MULTIDRUG RESISTANCE IN CANCER: PHARMACOLOGICAL STRATEGIES FROM BASIC RESEARCH TO CLINICAL ISSUES

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MULTIDRUG RESISTANCE IN CANCER: PHARMACOLOGICAL STRATEGIES FROM BASIC RESEARCH TO CLINICAL ISSUES

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More than 40 years ago, the observation that doxorubicin-resistant tumor cells were cross-resistant to several structurally different anticancer agents was the first step in the discovery of P-glycoprotein (P-gp).

P-gp belongs to the superfamily of ATP-binding cassette (ABC) transporters; its overexpression has become a therapeutic target for overcoming multidrug resistance in tumors. However, P-gp is also expressed in cells of normal tissues where it plays a physiological role, by protecting them from the toxic effects of xenobiotics. Also, ABCB1 gene polymorphisms may influence the response to anticancer drugs substrate of P-gp.

Several strategies to overcome P-gp tumor drug resistance have been suggested.

P-gp 'circumvention' is the most explored and is based on the coadministration of anticancer agents and pump inhibitors (P-gp modulators). Despite the positive findings obtained in preclinical studies, results of clinical trials are not yet successful and clinical research is still ongoing.

Other investigational approaches have been studied (e.g. P-gp targeting antibodies, use of antisense strategies or transcriptional regulators targeting ABCB1 gene expression) but their use is still circumscribed to the preclinical setting.

A further approach is represented by the encapsulation of P-gp substrate anticancer drugs into liposomes or nanoparticles. This strategy has shown higher efficacy in tumor previously treated with the free drug. The reasons explaining the increased efficacy of liposomal/nanoparticle-based drugs in Pgp-overexpressing tumors include the coating with specific

surfactants, the composition changes in the plasma membrane microdomains where P-gp is embedded, the direct impairment of P-gp catalytic mechanisms exerted by specific component of the liposomal shell, but are not yet fully understood. A second strategy to overcome P-gp tumor drug resistance is represented by exploiting the P-gp presence. Actually, P-gp-overexpressing cells show increased sensitivity (collateral sensitivity) to some drugs (e.g. verapamil, narcotic analgesics) and to some investigational compounds (e.g. NSC73306). P-gp-overexpressing cells are hypersensitive to reactive oxygen species, to agents perturbing the energetic metabolic pathways, changing the membrane compositions, reducing the efflux of endogenous toxic catabolites. However, the mechanisms explaining collateral sensitivity have not been fully elucidated.

Another approach to exploit P-gp is represented by ABCB1 gene transfer to transform bone marrow progenitor cells into a drug resistant state which may allow conventional or higher doses of anticancer drug substrates of P-gp to be administered safely after transplantation.

More recently the development and introduction in the clinics of anticancer drugs which are not substrates of P-gp (e.g. new microtubule modulators, topoisomerase inhibitors) has provided a new and promising strategy to overcome P-gp tumor drug resistance (P-gp 'evasion').

This 'research topic' issue aims at exploding the above mentioned matters, in particular by:

– retracing the history of the first researches on P-gp
– describing the physiological role of P-gp
– describing the molecular basis, structural features and mechanism of action of P-gp
– describing diagnostic laboratory methods useful to determine the expression of P-gp and its transporter function
– describing strategies to overcome tumor drug resistance due to P-gp and other ABC transporters
– indicating novel approaches to overcome P-gp multidrug resistance

ranging from basic research studies to pre-clinical/clinical studies.

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EDITORIAL
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Editorial: Multidrug resistance in cancer: pharmacological strategies from basic research to clinical issues

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Keywords: cancer, multidrug resistance, P-glycoprotein, reversing strategies

Tumor drug resistance is the leading cause of chemotherapy treatment failure. One of the more relevant mechanisms is represented by multidrug resistance (MDR) that leads to a reduced cellular accumulation of drugs due to increased efflux out of cells by the overexpression of several ATP-dependent efflux pumps or transporters. These proteins belong to the ATP-binding cassette (ABC) family and the most studied of them is P-glycoprotein (P-gp). Interestingly, P-gp acts as an efflux pump for various structurally unrelated anticancer agents (1, 2).

The aim of this issue, that includes nine contributes, is to highlight mechanistic aspects of the P-gp functions, provide information on the development of *in vitro* MDR tumor models, and describe potential strategies to overcome MDR.

Sharom (3) highlights the involvement of P-gp in a complex relationship with its lipid environment, which modulates the behavior of its substrates and many functions of the protein (e.g., ATP hydrolysis, drug binding, drug transport). Recently, some important principles governing P-gp–lipid and substrate–lipid interactions, and how these affect drug binding and transport, have been shown. In some cells, P-gp is associated with cholesterol-rich microdomains, which may modulate its functions. It is well known that the protein has also been proposed to operate as a drug translocase or flippase, moving its substrates from the inner to the outer leaflet of the membrane. The ability of substrates and modulators to interact with P-gp may depend on their ability to flip–flop between membrane leaflets. Membrane fluidizers and surfactants may reverse drug resistance, likely via an indirect mechanism.

On the other hand, Fu (4) introduces an interesting issue describing the localization of P-gp not only on the plasma membrane but also in many intracellular compartments (i.e., endoplasmic reticulum, Golgi, endosomes, lysosomes). P-gp can rapidly traffic and recycle among the intracellular compartments and between cellular organelles and plasma membrane, mainly via the indirect endosomal pathway. A role of cellular factors, such as Rab GTPases, in P-gp trafficking and recycling is also suggested.

McDermott et al. (5) provide a very detailed guide to the decision-making process for the development and ongoing maintenance of drug-resistant cancer cell lines. Relevant issues, such as the choice of the parental cell line, the strategy of cell exposure to anticancer drugs that has to mimic chemotherapy that patients receive in the clinical practice, as well as dose optimization, are discussed. Interestingly, McDermott et al. explore the heterogeneity of drug-resistant cell lines in relation to P-gp highlighting the complexity in developing P-gp resistant tumor models.

Until now, the scientific history of MDR-reversing strategies has been characterized by a repeated series of failures: indeed, the pharmacological inhibitors of the ABC transporters, such as P-gp, MDR-related proteins (MRPs), breast cancer resistance proteins (BCRP), have not reached the sufficient specificity and efficacy to be translated into the clinical practice.

Recently, however, the design of ABC transporters inhibitors has been progressively more refined, e.g., the creation of small libraries of compounds starting from versatile scaffolds or the use of dual

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effect drugs or multitarget drugs (i.e., chemotherapeutic drugs that are chemically conjugated with an ABC transporters inhibitor) can increase the selectivity and potency of transporter inhibitors and modulators, as described in the topic of Zinzi et al. (6). Although very innovative, these approaches do not solve the crucial challenge in the field of MDR-reversing strategy, i.e., achieving the maximal efficacy and selectivity against MDR cells. Recent high-throughput screenings of pharmacological libraries identified specific compounds, such as compounds increasing the generation of reactive oxygen species (ROS) and depleting cells of the anti-oxidant metabolite glutathione (GSH), which were unexpectedly more effective in ABC transporter-overexpressing cells than in ABC transporter-negative cells. This phenomenon is known as collateral sensitivity (7). The oxidative-mediated collateral sensitivity is, however, a multifaceted event in resistant tumors. In this topic, Gauthier et al. (8) demonstrate, for instance, that the GSH depletion induces apoptosis in chemoresistant cells overexpressing MRP1, but not in resistant cells overexpressing BCRP. This means that although MDR cells are generally more damaged than chemosensitive cells by oxidative stress, the degree of this damage is highly dependent on the spectrum of ABC transporters expressed by each tumor. Increasing the GSH efflux via ABC transporters is not the only strategy that can increase ROS in MDR cells. Changing the activity of redox sensitive factors that control anti-oxidant enzymes, phase 2 detoxifying enzymes, and stress response proteins, such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), apurinic-apyrimidinic endonuclease 1/redox factor 1 (APE-1/Ref-1), and forkhead box O (FoxO), can achieve the same goal. Interestingly, these transcription factors not only control the redox balance but also regulate the expression of specific ABC transporters as reported by Polimeni and Gazzano (9). Therein, their targeting may result in pleiotropic chemosensitizing benefits against MDR tumors.

A second approach to improve the selectivity of chemosensitizing agents against MDR cells is the use of nanoparticles, for the active targeting of chemotherapeutic drugs, chemosensitizing agents or siRNA within the resistant tumor as proposed in this topic by Conde et al. (10). Several open questions need to be solved before the translation of nanoparticle-based approaches into the clinical practice, such as the biocompatibility and the long-term safety of nanoparticles. Once the first phase I–phase II clinical trials and observational studies have been concluded, these issues will be clarified and nanoparticles may become useful tools also for the delivery of collateral sensitivity inducers or agents targeting redox sensitive factors within MDR cells.

Cancer stem cells (CSCs) exhibit several mechanisms of resistance against anticancer drugs that are mainly represented by the expression of ABC transporters and activation of different signaling pathways (e.g., Wnt/β-catenin signaling, Hedgehog, Notch, Akt/PKB). Thus, compounds able to modulate MDR on CSC membranes could induce cytotoxicity in these cells, as described by Zinzi et al. (11).

Two models have been suggested to explain the connection between MDR and CSCs: the "original" MDR model according to which, after exposure to the chemotherapeutic agent, only CSCs expressing ABC transporters repopulate the tumor, and the "acquired" MDR model according to which after chemotherapy, only CSCs survive and this population of survival cells, after mutations, originates new and more aggressive drug-resistant cell phenotypes. Thus, the combination of CSC targeting agents with novel or conventional cytotoxic drugs could lead to a potentiated effect. An innovative multimodal strategy, i.e., an approach in which specific CSC targeting drugs exert simultaneously the ability to circumvent tumor drug resistance (by ABC transporter modulation) and to exert cytotoxic activity toward CSCs and the corresponding differentiated tumor cells, may be hypothesized.

Targeting ABC transporters is a challenge not only limited to tumors cells but it also involves the tumor microenvironment in specific districts: as demonstrated in this topic by Adkins et al., breast cancer cells metastasizing within central nervous system are rich of P-gp and are surrounded by a complex vasculature expressing P-gp as well (12). This situation, which is common to other metastatic cancers, creates "MDR niches," making harder the full eradication of resistant cells and easier the tumor relapse. Finding compounds overcoming P-gp activity in both the tumor cell and the tumor microenvironment-associated cells is a future open challenge in the field of MDR-reversing strategies.

This topic has reviewed pharmacological strategies to overcome MDR. Fighting MDR involves multiple skills and know-how, including the ability to develop suitable *in vitro* drug-resistant tumor models, the understanding of the ABC transporter functions, the use of medicinal chemistry, the production of new nanomaterials, the analysis of the biochemical features of MDR cells, and the management of clinical trials. Such multidisciplinary approach is mandatory to open new perspectives against chemoresistant tumors.

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REVIEW ARTICLE
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Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: its role in modulating protein function

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Multidrug resistance in cancer is linked to expression of the P-glycoprotein multidrug transporter (Pgp, ABCB1), which exports many structurally diverse compounds from cells. Substrates first partition into the bilayer and then interact with a large flexible binding pocket within the transporter's transmembrane regions. Pgp has been described as a hydrophobic vacuum cleaner or an outwardly directed drug/lipid flippase. Recent X-ray crystal structures have shed some light on the nature of the drug-binding pocket and suggested routes by which substrates can enter it from the membrane. Detergents have profound effects on Pgp function, and several appear to be substrates. Biochemical and biophysical studies *in vitro*, some using purified reconstituted protein, have explored the effects of the membrane environment. They have demonstrated that Pgp is involved in a complex relationship with its lipid environment, which modulates the behavior of its substrates, as well as various functions of the protein, including ATP hydrolysis, drug binding, and drug transport. Membrane lipid composition and fluidity, phospholipid headgroup and acyl chain length all influence Pgp function. Recent studies focusing on thermodynamics and kinetics have revealed some important principles governing Pgp–lipid and substrate–lipid interactions, and how these affect drug-binding and transport. In some cells, Pgp is associated with cholesterol-rich microdomains, which may modulate its functions. The relationship between Pgp and cholesterol remains an open question; however, it clearly affects several aspects of its function in addition to substrate–membrane partitioning. The action of Pgp modulators appears to depend on their membrane permeability, and membrane fluidizers and surfactants reverse drug resistance, likely via an indirect mechanism. A detailed understanding of how the membrane affects Pgp substrates and Pgp's catalytic cycle may lead to new strategies to combat clinical drug resistance.

Keywords: P-glycoprotein (ABCB1), phospholipids, detergents, cholesterol, ATP hydrolysis, membrane partitioning, membrane transport, drug binding

INTRODUCTION

Many human cancers, including breast, kidney, and colon carcinomas, leukemias, multiple myeloma, and several pediatric cancers, develop multidrug resistance (MDR), which is a major obstacle to chemotherapeutic treatment (1, 2). MDR tumors are cross-resistant to a broad spectrum of structurally unrelated cytotoxic drugs, including the *Vinca* alkaloids (vinblastine, vincristine), anthracyclines (doxorubicin, daunorubicin), and taxanes. Physiologically, a complex network of ATP-binding cassette (ABC) proteins is involved in drug detoxification and protection of tissues from xenobiotics, including administered therapeutic drugs. Three of these ATP-driven drug efflux pumps are widely expressed in tumors, and have been linked to drug resistance; P-glycoprotein (Pgp; ABCB1), ABCG2 and ABCC1 (MRP1) (3, 4). Pgp and MRP1 exist as large single polypeptides, whereas ABCG2 is a "half-transporter" that functions as a homodimer. Together, these three proteins are able to carry out efflux of a wide range of anticancer drugs that are in common use clinically. The ability to inhibit the action of these drug efflux pumps has the potential to greatly improve the outcome of chemotherapy treatment, and the development of strategies to achieve this has been ongoing for many years.

Since Pgp was the first ABC drug efflux pump to be identified (5), and has been intensively studied for more than 35 years, it is the one we know most about. Hundreds of Pgp substrates have been identified over the years, including many clinically used drugs, chemotherapeutic agents, natural products, linear and cyclic peptides, amphiphiles, and fluorescent dyes (3, 4). The protein displays basal ATPase activity, which is often (but not always) stimulated by substrates, and it hydrolyzes ATP to power active transport, generating a drug concentration gradient across the membrane (6). Pgp and other ABC transporters are believed to operate by an alternating access model. The first step in transport involves binding of drug to the inward-facing conformation from the cytosolic side of the membrane. This is followed by a switch to the outward-facing conformation, which reorients the binding site to the extracellular side, resulting in drug release. ATP hydrolysis provides the energy for the switch between these two conformations. Despite recent

progress in determining the high resolution structure of Pgp, there are still many gaps in our understanding of how the protein functions at the molecular level.

It was evident some time ago that the host membrane plays a central role in Pgp-mediated MDR (7, 8), and the complexity of this relationship has become more apparent as our knowledge of the protein has advanced. First, the membrane has a profound effect on Pgp's lipophilic substrates, affecting both their lipid partitioning and transbilayer movement. Second, many aspects of Pgp structure (such as its stability and conformation) and function (including ATP binding, ATP hydrolysis, drug binding, and drug transport) are especially sensitive to the properties and composition of the surrounding membrane. It is important to understand how Pgp function is modulated by membrane properties, since this may influence transporter activity in tumors. Indeed, MDR was successfully reversed *in vitro* simply by altering the biophysical characteristics of the membrane (9). Changing membrane properties may thus be a useful approach for clinical reversal of MDR. The importance of the membrane in drug efflux also suggests additional approaches for development of new anti-cancer drugs and modulators with increased clinical effectiveness; for example, chemical modification of existing compounds may alter their interactions with the membrane, and their ability to access Pgp. If such strategies are to be successful, it is clearly important to have a detailed understanding of how the properties of the lipid environment affect all aspects of the Pgp catalytic cycle.

This review focuses on the complexity of the interactions of Pgp and its substrates with their membrane environment, the effect of these interactions on many different aspects of Pgp function, and how understanding them can help shed light on the molecular details of drug transport. Our current knowledge of Pgp has been gleaned from both *in vitro* and cellular studies of the transporter. The majority of these studies have been carried out with either rodent Pgp (Chinese hamster and mouse) or the human protein. The latter is clearly the clinically important homolog, but (as discussed further below) high resolution structures are currently available only for the mouse and *Caenorhabditis elegans* proteins. The rodent Pgps are highly active, relatively stable, and have proved extremely useful for *in vitro* studies of the structure and mechanism of action of the purified protein. The human protein, on the other hand, appears to be relatively unstable and is more difficult to express and purify [e.g., Ref. (10)]. The rodent Pgps are very closely related to the human homolog (e.g., 87% sequence identity and 93% sequence similarity between Chinese hamster and human Pgp), and only small differences in substrate specificity have been observed in cell-based transport assays. Recent work using purified hamster Pgp in a liposomal assay system to quantify drug interactions reported that results correlated very well with clinically relevant data on disposition of Pgp transport substrates in human subjects (11). Rodent Pgps thus remain very important models for understanding the function of the human protein.

