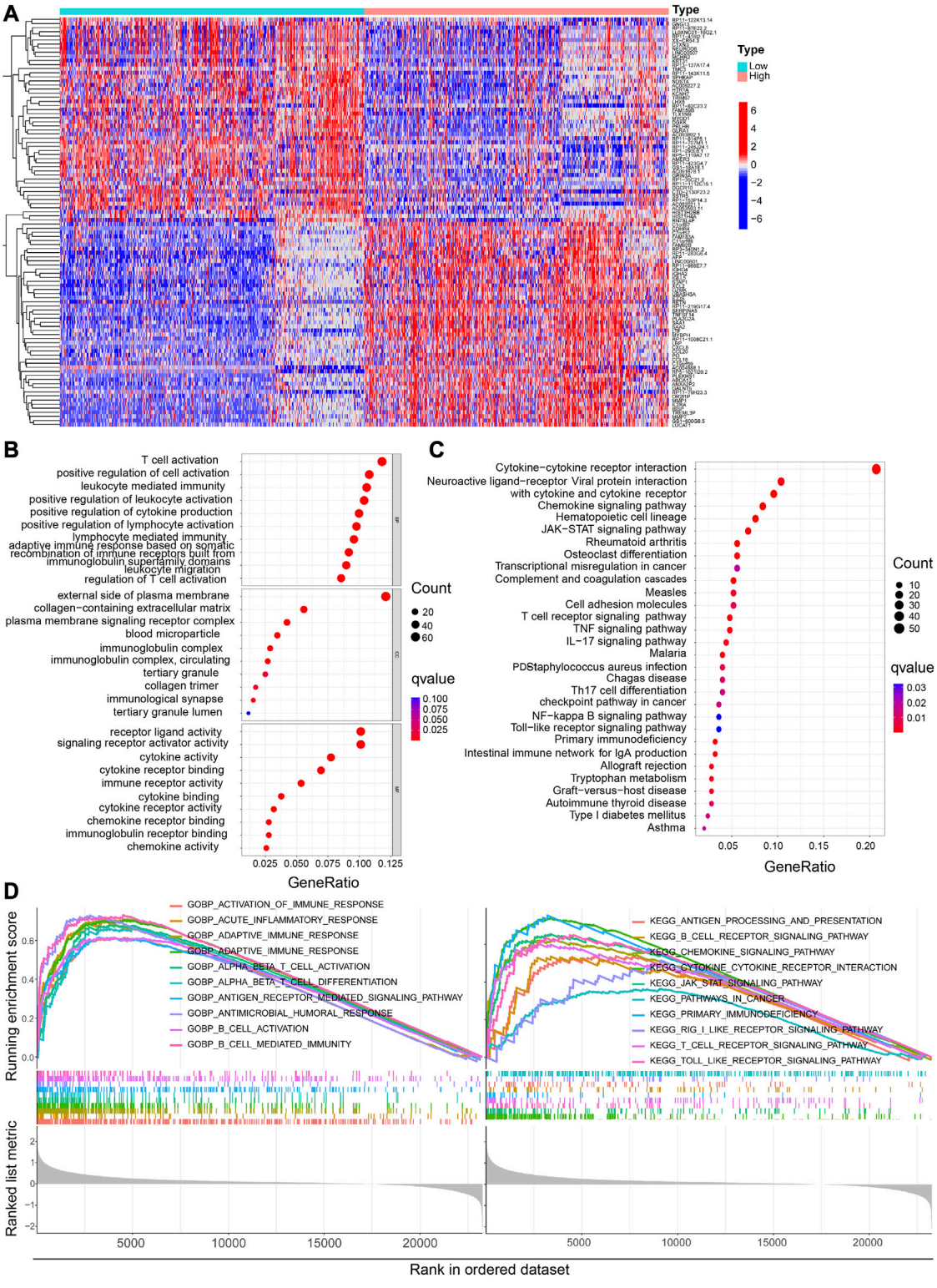

FIGURE 6
Functional analysis of DEGs between the high and low CP expression groups in the CGGA dataset. **(A)** Heatmaps of the differentially expressed genes between the high and low CP expression groups. GO **(B)** and KEGG **(C)** analyses of DEGs. **(D)** GSEA GO and KEGG enrichment analyses of the high and low CP expression groups in the CGGA dataset.

helps understand the mechanism of glioma occurrence and progression and provides molecular targeted therapeutic targets for glioma.

CP is an acute phase protein that is activated under a variety of circumstances, including inflammation, infection, diabetes, and trauma (Liu et al., 2022b). In the past few years, numerous

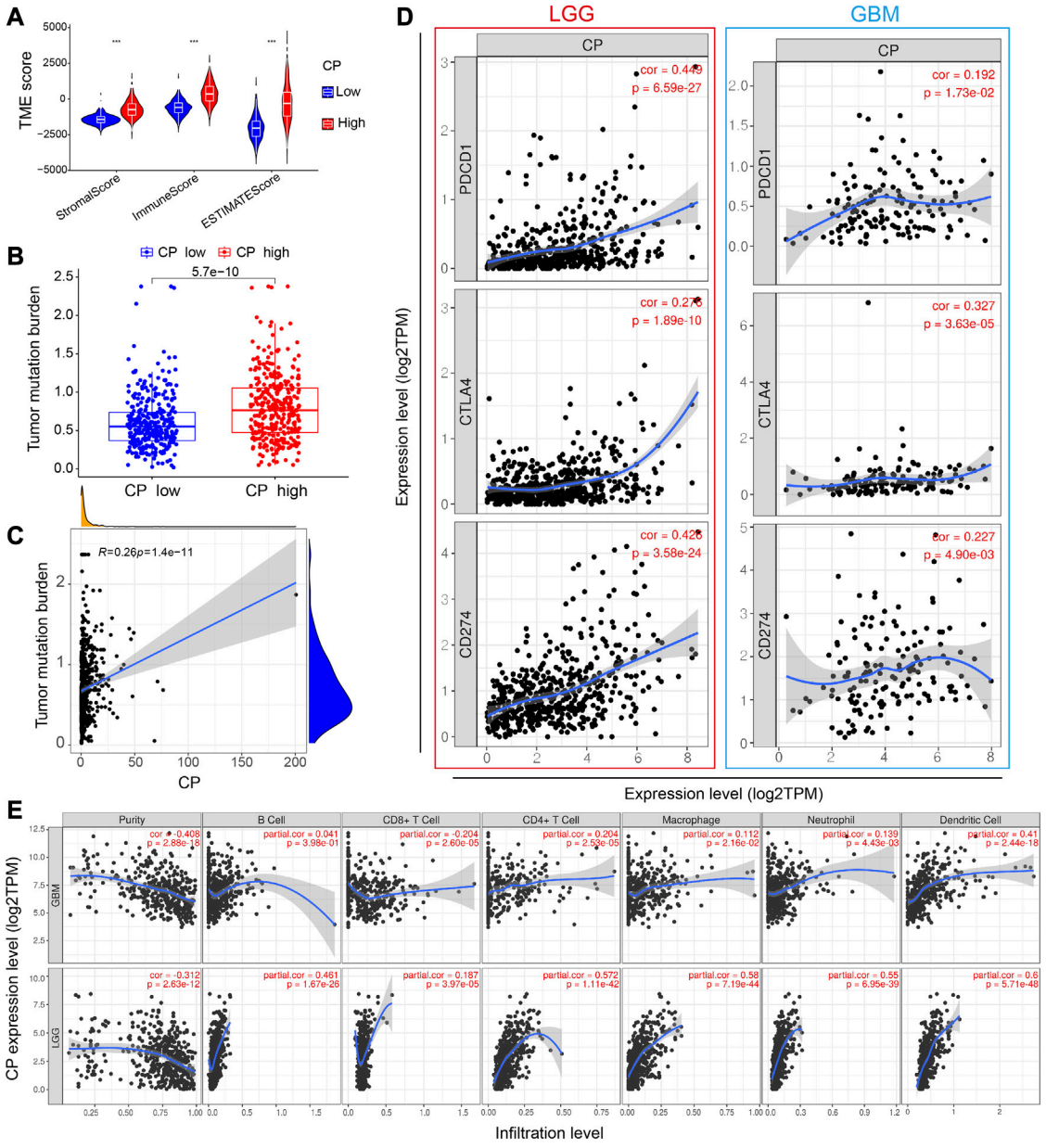

FIGURE 7
Analysis of the correlation between CP and immunity. **(A)** The TME scores between the high and low CP expression groups in the CGGA database. **(B)** The TMB between the high and low CP expression groups in the CGGA database. **(C)** The correlation between CP expression and TMB. **(D)** Scatterplots of the correlations between CP expression and PD-1, PD-L1 and CTLA-4 in LGG and GBM. **(E)** CP is significantly associated with tumor purity and is positively correlated with the infiltration of different immune cells according to the TIMER database.

physiological functions and activities of CP have been found, such as antioxidant activities, iron oxidase, copper transport, iron homeostasis management, and oxidation of organic amines (Hellman and Gitlin, 2002). Recent data implied that CP is linked to the onset and advancement of tumors. Lung (Dai et al., 2020; Schneider et al., 2022), epithelial ovarian (Schneider et al., 2022), colon (Lin et al., 2019), and bile duct tumors (Han et al., 2017) also have high serum CP levels. CP established the connection between the prognosis and severity of the above cancers via several molecular processes and signaling pathways. Intriguingly, in one study, the hypothesis suggested that it was associated with the local Fe homeostasis (Li et al., 2020). CP-ferroportin system (CP-Fpn) is the primary intracellular iron export pathway (Helman et al., 2023). Iron oxidation is another important function of CP. CP can transfer electrons to oxygen via regulating copper, which oxidizes Fe^{2+} to Fe^{3+} and enters the cell after binding to transferrin, thus providing sufficient iron for cell proliferation (Forciniti et al., 2020; Roy et al., 2022). Depletion of CP caused the accumulation of ferrous iron (Fe^{2+}) in cytoplasm. The accumulated Fe^{2+} mediates the

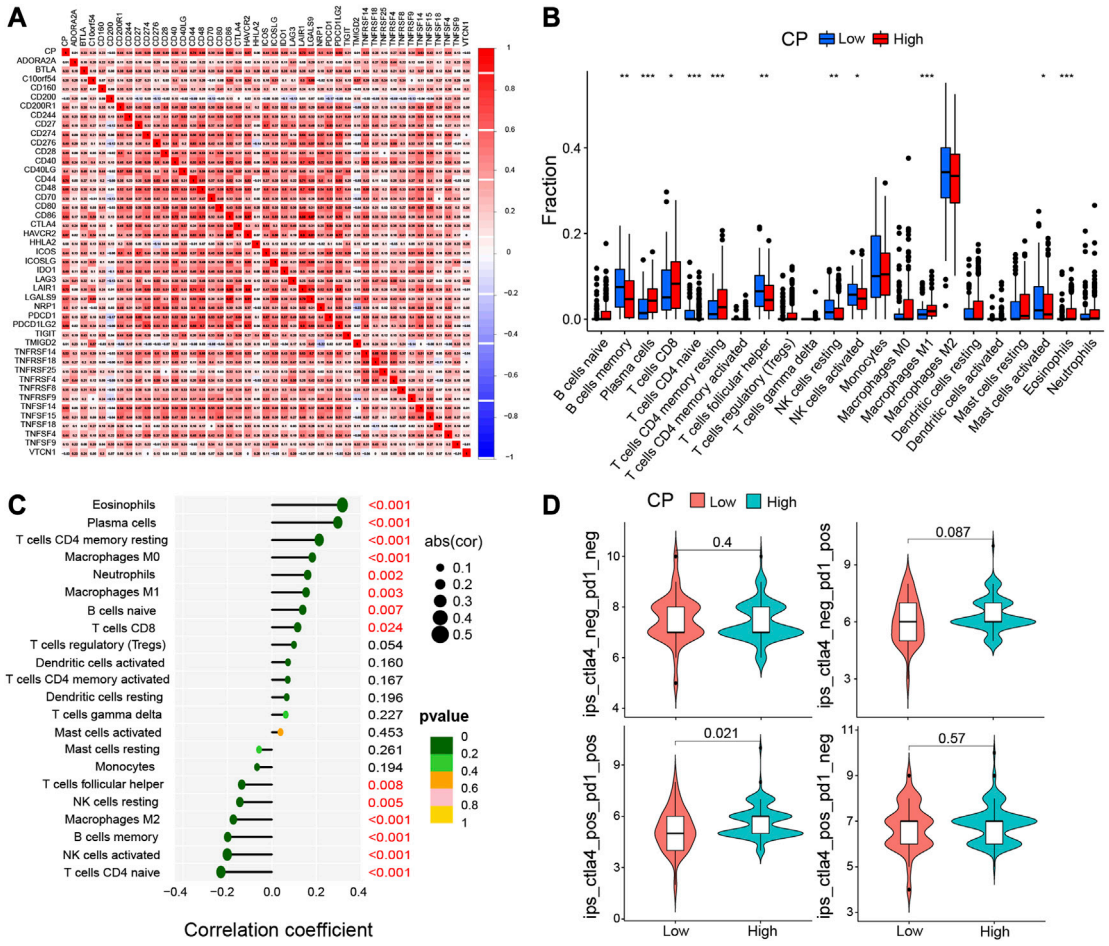

FIGURE 8
Immune cell infiltration analysis were calculated by the CIBERSORT algorithm and TIMER database. **(A)** In CGGA dataset, the correlation of each immune cell was analyzed. **(B)** The varied proportions of 22 subtypes of immune cells in the high and low CP groups in tumor samples. Horizontal and vertical axes represent TIICs and relative percentages, respectively. Blue and red colors represent the low and high CP expression groups, respectively. **(C)** The correlation between immune infiltrating cells and CP expression. The ordinate represents the name of the immune cell, and the abscissa represents the correlation coefficient. **(D)** In the GBM data set of the TCIA network platform (https://tcia.at/home), the efficacy of the high and low expression of CP in receiving the anti-PD-1 and anti-CTLA-4.

formation of reactive oxygen species *in vivo* by Fenton reaction, eventually leading to ferroptosis in cells (Huang et al., 2023a). In addition, CP expression is increased in tumor cells of different tumors like liver cancer, lung cancer, and melanoma, thus transferring electrons to oxygen and oxidizing Fe^{2+} to Fe^{3+} via regulating copper, thereby reducing intracellular Fe^{2+} and inhibiting ferroptosis (Li et al., 2020). Therefore, high CP expression in tumor cells inhibits ferroptosis, which reduces tumor cell death and promotes tumor progression. Although the role of CP in glioma is not clearly, CP is regarded as an attractive potential target for cancer treatment, and CP-inhibiting drugs are being investigated currently.

Here, we found that the CP expression was seen to be higher in tumors and their adjacent tissues compared to the normal tissue samples. CP expression was seen to increase significantly with an increasing glioma grade in the TCGA, CGGA, Gravendeel, and Rembrandt databases. Subsequent KM curves and statistical test analyses revealed that OS was significantly lower in the High-CP category than the Low-CP in the datasets. In addition, the clinical prognostic relevance of the CP in the patients suffering from glioma was explored. A higher CP expression was significantly correlated with the glioma grade, age, chemotherapy status, IDH mutations, PRS type, and 1p19q co-deletion status in patients with glioma. COX regression analysis in the TCGA and CGGA databases suggested that CP could serve as an independent prognostic factor that affected the prognosis of the patients suffering from glioma ($p < 0.001$). Therefore, CP could be regarded as an independent prognostic biomarker for glioma and could enable the development of the targeted precision oncology.

Cellular iron is involved in several metabolic processes and is an essential microelement for cell growth and metabolism. Compared to normal cells, tumor cells required a higher iron concentration to stimulate DNA synthesis and increase cell proliferation. Disruption of iron regulatory pathways can lead to iron accumulation, thus participating in tumor mutagenesis and promoting tumor growth.

For several malignancies, the degree of Fe homeostasis-linked gene expression has been used as both a prognostic indicator and a therapeutic target (Tsai et al., 2020). Some iron chelators, including DFX, desferrioxamine (DFO), Dp44mt, and triazepine, also induce apoptosis in several types of cancers and have been developed as antitumor drugs that display anticancer effects in malignancies like prostate cancer, lung cancer, neuroblastoma, leukemia, oral cancer, and breast cancer (Tan et al., 2023). In this study, genes and proteins interacting with CP were identified from the GeneMania and STRING datasets and found that iron metabolism-related genes such as SLC40A1 were closely related to CP.

TME plays a key role in the progression and invasion of gliomas. Several new treatment approaches have emerged in recent years for tumors, including immunotherapy (Hovhannisyan et al., 2023). However, the application status of immunotherapy in glioma is unsatisfactory due to disruption by the suppressive TME. During glioma progression, tumor cells evade evading host immunosurveillance and continue to grow. This process involves glioma-secreted cytokines that shape the intratumoral microenvironment and systematically alter the proliferation, differentiation, and function of immune cells in the body (Garner and de Visser, 2020). The cytokines present within the peritumoral environment are involved in the overall tumor progression process. They can not only cause immunosuppression of the tumor and evade the immune surveillance, but also promote angiogenesis and enhance the growth and invasiveness of tumor cells (Al Hrout et al., 2023). Therefore, a full understanding of the role of relevant cytokines in the TIME of glioma may provide an important theory for immunotherapy of glioma. Treatments targeting TME, like anti-PD-1 therapy and T cell immunotherapy, have achieved good outcomes. Whereas, the therapeutic effect of Immune Checkpoint inhibitors (ICBs) in glioma has been unpredictable and unfavorable, with only 8% of patients with GBM having a definite response (Arrieta et al., 2023). Currently, a growing body of data suggests that iron metabolism in TME is an important factor in maintaining cancer cell survival. Therefore, identifying iron metabolism-related factors affecting TME is crucial for the treatment of glioma.

In the present study, the relevant pathways and biological functions of CP were investigated using KEGG and GO pathway enrichment analyses to further investigate the mechanism of action of CP in glioma progression and its relationship with immune microenvironment. The results implied that CP was linked to immune function. In addition, the findings in this study revealed the correlation between the high CP expression in gliomas and increased infiltration of macrophages, B cells, neutrophils, dendritic cells, $CD4^+$ T cells, and $CD8^+$ T cells, with the help of the CiberSort and ESTIMATE algorithms and Timer portal. Immune and mesenchymal scores were computed for every glioma sample in CGGA to assess the proportion of immune and mesenchymal components in TME. TME scores were significantly elevated in the High-CP expression category compared to the Low-CP expression category. Based on the data derived from the TCGA database, the TMB scores were significantly elevated in the High-CP expression category compared to the Low-CP expression category. Additionally, the correlation of well-known T cell checkpoints like PD-L1, PD-1, and CTLA-4 with CP expression in the GEPIA cohort was further investigated. The findings indicated that the CP expression was related significantly to the PD-1, PD-L1, and CTLA-4 expression in LGG and GBM. In the GBM dataset of the TCIA web platform (https://tcia.at/home), patients in the high-CP expression group exhibited significant immune escape in CTLA-4 and PD-1 positive GBM tissues compared to the low-CP expression group after anti-PD-1 and CTLA-4 treatments. These findings further support the close correlation between CP expression and immune invasion, suggesting the involvement of CP in the escape of the immune cells in the glioma TME.

5 Conclusion

CP was confirmed to be an independent prognostic factor for predicting the prognosis of glioma patients through bioinformatics analyses of the glioma databases, and its specific mechanism may be related to tumor immunity. The present study still has many limitations. We focused on the analysis of the databases. Therefore, more *in vivo* and *in vitro* experiments need to be conducted for validating the above findings. In addition, the specific upstream and downstream pathways and mechanisms of CP need to be further explored. In conclusion, these findings suggest that CP could be regarded as a novel immune-linked therapeutic target for glioma. On the other hand, the exact role played by CP in TME deserves further investigation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

MJ, TD, and PH conceived and designed the study. JZ, FR, WL, SZ, and XJ performed the statistical analysis. MJ, TD, and YC wrote the manuscript. BC and YW revised the article. HC, YW, and PH provided financial support for this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1249650/full#supplementary-material

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Crosstalk between endoplasmic reticulum stress and multidrug-resistant cancers: hope or frustration

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Endoplasmic reticulum stress (ERS) is a kind of cell response for coping with hypoxia and other stresses. Pieces of evidence show that continuous stress can promote the occurrence, development, and drug resistance of tumors through the unfolded protein response. Therefore, the abnormal ac-tivation of ERS and its downstream signaling pathways not only can regulate tumor growth and metastasis but also profoundly affect the efficacy of antitumor therapy. Therefore, revealing the molecular mechanism of ERS may be expected to solve the problem of tumor multidrug resistance (MDR) and become a novel strategy for the treatment of refractory and recurrent tumors. This re-view summarized the mechanism of ERS and tumor MDR, reviewed the relationship between ERS and tumor MDR, introduced the research status of tumor tissue and ERS, and previewed the prospect of targeting ERS to improve the therapeutic effect of tumor MDR. This article aims to provide researchers and clinicians with new ideas and inspiration for basic antitumor treatment.

KEYWORDS

endoplasmic reticulum stress, unfolded protein response, multidrug resistance, apoptosis, molecular mechanism

1 Introduction

Malignant tumors are one of the most important public health problems in the world (Lin et al., 2021b). In the United States, 1,958,310 new cases and 609,820 die cases of invasive tumors are estimated to occur in 2023. The overall cancer incidence and mortality rates were 488.2/100,000 and 177.5/100,000 in males and 423.3/100,000 and 128.7/100,000 in females during 2020 (Siegel et al., 2023). China is the largest developing country in the world. According to statistics, China recorded an estimated 4,820,000 new cancer cases in 2022. China is the largest developing country in the world. According to statistics, China recorded an estimated 4,820,000 new cancer cases in 2022. In 2020, the overall cancer incidence in China was estimated to be 204.8/100,000. The cancer mortality rate was 163.9/100,000 in males and 98.1/100,000 in females (Qiu et al., 2021; Xia et al., 2022). Malignant tumors are not only a medical but also a complex problem composed of societal, family, and other aspects. With China's economic development and the increase in life expectancy, the rise in cancer patients is not an unexplained problem. Notably, the number of cancer deaths in China was five times higher than that in the United States in the same year. This phenomenon may not only be due to the level of medical care and decision-making but also the result of the economic situation of patients and the availability of medical resources.

The treatment of cancer has consistently been a major concern worldwide. In recent decades, with the rapid development in genetics and molecular biology, cancer treatment has gradually shifted toward precision medicine at the individual patient level. A personalized approach based on individual cancer genomic information has the potential to identify clinically viable target molecules and aid in the selection of appropriate therapies for individual patients. However, given the limited number of molecular targeted drugs, only a small proportion of patients can benefit from genetic analysis. Drug therapy remains the most important component of cancer treatment, especially for patients who have lost the opportunity to undergo surgery at the time of diagnosis. Drug therapy agents include chemotherapeutic agents, targeted agents, and immune checkpoint inhibitors. The continuous development of drug therapy has resulted in inspiration and motivation for the cure of tumors. However, the multidrug resistance (MDR) of tumor cells led to serious challenges in human survival. MDR refers to the cross-resistance of cancer cells to anticancer drugs with different structures and mechanisms of action (Assaraf et al., 2019). MDR is responsible for more than 90% of deaths in cancer patients receiving conventional chemotherapy or new targeted agents (Bukowski et al., 2020). Despite the billions of dollars that have been invested in tumor resistance research and the development of new anticancer therapies, MDR remains the greatest obstacle to tumor cure for patients without surgical opportunity. Chemotherapy, immunotherapy, or molecular targeted therapy may be effective initially, but surviving drug-resistant tumor cells will ultimately lead to tumor recurrence and treatment failure (Dhanyamraju et al., 2022).

The endoplasmic reticulum (ER) is an organelle in eukaryotic cells that plays a crucial role in protein folding, lipid biosynthesis, and calcium signaling. When cells experience stress conditions, such as nutrient deprivation, oxidative stress, and inflammatory responses, the capability of the ER to fold proteins is compromised. This results in the accumulation of unfolded or misfolded proteins in the ER cavity and triggers a signaling pathway called the unfolded protein response (UPR) (Ren et al., 2021). The UPR is a highly conserved cellular stress response pathway that alleviates endoplasmic reticulum stress (ERS). It involves the activation of three major signaling pathways, namely, protein kinase-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) (Kaufman, 2002). The activation of these pathways is ultimately a transcriptional program that restores protein folding homeostasis, reduces protein synthesis, and increases protein degradation. However, if ERS persists and becomes extremely severe, the UPR signaling pathway will change its role from a cytoprotective one to a promoter of apoptosis (Chen and Cubillos-Ruiz, 2021). Despite the substantial evidence on the persistence of ERS and UPR activation in numerous forms of cancer, whether these processes ultimately inhibit or promote tumor growth in patients remains inconclusive (Oakes and Papa, 2015). On the one hand, a variety of drugs exert antitumor effects through ERS-related signaling pathways. On the other hand, ERS is also related to the mechanism of tumor MDR, and its regulation can affect tumor resistance to antitumor drugs or reverse drug resistance (Nie et al., 2021). Although considerable work is needed before this finding can be used clinically, it gives new hope to patients with refractory or recurrent tumors. Therefore, this article reviews the research progress on multidrug-resistant cancers and ERS to provide novel ideas for basic and clinical researchers.

2 Mechanism of ERS

Since the 1980s, cellular stress has been known to induce the expressions of molecular chaperones in the inner reticulum (Koch et al., 1986), but it was not until the 1990s, when the first ERS sensor (IRE1) was discovered, that researchers paid attention to the UPR. Subsequently, two ERS receptors have been identified, namely, ATF6 and PERK; in addition, three ER transmembrane proteins collectively regulate the functions of thousands of genes involved in ER control while also regulating the rate of protein synthesis (Marciniak et al., 2022). Over the past decade, cellular alterations secondary to ERS have increasingly been recognized as central factors in the pathophysiology of human diseases. ERS and persistent UPR signaling have been documented well in tissues affected by diabetes, neurodegeneration, stroke, pulmonary fibrosis, viral infections, inflammatory diseases, cancer, and heart diseases (Oakes and Papa, 2015). The ability to fold proteins within the ER varies by cell type and often depends on the size of the ER; still, regardless of the ER size, cells operate at load limits and often encounter situations that impose workload on the ER beyond its capacity (Tabas and Ron, 2011). Moreover, a wide range of cellular disorders, such as hypoxia, nutrient deficiency, and inflammation, can affect the efficiency of protein folding in the ER and lead to the accumulation of misfolded proteins in this organelle. When the ER protein folding capacity is overwhelmed, ERS is then triggered. The mechanism of ERS mainly depends on the duration and severity of stimulation, that is, restoration of homeostasis or apoptosis and necrosis (Oakes and Papa, 2015). Various physiological and pathological stimuli can cause ERS, which triggers the UPR that transmits information on protein folding status to the nucleus and cytoplasm to regulate the protein folding capacity of the cell (Hetz et al., 2020). When mild to moderate (but persistent) ERS occurs, cells induce transcriptional and translational changes through the homeostatic UPR (hUPR), which promotes cell adaptation and improves cell survival. However, when ERS progresses to the point where the hUPR is insufficient to restore homeostasis, the UPR in the cell becomes dominated by the terminal UPR. This process actively initiates apoptosis and prevents sustained cell damage (Nie et al., 2021). The UPR is initiated by three ER-resident transmembrane proteins: IRE1α, PERK, and ATF6. In the absence of stress, these transmembrane proteins are inactivated when they bind to the ER lumen molecular chaperone glucose-regulated protein 78 (GRP78). During stress condition, the amount of unfolded proteins in the ER lumen increases, which triggers the dissociation of the three protein receptors from GRP78, which in turn activates downstream signaling pathways in a cascade (Fu et al., 2022b). Once activated, the three parallel UPR signaling pathways alter the rate of protein synthesis and trafficking to the ER through autophagy and ER-associated protein degradation (ERAD) pathways, regulate protein folding and maturation and quality control, and participate in protein trafficking and elimination of misfolded proteins (Hetz et al., 2020) (Figure 1).

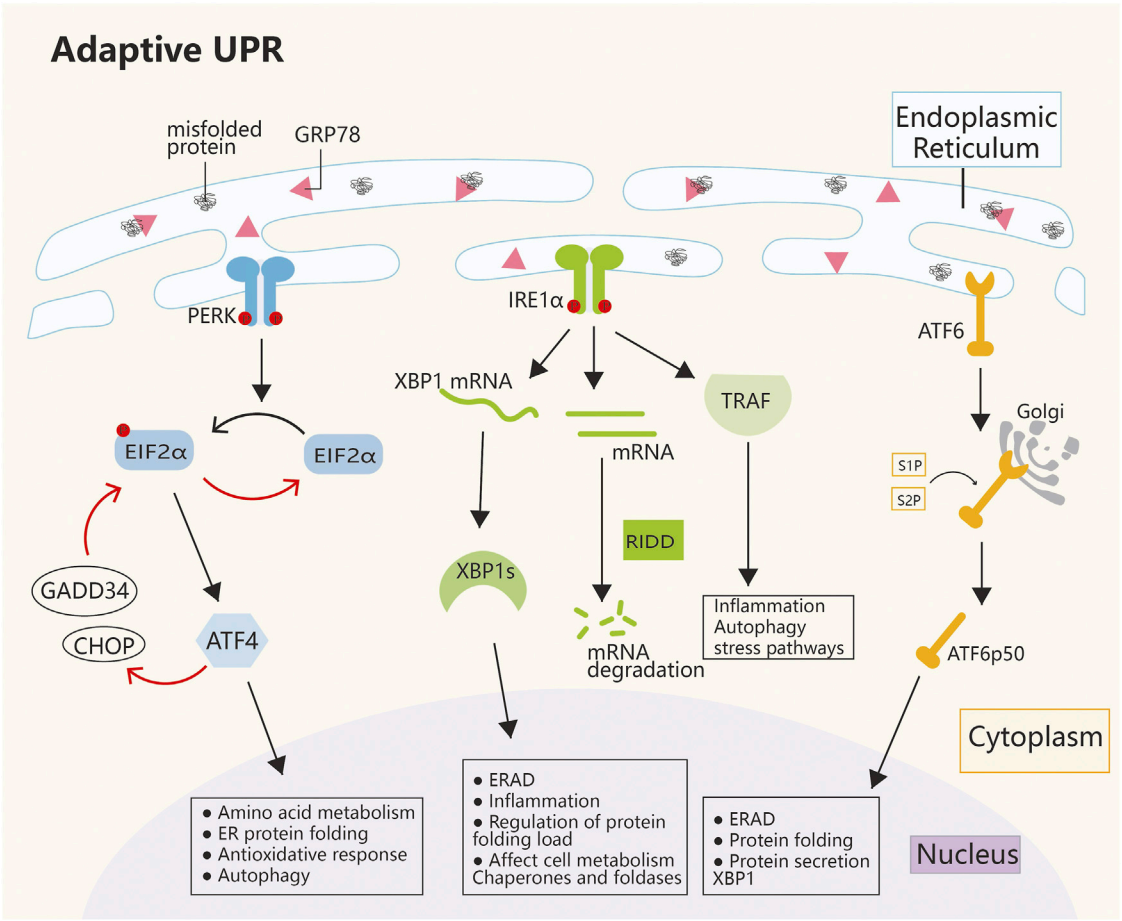

FIGURE 1
Mechanism of ERS. The main pathways of ERS are PERK, IRE1α, and ATF6. The three ERS sensors collaborate to coordinate the UPR signals. Under normal conditions, GRP78 is connected to the ERS sensor, which leaves it inactive. During ERS, GRP78 dissociates from the three transmembrane proteins on the ER membrane and activates their pathways. The IRE1α, PERK, and ATF6 pathways work together to regulate multiple genes, with the ultimate goal of restoring ER homeostasis and inducing cancer survival, angiogenesis, metastasis, and cell death resistance. ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; EIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage-inducible protein; GRP78, glucose-regulated protein 78; IRE1α, inositol-requiring enzyme 1α; PERK, protein kinase-like ER kinase; RIDD, regulated IRE1α-dependent decay; S1P, site-1 protease; S2P, site-2 protease; TRAF, tumor necrosis factor receptor-associated factor; XBP1, X-box binding protein 1.

2.1 PERK pathway

PERK is a transmembrane protein kinase on the ER membrane with serine/threonine protein kinase activity at the cytoplasmic terminus. When activated by the UPR, PERK dimerizes itself and phosphorylates eukaryotic translation initiation factor 2α (eIF2α). After phosphorylation, eIF2α is inactivated, and subsequent mRNA translation is arrested, which reduces the ER load (Starck et al., 2016; Wang et al., 2022). Phosphorylated eIF2α can selectively activate ATF4. As a transcription factor (TF), ATF4 can regulate a wide range of target genes; thereby, it indirectly regulates cellular antioxidative stress, the rate of protein synthesis, and cell apoptosis or autophagy (Gambardella et al., 2020; Read and Schröder, 2021). In ERS, ATF4 acts as a double-edge sword. On the one hand, ATF4 participates in a negative feedback loop and dephosphorylates eIF2α by activating GADD34 and C/EBP homologous protein (CHOP), which leads to the termination of stress response signaling and restoration of protein synthesis. Then, the homeostasis of cells is restored (Figure 1). On the other hand, if severe ER stress persists, ATF4 initiates the activation of caspase 8 expression after the activation of GADD34 and CHOP, which promotes apoptosis (Hetz et al., 2020). However, the reason for GADD34 and CHOP activation by ATF4 in cells in two completely different situations and the specific mechanism are still unclear. In addition, the molecule that plays a selective role in this regulatory process, which is believed to be an important direction of ERS research in the future, remains to be explored.

2.2 IRE1α pathway

IRE1α is a type 1 ER transmembrane protein kinase/endonuclease that oligomerizes and autophosphorylates in response to ER stress to activate RNase domains (Credle et al., 2005; Zhou et al., 2006). Under mild UPR conditions, IRE1α promotes the translation of XBP1 mRNA to produce activated

XBP1s, a TF that, upon entry into the nucleus, initiates the transcription of a number of genes (Grandjean et al., 2020). In addition, IRE1α activates the ERAD pathway. Moreover, IRE1α RNase can cleave ER-associated mRNA or noncoding functional RNA, which leads to their degradation through regulated IRE1-dependent decay (RIDD). This process results in the regulation of protein folding load, cellular metabolism, inflammation, and inflammasome signaling pathways, both of which alleviate ER stress and promote cell recovery to normal conditions. The IRE1α cytoplasmic domain also serves as a scaffold to recruit adaptor proteins, such as TRAF family members, to activate inflammatory responses under atypical ER stress conditions. (Figure 1). When ER stress is severe and persistent, IRE1α recruits TRAF2. Which further induces the activation of downstream c-Jun N-terminal kinase (JNK) and cell apoptosis. Alternatively, the cascade of caspase 8 or caspase 2 induces apoptosis after the activation of the RIDD pathway (Bashir et al., 2021; Lee et al., 2021).

2.3 ATF6 pathway

In response to ER stress, ATF6 is translocated from the ER to the Golgi and cleaved by S1P and S2P to release the basic leucine zipper-containing fragment ATF6p50. ATF6p50 then transports into the nucleus and activates the promoters of UPR target genes, which enhances its capability to process unfolded proteins (Ye et al., 2000). In addition, ATF6 can work with IRE1α to increase the transcription of XBP1 and enhance its capability to degrade unfolded proteins (Figure 1) (Bommiasamy et al., 2009). However, under severe ER stress, ATF6 activates CHOP-mediated apoptosis (Yang et al., 2020b). In recent years, considerable progress has been attained in the field of ERS, and researchers have revealed its key role in the pathophysiological process of diseases. The UPR is an ERS-related signaling pathway that is essential for determining cell fate (cell death or survival) in response to ER stress. Abnormal levels of ERS are closely related to different human diseases, including neurodegenerative diseases, obesity, diabetes, cancer, and autoimmune diseases. However, the mechanism of the survival to death transition under ERS is still unknown. The future challenge is to apply existing research results to develop drugs that can be safely used in clinical practice. To determine the human diseases that can be most effectively treated with these drugs, scholars must study the complexity of ER stress and its interactions with various cellular pathways to shed new light on future diagnostic, preventive, and therapeutic strategies for disease.

3 Multidrug-resistant cancers

With the continuous progress of modern medicine, the treatment of malignant tumors has changed greatly. From the earliest surgery or chemotherapy, treatment of malignant tumors has gradually evolved into a comprehensive therapy including surgery, chemotherapy, immunotherapy, molecular targeted therapy, etc. With the proposal and wide application of comprehensive therapy, the survival rate of patients has improved considerably (Siegel et al., 2023). However, tumor MDR greatly affects the prognosis of patients (Nie et al., 2022). Although resistance may develop in response to specific drugs or drug combinations, cross-resistance may confer resistance to drugs with different molecular targets or mechanisms of action. Thus, tumors can develop intrinsic resistance to agents that individuals have never been exposed to (Hanssen et al., 2021). Most deaths of cancer patients are ultimately attributed to tumor MDR (Bukowski et al., 2020). At present, tumor drug resistance is divided into primary and acquired drug resistances. Primary drug resistance refers to the innate resistance of tumor cells to a certain antitumor drug, regardless of whether they have been exposed to the drug. This type of resistance may be caused by the expressions of several mutant genes, abnormal cellular status of tumor cells, or rapid adaptation of tumor cells to the drug. Acquired drug resistance corresponds to the induction of drug resistance in tumor cells during tumor treatment, that is, tumor cells are sensitive to drugs at the initial use and later relapse and develop drug resistance (Chatterjee and Bivona, 2019). Nowadays, tumor MDR is no longer limited to traditional chemotherapy but has shown resistance to immunotherapy and molecular targeted therapy. The mechanism of tumor MDR is extremely complex, and the various mechanisms are not completely independent and often cross. Thus, overcoming this problem should not be limited to a specific or signaling pathway. Combination therapies may be needed to reduce and reverse tumor MDR.

4 Mechanism of MDR cancers

4.1 Interactions between cancer and drugs

Tumor cells can resist chemotherapeutic drugs by increasing efflux, decreasing absorption, or affecting metabolism (Figure 2). The Atp-binding cassette (ABC) transporter family is widely involved in the resistances of various tumor drugs as a drug efflux pump. It uses the energy of ATP hydrolysis to transfer chemotherapy drugs from cells to the outside of cells, reduce the concentration of intracellular drugs, and promote cell resistance. Out of the 48 ABC transporters, 19 can efflux anticancer drugs. ABC subfamily B member 1 (ABCB1), ABC subfamily C member 1 (ABCC1), and ABC subfamily G member 2 (ABCG2) are associated with MDR. They are also known as MDR-related proteins because they can nonspecifically efflux anthracyclines, taxanes, vinca alkaloids, tyrosine kinase inhibitors (TKIs), and other chemotherapy drugs. P-Glycoprotein (ABCB1) was the first identified and is the most studied protein. P-Glycoprotein is generally expressed in normal and tumor epithelial tissues, such as the brain, adrenal cortex, liver, kidney, and intestine. It is mainly responsible for the transport of compounds in a variety of structures but is highly expressed in numerous types of multidrug-resistant cancer cells. ABCC1 is also widely distributed in the kidney, adrenal gland, lung, pancreas, muscle, intestine, thyroid, and prostate. It can transport glutathione disulfides (such as cytotoxic drugs that bind to glutathione) and pump them out of cells. ABCC1 also plays a role in cellular redox homeostasis (Robey et al., 2018; Liu, 2019). On the one hand, it plays a role in protecting normal cells by transporting substrates across the biofilm. However, on the other hand, this property also allows them to become an umbrella for tumor cells.

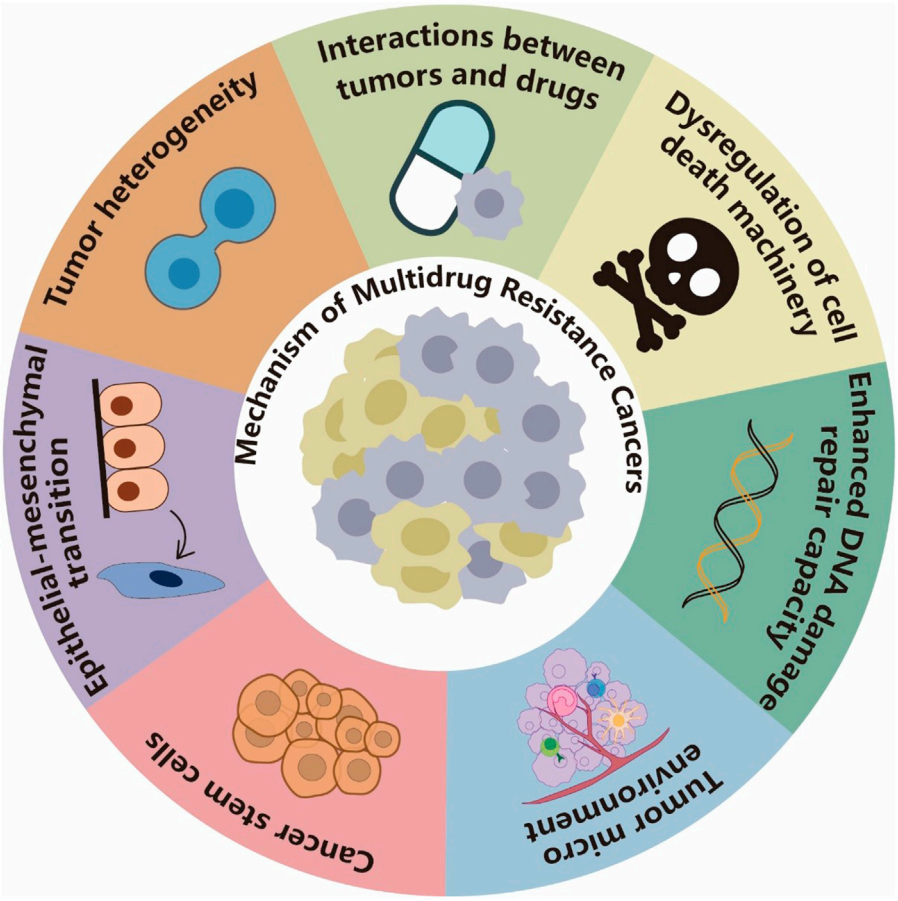

FIGURE 2
Mechanisms of MDR cancers. Tumor cells can resist drugs by increasing efflux, decreasing absorption, or affecting metabolism. They can also dysregulate the drug-induced cell death mechanism and repair DNA damage caused by chemotherapy drugs, which render the drugs less effective. The TME can mediate tumor MDR through multiple mechanisms, including preventing immune clearance of tumor cells, hindering drug absorption, and stimulating paracrine growth factors to promote cancer cell growth. Cancer stem cells (CSCs) develop MDR mainly through dormancy, epithelial–mesenchymal transition (EMT), MDR, and resistance to DNA damage-induced death. EMT and CSCs often share key signaling pathways and drug resistance phenotypes. EMT induction or EMT TF activation can endow tumor cells with stem cell-like characteristics, and the TME can also promote EMT-induced drug resistance. Tumor heterogeneity is usually a result of chromosomal instability, mutation, and epigenetic changes and considered one of the important reasons for tumor MDR.

Downregulation or abnormal binding of uptake transporters and changes in the tumor microenvironment (TME) may lead to decreased drug uptake. Mutations in the folate carrier gene of patients with acute lymphoblastic leukemia can reduce the binding of methotrexate to the transporter and lead to methotrexate resistance in patients (Wojtuszkiewicz et al., 2015). Platinum drug resistance is often caused by reduced drug accumulation of copper transporter 1 (CRT1), a member of the SLC subfamily 31, and several clinical studies have shown that decreased CTR1 expression is associated with reduced concentrations of platinum compounds in tumors and poor prognosis in patients with solid cancers treated with platinum therapy (Kalayda et al., 2012).

The mechanism of action of chemotherapeutic drugs can also rely on the metabolism of enzymes to exert anticancer effects. The upregulation of enzymes involved in drug metabolism can increase the rate of drug decomposition and reduce the efficacy, which leads to MDR. The overexpression of human cytochrome P450 CYP1B1 has been observed in a variety of malignant tumors. However, detecting this protein in normal tissues is difficult. The presence of CYP1B1 in cells reduces the sensitivity of tumors to anticancer drugs. Drug resistance of tumor cells can be reversed by coincubation of cells with the anticancer drug docetaxel (DTX) and cytochrome P450 CYP1 inhibitors (McFadyen et al., 2001).

4.2 Dysregulation of the cell death machinery

The cytotoxicity of antineoplastic drugs depends primarily on their capability to induce cell death. The main mechanisms of cell death comprise apoptosis, necrosis, and autophagy-related cell death. Chemotherapeutic agents induce cell death through a variety of molecular and cellular mechanisms, such as reactive oxygen species (ROS) induction, DNA damage, activation of proapoptotic receptors, induction of autophagy-related cell death, and immune cell effector responses. However, cancer cells undergo

constant evolution and adaptation, which confer them the ability to evade cell death (Figure 2) (Assaraf et al., 2019). Apoptosis can be activated by a mitochondrial pathway controlled by interactions between the proapoptotic and antiapoptotic members of the BCL2 family; the Bcl-2 antiapoptotic molecule is frequently upregulated in multidrug-resistant tumor cells to prevent drug-induced apoptosis (Maji et al., 2018; Zhang et al., 2021a). The P53 tumor suppressor gene is one of the most studied tumor suppressor genes and the most frequently mutated. It activates proapoptotic proteins, which, when mutated, prevent cell apoptosis or death, reduce sensitivity to antineoplastic drugs, drive cancer metastasis, and enhance MDR during cancer treatment (Yang et al., 2015; Cao et al., 2020). Numerous p53-regulated microRNAs (miRNAs) have been proposed to be involved in p53-regulated tumor functions. MiRNAs play an important role in cancer development, metastasis, angiogenesis, and MDR (Peng and Croce, 2016; Lampis et al., 2020). The miR-17-92 cluster is a novel target for p53-mediated transcriptional repression under hypoxic conditions. When down-regulating this cluster sensitizes cells to hypoxia-induced apoptosis, whereas its overexpression inhibits apoptosis. Therefore, tumor cells with increased miR-17-92 expression may escape hypoxia-induced apoptosis. The above results suggest that p53 and its regulated miRNA form a network, and cancer cells with dysregulated p53 or its target miRNA may have the ability to resist cell death, which results in MDR (Yan et al., 2009). Autophagy refers to the process by which cytoplasmic components are transported to lysosomes for extensive degradation in response to intracellular and extracellular stresses; this process is essential for cell survival in response to hypoxia, genomic instability, ER stress, and nutrient deprivation (Glick et al., 2010; Parzych and Klionsky, 2014). Autophagy often leads to MDR in tumors. On the one hand, autophagy removes damaged proteins and organelles from cancer cells to provide energy for their survival against anticancer therapy and avoid tumor cell death (Cordani and Somoza, 2019). On the other hand, autophagy promotes tumor cells to evade immune surveillance, which also allows tumor cells to survive (Gao et al., 2022). Therefore, autophagy participates in and promotes the development of tumor MDR, helps cancer cells escape apoptosis, and protects tumor cells from chemotherapy and targeted drugs (Onorati et al., 2018; Ferreira et al., 2021). According to some scholars, sustained autophagy activation leads to the turnover of proteins and organelles beyond the survival threshold, which can kill certain cancer cells with a high apoptosis threshold and thereby improve the therapeutic effect. However, to date, *in vivo* evidence of autophagic cell death in mammals is relatively limited; regardless the induction of drug-resistant tumor cell death by autophagic death remains an attractive but remains to be investigated therapeutic strategy (Yang et al., 2011; Choi, 2012).

4.3 Enhanced DNA damage repair capacity

Tumor cells may develop drug resistance through the enhancement of DNA damage repair pathways (Figure 2). Anticancer drugs, such as topoisomerase inhibitors, anthracyclines, and cisplatin, can induce different forms of DNA damage. Cisplatin exerts anticancer effects by inducing DNA double-stranded breaks (Kizek et al., 2012; Liu et al., 2019). After their chemotherapy drug-induced DNA damage, tumor cells activate a DNA repair mechanisms, which leads to the development of drug resistance due to the ability of cancer cells to repair DNA damage caused by chemotherapy drugs; this condition reduces the effectiveness of chemotherapy drugs (Trenner and Sartori, 2019). The role of base excision and mismatch repairs in 5-fluorouracil (5-FU) resistance has been demonstrated in numerous studies. At present, researchers are investigating the involvement of other repair pathways in the MDR of tumor cells, and a number of pathways, including homologous recombination (HR) and nonhomologous end joining pathways, have been discovered. Patients who initially respond to cisplatin treatment usually develop drug resistance due to the activation of HR DNA repair mechanisms (Sethy and Kundu, 2021; Sun et al., 2022b). YTH N6-methyladenosine RNA binding protein 1 (YTHDF1) is a m6A-binding protein that promotes the growth of breast cancer cells *in vitro* and *in vivo*. YTHDF1 promotes DNA replication and damage repair; thus, knockdown of YTHDF1 re-sensitizes breast cancer cells to doxorubicin, cisplatin, and olaparib (Sun et al., 2022b). Moreover, C-Jun activation domain-binding protein 1 (Jab1) positively regulates the DNA repair protein Rad51 in a p53-dependent manner. Moreover, the overexpression of Rad51 confers cell resistance to adriamycin and cisplatin in JAB1-deficient cells, and metformin can inhibit cisplatin-mediated upregulation of RAD51 by reducing the stability of RAD51 protein and increasing its ubiquitination, which improves cisplatin resistance (Lee et al., 2019; Liu et al., 2019). Tamoxifen-resistant breast cancer cells exhibit high-level expressions of BARD1 and BRCA1 genes, which are thought to contribute to the MDR of tumor cells to DNA-damaging chemotherapy drugs, including cisplatin and doxorubicin. Silencing BARD1 or BRCA1 expression or inhibition of BRCA1 phosphorylation by dinaciclib restores the sensitivity of tamoxifen-resistant cells to cisplatin (Zhu et al., 2018).

4.4 TME

The microenvironment around normal human tissues is an important barrier against tumors and can effectively inhibit tumor growth. Tumor cells colonizing normal tissues can change the microenvironment around tumor cells and form the TME by recruiting cancer-associated fibroblasts (CAFs), which regulate immune cells and their secreted factors and form neovascularization using vascular endothelial cells (Wu and Dai, 2017). Current studies have shown that the TME may mediate tumor MDR through various mechanisms, including preventing immune clearance of tumor cells, hindering drug absorption, and stimulating paracrine growth factors to promote cancer cell growth (Figure 2). The TME is generally composed of three parts: matrix components, cellular components, and soluble factors (Vasan et al., 2019). Tumor-associated fibroblasts are the main stromal components of the TME, and their high density in solid tumors increases tumor interstitial fluid pressure and hinders drug absorption. Several studies has also demonstrated the presence of CAFs in TME-mediated resistance, such as in MDR-associated esophageal squamous cell carcinoma patients, pancreatic cancer

patients resistant to gemcitabine, and gastric cancer patients resistant to 5-FU (Assaraf et al., 2019; Fiori et al., 2019; Wu et al., 2021). The tumor immune microenvironment is a subtopic in the study of the TME and has received extensive attention due to its participation in a wide variety of tumor biological processes. Immunosuppressive cells in the TME mainly include Treg cells, tumor-associated macrophages (TAMs), and MDSCs. These cells inhibit the activation, proliferation, and killing function of $CD8^+$ T cells by expressing coinhibitory molecules and secreting immunosuppressive factors, which eventually lead to the immune escape of tumor cells (Mao et al., 2021). Treg cells can inhibit the proliferation and activation of effector $CD8^+$ T cells through the expression of CD25, competitive binding of interleukin (IL)-2, secretion of IL-10 and transforming growth factor (TGF)-β, and other pathways that promote immune escape and lead to immunotherapy resistance (Sasidharan Nair and Elkord, 2018). TAMs can induce and maintain the immunosuppressive state of the TME through various pathways, such as the expression of immune checkpoint molecules (programmed death-ligand 1), production of immunosuppressive factors (TGF-β and IL-10), secretion of chemokines (CCL17 and CCL22), and abnormal metabolism of amino acids (Mantovani et al., 2017). A large number of soluble cytokines in the TME also enable tumors to evade immune surveillance. Soluble factors, such as TGF-β, vascular endothelial growth factor, chemokines, and inflammatory cytokines, constantly change and interact with each other to induce a complex network of changes. They jointly trigger functional changes in immune and tumor cells and participate in the induction of angiogenesis and interstitial fibrosis in the TME to promote its immunosuppressive nature. Thus, it leads to biological behaviors, such as malignant proliferation, invasion, metastasis, and drug resistance of tumors (Batlle and Massagué, 2019; Khalaf et al., 2021).