PGP IN HUMAN CANCER: ITS ROLE AND POTENTIAL MODULATION

P-glycoprotein expression is widespread in clinical cancer. The U.S. National Cancer Institute uses a panel of 60 tumor cell lines to identify and evaluate new anti-cancer agents. Expression of Pgp was detected in 39 of these cell lines, including renal and colon carcinomas, melanomas, and central nervous system tumors (12). Expression of Pgp was also highly correlated with resistance of these cell lines to anti-cancer drugs. Pgp has also been found in many human tumors (13, 14), with levels often increasing substantially after one or more rounds of chemotherapy, especially in acute myelogenous leukemia and lymphomas. Even very low levels of Pgp that are difficult to detect in tumor tissue significantly affect the sensitivity of cells to drugs. Although multiple mechanisms are undoubtedly responsible for drug resistance *in vivo* (15), Pgp is the single most important cause of MDR, representing an attractive target for intervention. Pgp expression has been linked to reduced responses to chemotherapy and poor clinical outcome for breast cancer, sarcomas, hematological malignancies such as leukemias, and pediatric cancers [reviewed in Refs. (1, 16)]. An important goal in cancer therapy has been the development of compounds that can effectively inhibit Pgp-mediated MDR. Such modulators (also known as chemosensitizers) have been identified based on their ability to reverse drug resistance in MDR cells *in vitro*. Pgp modulators commonly used in biochemical studies include verapamil and cyclosporin A. Since MDR is probably the single greatest barrier to successful chemotherapy, the ability to circumvent Pgp-mediated drug resistance could lead to improved treatment outcomes.

Three different strategies for defeating Pgp have been described (1); "engage" (co-administration of anti-cancer drugs and modulators), "evade" (the use of anti-cancer drugs that are poor Pgp substrates), and "exploit" (specific targeting of the Pgp molecule). Many clinical trials have been carried out to evaluate the hypothesis that co-administration of modulators would improve the effectiveness of chemotherapy drugs, thus leading to increased efficacy in patients [reviewed in Ref. (16)]. Early promising results obtained for pediatric cancers using cyclosporin A (17) generated optimism that this approach might be useful in tumors where Pgp expression is the primary cause of MDR. However, success in adult cancers has been elusive, in part because of poor clinical trial design and patient selection, and also because first and second generation modulators showed toxicity, sub-optimal effectiveness, and serious pharmacokinetic drug interactions (16, 18, 19). Since then, much more potent and less toxic third generation modulators have been developed to target Pgp specifically, such as LY336979 (zosuquidar) and XR9576 (tariquidar). However, they, too, failed to improve patient outcome in clinical trials [see Ref. (16)]. Several non-toxic plant natural products have also been proposed as MDR modulators, including curcumin (20), polyphenols (21), and flavonoids (22), however, these have not yet been tested in clinical trials. Given the disappointing outcome of the modulator strategy to date, it is not clear whether this line of attack will be successful. Designing novel approaches to specifically target Pgp in MDR will require detailed knowledge of the various steps in the catalytic cycle.

VACUUM CLEANER AND FLIPPASE MODELS FOR PGP FUNCTION

PGP AS A HYDROPHOBIC VACUUM CLEANER

The compounds that Pgp transports are typically lipophilic, so they accumulate within the lipid bilayer. Pgp substrates are also generally amphipathic molecules, and rather than distributing

uniformly in the hydrophobic core of the lipid bilayer, they align themselves in the interfacial region. NMR studies have shown that they tend to concentrate between the lipid headgroup and the first few carbon atoms of the lipid acyl chains (23). Early work strongly suggested that Pgp effluxes drugs directly from the membrane, rather than the aqueous phase (6). Acetoxymethyl ester derivatives of various fluorescent calcium and pH indicators entering intact cells from the extracellular side are intercepted and extruded without entering the cytosol (24). A Förster resonance energy transfer (FRET) study in which the transporter was photolabeled with the lipophilic probe iodonaphthalene-1-azide showed that the substrate doxorubicin was present within the membrane close to Pgp (25). This suggested that Pgp may interact with its substrates within the membrane and subsequently efflux them to the extracellular medium. These observations and others led to the proposal by Higgins and Gottesman that the transporter was a hydrophobic "vacuum cleaner" (**Figure 1**) responsible for removal of potentially harmful lipophilic compounds from the membrane (26). Later work showed that the rate of Hoechst 33342 efflux by Pgp was directly proportional to the membrane concentration of the fluorescent dye, but inversely proportional to its aqueous concentration, supporting the idea that transport takes place from the bilayer interior (27). In addition, studies employing a deletion mutant of Pgp showed that the transmembrane (TM) domains of the transporter were sufficient to bind drug substrates (28). The vacuum cleaner model is now widely accepted, and was a key concept in our understanding of the relationship between Pgp and its membrane environment.

Transport studies using reconstituted Pgp, plasma membrane vesicles, and intact MDR cells showed that several fluorescent substrates (Hoechst 33342, LDS-751, and a rhodamine derivative) were extracted from the cytoplasmic membrane leaflet (29–31). FRET studies designed to pinpoint the location of the binding sites for Hoechst 33342 and LDS-751 also showed that they were situated within the TM regions of Pgp, in the cytoplasmic leaflet of the membrane (32, 33). The recent X-ray crystal structures of Pgp from mouse (34) and *C. elegans* (35) have allowed a closer view of the potential routes that substrate molecules may follow from the bilayer inner leaflet into the binding pocket of the protein (see below).

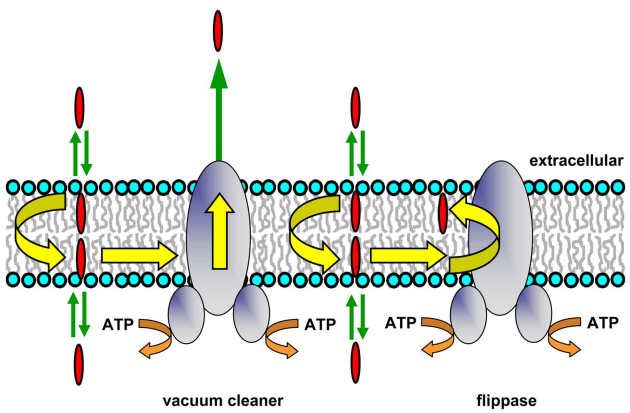

FIGURE 1 | Hydrophobic vacuum cleaner and flippase models of Pgp function. In the vacuum cleaner model, drugs partition into the membrane, spontaneously translocate to the cytoplasmic leaflet, and gain access to the Pgp substrate-binding pocket from within the bilayer interior. They are subsequently effluxed into the extracellular aqueous phase. In the flippase model, drugs partition into the membrane, spontaneously translocate to the inner leaflet, interact with the Pgp substrate-binding pocket, and are then flipped to the outer membrane leaflet. The drug concentration will be higher in the outer leaflet compared to the inner leaflet, and a concentration gradient is generated when drugs rapidly partition from the two membrane leaflets into the aqueous phase on either side of the membrane.

PGP AS A DRUG AND LIPID FLIPPASE

P-glycoprotein may be envisaged as expelling its substrates directly into the extracellular medium, which would have an energy cost for lipophilic species. The protein has also been proposed to operate as a drug translocase or flippase (**Figure 1**), moving its substrates from the inner to the outer leaflet of the membrane (26). This mechanism requires that drug molecules have a specific localization within each bilayer leaflet, rather than being randomly distributed in the hydrophobic core. An NMR study of Pgp substrates and modulators confirmed that this is the case, and showed that these molecules align with the phospholipid acyl chains (23). After reaching the outer leaflet, substrates would then either passively diffuse into the extracellular aqueous phase (a very fast process), or move back to the inner leaflet by spontaneous flip–flop. In order to maintain a substrate concentration gradient across the membrane, the rate of passive transbilayer flip–flop of substrate would need to be slower than the rate of Pgp-mediated flipping, so that its concentration remains higher in the outer leaflet. Indeed, the rate of drug movement between leaflets is variable, and can be quite slow, with half-times ranging from minutes to hours depending on the structure of the compound (36, 37). The flippase model involves delivery of drug to the outer leaflet, followed by rapid partitioning into the extracellular medium, while in the vacuum cleaner model, drug is delivered to the extracellular medium, followed by rapid partitioning into the outer leaflet. Since the same equilibrium state is reached in each case, it is not currently possible to distinguish experimentally between these two models, and they are not mutually exclusive. Energetic considerations suggest that release of a dehydrated lipophilic substrate into the outer membrane leaflet would be energetically more favorable than transfer into the aqueous phase followed by hydration, but it is possible that both of these locations are accessible to the substrate-binding pocket of Pgp during the transport process.

There is substantial evidence supporting the flippase model for Pgp function. Indeed, the protein is able to act as an outwardly directed flippase for several fluorescent phospholipid and glycosphingolipid molecules in both intact cells and reconstituted proteoliposomes. Cells overexpressing native or recombinant Pgp showed altered distribution of fluorescent phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), accumulated lower amounts of both short- and long-chain fluorescent phospholipid derivatives, and displayed increased outward transport of these analogs, which decreased after treatment with Pgp modulators (38–42). These observations strongly suggested that the lipids were Pgp substrates. Sharom and co-workers showed directly that purified Pgp reconstituted into proteoliposomes can act as a broad-specificity, outwardly directed flippase for a variety

of both short-chain and long-chain nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled phospholipids (43). Phospholipids with the fluorescent NBD group on both the acyl chain and the headgroup were translocated. Simple glycosphingolipids, such as glucosyl- and galactosylceramide were also flipped by reconstituted Pgp, as was lactosylceramide, although at a greatly reduced rate (44). Species with headgroups larger than two sugar residues are unlikely to be substrates. Like drug transport, phospholipid and glycolipid flippase activity required ATP hydrolysis, and was inhibited by the phosphate analog, *ortho*-vanadate. Flippase activity was inhibited in a concentration-dependent manner by known Pgp substrates, and inhibitory potency was highly correlated with their Pgp binding affinity, suggesting that drugs and membrane lipids follow the same route through the transporter. Taken together, these observations suggest that Pgp-mediated drug efflux probably takes place by a flippase-like mechanism.

INTERACTION OF LIPIDS AND LIPID-LIKE MOLECULES WITH PGP

Eckford and Sharom (45) used several functional screens to identify a number of lipid-based molecules that interact directly with Pgp with high affinity (**Figure 2**). These compounds resulted in inhibition of ATPase activity, competition for transport of two drugs, and competition for translocation of NBD–PC (16:0, 6:0), suggesting that they are substrates for Pgp. Included in these lipid species are platelet-activating factors (PAFs), ether–phospholipid signaling molecules that are probably endogenous substrates for the transporter (46, 47). Pgp is also able to bind and transport hexadecylphosphocholine (also known as miltefosine), an anti-cancer drug structurally related to PC (48). Other anti-cancer agents such as the alkyl phospholipids, edelfosine, and ilmofosine, and the related compounds, D-20133 and D-21266, also interact with the transporter, leading to resistance (45, 48). In support of these results, yeast cells expressing Pgp were previously reported to be resistant to the cytotoxicity of edelfosine (49). Pgp–lipid interactions thus have important consequences for cancer treatment using these lipid-based drugs.

Metabolic labeling of Pgp, followed by purification and organic solvent extraction, was used to isolate and identify the membrane lipids closely associated with Pgp (50). These proved to be enriched in PE and to a lesser extent phosphatidylserine (PS), but contained little PC or SM, despite the fact that these choline phospholipids are major components of the plasma membrane from which the transporter was solubilized. Thus Pgp associates preferentially with certain phospholipids; in this respect, it is interesting that PE and PS are enriched in the inner leaflet of the plasma membrane, whereas PC and SM are found primarily in the outer leaflet.

A recent mass spectrometry (MS) study (51) examined lipid interactions with Pgp using purified mouse protein in micelles of the detergent *n*-dodecyl-β-D-maltoside (DDM). Gas phase Pgp ions were released and retained only one to two detergent molecules, possibly in the binding pocket. MS could detect simultaneous binding of lipids, drugs, and nucleotides. Estimates of apparent K_d values, which were in the micromolar range, indicated that negatively charged lipids bind to Pgp more favorably than zwitterionic lipids, and both steric effects and headgroup charge modulated binding. Binding of the cyclic peptide, cyclosporin A, was observed to promote subsequent lipid binding. Interestingly, cardiolipin, a bulky anionic mitochondrial lipid, was also able to bind to Pgp. It was suggested that lipid molecules could interact with the transporter inside the large binding cavity and also at the interface between the protein and the lipid bilayer. This novel approach shows promise in the direct study of drug and lipid interactions with Pgp.

PHYSIOLOGICAL ROLE OF PGP–LIPID FLIPPASE ACTIVITY

The physiological significance of Pgp's lipid flippase activity is still the subject of speculation. The protein may be the primary means of exporting PAFs *in vivo*, by translocating them from the cytoplasmic to the extracellular membrane leaflet, from which they can diffuse into the aqueous external environment. Other probable endogenous Pgp substrates include steroid hormones such as aldosterone (52) and β-estradiol-17β-D-glucuronide (53). Indeed, the transporter is known to interact with several steroids (54, 55), and a role in transporting these hormones could explain Pgp expression in the adrenal gland. Pgp may also play a physiological role in the biosynthesis of complex glycosphingolipids in the Golgi by translocating glucosylceramide from the cytoplasmic to the luminal membrane leaflet. The protein most closely related to Pgp, ABCB4 (78% sequence similarity), acts primarily as a PC-specific flippase in the liver, exporting phospholipid into the bile (56, 57), although it can also transport some drugs at a low rate (58). Thus Pgp and ABCB4 may function in a similar manner.

It seems unlikely that the primary role of Pgp *in vivo* is that of a lipid flippase, because the rate of flipping is relatively low (43, 44), and Pgp could not rescue a knockout of ABCB4, even when both proteins were expressed in the liver canalicular membrane (59). However, differing expression levels of each protein could confound this observation. Another consideration is the existence of the plasma membrane aminophospholipid translocase, ATP8a1, which is a member of the P4-ATPase family. This protein normally translocates PE and PS to the inner leaflet, thus maintaining bilayer asymmetry (60). If Pgp moves these endogenous lipids from the inner to the outer leaflet at a significant rate, it would counteract the action of the translocase, resulting in a futile cycle of phospholipid flip–flop accompanied by wasteful hydrolysis of ATP.

Other mammalian ABC superfamily members are known to operate as physiological lipid transporters or flippases (61–63). Three proteins in the ABCA subfamily (ABCA1, ABCA7, and ABCA4) were shown to translocate fluorescent phospholipids in reconstituted systems (64). ABCA1 and ABCA7 transported lipids from the cytoplasmic to the extracellular leaflet, whereas ABCA4 is the only known eukaryotic ABC protein to move substrate in the opposite direction.

PGP STRUCTURE

Like other ABC proteins, Pgp comprises two homologous halves, each consisting of six TM segments, and two nucleotide-binding (NB) domains on the cytosolic side where ATP binds and is hydrolyzed (**Figure 3A**) (65, 66). The NB domains of ABC proteins contain three highly conserved sequences; the Walker A and B motifs (found in many ATP-binding proteins) and the C (or ABC signature) motif that is unique to this superfamily (67). It is now clear that nucleotide binding to ABC transporters is

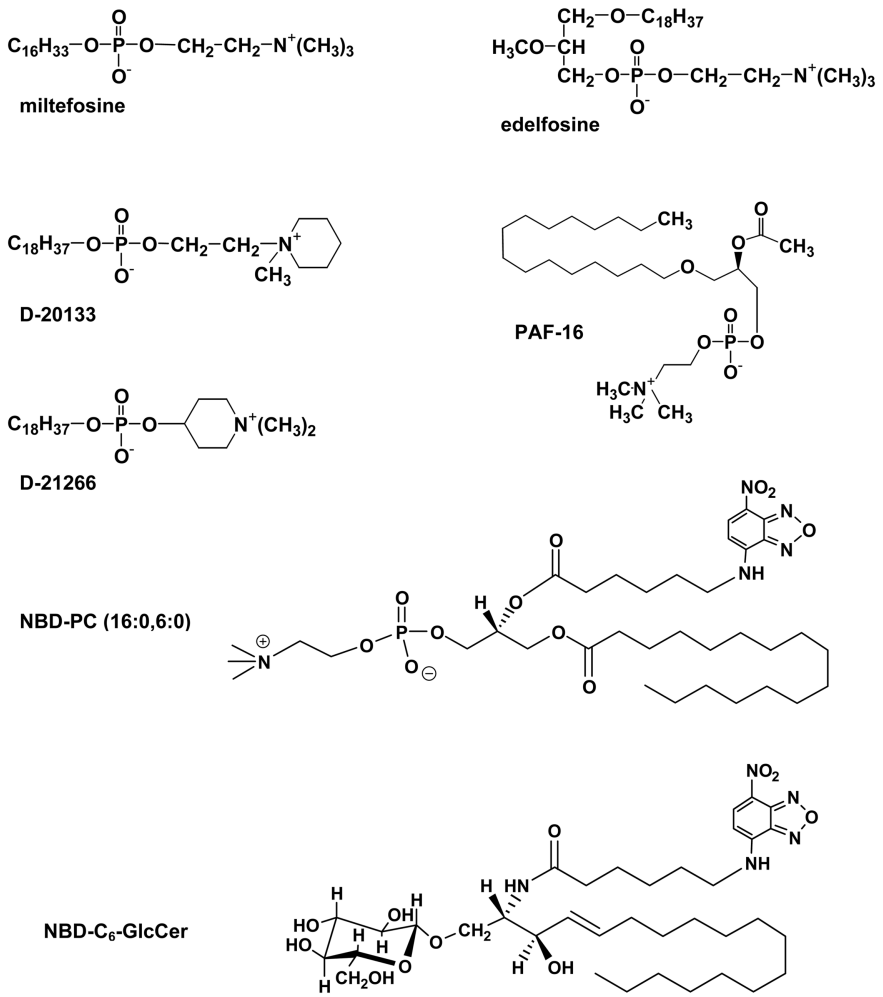

FIGURE 2 | Structures of some lipid-based Pgp substrates.

driven by dimerization of the NB domains (66, 68), which is essential for ATP-driven transport. The X-ray crystal structures of several isolated NB subunits of bacterial ABC proteins showed an interdigitated head-to-tail arrangement, a so-called sandwich dimer. Two ATP molecules are bound at the interface of the sandwich dimer, each interacting with the Walker A and B motifs of one NB domain and the C motif of the partner domain. These stable symmetric dimers are only observed when the NB domains are in an inactive state, either by mutation of an amino acid residue essential for catalysis, or in the absence of Mg^{2+} (69, 70). Thus, they probably do not represent a true catalytic intermediate.

Early electron microscopy (EM) studies provided the first glimpses of Pgp's three-dimensional structure [reviewed in Ref. (71)]. A medium-resolution EM structure of Chinese hamster Pgp at 8-Å resolution was obtained by cryo-electron crystallography of two-dimensional crystals (72). The protein contained bound nucleotide (AMP-PNP), and showed an asymmetric conformation. Other EM studies showed that the two NB domains of Pgp are closely apposed, leading to a closed conformation that was attributed to a sandwich dimer (73, 74).

In the past few years, important advances have been made in understanding the nature of the Pgp-drug-binding pocket [e.g., Ref. (32, 33, 75, 76)]. Mutagenesis studies suggested that the pocket was located at the interface between the two TM halves (77), and this was later confirmed by photoaffinity labeling in conjunction with MS (78, 79). The first X-ray crystal structures of mouse Pgp in an inward-facing conformation appeared in 2009 (34). The structures were determined in the absence of nucleotide, with and without bound cyclic peptide substrates, which are located in a drug-binding pocket within the TM regions of the protein. This large flexible cavity is made up of two bundles of six helices, each composed of portions from both the N- and C-terminal halves (**Figure 3B**). This phenomenon, known as domain swapping, has also been reported for other ABC exporters. The pocket is accessible from the cytoplasmic leaflet of the membrane, as expected. The drug-binding region is partially lined with aromatic and hydrophobic residues, which are proposed to bind substrates via

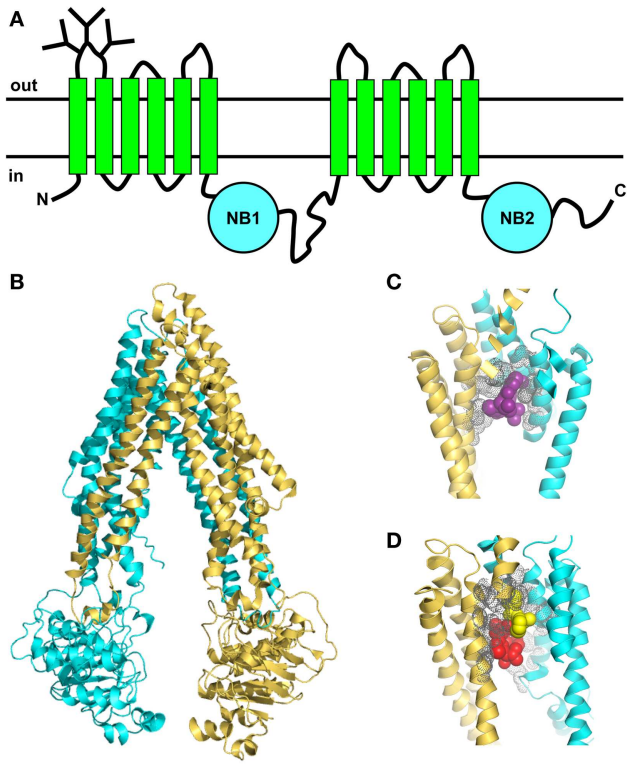

FIGURE 3 | Topology and X-ray crystal structures of Pgp. **(A)** The membrane topology shows two homologous halves, each with six TM segments and a NB domain on the cytoplasmic side, which binds and hydrolyzes ATP. **(B)** X-ray crystal structure of mouse Pgp (34) shows an inward-facing conformation in the absence of nucleotide (PDB 3G61). **(C,D)** The drug-binding pocket of mouse Pgp as seen in the X-ray crystal structure. **(C)** Close-up view (4.4 Å resolution) of one molecule of the cyclic peptide modulator QZ59-RRR (magenta, space-filling format) occupying the middle site inside the drug-binding pocket, with the volumes of nearby side-chains shown in gray (PDB 3G60). **(D)** Close-up view of two molecules of the cyclic peptide modulator QZ59-SSS occupying the upper and lower sites (yellow and red, respectively, space-filling format) inside the drug-binding pocket, with the volumes of nearby side-chains shown in gray (PDB 3G61).

hydrophobic and van der Waals interactions. Different stereoisomers of the same cyclic peptide substrate are held in different sub-sites by a unique set of interactions, and two molecules of the same substrate can fit into the pocket simultaneously, via different sets of interactions (**Figures 3C,D**). The presence of overlapping sub-sites inside the binding pocket helps to explain the extraordinary range of chemical structures that interact with Pgp. Although the mouse structures are not of very high resolution, they provided some useful information that will help to guide future studies. However, they have not settled any of the outstanding controversies in the field, such as the extent of the separation and movement of the NB domains during the catalytic cycle. Some new mouse Pgp structures show wider separations of the two NB domains (80). One structure shows a complex with a nanobody inhibitor, which appears to inhibit ATP hydrolysis by binding to an epitope on the N-terminal NB domain, thus preventing dimerization of the two domains.

A higher resolution structure (3.4 Δ) of *C. elegans* Pgp in an inward-facing conformation, without bound substrate or nucleotide, appeared in 2012 (35). It shows a portal leading from the cytoplasmic side of the bilayer to the central cavity where substrates are presumed to bind. This structure is compatible with decades of biochemical analysis on the human protein, and helps to explain perplexing functional data on the Phe335Ala mutant. Homology models of human Pgp derived from the *C. elegans* and mouse Pgp structures differ significantly in their orientation of TM helices 3–5, and recent work suggests that the *C. elegans* model is most likely correct (81).

Recent simulation studies of mouse Pgp in a model membrane showed a wide range of NB domain separations, underscoring the apparent high flexibility of the inward-facing protein conformation (82). Of great interest is the observation that the acyl chain of a membrane lipid slid into the cleft between TM helix 4 and TM helix 6, and remained there for the duration of the 50-ns simulation. The tip of the penetrating chain, which was more than 10 carbon atoms long, made contact with several amino acid residues lining the substrate-binding pocket that are known to be involved in drug binding. It is possible that the binding pocket of Pgp may be occupied by endogenous membrane phospholipids in the absence of drug substrates, in line with the idea that it also functions as a low activity lipid flippase. Occupation of the binding pocket by lipids might also explain the high basal ATPase activity observed for Pgp from mouse and Chinese hamster. If these lipids bind with relatively low affinity, they may be displaced by high affinity drug substrates, which would thus be transported preferentially.

To date, only inward-facing conformations of Pgp have been obtained using crystallographic studies, and there is still no nucleotide-bound structure available. Until these two "missing links" are obtained, it will be difficult to make progress on understanding the structural changes that take place during the catalytic and transport cycles. The difficulty in crystallizing Pgp in the outward-facing or nucleotide-bound forms might arise from either the high dynamic flexibility or low stability of these conformations.

MODEL SYSTEMS FOR STUDYING THE INTERPLAY BETWEEN PGP AND LIPIDS

The vacuum cleaner and flippase models predict that Pgp will be very sensitive to its membrane microenvironment. Because of the complexities of intact cells, they have limited usefulness in exploring the relationship between transporter function and membrane properties. Plasma membrane fluidity can be altered by growing cells in medium containing saturated fatty acids, which alters the endogenous lipid composition of cellular membranes. Membrane properties can also be changed by adding exogenous amphiphiles, or membrane rigidifying or fluidizing agents [e.g., Ref. (9)]. Experimental observables are typically restricted to indirect measures of Pgp function, such as total cellular drug uptake, or cell survival in the presence of drug.

Simpler model systems have provided unique opportunities to explore the structure and function of Pgp. Mammalian MDR cell lines selected with cytotoxic drugs can express very high levels of Pgp [up to 30% of the total plasma membrane protein

(83)], and have been popular choices as the starting material for model systems. Sealed inside-out plasma membrane vesicles have been used for studies of ATP-driven drug transport [see Ref. (6)], and also as a source of purified active protein for biochemical and spectroscopic characterization. These studies have typically relied on the use of exogenous agents to alter membrane fluidity. For example, the ability of Pgp in canalicular membrane vesicles to transport daunorubicin and vinblastine was reduced two- to four-fold by benzyl alcohol, a known membrane fluidizer (84).

Several research groups have reported successful purification of Chinese hamster, mouse, and human Pgp from drug-selected MDR cell lines, or cells expressing recombinant protein [reviewed in Ref. (6)]. The use of solubilizing detergents is clearly essential in the extraction and purification of Pgp from native cell membranes. The choice of detergent is a critical step, and is typically governed in the first instance by the necessity of maintaining various functions of the protein, including ATP binding, ATP hydrolysis, drug binding, and drug transport. A second consideration is the compatibility of the chosen detergent with reconstitution, if this is a desired goal (85). Detergents with very low critical micelle concentrations (CMCs), such as Triton X-100, are usually very difficult to remove by dialysis, gel filtration chromatography, or dilution, and are not a good choice for reconstitution. The mild detergents 3-[3-(cholamidopropyl) dimethyl amino]-1-propanesulfonate (CHAPS) and octyl-β-D-glucoside (OG) have relatively high CMC values, and have been used successfully for purification and functional reconstitution of both Chinese hamster and human Pgp from mammalian cells (86–89).

Purified Pgp of mouse or Chinese hamster origin typically displays high levels of both basal and drug-stimulated ATPase activity (2–3 μmol/min/mg), and can carry out ATP-dependent active transport of drugs and hydrophobic peptides after reconstitution [summarized in Ref. (90)]. However, the basal rate of ATP hydrolysis of purified human Pgp is much lower than that of the rodent proteins, and it appears to require the presence of both drug substrates and lipids for activity. The rodent and human proteins are highly homologous, and the underlying reason for this difference in behavior is not clear. Human Pgp was recently reconstituted into lipid nanodiscs composed of *Escherichia coli* lipids, where it retained robust levels of both basal and drug-stimulated ATPase activity (91). Surface plasmon resonance was used to probe conformational changes in Pgp associated with progression through the ATP hydrolysis cycle. This novel nanodisc platform may prove useful in the further study of human Pgp.

Many of the purified Pgp preparations described in the literature employ the addition of exogenous lipids to maintain its function. Very few research groups have isolated Pgp in the absence of added exogenous lipids (50, 92). This approach allowed the development of experimental protocols to reconstitute Pgp into proteoliposomes of defined phospholipids, either synthetic or natural mixtures (92–94). Using these model systems has permitted detailed investigation of how Pgp function is modulated by membrane properties, leading to unique insights into transporter behavior. Further details of these studies are provided below.

DETERGENT INTERACTIONS AND EFFECTS ON PGP FUNCTION

The interaction of Pgp with detergents has been examined from two different aspects. First, the effectiveness of different detergents in solubilization and functional reconstitution of Pgp has been studied by several groups. Coupled with this has been the investigation of how exposure to various detergents stabilizes or destabilizes the protein. Second, the role of detergents as specific Pgp substrates and modulators has been explored, with a view to probing the nature of their interactions with the drug-binding pocket.

Sharom and co-workers established a protocol for partial purification of Chinese hamster Pgp with high levels of ATPase activity using the zwitterionic detergent CHAPS (95), in the absence of any exogenously added lipids. This allowed subsequent exploration of the effects of various detergents on the protein's activity and stability (96). Only the high CMC detergents, CHAPS and OG, were able to preserve ATPase activity at higher concentrations (4 mM for OG, 10 mM for CHAPS), whereas Triton X-100, digitonin and SDS resulted in complete loss of activity at 100 μM levels. The ability of CHAPS to preserve Pgp ATPase activity, while other detergents caused loss of activity was later confirmed by Orlowski et al. (97). They noted that drug stimulation of ATPase activity was restored after dilution of detergent to a concentration below its CMC. Even after almost complete removal of CHAPS from purified Pgp by dialysis, 80–90% of the ATPase activity remained (96), suggesting that the protein still retained a substantial annual lipid layer around it, which provided protection from denaturation. Later work used metabolic labeling to show that highly purified Pgp in CHAPS solution retained 53–56 tightly bound phospholipids per protein molecule (50). This amount may be sufficient to surround the TM domains of the protein, thus assisting in maintaining its native conformation and function.

The non-ionic detergents DDM and zwittergent 3–12 were reported to inactivate Chinese hamster Pgp function at low concentrations, below their CMCs, while OG inhibited activity in the millimolar range (98). In all cases, loss of ATPase activity and reduced drug-binding activity were prevented by the addition of 0.2% w/w of a crude lipid mixture. It was suggested that these detergents disrupted the lipid–protein interface, which is essential for Pgp to maintain its functional conformation. Similarly, the non-ionic *n*-alkyl-β-D-maltosides and *n*-ethyleneglycol monododecyl ethers were reported to reduce Pgp ATPase activity at concentrations well below their CMC values (99).

Naito and Tsuruo carried out the purification of human Pgp from MDR K562 cells using a panel of 12 detergents, and assessed its function following reconstitution into proteoliposomes (100). They reported that only cholate, glycocholate, and taurocholate were able to extract the protein and maintain its activity. However, the measured ATPase and transport activities were extremely low, likely as a result of protein denaturation during immunoaffinity elution. The overall conclusion from all of these studies is that the sensitivity of Pgp to inactivation by detergents depends on the lipids present in its immediate environment.

P-glycoprotein was reported to be dimeric or multimeric in the plasma membrane of some MDR cells (101–103), however, the minimal functional unit of the protein appears to be a monomer (104). Poruchynsky and Ling reported that detergent extracts from hamster and human MDR cell lines contained Pgp in an oligomerized form (105). CHAPS resulted in a high proportion of oligomerized Pgp, whereas only monomers were found after SDS treatment, suggesting that the nature of the detergent is important in either the formation or preservation of oligomers. Both Pgp monomers and oligomers appeared functional, in that they bound photoactive nucleotide and drug analogs. However, other studies have failed to find any evidence of Pgp dimerization using both biochemical and genetic approaches (106). The existence and functional role, if any, of oligomeric forms of Pgp is, therefore, still not clear.

Doige and Sharom first noted that Triton X-100 stimulated Pgp ATPase activity at low concentrations and inhibited it at higher concentrations, suggesting that it interacted specifically with the transporter (96). Triton X-100 was also observed to inhibit photoaffinity labeling of Pgp by the substrate ^{3}H-azidopine at low concentrations (107, 108). It was later confirmed to be a high affinity substrate for Pgp ($K_d = \sim 0.4\,\mu M$) using fluorescence quenching of purified protein (109). Several cationic amphiphiles, including the detergents benzalkonium chloride, methylbenzethonium chloride, cetylpyridinium chloride, and dodecyltrimethylammonium chloride, also appear to be Pgp substrates (107, 108). Both drug-selected MDR cells and Pgp transfectants showed cross-resistance to these compounds, which was effectively sensitized by the modulator verapamil. The synthetic surfactant, nonylphenol ethoxylate, which is a common component of various household detergents, was identified as a constituent of human urine, and *in vitro* studies confirmed that it is also a Pgp substrate (110). These findings indicate that the compound is excreted into the urine *in vivo* by kidney Pgp, in keeping with the proposed physiological role of the transporter in elimination of potentially toxic compounds.