4.5 CSCs

CSCs are subsets of cancer cells with self-renewal and multidirectional differentiation properties. The CSCs theory states that tumor growth is driven by a small number of CSCs hidden in the cancer. This theory explains why tumor recurrence, metastasis, and MDR are almost inevitable after the initial successful chemotherapy or radiotherapy (Figure 2). This subset has been detected in most blood systems and solid tumors (Batlle and Clevers, 2017; Walcher et al., 2020). CSCs not only exhibit strong proliferation and differentiation abilities but are also considered a source of tumor heterogeneity (Nassar and Blanpain, 2016). Aldehyde dehydrogenase (ALDH) is a selective marker of CSCs in breast cancer, bladder cancer, embryonic rhabdomyosarcoma, head-and-neck squamous cell carcinoma, and lung cancer, and its high expression of ALDH causes resistance to a variety of chemotherapy and targeted agents, such as cisplatin, etoposide, fluorouracil, and gefitinib (Ginestier et al., 2007; Huang et al., 2013). According to current studies, CSCs mainly cause MDR through dormancy, EMT, MDR, and anti-DNA damage-induced death (Phi et al., 2018). After tumor formation, CSCs are considered quiescent or in G0 phase (Schmidt-Kittler et al., 2003). Chemotherapy in the traditional sense often causes irreversible damage to dividing tumor cells by interfering with or inhibiting DNA or RNA synthesis or inhibiting key enzymes required for DNA synthesis. However, most CSCs are in the G0 stage and are thus insensitive to chemotherapy drugs. In such cases, the use of chemotherapeutic drugs usually results in the elimination of tumor cells but enriches CSCs and hence allows the development of MDR (Kurtova et al., 2015). Nestin-ΔTK-IRES-GFP transgenic mice were used to label resting CSCs and glioma tumor cells in a mouse glioma model. After temozolomide treatment, the dividing tumor cells were effectively killed, but GFP-labeled resting CSCs proliferated rapidly (Chen et al., 2012). In addition, the ABC transporter family is involved in the MDR of CSCs, and a considerable number of ABC transporters are commonly overexpressed in cancer, especially in CSCs. CSCs alter DNA damage response (DDR) and repair pathways, and an efficient DDR leads to frequent radiation and chemical multiresistance (Huang et al., 2020).

4.6 EMT

EMT plays an important role in cancer progression, metastasis, and drug resistance (Figure 2). In this process, epithelial cells lose their apical–basal polarity and cell–cell adhesion and transfer to invasive mesenchymal cells, and although the specific links surrounding EMT and cancer metastasis remain to be further studied, the role of EMT in cancer drug resistance has been increasingly recognized (Du and Shim, 2016; Shibue and Weinberg, 2017). In the early 1990s, Sommers et al. observed EMT in two adriamycin-resistant MCF-7 cell lines and one vinblastine-resistant ZR-75-B-cell line (Sommers et al., 1992). Subsequently, studies have revealed that multidrug-resistant tumors, including pancreatic cancer, bladder cancer, breast cancer, etc., are often accompanied by EMT, and the signaling pathways that promote the EMT phenotype lead to MDR of tumors (Du and Shim, 2016). The EMT-mediated aggressive behavior of cancer cells can lead to tumor resistance to paclitaxel (PTX) and DTX. In this case, the upstream mediators of EMT, such as zinc finger E-box binding homeobox (ZEB)1/2, TGF-β, and miRNAs, are involved in regulating the response of cancer cells to PTX and DTX. On the contrary, the sensitivity of cancer cells to PTX and DTX can be restored after the inhibition of EMT by tumor suppressors (Ashrafizadeh et al., 2021). In lung cancer cells, gefitinib treatment can activate NOTCH-1 signaling, which results in an acquired EMT phenotype and treatment resistance (Xie et al., 2012). Although the link between EMT and multidrug-resistant tumors has been reported for a long time, the involved mechanisms remain elusive. One such mechanism is the remarkable similarity between the signaling pathways activated during EMT and those of CSCs (Huber et al., 2005). ZEB1 is a TF associated with EMT and can regulate CSCs self-renewal and drug resistance by regulating O-6-methylguanine methyltransferase via miR-200c and c-MYB (Siebzehnrubl et al., 2013). CSCs express various markers of normal stem cells, including their ability to survive in a dedifferentiated state (Gupta et al., 2019). However, cells undergoing EMT also have stem-like characteristics, and EMT and stem cell markers are co-expressed in tumor cells of patients with tumor metastasis (Oskarsson et al., 2014). In addition, EMT

induction or activation of EMT TFs endows tumor cells with stem-like characteristics (Puisieux et al., 2014). Altogether, EMT and CSCs often share key signaling pathways and drug-resistant phenotypes. Cells undergoing EMT similarly overexpress ABC transporters and consequently develop MDR (Du and Shim, 2016). The TME is also a factor mediating EMT-driven drug resistance. Hypoxia is another important TME that promotes cancer cells to undergo EMT and acquire drug resistance. The activation of hypoxia-inducible factor (HIF)-1α under hypoxic conditions promotes EMT in hepatocellular carcinoma (HCC) and induces drug resistance by increasing the expression of MDR1. Knockdown of HIF-1α reversed the EMT phenotype and abolished the drug resistance phenotype of HCC under hypoxic conditions, which further confirmed the role of hypoxia/HIF-1α in EMT-driven drug resistance (Jiao and Nan, 2012).

4.7 Tumor heterogeneity

Tumor heterogeneity refers to the changes in molecular biology or genes during tumor evolution, which lead to differences in the growth rate, invasion ability, and drug sensitivity of different tumor cells in the same tumor (Figure 2) (McGranahan and Swanton, 2017). Tumor heterogeneity can be manifested as spatial and temporal heterogeneities. Spatial heterogeneity describes the uneven distribution of genetically diverse tumor subsets in different disease sites or within a single disease site or tumor (Graf and Zavodszky, 2017). Temporal heterogeneity indicates the occurrence of tumors in patients at different stages, which may face different biological selection pressures; as a result, dynamic changes in individual genetic diversity occur over time (Dagogo-Jack and Shaw, 2018). Most tumors are complex ecosystems that evolve in under strong selection pressures from the microenvironment, including nutritional, metabolic, immune, and therapeutic components. A strong selection pressure promotes the diversification of malignant and benign (i.e., endothelial, mesenchymal, and immune) components of the TME, which eventually leads to a certain degree of tumor heterogeneity. This leads to aggressive disease progression and treatment resistance (Vitale et al., 2019). Chromosomal instability, inherited missense mutations, or epigenetic changes such as DNA methylation or histone modifications, can contribute to changes in tumor heterogeneity (Gorre et al., 2001; Stratton et al., 2009; Negrini et al., 2010). Tumor heterogeneity promotes MDR to epidermal growth factor receptor (EGFR) TKIs in lung cancer. In targeted therapy, the resistance gene mutations caused by tumor heterogeneity exhibit complexity. The difference in resistance mutation sites in the anaplastic lymphoma kinase (ALK) gene between different patients is complicated. Examples include G1202R, G1269A, L1196M, and F1174C (Katayama, 2017; Cooper et al., 2022), which can also exist as comutations of multiple drug resistance sites in the same patient, such as ALK and EGFR-L858R or ALK and BRAF comutation (Kim et al., 2013; Urbanska et al., 2020). Therefore, can targeting multiple signaling pathways in combination with antitumor strategies reduce the development of multidrug-resistant tumors?

5 Links between ERS and multidrug resistant tumors

Whether ERS is a favorable or an unfavorable factor for tumor MDR needs more research. At present, a huge number of evidence indicates the persistence of ERS in a variety of cancer types. However, whether ERS promotes or inhibits the development of patient tumors has been vigorously debated. On the one hand, the UPR is an adaptive response during ERS, and it relists the ER load by reducing the protein source and increasing the route of protein to restore cell homeostasis. Tumor cells can use this feature to promote the progression of malignant tumors and MDR. On the other hand, when overactivated ERS exceeds the threshold that cancer cells can withstand, it will activate proapoptotic pathways and induce cancer cell death (Oakes, 2020). Similar to the abovementioned mechanisms of tumor drug resistance, the mechanisms of ERS leading to tumor MDR are extremely rich and are often considered to be related to enhanced tumor drug excretion, which prevents the apoptosis of tumor cells through chemotherapy drugs, resistance to death through miRNA, and protective cell autophagy.

5.1 GRP78 and multidrug-resistant tumors

Three UPR-related transmembrane proteins are inactivated by GRP78 before ERS. During stress, the amount of unfolded protein in the ER lumen increases, which triggers the dissociation of the three protein receptors from GRP78. Thus, GRP78 can be considered as the initiating part of the entire ERS. GRP78 is a major ER molecular chaperone with Ca2+-binding and anti-apoptotic properties (Luo et al., 2006) and is therefore often considered to be associated with MDR in tumors. Compared with that in normal tissues, the expression of GRP78 is increased considerably in liver cancer, gastric cancer, breast cancer, renal cell carcinoma, and other tumors (Luo and Lee, 2013). GRP78 induces tumor MDR mainly through the following two pathways. On the one hand, it can reduce ER stress and cell apoptosis and therefore increase the resistance of tumor cells to chemotherapy and radiotherapy. GRP78 blocks cell apoptosis by binding and inactivating apoptotic components; GRP78 can bind BIK and caspase-7 in the ER and inhibit the cell apoptosis induced by CHOP (Reddy et al., 2003; Fu et al., 2007). On the other hand, GRP78 on the cell surface (sGRP78) transmits signals to promote the EMT and stemness of cancer cells, which results in MDR (Conner et al., 2020; Xia et al., 2021). In pancreatic cancer, downregulation of GRP78 reduced the clonogenic and self-renewal properties of pancreatic cancer cell lines *in vitro* (Dauer et al., 2019). Similarly, an increased level of GRP78 was found in gefitinib-resistant lung cancer cells, accompanied by the increase in EMT and CSCs characteristics (Liao et al., 2020). ER ribosome-binding protein 1 can enhance the expression of GRP78 and makes lung cancer cells resistant to various chemotherapy drugs, such as tunicamycin and doxorubicin (Tsai et al., 2013). The overexpression of GRP78 can also attenuate the activation of caspase-4 and caspase-7 and the induction of apoptosis by drugs, which leads to the resistance of melanoma to cisplatin and doxorubicin (Jiang et al., 2009). In addition, GRP78 has been identified as a positive regulator of the acquisition of sorafenib resistance in hepatocytes and a major

target for overcoming sorafenib resistance (Chiou et al., 2010). Numerous chemotherapy drugs combined with UPR inhibitors or activators can prevent cytoprotection and induce apoptosis, restore the sensitivity of cancer cells to chemotherapy drugs, and improve the efficacy of chemotherapy drugs. Given its importance in cancer cell resistance, GRP78 has also been a major target of anticancer therapy. The inhibition of glutamine-fructose-6-phosphate aminotransferase activity downregulates GRP78 expression and activates IRE1α, which leads to the increased sensitivity of non-small-cell lung cancer (NSCLC) cells to cisplatin and further initiates the apoptotic pathway (Chen et al., 2019). In pancreatic cancer cells, the combined treatment with siGRP78 (small-interfering RNA (siRNA) for GRP78) reduced the percentages of chemotherapeutic drug efflux to 27.1% and 2.9%–0.56% and 0.68%, respectively, compared with that of gemcitabine or PTX alone. This process is mediated by ABC transporters and regulated by the TF NRF2. NRF2 is a downstream target gene of PERK, and its activation upregulates the expressions of stress response proteins, drug metabolism enzymes, and ABCB1-encoded MDR1, which reverse the MDR of tumors (Salaroglio et al., 2017). Similarly, the inhibition of GRP78 expression by siRNA increases the apoptosis and sensitivity of breast cancer cells to chemotherapy and restores the anti-estrogen sensitivity of drug-resistant breast cancer cells (Yang et al., 2020a). In laryngeal squamous cell carcinoma (LSCC), the overexpression of miR-936 can substantially reduce the protein level of GPR78, inhibit the proliferation, migration, and invasion of LSCC cells, and improve sensitivity to doxorubicin and cisplatin (Lin et al., 2020). Overall, GRP78 has been extensively studied in multidrug-resistant tumors, and the findings identify GRP78 as a novel therapeutic target against MDR to chemotherapy in cancer cells.

5.2 PERK pathway and multidrug-resistant tumors

PERK often plays a key role in inducing apoptosis, which implies its close relationship with cancer treatment and drug resistance. PERK activates CHOP by modulating ATF4 expression through trans-autophosphorylation and phosphorylation of eIF2α after the dissociation of GRP78. CHOP promotes protective autophagy, which leads to drug resistance by inhibiting mammalian target of rapamycin (mTOR) complex 1 and promoting the expression of the ATG5–ATG12–ATG16L complex. CHOP is also a mediator of apoptosis. miR-146a induces drug resistance by inhibiting CHOP-mediated apoptosis. P-Glycoprotein, whose level increases after PERK activation, pumps several intracellular drugs out of tumor cells, which in turn reduces drug-induced apoptosis of tumor cells and leads to drug resistance (Cao et al., 2021). In terms of inhibiting tumor apoptosis, insulin resistance can lead to 5-FU resistance in HCC through activation of the PERK pathway and upregulation of Bcl-2 anti-apoptotic protein (Liu et al., 2016). In addition, the treatment of renal cell carcinoma cell lines with sunitinib *in vitro* increases GRP78 expression, which promotes the proliferation of renal carcinoma cells under hypoxia/hypoglycemia stress and resistance to apoptosis by stimulating PERK/eIF2α signaling (Correia de Sousa et al., 2023). In enhancing resistance to antitumor drug efflux, gene profiling demonstrated high levels of PERK in chemotherapy-resistant human colon cancer cells by. The related study further revealed a link between PERK and the nuclear receptor and TF Nrf2, which directly regulates the transcription of the ABC cassette transporter multidrug resistance protein 1 (MRP1). Targeting the PERK/Nrf2/MRP1 axis eliminated resistance to chemotherapy (Salaroglio et al., 2017). In terms of interaction with miRNA, the ATF4/PERK pathway also interacts with the long noncoding RNA (lncRNA) ZFAS1 signaling pathway, which is important in sorafenib resistance. Sorafenib, which is thought to resensitize cells to itself, may promote ZFAS1 activation by activating the PERK/ATF4 pathway and inhibiting PERK signaling in resistant HCC cells (Lin et al., 2021a). In addition, Golgin A2 pseudogene 10 (GOLGA2P10) is a pseudogene-derived lncRNA and is a nonfunctional residue formed during the evolution of a gene family. Pseudogenes are similar to normal genes but without normal function; they often exist in multiple gene families in eukaryotes. GOLGA2P10 is frequently upregulated in HCC tissues, induced by PERK/ATF4/CHOP signaling, and protects tumor cells from ER stress-induced apoptosis by regulating members of the Bcl-2 family of antiapoptotic proteins (Wu et al., 2020). In terms of drug resistance caused by CSCs, histone methyltransferase G9a is a potential target for epigenetic therapy of acute myeloid leukemia, and PERK/NRF2 signaling plays a key role in protecting leukemia stem cells (LSCs) from ROS-induced apoptosis, which confers LSCs with resistance to G9a inhibitors. Treatment with PERK/NRF2 or autophagy inhibitors overcomes the resistance to G9a inhibition and eliminates LSCs (Jang et al., 2020). Oral squamous cell carcinoma (OSCC) cells with high expression of CD10 also have CSC-related characteristics, which in turn affect tumor growth, EMT, and cisplatin resistance. CD10-positive cells secrete IL8 and promote cisplatin resistance in OSCC through the PERK signaling pathway (Pu et al., 2021). PERK promotes the binding of ATF4 to TRB3 through eIF2α phosphorylation, which inhibits the AKT/mTOR axis and increases basal autophagy in melanoma cells. The activation of the PERK-CHOP axis also promotes the transcription of autophagy genes through cooperation with ATF4 and C/EBPβ (Kong et al., 2022). The inhibition of PERK-dependent ERS using the PERK inhibitor GSK2606414 abolishes the resistance caused by BRAF-induced autophagy in melanoma cells (Ma et al., 2014). Similarly, vemurafenib induces resistance through autophagy in BRAF-mutant thyroid cancer cell lines. This autophagy is associated with the induction of eIF2α phosphorylation and CHOP expression by vemurafenib; in addition, autophagy inhibitors can effectively enhance the antitumor activity of vemurafenib in thyroid cancer and are tolerated well *in vivo* (Wang et al., 2017). One of the reasons for the high mortality rate of osteosarcoma is its easy resistance to chemotherapy drugs. Sestrin2, one of the most important cellular stress proteins, is highly expressed in surviving osteosarcoma cells after chemotherapy. Sestrin2 activates autophagy by inhibiting mTOR through the PERK–eIF2α–CHOP pathway and inhibits apoptosis through Bcl-2. In addition, a low sestrin2 expression can effectively reduce autophagy, increase p-mTOR expression, and reduce Bcl-2 expression in human osteosarcoma cells after chemotherapy of NU/NU mice. It can also promote the apoptosis of osteosarcoma cells (Tang et al., 2021). In a study related to pancreatic cancer, the use of TGF-β1

or cobalt chloride to simulate a severe hypoxic environment induced EMT in pancreatic ductal adenocarcinoma cells, and further treatment with acriflavine inhibited this conversion process. Gene enrichment analysis showed that by blocking eIF2a phosphorylation and reducing ATF4 translation, acriflavine inhibited the unfolded protein-responsive PERK/eIF2a/ATF4 pathway, that is, acriflavine restored the drug sensitivity of acquired drug-resistant pancreatic cancer cell lines. Therefore, targeting the PERK/eIF2a/ATF4 pathway can be used to inhibit EMT in pancreatic cancer cells. In return, the drug resistance of tumors can be reversed (Dekervel et al., 2017).

5.3 IRE1α pathway and multidrug -resistant tumors

As the most conserved signaling pathway in the UPR, IRE1α participates in the regulation of various stages of tumor development, and XBP1 plays an irreplaceable role in promoting cell survival and tumor MDR. Therefore, the close regulation of the IRE1α pathway can be used as an effective treatment of multidrug-resistant tumors (Shi et al., 2019). IRE1α is activated upon dissociation from GRP78, and the downstream XBP1 upregulates heat shock factor 1 (HSF1) expression. Meanwhile, HSF1 upregulates the expression of Bcl2-associated athanogene-3and stabilizes the expression of the antiapoptotic protein Mcl-1, which inhibits cell apoptosis and leads to drug resistance. In addition, IRE1α and ATF6 promote the expression of XBP1 and downstream HSF1. HSF1 promotes Bcl-1 expression through the receptor-interacting serine/threonine-protein kinase 1-mitogen-activated protein kinase (MAPK) 8/9 axis to induce protective autophagy, which leads to drug resistance. IRE1α also triggers Wnt signaling and nuclear factor (NF)-κB to promote tumor cell survival, which in turn reduces drug-induced apoptosis and results in MDR (Cao et al., 2021). Anticancer drugs, such as 5-FU, can activate the IRE1α-XBP1 pathway to induce the expressions of ABCB1, ABCC1 and ABCG2 in colon cancer cells. The inhibition of IRE1α RNase activity with the small-molecule 4μ8c suppresses the drug-induced expression of these ABC transporters and resensitizes 5-FU-resistant colon cancer cells to drug treatment (Gao et al., 2020). The overexpression of activated protein kinase (RACK1) prevents the apoptotic effect of sorafenib on HCC cells by upregulating XBP1 (Zhou et al., 2015). In addition, IRE1α can cleave and regulate miRNA (Kim and Croce, 2021), which is particularly interesting in the context of drug resistance. miRNA upregulation can confer resistance to 5-FU in HCC cell lines. miR-122 specifically targets the membrane transporter SLC7A1 that is associated with sorafenib resistance. The upregulation of miR-122 may reduce SLC7A1 expression and resensitize HCC cells to sorafenib treatment (Khaled et al., 2022). Sorafenib exposure of HCC cells can upregulate the IRE1α signaling pathway to induce autophagy. *In vitro* and *in vivo* studies showed that HCC cells were resensitized to ER stress-induced cell death when autophagy was inhibited (Shi et al., 2011). Tamoxifen has been widely used to reduce estrogen receptor (EsR)-positive breast cancer patients; however, approximately half of EsR-positive breast cancer patients exhibit chemotherapy resistance. XBP1s expression is highly correlated with EsR-positive breast cancer patients, and STF-083010 is an XBP1 splicing inhibitor. It can reverse the sensitivity of drug-resistant cells to tamoxifen (Shi et al., 2019). EsRβ reduces tumor survival in antiestrogen-sensitive and antiestrogen breast cancer cells. Some scholars believe that the upregulation of EsRβ can inhibit the expressions of IRE1 and XBP1, increase the sensitivity of tumor cells to tamoxifen, and cause the apoptosis of chemoresistant cells (Rajapaksa et al., 2015). In normal cells, IRE1 activity is impaired under sustained ERS, which leads to PERK-mediated apoptosis (Lin et al., 2007); however, in melanoma cells, IRE1 activity is maintained through the MEK/ERK pathway, which counteracts the PERK-mediated apoptosis (Tay et al., 2014). Meanwhile, PERK/eIF2α/ATF4 signaling protects chemotherapy-resistant hypoxic cells by inducing glutathione synthesis and reducing ROS accumulation. Activated ERS may also activate NF-κB and inhibitor of apoptosis (IAP) through the IRE1α–TRAF2 pathway, which leads to MDR; therefore, the use of IAP antagonists can enhance the effectiveness of melanoma therapy (El-Khattouti et al., 2016; Bai et al., 2021; Kong et al., 2022). The efficacy and safety of apatinib in the treatment of advanced gastric cancer and other tumors have been confirmed. Apatinib can induce autophagy in colorectal cancer cell lines through ERS, specifically through the IRE1α signaling pathway. Apatinib-induced protective autophagy has been considered a possible new drug resistance mechanism, and blocking autophagy can promote apoptosis in apatinib-treated colorectal cancer cell lines (Cheng et al., 2018). Sunitinib triggers protumor NF-kB activity through the IRE1α/TRAF2/IKKβ signaling axis, which promotes cell survival (Makhov et al., 2018). Tumor cells experiencing ERS can continue to propagate such condition in tumor-infiltrating leukocytes through paracrine signalling (Jiang et al., 2020), which contributes to the malignant progression and immune tolerance of the host and promotes drug resistance to immunotherapy. Cytokines in the TME, such as IL-4, IL-6, and IL-10, can lead to drug resistance through signal transducer and activator of transcription (STAT)3/6 activation of the IRE1α–XBP1 branch of macrophages (Yan et al., 2016). In addition, increased ROS production in tumor-infiltrating dendritic cells (TDCs) excessively activates IRE1/XBP1. Furthermore, such condition affects lipid metabolism and leads to the abnormal accumulation of liposomes and decreased ability of TDCs to cross-present antigen to T cells. This is also one of the reasons for tumor escape. Silencing XBP1 in TDCs using siRNA can restore its immunostimulatory activity *in situ* in immunotolerant TDCs (Cubillos-Ruiz et al., 2015). In conclusion, the IRE1α signaling pathway plays an irreplaceable role in MDR to chemotherapy and immunotherapy.

5.4 ATF6 pathway and multidrug -resistant tumors

ATF6 is the most mysterious of the three pathways, and the relationship between ATF6 and tumor MDR has not been widely explored. ATF6 can activate GRP78, which inhibits caspase-3 activation, maintains the stability of the ER and the internal environment, and leads to tumor resistance (Shen et al., 2002; Dong et al., 2004). The chemoresistance of ovarian cancer is related to the inhibitor of DNA binding 1 (ID1)-induced

autophagy. ID1 first activates NF-κB signaling by promoting the nuclear translocation of NF-κBp65, which enhances the expression and secretion of IL-6 in cancer cells. It subsequently activates STAT3 through protein phosphorylation of Y705, which promotes the transcription of ATF6 and subsequently induces ERS stress to promote autophagy. As a result, cancer cells develop resistance to cisplatin and PTX treatment. In addition, patients with high ID1 or ATF6 expression have poor overall and progression-free survival due to resistance to platinum therapy (Meng et al., 2020). Similarly, in tumors of the female reproductive system, ATF6 is highly expressed in cervical cancer cells. The upregulation of ATF6 in cervical cancer cells promotes their proliferation and inhibit apoptosis. ATF6 inhibits autophagy but promotes EMT through the MAPK signaling pathway, which is a possible reason for the chemoresistance of cervical cancer cells. The inhibition of ATF6 can promote apoptosis by inhibiting Bcl-2 and increasing the levels of caspase-3 (Liu et al., 2020). In recent studies related to chemotherapy resistance of gastric cancer, Janus kinase 2/STAT3 inhibitors reduced 5-FU resistance and autophagy through ATF6-mediated ERS (Ma and Wang, 2022). Compared with the other two pathways, studies on the ATF6 signaling pathway are limited, which means that this signaling pathway has great potential for research. Moreover, the ATF6 signaling pathway has shown great value in the study of reversing tumor drug resistance.

5.5 Enhancing ERS-mediated proapoptotic pathways in the treatment of multidrug-resistant tumors

The ERS-mediated proapoptotic pathway also offers a promising research direction for the treatment of multidrug-resistant tumors. Under prolonged ERS, the prosurvival function of the UPR is transformed into a proapoptotic signal and executed by mitochondria (Bhat et al., 2017). On the one hand, ER directly activates the apoptotic pathway through ERS-mediated calcium leakage into the cytoplasm, which leads to the activation of death effectors. ATF4, on the other hand, initiates apoptosis upon activation of GADD34 and CHOP. Therefore, the PERK/eIF2α/ATF4 pathway is often considered to play a key role in tumor progression and the development of cancer therapies (Chen et al., 2013; Cheng and Dong, 2018; Li et al., 2019). MCC1734, a derivative of coumarin, showed varying degrees of cytotoxicity against five multidrug-resistant cell lines expressing different resistance mechanisms and could not be pumped out of resistant cancer cells; thus, this compound shows promise in killing multidrug-resistant tumors. MCC1734 possibly exerts antitumor effects by upregulating the p-PERK, eIF2α, ATF4, and CHOP proapoptotic pathways in tumor cells (Lu et al., 2021). Similarly, lobaplatin promotes apoptosis and inhibits the proliferation of HCC by upregulating the PERK-eIF2α-ATF4-CHOP pathway (Li et al., 2019). The abnormal production of secreted mucins (MUCs) is an important feature of pancreatic ductal adenocarcinoma. The overexpressed mucins form a physical barrier to prevent drugs from reaching the target site. The transmembrane mucin MUC-4 is widely involved in the drug resistance in tumor cells. Silencing of MUC-4 gene expression in pancreatic cancer cells increases the rate of cell apoptosis induced by bortezomib through the mitochondrial pathway, which is mediated by the activated CHOP apoptotic pathway (Wissniowski et al., 2012). These results are expected to weaken the resistance of pancreatic cancer, colorectal cancer, and other tumors with a high mucin expression to chemotherapy drugs. In clear-cell renal cell carcinoma (ccRCC), sunitinib-resistant ccRCC cells showed considerably lower death-associated protein kinase 1 (DAPK1) mRNA and protein levels than sunitinib-sensitive ccRCC cells. The overexpression of DAPK1 enhances the apoptosis of sunitinib-resistant ccRCC cells through the ATF6-dependent ERS pathway (Song et al., 2020). Icariside II (IS) exhibits antitumor activity in various cancers, such as liver cancer, breast cancer, prostate cancer, and NSCLC (Xu et al., 2021). The combination therapy involving IS and cisplatin inhibits the proliferation of NSCLC cells and induces apoptosis through the activation of ERS by IS; these actions include the three branches of UPR signaling, namely, PERK, IRE1, and ATF6, and the downstream PERK-eIF2α-ATF4-CHOP pathway, which enhances cisplatin-induced apoptosis (Tang et al., 2022). In diffused large B-cell lymphoma, the overexpression of XBP1 greatly enhances ibrutinib-induced apoptosis in sensitive and resistant cells (Zhang et al., 2021b). Compared with ERS-related prosurvival pathways, studies on proapoptotic pathways are limited, but this does not affect their status and role in tumor MDR. Proapoptotic pathways remain a powerful tool in the addressing the problem of multidrug-resistant tumors.

5.6 Nanotherapeutic and multidrug-resistant tumors

In recent years, with the emergence of nanotechnology, nanocarrier drugs for the treatment of multidrug resistant tumors have been developed, and scientists are conducting extensive exploration and research along this direction. Nanomaterials refer to materials in the nanometer range of 1-100 nm, which have unique optical, magnetic and electrical properties (Cheng et al., 2021; Ashrafizadeh et al., 2023). Compared with traditional drugs, nanocarrier drugs can greatly improve the ability to selectively kill tumors and increase the therapeutic effect of drug-resistant tumors. For example, Quercetin still has anticancer effects on adriamycin and docetaxel resistant prostate cancer cells and can reverse drug resistance. Quercetin can reduce the expression of Bcl-2 protein and induce apoptosis of prostate cancer cells by activating IRE1α pro-apoptotic pathway. However, the main problem with quercetin is its low bioavailability and rapid metabolism. Encapsulation of quercetin into the Nano-vehicle agents either *in vivo* or *in vitro* could delay or prevent its metabolism, thereby maintaining high levels of quercetin in blood and other tissues for a long time (Liu et al., 2014; Hussain et al., 2021). At the same time, questions have been raised about the safety of nanomaterials, whether they can reach the target site smoothly and whether they have an effect on normal tissues. Luteinizing-hormone-releasing hormone (LHRH)-conjugated, polyethylene glycolylated (PEGylated), poly-lactide-co-glycolide nanocapsules conjugated to docetaxel and quercetin were formulated by researchers, The capsule was considered to be biodegradable, non-toxic, and able to target prostate cancer, and the results showed that the capsule exhibited reliable anti-tumor activity both *in vitro* and *in vivo* (Shitole et al., 2020). This combination gives us an important hint that chemotherapeutic drugs combined with ERS modulators and paired with nanocarriers may be a new weapon against drug-resistant tumors. In addition, in order to further improve the efficacy of anticancer drugs, the coupling of monoclonal antibodies with

cytotoxic drugs, called antibody-drug conjugates (ADCs), has been studied using trastuzumab (Tmab) in ADCs system. The results showed an improved therapeutic effect compared with Tmab alone (Nieto et al., 2020). Ginsenoside is a group of naturally occurring chemicals in ginseng extract. ginsenoside Rg3(Rg3) is one of the well-studied ginsenoside. Rg3 can promote the apoptosis of tumor cells through IRE1α, PERK and ATF6 pathways. Investigators developed a folate-targeted PEGylated cyclodextrin-based nanoparticle to co-deliver Rg3 and quercetin, more surprisingly, combine the resulting compound with anti-PD-L1 antibody achieved chemo-immunotherapy for colorectal cancer (Sun et al., 2022a). However, only a relatively small number of nanodrugs have been well developed and put into clinical use, and researchers still face problems such as low drug loading and premature drug leakage (Fu et al., 2022a). With the development of Artificial intelligence (AI), can nanodrugs be combined with AI technology. AI will be used to target and regulate the movement of nanocarriers and the release of chemotherapy, targeted drugs, immune checkpoint inhibitors and ERS modulators, so as to achieve the purpose of combined anti-multidrug resistant tumor treatment.

6 Discussion

At present, resistance to traditional chemotherapy, targeted therapy, or immunotherapy drugs is the most critical factor for tumor resistance or recurrence. Given the universality and complexity of drug resistance mechanisms, when tumor cells become resistant to one drug, they also usually develop different degrees of resistance to other drugs of the same type. Some tumor cells exhibit MDR, which greatly reduces the sensitivity of tumors to drugs and affects patient prognosis. Malignant cells utilize various strategies to proliferate under adverse conditions while suppressing the development of antitumor immune responses; in addition, the continuous activation of ERS sensors confers great tumorigenic, metastatic, and MDR capabilities to malignant cells (Cubillos-Ruiz et al., 2017). Multidrug-resistant tumors are still the main cause of death in cancer patients. Regardless, with the deepening of ERS research, the regulation of ERS prosurvival and proapoptotic pathways has become a tool against multidrug resistant tumors. However, the study of ERS-related multidrug resistant tumors still faces serious challenges, such as how GRP78 senses and measures protein metabolic stress and whether a specific choice should be selected from the three pathways when GRP78 in tumor cells faces the complex TME and generates ERS. It also indicates whether researchers can correctly and precisely select the signaling pathway of ERS leading to drug resistance. Second, researchers can determine whether a threshold exists for cells to identify prosurvival or proapoptotic pathways and whether researchers can detect and modulate that threshold. Thus, when ERS occurs, researchers can downregulate the threshold for tumor cells to switch to the proapoptotic pathway to allow more resistant cells to self-select the proapoptotic pathway and thus reverse tumor resistance. More importantly, when developing related drugs, a drug mechanism that ensures can kill multidrug-resistant cancer cells without affecting normal cells must be determined. Although numerous methods can be used to detect ERS levels *in vitro*, better methods for evaluating ERS *in vivo* are still lacking. Without sensitive and accurate detection indicators, the efficacy of drugs cannot be accurately detected, which results in treatment-related risks and harm. Moreover, compounds targeting ERS modulators can maintain a high degree of specificity and minimize side effects in preclinical or clinical applications. Finally, the types or characteristics of tumors that are prone to ERS-associated MDR remain to be elucidated. Although the research on ERS has achieved extremely rich results, only by dealing with the current bottlenecks can researchers ensure that ERS, which is a tool against MDR, will not become a double-edged sword. Therefore, researchers still need to elucidate the mechanism of ERS in tumor drug resistance and treatment to provide novel ideas for tumor treatment. Finally, the mechanisms of ERS that lead to drug resistance or treatment of drug-resistant tumors are not limited to a certain pathway or mechanism. Mechanisms are often interconnected and interact with each other and form an extremely complex network, which can also become an important direction of ERS research.

7 Conclusion

ERS plays an important role in various aspects of tumor MDR, and its mechanism and application are an important but difficult topic in current tumor research. Numerous studies have confirmed that ERS can promote tumor cell death, improve drug efficacy, and reverse drug resistance through synergistic effects with antitumor drugs. The core issue is the targeted regulation of ERS. The in-depth study of ERS also provides possible therapeutic targets for the treatment of multidrug-resistant tumors and new treatment strategies for drug-resistant patients. Before clinical application, a number of problems remain to be solved. Regardless, the in-depth study of ERS and the mechanism of tumor MDR will certainly create new opportunities for the diagnosis and treatment of tumors.

Author contributions

BQ: Project administration, Writing–original draft, Writing–review and editing. SW: Data curation, Methodology, Writing–review and editing. YD: Data curation, Writing–review and editing. CL: Data curation, Writing–review and editing. WL: Funding acquisition, Resources, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Updating the therapeutic role of ginsenosides in breast cancer: a bibliometrics study to an in-depth review

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Breast cancer is currently the most common malignancy and has a high mortality rate. Ginsenosides, the primary bioactive constituents of ginseng, have been shown to be highly effective against breast cancer both *in vitro* and *in vivo*. This study aims to comprehensively understand the mechanisms underlying the antineoplastic effects of ginsenosides on breast cancer. Through meticulous bibliometric analysis and an exhaustive review of pertinent research, we explore and summarize the mechanism of action of ginsenosides in treating breast cancer, including inducing apoptosis, autophagy, inhibiting epithelial-mesenchymal transition and metastasis, and regulating miRNA and lncRNA. This scholarly endeavor not only provides novel prospects for the application of ginsenosides in the treatment of breast cancer but also suggests future research directions for researchers.

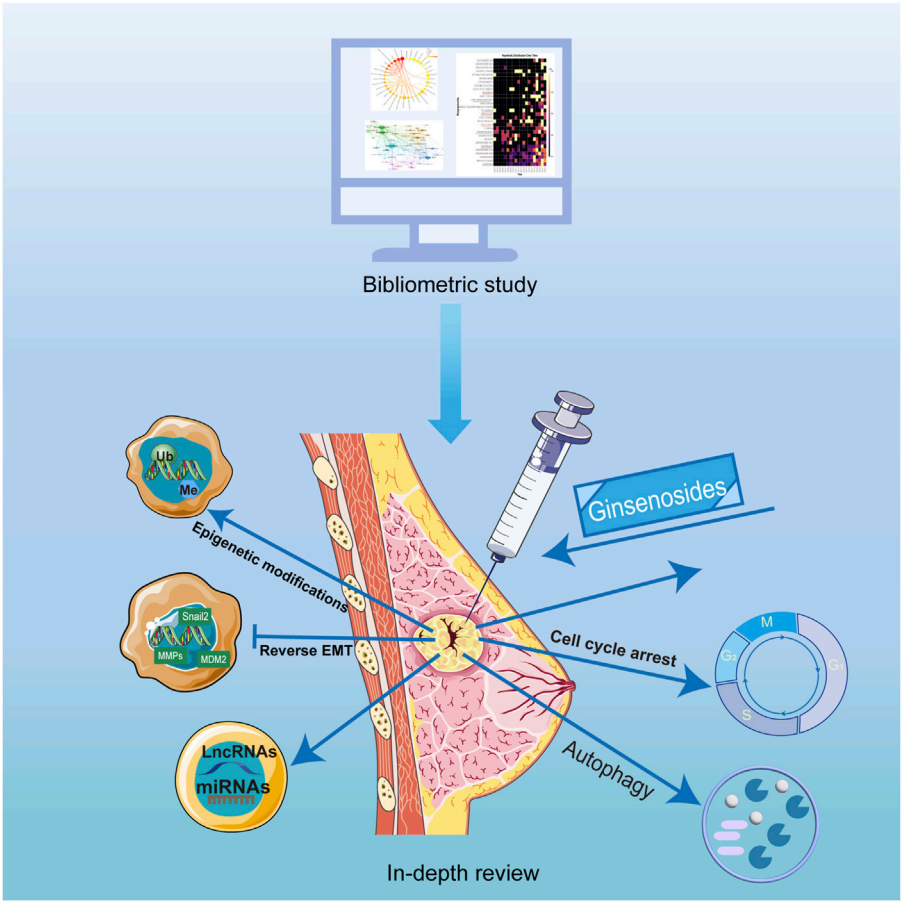
KEYWORDS

ginsenosides, breast cancer, bibliometrics, ginseng, review

1 Introduction

Breast cancer stands as the most prevalent neoplastic affliction globally, with higher rates in developed countries than in developing countries (Sung et al., 2021). Notably, the growth velocity in developing nations outpaces that of developed regions, a trend anticipated to persist (Lim et al., 2022). Compounding this issue, the incidence of breast cancer is increasing annually, and the affected demographic is increasingly skewed toward the younger populace (Fitzmaurice and Global Burden of Disease Cancer Collaboration, 2018). This has resulted in a huge public health burden as well as a huge physical and psychological toll on patients with breast cancer. With the advancement of medicine, not only surgery but also many other treatments have been applied to breast cancer, such as chemotherapy, targeted therapy, and endocrine therapy. These treatments have improved disease-free survival (DFS) and overall survival (OS) of breast cancer patients to some extent. Nevertheless, there are several adverse effects associated with these treatments, and not all breast cancer patients benefit from these treatments due to drug resistance. Especially for triple-negative breast cancer. Consequently, the survival of breast cancer patients remains a challenge. Therefore, it is necessary to develop new drugs to improve the DFS and OS of patients with breast cancer.

The pathologic process of breast cancer development broadly consists of four aspects: precancerous lesions, ductal carcinoma *in situ*, invasive breast cancer, and metastatic breast



GRAPHICAL ABSTRACT

cancer. Each stage involves multiple molecular mechanisms. Pre-cancerous breast lesions are mainly characterized by pathologic morphological and structural abnormalities compared to normal breast tissue (Ellis, 2010). Ductal carcinoma *in situ* (DCIS) is the earliest stage of breast cancer in which the abnormal cells are confined to the milk ducts of the breast. The development of DCIS generally begins through hereditary or acquired mutations that affect tumor suppressor genes (e.g., BRCA1, BRCA2) or oncogenic genes (e.g., HER2/neu), which disrupt cellular regulatory control and promotes unrestricted proliferation, resistance to apoptosis is enhanced (Solin, 2019). Invasive breast cancers include invasive ductal carcinoma and invasive lobular carcinoma, which differ pathologically from DCIS in that the growth of invasive breast cancers breaks through the basement membrane by a mechanism that is mainly due to the increased invasive potential of epithelial-mesenchymal transition (EMT). And invasive breast cancer has active angiogenesis, which promotes tumor growth (Keirsse et al., 2014). Metastatic breast cancer is a coordinated process of tumor cell invasion, circulating cell survival, and distant extravasation driven by EMT, extracellular matrix degradation, and immune evasion. In addition, with modern drug interventions, breast cancers with drug resistance emerge, and the mechanisms of resistance emergence are often related to alterations in the tumor microenvironment, epigenetic changes, EMT and immune evasion (Hashemi et al., 2022).

In recent years, natural compounds in Chinese medicine have gained the attention of many researchers due to their excellent efficacy and mild side effects. Among these, ginseng, an enduring perennial herb hailing from the Araliaceae family, is known for its ability to strengthen the body and improve longevity. Ginsenosides are its principal ingredients, which are thought to have anti-inflammatory, antioxidant, and anti-tumor properties. These ginsenosides are mainly classified into four distinct categories, namely, protopanaxadiol (ginsenosides Ra1, Ra2, Ra3, etc.), protopanaxatriol (ginsenosides Re, Rf, Rg1, etc.), C17 side-chain varied (Rg5, Rk1, Rh4, etc.) and oleanolic acid (ginsenosides R0, Rh3, R1, etc.). Numerous investigations demonstrated that ginsenosides had great potential for the treatment of breast cancer (Ratan et al., 2021). And it is very necessary to summarize and analyze these researches.

Bibliometrics is a way of using powerful statistical methods to retrospectively analyze and summarize research findings, calculate data correlations, and predict the development of future research (Li et al., 2018). Bibliometrics plays an important role in summarizing breast cancer research, ginseng research, and big data medical research (Özen Çınar, 2020, 2009–2018; Zeng et al., 2022). By encapsulating the evolving landscape of scholarly output, bibliometrics lays bare prevailing research focal points while illuminating the compass for impending inquiries. Therefore, we believe that bibliometrics is an appropriate strategy for exploring

research hotspots for ginsenosides in treating breast cancer. The bibliometric studies summarized current research hotspots and provided future research directions in specific areas. Therefore, we believe that bibliometrics is an appropriate strategy to summarize ginsenoside for breast cancer research as well as to discover research hotspots. However, to date, there are no bibliometric studies on ginsenosides for breast cancer in relevant articles. In this review, we first performed a bibliometric overview of ginsenosides in the treatment of breast cancer, followed by an in-depth analysis of the therapeutic effects of ginsenosides in breast cancer based on the bibliometric results.

2 Bibliometric research

2.1 Data and methods

2.1.1 Data download and filtering

The dataset utilized for the bibliometric analysis was obtained from the Web of Science (WoS) Core Collection. The search strategy was devised as follows: SU= [(Ginsenosides*) OR (ginsenoside*)] AND SU = [(breast cancer*) OR (breast carcinoma*)]. The language of the publication was restricted to English, and the time frame spanned from 1 January 2002, to 31 December 2022. The record contents of data were complete records and cited references. To avoid bias due to frequent database updates, all data retrieval and collection was completed within 1 day on 5 April 2023. Details of the search strategy were provided in Supplementary Table S1. The file format was plain text. We filtered the raw data through Bibliometrix (Version 3.13) in the R language (Version 4.2.1). The flow chart of the study of the therapeutic effect of ginsenosides on breast cancer was shown in Figure 1.

2.1.2 Bibliometric analysis and visualization

The main information was extracted using Bibliometrix and VOSviewer, including the annual number of publications, countries, and authors. The aforementioned variables were processed and visualized using Scimago Graphica (Version 2.0), R language, Origin 2022, and VOSviewer (version 1.6.11). For keyword clustering and co-occurrence analysis, we used Pajek, Bibexcel, and Vosviewer to visualize and analyze. The annual occurrences of keywords were visualized using R software.

2.2 General analysis

Based on our search strategy and screening, a total of 208 publications were collected that met the requirements. These publications were written by 1,057 authors from 302 organizations in 28 countries, and were published in 131 journals. Figure 2 displayed the temporal distribution of the number of publications in the area of research on ginsenosides for treating breast cancer. Overall, research on ginsenosides and breast cancer was rare before 2013, with an average of about 5 publications per year. However, the average number of publications per year from 2014 to 2022 reached about 17, indicating a fast growth rate a fast growth rate, and so far, the field of therapeutic effects of ginsenosides on breast cancer is increasingly being studied.

2.3 Distribution of countries/territories and institutions

A total of 28 countries contributed to this study. Table 1 presented the top 14 countries in terms of the number of articles published. Among them, China had the highest number of publications, followed by South Korea, the United States, Vietnam, and Japan, respectively. In terms of article citations, China unsurprisingly had the highest number of citations due to having a large number of publications. But the average number of citations was not high. In contrast, the UK, with only 3 publications, had an average of 60 citations, which reflected the high quality of the articles. Figure 3 presented a geographic bibliometric map based on a network of co-authorship relationships in the top 10 countries in terms of the number of articles published. It is noteworthy that although China and South Korea contributed the most articles, other countries such as the United States, Vietnam, and Japan also provided a significant number of articles. Moreover, in terms of citations, all articles were of relatively high quality.

A total of 302 institutions contributed to this research, Table 2 presented the top 10 institutions in terms of the number of publications. The Jilin University had the most publications but did not receive the highest number of citations. The highest number of citations and the average number of citations were obtained by Hong Kong Polytechnic University. Figure 4 demonstrated the collaborative relationships between multiple institutions. The Chinese Academy of Sciences collaborated the most with other institutions. However, in general, the institutions did not work very closely together.

2.4 Distribution of authors

Table 3 displayed the top 15 authors in terms of number of publications, with a minimum number of 4 publications. Among the highly productive authors, the one with the most publications was Fan Daidi, with a total of 23 publications from 2002 to 2022. Moreover, his articles received up to 158 citations, averaging 20 citations per article. Notably, an author named Wong Man-sau has received 325 citations despite having published only six relevant articles, indicating the high quality of her articles. Similar authors like Zhou Fang, Yang Deokchun, and Zhang Jingwei also gained high citations for their few articles.

Figure 5 illustrated the collaborative relationships among the authors, and we found that six main teams contributed to the research on ginsenosides for breast cancer. However, there was no strong cooperative communication relationship between the different teams, and the strengths of resources were not well integrated and utilized.

2.5 Keyword analysis

The keywords co-occurrence analysis illustrates the evolution process and hot topics. The keyword visualization in this review was presented as a co-occurrence network (Figure 6) and a heat map (Figure 7). For the co-occurrence network, keywords were divided

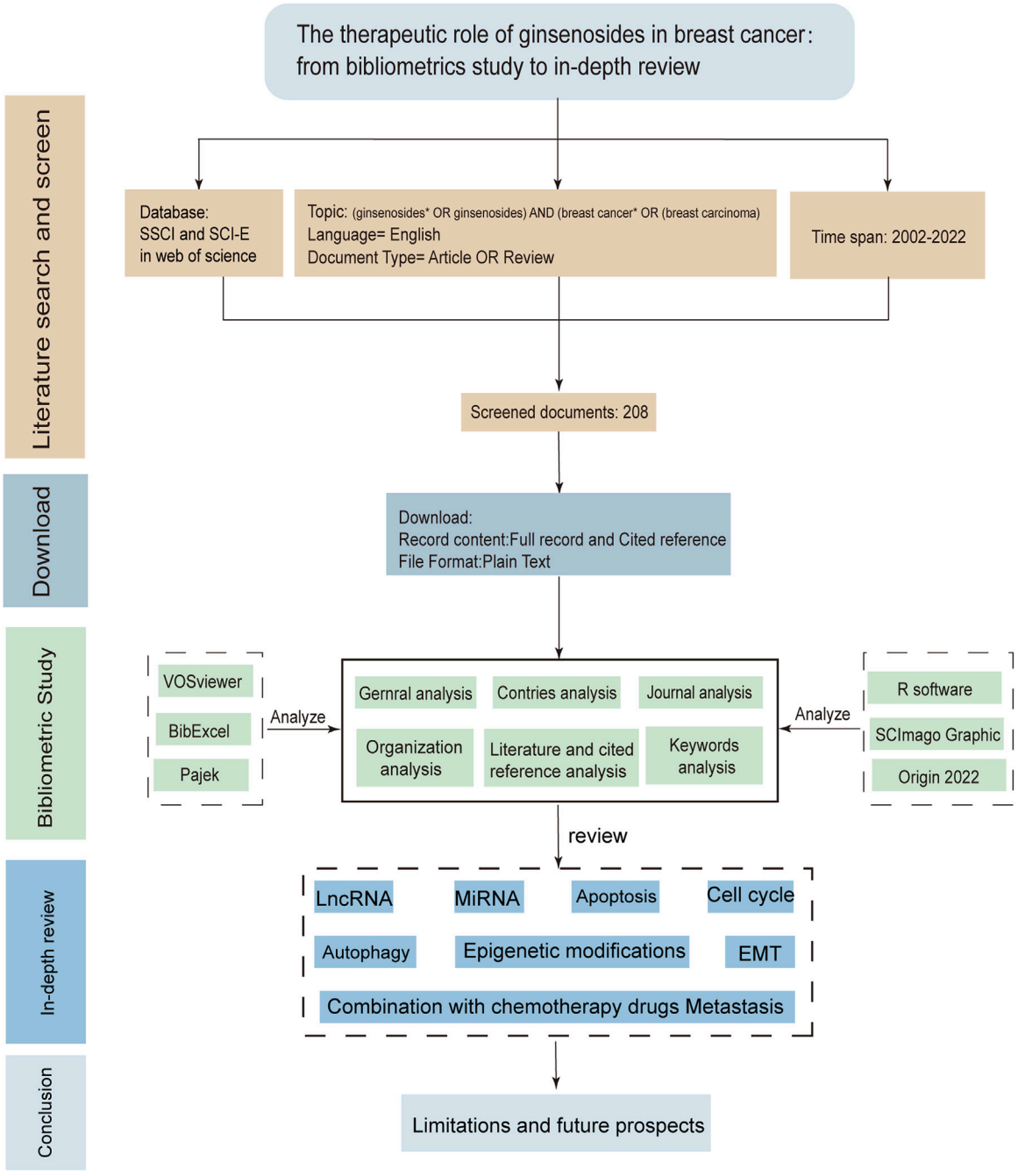


FIGURE 1
The flow chart of study of ginsenosides on breast cancer.

into various clusters represented by different colors based on the correlation. Notably, besides the keywords related to ginsenosides and breast cancer, "apoptosis" and "autophagy" were cross-cutting keywords in these clusters. It suggested that the way ginsenosides treat breast cancer was likely to be through the apoptotic, autophagic pathway. There were similar keywords like EMT, metastasis, cell cycle, etc. To understand the keywords evolution process, we used the keywords with high frequency to map the heat map of the distribution of the keywords over time. As shown in Figure 7, besides the keywords related to ginseng and breast cancer, the keywords like apoptosis, autophagy, metastasis, long non-coding RNA and microRNA appeared more frequently in recent years. These mechanisms may be a hot topic for future research. Preliminarily, these keywords may be the key pathway for ginsenosides in the treatment of breast cancer and these keywords appeared to be the hot topics in recent years. Therefore, we made a more in-depth study of ginsenosides for breast cancer based on these keywords.

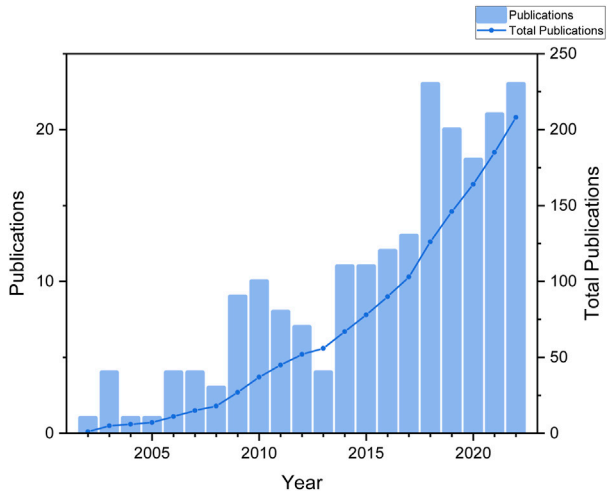


FIGURE 2
The number of publications by year.

3 The regulatory role of ginsenosides on breast cancer

3.1 Induction of apoptosis

Apoptosis, recognized as programmed cell death, represents an innate biological mechanism crucial for orchestrating equilibrium between cellular proliferation and demise within the organism. This process assumes the pivotal role of purging damaged or aberrant cells, preempting their potential deleterious impact. In breast cancer, the normal balance between cell growth and apoptosis is disrupted, leading to the uncontrolled growth and spread of cancer cells. Hence, instigating apoptosis within breast cancer cells emerges as a compelling therapeutic avenue, constituting a strategic maneuver for combating breast cancer and abrogating its propensity for metastatic expansion.

There are two main pathways for apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) (Wilken et al., 2011). These pathways contribute to the activation of caspases and cause cancer cell death. The promotion of apoptosis is a common feature of anti-cancer medications and has been a theme of anticancer drug research for decades. Many experiments demonstrated that ginsenosides induced apoptosis in different types of breast cancer cells through various mechanisms. The effect of ginsenosides in promoting apoptosis on breast cancer cells was shown in Figure 8; Table 4.

In terms of research on the induction of apoptosis in breast cancer cells, it was mainly the two types of ginsenosides, protopanaxadiol and C17 side-chain varied, that have been studied extensively. Signaling pathways play an influential role in apoptosis. As for the induction of apoptosis in breast cancer cells by ginsenosides, the PI3K/AKT signaling pathway was commonly involved. Ginsenoside Rk1 induced apoptosis in MDA-MB-231 cells by upregulating the expression of Bax and cytochrome C, cleaving caspases 3, 8, and 9, and decreasing the expression of Bcl-2 through the ROS/PI3K/Akt signaling pathway (Hong and Fan, 2019). Similarly, Ginsenoside Rg5 induced apoptosis in breast cancer cells by inhibiting the PI3K/Akt signaling pathway in a dose-dependent manner (Liu and Fan, 2018). Moreover, Ginsenoside Rp1 induced apoptosis in MCF-7, MDA-MB-231, and T-47D breast cancer cells through the insulin-like growth factor 1 receptor (IGF-1R)/Akt signaling pathway, thereby inhibiting breast the growth of breast cancer cells (Kang et al., 2011). Ginsenoside Rd inhibited the Akt/mTOR/p70S6K signaling pathway to promote apoptosis and

TABLE 1 The top 14 contries in terms of the number of publications.

Rank	Country	Publications	Total citations	Average citation
1	CHINA	132	3,800	29
2	SOUTH KOREA	49	1,662	34
3	USA	20	759	38
4	VIETNAM	7	222	32
5	JAPAN	6	197	33
6	AUSTRALIA	5	145	29
7	CANADA	4	151	38
8	INDIA	4	19	5
9	IRAN	4	65	16
10	ENGGLAND	3	179	60
11	RUSSIA	3	103	34
12	BRAZIL	2	91	46
13	EGYPT	2	23	12
14	PAKISTAN	2	41	21

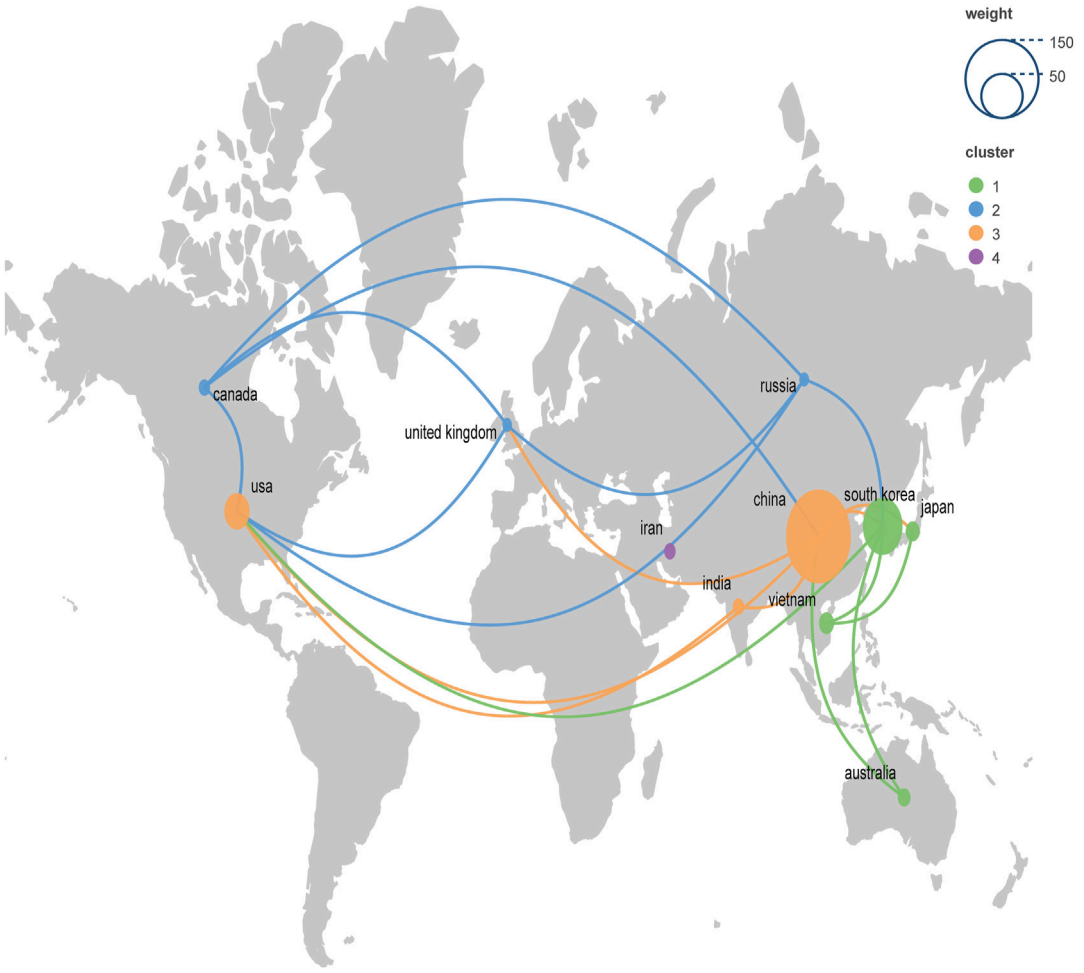


FIGURE 3
Geographic bibliometric map based on a network of co-authorship relationships in the top 10 countries in terms of number of articles published.

TABLE 2 The top 10 institutes in terms of the number of publications.

Rank	Institute	Publications	Citations	Average citation
1	Jilin University	11	170	15
2	China Pharmaceutical University	9	292	32
3	Chinese Academy of Sciences	9	310	34
4	Chungnam National University	9	172	19
5	Kyung Hee University	9	311	35
6	SEOUL National University	9	510	57
7	Hong Kong Polytechnic University	8	512	64
8	northwest university	8	158	20
9	Dongguo University	7	128	18
10	Fudan University	6	119	20

suppressed angiogenesis in MDA-MB-231 cells (Zhang et al., 2017). In addition, the NF-κb signaling pathway also regulates apoptosis. Some experiments demonstrated that ginsenoside Rg3 promoted apoptosis in triple-negative breast cancer cells by inhibiting the NF-κb signaling pathway (Kim et al., 2014; Yuan et al., 2017).

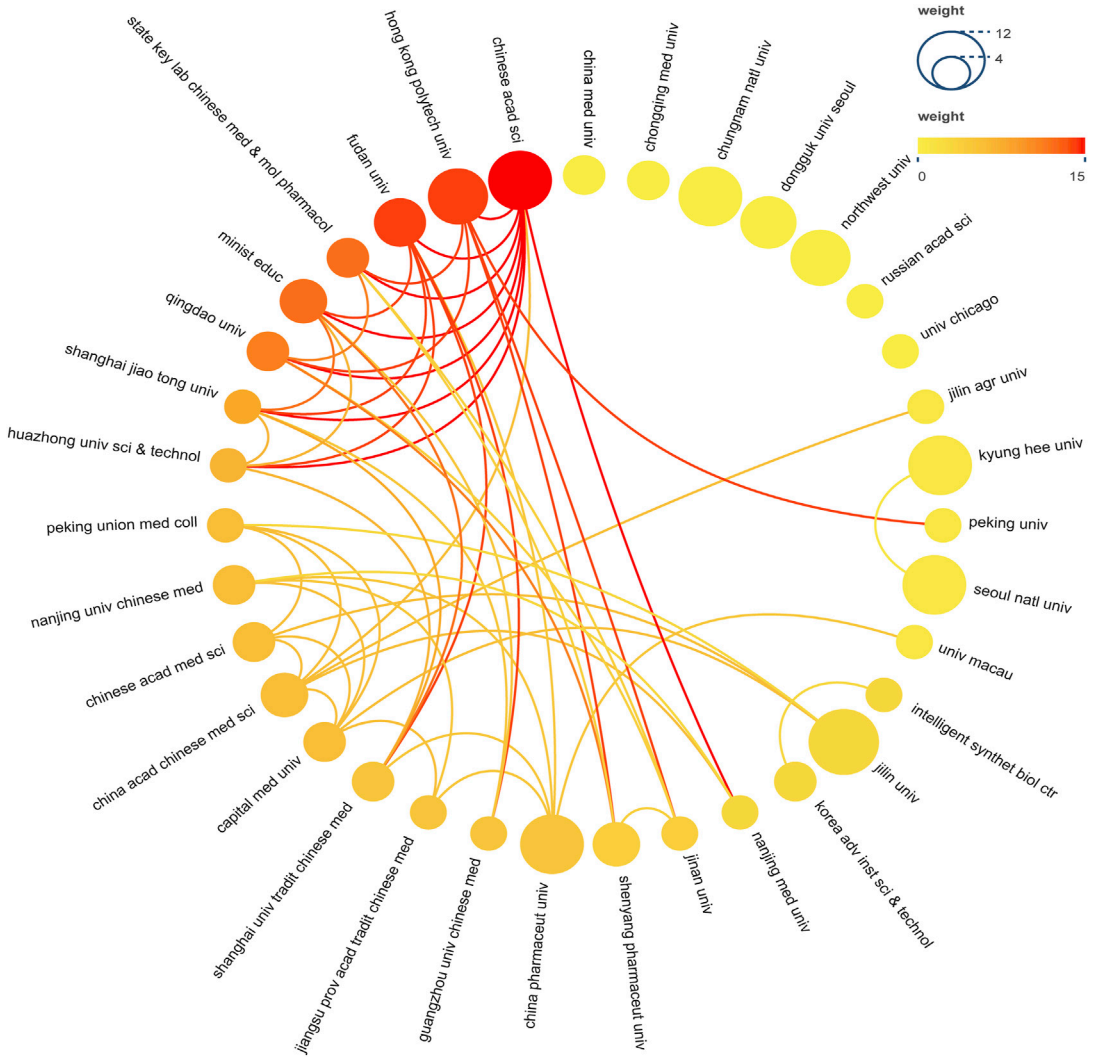


FIGURE 4
Institutional Cooperation Network Map. In the network, number of documents reflected by node size. The connection strength is reflected by the color.

Bcl-2/Bax/Caspase-3 is a signaling pathway closely related to apoptosis. Ginsenosides promote apoptosis in breast cancer cells by regulating the Bcl-2/Bax/Caspase-3 signaling pathway. Several experiments showed that ginsenoside Rh2 induced apoptosis in MCF-7 and MDA-MB-231 human breast cells (Choi et al., 2011), associated with mitochondria-mediated apoptosis. Besides, under the effect of ginsenoside Rh2, the expression of anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 was downregulated, whereas the expression of pro-apoptotic factors Bak, Bax, and Bim was upregulated, causing mitochondrial translocation of Bax and caspases activation. It is reported that ginsenoside Rh4 induced apoptosis of breast cancer cells by reducing Bcl-2, increasing Bax, and activating caspase-8, 3 (Duan et al., 2018). These observations highlight that ginsenosides induce apoptosis in various breast cancer cells and interact with multiple pathways and targets.

The NF-κb signaling pathway and PI3K/AKT signaling pathway are signaling pathways closely related to apoptosis. Consequently, prevailing investigations into ginsenoside-induced apoptosis in breast cancer predominantly converge upon these two pathways, while other apoptosis-related pathways, such as the MAPK signaling pathway and Wnt signaling pathway, have not been studied yet. The pathway of inducing apoptosis in breast cancer cells by ginsenosides is supposed to be multi-pathway and multi-targeted, but the current study is more limited to the classical pathway, so it is necessary to explore the mechanism of inducing apoptosis by ginsenosides from various aspects. In addition, other types of ginsenosides have been less studied in this aspect of apoptosis, and more research on other types of ginsenosides should be conducted.

3.2 Regulation of breast cancer cell cycle and proliferation

The cell cycle is a tightly regulated process that controls the growth and division of cells in the body. It consists of several distinct phases, including G1, S, G2 and M. In breast cancer, cell cycle

TABLE 3 The top 15 authors in terms of number of publications.

Rank	Author	Publications	Citations	Average citation
1	fan, daidi	8	158	20
2	heo, kyung-sun	7	92	13
3	kim, sun jung	7	128	18
4	jin, yujin	6	73	12
5	wong, man-sau	6	325	54
6	kim, hyeon woo	5	58	12
7	wang, guangji	5	181	36
8	zhou, fang	5	181	27
9	jeong, dawoon	4	108	12
10	myung, chang-seon	4	46	9
11	wang, jianxin	4	37	9
12	xia, jiaxuan	4	37	9
13	yang, deok chun	4	90	23
14	zhang, jingwei	4	125	31
15	zhu, ying	4	37	9

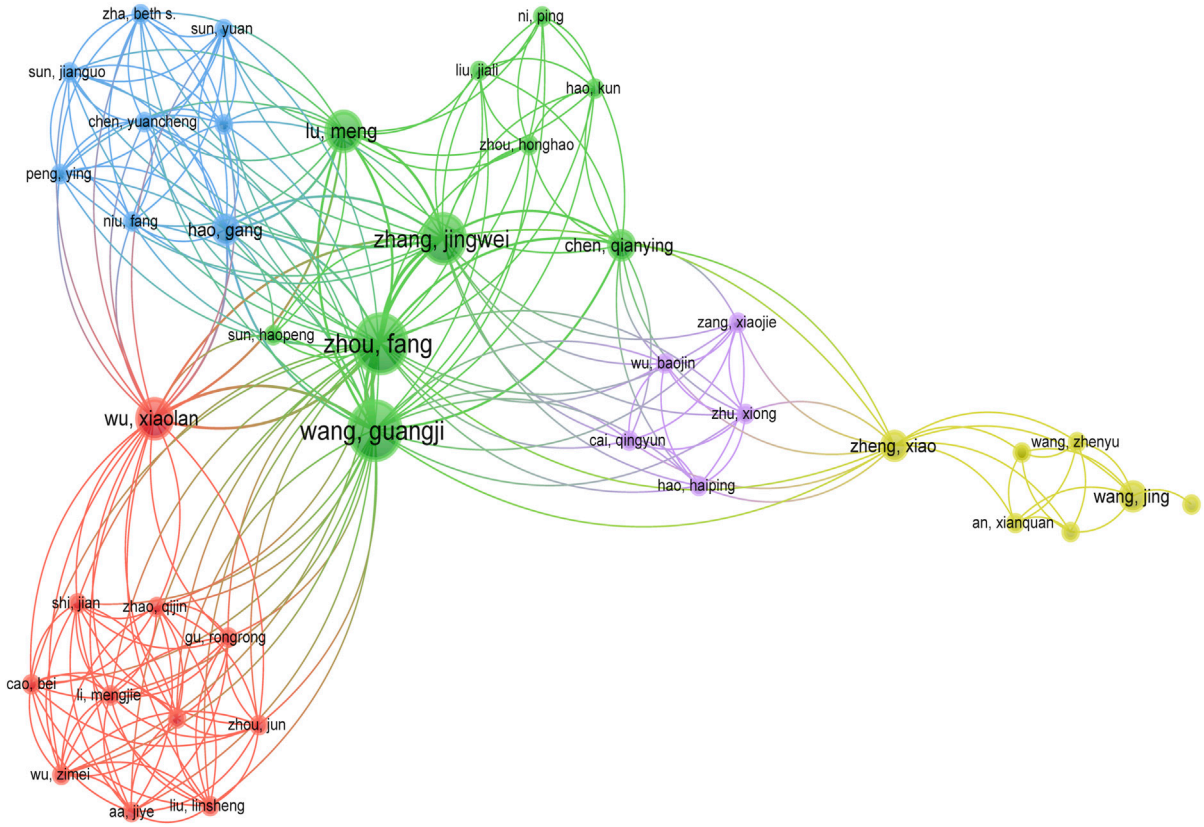


FIGURE 5
Co-authorship network map.

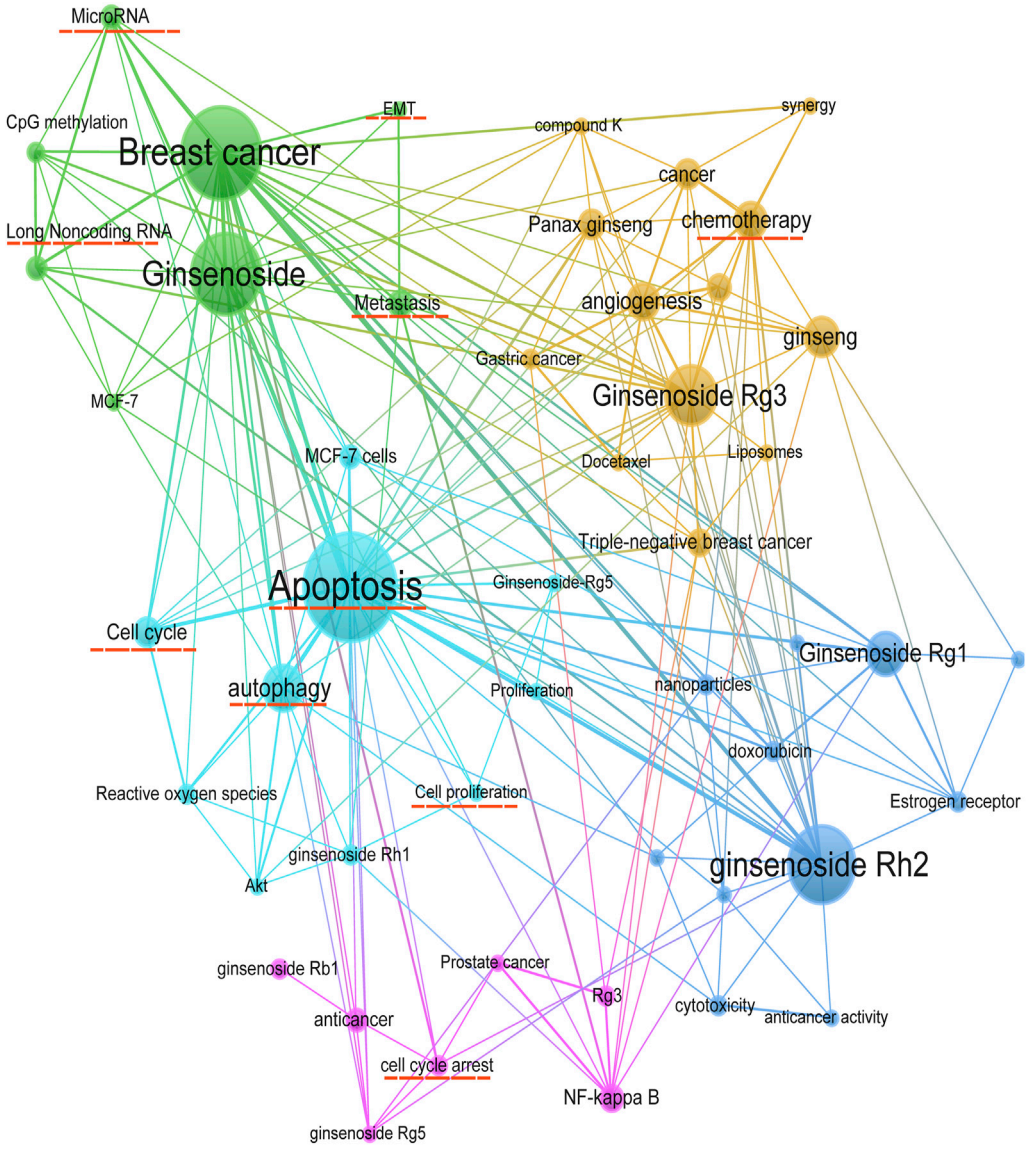


FIGURE 6
Keywords co-occurrence network.

deregulation leads to malignant proliferation of cancer cells. Specifically, mutations or alterations in genes that regulate the cell cycle can disrupt the normal progression of cells at different stages of the cycle, leading to abnormal proliferation and tumor formation. Many current breast cancer therapies, such as chemotherapy and endocrine therapy, slow or stop the growth of cancer cells by blocking the cell cycle.

Key regulators of this process are cell cycle-dependent kinases (CDKs) and cell cycle inhibitory proteins (CKIs). CDKs are proteins that promote cell cycle progression, while CKIs are proteins that inhibit cell cycle progression. When these regulatory proteins are not functioning properly, cells can begin to divide uncontrollably, leading to the growth of a tumor (Fry et al., 2004; Ding et al., 2020). Researchers envisioned blocking the cell cycle to stunt unlimited cell proliferation. Moreover, they have successfully developed CDK4/6 inhibitors to block the cell cycle and have shown promising results in the treatment of hormone receptor-positive breast cancer (Finn et al., 2015; Piezzo et al., 2020; Schettini et al., 2020). Numerous studies have demonstrated that ginsenosides inhibited various types of breast cancer cells. Ginsenosides Rk1 and Rp1 contributed to cell cycle arrest in triple-negative breast cancer cells (Kang et al., 2011; Hong and Fan, 2019). Ginsenosides Rh4, Rp1, Rg5, Rh2, 20(S)-Protopanaxadiol all induced cell cycle arrest in hormone receptor-positive breast cancer cells (Kang et al., 2011; Kim and Kim, 2015, 5; Duan et al., 2018; Zhang et al., 2018; Peng et al., 2022). More specifically, the mechanism of ginsenoside blocking the cell cycle in breast cancer affected the expression of CDKs and CKI. Ginsenoside Rg5 upregulated the expression of CKI, such as p53, p21, and p15, and downregulated the expression of cycle-dependent kinases, such as Cyclin D1, Cyclin E2, and CDK4 (Kim and Kim, 2015, 5). Therefore, these current studies revealed that ginsenosides induced cell cycle arrest by regulating cell cycle-related proteins. The effects of ginsenosides on the cell cycle of breast cancer were shown in Figure 9; Table 5.

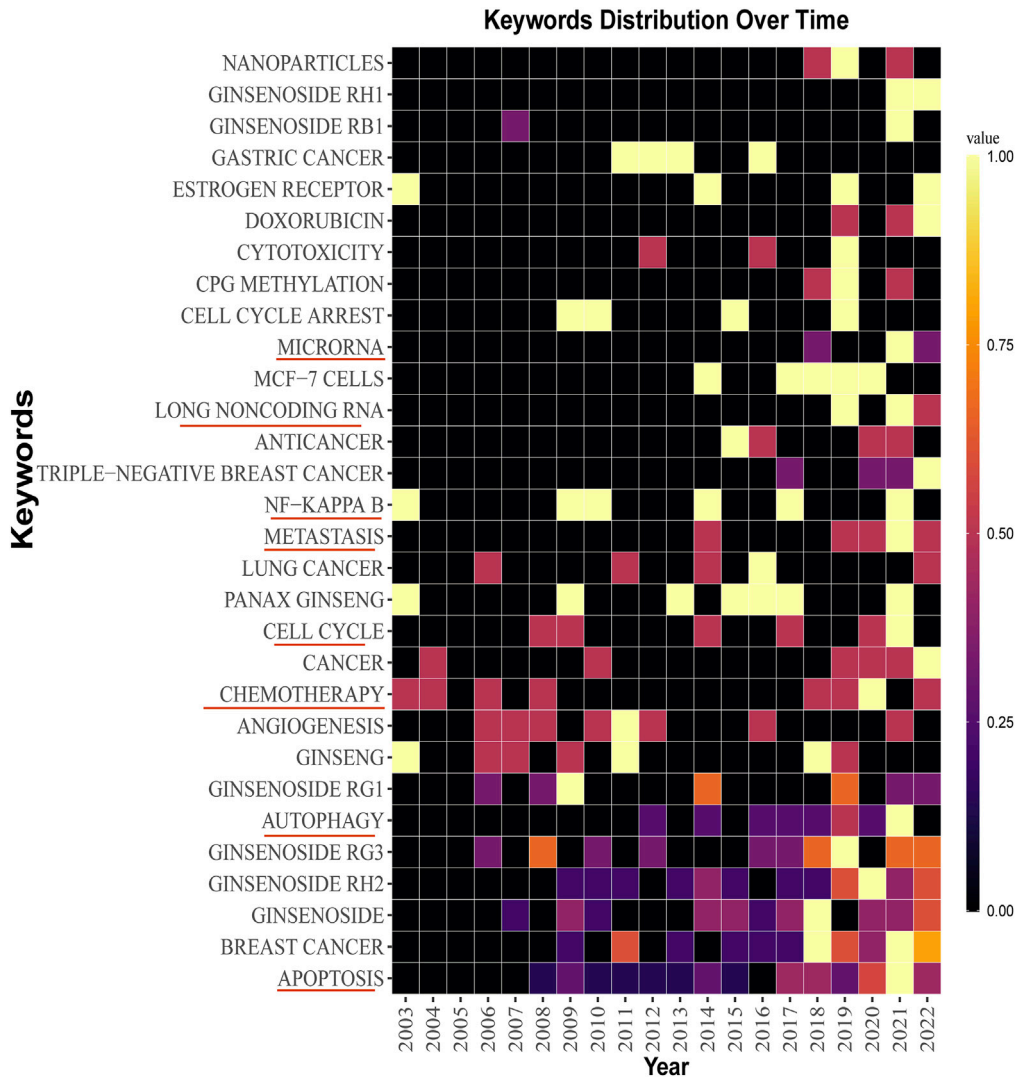


FIGURE 7
Keywords distribution over time.

Regulation of cell cycle to inhibit tumor growth is a fundamental property of antitumor drugs, but for the wide variety of ginsenosides, only a small number of ginsenosides with cell cycle regulation have been found in the current study. Moreover, some studies involved only one type of breast cancer. Therefore, further studies are needed to investigate the cell cycle regulatory effects of other types of ginsenosides on different types of breast cancer. In addition, the current study on the mechanism of ginsenosides regulating the cell cycle is not deep enough, and it is necessary to further elucidate the deep mechanism of ginsenosides regulating the cell cycle.

3.3 Induction of autophagy

Autophagy is the process of self-degradation of some intracellular components through the autophagic lysosomal pathway. On the one hand, autophagy induces apoptosis. When autophagy is overactivated, lysosomes degrade cellular components and induce cell death (Linder and Kögel, 2019). On the other hand, autophagy inhibits apoptosis. Autophagy provides nutrients to tumor cells in nutrient-deficient and low-oxygen environments and protects them from apoptosis (White, 2015; Onorati et al., 2018). The bi-nature of autophagy has attracted the attention of many scholars. Accumulating evidence showed that ginsenosides induced autophagy of breast cancer cells.

Ginsenoside Rg5 exerted a profound impact on breast cancer cell proliferation by invoking mitochondria-mediated autophagic cell death, as evidenced by a notable attenuation of cellular proliferation (Liu and Fan, 2018). Similarly, ginsenoside Rh1 and ginsenoside (20S)-protopanaxatriol induced non-protective autophagy and inhibited breast cancer cell proliferation through the PI3K/AKT pathway (Huynh et al., 2021; Li et al., 2021). Its mechanism was associated with the formation of autophagosomes and elevated expression levels of LC3BII, P62, and Atg proteins. In addition, these observations highlight that ginsenosides not only promote

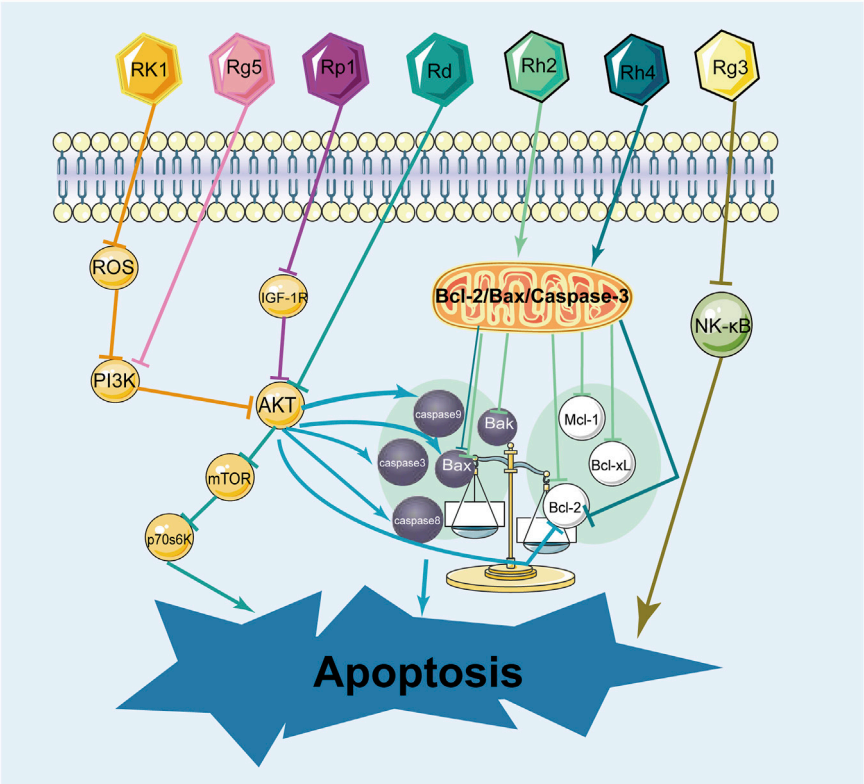


FIGURE 8
The mechanism of ginsenosides promoting apoptosis in breast cancer cells.

TABLE 4 Intervention mechanism of different ginsenosides on apoptosis in breast cancer cells.

Subtype	Saponins	The mechanism of inducing apoptosis	References
C17 side-chain varied	Ginsenoside Rk1	Inhibition of the ROS/PI3K/Akt signaling pathway decreased Bcl-2 expression, upregulated Bax and cytochrome C, cleaving caspases 3, 8, and 9 expression	Hong and Fan (2019)
C17 side-chain varied	Ginsenoside Rg5	Dose-dependent inhibition of PI3K/Akt signaling pathway	Liu and Fan (2018)
C17 side-chain varied	Ginsenoside Rp1	Inhibition of the IGF-1R/Akt signaling pathway	Kang et al. (2011)
C17 side-chain varied	Ginsenoside Rh4	Reduced Bcl-2, increased Bax and caspase-8, 3 expression	Duan et al. (2018)
Protopanaxadiol	Ginsenoside Rd	Inhibition of the Akt/mTOR/p70S6K signaling pathway	Zhang et al. (2017)
Protopanaxadiol	Ginsenoside Rg3	Inhibition of NF-κb signaling pathway	Kim et al. (2014)
			Yuan et al. (2017)
Protopanaxadiol	Ginsenoside Rh2	Downregulation of Bcl-2, Bcl-xL, and Mcl-1 and increased expression of Bak, Bax, and Bim resulted in mitochondrial translocation of Bax and activation of caspases	Choi et al. (2011)

apoptosis in breast cancer cells through the PI3K/AKT signaling pathway, but also induce cellular autophagy through this signaling pathway. Therefore, the PI3K/AKT signaling pathway is an important pathway for ginsenosides to exert autophagic effects.

As mentioned above, the relationship between autophagy and apoptosis is two-fold. In some cases, ginsenosides elicit autophagy that proves protective for cancer cells. An example is the case of ginsenoside F2, which both promotes apoptosis in breast cancer stem cells and induces autophagy within the same context. As mentioned above, the relationship between autophagy and apoptosis is two-fold. In some cases, ginsenosides elicit autophagy that proves protective for cancer cells. An example is the case of ginsenoside F2, which both promotes apoptosis in breast cancer stem cells and induces autophagy within the same

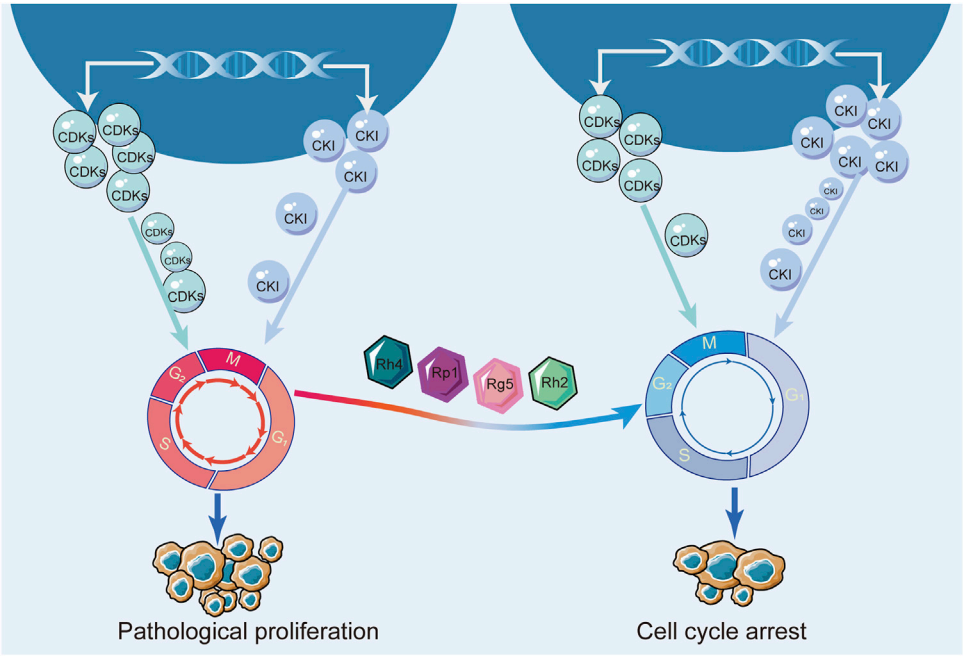


FIGURE 9
The mechanism of ginsenoside blocking breast cancer cell cycle.

TABLE 5 Various ginsenosides block the cell cycle in different types of breast cancer.

Subtype	Saponins	Types of breast cancer	References
C17 side-chain varied	Ginsenoside Rk1	Triple-negative breast cancer	Hong and Fan (2019)
C17 side-chain varied	Ginsenoside Rg5	Hormone receptor–positive breast cancer	Kim and Kim (2015)
C17 side-chain varied	Ginsenoside Rp1	Triple-negative breast cancer, Hormone receptor–positive breast cancer	Kang et al. (2011)
C17 side-chain varied	Ginsenoside Rh4	Hormone receptor–positive breast cancer	Duan et al. (2018)
Protopanaxadiol	Ginsenoside Rh2	Hormone receptor–positive breast cancer	Peng et al. (2022)
Protopanaxadiol	20(S)-Protopanaxadiol	Hormone receptor–positive breast cancer	Zhang et al. (2018)

context. (Mai et al., 2012). The evidence suggested that autophagy of breast cancer stem cells limits the effect of ginsenoside F2, which is a common problem of numerous anti-cancer drugs today. In other words, autophagy is closely related to drug resistance of tumors (Hu et al., 2022). However, some ginsenoside-induced protective autophagy is essential. The therapeutic prominence of trastuzumab in breast cancer treatment is undeniable. However, its clinical utility is constrained by its cardiotoxicity. A recent investigation unveiled the shielding potential of ginsenoside Rg2 against trastuzumab-triggered cardiotoxicity through the induction of autophagy in cardiomyocytes (Liu et al., 2021). This result illustrated ginsenoside Rg2 as a potential therapeutic agent to prevent cardiotoxicity associated with medications for treating breast cancer. The mechanism of autophagy induced by ginsenosides was shown in Figure 10; Table 6.

From the above studies, ginsenoside-induced autophagy exerted mostly anti-tumor effects on breast cancer. In addition, the protective autophagy induced by ginsenosides can also be used synergistically with anti-breast cancer drugs to reduce drug toxicity. This strategic integration holds the promise of expanding the therapeutic purview of ginsenosides within the realm of breast cancer treatment.

3.4 Inhibition of metastasis

Metastasis is the process by which cancer cells spread from the primary tumor to other parts of the body and is a major cause of mortality in breast cancer patients. Despite the wide range of treatments and medications available for breast cancer, it is reported that 20%–30% of breast cancer patients develop metastases after diagnosis or treatment of the primary tumor. This is a major concern, as metastases are often the cause of death in up to 90% of breast cancer cases (Yoshimaru et al., 2013). As a result, it is critical to focus on treatments that target

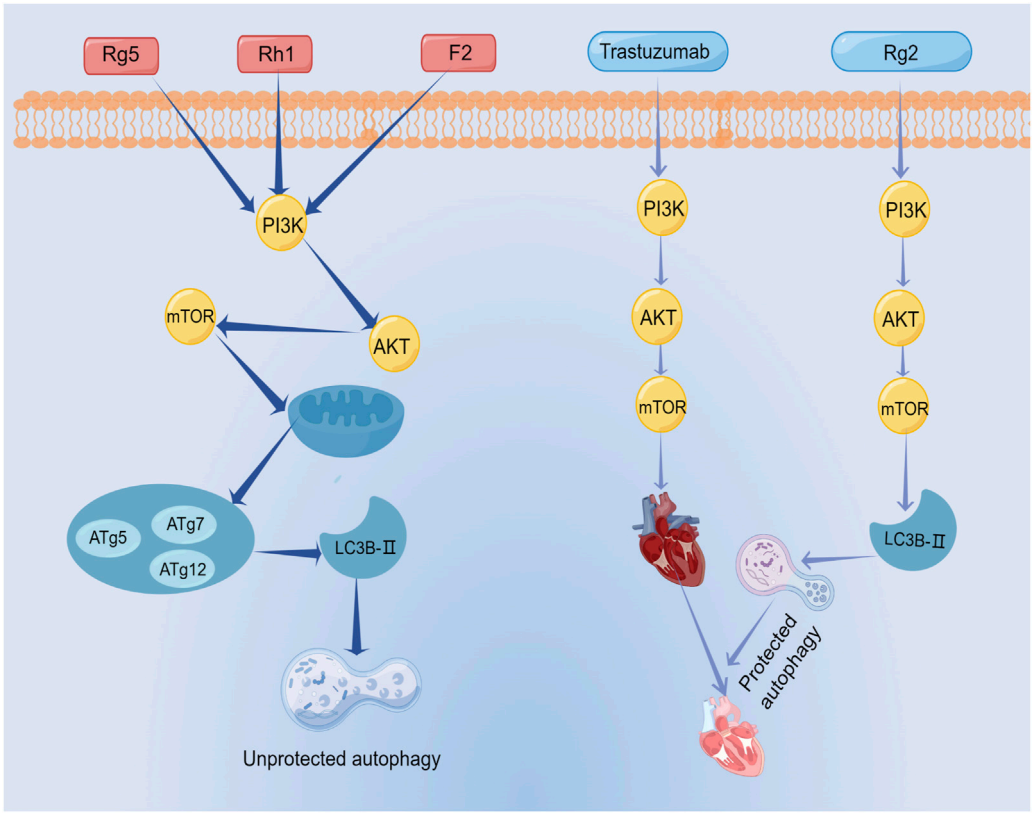


FIGURE 10
The mechanism of autophagy induced by ginsenosides.

TABLE 6 Induction of autophagy in breast cancer cells by ginsenosides.

Subtype	Saponins	Effects of autophagy	References
C17 side-chain varied	Ginsenoside Rg5	Inhibited the proliferation of breast cancer cells	Liu and Fan (2018)
C17 side-chain varied	Ginsenoside F2	Induced autophagy was protective of breast cancer stem cells	Mai et al. (2012)
Protopanaxatriol	Ginsenoside Rh1	Inhibited the proliferation of breast cancer cells	Huynh et al. (2021)
Protopanaxatriol	(20S)-Protopanaxatriol	Inhibited the proliferation of breast cancer cells	Li et al. (2021)
Protopanaxatriol	Ginsenoside Rg2	Induced autophagy prevented trastuzumab-induced cardiomyocytotoxicity	Liu et al. (2021)

metastases in order to improve outcomes for breast cancer patients.

There were only a few studies on ginsenosides against breast cancer metastasis and they were all focused on the category of protopanaxadiol. Circulating tumor cells, a type of tumor cell in the bloodstream, play a seed-like role in breast cancer metastasis. Metastatic niches are like soil. Multifunctional Rg3 liposomes loaded with doxorubicin was specifically targeted on circulating tumor cells and prevented the formation of metastatic niches by altering the immunosuppressive microenvironment (Xia et al., 2022). In another breast cancer study, ginsenoside Rd treatment resulted in a reduction in lung tumor lesions in a spontaneous and experimental model of metastasis. This mechanism was related to the fact that ginsenoside 2 decreased miR-18a expression and elevated Smad2 expression (Wang et al., 2016). In addition, a previous *in vivo* and *in vitro* study demonstrated that ginsenoside 20(S)-protopanaxadiol significantly inhibited the growth and lung metastasis of triple-negative breast cancer. The mechanism involved the inhibition of the EGFR-mediated MAPK signaling pathway and the reversal of EMT by ginsenoside 20(S)-protopanaxadiol.

The exploration of ginsenosides as potential suppressors of breast cancer metastasis appears to have been a less traversed avenue within the realm of scholarly inquiry. But breast cancer metastasis is very relevant to the prognosis of breast cancer patients. Therefore, we hope that more researchers would explore the inhibition of breast cancer metastasis by ginsenosides in the future.

3.5 Regulation of EMT

EMT is a process by which epithelial cells, which are normally tightly connected and organized in a specific tissue structure, transform into mesenchymal cells, which are more motile and invasive. EMT plays an important role in cancer progression by promoting invasion and metastasis. In breast cancer, EMT has been shown to play a critical role in the development of invasive and metastatic disease. During EMT, breast cancer cells lose their epithelial characteristics, such as cell-cell adhesion and polarity, and acquire mesenchymal characteristics, such as increased motility and invasiveness. This allows the cancer cells to break away from the primary tumor, invade surrounding tissues, and establish new tumors in distant organs (Mitra et al., 2015; Felipe Lima et al., 2016). Transcription factors such as Snail, Twist1, Slug, and ZEB1 can promote EMT by reducing or inhibiting the protein E-calmodulin. This leads to the conversion of epithelial cells into mesenchymal cells, allowing the cancer cells to spread more easily to other parts of the body (Uchikado et al., 2011; Wang et al., 2011; Deep et al., 2014). The strategic interception of EMT assumes a position of growing significance, materializing as a fulcrum for the prospect of forestalling and treating breast cancer metastasis.

Treatment with ginsenoside Rg1 has shown a notable impact on breast cancer tissues by downregulating SNAIL2 expression and significantly attenuating the expression of pivotal regulators of cell growth, including MAPK, EGFR, and TGF-β(Chu et al., 2020). This modulation is of particular importance since previous research has underscored the role of MAPK, EGFR, and TGF-β in fueling the progression of EMT, a pivotal driver of metastasis (Heldin et al., 2012; Serrano et al., 2014; Zhang et al., 2022b). A crucial driver of tumor invasion and metastasis is matrix metalloproteinases (MMPs), which facilitate the detachment of epithelial cells from their confines. Moreover, heightened MMP levels have been implicated in the degradation of E-calmodulin, a key mediator of EMT in cancer cells (Radisky and Radisky, 2010). Ginsenoside 20(S)-protopanaxadiol was reported to reduce the expression and activity of MMPs, while increasing the expression of tissue inhibitors of metalloproteinases (TIMPs), thus reinforcing its capacity to deter EMT progression (Peng et al., 2019). In addition, ginsenoside 25-OCH3-PPD inhibited EMT and prevented breast cancer metastasis by downregulating MDM2 (Wang et al., 2012, 2). MDM2 has been demonstrated to be closely associated with breast cancer invasion and metastasis. Moreover, MDM2 enhanced the invasion and migration of breast cancer cells by upregulating the expression of MMP-9. Therefore, MDM2 played a very important role in EMT (Rayburn et al., 2005; Chen et al., 2013, 9). Recognized as instrumental in fostering immune dysfunction and amplifying metastatic propensities, myeloid-derived suppressor cells (MDSCs) have emerged as a focal point of exploration in the context of tumor metastasis (Safarzadeh et al., 2018; Cole et al., 2021). Ginsenoside Rg3 has shown promise in attenuating cancer stemness and countering mesenchymal transformation by influencing MDSC modulation (Song et al., 2020). Many studies have demonstrated that ginsenosides can inhibit breast cancer EMT, but few articles have explored the deeper mechanisms of EMT inhibition. The mechanism of inhibiting EMT by ginsenosides was shown in Figure 11; Table 7.

3.6 Regulation of miRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs that play a vital role in the regulation of gene expression. They can act as either tumor suppressors or oncogenes, depending on the specific miRNA and the context in which it is expressed. In breast cancer, dysregulation of miRNAs has been implicated in the development and progression of the disease. Specifically, some miRNAs have been shown to be overexpressed in breast cancer to promote tumor growth and metastasis, while others are downregulated and may act as tumor suppressors. MiRNAs can affect various cellular processes involved in breast cancer development, such as cell proliferation, apoptosis, angiogenesis, invasion, and metastasis (Zhang et al., 2022a; Ismail et al., 2022). Overall, Targeting regulatory miRNAs has emerged as a promising strategy for the prevention and treatment of breast cancer.

Current studies only found that protopanaxadiol can interfere with miRNAs, thereby affecting apoptosis, proliferation, metastasis, and drug resistance in breast cancer cells. Ginsenoside Rh2 exhibited a role in upregulating miR-3614-3p expression, resulting in the inhibition of MCF-7 proliferation and the induction of apoptosis (Park et al., 2022). In addition, ginsenoside Rh2 downregulated miRNA-4425, inducing growth inhibition and apoptosis in various breast cancer cells (Park et al., 2021). Besides, ginsenoside Rh2 regulated miR-222, miR-34a, and miR-29a to attenuate chemoresistance in breast cancer (Wen et al., 2015). These observations highlighted the ability of ginsenoside Rh2 to target multiple miRNAs and thus contributed to therapeutic efficacy and reduced drug resistance as a potential enhancer of chemotherapy sensitivity. Several studies indicated that some oncogenes and tumor suppressor genes were under the control of miRNAs(Tong et al., 2016). Consequently, it is plausible that ginsenosides may mediate the upregulation of tumor suppressor genes and downregulation of oncogenes via miRNA modulation. EYA1, DACH1, and CHRM3 are considered as oncogenes. Ginsenoside Rg3 and Rd also showed an effect on the regulation of miRNAs. Ginsenoside Rg3 suppressed the expression of the three genes by regulating miRNA-424-5p (Kim et al., 2021). In another study, under the intervention of ginsenoside Rd, the expression of microRNA-18a decreased, whereas the expression of Smad2, an anti-oncogene, elevated significantly (Wang et al., 2016).

As seen with ginsenoside Rh2, one ginsenoside tends to modulate multiple miRNAs and thus exert multiple effects. However, there were few studies on ginsenosides regulating miRNAs, and ginsenosides regulating multiple miRNAs except Rh2 have not been found yet, but they certainly exist, and thus need to be further explored.

3.7 Regulation of lncRNA

Long non-coding RNAs (lncRNAs) are endogenously derived non-coding RNA molecules that exceed a length of 200 nucleotides. Operating through transcriptional and post-transcriptional regulatory mechanisms, lncRNAs actively participate in various cellular physiological processes. These

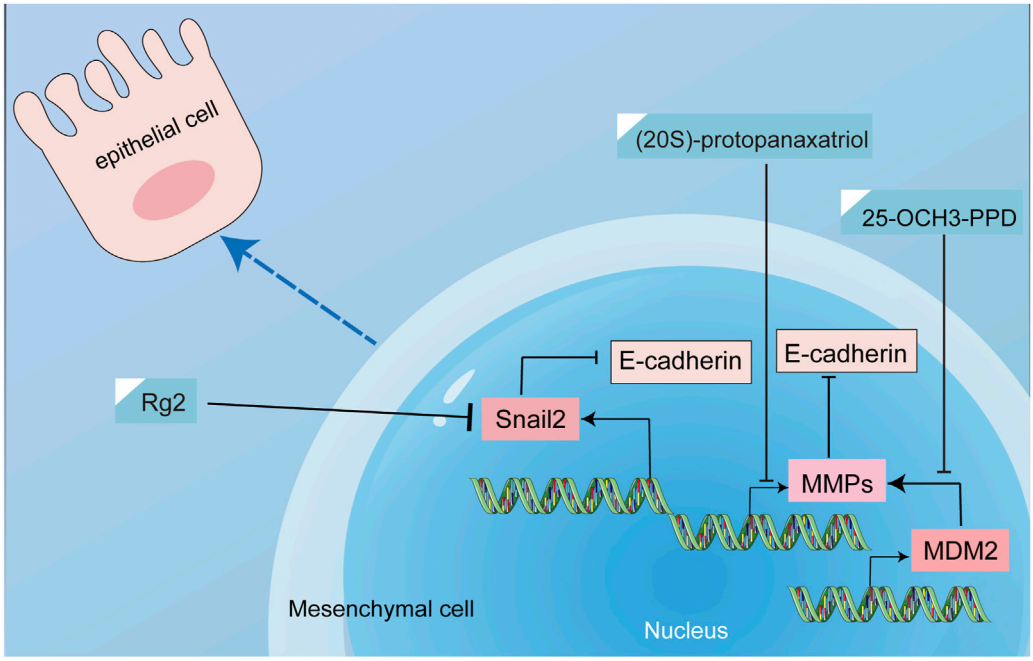


FIGURE 11
The mechanism of inhibiting EMT by ginsenosides.

TABLE 7 Interventional effects of ginsenosides on EMT in breast cancer.

Subtype	Saponins	The mechanism of inducing apoptosis	References
Protopanaxatriol	Ginsenoside Rg1	Reduced expression of SNAIL2, MAPK, EGFR and TGF-β in breast cancer tissues	Chu et al. (2020)
Protopanaxadiol	Ginsenoside 20(S)-protopanaxadiol	Decreased expression and activity of MMPs and increased expression of TIMPs	Peng et al. (2019)
Protopanaxadiol	Ginsenoside 25-OCH3-PPD	Reduced MDM2 expression and expression of EMT markers β-catenin, Twist, Vimentin, and Snail1	Wang et al. (2012)
Protopanaxadiol	Ginsenoside Rg3	Reduced MDSC expression and expression of EMT markers β-catenin and Vimentin	Song et al. (2020)

mechanisms encompass interactions with microRNAs (miRNAs), leading to the modulation of target gene regulation by miRNAs, as well as engagement in the competing endogenous RNA (ceRNA) network (Yang et al., 2016). Furthermore, it has been demonstrated that a variety of lncRNAs were aberrantly expressed in breast cancer tissues and cell lines, which were involved in regulating breast cancer cell proliferation, invasion, migration, apoptosis, and drug resistance (Liu et al., 2015). Taken together, in breast cancer, dysregulation of lncRNAs is associated with the development and progression of the disease. It is interesting to note that ginsenosides regulated lncRNAs to exert therapeutic effects on breast cancer.

The landscape of studies exploring the modulation of long non-coding RNAs (lncRNAs) by ginsenosides for breast cancer treatment is relatively sparse. Specifically, among the diverse array of ginsenosides, only protopanaxadiol compounds (Rg3 and Rh2) have been identified thus far for their capacity to regulate lncRNAs in breast cancer. Notably, a series of experiments have demonstrated the attenuation of tumor tissue and cell growth through the suppression of miRNA-4425 expression (Zhang et al., 2019; Lu et al., 2020). Moreover, Ginsenoside Rh2 has been observed to elevate the expression of the lncRNA STXBP5-AS1, which targets miRNA-4425, resulting in the induction of apoptosis and the inhibition of cell proliferation in breast cancer cells (Park et al., 2021). MiRNA-424-5p was reported to enhance chemotherapy sensitivity and regulate the cell cycle, apoptosis, and proliferation, playing a role as an important therapeutic player in breast cancer (Dastmalchi et al., 2021). A previous experiment reported that lncRNA ATXN8OS suppressed miRNA-424-5p expression. However, ginsenoside Rg3 prevented the oncogenic lncRNA ATXN8OS from repressing microRNA-424-5p (Kim et al., 2021). Additionally, a recent investigation has reported that ginsenosides Rh2 downregulate the activity of lncRNA CFAP20DC-AS1 while concurrently elevating the expression of miRNA-3614-3p, consequently prompting apoptosis and inhibiting cell proliferation in breast cancer cells (Park et al., 2022). Furthermore, ginsenoside Rh2 has

TABLE 8 Effects of ginsenosides in combination with chemotherapeutic agents.