Seelig's group further pursued the idea that detergents may be Pgp substrates using the non-ionic species Triton X-100, $C_{12}EO_8$ (dodecyloctaglycol), and Tween 80 (polysorbate-80) (111). The bell-shaped curves obtained for the effect of these detergents on Pgp ATPase activity suggested that they behaved like substrates, and interacted directly with Pgp with high affinity (K_d values $\sim 1\,\mu M$). Thermodynamic measurements showed that the ethoxyl groups of these detergents formed H-bonds with donor amino acid residues in the TM regions of Pgp, and their binding affinity increased with the number of these groups. Further investigation used a large series of polyoxyethylene alkylether detergents with varying numbers of methylene and ethoxyl residues (C_mEO_n), to systematically dissect the role of hydrophobic and H-bonding in binding of these compounds (112). Thermodynamic parameters indicated that detergent binding to the Pgp substrate cavity is driven exclusively by H-bonding or weak electrostatic forces, and not by hydrophobic interactions. Binding to Pgp is only achieved if the detergent has at least two hydrogen bond acceptor units within its chemical structure, supporting earlier work that used a large number of structurally diverse substrates (113). It is energetically unfavorable for the non-polar moiety of the detergent (the methylene chain) to enter the substrate-binding pocket, and it likely remains in contact with the hydrophobic lipid bilayer, where it may assist in flipping of the molecule to the other leaflet (112). This concept appears counter-intuitive, since the existence of hydrophobic and aromatic interactions between bound substrates and the surrounding cavity was suggested based on the X-ray crystal structures of mouse Pgp (34), and many QSAR studies have also stressed the importance of non-polar groups in binding to the transporter. However, the primary role of these groups may actually be to promote partitioning of the substrate into the bilayer, where it can then gain access to the protein's binding cavity.

Some detergents were reported to produce unexpectedly large down-modulation of Pgp ATPase activity, which was not a result of membrane disordering (114). The ratio of the free energy of detergent partitioning into the membrane (from K_{lip}) and the free energy of detergent binding to Pgp from within the membrane (from K_{dlip}) appeared to control the rate of ATP hydrolysis induced by the detergent, and probably the rate of its transport. Deviations from an optimal ratio of these free energies led to reduced rates of ATP hydrolysis and, by extension, transport. It was suggested that similar principles apply to drugs that are Pgp substrates and modulators.

PERTURBATION OF LIPID BILAYERS BY PGP

Early work on drug-selected MDR cell lines indicated that overexpression of Pgp led to secondary changes in the properties of their plasma membrane [summarized in Refs. (107, 115)]. It seemed likely that insertion of this large hydrophobic protein perturbs the physiochemical properties of the membrane, especially in cell lines where Pgp may represent as much as 20–30% of the total plasma membrane protein (83, 92). Loe and Sharom used the fluorescent probe merocyanine 540 to examine the local microenvironment in the plasma membrane of various CHO cell lines expressing Pgp (107). This negatively charged dye localizes in the outer leaflet of the plasma membrane, and the extent of its partitioning into membranes depending on the lipid packing density. Increasing levels of cellular drug resistance led to a progressive shift in the mean cell fluorescence to lower levels, indicating that the molecular packing of lipids in the outer leaflet of MDR cells increases with higher levels of Pgp expression.

The availability of purified Pgp led to further studies in reconstituted systems. Differential scanning calorimetry was first carried out by Romsicki and Sharom, using the synthetic phospholipid dimyristoyl-PC (DMPC) as the host lipid (116). Inclusion of increasing mole ratios of Pgp led to a decrease in the melting temperature of the bilayer, T_m. The cooperativity of the lipid melting transition was greatly reduced, and the transition enthalpy, ΔH, decreased linearly as the Pgp content increased to a lipid:protein ratio of 16:1 (w/w). Pgp was found to perturb a large number of bilayer phospholipids, preventing 375–485 of them from participating in the phase transition. As the Pgp content of the bilayers increased, the transition enthalpy was observed to increase again, an effect likely arising from either aggregation/oligomerization of the transporter, or a change in its mode of interaction with the bilayer. Oleinikov et al. explored the perturbing effects of Pgp in its native environment by forming monolayers from membrane fractions derived from a series of MDR cell lines in which the

Pgp content varied from 0 to 32% (w/w) (117). Compression of the Langmuir–Blodgett monolayers showed that 11 and 24% Pgp decreased monolayer stability without altering the surface area, while 32% Pgp increased both the stability and the surface area. Raman spectroscopy indicated that the presence of 32% Pgp reduced the lipid transition temperature by 7°C, and the number of perturbed lipids per protein was estimated at 400–500 (117), thus confirming the results seen in reconstituted systems (116).

Callaghan and co-workers reported that incorporation of Pgp into bilayers of PC–PE altered their overall fluidity, increased their permeability to polar compounds, and modified the packing organization of fluorescent lipid probes (115). The presence of the transporter also increased the flip–flop rate of short-chain phospholipids between bilayer leaflets, but this did not require ATP hydrolysis, so it likely takes place by an indirect mechanism.

Similar types of perturbing effects have previously been noted when other large hydrophobic proteins are inserted into lipid bilayers [e.g., Refs. (118, 119)]. Effects specific to Pgp may arise from its oligomerization behavior, and the ability of the transporter to interact with, and translocate, membrane lipids.

MODULATION OF PGP FUNCTIONS BY THE MEMBRANE

The availability of purified Pgp, both in detergent solution and reconstituted proteoliposomes, coupled with the development of biochemical and spectroscopic techniques for assessing many aspects of Pgp function, have greatly increased our understanding of the ways in which the lipid environment can modulate the transporter. The catalytic cycle of Pgp involves several steps, including nucleotide binding, drug binding from within the membrane, nucleotide hydrolysis, and drug translocation. Each of these steps may be subject to the influence of the surrounding membrane. In recent years, greater emphasis has been placed on the importance of measuring thermodynamic and kinetic constants for the various steps, since these provide a sound basis for construction of a detailed, experimentally testable model of the catalytic cycle.

DRUG-MEMBRANE PARTITIONING

P-glycoprotein substrates are typically lipid-soluble and amphipathic, and it is now clear that their binding to the protein takes place in two steps: partitioning into the lipid bilayer, followed by interaction with the substrate-binding pocket located within the TM domains. The presence of a drug within the membrane inner leaflet is thus a primary determinant of its recognition by the transporter. Amphipathic molecules are not randomly distributed in the bilayer, but orient themselves in an anisotropic manner at the lipid–water interface, parallel to the long axis of the membrane phospholipids (23, 120, 121). Their polar moieties interact with water and phospholipid headgroups, and (depending on the compound) their hydrophobic moieties may enter the non-polar core of the bilayer. Modeling studies suggest that a specific molecular conformation may also be required for partitioning of, for example, verapamil into the bilayer (120).

Since substrates gain access to Pgp from within the membrane, the lipid–water partition coefficient, K_{lip}, is an important molecular property affecting their interactions with the transporter. Lipid bilayers are multilayered, amphipathic structures (essentially highly ordered liquid crystals), and cannot be readily mimicked by isotropic systems such as octanol or olive oil. Experimental measurement of K_{lip} values using lipid bilayers is thus important for a quantitative understanding of the contribution of substrate partitioning to interaction with Pgp. K_{lip} values for Pgp substrates, whose structures are extremely diverse, range from 50 to 10^5 (111, 112, 114, 122–126). The high lipid–water partition coefficients of a series of polyoxyethylene alkylether detergents resulted in their concentration in a palmitoyloleoyl-PC (POPC) bilayer (112). Calorimetric measurements indicated that lipid partitioning was an entropy-driven hydrophobic event, driven by release of water molecules from the detergent when it entered the bilayer.

The passive diffusion and spontaneous flip–flop of small molecules across membranes decline exponentially with an increase in lateral packing density (127), which in turn depends on lipid composition and temperature. Native plasma membranes with high lateral packing density, such as these found in endothelial cells of the blood brain barrier, may thus make it easier for Pgp to establish a drug concentration gradient. Phospholipid bilayers exist in a rigid gel phase below the phase transition (melting) temperature, T_m, and a fluid liquid-crystalline phase above this temperature. These two phases are characterized by different packing densities, diffusion rates, fluidity, and lipid conformations. In an effort to understand the effects of membrane biophysical properties on partitioning of Pgp substrates, Clay and Sharom measured K_{lip} for three structurally unrelated Pgp substrates (LDS-751, H33342, and MK-571) in DMPC and palmitoylmyristoyl-PC (PMPC) bilayers over a temperature range that spanned T_m (122). Membrane partitioning of all three drugs was greatly favored in the fluid liquid-crystalline phase relative to the more rigid gel phase. The volume of the lipid bilayer increases by 4% when it melts (128), so drugs can fit more easily into the additional space present in the membrane in the liquid-crystalline phase, resulting in higher partitioning. The membrane partitioning of these three substrates was greatly affected by small changes in acyl chain length and lipid phase state, implying that such changes in membrane properties can readily modify Pgp's ability to bind drugs within the bilayer and transport them to the aqueous phase. Lipid rafts are small cholesterol- and glycosphingolipid-rich microdomains which display altered lipid composition, including acyl chain length, and also exist in a phase with reduced fluidity (the liquid-ordered phase, l_o), compared to the bulk membrane. Pgp appears to be located within rafts in some cell types (see below), and may move in and out of them in a dynamic fashion, thus potentially changing its access to drug substrates within the bilayer.

ATP BINDING AND HYDROLYSIS

Although the NB domains of Pgp are envisaged as being located in the cytoplasmic compartment, they appear to be almost completely dependent on the presence of membrane lipids for their catalytic activity. Early work showed that a Pgp-β-galactosidase fusion protein required phospholipids for ATPase activity (129). Detergent delipidation of Pgp results in complete loss of ATPase activity, which is readily and rapidly reversible by addition of lipids (96, 98, 130), suggesting that the loss of activity does not involve denaturation of the protein.

The ability of bulk exogenous phospholipids to protect Pgp catalytic activity from thermal inactivation was tested by Sharom

and co-workers, using partially purified Chinese hamster Pgp in CHAPS solution (96). Asolectin, a mixture of soybean phospholipids, provided complete protection of the ATPase activity at 0.25 mg/mL, whereas various PC species did not. PS was also able to maintain the ATPase activity, and various PE species, either alone or mixed with PC, actually stimulated activity. Restoration of ATPase activity following detergent delipidation with Triton X-100 and deoxycholate required a different set of phospholipids, likely reflecting the intimately associated annular lipids that Pgp prefers (96). In general, short, unsaturated fluid lipids were better able to restore activity than longer, saturated species. The ATPase activity of purified Chinese hamster Pgp in CHAPS solution was altered by incubation with various phospholipids, some of which stimulated activity, while others caused inhibition (50). The best stimulatory lipid, dipalmitoyl-PE, was a mixed activator, increasing V_{max} and decreasing the K_M for ATP. Exogenous lipids are probably able to exchange into the annular lipid region of Pgp in the presence of small amounts of detergent, thus modulating protein function. Mixed activation is a common observation with Pgp-drug substrates. The ability of phospholipids to modulate ATP hydrolysis could also be because they are actually transport substrates, as discussed previously in the context of the documented flippase activity of the transporter.

Urbatsch and Senior solubilized Chinese hamster Pgp using OG and attempted to purify it in the absence of exogenous lipids (86). This resulted in completely inactive protein that could not be restored by reconstitution. When Pgp was purified in the presence of three natural lipid mixtures, activity was maintained, and they were able to reconstitute it into proteoliposomes of these lipids (86). They noted different effects on the level of ATPase activity and its stimulation by drugs, depending on the host lipid. The level of drug stimulation, which may reflect coupling between drug binding and ATP hydrolysis, was also reported by other groups to be increased after reconstitution of Pgp (87, 131), as was high affinity drug binding (98).

To explore the effects of lipid melting on catalytic activity, purified Pgp was reconstituted into synthetic phospholipid bilayers with a defined melting temperature, composed of DMPC ($T_m = \sim$23(C) and PMPC ($T_m = \sim$28(C) (132). Both ATP binding and ATP hydrolysis were found to be modulated by the phase state of the membrane. The kinetic parameters and activation energy for ATP hydrolysis, as well as K_d for ATP binding, were significantly different above and below the bilayer melting temperature, and there was a sharp discontinuity at T_m. The binding affinity for ATP was higher in the fluid liquid-crystalline phase, whereas the K_M for ATP hydrolysis was lower in the rigid gel phase. No changes in these parameters were observed at the same temperatures with Pgp in detergent solution. Thus, the conformation and/or folding of the NB domains may be affected by the fluidity and/or packing density of the membrane, which in turn affects nucleotide binding and hydrolysis. The opposite effects observed for K_d and K_M are not contradictory, since these parameters can only be considered equivalent for a simple Michaelis–Menten reaction scheme, and the catalytic cycle for Pgp is likely much more complex, with multiple steps.

Using drug-induced modulation of ATPase activity as a surrogate measure of drug binding, Seelig and co-workers were able to estimate the thermodynamic parameters for ATP hydrolysis (133). They showed that although the membrane lateral packing density only modulates the overall ATPase activity by up to two-fold, it substantially affected the thermodynamics of the transporter, which operated in an enthalpy-driven manner at low packing densities, but was driven by entropy at high densities. Partitioning of highly lipophilic Pgp substrates into the bilayer, and their accumulation to high concentrations (see above), may increase the fluidity and reduce the lateral packing density of the membrane. However, this appears to have only small effects on the thermodynamics of ATPase activity (133).

A FRET study showed that the NB domains of Pgp lie close to the membrane surface (134). In addition, when expressed in *E. coli*, the C-terminal NB domain was associated with the membrane fraction, indicating that it might interact preferentially with lipid surfaces (135). Taken together, this suggests that Pgp's NB domains may be in physical contact with the membrane surface, much like peripheral proteins, explaining why membrane fluidity and packing density affect their function. The lipid requirement for catalytic activity may be interpreted in two ways. Either the lipids are needed for stabilization of the TM regions which in turn stabilize the NB domains, or the NB domains interact with the membrane directly and require phospholipids for their catalytic function and, possibly, their structural integrity.

Rodent and human Pgp appear to differ in terms of their stability and lipid requirements for activity. The human protein typically displays very low basal ATPase activity compared to the Chinese hamster protein, but shows much higher levels of drug-stimulated activity. Reconstitution into lipid is often necessary for observation of any ATPase activity at all [for example, see Ref. (136)]. The drug-stimulated activity of human Pgp also appears to be highly dependent on the presence of cholesterol (137). Kodan et al. carried out a systematic study of factors promoting the stability of human Pgp expressed in insect cells (10). They noted a remarkable stabilizing effect of cholesteryl hemisuccinate (CHS) on the stability of the protein's ATPase activity after DDM solubilization; only 50% of the activity remained after 1 h at 25°C in the absence of CHS, whereas 50% activity was still present after 30 days at 4°C in the presence of 0.02% CHS. CHS has been noted to stabilize other human membrane proteins after DDM solubilization (138).

DRUG BINDING

Measurement of equilibrium drug binding to Pgp in plasma membrane vesicles from MDR cells has been carried out using radiolabeled substrates and modulators (139). This approach is technically difficult because the hydrophobic compounds give rise to high levels of non-specific background association with the membrane. Fluorescence spectroscopic methods using purified Pgp in detergent solution and reconstituted proteoliposomes have allowed quantitative estimates of K_d for binding of many substrates and modulators, without the need to separate free and Pgp-bound drugs [reviewed in Refs. (140, 141)]. The affinity of Pgp for binding a large group of compounds with diverse structures covers a range of $\sim 10^4$, from 37 nM to160 μM.

Because of the difficulty of directly assessing drug binding, several groups have used drug or modulator stimulation of Pgp ATPase activity as a surrogate measure. Changing patterns of

substrate-induced ATPase stimulation in the presence of different lipids suggests that either drug binding itself, or the coupling between drug binding and ATP hydrolysis, is altered by the membrane environment. Following Pgp purification and reconstitution into three natural lipid mixtures, Urbatsch and Senior noted different effects on the level of stimulation of ATPase activity by three substrates and one modulator, depending on the host lipid (86). Other groups also reported that after reconstitution of Pgp the level of drug stimulation of ATP hydrolysis (87, 131), and the affinity of drug binding (98) were increased.

For a substrate to interact with Pgp, two distinct steps must take place. The drug must first partition from the aqueous phase into the bilayer, and then it must enter the binding pocket of the transporter. Seelig and co-workers proposed that Pgp functions optimally when the free energies for these two processes fall in a narrow range (114). The first step is defined by the lipid–water partition coefficient for the drug, K_{lip}, and the second step is defined by the dissociation constant for binding of the drug to Pgp within the membrane, K_{dlip} (see **Figure 4**). The apparent binding affinity of Pgp for drugs measured from the aqueous phase, K_d, is related to K_{dlip} by:

$$K_{dlip} = \frac{K_d}{\left(\frac{V_{lip}}{V} + \frac{1}{K_{lip}}\right)}$$

where V is the total volume of the system, and V_{lip} is the volume of lipid (122). Thus experimental measurement of K_{lip} and K_d can provide an estimate of the "true" affinity of Pgp for binding drugs from within the lipid bilayer.