Subtype	Saponins	Drugs combined with Ginsenoside	Effects	References
Protopanaxadiol	ginsenoside Rh2	Adriamycin	Inhibited adriamycin-induced expression of the oncogene ABCB1 in MCF-7/Adr cells; inhibited adriamycin-enhanced NF-κB binding to the human multidrug resistance (MDR1) promoter and reduced adriamycin resistance	Zhang et al. (2012)
Protopanaxadiol	Ginsenoside Rd	Doxorubicin	Reduced MDR1 expression in MCF-7/ADR cells and reversed doxorubicin resistance in MCF-7/ADR cells	Pokharel* et al. (2010)
Protopanaxadiol	Ginsenoside compound K	Cisplatin	Enhanced cisplatin-induced apoptosis and inhibition of proliferation and EMT in breast cancer cells	Zhang and Li (2016)
Protopanaxadiol	Ginsenoside Rg3	Paclitaxel	Inhibited the NF-κB signaling pathway, activated the cytotoxicity of paclitaxel, and enhanced the pro-apoptotic ability of paclitaxel	Yuan et al. (2017)

been demonstrated to inhibit breast cancer cell proliferation through the methylation modification of the promoter region of lncRNA C3orf67-AS1. And ginsenoside Rg3 regulated the methylation of lncRNA RFX3-AS1 and STXBP5-AS1, which affected the expression of their RFX3 and GRM1 target genes, thereby inhibiting the proliferation of breast cancer cells (Ham et al., 2019, 1).

Long non-coding RNAs (lncRNAs) represent a pivotal class of molecules governing diverse facets of breast cancer biology. The exploration of lncRNAs has emerged as a burgeoning field of research, signifying their growing significance. Presently, investigations into the interplay between ginsenosides and lncRNAs in the context of breast cancer therapeutics remain relatively limited. However, we anticipate a growing surge in future studies that delve into the interactions between ginsenosides and lncRNAs.

3.8 Regulation of epigenetic modifications

Epigenetic modifications refer to changes in gene expression that are not caused by changes in the underlying DNA sequence. These modifications include DNA methylation, histone modifications, ubiquitination, and so on. These mechanisms play a key role in regulating gene expression and cause genes to be either activated or silenced under different conditions (Kelly et al., 2010; Zhao and Shilatifard, 2019). Moreover, epigenetic modifications have great significance in the treatment of breast cancer (Abdel-Hafiz and Horwitz, 2015; Karami Fath et al., 2022). By regulating epigenetic modifications, it promotes the expression of oncogenes and silences pro-oncogenes, thereby effectively promoting apoptosis and inhibiting the further development of breast cancer. In conclusion, intervening in epigenetic modifications during breast cancer development can inhibit tumor growth and prevent metastasis.

Currently, studies found only one type of ginsenoside, the protopanaxadiol, to treat breast cancer through epigenetic modification. Notably, methylation stands as a pivotal factor in tumorigenesis and progression (Pouliot et al., 2015). Ginsenoside Rh2 was reported to reduce m6A RNA methylation by downregulating KIF26B expression in breast cancer cells (Hu et al., 2021). In another study, ginsenoside Rh2 induced hypomethylation of an entire chromosomal element LINE1. Besides, Rh2 downregulated highly methylated oncogenes (Lee et al., 2018). By genome-wide methylation analysis, ginsenoside Rg3 downregulated oncogenes by altering methylation levels (Ham et al., 2018, 3). These findings revealed that ginsenosides regulated the methylation levels of breast cancer-related genes, thereby inhibiting the growth of breast cancer cells. Ubiquitination degrades certain key oncoproteins, pro-metastatic proteins, and proteins associated with tumor drug resistance so that cancer patients benefit from them (Xiao et al., 2016; Tecalco-Cruz and Ramírez-Jarquín, 2018). Ginsenoside Rd was reported to increase the ubiquitination of MDR1 in MCF-7 cells, leading to a decrease in the expression of this MDR1 gene associated with multidrug resistance, which reduced resistance to chemotherapy drugs (Pokharel* et al., 2010).

The current findings only reported that ginsenosides can regulate breast cancer development and drug resistance through ubiquitination and methylation, two epigenetic modifications. However, other epigenetic modifications are equally important, such as acetylation, glycosylation, and phosphorylation. These modifications have not been reported, which is a blank that needs to be filled urgently in the current study of ginsenosides against breast cancer.

3.9 Combination with chemotherapy drugs

Chemotherapy, while a cornerstone in breast cancer treatment, is often limited by drug resistance and adverse effects that some patients encounter. Ginsenosides not only alleviated the adverse effects of chemotherapy drugs but also acted on breast cancer through multi-targeting, thus reducing the toxicity and increasing the effectiveness. Therefore, the combination of ginsenosides with chemotherapeutic agents has garnered attention as a judicious therapeutic strategy.

The only reported ginsenoside used in combination with chemotherapeutic agents is protopanaxadio. 20(S)-ginsenoside Rh2 curbed adriamycin-induced oncogene ABCB1 expression within MCF-7/Adr cells. Further effect lay in its suppression of adriamycin-amplified NF-κB's affinity for the human multidrug resistance (MDR1) promoter, effectively abating adriamycin resistance (Zhang et al., 2012, 2). Another study unearthed

the capabilities of ginsenosides Rd to quell MDR1 expression in MCF-7/ADR cells. Besides, ginsenoside Rd reversed doxorubicin resistance in MCF-7/ADR cells (Pokharel* et al., 2010). These results indicated that ginsenosides reversed breast cancer drug resistance, which helped chemotherapy drugs work better. In addition, the combination of ginsenosides and chemotherapeutic agents enhanced the anti-tumor activity. It has been reported that ginsenoside Rg3 activated the cytotoxicity of paclitaxel by inhibiting the NF-κB signaling pathway. Moreover, the union of ginsenoside Rg3 and paclitaxel elicited a more pronounced reduction in Bcl-2 protein expression, accompanied by an augmentation in Bax and Caspase-3 protein expression within breast cancer cells, thus fortifying the tumor-suppressive potential beyond that of paclitaxel monotherapy (Yuan et al., 2017). Similarly, ginsenoside compound K combined with cisplatin-induced apoptosis and inhibited the proliferation and EMT of breast cancer cells better than these two drugs alone (Zhang and Li, 2016). The effects of ginsenosides in combination with chemotherapeutic drugs were shown in Table 8.

These observations highlight that ginsenosides combined with chemotherapeutic agents achieved better efficacy than those used alone. These studies involved both *in vivo* and *in vitro* experiments, but the clinical application of ginsenosides must be supported by data from clinical research. Therefore, large-scale clinical trials should be conducted to provide data support for the clinical application of ginsenosides in combination with other chemotherapeutic agents for the treatment of breast cancer.

4 Conclusion

Breast cancer is the most prevalent cancer in women worldwide with a high rate of metastasis. With medical advances, the OS and DFS of patients with early-stage breast cancer have improved significantly. But not all breast cancer patients benefit from treatment, especially advanced breast cancer is still a very difficult problem. Ginseng is full of possibilities and interest, as it is considered to be a life-prolonging and health-enhancing medication and has received a lot of attention. Ginsenosides, as the main active ingredient of ginseng, have been widely explored for their therapeutic potential in breast cancer.

Through a combination of bibliometric analysis, this review delved into the therapeutic implications of ginsenosides in the context of breast cancer. The bibliometric investigation highlights the burgeoning prominence of ginsenoside intervention in breast cancer research. Through co-occurrence analysis of keywords, several potential mechanisms underlying ginsenoside action in breast cancer treatment have been unveiled. Subsequently, we conducted a more comprehensive exploration of these mechanisms. Numerous studies have revealed its potential mechanisms for treating breast cancer, including the promotion of apoptosis, autophagy, inhibition of cell proliferation, EMT, and metastasis, etc. Moreover, the combination of ginsenosides and chemotherapeutic drugs showed a synergistic effect. These mechanisms involve the inhibition of malignant proliferation, infiltration growth, metastasis, and drug resistance pathological processes of breast cancer. Among them, the promoting apoptosis and inhibiting proliferation mechanism of action of ginsenosides can inhibit the pathological behavior of malignant proliferation of breast cancer throughout the development of breast cancer from occurrence to progression. The mechanisms of ginsenosides inducing breast cancer cell autophagy, inhibiting EMT, metastasis, and regulating miRNAs, lncRNAs, and epigenetic modifications may be an important pathological process in preventing breast cancer cell infiltration and metastasis. These mechanisms are closely related to the clinical treatment of breast cancer, the reduction of drug resistance, the prevention of metastasis, and the prolongation of DFS and OS. We believe that with further research, ginsenosides will be clinically applied in these areas.

Despite the progress made in the pharmacology of ginsenosides against breast cancer, there are still some issues that need deeper exploration. First, while the quantity of research publications focusing on ginsenosides for breast cancer treatment exhibits an upward trend annually, the volume of papers released per year remains relatively modest. Additionally, based on the limitations of the current bibliometrics software, this paper can only present the data in the form of graphs and charts after analyzing the literature, and it cannot present specific statistical values, such as regression coefficients, significance, and so on. Second, most current studies have been conducted in animal models and at the cellular level, but there are very few large-scale randomized controlled clinical trials. Comprehensive systematic evaluations have yet to be extensively pursued. Third, several mechanism studies have focused on a single type of ginsenoside, particularly protopanaxadiol, for instance, research on the regulation of miRNAs, lncRNAs, and epigenetic mechanisms only involved protopanaxadiol, and without investigating the role of other classes of ginsenosides. Furthermore, in terms of mechanism, there is a lack of studies on other properties of ginsenosides on breast cancer, such as angiogenesis, cellular energy metabolism, and other aspects. In conclusion, a more comprehensive investigation is essential to advance the utilization of ginsenosides in clinical breast cancer treatment.

Author contributions

XD contributed to design and manuscript writing. XD, JW, CL, YZ, and LS were responsible for literature search, data acquisition, data analysis, and data visualization. AG provided valuable suggestions for data analysis, HF and LL reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Lipid metabolism as a target for cancer drug resistance: progress and prospects

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Cancer is the world's leading cause of human death today, and the treatment process of cancer is highly complex. Chemotherapy and targeted therapy are commonly used in cancer treatment, and the emergence of drug resistance is a significant problem in cancer treatment. Therefore, the mechanism of drug resistance during cancer treatment has become a hot issue in current research. A series of studies have found that lipid metabolism is closely related to cancer drug resistance. This paper details the changes of lipid metabolism in drug resistance and how lipid metabolism affects drug resistance. More importantly, most studies have reported that combination therapy may lead to changes in lipid-related metabolic pathways, which may reverse the development of cancer drug resistance and enhance or rescue the sensitivity to therapeutic drugs. This paper summarizes the progress of drug design targeting lipid metabolism in improving drug resistance, and providing new ideas and strategies for future tumor treatment. Therefore, this paper reviews the issues of combining medications with lipid metabolism and drug resistance.

KEYWORDS

drug resistance, lipid metabolism, combination drug, inhibitor, chemotherapy resistance, targeted therapy

1 Introduction

Lipid metabolism is a fundamental and intricate biochemical process within the human body. The primary biochemical process of lipid metabolism involves phospholipid and cholesterol metabolism, which are regulated by various factors such as insulin, glucagon, dietary nutrition, and enzymatic activities. Through this complex process, lipids are converted into essential components that are necessary for a wide range of biochemical reactions within the body (Liu et al., 2022).

The significance of lipid metabolism extends to the development, of cancer (Li et al., 2023), as cancer cells heavily rely on it to acquire the energy, biofilm constituents, and signaling molecules essential for their proliferation, survival, invasion, and metastasis (Bian et al., 2021). Unlike normal cells, cancer cells undergo a series of modifications in lipid metabolism, which can have a profound impact on the increased efflux of anti-tumor drugs and the modulation of apoptotic signaling pathways. These modifications consequently influence the development of tumor drug resistance.

Chemotherapy and targeted therapies currently serve as the primary treatment for cancer (Tse et al., 2021). These interventions hold the potential to improve the overall

survival and prognosis of cancer patients (Chen et al., 2023). However, the emergence of drug resistance presents a substantial clinical challenge that needs to be overcome. This challenge applies not only to the conventional chemotherapeutic agents commonly used in the initial stages but also to the targeted agents that are currently undergoing active development and investigation (Zeng et al., 2019).

The development of drug resistance in cancer is influenced by various factors, and its mechanisms can be broadly categorized into mutations in drug targets and metabolism, inhibition of apoptosis, activation of intracellular survival signaling pathways, enhanced DNA repair, immune evasion by cancer stem cells (CSCs), and metabolic abnormalities, and so on (Ramos and Bentires-Alj, 2015; Pan et al., 2016). While previous studies on cancer drug resistance mainly focused on genetic mutations and external factors, cancer metabolism have been as a new research focal point in recent years (Zhao et al., 2013; Ma and Zong, 2020). An increasing number of studies suggest that the development of resistance to chemotherapy and targeted therapies is closely linked to metabolic alterations, including lipid metabolism, which can affect the sensitivity of cancer cells to drugs.

Studies have indicated that the utilization of combination therapies targeting multiple pathways may effectively delay the development of therapeutic resistance (Fitzgerald et al., 2006). Combination drug therapy represents an emerging and more potent approach to treatment administration. By employing different mechanisms, drug combinations can collectively work towards achieving therapeutic objectives. Additional drugs can sequentially intervene in disease-related signaling pathways, either through the same or different routes, thereby producing synergistic effects (Yin et al., 2014). Moreover, synergistic drug combinations have the potential to reduce the required dosage of individual drugs within the mixture, consequently diminishing drug toxicity and mitigating the risk of drug resistance.

To further understand enhance our understanding of lipid metabolism and its connection to drug resistance and to investigate the relevance of drug combination, this review will focusconcentrate on five aspects: alterationkey areas: the modification of lipid metabolism in cancer cells, the association between phospholipid metabolism and drug resistance, the impact of cholesterol metabolism andon drug resistance, the involvement of microRNA andin lipid metabolism and drug resistance, and the significance of drug combination and lipid metabolism andin relation to lipid metabolism and drug resistance. Through exploring these aspects, we aim to gain deeper insights into the intricate relationship between lipid metabolism and the development of drug resistance.

2 Alteration of lipid metabolism in cancer cells

Extensive researches have been dedicated to exploring the intricate relationship between lipid metabolism and cancer development. Lipid metabolism plays a crucial role in providing signaling molecules (Boroughs and DeBerardinis, 2015), essential substrates for phospholipid synthesis (Zaidi et al., 2013), and metabolic fuels for mitochondrial oxidation (An et al., 2022). By modulating these pathways, lipid metabolism exerts control over the growth and proliferation of cancer cells. Cancer cells exhibit distinct patterns of nutrient uptake and utilization compared to normal cells, leading to a series of modifications in lipid metabolism. These metabolic alterations drive the growth and proliferation of cancer cells and even contribute to the development of resistance against conventional anticancer therapies.

Most normal cells primarily generate energy through mitochondrial oxidative phosphorylation, a process that involves the transfer of electrons from NADH or $FADH_2$ to O_2 via a series of mitochondrial electron carriers (Viña et al., 2009). However, in contrast to normal cells, many cancer cells rely on high-rate glycolytic and lactic acid fermentation pathways, a phenomenon known as the Warburg effect (Koppenol et al., 2011). Although aerobic glycolysis is less efficient in producing ATP compared to oxidative phosphorylation, it generates other metabolites that support tumor growth (Ashrafian, 2006). Lipid supply is crucial for the proliferation and survival of various cancer cells (Liang and Dai, 2022), and previous studies have demonstrated that cancer cells predominantly acquire lipids through the *de novo* fatty acid synthesis pathway (Baron et al., 2004). Activation of this pathway is believed to be necessary for carcinogenesis (Zhou et al., 2007).

Due to limited oxygen and extracellular nutrients, most cancer cells synthesize fatty acids *de novo*. The process of fatty acid synthesis occurs in the cytosol, with acetyl CoA serving as the starting material. However, acetyl CoA, although present in the mitochondria (Guertin and Wellen, 2023), cannot directly traverse the mitochondrial membrane, necessitating a transport mechanism to enter the cytosol. In contrast, citric acid produced in the tricarboxylic acid cycle (TCA) can cross the mitochondrial membrane and enter the cytosol. Within the cytosol, acetyl CoA is released from citrate by citrate lyase (ACLY) and participates in fatty acid synthesis. Acetyl CoA is subsequently converted to malonyl CoA by acetyl-CoA carboxylase (ACC), followed by the action of fatty acid synthase (FASN) in synthesizing lipids (Tanosaki et al., 2020) (Figure 1). Key regulators such as FASN and ACC are significantly upregulated in various human cancers, such as cervical cancer and breast cancer (Menendez and Lupu, 2017; Du et al., 2022).

Nevertheless, certain cell types, including proliferating fibroblasts, HeLa, and H460 cells (Yao et al., 2016), exhibit a preference for direct uptake of lipids from the extracellular environment rather than *de novo* synthesis. These cells convert exogenous lipids and lipoproteins into the necessary lipids for cell growth and proliferation, facilitated by fatty acid-binding proteins (FABPs) (Ntambi, 2022). According to these studies, lipid acquisition depends on the type of cell and microenvironment, whether through *de novo* fatty acid synthesis or alternative pathways, and contributes to tumorigenesis.

3 Phospholipid metabolism and drug resistance

Lipids containing phosphate are called phospholipids. Phospholipids can divide, which contain phosphate, play a crucial role in various biological processes. They can be categorized into two main groups: those made up of glycerol are

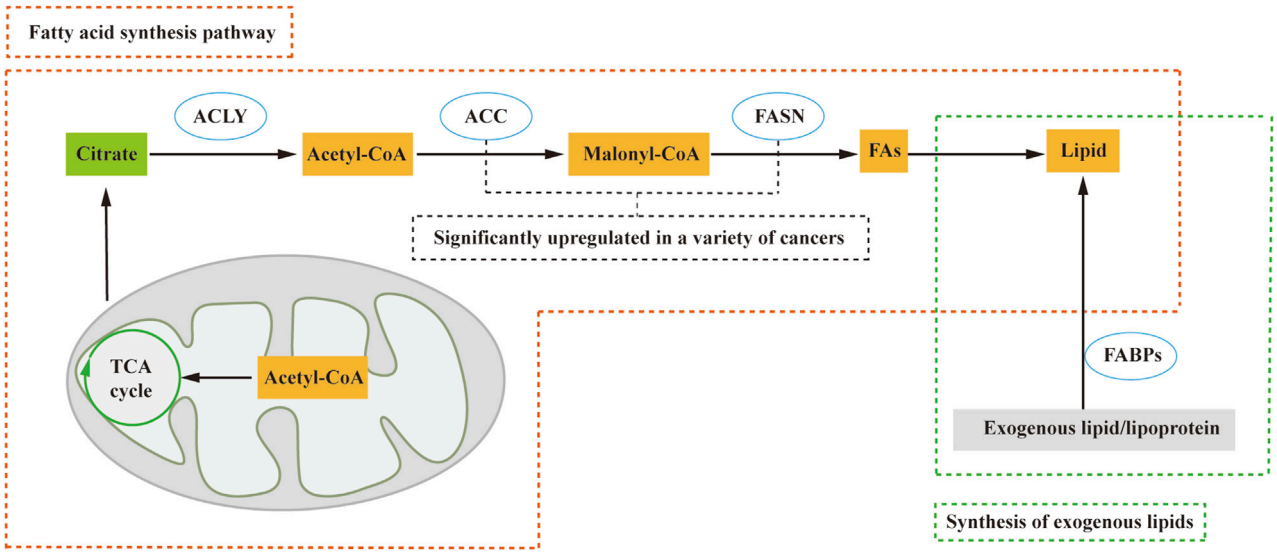

FIGURE 1
Lipid sources include the *de novo* fatty acid synthesis pathway and exogenous lipid uptake. In this process, citric acid from the tricarboxylic acid cycle (TCA cycle) crosses the mitochondrial membrane and enters the cytosol. Subsequently, ATP citrate lyase (ACLY) releases Acetyl-CoA, which plays a crucial role in the fatty acid synthesis pathway. Acetyl-CoA is then catalyzed by acetyl-CoA carboxylase (ACC) to form malonyl-CoA (Malony-CoA), and fatty acid synthase (FASN) further catalyzes the synthesis of fatty acids and, consequently, lipids. Additionally, fatty acid-binding proteins (FABPs) facilitate the conversion of exogenous lipids and lipoproteins into lipids. ACLY, ATP citrate lyase; ACC, acetyl-CoA carboxylase; FA, fatty acid; FABPs, fatty acid-binding proteins; FASN, fatty acid synthase; TCA cycle, tricarboxylic acid cycle.

called phosphoglycerides, composed of glycerol, and those made up of neurosphingosine are called sphingolipids, composed of sphingosine.

Glycerophospholipids are the most abundant phospholipids found in the body, serving multiple essential functions. They not only form the structural basis of biological membranes but also contribute to bile composition, act as surface-active substances, and play a role in protein recognition and cell membrane signaling. Glycerophospholipids can be classified into various categories based on their substitution groups, with some of the most important ones being phosphatidylcholine (PC) formed by choline and phosphatidic acid, phosphatidylethanolamine (PE) formed by ethanolamine and phosphatidic acid, phosphatidylserine (PS) formed by serine and phosphatidic acid, phosphatidylglycerol (PG) formed by glycerol and phosphatidic acid, and phosphatidylinositol (PI) formed by inositol and phosphatidic acid (Ntambi, 2022). The synthesis of glycerophospholipids occurs through three stages: raw material sourcing, activation, and glycerophospholipid generation. This process takes place in the endoplasmic reticulum of the cytoplasm, undergoes processing by the Golgi apparatus, and is ultimately utilized by tissue biofilms or secreted as lipoproteins. Glycerophospholipids can be synthesized in various body tissues, excluding mature erythrocytes. In living organisms, certain phospholipases can hydrolyze glycerophospholipids, and their degradation mainly involves hydrolysis catalyzed by different phospholipases in the body. During glycerophospholipid metabolism, several bioactive lipid molecules are generated, including inositol triphosphate, glycerol diacyl, arachidonic acid, phosphatidic acid, and lysophosphatidic acid. These lipid molecules, in turn, regulate diverse intracellular signaling pathways (Oude Weernink et al., 2007).

Sphingolipids, distinguished by the absence of glycerol and the presence of sphingomyelin, encompass sphingomyelin and glycosphingolipids. They are synthesized in various tissues throughout the body, with particularly high activity in brain tissues where they constitute a major component of neural tissue membranes. The synthesis of sphingolipids occurs within the endoplasmic reticulum. The breakdown of sphingolipids takes place through the hydrolysis of sphingolipids into choline phosphate and ceramide, catalyzed by phospholipase (Duan M. et al., 2022). Sphingomyelin, an important structural component of cell membranes, also serves as a precursor for various metabolites, including ceramide, ceramide-1-phosphate, sphingosine, sphingosine-1-phosphate, and glycosyl ceramide. These metabolites play crucial roles as bioactive lipid molecules involved in apoptosis and signaling pathways related to drug resistance.

3.1 Phospholipid metabolism and chemotherapy resistance

The fatty acid composition of phospholipids (PL) plays a crucial role in distinguishing between sensitive and resistant cells. Recent studies have highlighted the impact of acyltransferases on the fatty acid composition of PL, which can influence cancer chemosensitivity. For instance, lysophosphatidylcholine acyltransferase 2 (LPCAT2), an enzyme associated with lipid droplets, has been found to promote abnormal biosynthesis of phosphatidylcholine, leading to resistance to oxaliplatin and 5-fluorouracil in colorectal cancer. The underlying mechanism involves an enhanced anti-apoptotic response to endoplasmic

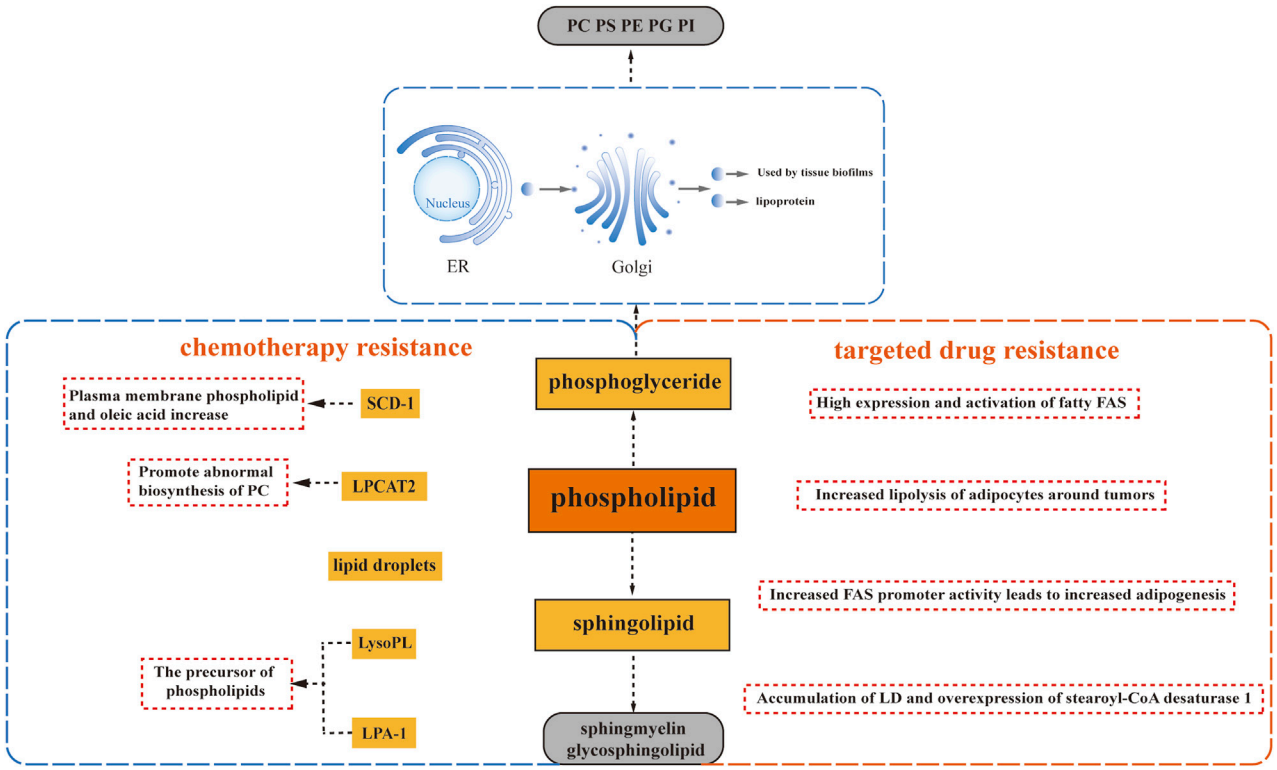

FIGURE 2
Phospholipid Metabolism and Drug Resistance. Phospholipid metabolism plays a crucial role in drug resistance, involving glycerophospholipids and sphingomyelins. Glycerophospholipids encompass phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI), each characterized by different substituents. Sphlngolipids comprise sphlngmyelins and glycosphingolipids. Several factors contribute to drug resistance: the abnormal biosynthesis of phosphatidylcholine driven by lysophosphatidylcholine acyltransferase 2 (LPCAT2), upregulation of Stearoyl-CoA desaturase 1 (SCD1) leading to lipid droplet accumulation, and the induction of drug resistance by precursors of phospholipids, namely, lysophospholipid (LysoPL) and lysophosphatidic acid receptor 1 (LPA-1).

reticulum stressors and improved resistance to immunogenic cell death induced by chemotherapy (Cotte et al., 2018). These findings suggest that LPCAT2 activity can modify the lipid composition of the endoplasmic reticulum and plasma membrane, thereby reducing sensitivity to endoplasmic reticulum stress and impairing recognition by the host immune system (Figure 2).

Additionally, progesterone has been found to upregulate Stearoyl-CoA desaturase 1 (SCD1), resulting in an enrichment of oleic acid in plasma membrane phospholipids. This enrichment has been correlated with docetaxel resistance, as it increases the mobility of the plasma membrane and alters its connection to the cytoskeleton. Consequently, cells become better adapted to drugs that target the cytoskeleton, such as docetaxel (Schlaepfer et al., 2012) (Figure 2). Moreover, studies have reported a link between enhanced lipid droplet formation and drug resistance. In progesterone-dependent breast cancer, increased intracellular accumulation of lipid droplets has been associated with docetaxel resistance since hydrophobic cytotoxic drugs, including docetaxel, are readily sequestered within lipid droplets (Schlaepfer et al., 2012). Similarly, a significant increase in neutral lipids within lipid droplets and the accumulation of free cholesterol in lysosomes have been observed in a variant of T-47D breast cancer cells resistant to the lipid-soluble drug tamoxifen (Hultsch et al., 2018). The presence of lipid droplets has also been identified in MCF7R cells with acquired resistance to doxorubicin (Morjani et al., 2001) (Figure 2).

Lysophospholipids (LysoPL), the direct precursors of phospholipids (PL), also play a role in mediating drug resistance. Interestingly, their mechanism of action is not solely dependent on the plasma membrane. A recent study demonstrated that LysoPL containing long saturated fatty acyl chains can induce drug resistance. This protective effect of LysoPL enables tumor cells to withstand DNA-damaging agents like cisplatin, representing a lipid-specific and drug-specific protective mechanism (Kramer et al., 2015) (Figure 2).

Similarly, lysophosphate-1 (LPA-1), a precursor shared by most phospholipids, has been implicated in reducing the effectiveness of adriamycin in inhibiting the viability of triple-negative MDA-MB-231 cells with paclitaxel (Samadi et al., 2009) (Figure 2). Furthermore, it has been observed that LPA-1 upregulates several multidrug efflux transport proteins, including MRP1, MRP2, MRP3, and BCRP, along with various antioxidant enzymes (Venkatraman et al., 2015) (Figure 2). This implies that LPA-1 activates at least two mechanisms that promote resistance to chemotherapy.

3.2 Phospholipid metabolism and targeted therapy resistance

Alterations in phospholipid-related metabolism are closely associated with the development of resistance to targeted drugs,

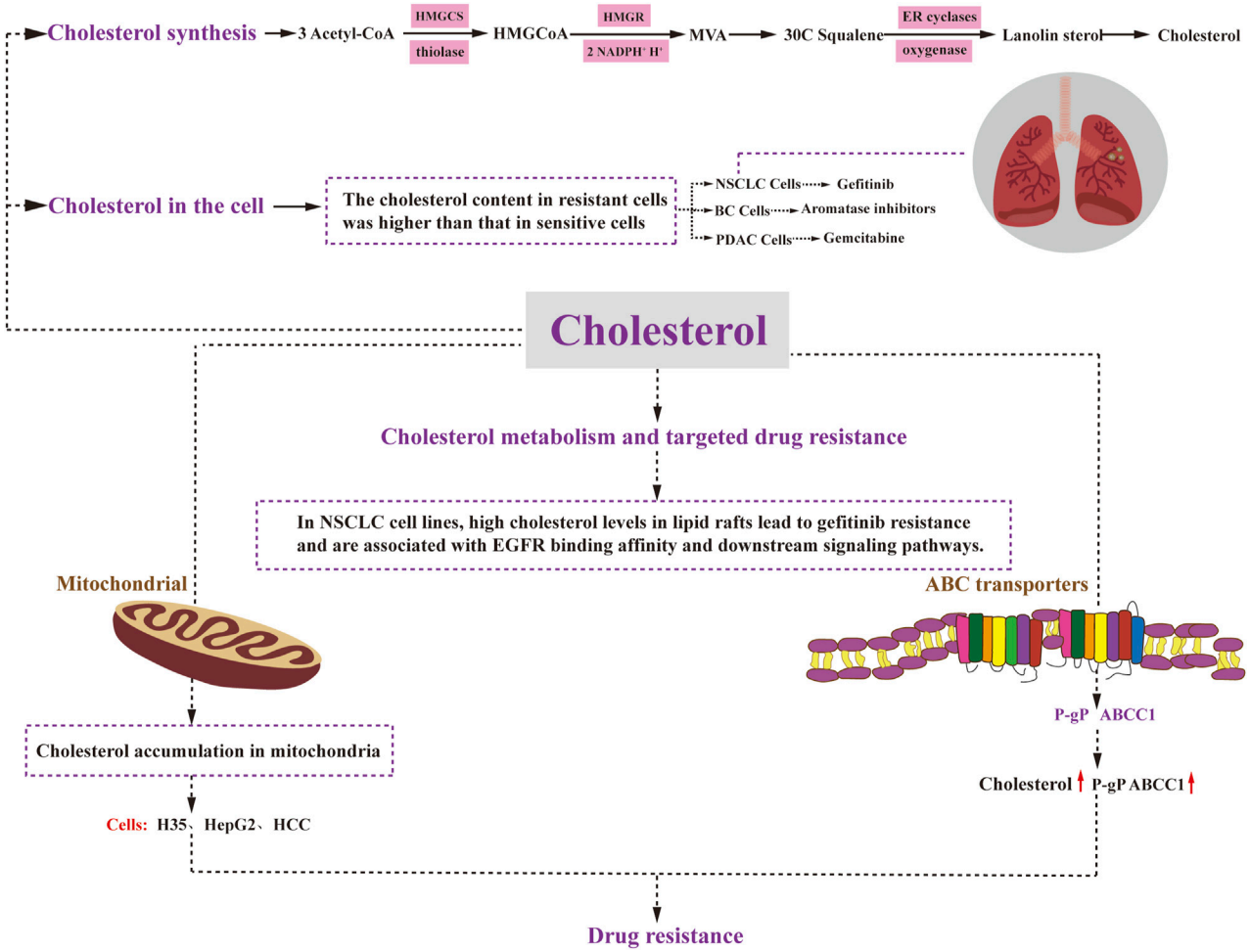

FIGURE 3
Cholesterol metabolism and drug resistance. Cholesterol synthesis involves three stages: HMGCoA generation, MVA generation, and cholesterol generation. Cholesterol plays a regulatory role in cancer cell resistance through various mechanisms. Elevated levels of mitochondrial cholesterol can contribute to resistance against apoptosis. Changes in the composition of cell membrane cholesterol can affect the function of ABC transporters, including P-glycoprotein and ABCC1. Moreover, alterations in cell membrane cholesterol content may impact the permeability of therapeutic agents and drug uptake. Ultimately, drug resistance in cancer cells affects critical cellular processes such as survival, proliferation, differentiation, and apoptosis. HMGCoA:3-Hydroxy-3-methylglutaryl coenzyme A; HMGCS: 3-Hydroxy-3-methylglutaryl coenzyme A synthetase; MVA: mevalonic acid; HMGR: HMGCoA reductase; ER cyclases: endoplasmic reticulum cyclases.

mirroring the pattern seen with many chemotherapeutic agents. In a comprehensive study conducted by Geneste *et al.*, it was revealed through both *in vitro* and *in vivo* validation that increased adipocyte lipolysis surrounding tumors contributes to the resistance of breast tumor cells to lapatinib-mediated cytotoxicity (Geneste et al., 2020) (Figure 2). Moreover, elevated expression and activation of adipogenic fatty acid synthase (FAS) were observed in breast, colon, and prostate cancers, leading to enhanced lipid droplet (LD) accumulation and synthesis of triacylglycerol (TAG) (Pandey et al., 2012; Wu et al., 2020).

Further investigations focusing on breast cancer cells have demonstrated that the development of trastuzumab resistance is associated with heightened adipogenesis due to increased FAS promoter activity (Menendez et al., 2004) (Figure 2). Similarly, the accumulation of LD and overexpression of stearoyl-CoA desaturase 1 (SCD1) were observed in non-small cell lung cancer (NSCLC) cells resistant to EGFR-tyrosine kinase inhibitors (TKIs) (Figure 2). Interestingly, the extent of LD accumulation resulting from upregulated adipogenesis was greater in EGFR/TKI-resistant cells with aberrantly activated EGFR signaling pathways than in cells harboring sensitive EGFR mutations. This observation aligns with the role of *de novo* adipogenesis driven by the upregulation of the receptor tyrosine kinase signaling pathway, particularly the maintenance of sterol regulatory element-binding protein (SREBP) activity (Butler et al., 2020).

4 Cholesterol metabolism and drug resistance

4.1 Cholesterol and its synthesis and metabolism

Cholesterol is a vital component of animal cell membranes, serving not only as a structural element but also as a precursor for

the synthesis of bile acids, vitamin D, and steroid hormones (Griffiths and Wang, 2022). Its physiological significance in the human body is diverse and essential (Rezen et al., 2011).

Cholesterol is synthesized in nearly all tissues of the body, with the liver being the primary site of synthesis, occurring predominantly in the cytosol and the endoplasmic reticulum. The process of cholesterol synthesis can be summarized into three stages (Figure 3). The first stage involves the production of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA): Within the cytosol, three molecules of acetyl CoA undergo catalysis by Thiolase and HMG-CoA synthase (HMGCS) to form HMG-CoA. This process is similar to the production of ketone bodies, although it occurs in a different intracellular location. The second stage encompasses the generation of mevalonic acid (MVA): HMG-CoA is converted by HMG-CoA reductase (HMGR), consuming two molecules of $NADPH^+$ and H^+ to produce mevalonic acid (MVA). This step is irreversible. The third stage involves the production of cholesterol: MVA is initially phosphorylated, decarboxylated, and dehydroxylated, ultimately leading to the synthesis of 30C squalene. This squalene is then catalyzed by endoplasmic reticulum cyclase and hydrogenase to generate lanolin sterols, which further undergo a series of multi-step reactions, including redox reactions, to ultimately yield cholesterol (Ačimovič and Rozman, 2013).

4.2 Cholesterol homeostasis and drug resistance

The association between cholesterol homeostasis and drug resistance has been extensively investigated (Duan Y. et al., 2022). Studies using data from the Cancer Genome Atlas have shown a correlation between cholesterol synthesis, decreased patient survival, and cancer progression (Weinstein et al., 2013). Understanding the role of cholesterol in drug resistance is crucial for overcoming challenges in cancer treatment (Wu et al., 2015). To elucidate the mechanisms underlying drug resistance, it is important to examine the involvement of cholesterol in this process.

Preclinical studies have provided compelling evidence linking cholesterol metabolism to drug resistance in various types of cancer, including prostate, lung, pancreatic, and breast cancers (Guillaumond et al., 2015; Nguyen et al., 2015; Zhan et al., 2019; El-Kenawi et al., 2021). Notably, in aggressive prostate cancer (CRPC), researchers have discovered that macrophage-derived cholesterol influences drug resistance during treatment by affecting its transportation and metabolism-related effects. This finding serves as a valuable model for understanding the mutation landscape of CRPC, supported by experimental data (El-Kenawi et al., 2021).

In non-small cell lung cancer, gefitinib-resistant cells have been found to exhibit significantly higher cholesterol levels compared to their gefitinib-sensitive counterparts (Zhan et al., 2019) (Figure 3). Similarly, in breast cancer, increased endogenous cholesterol biosynthesis in aromatase inhibitor-resistant cells leads to the activation of estrogen receptor-α, which subsequently diminishes the effectiveness of statins and impedes cell invasion (Nguyen et al., 2015) (Figure 3). In the case of pancreatic ductal adenocarcinoma (PDAC), disruption of low-density lipoprotein receptor (LDLR) internalization leads to alterations in free versus esterified cholesterol levels. This effect becomes more pronounced *in vivo* when gemcitabine (GEM) is administered (Guillaumond et al., 2015) (Figure 3). GEM-resistant PDAC cells exhibit elevated levels of cholesteryl ester (CE) compared to their sensitive counterparts (Li et al., 2018). Therefore, targeting LDLR or acyl coenzyme A cholesterol acyltransferase (ACAT) to limit CE accumulation holds promise for enhancing the efficacy of GEM against PDAC.

4.3 Mitochondrial cholesterol levels and drug resistance

Elevated levels of cholesterol in mitochondria have been demonstrated to contribute to resistance against apoptotic signaling, leading to chemotherapy resistance in cancer (Montero et al., 2008). Considering the crucial role of mitochondria in apoptosis regulation and chemotherapy response, several studies have highlighted the association between cholesterol accumulation and mitochondria-targeted chemoresistance in cancer cells. For instance, both rat H35 and human HepG2 and HCC cells exhibit resistance to anticancer agents that target mitochondria and induce the opening of mitochondrial permeability transition pores through various mechanisms (Le Bras et al., 2006) (Figure 3). Subsequent investigations have revealed that the resistance to chemotherapeutic agents in H35 and HepG2 cells can be reversed by treatment with lovastatin, an inhibitor of HMG-CoA reductase (HMGCR) that inhibits cholesterol resynthesis and prevents cholesterol accumulation in the mitochondrial membrane (Smith and Land, 2012). Moreover, a significant increase in mitochondrial cholesterol content has been observed in liver cancer tissues compared to normal tissues (Kramer et al., 2015). In this context, inhibition of the mevalonate pathway has been shown to reduce mitochondrial cholesterol content and enhance the hepatocyte response to the mitochondria-targeting drug doxorubicin, thereby sensitizing the cancer cells to chemotherapy (Montero et al., 2008). Additionally, mitochondria in hepatocellular carcinoma cells demonstrate resistance to mitochondrial membrane permeabilization and various other stimuli, with elevated cholesterol levels observed in all cases. Previous research has demonstrated that knockdown of the mitochondrial cholesterol transport polypeptide, steroid acute regulatory protein, which is upregulated in hepatocellular carcinoma cells, leads to reduced cholesterol synthesis in mitochondria and increased sensitivity of cells to chemotherapy (Domínguez-Pérez et al., 2019).

4.4 ABC transporter protein cholesterol levels and drug resistance

The significance of ABC transporter proteins in the development of drug resistance in various cancers has been extensively studied for several decades. Numerous studies have demonstrated that altering the cholesterol composition of cell membranes also affects the activity of these ABC transporter proteins Recent research has indicated that P-gP substrates may preferentially accumulate in cholesterol-rich regions of the

membrane, thus increasing P-gP transport activity (Subramanian et al., 2016). Consequently, elevated cholesterol levels can promote P-gP activity and mediate drug resistance. Consistent with this observation, in colon cancer cells, the quantity and transport activity of P-gP decrease as cholesterol synthesis is inhibited. Another ABC transporter protein, ABCC1, also appears to be regulated by cholesterol. Its function has been linked to its localization in cholesterol-rich membrane microstructure domains. When membrane cholesterol levels drop below 40%, ABCC1 partially relocates to the high-density fraction, resulting in reduced functionality (Marbeuf-Gueye et al., 2007). In summary, the localization and function of ABCC1 in cell membranes are regulated by cholesterol.

4.5 Cholesterol metabolism and drug resistance to targeted therapy

Patients with advanced non-small cell lung cancer (NSCLC) who are treated with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) experience significant clinical benefits. However, they inevitably develop acquired resistance. Research has demonstrated that high cholesterol levels in lipid rafts contribute to gefitinib resistance in NSCLC cell lines and are associated with altered EGFR binding affinity and downstream signaling pathways. This groundbreaking study highlights that elevated cholesterol levels in lipid rafts play a pivotal role in inducing gefitinib resistance in NSCLC cells by impacting EGFR phosphorylation, downstream signaling pathways, and EGFR-TKI affinity (Irwin et al., 2011) (Figure 3). Moreover, combination therapy with lovastatin has shown a synergistic inhibitory effect on gefitinib-resistant cells, making the combination of lovastatin and gefitinib a promising treatment strategy for patients with gefitinib resistance (Chen et al., 2018).

An increasing body of evidence highlights the crucial role of cholesterol in cancer development. Researchers have focused on investigating the impact of cholesterol on the acquisition of drug resistance in cancer. Elevated cholesterol levels and alterations in protein expression related to cholesterol metabolism have been observed in different types of drug-resistant cancer cells. Collectively, dysregulated cholesterol metabolism emerges as a fundamental factor contributing to the development of drug resistance in multiple cancer types.

5 MicroRNA-mediated lipid metabolism may affect drug resistance

MicroRNAs (miRNAs) are short, non-coding RNA molecules composed of approximately 22 nucleotides. They are encoded by endogenous genes and play a crucial role in the post-transcriptional regulation of gene expression in both plants and animals. MiRNAs have been widely implicated in various biological processes and are closely associated with the regulation of gene expression. In the context of lipid metabolism homeostasis, previous studies have revealed a close relationship between miRNAs and lipid metabolism. Specifically, several miRNAs, such as miR-33, miR-128–1, miR-144, and miR-148a, have been identified to target and suppress the expression of ABCA1 and ABCG1 transporter proteins. These findings have been demonstrated in cultured cells and further validated through *in vivo* experiments (Gerin et al., 2010; Horie et al., 2010; Goedeke et al., 2015; Wagschal et al., 2015). Since ABC transporter proteins have been implicated in the development of drug resistance, it is important to investigate whether the inhibitory effect of miRNAs on these transporter proteins is correlated with the emergence of drug resistance. Furthermore, miR-33a and miR-33b have been found to inhibit the synthesis of fatty acid oxidase, which leads to a reduction in intracellular lipid renewal (Dávalos et al., 2011; Gerin et al., 2010). These observations highlight the multifaceted role of miRNAs in lipid metabolism and suggest their potential involvement in modulating cellular responses to lipid-related therapies and drug resistance. Further research is needed to unravel the intricate mechanisms underlying the regulatory effects of miRNAs on lipid metabolism and drug resistance, providing valuable insights for the development of therapeutic strategies targeting these processes.

The liver, being the primary site of lipid metabolism in the body, plays a pivotal role in maintaining lipid homeostasis. Among the miRNAs involved in this process, miR-122 is particularly abundant in the liver (Chang et al., 2004). It exerts regulatory control over various genes associated with cholesterol and fatty acid synthesis (Krützfeldt et al., 2005; Esau et al., 2006), thereby influencing lipid metabolism. Another key player in regulating lipid metabolism is miR-27b, which acts as a central regulatory hub (Vickers et al., 2013), Its paralog, miR-27a, has also been identified as a regulator of lipid metabolism in the liver (Zhang et al., 2017). These miRNAs contribute to the fine-tuning of lipid synthesis and metabolism, ensuring the proper balance of lipids in hepatic cells. Furthermore, miR-223 has been found to inhibit cholesterol biosynthesis and reduce cholesterol levels. This miRNA adds to the repertoire of miRNAs involved in the regulation of lipid metabolism, highlighting their potential impact on cellular lipid profiles. Collectively, these miRNAs orchestrate a series of intricate associations with the development of drug resistance in cancer, potentially through their regulatory roles in lipid metabolism. Understanding the interplay between miRNAs, lipid metabolism, and drug resistance in cancer holds promise for the identification of novel therapeutic targets and strategies. Further investigation into these mechanisms will shed light on the underlying complexities of cancer biology and may pave the way for innovative approaches to combat drug resistance.

6 Combination of lipid metabolic pathways to alleviate tumor drug resistance

Combination therapy has emerged as a highly effective therapeutic approach employed in the treatment of various diseases (Han et al., 2017). Extensive research has demonstrated that combining multiple drugs offers several advantages over single-drug treatment, including enhanced efficacy, reduced toxicity, lower required doses with equal or improved effectiveness, and diminished development of drug resistance (Foucquier and Guedj, 2015). While single lipid-targeted medications can impede the growth and metastasis of cancer cells by inhibiting specific lipid-related

TABLE 1 Lipid metabolism-related combination therapy alleviates tumor resistance.

Pathway/ Enzyme	Lipid targeted drug	Drug combination	Vivo or vitro models	Effects
FAS	Orlistat	Trastuzumab	Chemotherapy-resistant ovarian cancer cells	Apoptosis increased significantly Menendez et al. (2006)
		Taxanes	Prostate resistant cell lines	Decreases viability increases apoptosis Souchek et al. (2017)
SCD1	A939572	Gefitinib	Lung cell lines	Reduces tumor progression and inhibits cancer cells She et al. (2019)
		Temsirolimus	Clear renal cell carcinoma cell lines	Decreases tumor cell proliferation and induction of apoptosis von Roemeling et al. (2013)
	SSI-4	Sorafenib	Sorafenib-resistant hepatocellular carcinoma cell lines	Increased sensitivity to sorafenib Ma et al. (2017)
	SSI-4	5-fluorouracil cisplatin	Gastric cells	Increased sensitivity to5-fluorouracil and cisplatin Wong et al. (2023)
	g-PPT	Gefitinib	TKI-resistant non-small cell lung cancer cell lines	Reverses resistance Huang et al. (2019)
LPCAT2	LPCAT2 or LD biogenesis inhibitor	Oxaliplatin	Colorectal cancer	Relieve drug resistance Cotte et al. (2018)
		5-fluorouracil		
Glycolytic ferment	PFKFB3	Carboplatin paclitaxel	Cervixcancer	Increased sensitivity to carboplatin and paclitaxel Mondal et al. (2019)
HMG-CoA reductase	Statins	Cytarabine, Daunorubicin, Doxorubicin, etc.	Colon cancer	Growth inhibition increased apoptosis Ding et al. (2017), Stirewalt et al. (2003)
			Breast cancer	
FABP	BMS309403	Carboplatin	Carboplatin-resistant ovarian cancer cell lines	Increased sensitivity to carboplatin Mukherjee et al. (2020)
CPT1	Etomoxir	Ara-C	Drug-resistant leukemia cells	Enhanced the cytotoxicity of Ara-C87 Salunkhe et al. (2020)

pathways, they often fall short in completely eradicating cancer cells. In contrast, combination therapy exploits the synergistic interactions between different drugs, working in tandem to achieve the ultimate objective of eliminating cancer cells. By simultaneously targeting multiple pathways or molecular targets involved in lipid metabolism, combination therapy exhibits a more comprehensive and potent anticancer effect. Numerous pharmacological inhibitors have been developed to target various lipid-metabolizing enzymes, and when combined with conventional therapies, they have demonstrated significant therapeutic efficacy (refer to Table 1 for examples). The rationale behind combination therapy lies in the complementary mechanisms of action and additive or synergistic effects that arise from targeting multiple points within the lipid metabolic pathways. Overall, combination therapy holds great promise as a powerful strategy to combat diseases, particularly in the context of lipid metabolism. The judicious selection and integration of lipid-targeted drugs in combination regimens can lead to improved treatment outcomes and offer new avenues for overcoming drug resistance. Continued exploration of optimal drug combinations and their mechanisms of action will undoubtedly contribute to advancements in therapeutic approaches for various disorders.

In the context of drug-resistant ovarian cancer, the combination of the FAS inhibitor orlistat and the specific Her-2 inhibitor trastuzumab has shown remarkable synergistic effects. *In vitro* studies demonstrated a substantial increase in apoptosis among chemotherapy-resistant ovarian cancer cells upon treatment with this combination (Menendez et al., 2006). Similarly, in prostate-resistant cell lines, the combination of orlistat with paclitaxel analogs exhibited reduced cell viability and increased apoptotic activity (Souchek et al., 2017). These findings highlight the potential of combination therapy in overcoming drug resistance and enhancing treatment outcomes in specific types of cancer. By concurrently targeting distinct molecular pathways or cellular processes, such as FAS inhibition and Her-2 blockade, orlistat and trastuzumab acted synergistically to induce apoptosis and impede the survival of drug-resistant ovarian cancer cells. Likewise, the combination of orlistat with paclitaxel analogs demonstrated enhanced efficacy in prostate-resistant cell lines, further underscoring the benefits of combining drugs with different mechanisms of action. The use of combination therapies holds great promise in addressing the challenges posed by drug resistance in cancer treatment. By exploiting synergistic interactions between drugs, these regimens offer the potential for improved therapeutic outcomes, reduced drug resistance, and enhanced patient responses. Continued research into optimal drug combinations and their underlying mechanisms will undoubtedly advance our understanding and implementation of combination therapies in combating drug-resistant cancers.

The SCD1 enzyme activity inhibitor A939572 has shown promising results in combination with gefitinib, a targeted therapy for lung cancer. When used together, A939572 and gefitinib significantly impeded tumor progression, inhibited

cancer cell growth, and exhibited favorable outcomes in an *in vivo* xenograft model (She et al., 2019). Similarly, in renal clear carcinoma cells and transplanted tumors, the combination of A939572 with tesirolimus demonstrated inhibitory effects on tumor cell proliferation and facilitated apoptosis both *in vitro* and *in vivo* (von Roemeling et al., 2013). Another SCD1 inhibitor, SSI-4, has also displayed potential in overcoming drug resistance in different cancer types. In hepatocellular carcinoma cells resistant to sorafenib, the combination of SSI-4 with sorafenib restored sensitivity to sorafenib and exhibited significant therapeutic benefits (Ma et al., 2017). Moreover, SSI-4 improved the sensitivity of gastric cancer-resistant cells to treatment with 5-fluorouracil and cisplatin (Wong et al., 2023). g-PPT, another SCD1 inhibitor, has demonstrated efficacy in reducing the synthesis of polyunsaturated fatty acids, inhibiting triglyceride (TG) synthesis, and preventing lipid droplet accumulation in cancer cells. In TKI-resistant non-small-cell lung cancer cells, the combination of g-PPT with gefitinib effectively countered drug resistance, promoting apoptosis and enhancing the therapeutic response (Huang et al., 2019). These findings highlight the potential of SCD1 inhibitors in combination with existing therapies for overcoming drug resistance and improving treatment outcomes in various cancer types. By targeting lipid metabolism pathways and modulating cellular processes, such combinations offer a promising approach to tackle drug resistance and enhance the efficacy of existing treatments. Continued research and clinical investigations are warranted to validate and further explore the potential benefits of these combination regimens in cancer therapy.