For reconstituted Pgp in three different lipid systems, a high-fluidity lipid (egg PC) gave a large increase in binding affinity (determined by fluorescence quenching) compared to a low-fluidity lipid (dipalmitoyl-PC; DPPC) for vinblastine and verapamil (6- to 15-fold reduction in K_d value), while daunorubicin showed a much smaller change (two-fold reduction) (124). K_d was also correlated with the value of K_{lip} for vinblastine and verapamil. The highest apparent binding affinity was observed for substrates that had the greatest partitioning into lipid, in other words, the highest concentration in the bilayer (**Figure 4**). This suggests that the bilayer drug concentration is a major determinant of binding affinity, in agreement with the vacuum cleaner model. Pgp may thus have a relatively low "true" affinity for binding within the bilayer (i.e., K_{dlip} is high), but the membrane highly concentrates its substrates. A large fraction of the free energy of drug binding to the protein from the aqueous phase was shown to be provided by the free energy of drug–lipid partitioning, on the order of 75% (122, 126). In concert with changes in binding affinity, different host lipids also produced an altered ATPase stimulation profile for verapamil. Higher drug-binding affinity led to higher levels of stimulation of activity, whereas the concentration of verapamil required for half-maximal stimulation remained unchanged (124).

The extent of partitioning of substrates into the membrane is thus important for their interaction with Pgp, because it essentially controls the effective concentration presented to the transporter. For example, the apparent binding affinity of *C. elegans* Pgp for paclitaxel and actinomycin D was increased 100- and 4,000-fold, respectively, when the protein was in a membrane environment compared to detergent solution (35). Seelig and co-workers were the first to address this idea directly, using the drug concentration required for half-maximal ATPase stimulation as an indirect measure of its binding affinity (142). By calculating the free energy of drug binding from the aqueous phase to Pgp in the membrane, and combining this with drug–lipid partitioning data, they were able to estimate the affinity of Pgp for binding three drugs from the lipid phase. K_{dlip} values were in the range 0.5–4 mM for three substrates (142), providing the first evidence that Pgp binds its substrates from the membrane with low affinity. Recent work by Clay and Sharom using reconstituted Pgp in DMPC and PMPC bilayers directly measured the K_d for binding to Pgp from the aqueous phase, and also the lipid–water partition coefficient for three structurally unrelated substrates (122). Estimated K_{dlip} values were high, in the range 9–70 mM, confirming that Pgp has an intrinsically weak interaction with substrates within the lipid bilayer.

Defined reconstituted systems provide a powerful tool to define the effect of bilayer physicochemical properties on drug binding. Romsicki and Sharom first made the interesting (and paradoxical) observation that the binding affinity for vinblastine, verapamil and daunorubicin was higher in gel phase DMPC bilayers, despite the fact that drug partitioning is substantially lower for gel phase lipid (124). More recent work using Pgp reconstituted into DMPC and PMPC bilayers showed that this relationship held true for the substrates H33342, LDS-751, and MK-571 (122); both K_d and K_{dlip} values are lower (i.e., binding affinity is higher) in rigid

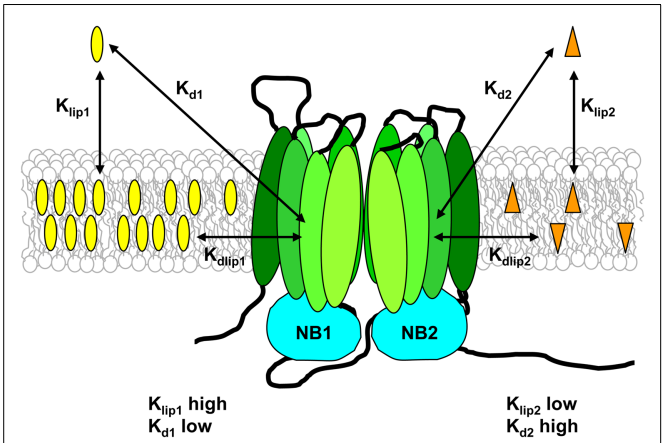

FIGURE 4 | Substrate–membrane relationships and their role in drug binding to Pgp. Pgp substrates partition into the lipid bilayer from the aqueous phase in accordance with their lipid–water partition coefficients (K_{lip}). The drug on the left of the figure has a high K_{lip} value and thus reaches a high concentration in the lipid phase, whereas the drug on the right of the figure has a low K_{lip} value, and therefore has a much lower membrane concentration. K_{lip} values measured for Pgp substrates are typically in the range 300–20,000. The apparent binding affinity (K_d) is determined using aqueous drug concentrations, and depends on both drug partitioning into the lipid phase and drug binding to the transporter from within the membrane. The intrinsic affinity of Pgp for binding substrate from the lipid bilayer (K_{dlip}) can be estimated from the values of K_{lip} and K_d, and is relatively low, in the range 0.5–70 mM.

gel phase bilayers. Van't Hoff plots of binding parameters over a wide temperature range showed different values of the binding free energy above and below the lipid phase transition. Since substrates must leave the lipid bilayer when they bind to Pgp, a less favorable drug–lipid interaction would encourage binding to the transporter. Analysis of the thermodynamic parameters for Pgp-drug binding in a manner which eliminated the contribution of the lipid was therefore carried out. Results indicated that both drug–lipid and drug–Pgp interactions contributed to the overall binding affinity, which was altered by both acyl chain length and lipid phase state, depending on the specific drug under consideration.

DRUG TRANSPORT

Early work showed that small molecule fluidizers and surfactants could reduce Pgp transport function in plasma membrane vesicles and intact MDR cells, leading to increased accumulation of drugs and reversal of drug resistance (9, 84, 143–145). Transport inhibition does not appear to involve direct interaction between these agents and Pgp, and is probably linked to increases in membrane fluidity or permeability (146, 147).

A transport study using Pgp reconstituted into bilayers of the synthetic phospholipid DMPC found that ATP-driven transport of [^{3}H]colchicine into reconstituted proteoliposomes determined by rapid filtration was more than two-fold higher at the T_m of 24°C than in the fluid liquid-crystalline phase at 32°C (8). Using Pgp in bilayers of DMPC and PMPC, the effect of bilayer phase state on the kinetics of ATP-driven transport was explored further (148). The initial rate of transport of the high affinity substrate tetramethylrosamine (TMR, a rhodamine derivative) was measured using a continuous real-time fluorescence assay over a time-scale of less than 1 min. Results showed that drug transport was modulated by the fluidity of the host membrane, with biphasic temperature dependence. The transport rate was high in rigid gel phase lipid, reached a maximum at the melting temperature of the bilayer, and then declined in fluid liquid-crystalline phase lipid. This behavior is unusual, as many membrane transport proteins show low or non-existent transport activity in rigid gel phase lipid, compared with activity in the fluid liquid-crystalline phase. Rigid gel phase lipid is poorly compressible, and it is thought that rate-limiting protein conformational changes are hindered in such an environment. It is possible that Pgp does not have a conformational barrier of this nature.

The rate of transport by Pgp likely depends directly on the drug concentration within the bilayer, and this, in turn, is controlled by the level of membrane partitioning of the substrate. Thus, the rate of drug transport may depend directly on the lipid–water partition coefficient, K_{lip}. Rogers and Davis reported that partitioning of various *p*-alkylphenols into DMPC bilayers also showed biphasic temperature dependence (149). The value of K_{lip} showed a maximum at T_m, declined substantially at temperatures higher than T_m, and remained constant, or declined slightly at temperatures lower than T_m, a pattern very similar to that seen for TMR transport by Pgp in bilayers of DMPC and PMPC (148). The temperature dependence of drug transport by Pgp may thus reflect the temperature dependence of K_{lip} for the substrate.

A recent study examined the effect of bilayer properties on Pgp-mediated transport of H33342 and LDS-751 in DMPC and PMPC bilayers, using real-time fluorescence measurements (122). Membrane partitioning data were also collected for these substrates, which allowed Pgp transport turnover numbers to be obtained in a reconstituted system for the first time. For both drugs, there is a clear discontinuity in transport rates above and below T_m, however, in contrast to the previous studies using TMR, higher transport rates were observed in the liquid-crystalline phase relative to the gel phase. Different temperature dependence of partitioning of the three substrates into gel and liquid-crystalline lipid bilayers may be responsible for this difference in behavior. LDS-751 and H33342 displayed the opposite behavior to TMR; their lipid–water partition coefficients increased with temperature in liquid-crystalline phase bilayers.

Overall, these studies clearly indicate that the membrane phase state is an important factor affecting Pgp's ability to transport drugs. One proposed strategy for reducing the transport activity of Pgp, thus reversing MDR, involves modifying the fluidity of the lipid environment (144). Since the effect of lipid fluidity on drug transport appears to vary depending on the specific substrate being transported, such an approach may not be feasible.

INTERACTIONS OF PGP WITH STEROLS

Cholesterol is a major component of mammalian plasma membranes, making up about 50% of the lipid content on a mole basis, and there have been many observations linking this sterol to Pgp function. Early work in intact cells suggesting the involvement of Pgp in cholesterol esterification was plagued by contradictions and confusion [reviewed in Ref. (123)] resulting from the use of Pgp modulators that later turned out to also inhibit cholesterol metabolism. Drug-selected MDR cells typically show changes in lipid composition, including cholesterol content, which may incidentally affect Pgp function. A study using cell lines where Pgp was inducible concluded that expression of the protein does not play a major role in cholesterol homeostasis (150), and that effects previously noted in drug-selected cells expressing Pgp might result from changes in other pathways that accompany selection. However, a possible physiological role for Pgp in intracellular cholesterol trafficking has been suggested (151). Using Pgp-deficient fibroblasts, replication of *Toxoplasma gondii* was shown to be critically dependent on Pgp, which played a role in the transport of host-derived cholesterol to the intracellular parasite.

Many studies investigating the role of cholesterol in Pgp function in intact cells and native membrane vesicles made use of methyl-β-cyclodextrin (MβCD), a cholesterol-extracting agent, to alter the amount of cholesterol in the plasma membrane (152–155). Essentially, all the cholesterol can be removed from membranes by this compound at a sufficiently high concentration, and inclusion complexes of MβCD with cholesterol can also be used to achieve controlled cholesterol repletion. However, MβCD was shown to directly inhibit the catalytic activity of Pgp, independent of its ability to extract cholesterol from membranes (123), calling into question the conclusions of studies that used this reagent.

Many groups have reported that the presence of cholesterol affects the catalytic activity of Pgp and its stimulation by substrates and modulators. When mouse Pgp was partially purified by hydroxyapatite FPLC using SDS, reconstitution into bilayers without cholesterol was reported to result in four- to five-fold

reduced ATPase activity relative to bilayers where 20–40% cholesterol was incorporated (156). In addition, no stimulation of the activity by verapamil was observed unless cholesterol was included in the bilayer. Further studies showed that the lipids α-tocopherol and DPPC could also enhance verapamil stimulation, although they had no effect on basal ATPase activity (157). However, the ATPase activity of this Pgp preparation was very low, and it is possible that it was either partially denatured or not properly folded because of the somewhat harsh solubilization conditions, which likely strip away endogenous lipids. Callaghan and co-workers reported that the basal ATPase activity of partially purified hamster Pgp in PC–PE liposomes was increased ~two-fold on incorporation of 0–30% cholesterol (115). The fold-stimulation of ATPase activity induced by verapamil was reduced at higher cholesterol levels, although the absolute level of activity increased. Eckford and Sharom found that cholesterol had only a modest overall effect on catalytic activity. Purified Chinese hamster Pgp reconstituted into DMPC bilayers showed a small increase in basal ATPase activity, but a decrease in the level of verapamil-stimulated activity, with increasing cholesterol content from 0 to 30% (123). Cholesterol at high concentrations was able to partially restore the activity of delipidated Pgp, but less effectively than either PC or a PC-cholesterol mixture. Using purified reconstituted human Pgp, Kimura et al. reported that increasing cholesterol in the bilayer from 0 to 20% increased basal ATPase activity and modulated drug-stimulated activity (137).

However, cholesterol is not strictly required for Pgp function. Pgp purified using CHAPS is fully active (ATPase activity and drug transport) in proteoliposomes composed of synthetic phospholipids in the absence of added cholesterol [for example, see Refs. (122, 123, 132, 148)]. Perhaps this is because CHAPS-purified Pgp appears to retain an annular phospholipid layer around it (50). Alternatively, the sterol-like structure of CHAPS may interact with Pgp in a favorable manner, mimicking cholesterol. However, CHAPS does not stimulate Pgp ATPase activity like other detergents that are known to be substrates (96). It seems likely that the effects of cholesterol on both basal and drug-stimulated ATPase activity will differ depending on the specific detergent used to solubilize the protein, the extent to which endogenous lipids have been removed, and the lipid mixture used for reconstitution.

Cholesterol is well known to alter membrane packing, order, and fluidity. Such changes in the local environment may directly modulate the ability of Pgp to bind and hydrolyze ATP, and to transport drugs. Physicochemical changes in the membrane induced by cholesterol may also affect the concentration of drugs in the bilayer by changing their lipid–water partition coefficients. The inclusion of 30% cholesterol in DMPC proteoliposomes reduced Pgp's binding affinity for ATP by ~2.5-fold (123). This is in agreement with a previous report that ATP-binding affinity decreased in bilayers with lower fluidity (132). Photoaffinity labeling of Pgp by the substrate [^{3}H]azidopine was altered by yeast sterols, and cholesterol, suggesting that these lipids affected drug binding (158, 159). Later studies using fluorescence quenching to quantify drug interactions with purified reconstituted Pgp found that inclusion of cholesterol in the bilayer alters binding affinity (123, 124). The effects were dependent on the specific substrate used, with some drugs (e.g., vinblastine) showing a 10-fold increase in K_d value as cholesterol levels increased from 0 to 30%, while others (e.g., daunorubicin) showed essentially no change (123). These complex effects of cholesterol are different for individual drugs, and may be related to drug–lipid partitioning. Eckford and Sharom found that cholesterol had a large effect on drug partitioning into lipid bilayers, reducing the K_{lip} value by two- to nine-fold for several common Pgp substrates, with the exception of Hoechst 33342, which displayed a ~two-fold increase in K_{lip} (123). These differences may arise from the specific localization within the bilayer of drugs and cholesterol, which will modify the properties of different regions of the membrane selectively.

A water-soluble cholesterol analog, polyoxyethylcholesteryl sebacate, competed with daunorubicin for binding to Pgp (160), and cholesterol co-eluted with purified Pgp in detergent (137), however, it is still not clear whether these interactions are specific. Using the kinetics of drug-stimulated ATPase activity as a surrogate measure for drug-binding affinity, Kimura et al. reported that the presence of cholesterol in reconstituted proteoliposomes increased Pgp's binding affinity for small molecular mass drugs (<500 Da), but not for larger drugs (800–900 Da) (137). They proposed that cholesterol may occupy the substrate-binding pocket simultaneously with smaller drug molecules, filling the empty space and thus promoting their binding (161). However, when drug binding to reconstituted Pgp was measured directly using fluorescence quenching, no relationship between drug size and the effect of cholesterol on binding affinity was found (123). A large drug (vinblastine) showed a large reduction in binding affinity at high bilayer cholesterol, whereas a small drug (daunorubicin) showed little change in affinity.

The effect of cholesterol on the ability of Pgp to flip fluorescent NBD–PC and transport three different substrates were explored in reconstituted proteoliposomes (123). A modest decrease in flippase activity was noted at 20–30% cholesterol, and the ability of vinblastine to compete for flippase activity was enhanced. Inclusion of cholesterol in the bilayer showed biphasic effects on the initial rate of transport of TMR and Hoechst 33342. Net transport into proteoliposomes is a balance between inward active pumping of substrate by Pgp and passive outward efflux through the bilayer. These results were explained by cholesterol altering the membrane permeability and partitioning of substrates, which affect transport indirectly (123).

Whether Pgp is directly involved in outward movement of cholesterol across the plasma membrane has been the subject of debate. The effect of Pgp on the transbilayer distribution of cholesterol in native membrane vesicles was monitored using cholesterol oxidation by cholesterol oxidase. (152). It was concluded that Pgp was responsible for ATP-dependent transport of cholesterol from the cytoplasmic to the extracellular leaflet of the membrane. However, the estimated rate of spontaneous cholesterol movement between membrane leaflets was very slow, with a half-time of ~10 min. This contradicts other reports of very fast intrinsic flip–flop [reviewed in Ref. (162)], which suggest that cholesterol does not require a transport protein to move between bilayer leaflets. Eckford and Sharom confirmed rapid flip–flop of two cholesterol analogs in native membrane vesicles and proteoliposomes, faster than the time-resolution of the cholesterol oxidase assay, and also failed to show Pgp-mediated flip–flop of

cholesterol (123). Since Pgp mediates ATP-dependent translocation of SM (44), which has a high affinity for cholesterol (163), it may indirectly influence cholesterol distribution in membranes. It is unlikely that Pgp is directly involved in moving cholesterol between membrane leaflets.