In colorectal cancer, increased LPCAT2-mediated lipid droplet (LD) production has been linked to resistance against oxaliplatin and 5-fluorouracil (Cotte et al., 2018). In subsequent *in vivo* experiments using a colon cancer mouse model, the administration of LPCAT2 or LD biogenesis inhibitors resulted in tumor regression and increased survival, indicating the significant improvement of LPCAT2-mediated drug resistance. Similarly, in mice with ovarian and cervical cancers that were insensitive to carboplatin and paclitaxel, the use of the glycolytic enzyme inhibitor PFKFB3 showed promising results. By indirectly blocking LD biogenesis and lipid autophagy, PFKFB3 alleviated resistance to carboplatin and paclitaxel, thereby enhancing their effectiveness (Mondal et al., 2019). Furthermore, studies conducted on rat H35 and human HepG2 cells, known to be resistant to various antitumor agents, revealed the potential of lovastatin in reversing chemotherapeutic resistance (Le Bras et al., 2006). Lovastatin, through its inhibition of HMGCR, a key enzyme in cholesterol synthesis, effectively prevented the accumulation of cholesterol in the mitochondrial membrane. This inhibition of cholesterol synthesis in mitochondria by lovastatin led to the restoration of sensitivity to chemotherapeutic agents in H35 and HepG2 cells. These findings underscore the significance of targeting lipid metabolism pathways and LD biogenesis to combat drug resistance in cancer cells. Inhibition of LPCAT2-mediated LD production or modulation of glycolytic enzymes and cholesterol synthesis holds promise for overcoming resistance to specific chemotherapeutic agents and improving treatment outcomes. Further research is needed to explore the full potential of these approaches and their applicability in clinical settings.

Moreover, statins, inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme, have demonstrated the ability to inhibit cancer cell growth and induce apoptosis in various types of tumor cell lines. When used in combination with drugs like cytarabine, erythromycin, and doxorubicin, statins have shown promising results in xenograft models of colon cancer, breast cancer, and other malignancies (Stirewalt et al., 2003; Ding et al., 2017). These findings suggest the potential of statins as adjunctive therapy to enhance the efficacy of existing chemotherapeutic agents. Another noteworthy inhibitor, BMS309403, which targets fatty acid-binding proteins (FABPs), has shown promise in overcoming resistance to carboplatin in ovarian cancer. When combined with carboplatin, BMS309403 significantly increased the sensitivity of carboplatin-resistant cells, offering a potential strategy to overcome resistance in this context (Mukherjee et al., 2020). These findings highlight the potential of combining statins or FABP inhibitors with conventional chemotherapeutic agents to improve treatment outcomes and overcome drug resistance in various cancer types. Further research and clinical studies are warranted to explore the full therapeutic potential and safety profile of these combinations.

Another interesting study focused on leukemia cells that had developed resistance to the chemotherapeutic agent arabinofuranosylcytosine (Ara-C). In these resistant cells, it was found that Ara-C preferentially drove the tricarboxylic acid (TCA) cycle through fatty acids (FAs), relying less on glucose metabolism. This metabolic adaptation led to enhanced oxidative phosphorylation in the mitochondria (OXPHOS), which contributed to the cells' drug resistance (Farge et al., 2017). Interestingly, the entry of long-chain fatty acids (LCFAs) into the mitochondria requires the involvement of carnitine palmitoyltransferase (CPT), which consists of two isoforms, CPT1 and CPT2 (Wang et al., 2020). In subsequent experiments, researchers discovered that blocking CPT1 using etomoxir, a specific inhibitor, disrupted the OXPHOS status and significantly potentiated the cytotoxic effect of Ara-C on drug-resistant leukemia cells (Salunkhe et al., 2020). This finding suggests that targeting fatty acid metabolism through CPT1 inhibition could be a promising approach to sensitize resistant leukemia cells to Ara-C treatment. These findings shed light on the metabolic reprogramming occurring in drug-resistant leukemia cells and highlight the potential of targeting specific metabolic pathways, such as fatty acid metabolism, to overcome drug resistance and improve therapeutic outcomes. Further investigations are needed to validate these findings and explore the clinical implications of modulating fatty acid metabolism in the context of leukemia treatment.

Lipid metabolism plays a crucial role in the development of resistance to anti-angiogenic drugs (AAD). Researchers have identified that combining anti-angiogenic therapy with lipid metabolism inhibitors could potentially overcome the emergence of AAD resistance. Preclinical studies have demonstrated that tumors with identical genetic backgrounds but implanted in different locations exhibit varying responses to AAD treatment (Iwamoto et al., 2018). For instance, hepatocellular carcinoma (HCC) growing in a steatotic (fatty) liver becomes resistant to anti-angiogenic therapy, while HCC growing in a non-steatotic liver remains sensitive. These findings highlight the influence of the adipose tissue environment on AAD resistance. Tumors growing

in a fatty environment are typically more hypoxic compared to those developing in non-adipose tissues. Hypoxia induced by AAD leads to three significant alterations in lipid metabolism. Firstly, it promotes lipolysis in adipocytes, leading to the release of metabolites such as glycerol and free fatty acids (FFAs) (Nieman et al., 2011). Secondly, cancer cells respond to hypoxia by upregulating the expression of fatty acid translocase (CD36), facilitating increased uptake of FFAs by the tumor cells (Choi et al., 2018). Lastly, cancer cells undergo metabolic reprogramming, activating the β-oxidation pathway to generate energy from FFAs, thereby promoting tumor growth and metastasis (Park et al., 2016). Building upon these findings, combining AAD with inhibitors targeting lipid metabolism holds promise in alleviating and overcoming the development of AAD resistance. By targeting the metabolic adaptations occurring in the tumor microenvironment, this combination approach has the potential to enhance the effectiveness of anti-angiogenic therapy and improve treatment outcomes. However, further research and clinical investigations are necessary to validate these findings and determine the optimal strategies for combining AAD with lipid metabolism inhibitors in the clinical setting.

7 Summary and discussion

Malignant tumors pose a significant global health threat, and their incidence continues to rise each year (Ferlay et al., 2018). While medical advancements, have greatly improved the overall survival rates (Weiss et al., 2022), drug resistance complicates treatment strategies. Thus, overcoming drug resistance has become a critical issue in anticancer therapy. Recent studies have highlighted the role of lipid metabolism in influencing drug resistance (Kramer et al., 2015; Wu et al., 2015; Cotte et al., 2018). It has been shown that lipid-related processes can impact drug efficacy by affecting drug diffusion, altering membrane permeability, influencing mitochondrial function, and modulating the activity of ABC transporter proteins (Hegedüs et al., 2015). Upregulation of LPCAT2 and SCD1(Schlaepfer et al., 2012; Cotte et al., 2018), enhanced lipid droplet formation (Hultsch et al., 2018), PL precursor LysoPL and LPA-1 in phospholipid metabolism (Kramer et al., 2015) are all associated with drug resistance. Cholesterol metabolism affects drug penetration, absorption, and drug resistance by affecting mitochondrial cholesterol level (Le Bras et al., 2006), ABC transporter activity (Waghray and Zhang, 2018) and cell membrane cholesterol content (Subramanian et al., 2016). This review details the changes of lipid metabolism in drug resistance and how lipid metabolism affects drug resistance.

Further studies have shown that while single lipid-targeting drugs can hinder cancer cell growth and metastasis by inhibiting specific lipid-related pathways, they often fail to completely eradicate cancer cells (Han et al., 2017). In contrast, combination therapy utilizes synergistic interactions between different drugs that work together to reach the ultimate goal of eliminating cancer cells. Combination therapy shows a more comprehensive and effective anti-cancer effect by simultaneously targeting multiple pathways or molecular targets of lipid metabolism (Foucquier and Guedj, 2015). This review summarizes the progress of drug design targeting lipid metabolism in improving drug resistance.

The advantages of drug combination and our understanding of lipid metabolism suggest that we can continue to explore the synergies between targeted drugs of lipid metabolism and traditional anticancer drugs, so as to innovate new therapeutic approaches to improve the efficacy of anticancer drugs and mitigate the emergence of drug resistance for the benefit of patients. Therefore, further research in this area is essential to uncover the complexity of lipid metabolism during tumor resistance and to optimize the implementation of combination therapy strategies.

Author contributions

Z'aW: Writing–original draft. YW: Writing–review and editing. ZL: Writing–review and editing. WX: Writing–review and editing. SH: Funding acquisition, Writing–review and editing. XK: Conceptualization, Funding acquisition, Supervision, Writing–review and editing, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cytotoxicity and reversal effect of sertraline, fluoxetine, and citalopram on MRP1- and MRP7-mediated MDR

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Cancer is one of the leading causes of death worldwide, and the development of resistance to chemotherapy drugs is a major challenge in treating malignancies. In recent years, researchers have focused on understanding the mechanisms of multidrug resistance (MDR) in cancer cells and have identified the overexpression of ATP-binding cassette (ABC) transporters, including ABCC1/MRP1 and ABCC10/MRP7, as a key factor in the development of MDR. In this study, we aimed to investigate whether three drugs (sertraline, fluoxetine, and citalopram) from the selective serotonin reuptake inhibitor (SSRI) family, commonly used as antidepressants, could be repurposed as inhibitors of MRP1 and MRP7 transporters and reverse MDR in cancer cells. Using a combination of *in silico* predictions and *in vitro* validations, we analyzed the interaction of MRP1 and MRP7 with the drugs and evaluated their ability to hinder cell resistance. We used computational tools to identify and analyze the binding site of these three molecules and determine their binding energy. Subsequently, we conducted experimental assays to assess cell viability when treated with various standard chemotherapies, both with and without the presence of SSRI inhibitors. Our results show that all three SSRI drugs exhibited inhibitory/reversal effects in the presence of chemotherapies on both MRP1-overexpressed cells and MRP7-overexpressed cells, suggesting that these medications have the potential to be repurposed to target MDR in cancer cells. These findings may open the door to using FDA-approved medications in combination therapy protocols to treat highly resistant malignancies and improve the efficacy of chemotherapy treatment. Our research highlights the importance of investigating and repurposing existing drugs to overcome MDR in cancer treatment.

KEYWORDS

cancer, drug resistance, SSRI family, ABC transporters, ABCC1/MRP1, ABCC10/MRP7

1 Introduction

Cancer is a global health concern, with an estimated 19.3 million new cases and almost 10 million deaths reported in 2020 (Sung et al., 2021). Chemotherapy is a common treatment for cancer, but its success depends on reaching an effective anticancer drug concentration inside the cancer cells. Unfortunately, cancers are capable to develop resistance to a variety of chemotherapeutic agents, severely reducing the efficiency of the treatment. Research in recent decades has been focused on understanding the phenomenon of multidrug resistance (MDR) in cancer cells (Nooter and Stoter, 1996; Wu et al., 2011). One of the main causes of MDR is the overexpression of transporters on the membrane of cancer cells that actively export the anti-cancer drugs out of the tumor cells, thereby increasing their survival rates (Leslie et al., 2001; Austin Doyle and Ross, 2003; Thomas and Coley, 2003; Leslie et al., 2005; Munoz et al., 2007). Those pumps belong to the ATP-Binding Cassette (ABC) transporters superfamily, which its members utilize binding and hydrolysis of ATP to translocate substrates across membranes (Tiwari et al., 2011; Wilkens, 2015).

The human genome organization divides the currently 49 known human ABC transporters into seven subfamilies, from ABCA to ABCG. The subfamily ABCC contains 13 members, nine of which are referred to as Multidrug Resistance Proteins (MRPs) (Dean and Allikmets, 2001). Based on their structures, the MRPs are classified as short MRPs (MRP4, MRP5, MRP8, and MRP9) and long MRPs (MRP1, MRP2, MRP3, MRP6, and MRP7). The structure of MRPs is composed of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) that are responsible for binding and hydrolyzing ATP. The NBDs are located on the cytoplasmic side of the membrane, while the TMDs span the lipid bilayer and form the substrate-binding site. The NBDs and the TMDs are connected by a linker region (Wilkens, 2015). The long MRPs are characterized by an additional fifth domain, TMD0, located at the N-terminus of the transporters (Johnson and Chen, 2018). Two of the MRPs, ABCC1/MRP1, and ABCC10/MRP7 have been found to be highly expressed in a variety of tissues, such as the liver, kidney, and brain (Kruh and Belinsky, 2003). They have also been shown to transport a wide range of substrates, including drugs, toxins, and other small molecules. They actively extrude glucuronide conjugates such as estradiol-17-beta-o-glucuronide, glutathione conjugates, such as leukotriene C4 (LTC4), and other xenobiotics (Leier et al., 1994; Stride et al., 1997; Sjölinder et al., 1999; Chen et al., 2003; Hopper-Borge et al., 2004). Both mediate MDR in cancer cells by preventing the intracellular accumulation of anticancer drugs (Stride et al., 1997; Hopper-Borge et al., 2004; Malofeeva et al., 2012).

Inhibiting the function of ABC transporters using small molecules is considered a potential strategy for reversing MDR (Palmeira et al., 2012; Peña-Solórzano et al., 2017; Stefan and Wiese, 2019). Chemosensitizers are drugs that are designed to make cancer cells more susceptible to chemotherapy. By using chemosensitizers in combination with chemotherapy, the efficacy of treatment can be improved, and resistance can be overcome (Krishna and Mayer, 2000). Previous studies have demonstrated that drugs that are not specifically designed for cancer treatment can have anti-cancer effects and thus can be repurposed: Metformin, a drug used to treat type 2 diabetes, has been shown to have anti-cancer properties and has been found to increase the sensitivity of cancer cells to chemotherapy in preclinical and clinical studies (Aljofan and Riethmacher, 2019; Cheng et al., 2020; Urpilainen et al., 2020); Sildenafil, a phosphodiesterase-5 inhibitor used to treat erectile dysfunction, can sensitize cancer cells to chemotherapy and radiation therapy, making them more vulnerable to treatment (Das et al., 2010; Di et al., 2010; Pantziarka et al., 2018; Cruz-Burgos et al., 2021); Statins, drugs used to lower cholesterol levels, have also been found to have potential as chemosensitizers, by increasing the sensitivity of cancer cells to chemotherapy, leading to improved treatment outcomes (Oku et al., 2015; Mallappa et al., 2019).

Selective Serotonin Reuptake inhibitors (SSRIs) are a class of medications commonly used to treat depression, anxiety, and other mental health conditions. These medications work by increasing the levels of the neurotransmitter serotonin in the brain, which helps to reduce symptoms of mental health conditions. They are generally considered to have fewer side effects and be less dangerous than older-generation antidepressants, such as tricyclic antidepressants, because they specifically target the serotonin transporter, rather than affecting a wide range of neurotransmitters (Wang et al., 2018). It was previously shown that drugs from the SSRI family exhibit anti-cancer properties (Nordenberg et al., 1999; Serafeim et al., 2003; Argov et al., 2009; Kast et al., 2013; Zhang et al., 2022). Moreover, it was suggested that SSRI members can be repositioned for cancer treatment (Peer and Margalit, 2006; Drinberg et al., 2014; Baú-Carneiro et al., 2022). For example, fluoxetine (Prozac) was found to be unique among other repurposed chemosensitizers, in that it can effectively target MDR cells at low, safe doses that are well below the levels considered safe for humans. It was proposed this could potentially merit a separate classification for fluoxetine, and maybe other SSRIs, as a fourth-generation chemosensitizer (Peer and Margalit, 2006). Based on these findings, we sought to investigate whether members of the SSRI family, sertraline, fluoxetine, and citalopram, could modulate resistance in cells overexpressing MRP1 and MRP7 transporters.

In this study, we use a multi-disciplinary approach to investigate the protein-ligand interaction of MRP1 and MRP7 with these three SSRI medications and evaluate their ability to counter cell resistance. We used *in silico* tools to predict the binding site of each molecule and performed energy calculations to evaluate the complexes' stability. Then, using *in vitro* assays we were able to examine cancer cell viability using each of the molecules combined with different commonly used chemotherapies. Our results demonstrate that all three SSRI drugs had inhibitory/reversal effects on both MRP1-overexpressed cells and MRP7-overexpressed cells. Hence, our findings suggest that these FDA-approved medications may have potential therapeutic benefits when used in combination therapy protocols to treat highly resistant malignancies. Although there are several MRP1 inhibitors that have been investigated in preclinical and clinical studies, there are currently no MRP1 inhibitors that are approved for clinical use as chemosensitizers (Wang et al., 2021a). Given the high costs and the low success rates associated with traditional drug development schemes, repurposing existing drugs for use as chemosensitizers is a cost-effective and efficient approach to identifying new chemosensitizers. The study opens new possibilities to repurpose these drugs to address this unmet medical need.

2 Materials and methods

2.1 Protein structures and evaluation

The hMRP1 (https://alphafold.ebi.ac.uk/entry/P33527) and hMRP7 (https://alphafold.ebi.ac.uk/entry/Q5T3U5) structures were predicted using AlphaFold (Jumper et al., 2021). Despite the availability of cryo-EM structures of bovine MRP1 in complex with and without the ligand LTC4 (Johnson and Chen, 2017), no high-resolution human MRP1 structure has been determined to date. These structures also contain missing residues, such as an 88-residue gap between residues 868 and 955. The AlphaFold prediction for MRP1 closely resembles the bovine MRP1 structure in its bound state, with an RMSD value of 1.707 Å. As no high-resolution human MRP7 structure is currently available, we also utilized the AlphaFold algorithm to model this protein.

Evaluation of the AlphaFold predicted model protein structures was performed using pLDDT (predicted Local Distance Difference Test) (Jumper et al., 2021), ProSA (Sippl, 1993), ERRAT (Colovos and Yeates, 1993), Procheck 3.5.4 (Laskowski et al., 1993), and assessment tools (clashscore and MolProbity (Williams et al., 2018)) integrated within the SWISS-MODEL workspace (Waterhouse et al., 2018).

2.2 Protein structure network elastic network model (PSN-ENM)

Protein Structure Network (PSN) and Elastic Network Model (ENM) are computational biology techniques used to study the dynamic behavior of proteins (Raimondi et al., 2013). In the PSN model, the protein structure is depicted as a network of nodes and edges. The nodes correspond to amino acid residues, while the edges represent their interactions. The ENM is a coarse-grained model of the protein structure that simplifies the interactions between atoms. It enables the calculation of the normal modes of protein motion, which offer insight into the protein's dynamics and flexibility. We used the WebPSN (Seeber et al., 2015; Felline et al., 2020) for calculating the interactions between the residues in the protein and mapping these interactions onto a network (Seeber et al., 2015; Felline et al., 2020). The resulting network representation of the protein structure was applied to compare MRP1 and MRP7 and identify critical residues and regions at the TMDs.

2.3 Normal mode analysis (NMA)

NMA was performed to characterize the inherent flexibility of MRP1 and MRP7 using DynaMut (https://biosig.lab.uq.edu.au/dynamut/). This extracts the atomic displacement of the Cα atoms and their relative motion amplitude in order to account for their intrinsic motions (Rodrigues et al., 2018). Statistical comparison between the obtained flexibility measures was calculated using Student's t-test.

2.4 Protein preparation and grid generation for docking

Bond orders were assigned, hydrogen atoms were added, water molecules were removed beyond 5 Å of the molecule, and energy minimization was applied using the OPLS4 force field. A receptor grid for all the ligands was generated using the Glide application (Schrodinger Release, 2021a) of Maestro (Schrodinger Release, 2021b) within Schrödinger suite (Schrodinger Release, 2021c) by specifying the highest score binding (active) site residues identified by the "SiteMap" tool (Schrodinger Release, 2021d).

2.5 Ligand preparation for docking

Three FDA-approved drugs from the SSRI family (Sertraline, Fluoxetine, and Citalopram) and two known chemotherapeutic drugs (Doxorubicin and Vincristine) were selected for molecular docking. PubChem database (https://pubchem.ncbi.nlm.nih.gov) was used to extract the chemical structure of the selected molecules. Using the LigPrep module (Schrodinger Release, 2021e) from the Maestro (Schrodinger Release, 2021f), the ligands' structures were optimized using the OPLS4 force field, with respect to energy, chirality, and ionization state. Once all the ligands were optimized, they were further considered for docking studies.

The pKa values of Sertraline, Fluoxetine, and Citalopram, which were determined using Chemaxon (https://chemaxon.com) lie within the range of 9–10. At a physiological pH (7.35–7.45), it is likely that these three molecules would exist predominantly in their protonated (acidic) form and would have a positive charge.

2.6 Molecular docking

Flexible docking was performed on a defined receptor grid using the extra precision mode (XP) in the Glide application (Schrodinger Release, 2021a) of Maestro (Schrodinger Release, 2021b). The glide energy was used to rank the various docking poses and identify the ones that are likely to represent the most favorable binding poses in the protein-ligand complexes. The structure output format was set to a pose viewer file to view the output of the resulting docking studies from a pose viewer.

2.7 Halogen bonds

To analyze the existence of halogen bonds between Sertraline, Fluoxetine, and Citalopram to MRP1 and MRP7, we used Protein–Ligand Interaction Profiler (PLIP) (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index). PLIP is a Python-based open-source software that provides a detailed report on the intermolecular interactions between a protein and its ligand. It identifies different types of interactions such as hydrogen bonds, salt bridges, pi-stacking, hydrophobic contacts and halogen bonds (Salentin et al., 2015; Adasme et al., 2021).

2.8 Molecular mechanics-generalized born surface area (MM-GBSA) calculations

MM-GBSA calculations were performed using two different tools: the Prime module (Schrodinger Release, 2021f) in the Schrödinger Maestro (Schrodinger Release, 2021e) and the fastDRH (http://cadd.zju.edu.cn/fastdrh/overview) (Wang et al., 2022). These tools were used to compute the binding energies using the MM-GBSA method, which involves two equations:

$$\Delta G_{bind} = G_{Complex} - \left(G_{Receptor} + G_{Ligand}\right) \quad (1)$$

The MM-GBSA free energy of binding (ΔG_{bind}) is calculated as the difference between the free energy of the complex ($G_{Complex}$), and the sum of the free energies of the receptor ($G_{Receptor}$), and the ligand (G_{Ligand}), in solution (Genheden and Ryde, 2015).

$$G_{Molecule} = \Delta E_{MM} + \Delta G_{GB} + \Delta G_{SA} - T\Delta S \quad (2)$$

Equation 2 represents the free energy of the molecule. It takes into account the change in internal energy of the molecule (ΔE_{MM}) calculated using molecular mechanics, the change in solvation energy of the molecule (ΔG_{GB}) calculated using the generalized Born surface area method, the change in surface area of the molecule (ΔG_{SA}), and the change in entropy of the molecule ($T\Delta S$) (Genheden and Ryde, 2015).

2.9 Materials

Sertraline, fluoxetine, and citalopram were purchased from TCI America (Portland, OR, USA). Doxorubicin was purchased from LC laboratories (Woburn, MA, USA). Paclitaxel, vincristine, and cisplatin were purchased from Alfa Aesar (Tewksbury, MA, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffer saline (PBS), Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 with L-glutamine medium (RPMI-1640), fetal bovine serum (FBS), penicillin/streptomycin and trypsin-EDTA 0.25% were purchased from Hyclone (Waltham, MA, USA).

2.10 Cell lines and cell culture

This study used the human epidermoid carcinoma cell line KB-3-1 as the drug-sensitive cell line, and its MRP1-overexpressing cell line KB/CV60 (Supplementary Figure S1A), which was maintained in the medium with 1 μg/mL of cepharanthine and 60 ng/mL of vincristine (Aoki et al., 2001); and the MRP7-overexpressing SKOV3/MRP7 cell line (Supplementary Figure S1B) by transfecting recombinant pcDNA3.1/MRP7 plasmids and the parental human ovarian adenocarcinoma cell line SKOV3 (Wang et al., 2021b) were also used. All cell lines were grown as adherent monolayers in an essential medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humid atmosphere incubator at 37 °C with 5% CO_2. KB-3-1 and KB/CV60 were cultured in DMEM, and SKOV3 and SKOV3/MPR7 were cultured in RPMI-1640.

2.11 Cytotoxicity determination by MTT assay

The cytotoxicity was analyzed using a slightly modified MTT assay as previously described (Wang et al., 2021c). Cells were collected and resuspended at a final concentration of 5×10^3 cells/well for all four kinds of cell lines. Paclitaxel, doxorubicin, and vincristine, known as MRP7 or MRP1 substrate drugs, were used as positive controls for different cell lines, and cisplatin as non-substrate for both MRP7 and MRP1 was used as the negative control. The absorbance was determined at 570 nm by the accuSkan GO UV/Vis Microplate Spectrophotometer (Fisher Sci., Fair Lawn, NJ). The IC_{50} values were calculated from the survival curves.

3 Results

In this study, we aimed to determine if three drugs from the SSRI family (sertraline, fluoxetine, and citalopram), commonly used as antidepressants (Figure 1, right panel), can be repurposed as inhibitors of MRP1 and MRP7 transporters (Figure 1, left panel) to overcome MDR in cancer cells.

3.1 Quality assessments and flexibility of the human MRP1 and MRP7 AlphaFold's structures

The AI-generated models of the human MRP1 and MRP7 were evaluated by validation means for their quality. AlphaFold generates a confidence score for each residue, known as pLDDT, which ranges from 0 to 100. The higher the pLDDT score, the more confidence the model has in its prediction. The pLDDT scores for the entire proteins' sequences are 83.01 ± 16.10 and 81.66 ± 16.54 for MRP1 and MRP7, respectively. When considering only the core of the protein (TMD1+2 and NBD1+2), the pLDDT score increases to 87.63 ± 7.54 and 85.63 ± 13.42 for MRP1 and MRP7, respectively. The modeling accuracy is highly supported by the elevated confidence levels, which in turn significantly enhance the likelihood of correctly localizing binding sites (Jumper et al., 2021). ProSA analysis of MRP1 and MRP7 structures gave Z-scores of −13.46 and −15.17, respectively, indicating their structures lie in the range compared to similar-sized structures in the PDB database. ERRAT analysis revealed an overall quality of 97.16% and 95.99% for the MRP1 and MRP7 structures, respectively, when any value above 95% indicates a high confidence level. All bond lengths, backbone, and rotamer angles were in high agreement with standard values. For both proteins, no major stereochemical clashes or bad contacts for main-chain or side-chain parameters were detected. The clashscore and the MolProbity score were very low (1.13 and 1.31 respectively for MRP1, and 0.48 and 0.98 for MRP7), indicating high-quality models. Ramachandran plot for MRP1 showed that 99.7% of the residues lie in allowed regions, from which 92.8% in the most favored regions, whereas for the MRP7 99.8% of the residues lie in allowed regions, from which

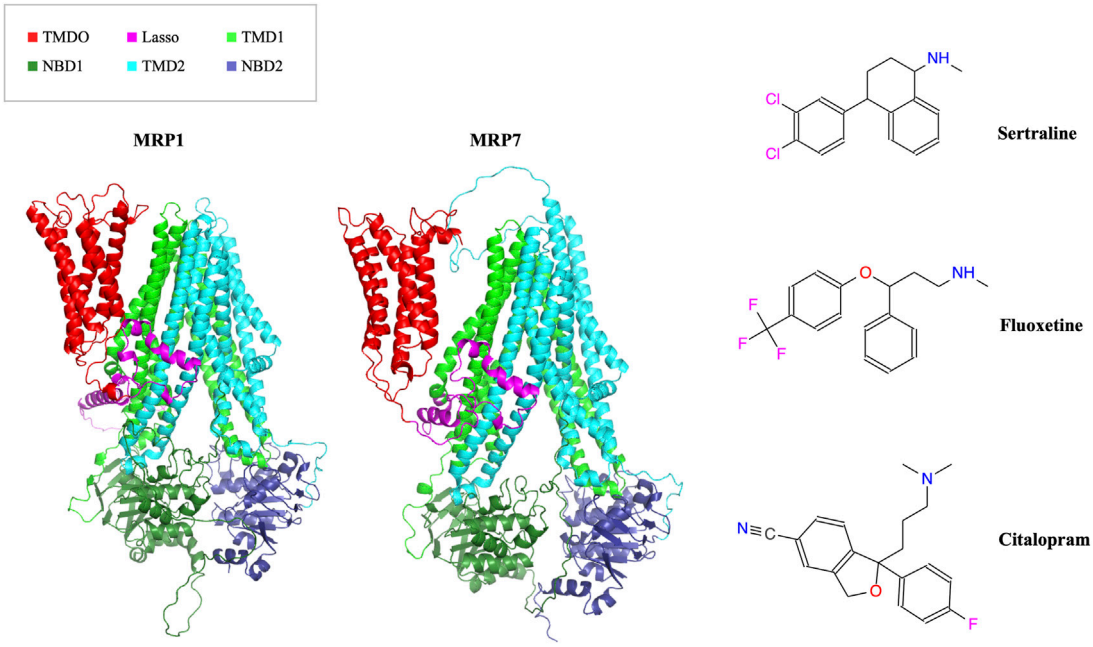

FIGURE 1
In the left panel, the AlphaFold model structures of MRP1 and MRP7 are presented in a cartoon representation viewed from within the membrane plane. Protein's domains: TMD0, Lasso motif, TMD1, NBD1, TMD2, and NBD2 are colored in red, pink, light green, dark green, cyan, and blue, respectively. In the right panel, the molecular structures of three clinically used SSRI medications (sertraline, fluoxetine, and citalopram) are shown.

92.6% in the most favored regions. Overall, according to our quality assessment analyses the AlphaFold-generated models of both proteins are considered reliable structures.

NMA was used to capture the intrinsic flexibility of MRP1 and MRP7. Our results suggest that MRP1 is much more flexible than MRP7 (p-value is <.00001), and it appears to possess a greater degree of conformational adaptability. This is evidenced by having dynamic regions that facilitate its capacity to interact with and transport a diverse range of substrates. Conversely, MRP7 exhibits a comparatively rigid architecture, featuring fewer flexible regions, which may limit its substrate selectivity and transport efficacy.

3.2 Comparison of the human MRP1 and MRP7 using the PSN-ENM approach

In this study, we employed the PSN-ENM (Protein Structure Network - Elastic Network Model) approach to compare the human MRP1 and MRP7 as receptors, with the aim of identifying similarities and differences between these two proteins. We conducted two types of analysis: hubs analysis and meta-path analysis, both of which yielded significant insights.

Hubs analysis refers to the identification of specific residues in a protein that have a relatively large number of connections or interactions with other residues in the same protein. These interactions can be in the form of hydrogen bonds, salt bridges, van der Waals forces, and hydrophobic interactions. We found that MRP1 and MRP7 have distinct sets of hubs, with 234 and 251 total hubs, respectively, and 111 shared hubs between the two proteins. Our analysis identified 46 shared hubs between MRP1 and MRP7 in the transmembrane domains TMD1 and TMD2, which are likely to be the "hot spots" that interact with potential ligands (Figure 2, left panel). For instance, we found that certain residues in MRP1 (K332, M338, P343, H382, and Y384) correspond to specific residues in MRP7 (K292, L298, P303, H342, and Y344). On the other hand, unique hubs for each protein are likely to be crucial in determining their binding specificity. For example, we found that H335, F385, and Q450 are unique hubs for MRP1, while Q1156 is a unique hub for MRP7.

Meta-path analysis refers to the identification of sequences of residues within a protein that are connected by specific interactions. Our meta-path analysis revealed that MRP1 and MRP7 had different dominant meta-paths. Specifically, we found that MRP1 had more diverse and complex meta-paths than MRP7, with 38 links compared to 28 links in MRP7. Furthermore, only four links and eight nodes were shared by both meta-paths, indicating differences in their inner pathways of communication between residues. The common links for the two proteins are I591-P343, L588-P343, G1207-T1241, and E1204-T1241 in MRP1 and M546-P303, L543-P303, G1159-T1195, and Q1156-T1195 in MRP7, respectively. The mutual nodes within the meta-paths are P343, H382, F583, L588, I591, E1204, G1207, and T1241 in MRP1 and P303, N342, F538, L543, M546, Q1156, and T1195 in MRP7, respectively.

The classification of unique hubs and meta-paths through our analysis can aid in the identification and selection of key residues for drug design targeting MRP1 and MRP7 proteins.

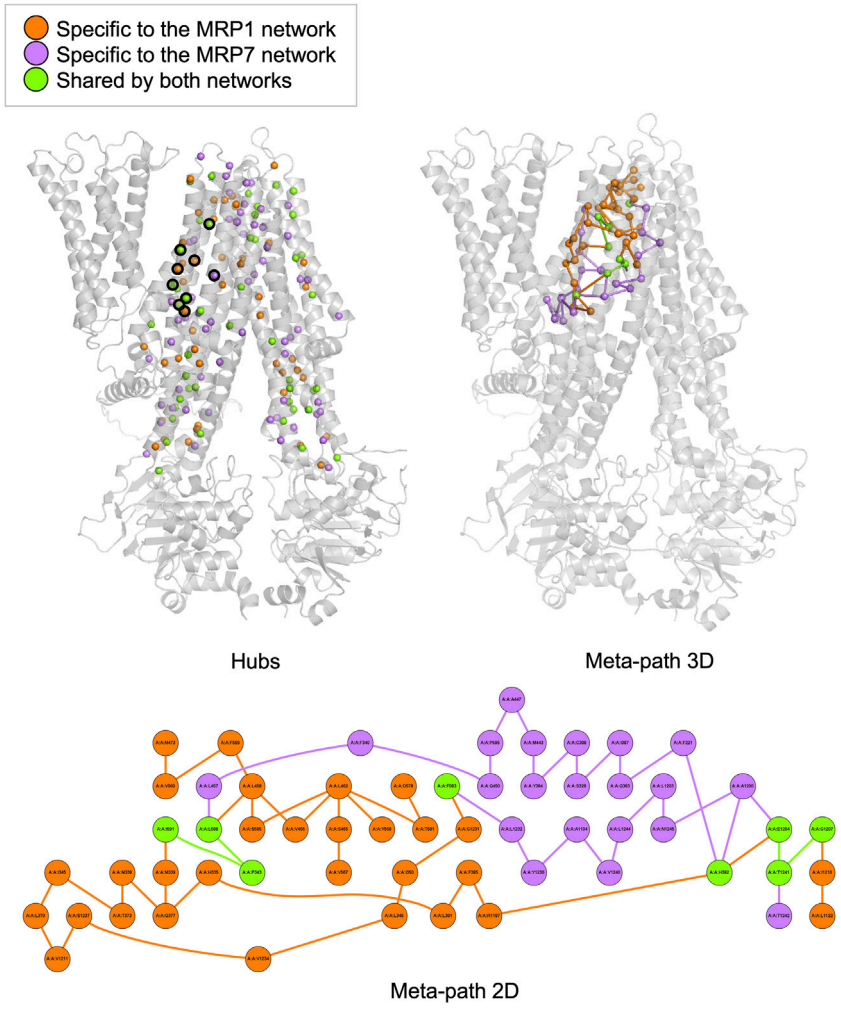

FIGURE 2
Hubs and meta-path analysis projection at the MRP1 structure (as a representative for both MRP1 and MRP7 structures) colored by the following color code: orange - specific for MRP1, purple - specific for MRP7 and green - mutual for both proteins. A 3D representation of MRP1 shows the hubs located in TMD1 and TMD2 as spheres (left panel). Hubs discussed in the text are surrounded by a black frame. A 3D representation of MRP1 shows the meta-path as a series of lines connecting the different nodes (right panel). A 2D representation of the meta-path presents a more detailed view of the interactions between residues along communication pathways in MRP1 (lower panel).

3.3 Identifying binding pockets of sertraline, fluoxetine, and citalopram with MRP1 and MRP7 through molecular docking

We utilized molecular docking to analyze the binding of sertraline, fluoxetine, and citalopram with MRP1 and MRP7, resulting in a total of six protein-ligand complexes. Docking was performed on a defined receptor grid using the extra precision mode (XP) in the Glide application as described in the Methods section. The binding site of MRP1 to the three inhibitors is mainly composed of hydrophobic and polar residues (Figure 3, left panel). The hydrophobic residues involve in sertraline binding are P343, M338, M339, L370, A374, A587, I591, and L1238. Additionally, there are polar regions composed of the following residues: Q377, N590, S1235, Q1239, T1241, and T1242. G342 is also part of the sertraline binding site. The binding site of fluoxetine (Figure 3, left panel, middle row) is also composed of hydrophobic and polar residues as well as charged ones. The hydrophobic residues involve in fluoxetine binding are M339, L381, Y384, F385, A587, I591, and F594. In addition, there are polar regions composed of the following residues: H335, T378, Q450, N590, Q1239, T1241, and T1242. The positively charged K332 and the negatively charged E1204 are also stabilizing fluoxetine in the binding site. Similarly, the binding site of citalopram (Figure 3, left panel, bottom row) is composed of hydrophobic, polar, and charged residues. The hydrophobic residues involve in citalopram binding are M339, A374, L381, Y384, F385, I591, F594, and W1246. The polar residues involve in citalopram binding are HIS335, Q450, N590, Q377, T378, H382, N1208, T1241, and T1242. The positively charged K332 and R593 as well as the negatively charged E1204 also play a role in the fluoxetine binding.

The binding site of MRP7 to sertraline shares similarities with MRP1, as it is mainly composed of hydrophobic and polar residues. However, the binding site of MRP7 includes five glycine residues,

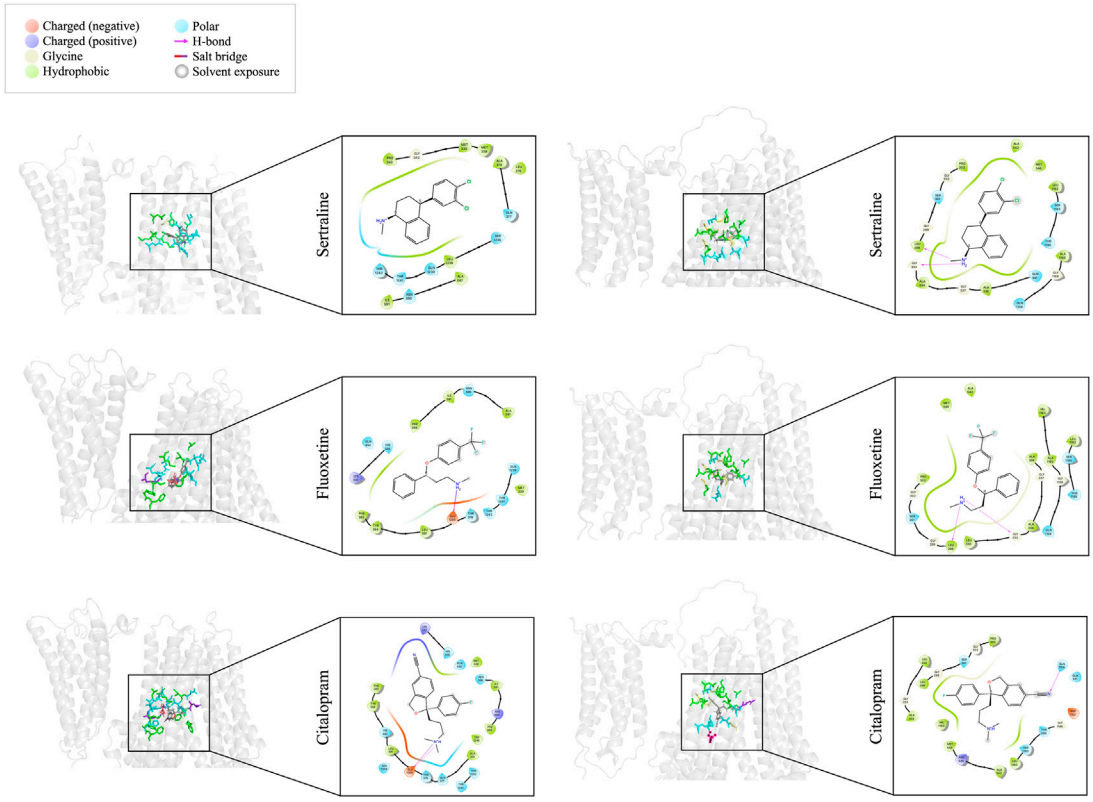

FIGURE 3
Docking interactions of sertraline, fluoxetine, and citalopram with MRP1 (left panel) and MRP7 (right panel). On the left side of each panel, a 3D cartoon representation of the extracellular part of the protein (in light gray), bound to the different drugs (sticks representation in dark gray) is presented. Residues involved in the binding of each drug are colored as described in the color legend. On the right side of each panel, a 2D representation of the different interactions of the specific drug in the binding site, including residues' names and positions, are colored based on the color legend.

whereas only one glycine residue was involved in the interactions of MRP1 (Figure 3, right panel, top row). The hydrophobic residues involve in sertraline binding are L298, P303, A334, A338, A542, M546, A1160, and L1192. The polar regions are composed of the following residues: S301, Q341, Q1156, S1193, and T1195. The glycine residues that are involved in the binding are in positions 299, 302, 333, 337, and 1159. Similarly, the binding site of fluoxetine to MRP7 (Figure 3, right panel, middle row) is composed of hydrophobic residues, polar residues, and glycine residues. The hydrophobic residues involve in fluoxetine binding are L298, P303, L330, A334, A338, A542, M546, A1160, V1163, and L1192. The polar regions are composed of the following residues: S301, Q1156, S1193, and T1195. The glycine residues are the same as in the sertraline binding site. The binding site of citalopram to MRP7 (Figure 3, right panel, bottom row) is composed of hydrophobic, polar, charged, and glycine residues. The hydrophobic residues involve in citalopram binding are the same as for fluoxetine, except for A338 and A1160. The polar residues involved in citalopram binding are the same as for sertraline. The glycine residues that are involved in the binding are in positions 299, 302, 333, and 1196. The positively charged R545 and the negatively charged D1152 are also stabilizing the citalopram in the binding site.

3.4 Comparison of the interacting residues in the binding sites of MRP1 and MRP7

The amino acids of MRP1 and MRP7 that are involved in the binding of the drugs sertraline, fluoxetine, and citalopram are summarized in Table 1. From the proteins' perspective: MRP1 and MRP7 share three residues that interact with all the drugs: M339, I591, and T1241 in MRP1 correspond to G299, M546, and T1195 in MRP7 (Table 1, dark gray). For MRP1, residues N590 and T1242 are involved in binding all three ligands but not for MRP7 (Table 1, light gray). For MRP7 solely, the residues that are involved in the binding of all three ligands are L298, S301, G302, P303, A334, A542, Q1156, L1192, and S1193 (Table 1, light gray). From the drugs' perspective, there are 10 mutual residues in MRP1 and MRP7 that bind sertraline: M338/L298, M339/G299, G342/G302, P343/P303, A374/A334, Q377/G337, A587/A542, I591/M546, L1238/L1192, Q1239/S1193, and T1241/T1195 for MRP1/MRP7 respectively; seven mutual residues in MRP1 and MRP7 that bind fluoxetine: M339/G299, R378/A338, A587/A542, I591/M546, E1204/Q1156, Q1239/S1193, and T1241/T1195; eight mutual residues in MRP1 and MRP7 that bind citalopram: M339/G299, A374/A334, L381/Q341, N590/R545, I591/M546, E1204/Q1156, T1241/T1195, and T1242/G1196.

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TABLE 1 The interacting residues involved in binding the three inhibitors, sertraline, fluoxetine, and citalopram, to the proteins MRP1 and MRP7. Each row presents a different amino acid and its position, and each column presents a different drug. The presence of an amino acid is indicated by a letter and a number, and its absence is indicated by a hyphen. The MRP1 and MRP7 were aligned using PyMOL, and the table uses color coding to highlight residues found to bind the inhibitors. Residues that bind the three inhibitors in the same protein are colored in light gray, and residues that bind the three inhibitors in both proteins are colored in dark gray. Residues binding the same drug for both proteins are highlighted in bold.

MRP1			MRP7		
Sertraline	Fluoxetine	Citalopram	Sertraline	Fluoxetine	Citalopram
-	K332	K332	-	-	-
-	H335	H335	-	-	-
M338	-	-	**L298**	L298	L298
M339	**M339**	**M339**	**G299**	**G299**	**G299**
-	-	-	S301	S301	S301
G342	-	-	**G302**	G302	G302
P343	-	-	**P303**	P303	P303
L370	-	-	-	L330	L330
-	-	-	G333	-	G333
A374	-	**A374**	**A334**	A334	**A334**
Q377	-	Q377	**G337**	G337	-
-	**T378**	T378	A338	**A338**	-
-	L381	**L381**	Q341	-	**Q341**
-	-	H382	-	-	-
-	Y384	Y384	-	-	-
-	F385	F385	-	-	-
-	Q450	Q450	-	-	-
A587	**A587**	-	**A542**	**A542**	A542
N590	N590	**N590**	-	-	**R545**
I591	**I591**	**I591**	**M546**	**M546**	**M546**
-	-	R593	-	-	-
-	F594	F594	-	-	-
-	-	-	-	-	D1152
-	**E1204**	**E1204**	Q1156	**Q1156**	**Q1156**
-	-	-	G1159	G1159	-
-	-	N1208	A1160	A1160	-
-	-	-	-	V1163	-
S1235	-	-	-	-	-
L1238	-	-	**L1192**	L1192	L1192
Q1239	**Q1239**	-	**S1193**	**S1193**	S1193
T1241	**T1241**	**T1241**	**T1195**	**T1195**	**T1195**
T1242	T1242	**T1242**	-	-	**G1196**
-	-	W1246	-	-	-

TABLE 2 Halogen bonds formed by sertraline and citalopram with MRP1 and MRP7. Each row in the table includes specific details about the protein residues that are involved in the binding, the atoms of the ligand that participate in the interaction, and the geometry of the bond. All atom and residue numbering are in accordance with the numbering in the corresponding PDB file given as input (post-docking complex).

	Residue	AA	Distance (Å)	Donor angle	Acceptor angle	Donor atom	Acceptor atom
MRP1-SER	370	LEU	4.80	161.40	143.74	2 [Cl]	3065 [O]
	1241	THR	4.96	149.65	92.00	1 [Cl]	9826 [Oγ]
MRP1-CIT	593	ARG	3.22	140.51	114.03	1 [F]	4827 [Nε]
MRP7-CIT	330	LEU	3.87	151.46	145.53	1 [F]	2547 [O]

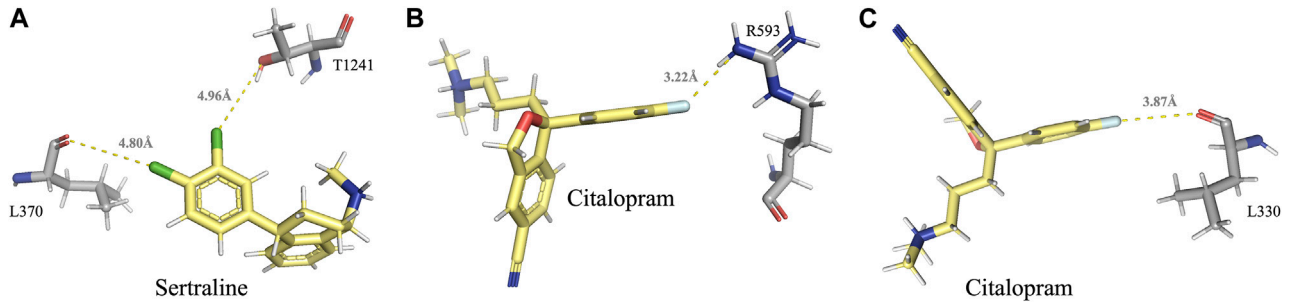

FIGURE 4
Visual representation of the halogen bonds formed by sertraline and citalopram with MRP1 and MRP7, as elaborated in table 2. **(A)** Halogen bonds formed between the chlorine atoms of sertraline and the backbone carbonyl oxygen atom of L370 and the side-chain hydroxyl oxygen atom of T1241. **(B)** A halogen bond formed between the fluorine atom of citalopram and the guanidinium group of R593. **(C)** A halogen bond formed between the fluorine atom of citalopram and the backbone carbonyl oxygen of L330. Residues of MRP1 and MRP7 are colored in gray, and the drugs are colored in yellow.

MRP1 and MRP7 share a significant amount of corresponding residues, involved in the binding of sertraline, fluoxetine, and citalopram. This suggests that both MRP1 and MRP7 may bind these drugs in similar ways and may have similar mechanisms of drug resistance. The amino acids that exclusively interact with the drugs only in one protein can provide crucial insights into the specific interactions that facilitate the binding of these drugs to MRP1, in contrast to MRP7.

3.5 Halogen-mediated interactions

A halogen bond is a type of intermolecular interaction that results from the electrostatic interaction between a halogen atom and a negatively charged species, such as a lone pair of electrons or a negative ion. XB donors can include iodine, bromine, chlorine, and, in certain cases, fluorine. Although the fluorine atom is not a very effective XB donor, it can exhibit a positive σ-hole when bound to another fluorine atom or attached to O, N, C, or other atoms that possess strong electron-withdrawing substituents (Cavallo et al., 2016). Halogen bonds share similarities with hydrogen bonds in terms of strength and directionality; however, they possess a longer range (in some cases up to 6.0 Å) and exhibit greater directionality. In protein-ligand complexes, halogen bonding interactions can arise between a halogen-containing ligand and any available Lewis base within the protein's binding site (Wilcken et al., 2013). Sertraline, fluoxetine, and citalopram share a common feature in their chemical structure - the presence of halogen groups. Specifically, sertraline has a dichlorophenyl group, fluoxetine has a trifluoromethyl group, and citalopram has a fluorophenyl group. Our analyses have shown that these groups play a significant role in the interactions with the MRPs. All halogen bonds were analyzed using Protein–Ligand Interaction Profiler (PLIP) and subsequently validated for correct orientation and bond length using PyMOL. Sertraline's chlorine atoms interact with the backbone carbonyl oxygen atom of L370 ($d_{F\leftrightarrow O}$ = 4.80 Å) and with the side-chain hydroxyl oxygen atom of T1241 ($d_{F\leftrightarrow O}$ = 4.96 Å) in MRP1. Citalopram's fluorine atom forms a halogen bond with the guanidinium group of R593 in MRP1 ($d_{F\leftrightarrow N}$ = 3.22 Å) and with the backbone carbonyl oxygen of L330 in MRP7 (dF↔O = 3.87 Å) (Table 2; Figure 4).

3.6 Energy calculations of the six complexes

To evaluate the binding affinity of these complexes, we used three different methods for energy calculations: Glide energy, Prime MM-GBSA calculations, and fastDRH MM-GBSA calculations (Table 3). The binding free energy value serves as an indicator of the binding strength between the ligand and

TABLE 3 The energy values (kcal/mol units) of the three SSRI drugs (sertraline, fluoxetine, and citalopram) bound to MRP1 and MRP7, as calculated by three different methods: Glide Energy, MM-GBSA using Prime, and MM-GBSA using fastDRH. A more favorable binding position is indicated by a lower energy value. The table presents the top-ranked pose/most favorable docking conformer for each protein-ligand complex.

	Glide energy		MM-GBSA (Prime)		MM-GBSA (fastDRH)	
Protein	MRP1	MRP7	MRP1	MRP7	MRP1	MRP7
Drug						
Sertraline	−33.516	−31.386	−40.35	−43.17	−31.22	−25.66
Fluoxetine	−30.592	−36.024	−33.64	−37.24	−22.60	−30.87
Citalopram	−35.401	−33.102	−42.70	−35.83	−28.21	−25.67

protein, wherein a lower value corresponds to a more favorable docking orientation and a stronger binding interaction. A negative value signifies that the free energy of the protein-ligand complex is lower than that of the individual components when they are present in a solution, indicating greater stability and a higher probability of being observed in nature.

The Glide energy scores were analyzed to measure the quality of the docking pose, considering various factors such as the shape complementarity between the ligand and the protein binding site, the van der Waals interactions strength, and the solvation energy of the complex. All six complexes had comparable Glide energy scores ranging from −36.024 to −30.592 kcal/mol. The MRP1-sertraline and MRP1-citalopram complexes had better docking scores than the MRP7 complexes. However, the MRP7-fluoxetine complex had a better score than the MRP1 complex. Among the six complexes, the MRP7-fluoxetine complex achieved the best Glide energy score, whereas the MRP1-fluoxetine complex had the least favorable score.

The Prime MM-GBSA calculations were performed to validate the docking results further. All inhibitors had relatively similar binding free energy (ΔG_{Bind}) values ranging from −43.17 to −33.64 kcal/mol. The MRP7-sertraline and MRP7-fluoxetine complexes had lower free energy of binding values than the MRP1 complexes. However, the MRP1-citalopram complex had a lower free energy of binding value than the MRP7 complex. Among the complexes, the MRP7-sertraline complex had the most negative free energy of binding value (−43.17 kcal/mol), whereas the MRP1-fluoxetine complex had the least negative value (−33.64 kcal/mol).