LIPID RAFTS AND PGP

The cholesterol content of the plasma membrane is highly regulated and relatively invariant. However, some domains are selectively enriched in cholesterol, namely lipid rafts and caveolae, which are characterized by the presence of higher lipid order, lower density, and detergent resistance (164). In intact cells, dynamic movement of Pgp into and out of these more ordered, cholesterol-rich domains could potentially regulate its functions. Pgp has been proposed to localize in low density raft microdomains and caveolae in some cell and tissue types [for a detailed list, see Ref. (165)]. However, in one MDR cell line, Pgp was present in intermediate density Brij-96 domains that were biochemically and physically distinct from both classical low density lipid rafts and caveolae (166), and in another case, Pgp was found not to be associated with lipid rafts at all (167). Thus, any proposed role for these domains in functional modulation of Pgp must necessarily be cell type-specific. Typically, treatments that break up lipid rafts (e.g., MβCD) result in loss of Pgp from raft domains, and a decrease in its function [for example, see Ref. (168)]. However, interpretation of these results is complicated by the fact that MβCD has secondary effects besides cholesterol sequestration; it can extract other membrane components such as phospholipids (169), and it is also known to inhibit Pgp catalytic function directly (123). One other source of variation in assessing the association of Pgp with these domains is the methodology used to isolate lipid rafts. Several different detergents have been commonly used for their extraction from intact cells, including Triton X-100, Brij-96, and Lubrol, and detergent-free approaches using carbonate have also been employed [see Ref. (165)]. It has been suggested that lipid rafts domains are concentric layered structures, with different sensitivities to detergent extraction at their periphery compared to the central core (170). This may explain why Pgp is typically strongly associated with Lubrol- or Brij-96-based rafts, but less so with Triton X-100 rafts (171).

Perhaps not surprisingly, Callaghan and co-workers showed that Pgp was fully functional after reconstitution into liquid-ordered (l_o phase) membranes rich in sphingolipids and cholesterol, i.e., with a typical "lipid raft" composition (172). The main difference noted between Pgp behavior in raft-like proteoliposomes and in those composed of PC alone was that drugs were able to stimulate or inhibit ATPase activity at lower concentrations. This is compatible with reports that Pgp binds its substrates more tightly in less fluid lipid bilayers (122, 124).

DETERGENTS AND MEMBRANE FLUIDIZERS AS PGP MODULATORS

Lipophilic compounds cross lipid bilayers by a three-step process involving partitioning into the interfacial region, diffusion through the hydrophobic core, and desorption from the opposite side of the membrane. Since many compounds, including Pgp substrates, are localized in specific regions of a lipid bilayer, the second step may be thought of as flip–flop (173), and is the slowest, rate-limiting step for movement of amphiphilic species across membranes. For example, doxorubicin flip–flop across lipid bilayers occurs with a half-time of ~1 min (174). The ability of substrates and modulators to interact with Pgp may depend on their ability to flip–flop between membrane leaflets. For example, some positively charged drugs and peptide modulators cannot interact with Pgp in intact cells if supplied on the extracellular side, probably because they have a very low rate of flip–flop to the inner leaflet (175). However, they can interact with Pgp in membrane vesicles, where the cytoplasmic leaflet is accessible. In support of this idea, substrate concentrations were found to be significantly lower in the cytosolic leaflet of intact cells expressing Pgp than in the cytosolic leaflet of inside-out membrane vesicles (176).

P-glycoprotein may handle classical modulators in exactly the same way as drugs, i.e., they are transported with hydrolysis of ATP (37, 177). The difference in the behavior of modulators and drugs may be related to their rate of flip–flop across the membrane (37). Pgp substrates were found to cross lipid bilayers relatively slowly (half-time for rhodamine 123 of ~3 min), while the transbilayer diffusion rates of several modulators were extremely fast. Pgp moves drugs and modulators (those that are transported) to the outer membrane leaflet, or they re-enter it after being moved to the extracellular medium. The rate of flip–flop to the inner leaflet is proposed to be slow for substrates, so that Pgp can keep pace, establish a drug concentration gradient across the membrane, and ultimately cause drug resistance. The long residence time of substrates in the inner leaflet also allows more opportunity for interaction with Pgp (174). For modulators, the rate of transbilayer flip–flop is so rapid that Pgp cannot keep pace or establish a concentration gradient, and MDR cells are not resistant to them. Work by Seelig and co-workers confirmed these ideas (176). Pgp in intact cells was found to transport substrates at a rate proportional to that of ATP hydrolysis, however, it could only prevent substrates from entering the cytosol if their rate of diffusion across the lipid bilayer was slow, in a range similar to that of Pgp-mediated efflux. This model suggests that for a modulator to be effective, it should bind to Pgp with high affinity and also have a high transbilayer diffusion rate (36, 177). If a compound modifies membrane properties to increase the rate of transbilayer movement of a drug sufficiently, it may be able to circumvent resistance without interacting specifically with Pgp. Thus, a second class of Pgp modulators may exist, consisting of agents such as surfactants and membrane fluidizers. In fact, several compounds that fall into this category are already known to reverse MDR (e.g., Pluronic block copolymers, Cremophor EL, Solutol HS15, Tween 80) (143, 178). Surfactants are ubiquitous in cleaning products, and are also present as additives in food and cosmetics. Since they are typically of very low toxicity, they may represent a useful class of Pgp modulators.

Evidence for the idea that Pgp-mediated MDR can be modulated by acceleration of passive drug permeation across the plasma membrane is contradictory. Tween 80 and Cremophor EL inhibited Pgp function in cell monolayers, increasing the apical-to-basolateral permeability and decreasing the basolateral-to-apical permeability of the substrate rhodamine 123 (179). These effects were related to the ability of the surfactants to increase membrane

fluidity, in contrast with OG, which did not either modulate membrane fluidity or affect Pgp transport. However, polyoxyethylene surfactants that reversed MDR actually decreased lipid fluidity in plasma membrane vesicles from MDR cells, as assessed using several fluorescent probes (147), whereas surfactants that did not reverse MDR did not influence membrane fluidity. Low concentrations of Pluronic 61 were found to greatly increase the rates of phospholipid flip–flop and transbilayer movement of doxorubicin in lipid bilayers (180). Anesthetics (benzyl alcohol, chloroform, and diethylether) and non-ionic detergents (Tween-20, Nonidet P-40, and Triton X-100), increase membrane fluidity and the rate of transbilayer drug flip–flop (144). These compounds also abolish Pgp ATPase activity and drug binding, possibly by increasing membrane fluidity. Recent work in intact cells reported different results from experiments using liposomes and plasma membrane vesicles (146). Anesthetics were found to modulate MDR by accelerating transbilayer drug movement, whereas Pluronic P85, Tween-20, Triton X-100, and Cremophor EL had no effect on drug movement, and modulated MDR by inhibiting Pgp-mediated efflux. No correlation was found between the ability of surfactants to accelerate drug movement and their membrane fluidizing effects (146). Thus the molecular mechanism by which surfactants and membrane fluidizers inhibit the action of Pgp is still very much an open question.

CONCLUSION AND FUTURE PROSPECTS

P-glycoprotein is an unusual transporter, and various aspects of its function appear to be modulated by the lipid environment in novel and complex ways. Its mode of action as a hydrophobic vacuum cleaner and lipid/drug flippase make it especially sensitive to the properties of the surrounding lipid bilayer. Membrane composition, fluidity, and phase state all appear to be important parameters affecting Pgp stability, ATP binding, ATP hydrolysis, drug binding, and drug transport. Substrate interactions with the lipid bilayer play a critical role in the overall process of drug binding to Pgp, and are also modulated by the physicochemical properties of the membrane. It has been proposed that changing the properties of the host membrane may be a useful approach for clinical modulation of MDR. Clearly, an enhanced understanding of how all aspects of the Pgp catalytic cycle are affected by the local lipid microenvironment is essential if this strategy is to be successful. For example, the relationship between membrane partitioning and the binding affinity of Pgp substrates suggests one way to reduce or circumvent drug resistance. If a chemotherapeutic drug can be chemically modified to reduce its lipid–water partition coefficient, the ability of Pgp to transport it might be reduced. This would allow the drug to reach its intracellular targets, and thus increase its clinical effectiveness. Recent work on Pgp-membrane interactions has advanced to the point where it is now possible to measure thermodynamic and kinetic constants for the various steps of the catalytic cycle in model systems. Although the relationship between Pgp and its membrane environment is likely to be much more complex in living cells, this approach may provide a rational basis for novel strategies to overcome Pgp-mediated MDR in human tumors. It may also lead to a better understanding of the molecular mechanism of this enigmatic transporter.

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Where is it and how does it get there – intracellular localization and traffic of P-glycoprotein

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P-glycoprotein (P-gp), an ATP-binding cassette, is able to transport structurally and chemically unrelated substrates. Over-expression of P-gp in cancer cells significantly decreases the intercellular amount of anticancer drugs, and results in multidrug resistance in cancer cells, a major obstacle in cancer chemotherapy. P-gp is mainly localized on the plasma membrane and functions as a drug efflux pump; however, P-gp is also localized in many intracellular compartments, such as endoplasmic reticulum, Golgi, endosomes, and lysosomes. P-gp moves between the intracellular compartments and the plasma membrane in a microtubule-actin dependent manner. This review highlights our current understanding of (1) the intracellular localization of P-gp; (2) the traffic and cycling pathways among the cellular compartments as well as between these compartments and the plasma membrane; and (3) the cellular factors regulating P-gp traffic and cycling. This review also presents a potential implication in overcoming P-gp-mediated multidrug resistance by targeting P-gp traffic and cycling pathways and impairing P-gp localization on the plasma membrane.

Keywords: P-glycoprotein, intracellular localization, traffic, recycling, cell polarization, multidrug resistance in cancer

INTRODUCTION

P-glycoprotein (P-gp), a 170 kDa membrane protein, is a member of sub-family B of the ATP-binding cassette (ABC) transporter superfamily, and is also called ABCB1. P-gp has two structurally identical halves. Its N-terminal half contains six transmembrane domains, followed by a large cytoplasmic domain with an ATP-binding site. Similarly, the C-terminal half also has six transmembrane domains and an ATP-binding site (1, 2). Plasma membrane located P-gp is able to transport many chemically and structurally unrelated substrates out of the cells, and acts as an efflux pump (1, 2). P-gp is primarily expressed in the liver, kidney, gastrointestinal tract, and blood brain barrier. P-gp is located on the canalicular apical membrane of hepatocytes in the liver; on the brush border of proximal tubule cells in the kidney; and on the apical membrane of mucosal cells in the small intestine (3). Given the transporting function of P-gp, these tissue distributions allow P-gp to excrete endogenous metabolites, exogenous substrates, and toxins into the urine, bile, and feces. Thus, P-gp can protect the organism as well as eliminate cellular wastes (3, 4). Furthermore, another essential localization of P-gp is on the luminal surface of capillary endothelial cells of the blood brain barrier which prevents cytotoxins from penetrating the endothelium and protects brain (5).

Although animal well-being, normal physiological function, and life span were not affected after P-gp was knocked out in mice, higher drug sensitivity and increased drug side effect/toxicity occurred (6). While knocking out P-gp appears to be less problematic, over-expression of P-gp causes major concerns in clinical oncology. The most notable consequence of over-expression of P-gp in clinic is to cause multiple drug resistance (MDR) in cancer chemotherapy (2). Given P-gp has a structurally broad range of substrates, the occurrence of MDR during chemotherapy is one of the big challenges for successful cancer treatment in clinic. MDR can be either intrinsic, occurring in cancers that have not been exposed to chemotherapy before but derived from tissue naturally expressing P-gp (e.g., liver, kidney, intestinal cancers), or required MDR, which develops after cancers are treated with chemotherapy (7). Nearly half of human cancers express P-gp at levels sufficient to develop MDR. The likelihood of failure in chemotherapy is increased when P-gp expression is upregulated during therapy (8).

INTRACELLULAR LOCALIZATION OF P-gp

P-glycoprotein is primarily localized on the plasma membrane for its efflux function, however, it is also localized intracellularly (9, 10). Using immunofluorescence and over-expression of P-gp-GFP fusion protein approaches, co-localization results revealed that P-gp is localized in many cellular organelles, including endoplasmic reticulum (ER) (9), Golgi (9), early endosome (11–13), recycling endosome (12), later endosome, lysosome (9, 11), and proteasome (14) (**Figure 1**). These intracellular localizations link to synthesis (ER), modification (Golgi), traffic/recycling (Golgi and endosomes), and degradation (lysosome and proteasome) sites for P-gp. Although one study suggests P-gp is also located in mitochondria in doxorubicin-resistant K562 human leukemia cells (15), others reveal that P-gp is not presented in mitochondria either in MCF-7 (ADR) human breast cancer and KB-V1 human cervix carcinoma drug resistant cell lines (16) or in primary rat hepatocytes (17). Furthermore, transient transfection of P-gp-GFP in cancer cells reveals that the ER and Golgi localization of P-gp appears to be transient, suggesting that P-gp can rapidly traffic to the endosomal compartment and the plasma membrane

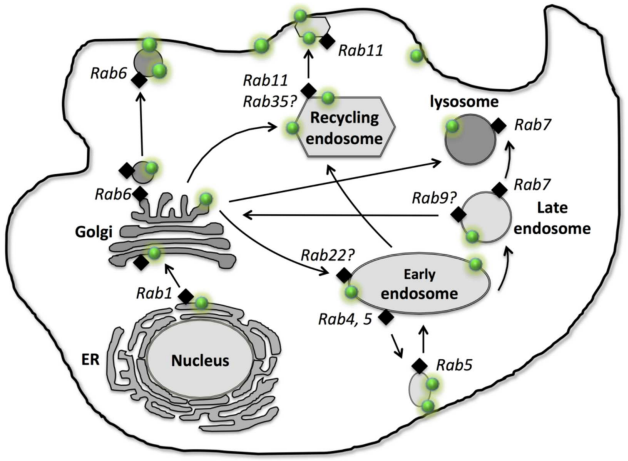

FIGURE 1 | Intracellular localization, traffic, and recycling of P-glycoprotein. P-gp is shown as "green dot ●." Different Rab GTPases that are involved in P-gp traffic and recycling are indicated as "black diamond ◆." Arrows represent the traffic and recycling path among the intracellular organelles and between the cellular organelles and the plasma membrane.

localization after it is synthesized in ER and modified in Golgi. This rapid transport to the membrane localization explains why less ER or Golgi localization can be observed in the stable cell line which is overexpressed with P-gp-GFP. It is possible that activity of P-gp synthesis remains at a relatively low level due to the very long half-life of P-gp (14–17 h) in the stable cell line (18). Similarly, the degradation localization (lysosome) also appears to be less common within the cells compared to the endosomal localization which is involved in constantly trafficking/recycling P-gp between the cellular pool and the plasma membrane (11).

INTRACELLULAR TRAFFIC AND RECYCLING OF P-gp

After synthesis in ER, P-gp first needs to be correctly folded before exit from ER and entry to Golgi for modification. Currently, very little information is available about the exact regulatory process for P-gp folding in ER. Glycoprotein glucosyl transferase (UGGT) is able to sense the folding states of glycoproteins, resulting in mis-folded glycoprotein rebinding calnexin (a chaperon for protein folding) and going through re-folding cycles or being rapidly degraded via endoplasmic-reticulum-associated protein degradation (ERAD) (19). Thus, UGGT may play a potential role in recognizing the folding of P-gp. Moreover, a study suggests that SPTLC1 (Serine palmitoyltransferase enzyme 1) is able to interact with ABCA1 and cause ER retention of ABCA1, revealing the role of SPTLC1 in ER exit of ABC transporters (20). Furthermore, the formation of disulfide bonds is a critical step in the maturation of the majority of the proteins inside ER (21). Studies showed that two other ABC transporters, ABCB6 and ABCC8, form the disulfide bonds between highly conserved cysteine, which is important for these ABC transporters to exit ER and traffic to the plasma membrane (22). We still lack direct evidence of how P-gp export at the ER – the first step of its trafficking along the biosynthetic secretory pathway. In mammalian cells, the ER export occurs via Coat Protein II (COPII)-coated vesicles. COPII vesicles bud from the ER and are able to fuse to ER-Golgi intermediated compartment (23). Studies reveal that COPII plays an essential role in exporting ABCB1 and ABCC7 (cystic fibrosis transmembrane conductance regulator, CFTR) from ER to Golgi (24, 25), suggesting COPII may regulate ER export of P-gp as well. Golgi is involved in the biosynthesis of glycan chains of glycoproteins, the 150 kDa P-gp is transported to the Golgi and glycosylated as the 170 kDa mature protein (26) (**Figure 1**).

After its modification in Golgi, the 170 kDa mature P-gp traffics to the plasma membrane. Membrane proteins can traffic to the plasma membrane via either the constitutive pathway which involves membrane protein-containing vesicles moving directly to the plasma membrane (27, 28) or the endosomal pathway in which protein-containing vesicles are first transported to endosomal compartments to establish the intracellular pool, and then traffic to the plasma membrane (29). In both cases, the cytoskeleton is needed for the traffic of these membrane protein vesicles (30). The *trans*-Golgi network (TGN) is a major sorting site for proteins trafficking to the plasma membrane and endosomal pathway. A study suggests that membrane proteins use *N*-glycan chains as sorting determinants (31); whether this also applies to P-gp needs to be investigated. However, some studies show that N-glycosylation at amino acid residue asparagine 596 at third extracellular loop is not necessary for ABCG2 traffic (32), and the immature core-glycosylated CFTR (ABCC7) can be transported to the plasma membrane and is functional (33).

In the non-polarized cancer cells, P-gp was reported localized in EEA1 and Rab5 positive early endosome which serves as an intracellular reservoir prior to P-gp moving to the plasma membrane (11), suggesting that P-gp can traffic to the plasma membrane via the indirect endosomal pathway. Furthermore, immunofluorescence study showed that P-gp is also localized in lysosome after transient expression of human P-gp-GFP in HeLa cells or in the human breast cancer MCF-7 cells, which are stably expressed with P-gp-GFP, suggesting P-gp can be moved to the lysosomal degradation compartment, presumably through the early and late endosome (12). However, an immunofluorescence study reveals that P-gp is not localized in Rab11 positive recycling endosomes in human breast cancer MCF-7 cells, which stably express P-gp-GFP (11). In the polarized WIFB9 cells as well as hepatocytes, apical ABC transporters (e.g., P-gp, ABCB11) move to the apical membrane via Rab11a recycling endosomes and recycle between the apical membrane and the Rab11a positive intracellular endosomal pool (29, 34) (**Figure 1**). These studies suggest that P-gp traffics and recycles via different endosomal pathways (early endosome vs. recycling endosome) in non-polarized cells (e.g., cancer cells) and polarized cells.

REGULATION OF P-gp TRAFFIC AND RECYCLING – ROLE OF Rab GTPases

Rab GTPases, the largest branch of small GTPase, are known to regulate vesicular transport in exocytosis, endocytosis, and recycling by controlling many steps in membrane trafficking such as vesicle formation, movement, uncoating, docking, and fusion (35). So far more than 70 Rab GTPases have been identified in humans (35). Each Rab protein is believed to be specifically associated

with a particular organelle or pathway (35). Currently, few studies revealed the role of Rab proteins in P-gp trafficking, thus, the more general involvement of Rab proteins in ABC transporters trafficking and recycling will be discussed.

Rab1, 2, and 6 are localized in ER and Golgi and regulate vesicle transport along the ER-Golgi biosynthetic pathway. Rab1, 2 regulate vesicle movement from ER to Golgi (35, 36), while Rab6 is involved in Golgi to the cell surface for exocytosis of newly synthesized proteins and lipids (37). Study showed that P-gp was predominantly intracellular, largely in Rab6-containing Golgi vesicles and Golgi cisternae (7), suggesting Rab6 may regulate P-gp traffic directly from Golgi to the plasma membrane (**Figure 1**).

Along the endosomal trafficking pathway, Rab11 and Rab13 are involved in membrane protein traffic from Golgi to the recycling endosome (38–40). Rab11a was shown to regulate P-gp and ABCB11 traffic to the apical membrane in polarized WIFB9 cells (29, 34) (**Figure 1**). Furthermore, studies showed that Rab11a was also needed for both WT-CFTR and ΔF508-CFTR to undergo trafficking to the apical recycling compartment in polarized human airway epithelia cells (41) as well as polarized intestinal epithelial cells (42). These studies reveal that, in polarized cells, P-gp traffics to the apical membrane via the Rab11a positive recycling endosome. However, in non-polarized MCF-7 cells, stable expressed P-gp-EGFP did not co-localize with Rab11 positive recycling endosome (11), suggesting P-gp does not traffic to the cell surface via the Rab11a positive recycling endosome. Evidence indicates that membrane trafficking of CFTR is cell type-specific and it differs in polarized human airway epithelial cells such as CFBE41o- cells and in non-polarized fibroblasts such as BHK-21 cells (41). Thus, Rab11a may have a differential role in P-gp trafficking in non-polarized and polarized cells. Other Rab proteins, such as Rab17, Rab25, Rab35, and Rab40 are also localized in recycling endosome (36, 43). Rab35 is shown to play important role in insulin-stimulated GLUT4 (Glucose transporter type 4) translocation in adipocytes (44). Further studies are needed to investigate the role of Rab11 as well as other Rab proteins (e.g., Rab17, Rab25, Rab35, and Rab40) in P-gp traffic in both polarized and non-polarized cells.

Early endosomal pathway is shown to be involved in P-gp trafficking and recycling. Among many of the early endosomal Rab proteins, Rab4 and Rab5 are known to regulate P-gp trafficking and recycling in many cancer cells (12, 13, 45). Over-expression of dominant-negative Rab5 mutant (S34N-Rab5) results in large intracellular accumulation of P-gp-EGFP in non-polarized Hela cells, and similar cellular accumulation of wild type P-gp in multidrug resistant MCF-7/Adr cells, revealing that Rab5 regulates P-gp exocytosis from the endosome compartment (such as early endosome) to the plasma membrane in non-polarized cells (12). In contrast, another study in colon cancer cells LS174T, demonstrated that over-expression of wild-type Rab5 resulted in recycling P-gp from the plasma membrane into intracellular compartments, suggesting that Rab5 regulates P-gp endocytosis instead of exocytosis (13), however, this study did not investigate the polarization status of the cell culture of LS174T, which is shown to be polarized in normal culture condition (46). Thus, the differential role of Rab5 in P-gp trafficking and recycling in these cancer cells may be related to the difference in polarization (**Figure 1**).

Rab4 is also localized in early endosome and shown to regulate P-gp exocytosis in drug resistant leukemia cells, K562ADR (45). Cell surface expression of P-gp decreased after over-expression of GFP-Rab4 or constitutively active Rab4Q72L mutant, but not dominant-negative Rab4S27N mutant or Rab14 in the K562ADR cells, suggesting that Rab4 regulates P-gp trafficking to the plasma membrane from endosomal compartments (45). However, in HeLa cells, the intracellular localization of P-gp-EGFP was not affected when there was over-expression of either wild-type Rab4 or dominant-negative mutant N121I-Rab4 respectively (12), indicating Rab4 does not effect P-gp trafficking. The different cell lines used in these studies may be the reason for obtaining different results. Given many other Rab proteins, such as Rab10, 14, 15, 17, 22, and 23 are localized on early endosome (43), it is likely these Rab proteins can also regulate the trafficking and recycling of P-gp between the early endosomal compartment and the plasma membrane in a cell and tissue type-specific manner.

Transmembrane proteins can be transported from late endosome to lysosome which is responsible for degradation of the membrane proteins (47). P-gp is localized in Lamp-2 positive lysosome, revealing its lysosomal dependent degradation pathway (11). Rab7 is localized on late endosome and lysosome, and is essential for later endocytic membrane trafficking from late endosome to lysosome (48, 49), while Rab9 is localized on later endosome and responsible for transit from later endosome to the TGN (50). Although very little is known about the role of Rab7 and 9 on later endocytic membrane trafficking of P-gp, Rab7 and 9 are shown to regulate other ABC transporter trafficking (e.g., CFTR). Using over-expression of wild type and mutant Rab GTPases, a study revealed that CFTR could enter Rab7-dependent late endosomal traffic or Rab9-mediated translocation to the TGN (51), suggesting the role of both Rab7 and 9 in later endocytic trafficking of CFTR. However, the potential roles of Rab7 and 9 on P-gp trafficking need to be investigated (**Figure 1**).

CLINICAL IMPLICATIONS

P-glycoprotein plays an important role in drug excretion and is one of the main causes for MDR in cancer chemotherapy. Different generations of P-gp inhibitors are developed and enter into preclinical and clinical studies. The first generation of P-gp inhibitors, such as verapamil and cyclosporin A, are active substrates of P-gp. Both verapamil and cyclosporin A cause side effects in patients due to high dose of the drugs are need for their inhibition of P-gp (52, 53). The second generation of P-gp inhibitors include valspodar (PSC 833), dexverapamil, and dofequidar fumarate. However, these second generation inhibitors also inhibit drug metabolism enzymes and other ABC transporters, which results in impaired drug metabolism and elimination (54–56). The third generation of P-gp inhibitors, which are currently undergoing clinical trials, include zosuquidar (LY335979), elacridar (GF120918), CBT-1, and XR9576 (57–60). However, some of the trials are unsuccessful in improving therapeutic efficacy (61). For example, trial was stopped in patient with non-small-cell lung cancer due to chemotherapy-related toxicity after administration of XR9576 (60). Thus, there

is an urgent need to develop innovative strategies to overcome P-gp-mediated MDR in cancer chemotherapy.

Given P-gp needs to be transported to the plasma membrane so as to efflux the anticancer drugs out of cells, blocking the trafficking of P-gp to its final destination – the plasma membrane location – can be an innovative approach to overcome MDR and improve therapy. There are multiple potential targets along the P-gp traffic pathway. Study revealed that inhibition of P-gp maturation resulted in accumulation of P-gp in Golgi, and this immature P-gp in Golgi was inactive and presumably led to degradation. Consequently there was an increased cellular accumulation of P-gp substrate (62). Experiments also reveal that blocking P-gp traffic to the plasma membrane by interrupting the cytoskeleton highway or modulating Rab activation can cause increased intracellular accumulation of P-gp, resulting in more intracellular retention of anticancer drug (9, 11, 12). Although the intracellular P-gp that is trapped on the way to the plasma membrane remains active, it is likely that intracellular P-gp do not contribute to drug resistance (63). It is essential to identify the regulatory effectors, such as specific Rab GTPases or Rab binding proteins for P-gp traffic and recycling, and to screen potential candidates for targeting these effectors. Therefore, more studies are needed to understand the molecular and cellular mechanisms of P-gp intracellular traffic/cycling and its regulatory factors.

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In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: a practical guide with case studies

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The development of a drug-resistant cell line can take from 3 to 18 months. However, little is published on the methodology of this development process. This article will discuss key decisions to be made prior to starting resistant cell line development; the choice of parent cell line, dose of selecting agent, treatment interval, and optimizing the dose of drug for the parent cell line. Clinically relevant drug-resistant cell lines are developed by mimicking the conditions cancer patients experience during chemotherapy and cell lines display between two- and eight-fold resistance compared to their parental cell line. Doses of drug administered are low, and a pulsed treatment strategy is often used where the cells recover in drug-free media. High-level laboratory models are developed with the aim of understanding potential mechanisms of resistance to chemotherapy agents. Doses of drug are higher and escalated over time. It is common to have difficulty developing stable clinically relevant drug-resistant cell lines. A comparative selection strategy of multiple cell lines or multiple chemotherapeutic agents mitigates this risk and gives insight into which agents or type of cell line develops resistance easily. Successful selection strategies from our research are presented. Pulsed-selection produced platinum or taxane-resistant large cell lung cancer (H1299 and H460) and temozolomide-resistant melanoma (Malme-3M and HT144) cell lines. Continuous selection produced a lapatinib-resistant breast cancer cell line (HCC1954). Techniques for maintaining drug-resistant cell lines are outlined including; maintaining cells with chemotherapy, pulse treating with chemotherapy, or returning to master drug-resistant stocks. The heterogeneity of drug-resistant models produced from the same parent cell line with the same chemotherapy agent is explored with reference to P-glycoprotein. Heterogeneity in drug-resistant cell lines reflects the heterogeneity that can occur in clinical drug resistance.

Keywords: chemotherapy, cancer, drug-resistance, cell lines, selection strategy

INTRODUCTION AND HISTORICAL PERSPECTIVE

The development of chemotherapy drug-resistant cancer cell lines is a long established approach for investigating the mechanisms of cytotoxicity and resistance to chemotherapy agents. One of the first publications to describe the development of an anti-cancer drug-resistant *in vitro* model, which exhibited acquired resistance to a chemotherapy drug, was published in 1970 (1). Resistant cell lines were developed from parental Chinese hamster cells using a stepwise increase in treatment dose with actinomycin D. This induced 2500-fold greater resistance to the drug than that observed in the parental cells. These resistant cell lines were also cross resistant to other chemotherapy drugs such as vinblastine and daunorubicin. Some earlier drug-resistant cell lines were developed in the 1950 and 1960s using *in vivo* mouse models, including models resistant to methotrexate (2, 3), vinblastine, terephthalanilide (4), and the guanine analog, 8-azaguanine (5).

Publications in this research field usually place little emphasis on how the drug-resistant cell lines were established in the laboratory. The development of drug-resistant cell lines can take anything from 3 to 18 months in the laboratory and many decisions are taken along this journey. This review summarizes the major methodological approaches for developing drug-resistant

Abbreviations: 5-FU, fluorouracil; ADR, adriamycin; AUC, area under concentration time curve; CHL, chlorambucil; CIS, cisplatin; CR, complete response; CSC, cancer stem cells; CYC, cyclophosphamide; DNR, daunorubicin; DOCE, docetaxel; DOX, doxorubicin; EPI, epirubicin; ETO, etoposide; GFR, glomerular filtration rate; IC_{50}, 50% inhibitory concentration; IFO, ifosfamide; IV, intravenous; MEL, melphalan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, no response; OX, oxaliplatin; PARP, poly (ADP-ribose) polymerase; PD, progressive disease; P-gp, P-glycoprotein; PR, partial response; PRED, prednimustine; RAD, radiation; SCLC, small cell lung cancer; TAX, paclitaxel; THI, thiotepa; UNK, unknown; VINC, vincristine; VIND, vindesine.

cell lines *in vitro* with reference to the literature and includes several case studies from our experience.

IC_{50} VALUES AND FOLD RESISTANCE

Drug-resistant cell models are developed in the laboratory by repeatedly exposing cancer cells growing in cell culture to drugs. The surviving daughter resistant cells are then compared to the parental sensitive cells using combination cell viability/proliferation assays such as the MTT (6), acid phosphatase (6), or clonogenic assays (7). The sensitivity of these paired cell lines is usually determined by exposing them to a range of drug concentrations and then assessing cell viability. The IC_{50} (drug concentration causing 50% growth inhibition) for these paired cell lines can be used to determine the increase in resistance known as fold resistance by the following equation:

$$\text{Fold Resistance} = IC_{50} \text{ of Resistant Cell Line}/IC_{50} \text{ of Parental Cell Line}$$

WHAT IS A CLINICALLY RELEVANT LEVEL OF RESISTANCE?

To determine the level of drug resistance that occurs in the clinical treatment of cancer we can compare cell lines that have been established from cancer patients before and after chemotherapy (**Table 1**) (8–14). The majority of cell lines listed in **Table 1** developed from patients post-chemotherapy show a two- to five-fold increase in resistance to the agents the patients were treated with, based on a comparison of IC_{50} values. Three cell lines had higher levels of resistance but these were still relatively low-level at ~8–12-fold higher than the parental cells (PEO4, SK-3, and GLC-16).

CLINICALLY RELEVANT VS. HIGH-LEVEL LABORATORY MODELS

For the purposes of this review we will divide drug-resistant cell models into two categories: clinically relevant models or high-level laboratory models. Both types of models have their advantages and disadvantages for research.

Clinically relevant models are developed with the aim of trying to mimic the conditions cancer patients experience during chemotherapy. Doses of drug are lower, and a pulsed treatment strategy is often used where the cells recover in drug-free media. This mimics the cycles of chemotherapy a patient receives in the clinic. Disadvantages to clinically relevant models can include unstable resistance, very low-level resistance, and small molecular changes to detect and analyze. Based on the cell lines derived from the patients before and after chemotherapy shown in **Table 1**; we have defined clinically relevant resistance as a two- to five-fold increase from the IC_{50} value of the parent cell line. Examples of clinically relevant models are shown in **Table 2** (15–21).

High-level laboratory models are developed with the aim of understanding potential mechanisms of toxicity and resistance to chemotherapy agents. Doses of drug are often high and treatment doses are escalated over time. Cells are frequently grown continually in the presence of drug or highly drug-resistant clones are selected from a mixed population. In some earlier drug-resistant models, mutagenesis was also induced prior to drug treatment (22,

Table 1 | Cell lines established from cancer patients before and after chemotherapy.