In addition, we used the fastDRH software, an independent tool from the Schrödinger suite, to compute the binding energy score. The results showed that the MM-GBSA binding energy using fastDRH ranged from −31.22 to −22.6 kcal/mol, with a variation similar to that obtained by the Glide energy and the MM-GBSA using Prime. The MRP1-sertraline and MRP1-citalopram complexes exhibited lower free energy of binding values than the MRP7 complexes, but the MRP7-fluoxetine complex had a lower value than the MRP1 complex. The MRP1-sertraline complex displayed the most negative value (−31.22 kcal/mol), whereas the MRP1-fluoxetine complex had the least negative value (−22.60 kcal/mol). The slight difference in the values between the two methods for MM-GBSA calculations is algorithm dependent.

3.7 The effect of sertraline, fluoxetine, and citalopram on the efficacy of antineoplastic drugs in MRP1- and MRP7-Mediated MDR cell lines

An MTT assay was performed to examine the cell viability of sertraline, fluoxetine, and citalopram on the pair of KB-3-1 and KB/CV60 cells (Figure 5) and the pair of SKOV3 and SKOV3/MRP7 cells (Figure 6), respectively. After that, the concentrations of sertraline, fluoxetine, and citalopram with no toxic (lower than IC_{20}) were chosen to test the potential reversal effect by the MTT assay of substrate drugs with or without an inhibitor. Table 4 shows that the decreased cytotoxicity of the substrate drugs, vincristine, and doxorubicin, can be enhanced to a similar level with the known MRP1 inhibitor ONO-1078 and even similar to the cytotoxicity in the parental KB-3-1 cells. At the same time, the decreased cytotoxicity of the substrate drug, paclitaxel, can be enhanced to a similar level with the known MRP7 inhibitor cepharanthine and even similar to the cytotoxicity in the parental SKOV3 cells (Table 5). In addition, all three drugs and ONO-1078 and cepharanthine did not alter the cytotoxicity of the non-substrate drug, cisplatin, in either the drug-resistant cells or the parental cells.

4 Discussion

ABC transporters, which are responsible for the efflux of drugs from cells, are considered promising targets for drug discovery. By developing inhibitors of these transporters, multidrug resistance (MDR) can potentially be overcome and drug accumulation in cells can be improved. This study aims to investigate the repurposing of FDA-approved medications, specifically drugs from the SSRI family that are commonly used as antidepressants, to address drug resistance caused by ABCC1/MRP1 and ABCC10/MRP7, when exposed to chemotherapy. The research approach combines *in silico* predictions with *in vitro* validations to achieve synergy in the results obtained.

To examine the molecular characteristics of the human MRP1 and MRP7 in an atomic-scale resolution, it is necessary to analyze their structures. We previously generated these structures by homology modeling and subsequent molecular dynamics simulations (Amram et al., 2014; Bin Kanner et al., 2021; Wang et al., 2021d). However, our models were based on the cryo-EM bovine MRP1 that lacked stretches of tenths of residues and TMD0 or on the SAV1866 bacterial transporter. Therefore, in

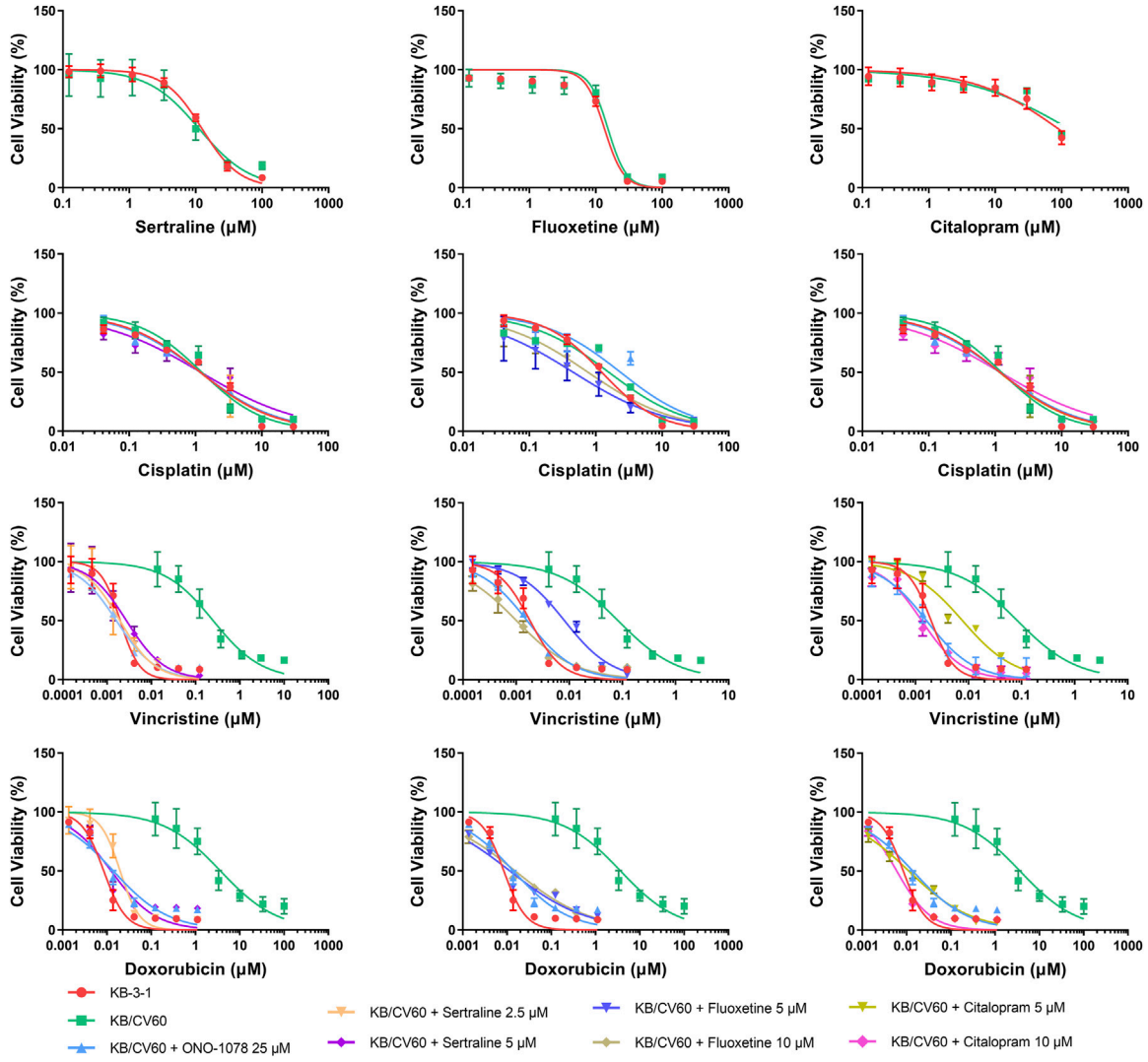

FIGURE 5
MTT assay showing the ability of sertraline, fluoxetine, and citalopram to reverse MDR mediated by MRP1 over-expressed in KB/CV60 cells. Mean ± SD, n = 3.

our current study, we opted to use the AlphaFold-generated MRP1 and MRP7 human structures as they include the missing gaps from the bovine MRP1 structure and TMD0. AlphaFold is currently a prominent source of protein structures that have not been experimentally determined. The AlphaFold models of the human MRP1 and MRP7 are consistent with the topological distinctive architecture of long eukaryotic ABCC transporters, as elaborated in Figure 1, left panel. By conducting comprehensive quality assessment analyses, we have determined that the AlphaFold-generated models can be considered robust and sound structures that are suitable for further in-depth structural and functional applications. Noteworthy, MRP1 and MRP7 possess structural differences, that may account for their distinct substrate specificities and transport activities.

Before performing docking experiments, we utilized the PSN-ENM method to gain a comprehensive understanding of the distinct characteristics and similarities between MRP1 and MRP7 and to reveal the unique binding modes of these two proteins. Specifically, we focused on the TMDs domains, which are of high interest due to their role in substrate binding and translocation. Two network analyses were conducted: hubs and meta-path. Hubs analysis identified highly connected nodes within the protein network, which may correspond to residues or regions of the protein that are involved in multiple interactions. Hubs that are within the TMDs have a high potential to be involved in ligand binding and to serve as druggable sites. Meta-path analysis involves examining the influence of different types of paths between nodes, providing insights into functional relationships between residues and how they contribute to protein function and stability.

We analyzed the networks of MRP1 and MRP7 to identify similarities and differences between the two proteins. The common hubs and links can be considered points of convergence in the communication pathways between residues within MRP1 and MRP7, revealing important similarities in the functional relationships between residues within these two proteins. We

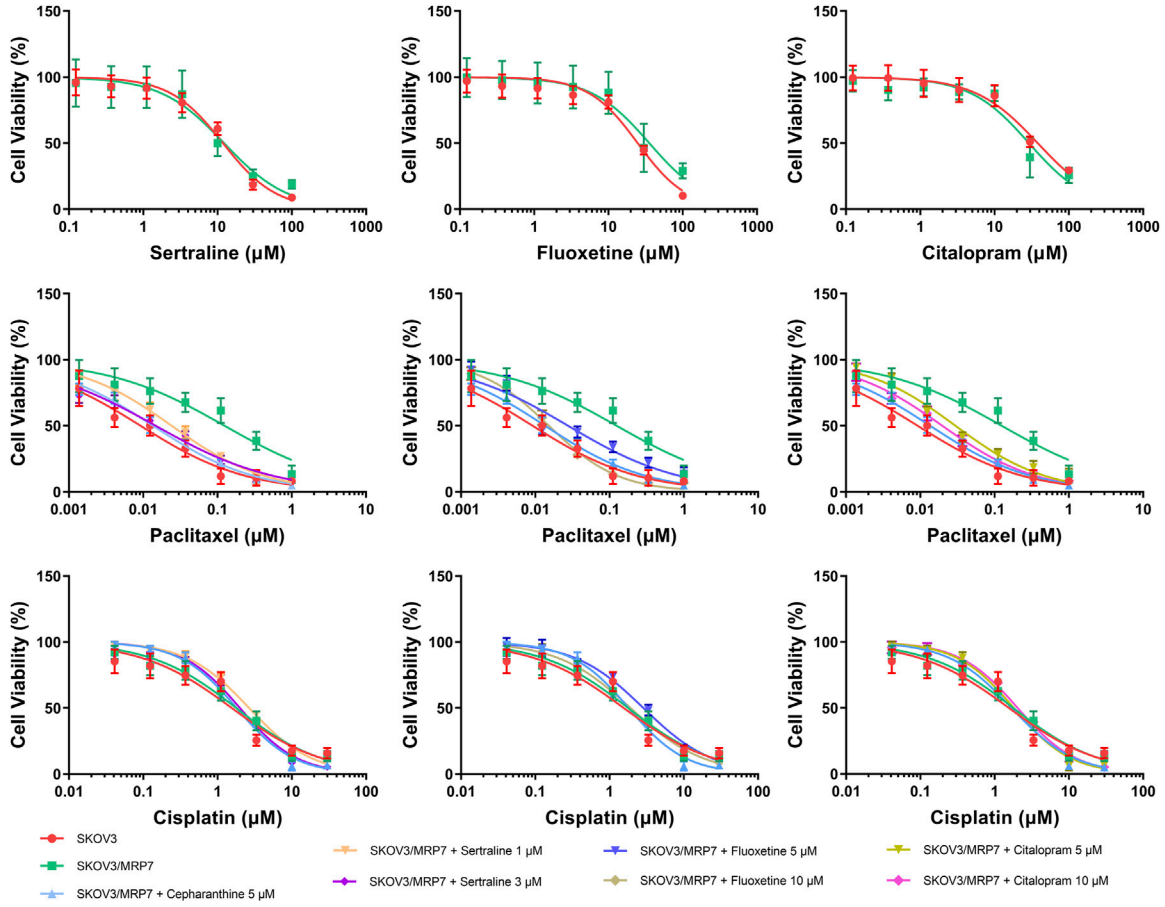

FIGURE 6
MTT assay showing the ability of sertraline, fluoxetine, and citalopram to reverse MDR mediated by MRP7 over-expressed in SKOV3/MRP7 cells. Mean ± SD, n = 3.

located 46 shared hubs in TMD1 and TMD2 that correspond to interacting residues, including K332/K292, M338/L298, P343/P303, H382/H342, and Y384/Y344 in MRP1/MRP7, respectively. These hubs were later found in the docking experiments to bind the SSRI drugs, which provided additional confirmation of our docking results. Surprisingly, in the meta-path analysis, we found only four links and eight nodes shared by both meta-paths. Six out of eight of the mutual regular nodes (not hubs) within the meta-paths were also found later in the docking experiments to bind the SSRI drugs: P343/P303, H382/N342, I591/M546, E1204/Q1156, G1207/G1159, and T1241/T1195 in MRP1/MRP7, respectively. Although the mutual node F583/F538 is not part of the binding site, it is specifically interesting since we previously reported that the point mutation F583A results in a long-range allosteric impact, that propagates across the membrane, rendering the protein inactive (Weigl et al., 2018; Bin Kanner et al., 2021). The unique hubs and links in the networks of MRP1 and MRP7 suggested differences in their binding specificity, and analyzing these unique links could identify key residues or interactions specific to each protein. For instance, the protein-specific hubs include H335, F385, and Q450 for MRP1, and Q1156 for MRP7. Our docking analyses further confirmed that these residues were involved in ligand binding only in one protein.

Molecular docking was performed using Maestro by Schrödinger suite to investigate the binding modes of sertraline, fluoxetine, and citalopram to MRP1 and MRP7 (Figure 3). It is imperative to visually evaluate the docking poses and consider other factors, such as the strength and type of chemical interactions between the protein and ligand, the conformational stability of the complex, and the biological relevance of the docking pose. In the case of MRP1, the binding sites of the drugs comprised mainly of hydrophobic and polar residues, with some charged residues also involved in binding (Figure 3, left panel). This aligns with previous studies, which divided the binding pocket into two sections: a positively charged region (the P-pocket) and a primarily hydrophobic area (the H-pocket) (Johnson and Chen, 2017; He et al., 2021). While the binding sites of MRP7 and MRP1 share hydrophobic and polar residues, MRP7's binding site contains five glycine residues, whereas MRP1's binding site has only one (as shown in Figure 3, right panel). Glycine residues are thought to play a role in enhancing flexibility at enzyme active sites (Yan and Sun, 1997). The presence of glycine residues in MRP7's binding site confers localized flexibility, presumably facilitating the binding of a variety of ligands and compensating for the protein's relative overall rigidity in comparison to MRP1. The different orientations of the drugs bound to each protein, as depicted in Figure 3 (left panel vs

TABLE 4 Effect of sertraline, fluoxetine, and citalopram on reversing MRP1-mediated MDR in KB/CV60 cells. Resistance Fold was calculated by dividing the IC_{50} value of a substrate drug into drug-resistant KB/CV60 cells in the presence or absence of an inhibitor (sertraline, fluoxetine, citalopram, or ONO-1078) by the IC_{50} value of parental KB-3-1 cells without an inhibitor.

Treatment	IC_{50} (Mean ± SD, nM) [Resistance fold]	
	KB-3-1	KB/CV60
Vincristine	1.98 ± 0.27 [1.00]	249.6 ± 27.73 [126.06]
VCR + Sertraline (2.5 μM)	1.96 ± 0.33 [0.99]	1.74 ± 0.18 [0.88]
VCR + Sertraline (5 μM)	1.74 ± 0.26 [0.88]	1.97 ± 0.27 [0.99]
VCR + Fluoxetine (5 μM)	1.83 ± 0.22 [0.92]	12.82 ± 2.13 [6.47]
VCR + Fluoxetine (10 μM)	1.61 ± 0.18 [0.81]	1.56 ± 0.17 [0.79]
VCR + Citalopram (5 μM)	1.98 ± 0.27 [1.00]	8.34 ± 1.61 [4.21]
VCR + Citalopram (10 μM)	1.77 ± 0.14 [0.89]	1.51 ± 0.26 [0.76]
VCR + ONO-1078 (20 μM)	1.62 ± 0.23 [0.82]	1.77 ± 0.21 [0.89]
Doxorubicin	8.48 ± 1.30 [1.00]	672.3 ± 88.50 [79.28]
DOX + Sertraline (2.5 μM)	9.18 ± 1.77 [1.08]	19.85 ± 2.08 [2.34]
DOX + Sertraline (5 μM)	8.19 ± 0.94 [0.97]	12.50 ± 1.53 [1.47]
DOX + Fluoxetine (5 μM)	8.67 ± 1.09 [1.02]	16.75 ± 1.63 [1.98]
DOX + Fluoxetine (10 μM)	9.28 ± 1.68 [1.09]	12.57 ± 1.56 [1.48]
DOX + Citalopram (5 μM)	8.40 ± 0.92 [0.99]	9.33 ± 1.04 [1.10]
DOX + Citalopram (10 μM)	6.05 ± 0.97 [0.71]	6.07 ± 0.84 [0.72]
DOX + ONO-1078 (20 μM)	8.98 ± 0.74 [1.06]	7.25 ± 0.92 [0.85]
Cisplatin	1778 ± 253.4 [1.00]	1839 ± 230.4 [1.03]
Cisplatin + Sertraline (2.5 μM)	1672 ± 249.4 [0.94]	1755 ± 198.5 [0.99]
Cisplatin + Sertraline (5 μM)	1852 ± 242.9 [1.04]	1794 ± 172.9 [1.01]
Cisplatin + Fluoxetine (5 μM)	1868 ± 162.6 [1.05]	1842 ± 191.0 [1.04]
Cisplatin + Fluoxetine (10 μM)	1725 ± 192.2 [0.97]	1827 ± 238.4 [1.03]
Cisplatin + Citalopram (5 μM)	1795 ± 177.5 [1.01]	1688 ± 152.7 [0.95]
Cisplatin + Citalopram (10 μM)	1758 ± 181.5 [0.99]	1776 ± 215.7 [1.00]
Cisplatin + ONO-1078 (20 μM)	1833 ± 107.7 [1.03]	1769 ± 229.0 [0.99]

Values in the table are represented as means ± SD determined from at least three independent experiments performed in triplicate.

right panel), corroborate our observation of distinct levels of flexibility between the two proteins.

We identified several key amino acid residues in MRP1 and MRP7 that are involved in binding all three drugs, as well as some residues that were specific to certain drugs, as elaborated in Table 1. To evaluate the biological relevance of the docking pose, it is important to determine whether the docking pose is consistent with known biological data and if it is likely to be biologically active. In the ligand-bound bovine cryo-EM structures of MRP1 (Johnson and Chen, 2017; Pietz et al., 2023), important residues involved both in LTC4 and macrocyclic peptide binding were identified, including K332 and H335 (forming hydrogen bonds) and L381, F385, and F594 (forming hydrophobic contacts). It has been demonstrated that the transport of many substrates is significantly reduced or completely lost when these residues are mutated (Haimeur et al., 2002; Campbell et al., 2004; Haimeur et al., 2004; Situ et al., 2004). Similarly, the mutation of W1246 led to a loss of drug resistance and specifically affected the transport of organic anions (Ito et al., 2001). The outward-facing bovine cryo-EM structure study revealed that local structural changes in the substrate-binding site are necessary for substrate recognition and release outside the cell. Several residues, including F594 and Y1242 (corresponding to T1242 in humans), reposition their side chains to coordinate LTC4 during the transition from the apo to substrate-bound conformation. F583 was also found to be essential in exposing the translocation pathway to the extracellular space. In a study on the functional sites in human MRP1, P343, H382, Q450, F583, and R593 in TMD1, and S1235 in TMD2 were found to have relatively high ΔΔG values, highlighting their importance as key residues (He et al., 2021). The mutation N590A was reported to decrease the affinity of hMRP1 for LTC4 and substantially reduced the binding of ATP to NBD1 (Zhang et al., 2004). For MRP7, we previously

TABLE 5 Effect of sertraline, fluoxetine, and citalopram on reversing MRP7-mediated MDR in SKOV3/MRP7 cells. Resistance Fold was calculated by dividing the IC_{50} value of a substrate drug into drug-resistant SKOV3/MRP7 cells in the presence or absence of an inhibitor (sertraline, fluoxetine, citalopram, or cepharanthine) by the IC_{50} value of parental SKOV3 cells without an inhibitor.

Treatment	IC_{50} (Mean ± SD, nM) [Resistance fold]	
	SKOV3	SKOV3/MRP7
Paclitaxel	9.21 ± 0.998 [1.00]	124.6 ± 23.8 [13.5]
PTX + Sertraline (1 μM)	8.55 ± 0.824 [0.93]	19.5 ± 2.16 [2.12]
PTX + Sertraline (3 μM)	8.57 ± 0.853 [0.93]	13.6 ± 2.13 [1.45]
PTX + Fluoxetine (5 μM)	9.53 ± 0.987 [1.03]	27.6 ± 2.52 [3.00]
PTX + Fluoxetine (10 μM)	9.20 ± 0.962 [1.00]	16.7 ± 1.85 [1.81]
PTX + Citalopram (5 μM)	9.28 ± 0.984 [1.01]	17.8 ± 1.82 [1.94]
PTX + Citalopram (10 μM)	9.96 ± 1.08 [1.08]	15.2 ± 1.71 [1.65]
PTX + Cepharanthine (5 μM)	8.68 ± 0.952 [0.94]	10.6 ± 1.17 [1.15]
Cisplatin	1752 ± 178 [1.00]	1787 ± 175 [1.02]
Cisplatin + Sertraline (1 μM)	1656 ± 183 [0.95]	1793 ± 193 [1.02]
Cisplatin + Sertraline (3 μM)	1784 ± 175 [1.02]	1786 ± 181 [1.02]
Cisplatin + Fluoxetine (5 μM)	1812 ± 185 [1.03]	1816 ± 188 [1.04]
Cisplatin + Fluoxetine (10 μM)	1766 ± 175 [1.01]	1789 ± 196 [1.02]
Cisplatin + Citalopram (5 μM)	1872 ± 185 [1.07]	1857 ± 199 [1.06]
Cisplatin + Citalopram (10 μM)	1794 ± 198 [1.02]	1713 ± 184 [0.98]
Cisplatin + Cepharanthine (5 μM)	1846 ± 187 [1.05]	1732 ± 183 [0.99]

Values in the table are represented as means ± SD determined from at least three independent experiments performed in triplicate.

predicted the potential binding pocket of inward-facing MRP7 based on the homology modeling of the bovine MRP1 cryo-EM structure (Wang et al., 2021d). In that study, we suggested that A542, R545, M546, and D1152 were residues involved in ligand binding. Furthermore, using docking experiments we presented the interaction of Paclitaxel and Methotrexate with the following residues: G299, A334, G337, A338, Q341, R545, M546, D1152, Q1156, L1192, S1193, T1195, G1196.

Halogenated compounds, including both synthetic and naturally occurring substances, have become increasingly popular in recent years for their diverse range of biological activities. The incorporation of halogen groups in the design of therapeutic agents has led to the development of innovative drugs with improved pharmacological properties (Benedetto Tiz et al., 2022). Accordingly, we found that sertraline and citalopram interact with various residues in the binding pocket of the MRPs through halogen bonds (as elaborated in the results section). This finding is consistent with previous studies showing that halogen-containing molecules can interact with ABC transporters. For instance, halogenated chalcones exhibited high binding affinity to P-gp (Bois et al., 1998), halogenated methylpurines were used as a substrate for MRP1 (Okamura et al., 2009; Zoufal et al., 2019), and halogenated derivatives of flavone-based compounds showed MDR-reversing capacity in MRP1 expressing cells (Mavel et al., 2006). Our results suggest that the presence of halogen groups in the chemical structure of SSRIs may contribute to their ability to inhibit MRPs. This insight provides valuable information on the mechanism of action of halogenated drugs and their potential therapeutic applications.

To determine the binding energy between the proteins and each of the drugs, three energy calculation methods were used: glide energy, MM-GBSA using Prime, and MM-GBSA using fastDRH. It is important to note that the values provided in Table 3 are based on the approximations used in the Glide software of Maestro and the MM-GBSA formalism. Therefore, these values should be considered estimates that indicate qualitative trends rather than precise quantitative values. The results revealed several important trends. First, the differences between the drugs were relatively small in each method, suggesting that all the ligands bind with similar affinity to the proteins. Second, the binding energy of the drugs to the two proteins differed, indicating that MRP1 and MRP7 may have slightly different binding characteristics for the three drugs. Furthermore, in two of the three methods, citalopram had the best score for MRP1, and fluoxetine had the best score for MRP7. Apart from MRP7-fluoxetine, the MRP1-ligand complexes consistently received higher rankings than the MRP7 complexes in most of the evaluations. This observation could be attributed to the innate flexibility of the proteins, as indicated by our NMA analysis. Specifically, MRP1 demonstrated greater structural flexibility, featuring numerous highly mobile regions that could facilitate its ability to bind and transport a diverse range of substrates. Conversely, MRP7 exhibited a more rigid structure, with fewer flexible

regions, which could limit its substrate specificity and transport efficiency. Finally, the results suggest that different energy calculation methods may yield comparable outcomes, indicating the reliability of the findings.

In the *in vitro* study, the cytotoxicity of sertraline, fluoxetine, and citalopram were first assessed in MRP1- or MRP7-overexpressing cell lines, and the non-toxic concentrations were used in the following reversal studies. The results indicated that the drug resistance of the KB/CV60 and SKOV3/MRP7 were significantly resensitized after the co-incubation with sertraline, fluoxetine, and citalopram. However, the sensitivity of the parental cells were not affected. Meanwhile, no difference was suggested between the IC_{50} of cisplatin, which is not a substrate for either MRP1 or MRP7. Different chemotherapies were used in order to examine the potential inhibitory effect of the chemosensitizers. These drugs were chosen because they represent different modes of action: paclitaxel and vincristine are microtubule-targeting agents, cisplatin forms covalent bonds with DNA and by that causes intra-strand and inter-strand cross-links that interfere with DNA replication and transcription, and doxorubicin intercalates into DNA and inhibiting topoisomerase II. Taken together, the *in vitro* study results demonstrated the potential capability of sertraline, fluoxetine, and citalopram on reversing MRP1- and MRP7-related MDR.

The development of a new anti-cancer drug is a time-consuming process, taking up to 15 years and costing around $650 million (Prasad and Mailankody, 2017; Antoszczak et al., 2020). Therefore, it is reasonable to explore alternative strategies, such as drug repurposing. Our study examined the potential of repurposing drugs that have already received regulatory approval for one disease to treat alternative therapeutic applications for different medical conditions. Specifically, we explored the potential of SSRIs, which are primarily used to inhibit serotonin reuptake, as inhibitors of ABC transporters. Since cancer patients may already be taking SSRIs for depression and anxiety, we investigated whether these drugs could also act as chemosensitizers for MRP1 and MRP7, extending their potential benefits. Our findings were supported by clinical studies, including evidence that sertraline can enhance the effects of vincristine and doxorubicin (Amit et al., 2009) and inhibit the growth of colon cancer cells in colorectal cancer-xenografted mice (Gil-Ad et al., 2008). Interestingly, not only MRP1 and MPR7 but also P-gp serve as a target for chemosensitizers such as sertraline, fluoxetine (O'Brien et al., 2012), and citalopram (Uhr and Grauer, 2003; Uhr et al., 2008).

Our study makes a valuable contribution to the existing body of knowledge in this area of research from several aspects. First and foremost, it demonstrates that FDA-approved drugs can be repurposed for treating cancer, specifically overcoming chemotherapy resistance, saving valuable time and money. Second, our results suggest that the incorporation of halogen groups in the design of novel therapeutic agents could be a promising strategy to improve their pharmacological properties and aid in the development of more potent and selective MRPs inhibitors. In addition, to the best of our knowledge, this is the first study to use the predicted structures of the human MRP1 and MRP7 generated by AlphaFold. This is particularly significant for the human MRP7, one of the least studied members of the ABCC subfamily, which currently has no high-resolution structure available.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1290255/full#supplementary-material

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Genome-wide CRISPR/Cas9 screening for drug resistance in tumors

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Genome-wide clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) screening is a simple screening method for locating loci under specific conditions, and it has been utilized in tumor drug resistance research for finding potential drug resistance-associated genes. This screening strategy has significant implications for further treatment of malignancies with acquired drug resistance. In recent years, studies involving genome-wide CRISPR/Cas9 screening have gradually increased. Here we review the recent application of genome-wide CRISPR/Cas9 screening for drug resistance, involving mitogen-activated protein kinase (MAPK) pathway inhibitors, poly (ADP-ribose) polymerase inhibitors (PARPi), alkylating agents, mitotic inhibitors, antimetabolites, immune checkpoint inhibitors (ICIs), and cyclin-dependent kinase inhibitors (CDKI). We summarize drug resistance pathways such as the KEAP1/Nrf2 pathway MAPK pathway, and NF-κB pathway. Also, we analyze the limitations and conditions for the application of genome-wide CRISPR/Cas9 screening techniques.

KEYWORDS

drug resistance, tumors, genome-wide CRISPR/Cas9 screening, MAPK pathway inhibitors, PARP inhibitors

1 Introduction

Cancer remains a persistent disease, and treating it will continue to be a formidable challenge. Although molecularly targeted drugs, cellular immunotherapy, and combination approaches have significantly improved cancer prognosis, drug resistance in some tumors still greatly impacts patient outlook.

Drug resistance in tumors is a widespread clinical issue that can be primarily attributed to tumor heterogeneity and the kinetics of cancer cell growth and burden. During progression, tumor cells acquire mutant clones and grow exponentially at low tumor loads. Under drug stress, cells with partially drug-resistant mutations survive and increase exponentially in value. Additionally, new mutations may arise in response to the drug, leading to acquired resistance in the cells (Gottesman, 2002). The typical mechanisms of drug resistance in cancer cells include

activation of signaling pathways, loss of function of apoptotic proteins or cancer suppressor genes, the tumor microenvironment and immunology, regulation of microRNAs, secondary mutations affecting drug targets, activation of critical downstream signals, and involvement of histological phenotypes (Ward et al., 2021). It seems that in the presence of drug pressure, genetic mutations occur in tumor cells, which then impact the aforementioned mechanisms to develop drug resistance and promote survival. Given the significance of genetic alterations in cancer drug resistance, gene therapy is a promising new treatment strategy. Compared to conventional treatments such as chemotherapy, gene therapy has fewer adverse effects and offers the potential for a cure (Akbari Kordkheyli et al., 2022). Genetic loss-of-function (LOF) and gain-of-function (GOF) screening is a crucial gene therapy approach that can identify cancer-selective vulnerabilities and holds promise for identifying new therapeutic targets for drug-resistant cancers (Munoz et al., 2016). RNA interference-based screening has been utilized for cancer therapy target identification (Ling et al., 2018), yet its effectiveness is commonly hindered by off-target effects (Munoz et al., 2016). While CRISPR/Cas9 is an efficient and innovative technique for editing genes, it has been utilized to identify mutations responsible for tumor drug resistance (Akbari Kordkheyli et al., 2022). This article reviews the technical characteristics of CRISPR/Cas9, its application in tumor drug resistance, and its limitations. The aim is to offer new ideas for the study of tumor drug resistance using CRISPR/Cas9.

2 CRISPR/Cas9 technologies

CRISPR was discovered in prokaryotes and used to counteract exogenous DNA during evolution (Mojica and Montoliu, 2016). The system contains two main components: the single-guide RNA (sgRNA) and Cas9 protein. The 20 bp length sgRNA can recognize the target DNA sequence. The Cas 9 protein, as an endonuclease, can create DNA double-strand breaks (DSBs), which results in non-homologous end-joining or homology-directed repair. Non-homologous end-joining often introduces new bases and leads to insertions and/or deletions (indels) and inactivation of genes, thus enabling targeted gene editing (Hsu et al., 2014). Modifications to the Cas9 protein add more functionality to the CRISPR system. CRISPRa (CRISPR activation) and CRISPRi (CRISPR interference) are two systems based on modified Cas9 protein. The deactivated Cas9 lost its nuclease activity. Instead, it can recruit transcriptional activators or repressors to promote or interfere with gene expression (Konermann et al., 2015).

As the number of sgRNA increases, the scale of libraries increases from small libraries like the kinase library and epigenetic library to genome-wide libraries. Researchers can analyze genetic perturbations on a genome-wide scale in one screening (Han et al., 2016; Yu and Yusa, 2019; Wan et al., 2021).

3 The mechanism and strategy of CRISPR/Cas9 screening

The most common library is GeCKO (Genome-scale CRISPR Knock-Out) and SAM (synergistic activation mediator), both of which were provided by Zhang (Sanjana et al., 2014; Joung et al., 2017b). Different sgRNAs serve as molecular tags in single cells. The abundance of sgRNAs varies in different subclones in cells. DNA extraction was performed after incubation for different passages and sgRNAs were sequenced. As cells were introduced with different sgRNAs, the knockdown (or overexpression) of drug-promoting and drug-suppressing genes caused changes in drug resistance, leading to differences in cellular growth activity, resulting in different read counts of sgRNA (Li et al., 2014). The read count differences between control and experimental groups were analyzed, and bioinformatic technology and other experiments can be utilized for further inquiry (Shalem et al., 2015). A brief overview of this strategy is depicted in Figure 1.

The screening can be divided into GOF and LOF depending on whether the library is a CRISPRa or a CRISPRi library. In a single screening based on viability, both positive selection and negative selection can be achieved, depending on the increase and decrease in the number of cell populations. The model choice has been well illustrated and the pros and cons of each screening method have been discussed (Doench, 2018; Sharma and Petsalaki, 2018). The model choice depends on the phenotype and perturbation of the system. A pre-well-designed model before screening is of great importance to the next validation of the top-hit genes.

Comprehensively, genome-wide CRISPR/Cas9 knockout (inhibition) or activation screening can locate the gene perturbation caused by drugs. In this review, most studies focus on several notable genes and investigate one gene with drug resistance *in vivo* and *in vitro*.

4 Genome-wide CRISPR/Cas9 screening for drug resistance in tumors

In research on drug resistance in tumors, genome-wide CRISPR/Cas9 screening for drug resistance in tumors serves as a powerful initial screening tool for potential drug resistance-related genes. This is of great significance for further treatment of malignant tumors that are resistant to targeted or chemotherapeutic drugs.

We collected data from PubMed by using specific keywords such as CRISPR in combination with other related keywords including pancreatic, lung, liver, gastric, breast, bladder, colon, renal, bone, glioma, ovarian, testicular, cancer, tumor, and malignancy. Only articles focused on drug resistance with genome-wide CRISPR/Cas9 screening were included in this review. After skimming titles and abstracts, 64 articles were included in this review and will be introduced in line with different kinds of drugs, and the summary table is listed in Supplementary Tables S1–S9. We hope that the results will shed light on the resistance mechanism of different drugs.

We focused on the most commonly used drugs in clinical practice and those with the highest number of studies. The drugs involved in this review are MAPK pathway inhibitors, poly (ADP-ribose) polymerase inhibitors (PARPi), antimetabolites, alkylating agents, mitotic inhibitors, immune checkpoint inhibitors (ICIs), and (cyclin-dependent kinase inhibitors) CDKI.

4.1 MAPK signaling inhibitors

MAPK signaling inhibitors are commonly used in clinical practice. Many drugs have been designed to target the RTK

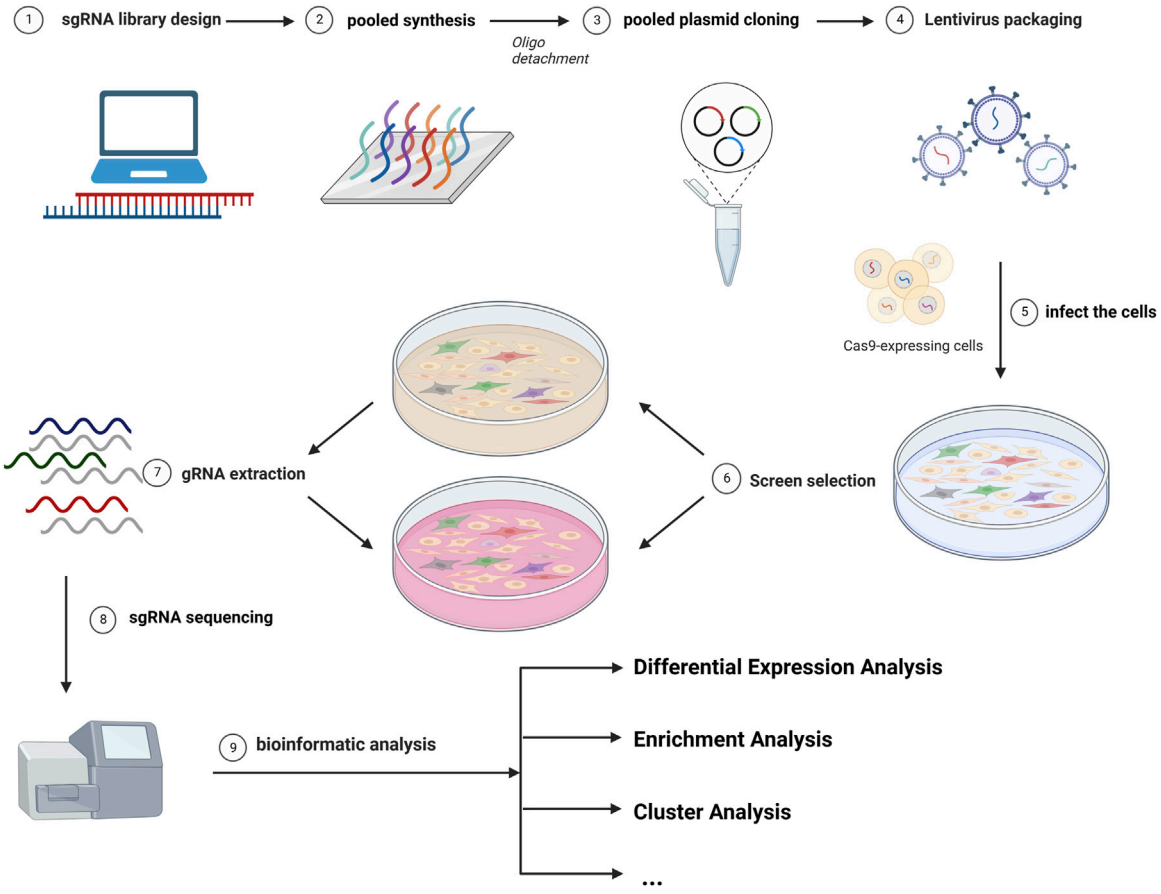

FIGURE 1
The diagram of common CRISPR/Cas9 screening. The screen workflows include the design of the library, the infection of cells, the selection of cells, the gRNA extraction, and the analysis of the sgRNA distribution.

(receptor tyrosine kinase)- RAS (rat sarcoma)- RAF (rapidly accelerated fibrosarcoma)-MEK (mitogen-activated protein kinase)-ERK (extracellular-signal-regulated protein kinase) pathway, such as RTK inhibitors, BRAF (B-Raf proto-oncogene) inhibitors, and MEK inhibitors. They act primarily on single or multiple sites such as RTK, Raf1, RafB, and MEK proteins, inhibiting their phosphorylation and blocking signaling. They inhibit angiogenesis, proliferation, and tumor growth (Cargnello and Roux, 2011). Targeted agents are more efficient than traditional chemotherapeutics. However, the clinical efficacy of targeted drugs is unsatisfactory (Zhai and Sun, 2013), and the current genome-wide CRISPR/Cas9 screening model reveals some of the resistance mechanisms. The mechanism of MAPK signaling pathway inhibitors resistance was summarized in Figure 2.

4.1.1 Receptor tyrosine kinase inhibitors

RTK is a class of transmembrane proteins with intrinsic phosphotyrosine kinase activity, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), MET proto-oncogene (MET), and KIT proto-oncogene (KIT) (Regad, 2015). They mainly receive extracellular signals and regulate cell proliferation, differentiation, and survival. Common RTK inhibitors include erlotinib, lapatinib, and gefitinib. Multi-targeted kinase inhibitors include sorafenib, lenvatinib, and regorafenib.

Erlotinib, the first-generation tyrosine kinase inhibitor (TKI), is mainly used in lung and pancreatic cancer. Terai et al. (2021) identified SHOC2 (leucine-rich repeat scaffold protein) as a drug-resistance gene in lung cancer. SHOC2 affects the sensitivity to EGFR–TKIs in NSCLC (non-small cell lung carcinoma) cells via the SHOC2/MRAS (muscle RAS oncogene)/PP1c (protein phosphatase 1) and SHOC2/SCRIB (scribble planar cell polarity protein) pathways. The mutant SHOC2-MRAS-PP1c complex was later identified to enhance holophosphatase activity (Kwon et al., 2022). Zeng et al. (2019) discovered RIC8A (RIC8 guanine nucleotide exchange factor A) and ARIH2 (Ariadne RBR E3 ubiquitin protein ligase 2) as genes for erlotinib resistance in NSCLC cells. Knockout of RIC8A, essential for G-alpha protein activation, enhanced EGFR TKI-induced cell death, as well as knockout of ARIH2 which conferred resistance to EGFR inhibition by promoting *de novo* protein synthesis through methionyl aminopeptidase 2. By using gefitinib as screening pressure, Georgiou et al. (2020) identified the role of NF1 (neurofibromin 1) in EFGR inhibitor resistance. NF1-mutant colorectal cancer cell lines are resistant to EGFR inhibitors, indicating that loss of NF-1 could be a biomarker for assessing the application of EGFR inhibitors.

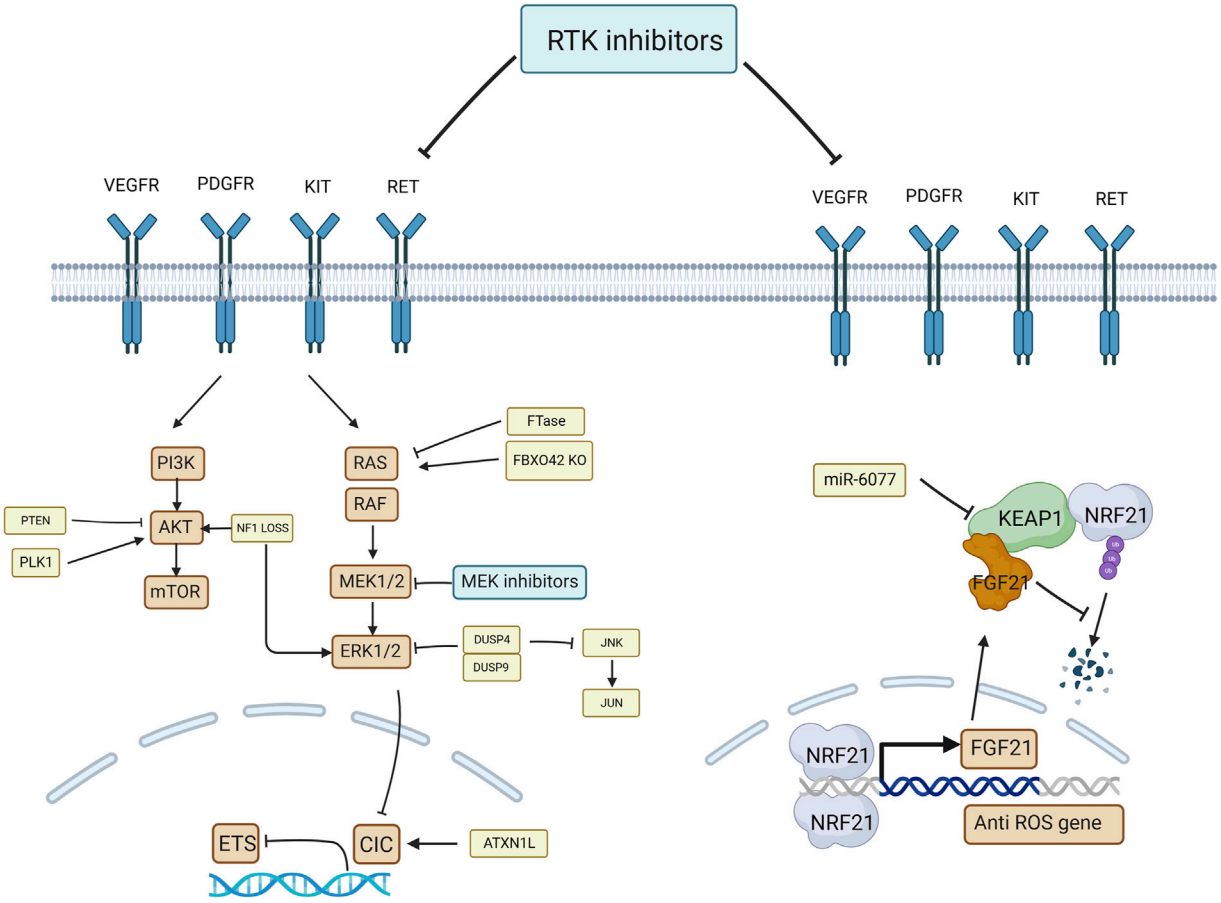

FIGURE 2
The diagram of the mechanism of MAPK signaling pathway inhibitors. The drug resistance gene involves FBXO42, PLK1, PTEN, DUSP4/9, ATXN1L, and miR-6077. They reverse the drug resistance by reactivating the MAPK pathway or by promoting the transcription of genes that reduce ROS.

Cell cycle proteins and ubiquitination have likewise been found to be associated with erlotinib resistance, which was identified in the screening by Lee J. et al. (2021). Chemical inhibitors targeting genes in these two pathways, nutlin-3 and carfilzomib, in combination with erlotinib reduce the development of erlotinib resistance.

Tumor cells can also acquire a drug-resistant phenotype to RTK inhibitors by EMT (epithelial to mesenchymal transition). Raoof et al. (2019) discovered that FGFR plays a role in tumor mesenchymal cell resistance to a third-generation TKI: EGF816. FGFR signaling was found to be necessary for the survival of epithelial and drug-sensitive cells when undergoing an EMT-like process during the first exposure to EGFR inhibitors, suggesting that dual EGFR + FGFR inhibition may be a promising strategy to prevent the emergence of resistant clones.

4.1.2 Multi-targeted RTK inhibitors

Multi-targeted RTK inhibitors such as sorafenib, lenvatinib, regorafenib, and lapatinib, target more than one kind of RTK, which theoretically inhibit MAPK signaling pathway more effectively, but they also face the dilemma of drug resistance.

A more plausible mechanism is the reactivation of the MAPK/ERK pathway. Lu et al. (2021) screened NF1 and DUSP9 (dual specificity phosphatase 9) by LOF as genes associated with lenvatinib resistance in HCC (hepatocellular carcinoma). Lenvatinib exerts its therapeutic effect mainly by inhibiting kinases in the PI3K (Phosphoinositide 3-Kinase)/AKT (Protein Kinase B) and MEK/ERK signaling pathways. Knockdown of NF1 and DUSP9 increased cell resistance and enhanced cell proliferation and migration. NF1 deletion induced phosphorylation of PI3K/AKT and MAPK/ERK pathways, leading to activation of the pathway and induced lenvatinib resistance. Whereas (Huang et al., 2022) identified DUSP4 (dual specificity phosphatase 4) as an HCC lenvatinib resistance gene through LOF screening, and, similarly, DUSP4 knockdown was found to lead to cellular resistance through activation of the MAPK/ERK pathway. DUSP4 is a member of the bispecific protein phosphatase subfamily involved in the inactivation of the corresponding target kinase, including the MAPK cascade, and inhibition of DUSP4 increased the phosphorylation level of ERK. Lenvatinib blockade of upstream MAPK is not sufficient to inhibit the downstream rephosphorylation of ERK. A combination of lenvatinib and MEK inhibitors is suggested as a possible treatment modality to overcome lenvatinib resistance. A similar mechanism was also found in lapatinib, which is also a multi-targeted RTK inhibitor. Ning et al. (2021) found that loss-of-function mutations in C-terminal Src kinase and PTEN (phosphatase and tensin homolog) in gastric

cancer reactivated MAPK and PI3K pathways, leading to lapatinib resistance.

Makhov et al. (2020) found FTase (farnesyltransferase)-dependent cellular factors (a cytokine that acts on RAS proteins) to be associated with sunitinib resistance in clear cell renal cell carcinoma. Combination therapy with lonafarnib, an FTase inhibitor, may potentiate the anti-tumor efficacy of sunitinib through two potential mechanisms: 1) suppression of Rheb-dependent mTOR complex1 activation and 2) dysregulation of lysosomal sequestration of TKIs.

Oxidative stress has been reported to be related to TKI resistance. Many drugs can induce ROS (reactive oxygen species). Zheng et al. (2019) found that KEAP1 (kelch-like ECH-associated protein 1)/Nrf2 (Nuclear factor erythroid 2-related factor 2) affects sorafenib resistance through the ROS pathway. Knockout of KEAP1, a Cul3-based E3 ligase, increased cell survival to sorafenib-resistant treatment by targeting the transcription factor Nrf2 and degradation. Knockdown of KEAP1 led to activation of the cellular Nrf2 pathway that controls the expression of antioxidant genes, resulting in higher resistance to oxidative stress caused by sorafenib. Chen et al. (2022) also identified KEAP1 as a drug resistance-associated gene and FGF21 (fibroblast growth factor 21) as a downstream factor of Nrf2. Upregulation of Nrf2 leads to an increase in FGF21, causing an increase in cellular antioxidant capacity, while FGF21 promotes the transcription of Nrf21 by inhibiting the ubiquitination of Nrf2, leading to high levels of positive Nrf2 feedback, thus promoting a stable state of drug resistance. The KEAP1/Nrf2 pathway is also found in TKI-resistant cells in lung cancer. Krall et al. (2017) investigated the inhibitory effects of EGFR, ALK (anaplastic lymphoma kinase), BRAF, and MEK in lung cancer and found that KEAP1 deletion modulated multiple sensitivities lung cancers with EGFR, ALK, BRAF, and KRAS or NRAS mutations. KEAP1 deletion reduced the ubiquitination of Nrf2 degradation. KEAP1 deficiency promotes cell survival and increases glutathione synthesis thus reducing the drug-induced ROS. In addition, KEAP1 was found to be associated with the miR-6077-mediated pathway for cisplatin/pemetrexed resistance by Bi et al. (2022).

Cellular autophagy, a mechanism of cellular protection in harsh environments, plays a role in TKI resistance in HCC. Li et al. (2021) selected MTX1 (metaxin 1) as a sorafenib resistance-associated gene in HCC through GOF screening. Significant overexpression of MTX1, leading to sorafenib resistance, was associated with poor prognosis and accelerated the proliferation of HCC cells. MTX1 increased drug resistance by antagonizing the inhibitory effect of CDGSH iron sulfur domain 1 on cellular autophagy, which enhances cellular autophagy and increases drug resistance in HCC. The team further identified miR-15a and miR-20b as relevant genes for sorafenib resistance through LOF screening and found that the silencing of miR-15a or miR-20b effectively promoted HCC cell survival and drug resistance in the presence of sorafenib. They both target CDC37L1 (the cell division cycle 37 like 1), which acts as a molecular chaperone in enhancing the interaction between HSP90 and PPIA (peptidylprolyl isomerase A). The silencing of both facilitated the stability of PPIA. Namely, the downregulation of miR-15a and miR-20b promotes the resistance of sorafenib to HCC by enhancing the binding of HSP90 to PPIA via CDC37L1 (Li et al., 2022).

Sun et al. (2018) identified SGOL1 (shugoshin 1), which encodes a mitosis-related protein gene, as a relevant gene for sorafenib resistance in HCC cells by introducing a GECKOv2 sgRNA library into HUH7 cells. SGOL1 knockout can reduce apoptosis and the cytotoxicity of sorafenib. The SGOL1 protein was disabled for the subsequent sister-chromatid segregation at inner centromeres (Zhang and Liu, 2020), suggesting that SGOL1 may modulate drug resistance by regulating chromosome segregation.

Metabolic alternation also plays a role in drug resistance. By performing a genome-wide screening for GECKOv2 in the MHCC97L cell line, Wei et al. (2019) found that PHGDH (phosphoglycerate dehydrogenase), an enzyme of the SSP (serine synthesis pathway), catalyzes the change from 3PG to 3PHP, and knockdown of the PHGDH gene sensitized the HCC cell line to sorafenib. HCC cells respond to oxidative stress induced by sorafenib treatment by increasing PHGDH expression. Activation of SSP is a common mechanism of TKI resistance and targeting SSP through PHGDH inhibitors is one way to treat TKI-resistant HCC. Similarly, Sofer et al. (2022) identified hexokinase 1 and integrin subunit beta 5 as regorafenib resistance-associated genes in a GOF screening. Hexokinase 1 catalyzes the first step of glycolysis, the conversion of glucose to glucose-6-phosphate, suggesting that changes in glucose metabolism underlie drug resistance and that glycolysis inhibitors may improve TKI efficiency.

On the other hand, Cai et al. (2020) used GOF screening to correlate lipid metabolism with cellular resistance and screened LRP8 (LDL receptor-related protein 8) as a sorafenib resistance-associated gene, which encodes a member of the low-density lipoprotein receptor family and functions as a receptor for the cholesterol transporter protein apolipoprotein E. Overexpression of LRP8 inhibited apoptosis and increased sorafenib resistance, whereas knockdown reduced cellular resistance. High levels of LRP8 expression correlated with patient prognosis, and overexpression of LRP8 was found to increase b-catenin levels, and the ApoE-LRP8 pathway was suggested to be a resistance-related pathway in HCC.

4.1.3 RAF inhibitor

RAF inhibitors, such as vemurafenib, mainly inhibit cells with BRAF V600E mutations. In most patients, resistance emerges within a few months. 13 novel genes were identified by Goh et al. (2021) in screening for vemurafenib resistance in melanoma cells. Among them, NF1, CUL3, and NF2 are associated with the MAPK pathway, and the remaining genes are related to epigenetics, cell cycle, telomeres, etc. Joung et al. (2017a) identified 11 LncRNA loci in melanoma cells. Gautron et al. (2021) identified SMAD3, BIRC3, and SLC9A5 in melanoma as key actors of BRAF inhibitor resistance. SMAD3 plays a key role in melanoma resistance to treatment by promoting an EMT-like process, and their results suggest that the regulation of BRAF inhibitor resistance gene expression is multiparametric.

4.1.4 MEK1/2 protein inhibitors

Trametinib, a MEK inhibitor, functions as an allosteric, ATP noncompetitive inhibitor with nanomolar activity against both MEK1 and MEK2 kinases. It can inhibit cell proliferation, cause cell cycle arrest in the G1 phase, and induce apoptosis. Wang et al. (2017) performed a genome-wide screening in KRAS-mutated pancreatic adenocarcinoma to investigate RTK-RAS-MAPK

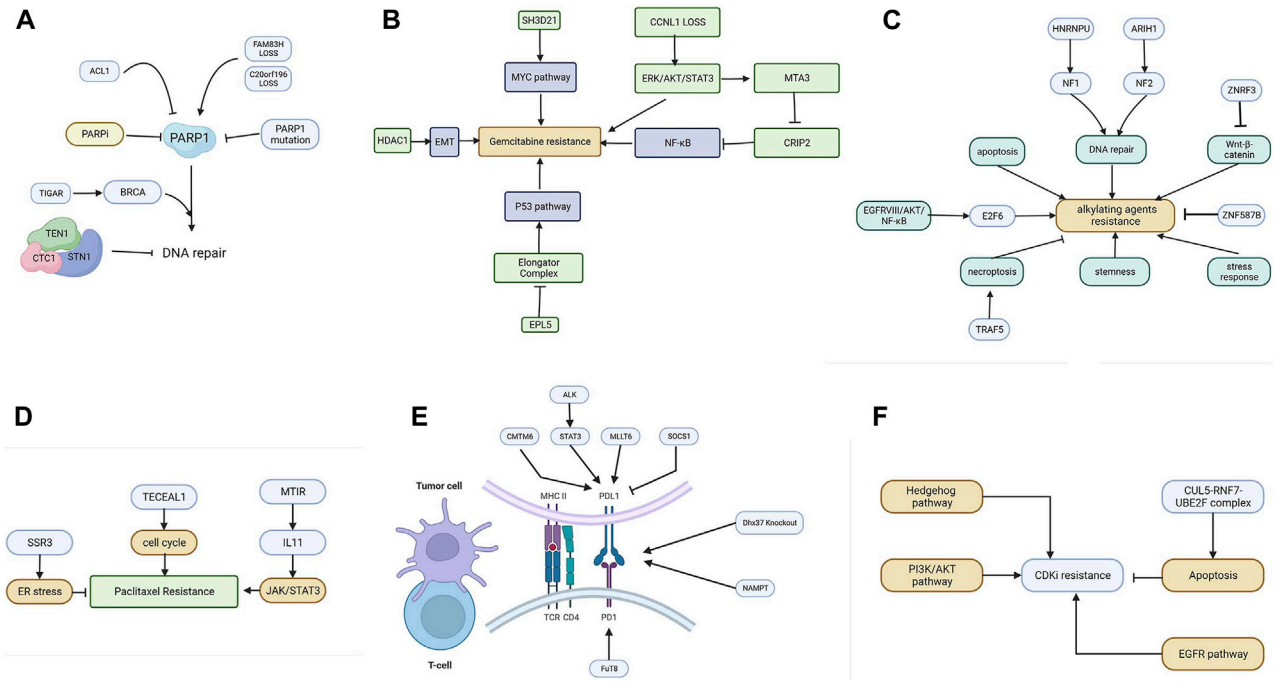

FIGURE 3
(A) The resistance mechanism of PARP inhibitors. **(B)** Various genes promote gemcitabine resistance by activating the EMT, NF-κB, MYC, and P53 pathways. **(C)** The resistance mechanism of alkylating agents includes the enhanced pathway of DNA repair, apoptosis, Wnt-β catenin, NF-κB, stemness, and stress response. **(D)** Cell cycle, JAK/STAT3, and ER stress are involved in mitotic inhibitor resistance. **(E)** The resistance mechanism of ICIs. Tumor cells can upregulate PD-L1 to inhibit T-cells via CMTM6, ALK, MLLT6, and SOCS1. **(F)** The resistance mechanism of CDKI. The Hedgehog pathway, PI3K/AKT pathway, apoptosis, and EGFR pathway are involved in CDKI resistance.

pathway reactivation and found that gene ATXN1L deletion caused a decrease in protein CIC (capicua transcriptional repressor), leading to increased cellular resistance, and determined the ATXN1L-CIC-ETS transcription factor axis to be a mediator of resistance to MAPK inhibitors. The ERN1-JNK-JUN pathway is present in KRAS mutant colorectal cancers and is involved in regulating MEK inhibitor resistance in colon cancer (Šuštić et al., 2018). It is emphasized that JUN-activated kinases, TAK1 and JNK, may be important loci for MEK inhibitors in KRAS-mutant cancer cells.

FBXO42 (F-box protein 42) is an E3 ubiquitin ligase associated with trametinib resistance in NRAS-mutated melanoma cells. Knockdown of FBXO42 increases the TAK1 signaling pathway, which may promote an increase in active P38, leading to an enhancement of the ERK signaling pathway. This suggests that the concomitant use of MEK inhibitors with TAK1 inhibitors can improve the efficacy of MEK inhibitors (Nagler et al., 2020). Yu et al. (2022) found that GRB7 (growth factor receptor bound protein 7) effectively increased major resistance to MEK inhibitors via the RTK pathway in KRAS-mutated colon cancer. PLK1 (Polo-like kinase 1) is the major interacting kinase of GRB7. The combination of PLK1 and MEK inhibitors could synergistically inhibit CRC cell proliferation and induce apoptosis *in vitro* and *in vivo*.

4.2 Poly (ADP-ribose) polymerase inhibitors

The mechanism of PARP inhibitors is related to the concept of synthetic lethality. Protein PARP1 (poly (ADP-ribose) polymerase 1) is mainly responsible for the repair of DSBs. PARPi targeting homologous recombination-deficient tumors hold great promise for the treatment of tumors with damaging mutations in BRCA1/2 or other homologous recombination factors (Noordermeer and van Attikum, 2019). Unfortunately, PARPi resistance has proven to be a major clinical problem. The use of genome-wide CRISPR/Cas9 screening techniques helps us to better understand the cell response to PARPi. The mechanism of PARPi is summarized in Figure 3A.

The resistance mechanism of PARPi mainly focuses on the promotion of DNA repair. Barazas et al. (2018) performed LOF screening in a BRCA1-deficient cell line and found that the deletion of members of the CTC1-STN1-TEN1 complex led to PARPi resistance in BRCA1-deficient cells *in vitro* and *in vivo* by enhancing the repair of DSBs. Dev et al. (2018) identified two proteins, C20orf196 and FAM35A, in BRCA1-deficient cells by performing a whole genome screening in breast cancer. The inactivation of the two proteins resulted in strong PARP inhibitor resistance by forming a complex, “Shieldin” (SHLD1/2), which promotes DSBs end-joining.

Mutation of the PARP1 protein that results in drug resistance has been illustrated. By picking resistant clones in genome-wide screening in embryonic cells, Pettitt et al. (2018) found PARP1 point mutations leading to PARP inhibitor resistance in ovarian cancer. Mutations both within and outside of the PARP1 DNA-binding zinc-finger domains cause PARPi resistance and alter PARP1 trapping, suggesting that PARP1 intramolecular interactions may influence PARP-mediated cytotoxicity.

Zimmermann et al. (2018) performed three screenings with the TKOv1 library and discovered that mutations in three genes encoding ribonuclease H2 sensitized cells to PARP inhibition and the manipulation of genomic ribonucleotide processing may contribute to the treatment with PARPi.

Fang et al. (2019) found that TIGAR (TP53-induced glycolysis regulatory phosphatase), encoded by C12orf5, regulates PARP1 resistance in ovarian cancer, and high expression of TIGAR is associated with poor prognosis. TIGAR knockdown enhanced the sensitivity of cancer cells to olaparib through the downregulation of BRCA1 and Fanconi anemia depletion pathways and increased the sensitization of these cells by affecting metabolic pathways and increasing the cytotoxic effects of olaparib.

Juhász et al. (2020) identified ALC1 (amplification in liver cancer1) as a modulator of PARP inhibitor. ALC1 can remove inactive PARP1 through binding to PARylated chromatin, thus the overexpression of ALC1 reduces the trapping of inhibited PARP1 and decreases the sensitivity of BRCA-deficient cells to PARP inhibitors.

Autophagy plays an important role in treatment with olaparib and is associated with its resistance. In prostate cancer, Ipsen et al. (2022) determined that deletion of PARP1, ARH3 (ADP-ribosylserine hydrolase), tryptophan 5-monooxygenase activation protein epsilon, and ubiquitin protein ligase E3 component n-recognin 5 resulted in olaparib resistance, where PARP1 or ARH3 knockdown resulted in reduced autophagy and increased cellular resistance, suggesting that low ARH3 expression is an independent prognostic indicator.

4.3 Antimetabolites

Gemcitabine, 2′,2′-difluoro-2′-deoxycytidine, is currently used in a variety of solid tumor cancers. It is delivered into cells via membrane nucleoside transport proteins (hENTs and hCNTs). It undergoes complex conversion to the nucleotides gemcitabine diphosphate and triphosphate (Mini et al., 2006). The triphosphate competes with deoxycytidine triphosphate, which leads to inhibitory DNA synthesis. Several screenings have been performed to elucidate the mechanism of gemcitabine resistance. The mechanisms are summarized in Figure 3B.

Bakke et al. (2019) identified PSMA6 (proteasome 20S subunit alpha 6) in pancreatic cell lines and validated its role in pancreatic cancer cell lines and found that the knockout of PSMA6, as a proteasomal subunit of the 20S core complex, can induce apoptosis in cells.

SH3D21 (SH3 domain containing 21) (Masoudi et al., 2019) was identified as a drug resistance maintenance gene in the Panc1 cell line, and knockdown of SH3D21 resulted in increased sensitivity of pancreatic cancer cells to gemcitabine, and it is hypothesized that the MYC pathway is associated with gemcitabine resistance. Yang et al. (2022) screened for DCK (deoxycytidine kinase) and cyclin L1 and found that deletion of CCNL1 activated the ERK/AKT/STAT3 survival pathway, leading to cellular resistance to gemcitabine treatment.

The MTA3 (the metastasis associated 1 family member 3)-CRIP2 (cysteine-rich protein 2)-NF-κB pathway was associated with gemcitabine resistance. Wu et al. (2022) screened MTA3 through genome-wide overexpression as part of the NuRD transcriptional repressor complex. Downregulation of the MTA3 gene resulted in increased cellular sensitivity to gemcitabine. It was further determined that MTA3 primarily represses CRIP2 transcription whereas CRIP2, a transcriptional repressor, primarily suppresses tumorigenesis by inhibiting NF-κB signaling to suppress tumorigenesis. Sarr et al. (2019) found a role for pyrimidine metabolism in NUC-1031 resistance, which is a phosphoramidite transformation of gemcitabine, mainly through the DCK and dCTP pyrophosphatase 1, and concluded that DCK levels were associated with patient prognosis.

Xu et al. (2019) found that the elongator complex was associated with gemcitabine resistance. They identified ELP5 (elongator acetyltransferase complex subunit 5) in gallbladder cancer. The loss of ELP5 compromised the integrity and stability of the elongator complex and abrogated wobble U34 tRNA modification, which interfered with the translation of hnRNPQ (heterogeneous nuclear ribonucleoprotein Q) mRNA, which in return regulates cells through the P53 pathway. The elongator/hnRNPQ/P53 axis may control gemcitabine sensitivity.

Ramaker et al. (2021) performed CRISPR screening among four classes of chemotherapeutic agents (gemcitabine, oxaliplatin, irinotecan, and 5-fluorouracil), and found that HDAC1 (histone deacetylase 1) and ABCG2 (ATP binding cassette subfamily G member 2) serve as four common drug resistance genes, with HDAC1 overexpression leading to drug resistance associated with EMT, while ABCG2 was found to be a general resistance mechanism mainly through the cells that pump the drug.

4.4 Alkylating agents

Common alkylating agents include cisplatin, oxaliplatin (a third-generation platinum-based chemotherapeutic agent), and temozolomide. The pharmacological mechanism of alkylating agents involves the inhibition of DNA replication and transcription through internal and inter-strand crosslinks resulting from binding to DNA (Wang L. et al., 2021), followed by the induction of damage to double-stranded DNA. Cisplatin is a first-generation platinum drug used as a first-line therapy in clinical practice with a good inhibitory effect on solid tumors. Figure 3C summarizes the resistance mechanism of common alkylating agents.

Non-coding RNAs also play a role in regulating drug-resistant cisplatin resistance. Bi et al. (2022) found that miR-6077 promoted cisplatin/pemetrexed resistance in lung adenocarcinoma through the cyclin-dependent kinase inhibitor 1A and KEAP1 pathways. Goodspeed et al. (2019) found MSH2 (mutS homolog 2) in bladder cancer that led to *in vitro* resistance to cisplatin via the hyaluronan-mediated motility receptor pathway and that patients with low MSH2 levels had a poor prognosis when receiving platinum-based chemotherapy. Also in bladder cancer, Shi et al. (2022) found that HNRNPU (heterogeneous nuclear ribonucleoprotein U) knockdown inhibited cell proliferation, invasion, and migration. Furthermore, the deletion of HNRNPU promoted the sensitivity of T24 cells to cisplatin, mainly associated with S cell cycle phase blockage, and in addition, HNRNPU was found to regulate chemosensitivity by affecting the expression of NF1.

In melanoma, zinc and ring finger 3, an ubiquitin ligase known to be a targeting and negative feedback regulator of Wnt-β catenin signaling enhanced cisplatin resistance in normal and melanoma cells independently of b-catenin. ARIH1 (Ariadne1 homolog), another ubiquitin ligase, also enhanced cisplatin resistance in normal and melanoma cells by modulating ARIH1, and the tumor suppressor neurofibrillary protein 2, NF2, enhanced cisplatin resistance in melanoma, but not in normal cells (Ko and Li, 2019).

Lan et al. (2021) identified M2 tumor-associated macrophages as an important mediator of oxaliplatin resistance acquisition in colorectal cancer. Moreover, TNF receptor-associated factor 5 mediates oxaliplatin resistance in CRC triggered by methyltransferase 3.

In ovarian cancer, Ouyang et al. (2019) found that downregulation of SULF1 (sulfatase 1) resulted in diminished cisplatin-induced cytotoxicity. SULF1 may regulate cell signaling by altering the sulfated state of the acetyl heparan sulfate chain, thereby affecting platinum sensitivity; in addition, knockdown of ZNF587B, a C2H2-type zinc finger protein, significantly reduced cisplatin sensitivity in ovarian cancer cells. Also in ovarian cancer, Stover et al. (2019) performed systematic overexpression and inhibition of BCL-XL, BCL-W, MCL1 (myeloid cell leukemia sequence 1), or BCL-2 and found that overexpression of anti-apoptotic proteins increased cisplatin and paclitaxel resistance.

Skripova et al. (2022) performed a screening for cisplatin and oxaliplatin resistance in pancreatic cancer. The genes were associated with DNA repair, cell cycle regulation, components of detoxification and antioxidant systems, and intracellular signaling pathways. The results also identified genes previously associated with platinum drug sensitivity/resistance, demonstrating the adequacy of the CRISPR/Cas9 screening approach in the search for regulators of drug sensitivity.

Temozolomide (Lee, 2016) (TMZ) is an oral alkylating agent used to treat glioblastoma multiforme and astrocytoma. However, at least 50% of TMZ-treated patients do not respond to TMZ. This is mainly due to the overexpression of O-6-methylguanine-DNA methyltransferase and the lack of DNA repair pathways in glioblastoma cells.

Huang et al. (2019) identified in glioblastoma that NF-κB/E2F6 (E2F transcription factor 6) was responsible for EGFRvIII-associated temozolomide resistance and E2F6, under the control of the EGFRvIII/AKT/NF-κB pathway, showed a promising therapeutic target for TMZ resistance.

MacLeod et al. (2019) described genome-wide CRISPR/Cas9 screening, identifying genetic vulnerabilities in a panel of patient-derived glioblastoma stem cell cultures. Regulators of stemness (genes such as SOX2, SOX9, DOT1L, and SOCS3) and stress response (UFMylation and endoplasmic reticulum-associated protein degradation pathway) govern the growth of glioblastoma stem cells. Chemogenomic screening using temozolomide identified modulators of sensitivity to chemotherapy. In the over-expression group, the Nrf2 and Wnt pathways were involved in TMZ resistance, and overexpression of frizzled class receptor 6, catenin beta 1, or Nrf2 genes significantly increased cell survival (Rocha et al., 2020).

4.5 Mitotic inhibitors

One of the most well-known mitotic inhibitors is paclitaxel (PTX). PTX (Abu Samaan et al., 2019) is widely used in the treatment of various types of malignant diseases. The mechanism of PTX action represents several ways in which PTX affects cellular processes leading to programmed cell death. PTX is frequently used as a front-line therapeutic agent in breast cancer. Unfortunately, the resistance of BC to PTX therapy is a major barrier to clinical use and one of the leading causes of death associated with treatment failure. Factors that contribute to PTX resistance are ABC transporter proteins, microRNAs, or mutations in certain genes. The resistance patterns are summarized in Figure 3D.

Rushworth et al. (2020) identified 17 candidate genes in prostate cancer whose inhibition may enhance the efficacy of docetaxel, with TCEAL1 (transcription elongation factor A-like 1) being the preferred candidate. Deletion of TCEAL1 leads to altered cell cycle, increased sub-G1 cell death, and increased polyploidy.

Paclitaxel resistance is a major concern in the treatment of patients with breast cancer, and (Lian et al., 2020) showed that expression of MEF2-interacting transcriptional repressor can increase the level of interleukin 11 and activate the downstream JAK/STAT3 signaling in triple-negative breast cancer, which can lead to paclitaxel resistance.

Dmello et al. (2022) found that the endoplasmic reticulum protein SSR3 (signal sequence receptor subunit 3) was associated with paclitaxel resistance in breast cancer, and that knockdown of SSR3 made cells resistant to PTX, while overexpression made them sensitive to PTX. The mechanism is that SSR3 confers susceptibility to PTX by regulating the phosphorylation of ER stress sensor IRE1α.

4.6 Immune checkpoint inhibitors

Tumor cells escape from immune surveillance and progress through different mechanisms, including activation of immune checkpoint pathways that suppress antitumor immune responses. ICIs enact anti-tumor functions by interrupting co-inhibitory signaling pathways and promoting immune-mediated elimination of tumor cells. Figure 3E summarizes the network of resistance in tumor and immune cells.

Immune checkpoint inhibitors target different axes including cytotoxic T lymphocyte antigen 4, PD-1/PD-L1 (programmed death protein 1/programmed death ligand 1), B-and T-lymphocyte attenuator, T cell immunoglobulin and mucin-containing molecule 3, T cell immunoreceptor with Ig and ITIM domains, V-domain Ig suppressor of T-cell activation, lymphocyte activation gene-3, and indoleamine 2,3-dioxygenase 1 (Lee J. B. et al., 2021). While resistance frequently occurs in patients treated with conventional cancer therapies and targeted therapies, in large subsets of patients treated with ICIs, long-lasting immunologic memory is commonly identified (Jenkins et al., 2018). However, the emergence of acquired resistance is observed in longer follow-up clinical trial populations.

Genome-wide CRISPR screening for ICIs can be performed both in immune cells and tumor cells to locate related axes that regulate acquired resistance.

Deficiencies in cancer cell antigen presentation are the main mechanism of ICI resistance. Dong et al. (2019) performed *in vivo* and *in vitro* CRISPR screening in CD8 T cells and found that knockdown of DEAH-box helicase 37 enhanced the efficacy of antigen-specific CD8 T cells against triple-negative breast cancer *in vivo*. Wang Y. et al. (2021) determined that nicotinamide phosphotransferase is required for T-cell activation and demonstrated that NAD + supplementary significantly enhanced anti-PD-1 immunotherapy in a murine solid tumor model. Okada et al. (2017) found that the inhibition of fucosyltransferase 8, by genetic ablation or pharmacologic inhibition, could reduce cell-surface expression of PD-1 and enhance T-cell activation, leading to more efficient tumor eradication.

A screening in melanoma cells by Manguso et al. (2017) found that the deletion of protein tyrosine phosphatase protein tyrosine phosphatase non-receptor type 2 (PTPN2) enhances interferon-γ-mediated antigen presentation and growth inhibition to improve the efficacy of immunotherapy. Burr et al. (2017) found in a genome-wide screening that CMTM6 (CKLF-like MARVEL transmembrane domain containing 6 protein) could bind PD-L1 and maintain its expression on the cell membrane surface, and that the reduction of CMTM6 reduced PD-L1 expression and attenuated tumor suppression of T cells. Gu et al. (2021) found that TNF receptor-associated factor 3, with its regulation of the NF-κB pathway, led to a decrease in the MHC-I-specific negative regulator TRAF, which sensitized cancer cells to antigen-specific T cell-driven cytotoxicity. This finding may be useful in the treatment of MHC-I-suppressed tumors. Using genome-wide CRISPR and metabolic inhibitor screening, Zhang et al. (2019) demonstrated that ALK activates STAT3 and ultimately induces PD-L1 expression through the effect of interferon regulatory factor 4 and basic leucine zipper ATF-like transcription factor 3 on the enhancer region of the PD-L1 gene. Suresh et al. (2020) identified that impairment of heme production activated an integrated stress response that allowed bypassing of the inhibitory upstream open reading frame in the PD-L1 5' UTR, thereby enhancing PD-L1 translation and suppressing anti-tumor immunity. Wroblewska et al. (2018) developed a barcoding system that operates at the protein level and identified SOCS1 (suppressor of cytokine signaling 1) as a negative regulator of PD-L1. Frequent loss of interferon regulatory factor 2, resulting in reduced MHC I antigen presentation and increased PD-L1 expression leading to immune escape (Kriegsman et al., 2019). Sreevalsan et al. (2020) found that the transcriptional regulator MLLT6 (myeloid/lymphoid or mixed-lineage leukemia; translocated to, 6) is required for efficient PD-L1 protein expression and cell surface presentation in cancer cells. Depletion of MLLT6 reduced the inhibition of CD8 cytotoxic T cell-mediated cytolysis. Taken together, the above shows that CRISPR not only has a screening function in the drug-tumor cell model but also helps to screen immune cells and tumor cells for regulatory factors in immunotherapy.

4.7 CDK inhibitors

Cell cycle regulation depends on three major regulators, namely, cyclin, CDK (cell cycle-dependent protein kinases), and CDKI, and the normal function of the cell cycle depends mainly on the temporal activation of various CDKs and their phosphorylation modifying the corresponding substrate protein kinase complexes of cyclins and CDK. The mechanism of CDKI is shown in Figure 3F.

The first generation of CDKIs, such as flavopiridol and roscovitine, demonstrated disappointing effects in clinical trials due to their defects in targeting specific CDKs, while the third generation of CDK4/6 inhibitors, such as palbociclib, abemaciclib, and ribociclib, had satisfactory results in advanced or metastatic breast cancer.

Tong et al. (2019) found differential genes in bladder cancer were mainly enriched in the receptor tyrosine kinase, PI3K-Akt, Ras/MAPK, JAK/STAT, or Wnt signaling pathways and found that inhibitors targeting RTKs, PI3K-AKT and Ras/MAPK had synergistic effects in combination with palbociclib. Kabir et al. (2019) found that the cullin 5 ubiquitin ligase complex mediated the resistance of lung cancer cells to cyclin-dependent kinase 9 and MCL1. Daggubati et al. (2021) performed a CDK4/6 inhibitor screening in medulloblastoma and identified reduced ribosomal protein expression as the basis of resistance to CDK6 inhibition in Hedgehog-associated medulloblastoma cells, leading to ER stress and activation of the unfolded protein response. This increases the activity of enzymes that produce the smooth-activated sterol lipids which maintain HH signaling in medulloblastoma. Upregulation of RTK-RAS-RAF and RTK-PI3K-AKT signaling cascade activity in NRAS mutant melanoma leads to resistance to combined inhibition of MEK1/2 and CDK4/6 (Hayes et al., 2019). Mayayo-Peralta et al. (2021) identified activation of the EGFR pathway in CDK4/6-resistant cells in breast cancer, raising the possibility of using receptor tyrosine kinase signaling cascade inhibitors to target CDK4/6 inhibitor resistance.

5 Discussion

The above results demonstrate the power and convenience of genome-wide CRISPR/Cas9 screening as a screening technique. Based on our data, numerous drug-resistance genes have been identified in multiple tumors based on CRISPR/Cas9 screening technology, which affects the cellular response to chemotherapeutic agents through various pathways. The most abundantly studied are MAPK pathway inhibitors, and the common mechanisms of resistance are the reactivation of MAPK pathways such as DUSP7, NF2, and the KEAP1/Nrf2 pathway, which are present in multiple cancer resistance models. Despite the variation in results between studies, individual genes have been validated *in vitro* and *in vivo* and do correlate with cellular drug resistance. This suggests that the acquisition of cellular drug resistance phenotypes is often a multi-pathway, multi-omics alteration, such as metabolism, EMT, and signal transduction.

However, CRISPR/Cas9 screening still has some shortcomings that need to be addressed. The genome-wide screening technique is highly influenced by cell heterogeneity, resulting in different sgRNA distributions even within identical studies, cell lines, and drug treatments (Sun et al., 2018; Zheng et al., 2019). The screening results also depend on several factors such as the construction of the sgRNA library, the treatment of the control group, the cell response to the delivery system, the multiplicity of infection, and the random genetic drift of the cell line (Doench, 2018). The most important of these is the establishment of the sgRNA library since the downstream data analysis mainly focuses on the distribution of the sgRNA in the

genome by sequencing. The off-target effect, the efficiency of the knockout effect, the drug concentration, and the time length for drug exposure should also be taken into consideration.

A model of cellular alteration to drug resistance suggests that cells acquire drug resistance partially through alterations in the epigenome in the early stages and phenotypic alterations in drug resistance through alterations in the genome, transcriptome, and metabolome in the later stages (Wang et al., 2023). To better illustrate this genetic alternation pattern, a more comprehensive approach can be done by single-cell screening. Single-cell CRISPR/Cas9 screening enables the detection of both the sgRNA distribution and the transcriptome data and is an exciting technology but also has a high sequencing cost (Wang and Wang, 2017). Another screening method is arrayed screening. While all sgRNAs are introduced into a single culture dish in a pooled screening, array screening introduces each sgRNA into cells in a single well on a platform. This technology allows for the selection of phenotypes not limited to cell viability, such as subcellular localization and morphometric (Datlinger et al., 2017). The application for different screening methods depends on the selection criteria. Pooled screening, due to its lower cost and ease of operation, has become the most used method for drug resistance research.

Also, targeting partial genomic screening has higher robustness than whole-genome screening (Zhong et al., 2022), and whole-genome screening tends to cover most of the differential genes, but this methodology may not uncover very strongly selected genes and may be dependent on factors such as *in vitro* drug metabolism effects (Sarr et al., 2019). For genome-wide screening assays, targeted experiments on the screened genes are required to draw more rigorous conclusions.

Therefore, treatments targeting a single gene (e.g., single-point targeted drugs) may eventually lead to the emergence of new drug-resistant genes, and the change in tumor drug resistance can only be to a certain extent and cannot fundamentally change the cellular drug resistance. If we want to reverse the process of tumor drug resistance, it is essentially a process of reversing entropy, which requires precise modulation of the dysregulated gene within the tumor to reprogram the expression network, which is currently pending the emergence of new technologies.

Genome-wide CRISPR/Cas9 screening technology is a powerful tool for screening unknown drug resistance genes and has particularly progressed in different cancer studies, especially in targeted drug resistance studies. Drug resistance in tumor cells is often caused by multiple gene mutations. Meta-analysis against genome-wide CRISPR/Cas9 drug resistance screening models can perhaps construct a gene-related network to reveal the drug resistance network in tumors.

CRISPR/Cas9 technology currently has powerful capabilities as a gene editing technology, and genome-wide screening technology is also a credible and convenient means of screening cellular drug resistance-associated genes. More data from CRISPR/Cas9 screening will help reveal cellular response to drugs.

Author contributions

ZZ: Writing–original draft, Writing–review and editing. HW: Writing–review and editing. QY: Writing–review and editing. JC: Writing–review and editing. YC: Writing–review and editing. SR: Writing–review and editing. JY: Data curation, Validation, Writing–review and editing. ZW: Data curation, Validation, Visualization, Writing–review and editing. MH: Supervision, Validation, Visualization, Writing–review and editing. SH: Writing–review and editing. QZ: Funding acquisition, Investigation, Writing–review and editing. CZ: Writing–review and editing. BH: Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Glossary

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR associated nuclease 9
LOF	Loss-of-function
GOF	Gain-of-function
sgRNA	Single-guide RNA
DSBs	DNA double-strand break
CRISPRa	CRISPR activation
CRISPRi	CRISPR inhibition
GECKO	Genome-scale CRISPR Knock-Out
SAM	Synergistic activation mediator
MAPK	Mitogen-Activated Protein Kinase
PARPi	Poly (ADP-ribose) polymerase inhibitors
ICIs	Immune checkpoint inhibitors
CDKI	Cyclin-dependent kinase inhibitors
RKT	Receptor tyrosine kinase
RAS	Rat sarcoma
RAF	Rapidly Accelerated Fibrosarcoma
MEK	Mitogen-activated protein kinase
ERK	Extracellular-signal regulated protein kinase
BRAF	B-Raf proto-oncogene
EGFR	Epidermal growth factor receptor
VEGFR	Vascular endothelial growth factor receptor
FGFR	Fibroblast Growth Factor Receptor
MET	MET proto-oncogene
KIT	KIT proto-oncogene
TKI	Tyrosine kinase inhibitor
SHOC2	SHOC2 leucine-rich repeat scaffold protein
MRAS	Muscle RAS oncogene
PP1c	Protein phosphatase 1
SCRIB	Scribble planar cell polarity protein
NSCLC	Non-Small Cell Lung Carcinoma
RIC8A	RIC8 guanine nucleotide exchange factor A
ARIH2	Ariadne RBR E3 ubiquitin protein ligase 2
EMT	Epithelial to mesenchymal transition
NF1	Neurofibromin 1
DUSP9	Dual specificity phosphatase 9
HCC	Hepatocellular carcinoma
PI3K	Phosphoinositide 3-Kinase
AKT	Protein Kinase B
DUSP4	Dual specificity phosphatase 4
PTEN	Phosphatase and tensin homolog
FTase	Farnesyltransferase
ROS	Reactive oxygen species
KEAP1	Kelch-like ECH associated protein 1
Nrf2	Nuclear factor erythroid 2-related factor 2
FGF21	Fibroblast growth factor 21
ALK	Anaplastic lymphoma kinase
MTX1	Metaxin 1
CDC37L1	Cell division cycle 37 like 1
PPIA	Peptidylprolyl isomerase A
SGOL1	Shugoshin 1
PHGDH	Phosphoglycerate dehydrogenase
SSP	Serine synthesis pathway
LRP8	LDL receptor-related protein
CIC	Capicua transcriptional repressor
FBXO42	F-box protein 42
GRB7	Growth factor receptor bound protein 7
PLK1	Polo-like kinase 1
PARP1	[oly (ADP-ribose) polymerase 1
TIGAR	TP53-induced glycolysis regulatory phosphatase
ALC1	Amplification in liver cancer1
ARH3	ADP-ribosylserine hydrolase
PSMA6	Proteasome 20S subunit alpha 6
SH3D21	SH3 domain containing 21
DCK	Deoxycytidine kinase
MTA3	The metastasis associated 1 family member 3
CRIP2	Cysteine-rich protein 2
ELP5	Elongator acetyltransferase complex subunit 5
HnRNPQ	Heterogeneous nuclear ribonucleoprotein Q
HDAC1	Histone deacetylase 1
ABCG2	ATP binding cassette subfamily G member 2
MSH2	MutS homolog 2
ARIH1	Ariadne1 homolog
SULF1	Sulfatase 1
TMZ	Temozolomide
E2F6	E2F transcription factor 6
PTX	Paclitaxel
TCEAL1	Transcription elongation factor A-like 1

SSR3	Signal sequence receptor subunit 3
PD-1/PD-L1	Programmed death protein 1/programmed death ligand 1
CMTM6	CKLF like MARVEL transmembrane domain containing 6 protein
SOCS1	Suppressor of cytokine signaling 1
MLLT6	Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 6
CDK	Cell cycle-dependent protein kinases
MCL1	Myeloid cell leukemia sequence 1

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Disitamab Vedotin (RC48) for HER2-positive advanced breast cancer: a case report and literature review

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Background/aim: Human epidermal growth factor receptor 2 (HER2)-positive breast cancer is associated with a higher risk of metastasis and poorer overall survival (OS) due to HER2 gene overexpression/amplification. Although anti-HER2 targeted therapy has shown survival benefits in HER2-positive advanced breast cancer (ABC) patients, long-term treatment often leads to drug resistance, complicating further treatment options. RC48, an antibody-drug conjugate (ADC), combines the benefits of antibody targeting with the cytotoxic effects of a small molecule drug.

Case report: We present a case involving a female patient with HER2-positive ABC who developed drug resistance and disease progression following multi-line anti-HER2 targeted therapy. In this instance, RC48 exhibited anti-tumor activity in an ABC patient resistant to HER2-targeted therapy. After eight treatment cycles with 120 mg of RC48, the tumor size decreased and stabilized.

Conclusion: This case report underscores the potential clinical value of RC48 as a promising treatment alternative for patients resistant to HER2 targeted therapies.

KEYWORDS

Disitamab Vedotin (RC48), advanced breast cancer, human epidermal growth factor receptor 2, targeted therapy, tumor resistance, case report

1 Introduction

Breast cancer is the most common malignant tumor worldwide, with the highest incidence and the second highest mortality in women (1). Human epidermal growth factor receptor 2 (HER2)-positive breast cancer represents approximately 15-20% of all breast cancer cases. This tumor subtype, HER2-positive breast cancer, is more invasive, prone to metastasis, and associated with poorer prognosis (2). Anti-HER2 therapy can greatly improve the survival rate of patients; however, after multi-line treatment, the majority of advanced breast cancer (ABC) patients eventually develop drug resistance, leading to disease progression, and the limited subsequent treatment options (3). Currently, the mechanisms underlying resistance to anti-HER2 targeted therapy are under study. Research has indicated that resistance may be associated with spatial effects (structural mutations of the HER2 protein), overexpression of tyrosine kinase receptors [such as the insulin-like growth factor receptor (IGFR)], and mutations in the HER2 downstream signaling pathway (4). Overcoming resistance to anti-HER2 targeted therapy is crucial, and identifying new effective treatment strategies can provide valuable insights for clinical practice.

Currently, the primary treatment for HER2-positive ABC involves anti-HER2 targeted therapy drugs, often utilizing a combination of trastuzumab and pertuzumab as first-line treatment (3). The combination of trastuzumab and pertuzumab synergistically enhances the inhibition of the HER2 pathway (5).

The study of von Minckwitz et al. (6) showed that the HER2 pathway could be further inhibited when the first-line treatment disease progresses, and subsequent targeted therapy still has application value. Trastuzumab emtansine (T-DM1) is a novel antibody-drug conjugate (ADC). Verma et al. (7) assessed the utility of T-DM1 in patients who experienced disease progression following trastuzumab treatment. The T-DM1 treatment group had an objective response rate (ORR) of 43.6%, a median progression-free survival (PFS) of 9.6 months, and overall survival (OS) of 30.9 months, with $P < 0.0017$. These results indicate that T-DM1 could serve as a preferred drug for targeted therapy after drug resistance develops. DS-8201 (T-DXd) is another novel ADC drug. In a phase III clinical trial (NCT03529110), T-DXd treatment demonstrated significant OS and PFS benefits (28.8 months vs. 6.8 months) compared to T-DM1, and T-DXd substantially reduced the risk of death by 36% (HR, 0.64) (8).

Disitamab Vedotin (RC48) is an ADC with a higher affinity for HER2 than the targeted drug trastuzumab (KD, 5.0E-10M vs KD, 1.9E-09M) (9). By utilizing antibodies, RC48 can block the downstream signaling pathway activated by HER2, work in conjunction with cytotoxic drugs to kill tumor cells and interfere with the transcription, division, proliferation, and growth of cancer cells to exert anti-tumor effects. In a clinical trial (NCT02881190), RC48 demonstrated stronger antitumor activity in HER2-positive breast cancer, gastric cancer, and trastuzumab and lapatinib-resistant xenograft tumor models than the FDA-approved T-DM1 (10, 11).

However, to the best of our knowledge, there have been limited studies reporting the efficacy of RC48 monotherapy in ABC patients resistant to HER2-targeted therapy. Therefore, we present a case of a female patient with HER2-positive ABC who developed resistance to multiple lines of anti-HER2 therapy. This patient experienced significant benefits and ultimately became eligible for surgery following treatment with RC48. This case report offers new insights into addressing resistance to HER2 targeted therapy in clinical practice.

2 Case report

A 54-year-old female patient was admitted to an external hospital on December 15, 2019 due to the discovery of a right breast tumor for 2 weeks. A physical examination revealed a tumor in the upper quadrant of the right breast. Ultrasound-guided right breast tumor biopsy pathology indicated non-specific invasive breast cancer in the right breast; immunohistochemical staining results showed that estrogen receptor (ER) (70% weak-moderate intensity +), progesterone receptor (PR) (40% weak-moderate intensity +), HER2 (3 +), proliferation index (Ki67) (80%); right axillary lymph node biopsy cytology showed cancer metastasis. The patient was diagnosed with right breast invasive breast cancer (cT4N1M0 stage III). The patient began anti-tumor treatment in July 2020 after half a year, and had been treated with multiple lines. The treatment is shown in Table 1.

On July 14, 2020, the patient began neoadjuvant chemotherapy, receiving two cycles of albumin-bound paclitaxel combined with fluorouracil (specific dose: albumin-bound paclitaxel 125mg/m2 d1 d8 ivgtt, 5FU 500mg/m2 d1 ivgtt, with 21 days per cycle), during which the tumor continued to grow. On August 27, 2020, the patient underwent a PET-CT examination, which revealed right breast cancer with right axillary lymph node metastasis and a response of progressive disease (PD).

On September 1, 2020, the neoadjuvant treatment regimen was changed to albumin-bound paclitaxel, carboplatin, and trastuzumab for two cycles (actual dose: albumin-bound paclitaxel 200mg d1, d8 ivgtt, carboplatin 700mg d1 ivgtt, trastuzumab 580mg for the first dose, followed by 430mg ivgtt d1 q21d). Since the tumor did not shrink after two chemotherapy cycles, pertuzumab (initially 840mg, followed by 420mg ivgtt d1) was added for two cycles. After completing four treatment cycles, the PET-CT findings from November 20, 2020, were juxtaposed with the data from August 27, 2020, for comparative analysis. The right breast tumor had reduced in size, and the right axillary lymph node had also decreased in size (Figure 1), achieving the best response of partial response (PR). For economic reasons, the patient discontinued treatment on their own accord.

In September 2021, the right breast tumor locally enlarged and ulcerated. A chest CT scan revealed multiple enlarged lymph nodes in the bilateral axilla, which were larger and more numerous than before. On September 27, 2021, the patient began first-line treatment with two cycles of albumin-bound paclitaxel, capecitabine, trastuzumab, and pertuzumab (actual dose: albumin-bound paclitaxel 200mg d1, d8 ivgtt, capecitabine 1000mg/m2 d1-d14 bid po, trastuzumab 430mg ivgtt d1 q21d, pertuzumab 420mg ivgtt d1). Due to economic factors, the

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TABLE 1 Treatment of HER2-positive ABC patients.

Time range	Treatment lines	Treatment regimen	Best response	PFS (months)
Jul 2020 — Aug 2020	Neoadjuvant	Albumin Paclitaxel + Fluorouracil X2	PD	1.5
Sept 2020— Nov 2020	Neoadjuvant	Albumin Paclitaxel + Carboplatin + Trastuzumab + Patuzumab X4	PR	12
Dec-2020 — Sept 2021	N/A	The patient discontinued treatment due to economic factors	N/A	N/A
Sept 2021 — Dec 2021	First-line	Albumin paclitaxel + capecitabine + trastuzumab + pertuzumab X2; Docetaxel + trastuzumab + pertuzumab X2	PD	3
Dec 2021 — Mar 2022	Second-line	Capecitabine + pyrotinib + trastuzumab + pertuzumab X4	PD	3
Mar 2022 — Jun 2022	Third-line	Inetetamab + Vinorelbine + cisplatin X2	PD	2
Jul 2022 — Oct 2022	Fourth-line	RC48 (2.0mg/kg) 120mg X8 (July 6th—Oct 21st)	PR	4
Nov 2022	Surgery	Modified radical mastectomy of right breast	NA	NA
Nov 2022 — Today	Maintenance	Trastuzumab + pyrotinib + abemaciclib + fulvestrant	SD	8

PD, progressive disease; PR, partial remission; SD, stable disease; NA, not applicable.

chemotherapy drug was switched to docetaxel, and the actual medication regimen was docetaxel, trastuzumab, and pertuzumab for two cycles. After four cycles of treatment, the tumor was visibly larger, and the bleeding became severe. A chest CT scan showed that the bilateral axillary lymph nodes had increased in size compared to before, with a response of PD.

Due to disease progression following first-line treatment, the patient underwent second-line treatment on December 27, 2021, with four cycles of capecitabine, pyrotinib, trastuzumab, and pertuzumab (actual dose: capecitabine 1000mg/m2 d1-d14 bid po, pyrotinib 400mg qd po, trastuzumab 430mg ivgtt d1 q21d, pertuzumab 420mg ivgtt d1). During treatment, the tumor

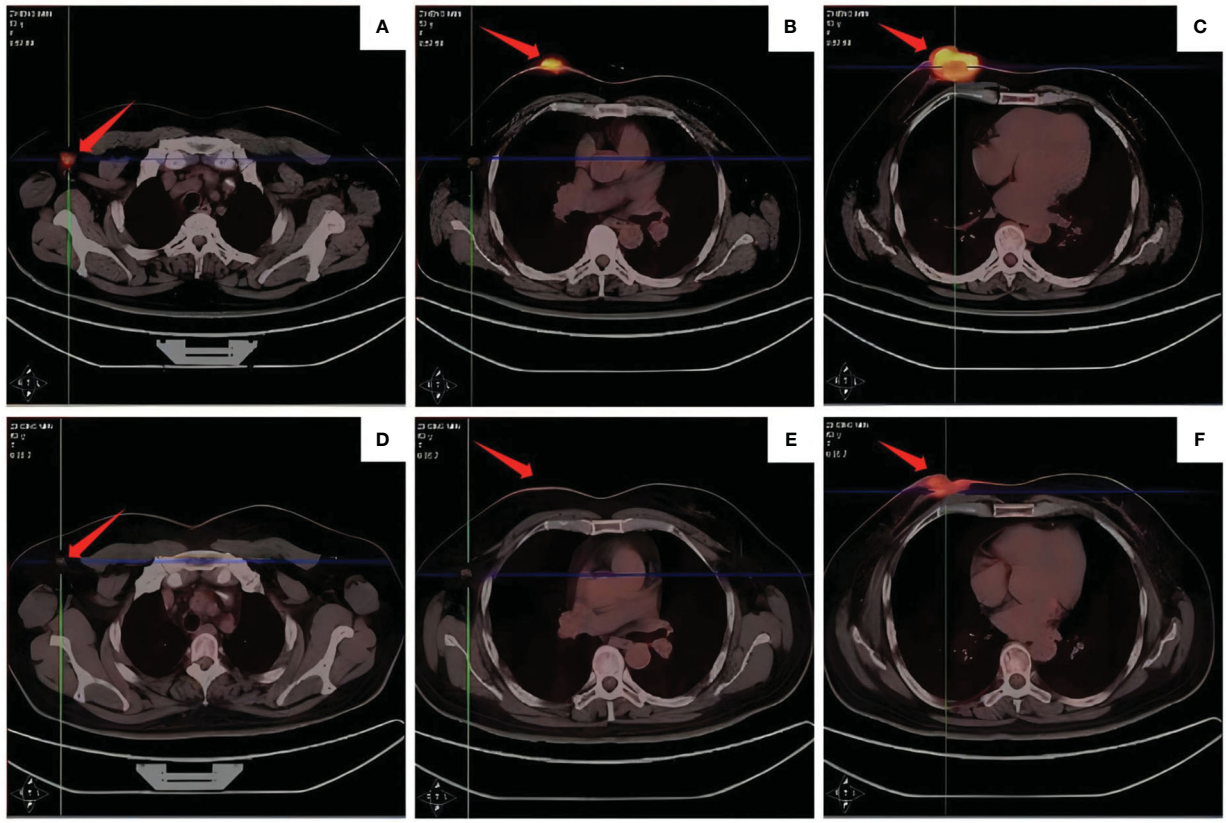

FIGURE 1
PET-CT results of patients at different time. On August 27, 2020, **(A, B)** multiple lymph nodes in the right axilla were enlarged, with a large diameter of about 1.5 cm, SUVmax: 9.1; **(C)** 3.7 cm x 3.8 cm tumor was seen in the right breast nipple, SUVmax: 22.4; **(D-F)** after four cycles of treatment (November 20, 2020), the right breast tumor and axillary lymph nodes were smaller than before.

ruptured, and bleeding was stopped using an external application of Yunnan Baiyao powder. On March 17, 2022, a chest CT scan was reviewed, showing that the bilateral axillary lymph nodes had increased in size compared to before, with a response of PD. Considering the possibility of drug resistance, the patient was advised to undergo genetic testing. The results revealed a PIK3CA exon 10 c.1624G>A p. E542K missense mutation, but related targeted drugs were not yet available to us.

On March 18, 2022, the patient underwent third-line chemotherapy with two cycles of Inetetamab, vinorelbine, and cisplatin (specific drugs: Inetetamab 550mg ivgtt d1 q21d, vinorelbine 40mg ivgtt d1, d5 q21d, cisplatin 60mg ivgtt d1 q21d). On June 9, 2022, PET-CT results showed that the original right breast tumor had significantly increased in size compared to before, and the original right axillary lymph nodes were larger and more numerous than before. New lymph nodes with increased FDG metabolism appeared in the bilateral chest wall, right internal mammary region, and left axilla, indicating disease progression once again, with a response of PD.

On June 16, 2022, a right axillary lymph node biopsy revealed poorly differentiated carcinoma. Immunohistochemistry results were as follows: ER (3 + 80%), PR (–), HER2 (3+), and Ki-67 (+ 2%). The immunohistochemical results confirmed that the patient still had HER2 overexpression. Considering the chemotherapy and targeted resistance after previous multi-line chemotherapy, the patient was given RC48 as a fourth-line treatment, which is administered in a cycle every 14 days with the treatment being given on the first day of each cycle. On July 6, 2022, eight cycles of RC48 treatment were initiated, with the planned medication regimen as follows: RC48 (2.0 mg/kg) 120mg ivgtt d1 q14d. Before treatment, the patient experienced significant pain and required analgesic drugs for pain control. After RC48 treatment, the pain considerably improved, and the dosage of analgesics was significantly reduced. During the treatment period, the ulcerated tumor gradually healed (Figure 2A), the patient tolerated the treatment well, and no obvious adverse reactions occurred. After six cycles, a chest CT scan showed that the breast tumor and bilateral axillary lymph nodes had significantly reduced in size, with the best response of PR (Figure 2B).

In order to explore the improvement in quality of life brought about by RC48, we used the FACT-B scale to evaluate the patient-reported outcomes (PROs). After using RC48, the patient experienced significant improvements in the five functional areas and overall health compared to their previous quality of life (Figure 3A). In the process of multi-line anti-HER-2 treatment, the patient often has adverse reactions such as bone marrow suppression and limb end numbness, among which nausea and vomiting most affect the quality of life. During the treatment with

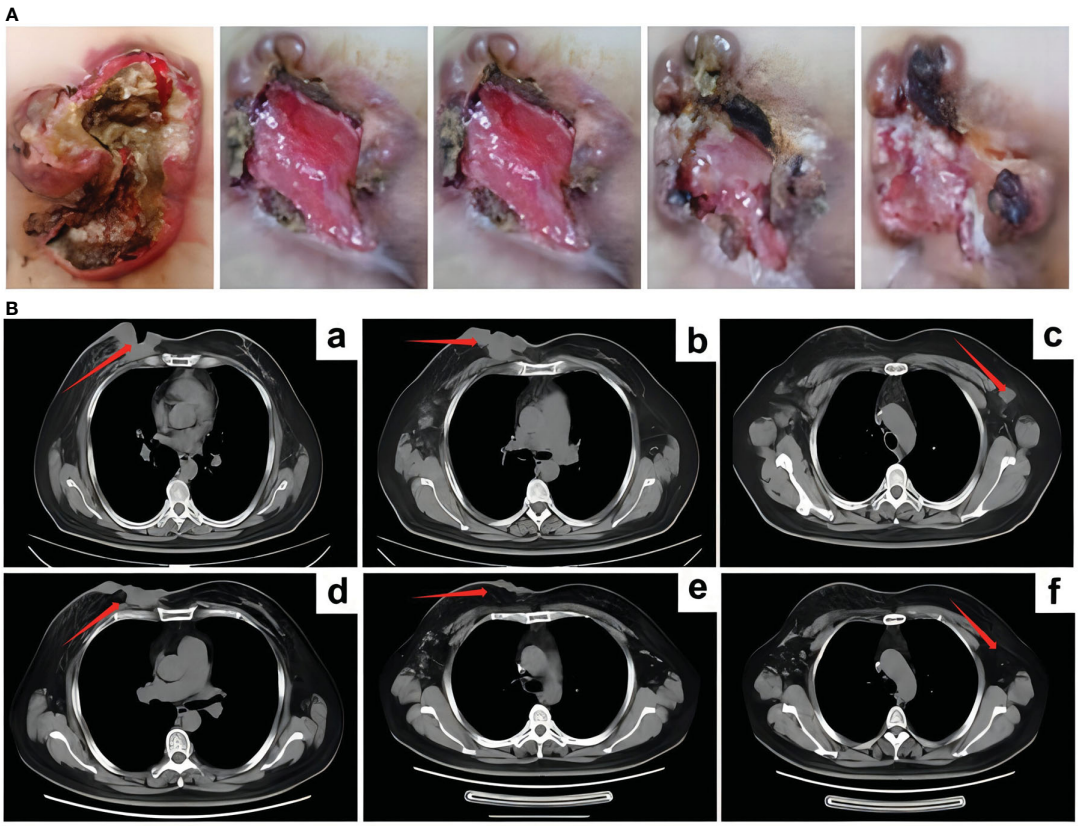

FIGURE 2
The curative effect of RC48 treatment. **(A)** After RC48 treatment, the tumor rupture was smaller than before. **(B)** CT efficacy evaluation after 6 cycles of RC48 treatment. (a-c) Patient status before RC48 treatment; (d-f) after RC48 treatment, the breast tumor and bilateral axillary lymph nodes were significantly reduced.

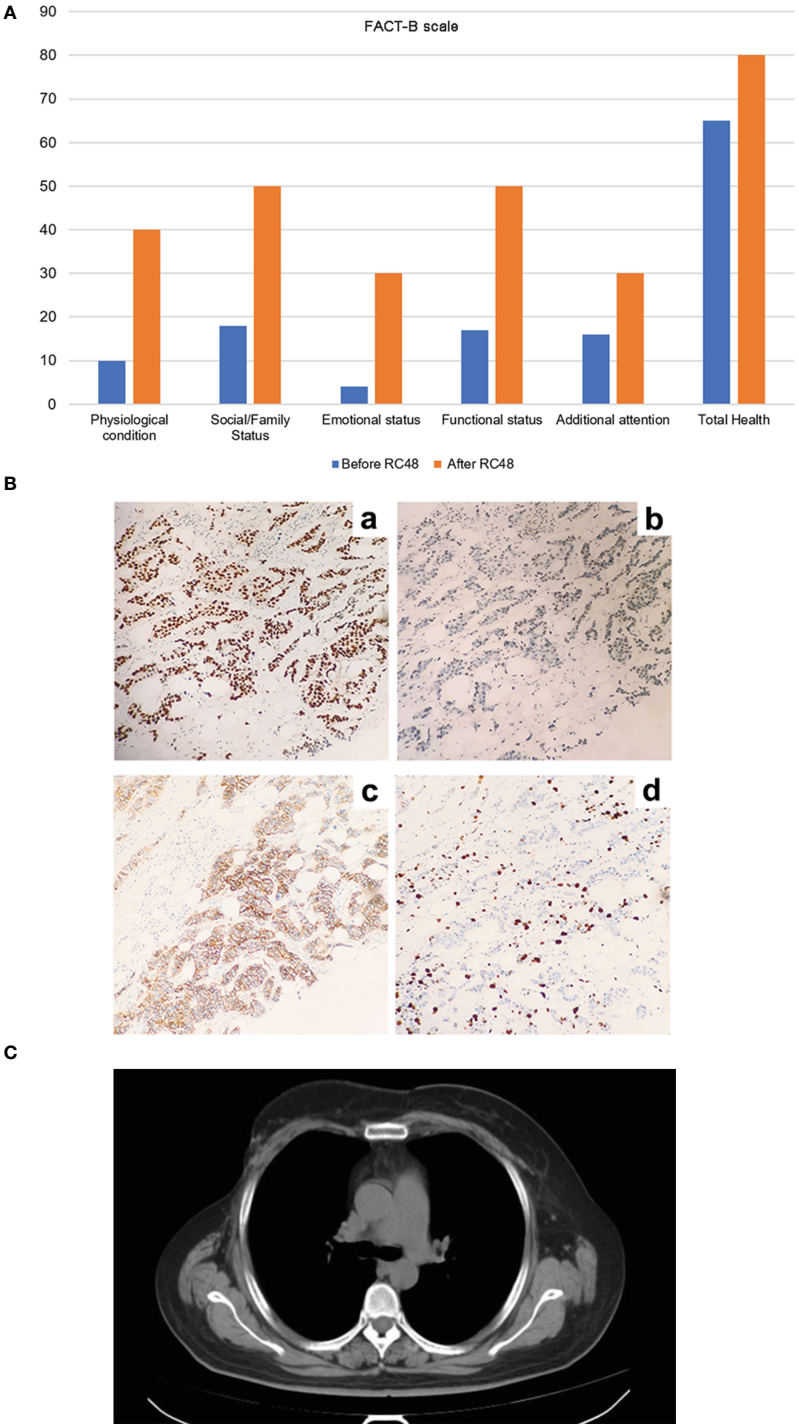

FIGURE 3
The improvement of patient's life quality and postoperative immunohistochemical results of right breast cancer after RC48 treatment, as well as the CT results after half a year (July 12, 2023). **(A)** The five functional areas and overall health level of patients were significantly improved, and the quality of life was significantly improved. **(B)** The postoperative immunohistochemical results of right breast cancer. (a) ER (2 + 60%), (b) PR (5%), (c) HER2 (2 +), (d) Ki67 proliferation index (60%). **(C)** The patient 's CT results showed no enlarged lymph nodes, and the condition was stable.

RC48, the patient feels good about herself, no obvious systemic adverse reactions occurred, and the overall quality of life was significantly improved.

After six cycles of RC48 treatment, the tumor shrank and stabilized, with the 7th and 8th cycles continuing to show stability. In order to further improve the quality of life, a multidisciplinary team of breast cancer specialists (MDT) recommended a modified radical mastectomy of the right breast cancer, which was performed on November 21, 2022. Postoperative pathology revealed non-specific invasive carcinoma in the right breast and metastatic carcinoma in the right axillary and subclavian lymph nodes. Immunohistochemical results for the right breast

lesions showed: ER (2 + 60%), PR (+ 5%), HER2 (2+), and Ki67 (60%) (Figure 3B); HER2 FISH was positive.

Considering that the patient's contralateral axillary enlarged lymph node was a metastatic lymph node, it was recommended that the patient continue maintenance treatment after surgery. The patient's postoperative pathology was positive for hormone receptor (HR+) and HER2 overexpression. The recommended treatment was trastuzumab + pyrotinib targeted therapy, combined with abemaciclib + fulvestrant targeted dual pathway endocrine therapy (specific medications: trastuzumab 430mg ivgtt d1 q21d, pyrotinib 400mg qd po, abemaciclib 50mg bid po, fulvestrant 500mg intramuscular injection). The patient's condition was stable half a year after operation. The CT results showed that the right breast was changed after operation (Figure 3C).

3 Discussion

HER2 is a transmembrane protein with tyrosine kinase activity. It mediates signal transduction activation and downstream signaling pathways through heterodimer and tyrosine kinase autophosphorylation (12). HER2-positive tumors are prone to metastasis, and most have a poor prognosis. Anti-HER2 targeted therapy can greatly improve the survival rate of breast cancer (13). Trastuzumab combined with chemotherapy in the treatment of HER2-positive ABC patients can significantly prolong the disease-free survival (DFS) and OS. However, most patients may develop drug resistance after treatment. More than 70% of HER2-positive breast cancer patients have tumor recurrence, lymph node metastasis, etc (4).

The issue of drug resistance is crucial for patients' prognosis. Currently, the mechanisms of targeted therapy resistance mainly involve changes in the HER2 receptor molecular structure, alterations in the PI3K/AKT/mTOR signaling pathway, and influences of the HER family on downstream signaling pathways. Other mechanisms include incomplete blocking of the HER2 signaling pathway, involvement of immune mechanisms, and changes in oncogenes/tumor suppressor genes (14–19). The patient's genetic test suggested a missense mutation in PIK3CA, which may be the cause of drug resistance. However, due to the unavailability of related signaling pathway inhibitors, targeted treatment was not possible.

In addition, other factors affecting drug resistance and efficacy, which are clinically important, should not be overlooked. Our case report highlights a treatment gap in one patient, which may have also contributed to the observed drug resistance. Due to financial difficulties and adherence issues, our patient discontinued treatment, which may lead to a decrease in drug efficacy and the development of resistance, especially to targeted therapies such as anti-tumor necrosis factor-2 (anti-HER-2). Suspension of treatment after initial efficacy of neoadjuvant therapy may lead to the development of drug resistance in tumor cells, which may affect the efficacy of subsequent anti-HER-2 therapy. In addition, the coronavirus pandemic poses a healthcare challenge. Our patient's treatment interruption coincided with the coronavirus epidemic. Although the treatment interruption was not directly caused by the epidemic, the economic impact may have exacerbated the patient's financial stress, which may have influenced her decision to continue treatment.

RC48 is an antibody-coupled drug with both antibody targeting and small molecule drug killing properties. Relevant clinical studies have confirmed its efficacy in the treatment of ABC. Two phase-I clinical studies, NCT02881138 and NCT03052634 (20, 21), evaluated the efficacy of RC48 in patients with HER2-positive (IHC 3+ or IHC 2+ and FISH amplification) locally advanced or metastatic breast cancer. We performed a combined analysis of the two studies (20, 21) to assess the efficacy. Among the 70 patients treated with RC48, the ORR was 31.4% (22/70), the clinical benefit rate (CBR) was 38.6%, and the median PFS was 5.8 months. Among the 64 patients receiving ≥ 1.5 mg/kg dose, the ORR was 34.4% (22/64), and the median PFS was 6.2 months. The ORR of patients receiving 1.5 mg/kg, 2.0 mg/kg, and 2.5 mg/kg doses was 22.2%, 42.9%, and 36.0%, respectively, and the median PFS was 6.2 months, 6.0 months, and 6.3 months, respectively. It can be observed that RC48 demonstrates a favorable effect in HER2 positive metastatic breast cancer, and 2.0 mg/kg Q2W is the optimal choice. The author's intention here is to convey an integrated analysis of the two aforementioned studies.

In another phase I clinical study (NCT02881190) (11), 57 patients with solid tumors were enrolled and treated with RC48 at a dose of 2.0 mg/kg Q2W. The ORR and disease control rate (DCR) were 21.0% (12/57) and 49.1% (28/57), respectively. Notably, the efficacy of RC48 in HER2+/FISH- patients was similar to that of IHC2+/FISH+ and IHC3+ patients, with ORRs of 35.7% (5/14), 20.0% (2/10), and 13.6% (3/22), respectively. RC48 also demonstrated promising efficacy in patients who had not previously received HER2-targeted therapy, with an ORR of 15.0% (3/20) and a DCR of 45.0% (9/20). These results suggest that RC48 may have a beneficial effect on HER2-positive or low-expressing solid tumors, further highlighting its potential as a therapeutic option for these patients.

The study by Parise C et al. (22) demonstrated that approximately 50% of HER2-positive breast cancer patients also express hormone receptors (HR), that is, HER2+/HR+ or HER2+/ER+/PR+, accounting for 10%-15% of all breast cancers. HER2 and ER-mediated signaling pathways in breast cancer patients intersect at multiple nodes. ER can affect apoptosis by activating downstream pathways such as HER2, thereby influencing the efficacy of anti-HER2 targeted therapy (23). The Monarch HER study included ABC patients who received at least two anti-HER2 regimens and those who did not receive CDK4/6 inhibitors and fulvestrant. The results showed that, compared to standard chemotherapy plus trastuzumab, abemaciclib plus trastuzumab plus fulvestrant significantly improved PFS in HER2+/ER+ ABC patients resistant to anti-HER2 therapy (8.3 months vs. 5.7 months, P=0.051) (24). According to the final OS results presented at the European Society for Medical Oncology (ESMO) in 2022 (25), abemaciclib plus trastuzumab combined with or without fulvestrant significantly improved the OS of HR+/HER2+ ABC patients compared to standard chemotherapy plus trastuzumab (31.1 months vs. 20.7 months). These findings provide guidance for our postoperative maintenance therapy.

For HER2-positive breast cancer patients, chemotherapy combined with targeted therapy is predominantly used in first-line treatment, significantly prolonging survival and improving the quality of life (26). In our case, the patient was initially diagnosed with invasive breast cancer and axillary lymph node metastasis. Immunohistochemical results showed HER2 (3+), indicating that dual-target combined chemotherapy could provide the greatest benefits (27). The patient experienced disease progression after neoadjuvant therapy and third-line anti-HER2 combined chemotherapy, and the local tumor ulceration severely impacted her quality of life. Faced with multiple drug resistance, we opted for 8 cycles of RC48 treatment. The tumor shrank significantly, the ulceration improved considerably, the patient became eligible for surgical treatment, and her quality of life improved substantially. For postoperative treatment, we employed trastuzumab + pyrotinib targeted therapy in conjunction with abemaciclib + fulvestrant maintenance therapy. This approach resulted in acceptable tumor control and a stable condition for the patient. However, if the patient experiences a relapse, determining the subsequent treatment plan will be a significant challenge.

We report the successful treatment of a HER2-positive ABC patient using RC48 monotherapy. After the failure of multiple lines of anti-HER2 treatment, the use of ADC drugs was proved effective, providing the opportunity for surgical treatment and improving the patient's quality of life. Our case offers new therapeutic options for other ABC patients resistant to HER2 treatment, holding clinical reference value and presenting new treatment ideas for patients with refractory HER2-targeted therapy resistance.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of First Affiliated Hospital of Dalian Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YL: Conceptualization, Data curation, Methodology, Writing – original draft. JZ: Conceptualization, Software, Writing – original draft. ZC: Validation, Writing – review & editing. XG: Validation, Writing – review & editing. LZ: Validation, Writing – review & editing. ZL: Formal Analysis, Writing – review & editing. XW: Formal Analysis, Writing – review & editing. PY: Investigation, Writing – review & editing. JL: Conceptualization, Writing – review & editing. FF: Conceptualization, Resources, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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NOP58 induction potentiates chemoresistance of colorectal cancer cells through aerobic glycolysis as evidenced by proteomics analysis

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Introduction: The majority of individuals diagnosed with advanced colorectal cancer (CRC) will ultimately acquire resistance to 5-FU treatment. An increasing amount of evidence indicates that aerobic glycolysis performs a significant function in the progression and resistance of CRC. Nevertheless, the fundamental mechanisms remain to be fully understood.

Methods: Proteomic analysis of 5-FU resistant CRC cells was implemented to identify and determine potential difference expression protein.

Results: These proteins may exhibit resistance mechanisms that are potentially linked to the process of aerobic glycolysis. Herein, we found that nucleolar protein 58 (NOP58) has been overexpressed within two 5-FU resistant CRC cells, 116-5FuR and Lovo-5FuR. Meanwhile, the glycolysis rate of drug-resistant cancer cells has increased. NOP58 knockdown decreased glycolysis and enhanced the sensitivity of 116-5FuR and Lovo-5FuR cells to 5FU.

Conclusion: The proteomic analysis of chemoresistance identifies a new target involved in the cellular adaption to 5-FU and therefore highlights a possible new therapeutic strategy to overcome this resistance.

KEYWORDS

colorectal cancer, chemoresistance, aerobic glycolysis, 5-FU, NOP58

1 Introduction

Colorectal cancer (CRC) ranks as the third most common prevalent form of cancer globally and it holds the second position in terms of death associated with malignancies (Sung et al., 2021; Heide et al., 2022; Zhuo et al., 2023). Chemotherapy is considered the primary therapeutic approach for individuals with CRC who have had disease progression

and metastasis. One often employed strategy involves the administration of 5-fluorouracil (5-FU), either as a standalone agent or as a crucial constituent of systemic chemotherapy, having the purpose of treating CRC (Correale et al., 2004; Rosmarin et al., 2014; Huang et al., 2023; Li et al., 2023). Nevertheless, drug resistance is the greatest challenge for 5-FU treatment. Hence, it is crucial to elucidate the fundamental mechanism responsible for 5-FU resistance.

Cancer cells have enhanced glycolysis as a means to generate ATP, resulting in a transfer in the primary location of energy generation from the mitochondria to the cytosol (DeBerardinis and Chandel, 2016; Kim et al., 2020), which is called the Warburg effect (Kato et al., 2018; Duan et al., 2023). The metabolic changes described herein perform an essential function in facilitating the provision of energy and serving as a source of building blocks, thereby significantly contributing to the evolution of tumors and the development of resistance to chemotherapy (Montal et al., 2015; Shukla et al., 2017; Romani et al., 2022). A rising body of studies suggests that the suppression of glycolysis is a highly effective means of inducing death in multidrug-resistant cells. This finding suggests that directing efforts on glycolysis could serve as a promising and innovative technique for overcoming multidrug resistance (Xu et al., 2005; Li et al., 2022).

Nucleolar protein 58 (NOP58) ribonucleoprotein, performs a crucial function in the maintenance of cellular homeostasis by serving as a critical component for multiple box C/D short nucleolar RNAs (Zhou et al., 2017; Ojha et al., 2020; Abel et al., 2021). Previous studies have provided evidence indicating that NOP58 has the capacity to enhance the stability of family with sequence similarity 83 member A (FAM83A) mRNA, hence facilitating the advancement of tumor growth (He and Yu, 2019), while the suppression of NOP58 was demonstrated to impede the proliferation of cancer cells and reduce their oncogenic properties (Su et al., 2014). Moreover, NOP58 has the ability to directly engage with lncRNA ZFAS1, hence facilitating its functional activation, and this complex ultimately contribute to CRC tumorigenesis (Wu et al., 2020). While the previous research has shown the molecular mechanism of NOP58, its potential involvement in chemoresistance is still unknown.

Hence, the primary purpose of the current research was to characterize the differences between the 5-FU resistant cell lines and wide type on the proteomic level. Here, we showed a metabolic reprogramming mechanism that enables CRC cells to develop resistance to 5-FU treatment. The expression of NOP58 was observed to be significantly increased in 5-FU resistant cell and silencing NOP58 increased the sensitivity to 5-FU through regulation of glycolysis. The current study purpose is to investigate the effect of NOP58 on colon cancer cells that exhibit resistance to 5-FU, thereby offering a novel theoretical framework for potential therapeutic strategies for individuals with 5-FU resistance.

2 Materials and methods

2.1 5-FU resistance cell model construction

HCT116 and Lovo cell lines underwent a progressive increase in 5-FU concentration, ranging from 1 μg/mL to 10 μg/mL, in a continuous manner. The assessment of resistance to 5-FU was conducted at various doses through the determination of the IC50 utilizing MTS assays. The resistance to 5-FU was operationally defined as a resistance index (RI, IC50 of the 5-FU-R cells/IC50 of the WT cells) > 5. This definition was applied following a 6-month period of 5-FU treatment.

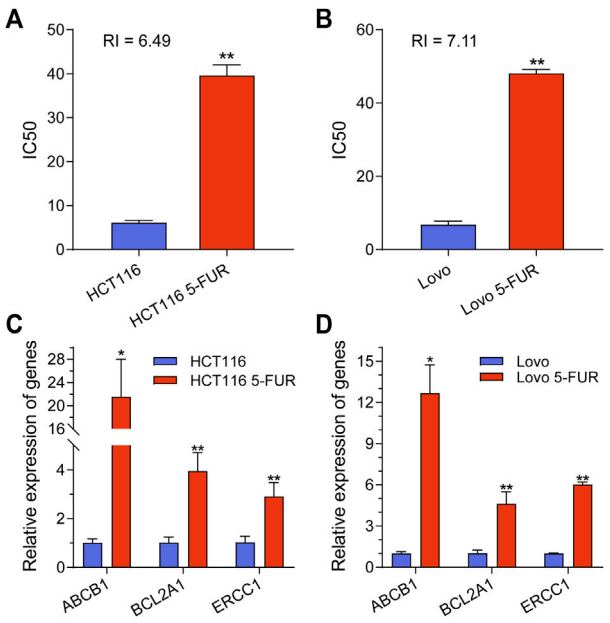

FIGURE 1
Construction and classification of 5-FU-resistant CRC cell lines. **(A)** IC50 values for parental and 5FU-resistant cells of HCT116 cell. **(B)** IC50 values for parental and 5FU-resistant cells of Lovo cell. **(C)** The mRNA expression level of ABCB1, BCL2A1 and ERCC1 in parental and 5-FU-resistant cells of HCT116. **(D)** The mRNA expression level of ABCB1, BCL2A1 and ERCC1 in parental and 5-FU-resistant cells of Lovo. $^{**}p < 0.01$, $^{*}p < 0.05$.

2.2 MTS assay

Cells that had undergone pre-treatment under different circumstances were collected and subsequently inoculated within 96-well plates at 4000 cells per well, reaching the final volume of 100 μL per well. Following a 12 h incubation period, 100 μL of the specified concentration of 5-FU was introduced into the wells of 96-well plates. Following a 48 h treatment period, a volume of 13 μL of MTS solution (Sigma; #75-79-6) solution (2 mg/mL) was introduced to all wells and subsequently underwent incubation for a duration of 2 h at 37°C. Using a microplate reader, absorbance was measured at 492 nm.

2.3 Mass spectrometry-based proteome profiling

2.3.1 Protein isolation and digestion

The RIPA buffer (Thermo Fisher Scientific, 89901) was introduced to both the 5-FU-R and WT cells in order to facilitate protein extraction. Subsequently, sonication was

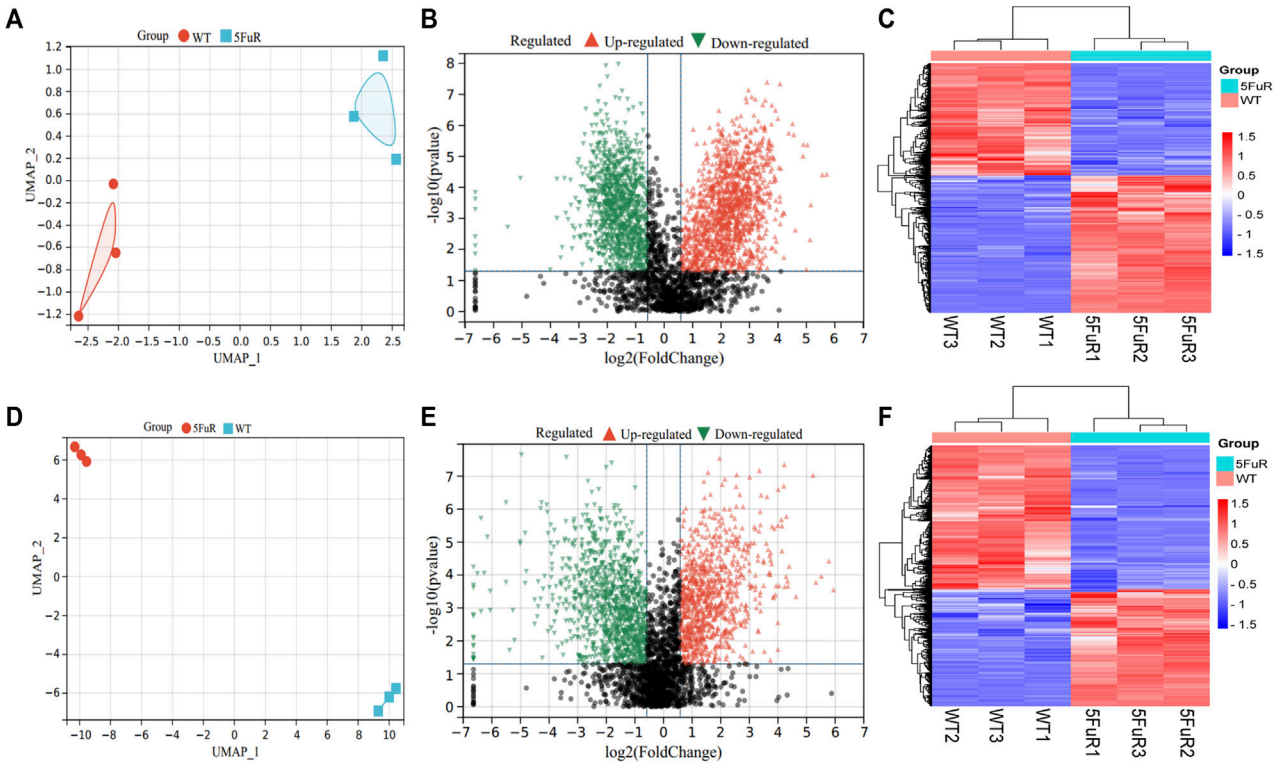

FIGURE 2
Differential expressed genes in 5-FU resistant CRC cells. **(A, D)** UMAP analysis of the proteomic data from parental and 5FU-resistant cells of HCT116 **(A)** and Lovo **(D)**. **(B, E)** Volcano plots showing the proteins with a statistically significant decreasing and increasing abundance in HCT116-5FuR **(B)** and Lovo-5FuR **(E)**. **(C, F)** The heatmap indicates the proteins that are expressed differentially between the parental and 5FU-resistant cells of the HCT116 cell line **(C)** and Lovo **(F)** cells.

performed for a total of 6 cycles, with each cycle consisting of 5 s on and 5 s off. The proteins were subsequently subjected to denaturation at 95°C for 2 min. The insoluble fragment was separated from the solution using centrifugation at 12000 *g* for a duration of 10 min. The supernatant was then used in the proteomic investigation. The protein content was determined utilizing BCA kit (Thermo Fisher Scientific, 23227).

The protein digesting process employed the filter-aided sample preparation (FASP) approach. In this study, proteins have been transferred to 10 kDa centrifugal filter tubes (Thermo Fisher Scientific, 88513) and subjected to a series of treatments. Firstly, the disulfide bond was disrupted by incubating the proteins via 50 mM DTT (Sigma-Aldrich, D9163) in 300 μL of UA buffer (8 M urea (Sigma-Aldrich, U0631) in 0.1 M Tris-HCl (Thermo Fisher Scientific, 15506017), pH 8.5) at 37°C for 30 min. Afterwards, the proteins were alkylated by treating them via 50 mM IAA (Sigma-Aldrich, I6125) in 300 μL of UA buffer for a duration of 30 min within the absence of light. Following this, the proteins were washed three times with 300 μL of UA buffer, and then underwent hydration two times using 300 μL of 50 mM NH4HCO3(Sigma-Aldrich, 11213). The aforementioned stages were subjected to centrifugation at 12000 *g* at a temperature of 25°C. The proteins were subjected to enzymatic digestion using trypsin (Thermo Fisher Scientific, 90058) at 1:100 (w/w) in 50 mM NH4HCO3, and the digestion process was carried out at 37°C for 18 h. Following the process of digestion, the elution of peptides was achieved through the utilization of centrifugation. Following that, the peptides underwent purification and extraction utilizing custom-made C18 Tips (Empore, 98060402173) in a solution consisting of 80% ACN and 2% TFA. The peptides underwent lyophilization and were subsequently acidified using a 0.1% FA solution. Using the BCA peptide quantification kit (Thermo Fisher Scientific, 23275), the concentration of the peptide was measured.

2.3.2 Proteomic analysis

In order to perform proteomic analysis, the peptides (1 μg each specimen) were introduced into a nanoflow HPLC Easy-nLC1200 system (Thermo Fisher Scientific), utilizing a 90 min LC gradient at 300 nL/min. 0.1% (v/v) FA was diluted in H2O to prepare Buffer A, while Buffer B was prepared by diluting 0.1% (v/v) FA in 80%ACN. The gradient was established according to the following parameters: 2%–8% B in 1 min; 8%–28% B in 60 min; 28%–37% B in 14 min; 37%–100% B in 5 min; 100% B in 10 min. The Q Exactive HF mass spectrometer (Thermo Fisher Scientific) was utilized for the proteomic analyses. In positive ion mode, the spray voltage was set at 2100 V, while the temperature of the ion transfer tube was kept constant at 320°C. The data-dependent acquisition method was implemented via Xcalibur software, specifically utilizing the profile spectrum data type. The MS1 full scan was configured with 60000 at m/z 200, AGC target 3e6, and maximum IT 20 ms using the orbitrap mass analyzer

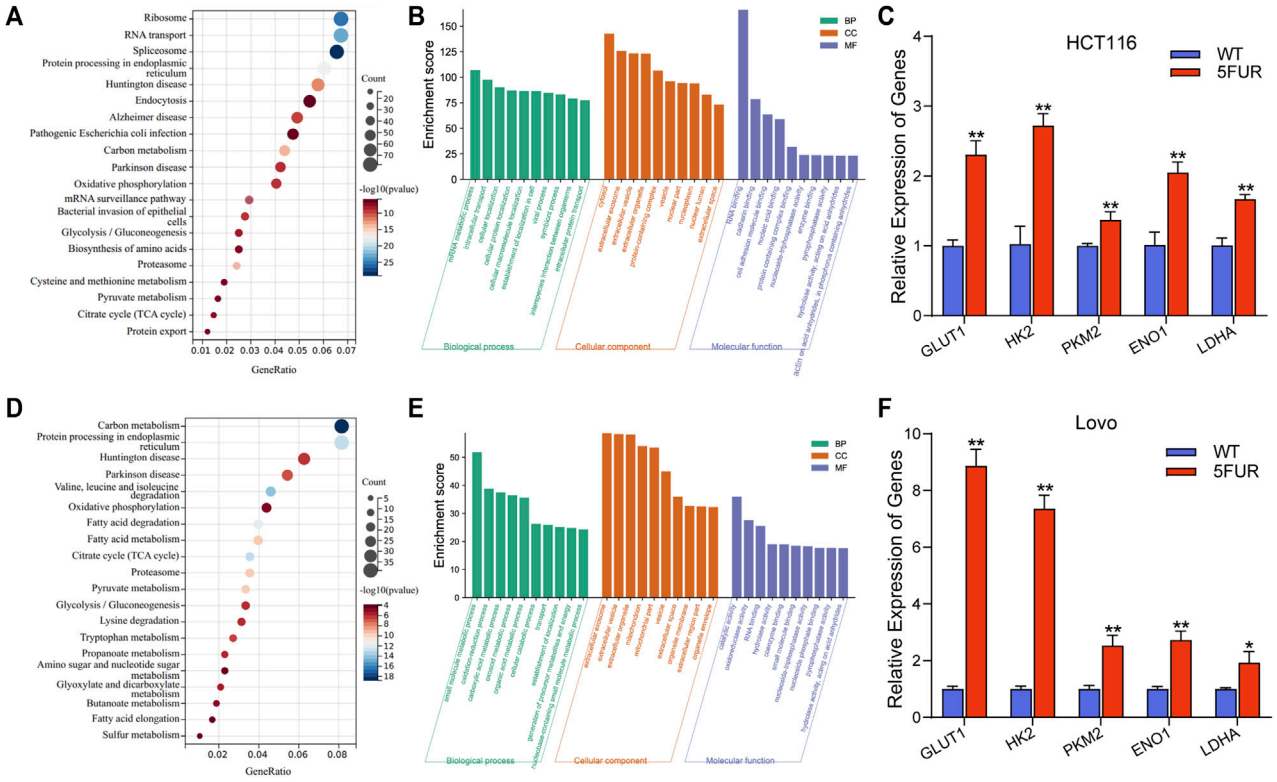

FIGURE 3
Resistant cells display increased glycolysis. **(A)** KEGG showing the significant enrichment of the top 20 pathways in HCT116-5FuR. **(B)** GO analysis of differentially expressed terms in parental and 5FU-resistant HCT116. **(D)** KEGG showing the significant enrichment of the top 20 pathways in Lovo-5FuR. **(E)** GO analysis of differentially expressed terms in parental and 5FU-resistant Lovo. **(C, F)** The mRNA expression of glycolytic enzymes HK2, PKM2, GLUT1, LDHA and ENO1 in parental and 5FU-resistant cells of HCT116 **(C)** and Lovo **(F)**. $^{**}p < 0.01$, $^{*}p < 0.05$.

(350–1500 m/z). This was subsequent by the acquisition of "top 20" MS2 scans by HCD fragmentation, which were performed at 15000 atm/z 200, an AGC target of 1e5, and a maximum IT time of 45 ms. 110,0 m/z was determined to be the initial mass of the MS2 spectrum. The isolation window was established at a value of 1.6 m/z. The experimental conditions included a normalized collision energy (NCE) of 27% and a dynamic exclusion duration of 45 s. Precursors possessing a charge of 1, 8, and >8 was omitted from the MS2 analysis.

2.4 Database searching of MS data

The initial processing of data was conducted in Proteome Discoverer 2.2, utilizing an ion-current-based label-free quantification approach. Using Sequest HT with a maximum mass tolerance of 10 ppm for the parent ion and a fragment tolerance of 0.02 Da for tandem mass spectrometry, peptides were identified. The UniProtSwissProt Human canonical database (downloaded on Uniprot, 2022) was utilized to conduct a comprehensive search of all available data. The static modification of cysteines through carbamido methylation was taken into consideration, whereas the potential variable modifications of protein N-termini through acetylation and methionine through oxidation were examined. The application of false discovery rate calculations was employed to conduct multiple testing correction. The false discovery rate threshold for both peptide spectral matches was set at 1%, (which were determined utilizing Percolator), and the peptide group levels. By performing pairwise analysis on individual peptides, the quantification ratios of each peptide were determined and afterwards calculating the average for both peptide groups and protein levels. The determination of significance was thereafter conducted through the utilization of analysis of variance, which was in accordance with the assessment of peptide background at the peptide group and protein levels.

2.5 Knockdown of NOP58

GenePharma (Suzhou, China) was the supplier of the small interfering RNA (siRNA) and negative control (NC) targeting NOP58 (the sequence of siRNA in Supplementary Table S1). The transfection procedure was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer guidelines.

2.6 Total RNA extraction and real-time quantitative PCR (RT-qPCR)

The total RNA extraction was conducted utilizing Trizol reagent (Invitrogen) following the manufacturer's guidelines. The total RNA that was obtained was quantified using a Nanodrop spectrophotometer,

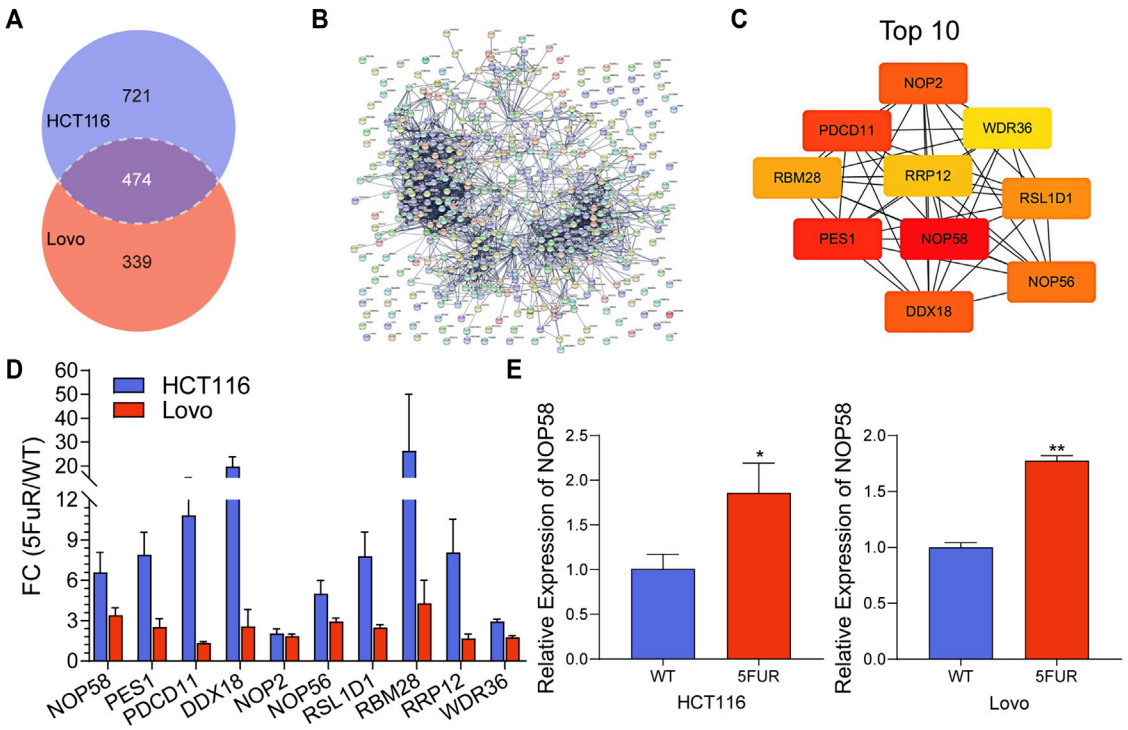

FIGURE 4
NOP58 is associated with CRC resistance. **(A)** The Venn diagram displayed the proteins that were found to be shared across the differentially expressed proteins in the HCT116-5FuR and Lovo-5FuR. **(B)** PPI network analysis of the overlapped proteins in **(A)**. **(C)** Top 10 overlapping protein hub genes in **(B)**. **(D)** The actually fold change expression of hub genes in HCT116-5FuR and Lovo-5FuR proteomic data. **(E)** qPCR showing the relative expression of NOP58 in parental and 5FU-resistant cells of HCT116 and Lovo. *$p < 0.05$.

and subsequently converted into cDNA using reverse transcription. The relative quantification of mRNA was conducted using the $2^{-\Delta\Delta Ct}$ approach, with the gene Actin functioning as an internal reference. The sequence of primers is shown in the Supplementary Table S2.

2.7 Statistical analysis

The experimental data is reported as the mean ± standard deviation (SD) obtained from three separate and independent studies. The data were subjected to analysis utilizing SPSS 13.0. One-way ANOVA or the Student's t-test was utilized in the statistical analysis. Statistical significance was established at a *p*-value <0.05.

3 Results

3.1 Construction and classification of 5-FU-resistant CRC cell lines

In order to examine the underlying mechanism of 5-FU resistance within CRC, two CRC cell line models (HCT116 and Lovo) with acquired resistance to 5-FU were created. During the course of roughly 8 months, wild-type (WT) cells were exposed to a cultivation method in which the concentration of 5-FU was gradually raised. The half maximum inhibitory concentration (IC50) was determined, revealing that 5-FU resistance (5-FU-R) cells exhibited resistance indexes ranging from 5 to 8 following 5-FU induction. These resistance indexes were assessed subsequent to a 2 weeks period of drug removal (Figures 1A,B). Moreover, drug-resistant associated proteins were detected. The results showed that ABCB1, BCL2A1, and ERCC1 were significantly increased in 5-FU-R CRC cells compared to WT cells (Figures 1C,D). The collective results of this study demonstrate that 5-FU-R CRC cells were successfully established *in vitro*.

3.2 Differential genes in 5-FU resistant CRC cells

To illustrate the mechanism of 5-FU resistance in CRC, the HCT116-5FuR and Lovo-5FuR and their corresponding parental cell lines underwent analysis using protein mass spectrometry. In the HCT116 and Lovo cell lines, a total number of 3683 and 3402 proteins were respectively discovered. Visualization of the abundance of proteins by UMAP (Methods) differentiated the proteome profiles, which clearly discriminated the proteomes of the WT and resistance (Figures 2A,D). In accordance with the outcomes obtained from protein mass spectrometry analysis, volcano plot and heatmap revealed 2175 proteins were significantly differentially expressed in HCT116-5FuR, in which 1195 underwent

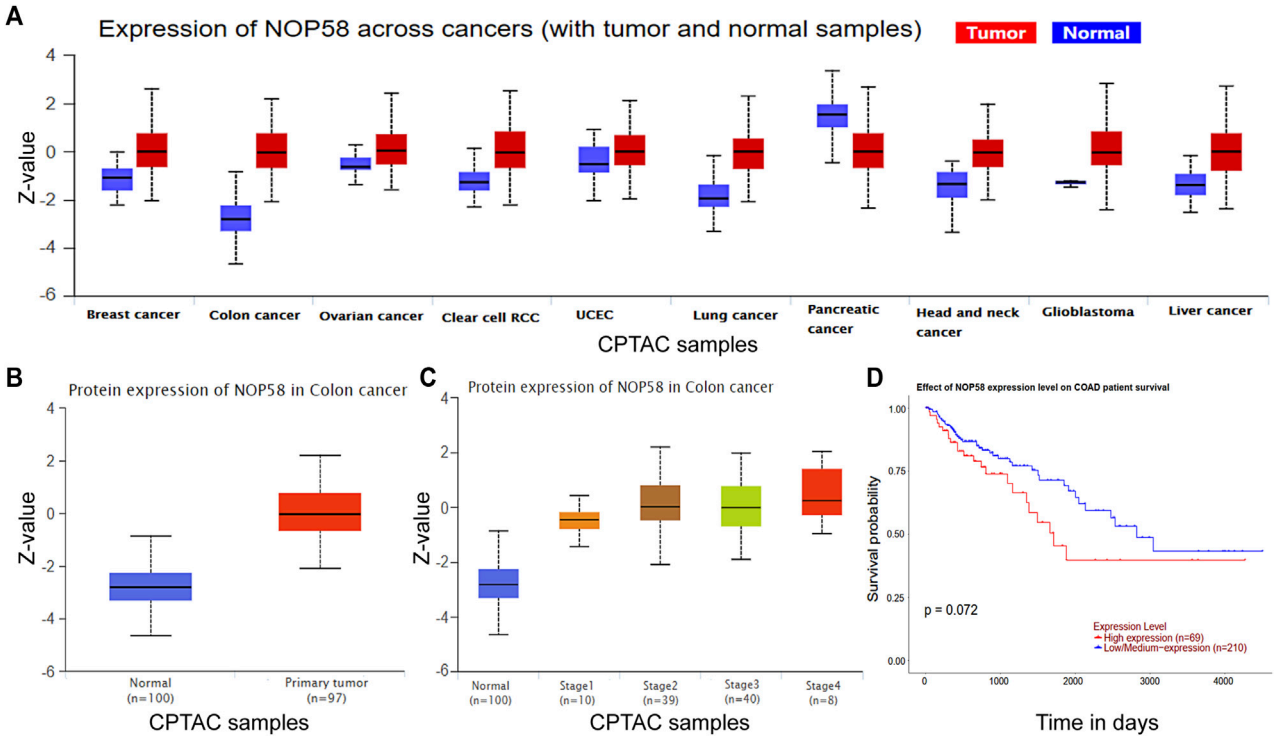

FIGURE 5
NOP58 is correlated with CRC development. **(A)** The CPTAC dataset showing the expression of NOP58 across cancers. **(B)** NOP58 protein expression in colon cancer. **(C)** NOP58 protein expression in different stages of colon cancer. **(D)** Kaplan–Meier survival curves of patients with COAD according to NOP58 expression.

upregulation and 980 underwent downregulation (|fold change| >1.5, $p < 0.05$) (Figures 2B,C). Moreover, 813 highly expressed proteins and 998 downregulated proteins in Lovo-5FuR underwent screening (|fold change|>1.5, $p < 0.05$) (Figures 2E,F).

3.3 Resistant cells display increased glycolysis

The proteins that exhibit variable expression possess a range of molecular and biological functions, as discovered by the examination of KEGG and GO databases. The proteins in the HCT116-5FuR exhibited a notable enrichment primarily in metabolism, including Oxidative phosphorylation, Glycolysis/Gluconeogenesis and Citrate cycle (TCA cycle) (Figures 3A,B). Meanwhile, we found that the proteins in Lovo-5FuR were primarily enriched in Glycolysis/Gluconeogenesis (Figures 3D,E). Based on prior investigations, there exists a correlation between drug resistance and glycolysis. Consequently, our study focused on the examination of glycolysis in HCT116-5FuR and Lovo-5FuR cells. qPCR was conducted in order to ascertain Hexokinase 2 (HK2), Pyruvate kinase M2(PKM2), Glucose transporter type1 (GLUT1), lactate dehydrogenase A (LDHA), and α-Enolase (ENO1) expression level, which are recognized as pivotal enzymes involved in the glycolytic pathway. According to Figures 3C,F, the key enzymes were highly expressed in 5-FU resistant CRC cells, contrasted to the matched WT cells. The results of our study indicate that there was an observed elevation in glycolytic activity within colon cancer cells that had developed resistance to 5-FU treatment.

3.4 NOP58 is associated with CRC resistance

In addition, 474 proteins overlapped between the upregulated proteins in HCT116-5FuR and Lovo-5FuR (Figure 4A). To further investigate the probable pathways, a study of protein-protein interactions (PPI) network was conducted on the subset of 474 proteins using the STRING database (Figure 4B). In addition, a comprehensive analysis was conducted to identify the top 10 hub genes, among which NOP58 emerged as the highest ranking gene (Figure 4C). We integrated the expression abundance of the top 10 hub genes in proteome of HCT116-5FuR and Lovo-5FuR (Figure 4D). In order to investigate the potential implications of NOP58 in the progression of drug resistance in colon cancer cells, we conducted an analysis to determine the expression levels of NOP58 in both HCT116-5FuR and Lovo-5FuR cell lines. According to Figure 4E, the mRNA levels of NOP58 were increased in HCT116-5FuR cells compared with HCT116 cells and the Lovo-5FuR cells demonstrated a notable elevation in mRNA levels of NOP58 in comparison to the Lovo cells. Taken together, NOP58 plays a vital role in CRC resistance.

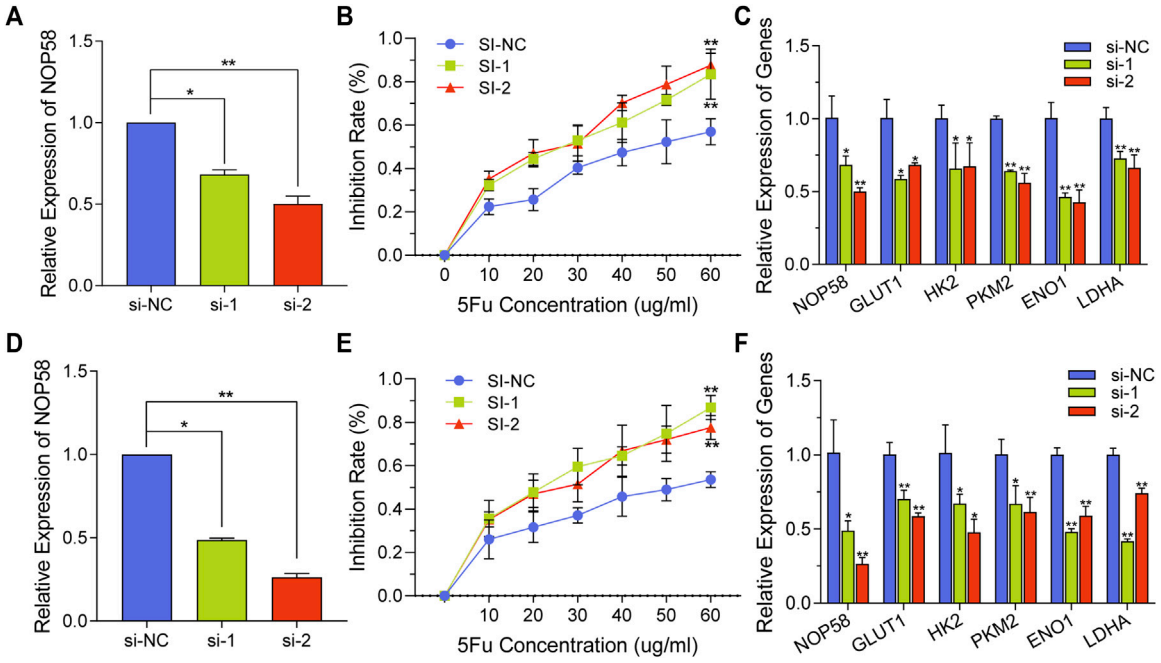

FIGURE 6
NOP58 knockdown overcomes the resistance to 5-FU by regulating glycolysis. **(A, D)** qPCR analysis demonstrating NOP58 expression level within HCT116-5FuR **(A)** and Lovo-5FuR **(D)** after the NOP58 siRNA or negative control siRNA (NC) were transfected. **(B, E)** The IC50 extents of HCT116-5FuR **(B)** and Lovo-5FuR **(E)** cells response to 5-Fu with or without NOP58 suppression. **(C, F)** The mRNA expression of glycolytic enzymes HK2, PKM2, GLUT1, LDHA and ENO1 in HCT116-5FuR **(C)** and Lovo-5FuR **(F)** transfected with negative control siRNA or NOP58 siRNA. $^{**}p < 0.01$, $^{*}p < 0.05$.

3.5 NOP58 is correlated with CRC development

We studied the NOP58 expression on series of cancers by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (https://proteomics.cancer.gov/programs/cptac) in the UALCAN web, which revealed that NOP58 underwent overexpression across colon cancer, clear cell renal cell carcinoma (RCC), breast cancer, lung cancer, glioblastoma and liver cancer, head and neck cancer (Figure 5A). Furthermore, according to the CPTAC database, an elevation in the expression of NOP58 protein has been identified in CRC tissues in comparison to normal colorectal tissues (Figures 5B, C). Additionally, a strong positive association was seen between elevated levels of NOP58 and decreased overall survival rates among patients diagnosed with CRC (Figure 5D). Overall, NOP58 promoted the progression of colorectal cancer.

3.6 NOP58 knockdown overcomes the resistance to 5-FU by regulating glycolysis

To investigate the impact of NOP58 on drug resistance, si-NOP58 or si-Con was transfected into HCT116-5FuR and Lovo-5FuR cells. Following a 48 h incubation period, qPCR was conducted, revealing that the introduction of si-NOP58 successfully suppressed the expression of NOP58 in both HCT116-5FuR and Lovo-5FuR cell lines (Figures 6A,D). HCT116-5FuR and Lovo-5FuR cells with or without transfections were subjected to incubation with varying amounts of 5-FU (10, 20, 30, 40, 50, 60 μg/mL) for 48 h. The IC50 value for 5-FU has been reduced in HCT116-5FuR and Lovo-5FuR underwent transfection with si-NOP58 in comparison to that in cells transfected via si-Con (Figures 6B,E). Furthermore, we conducted an assessment of the impact of si-NOP58 on the glycolytic pathway in CRC cells that had developed resistance to 5-FU. According to Figures 6C,F, si-NOP58 treatment caused decreased expression level of HK2, GLUT1, PKM2, ENO1, and LDHA. The findings of the study demonstrated that the inhibition of NOP58 expression resulted in a suppression of glycolysis in both HCT116-5FuR and Lovo-5FuR cells. Additionally, this silencing of NOP58 expression resulted in an elevated sensitivity to 5-FU.

4 Discussion

For almost 6 decades, 5-FU has served as the primary medication for single-drug and multi-drug chemotherapy, demonstrating its efficacy as a systemic treatment for the management of advanced and metastatic CRC (Correale et al., 2004; Rosmarin et al., 2014). However, a specific number of patients still acquired 5-FU resistance (Del Rio et al., 2007). Hence, the identification of ways to overcome resistance to 5-FU poses significant hurdles in the realm of clinical practice. Multiple pathways of resistance to 5-FU have been documented, encompassing alterations across the proportion of drug influx or efflux (Mansoori et al., 2017), intratumor heterogeneity (Mansoori et al., 2017), epigenetic

factors (Kreso et al., 2013), tumor microenvironment (Gatenby et al., 2010), and 5-FU metabolic enzymes (Longley et al., 2003). The current investigation aims to provide a comprehensive understanding of the fundamental mechanism behind 5-FU resistance in CRC by examining its role in promoting glycolytic metabolism.

NOP58 was previously reported to contribute to maturation, stability, and localization of snoRNAs (Liang et al., 2019). We found that NOP58 was significantly upregulated within 5-FU resistant CRC cells. In addition, the downregulation of NOP58 expression led to elevated sensitivity of CRC cells to 5-FU, and decreased the expression of glycolytic enzymes. The implementation of this procedure may have had an impact on the responsiveness of cells treated with 5-FU chemotherapy.

An essential component of metabolic reprogramming in cancers is the heightened reliance on glycolysis as a means of energy production (DeBerardinis and Chandel, 2016). Furthermore, the high rate of glycolytic flow not only serves as an energy source but also offers a diverse range of raw materials for biosynthetic processes (Montal et al., 2015). The phenomenon of increased aerobic glycolysis performs a significant function in the proliferation of tumors, as it confers cancer cells with advantageous growth capabilities and resistance to therapeutic interventions (Shukla et al., 2017). The enzymes that perform a regulatory function in glycolysis have also been linked to the promotion of drug resistance across tumors (Xu et al., 2005; Bhattacharya et al., 2016).

The phenomenon of drug resistance in CRC cells towards vincristine and oxaliplatin can be effectively addressed by the genetic manipulation of polypyrimidine tract binding protein 1 (PTBP1) by means of knockout techniques (Cheng et al., 2018), a key regulator of the glycolytic pathway. The enzyme HK2 plays a crucial role in initiating the primary step that regulates the rate of glucose metabolism. Furthermore, the compound 2-DG, which acts as an inhibitor of HK2, has demonstrated the ability to counteract drug resistance in several *in vitro* models (Shi et al., 2019). The suppression of MCT1 significantly enhanced the chemosensitivity of human osteosarcoma cells (Zhao et al., 2014). The findings suggests that the suppression of glycolysis by the targeting of key enzymes involved in the glycolytic process could serve as a promising treatment strategy for effectively overcoming medication resistance across various cases.

In general, several techniques centered around molecular targets were suggested in order to counteract 5-FU resistance in CRC and enhance the efficacy of 5-FU treatment. Nevertheless, these endeavors have not yielded favorable outcomes. We demonstrated that NOP58 induced chemoresistance through glycolysis pathway. The current investigation offers a novel therapeutic strategy for the precise management of CRC individuals suffering from resistance to 5-FU therapy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

FW: Conceptualization, Data curation, Formal Analysis, Investigation, Writing–original draft. BY: Conceptualization, Supervision, Writing–original draft. QY: Investigation, Methodology, Writing–original draft. GaW: Investigation, Methodology, Writing–original draft. BL: Investigation, Methodology, Writing–original draft. GG: Conceptualization, Data curation, Validation, Writing–original draft. HW: Data curation, Formal Analysis, Writing–original draft. HS: Investigation, Methodology, Writing–original draft. SL: Conceptualization, Writing–review and editing. CM: Conceptualization, Writing–review and editing. XJ: Conceptualization, Visualization, Writing–review and editing. GiW: Funding acquisition, Visualization, Writing–review and editing. BC: Conceptualization, Funding acquisition, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1295422/full#supplementary-material

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