Cancer type	Parent cell line (established)	Chemotherapy received	Resistant cell line (established)	Fold resistance to chemotherapy received	Reference
Lung	EBC-2 (18th September 1997)	CIS, IFO, VIND	EBC-2/R (4th October 1997)	CIS – 2.3, IFO[a] – 3.2, VIND – 0.77	(8)
	SK-1 (August 1986)	CYC, ADR, ETO, VINC, RAD	SK-2 (March 1987)	ADR – 1.2, ETO – 1.2, CYC[b] – 1.3	(10)
		CIS, ETO	SK-3 (May 1987)	CIS – 8.6, ETO – 6.2	
	TM1 (April 1987)	CYC, ADR, ETO, VINC	TM2 (September 1987)	CYC[b] – 5.4, ADR – 3.0, ETO – 3.5	
	GLC-14 (December 1984)	CYC, DOX, ETO	GLC-16 (October 1985)	DOX – 3.18, ETO – 12.1	(11)
Neuroblastoma	KP-N-AY (October 1984)	ADR, CIS, CYC, VINC	KP-N-AYR (December 1985)	ADR – 3.0, CIS – 2.7	(9)
Ovarian	PEO1 (February 1982)	CIS, CHL, 5-FU	PEO4 (November 1982)	CIS – 8.72	(12, 13)
		CIS, CHL, 5-FU	PEO6 (February 1983)	CIS – 4.64	(12, 13)
	PEA1	CIS, PRED	PEA2	CIS – 4.30	(13, 14)
	PEO14	CIS, CHL	PEO23	CIS – 4.48	(13, 14)

ADR, adriamycin; CIS, cisplatin; CHL, chlorambucil; CYC, cyclophosphamide; DOX, doxorubicin; ETO, etoposide; 5-FU, fluorouracil; IFO, ifosfamide; RAD, radiation; PRED, prednimustine; VINC, vincristine; VIND, vindesine.

[a] *Used 4-hydroperoxy ifosfamide (the active form of ifosfamide).*

[b] *Used 4-hydroperoxycyclophosphamide (the active form of cyclophosphamide).*

Table 2 | Different selection strategies and classification of resulting drug-resistant cell lines.

Cancer type	Parent cell line	Selecting agent	Exposure	Dose	Population	Resistant cell line	Fold resistance to selecting agent	Development time (months)	Classification	Reference
Cervical	KB-3-1	CIS	Continuous	Stepwise and	Whole	KBCP10	1152	UNK	High-level lab	(22, 23)
		COL		mutagenesis	Cloned	KB-8-5-11	40	UNK	High-level lab	(24)
Leukemia	CCRF-CEM	EPI	Pulse	Constant	Whole	CEM/E25	7	UNK	Clinically relevant	(15, 16)
			Continuous	Stepwise	Whole	CEM/E1000	94	8 from E25	High-level lab	
	K562	DNR	Pulse	Stepwise	Whole	K562/DNR	3	2	Clinically relevant	(17)
Lung	DLKP	ADR	Continuous	Stepwise	Whole	DLKP-A	322	18	High-level lab	(25)
	A549	PAC	Pulse	Constant	Whole	A549-txl	5.5	2.5	Clinically relevant	(18)
	SKLU1					SKLU1-txl	5.0		Clinically relevant	
	SKMES1	PAC				SKMES1-txl	24.7		High-level lab	
		DOCE				SKMES1-Txt	29.1		High-level lab	
	DMS53	PAC				DMS53-txl	6.3		Clinically relevant	
		DOCE				DMS53-Txt	1.8		Clinically relevant	
	DLRP	DOCE				DLRP-Txt	4.1		Clinically relevant	
	H69	CIS	Pulse	Constant	Whole	H69CIS200	1.5–2	8	Clinically relevant	(19, 20)
		OX				H69OX400				
Ovarian	IGROV-1	CIS	Pulse	Stepwise	Whole	IGROVCDDP	8.41		Clinically relevant	(21)

ADR, adriamycin; CIS, cisplatin; DNR, daunorubicin; DOCE, docetaxel; EPI, epirubicin; OX, oxaliplatin; UNK, unknown.

23). High-level models are often more stably resistant and therefore easier to maintain in culture for an ongoing research project. Levels of resistance are often higher and as such molecular changes associated with the mechanism of resistance are larger and easier to identify. The disadvantage of these models is the higher the level of resistance the less relevant the model becomes to the clinic. Examples of high-level laboratory models are shown in **Table 2** (15, 16, 18, 22–25).

PLANNING A SELECTION STRATEGY FOR DRUG-RESISTANT CELL LINES

CHOICE OF PARENTAL CELL LINE

Choosing a parental cell line is very important as it is the basis of all the subsequent experiments. The parental cell line should be very easy to maintain in cell culture as resistant variants usually become more challenging to grow. Ideally, the researchers performing the drug-resistant selection in the laboratory should be very familiar with growing the parental cells. Researchers experienced in growing a particular cell line will have more of an idea of when the cells need to be subcultured and when it is best to leave them. This experience is important when deciding when to subculture cells recovering from the drug treatment.

It is also important to consider the patient from whom the cell line is derived. If possible, it is good to choose a chemotherapy and radiation naïve cell line. Previous treatment with chemotherapeutic agents and radiation may have already caused changes in resistance pathways, and increased expression of drug resistance markers that may not be relevant to the agent being studied. However, chemotherapy and radiation naïve cell lines are relatively rare. As an alternative to a chemotherapy naïve cell line, choose a cell line with a relatively low baseline IC_{50} value for the drug of interest as a two- to five-fold increase in resistance will result in an IC_{50} of the daughter resistant cell line remaining within the clinically relevant range. **Table 3** shows the clinical characteristics of some commonly used ovarian cancer cell lines as an example of the kind of information that is available for cell lines [(12, 14, 26–38); Sikic, personal communication]. In the case of ovarian cancer, the majority of cell lines commonly used in research are derived from metastatic ascites, and are not chemonaïve (**Table 3**).

EXPOSURE TO CHEMOTHERAPY AGENT

The researcher needs to decide what kind of model they are trying to develop, a clinically relevant model or a high-level laboratory model. A clinically relevant model is informed by data gathered from the clinical administration of drug and usually has minimal escalation of the treatment dose. The sky is the limit for a high-level laboratory model where dose escalation is used extensively to achieve a large fold resistance. However, the solubility of the selecting agent will be final limiting factor in how much drug can be applied to cancer cells. Doses that approach the limit of solubility will not be in the clinically relevant range.

The reality is that most selection strategies start out with a clinically relevant strategy and then are escalated within the clinical range and escalated further again beyond the clinical dose range to make a high-level model. The main reasons for this approach are the stability of the resistance phenotype produced and that the resistance established in the daughter cell line is statistically significant when compared to the parent cell line.

Table 3 | Clinical characteristics of ovarian tumors from which ovarian cell lines were established.

Cell line	Original tumor histology	Isolated from	Treatment received pre-isolation	Response	Reference
59M	Endometrioid/clear cell	Ascites	None	N/A	(26)
EFO27	Mucinous	Solid metastasis	None	N/A	(27)
ES2	Serous/clear cell	Primary tumor	None	N/A	[(28); Sikic, personal communication]
FUOV1	Serous	Primary tumor	None	N/A	(29)
HEY	Serous	Peritoneal deposit and xenograft	Radiotherapy, radium	CR	(26, 30)
HOC1	Serous	Ascites	MEL, CIS, ADR, CYC	PR, PR	(31, 39)
HOC8	Serous	Ascites	MEL	PR	(32, 33)
IGROV-1	Endometrioid/clear cell	Primary tumor	None	N/A	(34)
OAW28	Adenocarcinoma	Ascites	CIS, MEL	NR, NR	(26)
OAW42	Serous	Ascites	CIS	CR	(26)
OC316	Serous	Ascites	CIS, ETO, CYC, TAX	PD, SD	(35)
OVCAR3	Serous	Ascites	CYC, CIS, DOX	Unknown	(26, 36, 37)
PEA1	Adenocarcinoma	Pleural effusion	None	N/A	(14)
PEO1	Serous	Ascites	CIS, CHL, 5-FU	CR	(12, 14)
PEO14	Serous	Ascites	None	N/A	(14)
SKOV3	Adenocarcinoma	Ascites	THI	Unknown	(26)
SNU251	Endometrioid	Ascites	CYC, ADR, CIS	Unknown	(38)

ADR, adriamycin; CIS, cisplatin; CHL, chlorambucil; CR, complete response; CYC, cyclophosphamide; DOX, doxorubicin; ETO, etoposide; MEL, melphalan; N/A, not applicable; NR, no response; PD, progressive disease; PR, partial response; TAX, paclitaxel; THI, thiotepa.

Cell lines are frequently cultured in the presence of antibiotics in many laboratories. When establishing a new drug-resistant model, we recommend not using antibiotics as this does not mimic the clinical situation, cancer patients are not continually treated with antibiotics. Resistance mechanisms produced in the presence of antibiotics may not reflect clinical drug resistance.

Pharmacokinetics and drug stability

In order to produce a clinically relevant model of drug resistance, it is important to research how the chemotherapy agent is administered in the clinical treatment of cancer. The amount of chemotherapy administered intravenously (IV) is often expressed in the units milligrams per square meter. These can be converted to micrograms per milliliter or micromolar by consulting pharmacokinetic studies on the drug where the concentration achieved in the bloodstream is measured.

Chemotherapy administered by IV is often given in cycles where the patient receives the drug on a weekly or monthly basis. A pulsed-selection strategy where the cells are treated with drug and then the surviving population are allowed to recover in drug-free media mimics this clinical scenario. Pharmacokinetic studies will give a broad range of doses achieved in the bloodstream, the highest immediately after the bolus of drug is administered to the patient, this then drops over the next hours and days depending on the rate of excretion of the drug. This gives a broad dose range to define the clinical relevance of the dose of drug used in the development of a drug-resistant model. A higher dose for several hours could model the bolus of drug, a lower dose for a several days could model the longer excretion of the drug. Following an intravenous bolus injection of 100 mg/m^2 cisplatin a peak-plasma level of ~6 μg/mL is reached but this quickly drops to <2 μg/mL after 2 h (40) Clearance of cisplatin from the body is triphasic where the distribution half-life is 13 min, the elimination half-life is 43 min, and the terminal half-life is 5.4 days (41). After 24 h, 25% of the initial cisplatin dose has been eliminated from the body with renal clearance accounting for 90%.

Carboplatin has a similar mechanism of action to cisplatin but needs a 20–40-fold higher dose to exhibit the same cytotoxicity as cisplatin. However, only a 10-fold increase in carboplatin dose is required to reach similar intracellular platinum concentrations (42). After intravenous bolus injection of 375 mg/m^2 carboplatin peak-plasma levels of ~39 μg/mL are achieved, which drops to 9 μg/mL within 2 h (43). Clearance of carboplatin has a distribution half-life of 22 min, an elimination half-life of 116 min, and a terminal half-life of 5.8 days (44). Clearance of carboplatin from the body is primarily by the urine as unchanged drug. After 24 h, 90% clearance is achieved. Carboplatin does not have significant excretion from the renal tubules as seen for cisplatin, instead the glomerular filtrate accounts for the vast majority of elimination. For this reason, glomerular filtration rate (GFR) is linearly related to total renal clearance giving relatively simple pharmacokinetics for carboplatin. Even at high doses evidence suggests that carboplatin has linear pharmacokinetics (45). A formula called the "Calvert formula" has been derived, which is based on the GFR and is used to provide a suitable dose for patients in relation to an area under concentration time curve (AUC) value. AUC is the ratio of the amount of drug that reaches the systemic circulation and the clearance of the drug, which correlates to its clinical efficiency and toxicity. This formula has been validated in a perspective study (46). Conventional doses of carboplatin administered to patients generally are aimed at giving an AUC value of between 5 and 7 mg/mL/min.

The amount of chemotherapy administered orally is usually expressed in the unit milligrams per day. Again pharmacokinetic

studies can be used to convert this to a concentration in the bloodstream. A continuous treatment strategy where the cells are cultured constantly in the presence of drug can be clinically relevant for an oral drug given daily or twice daily as a relatively constant amount of the drug is present. Olaparib is a member of the poly (ADP-ribose) polymerase (PARP) inhibitor class of drugs and is administered orally. The maximum tolerated dose of olaparib is 400 mg twice daily. Absorption is rapid and its peak-plasma concentration is reached within 1–3 h. Plasma levels then decline biphasically and it has a terminal elimination half-life of ~5–7 h (47). A phase 1 study on Japanese patients found that peak-plasma values for a single dose of 400 mg olaparib was ~7 μg/mL, which dropped below 0.1 μg/mL after 50 h. For a dose of 400 mg administered twice daily for 15 days, peak-plasma concentrations were found to be similar. The half-life of olaparib was recorded to be between 7 and 11 h across doses ranging from 100 to 400 mg (48).

The chemical stability of drugs used in establishing drug-resistant cell line models is also an important consideration when designing a selection strategy. For example, temozolomide an alkylating agent used in the treatment of glioblastoma and metastatic melanoma when in its active state, has a half-life of 25 and 60 min for the first and second phases (49) whilst docetaxel a microtubule destabilizing agent has a half-life of 12 h (50). Lapatinib, a dual EGFR HER2 inhibitor used in the treatment of HER2-positive breast cancer has a half-life of 24 h (51) whilst the monoclonal HER2 antibody trastuzumab also used in HER2-positive breast cancer has a half-life of over 5 days (52). Drugs with a shorter half-life will have to be dealt with carefully to ensure that cancer cells receive the maximal benefit from drug dosing. Also drugs with a long half-life should be removed from cells long before the models are to be used in experiments. This ensures that residual drug will not remain in the cells and effect proliferation assays comparing survival between the parental and resistant cells.

Optimization of treatment dose in parental cell line

The dose of drug used must be optimized for the parental cell line selected for use in developing the resistant model. A cytotoxicity assay in the parental cell line can be used to determine a suitable dose range. This dose range can then be compared to the pharmacokinetic information for the drug of interest. The rate of recovery from drug treatment is just as important as the IC_{50}; as the rate of recovery can be different between agents even if an equivalently cytotoxic dose is administered to cells. **Figure 1** shows the recovery of two ovarian cancer cell lines (OVCAR8 and UPN251) from equivalently cytotoxic doses of carboplatin and paclitaxel. The recovery from paclitaxel is much faster than carboplatin.

The chemotherapeutic drug paclitaxel is frequently given at a dose of 175 mg/m^2 as a single agent (53, 54). Pharmacokinetic studies for this dose show peak-plasma concentrations as high as 10,000 ng/mL but drop off quickly after 24 h to 50 ng/mL and below (55, 56). In the development of platinum/taxane-resistant OVCAR8 and UPN251 ovarian cancer cells, treatment doses were chosen trailed over the range of IC_{20}–IC_{80}, and were consistent with doses used in the clinical setting. Paclitaxel doses tested for OVCAR8 and UPN251 were from a range of 2.3–14 and 10–100 ng/mL. Carboplatin doses tested for OVCAR8 and UPN251 were 2.3–18.5 and 0.7–2 μg/mL, respectively. The final chosen doses of paclitaxel and carboplatin displayed an initially large percentage cell death or growth inhibition compared to a control grown in drug-free media. Carboplatin doses of 4 and 2 μg/mL and paclitaxel doses of 12 and 60 ng/mL were chosen for OVCAR8 and UPN251, respectively (**Figure 1**). After treatment with the selected doses and removal of the drug, the cells were able to return to logarithmic growth ensuring the selection of resistant cell sub populations.

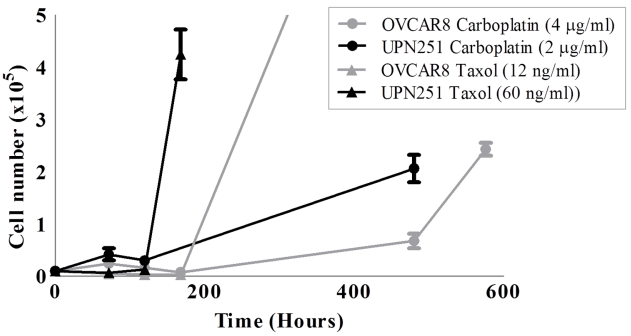

FIGURE 1 | Recovery of OVCAR8 and UPN251 from carboplatin or paclitaxel treatment. 1×10^4 cells were treated for 3 days with the indicated doses of either carboplatin or paclitaxel and recovery monitored as described in Section "Methods."

In the development of platinum-resistant H69 small cell lung cancer (SCLC) cells, treatment doses were chosen in the range of IC_{10}–IC_{40}, and were consistent with doses used in the clinical setting (19). Two exposure times and doses were used for cisplatin and oxaliplatin reflecting differing pharmacokinetic phases of the administration of platinum drugs; 2-h treatments at 1–8 μg/mL and 4-day treatments at 0.2–1.6 μg/mL. The lowest drug concentration treatments all produced 20–30% cell death and growth arrest in H69 cells. Drug-treated cells increased in size and did not aggregate in typical SCLC clumping morphology. Surviving cultures were then retreated when their normal growth rate and clumping morphology had returned, ~3–4 weeks later.

POPULATION DYNAMICS

In most selection strategies the whole population of cells remains as one group throughout the selection, no cloning or other separation methods are used. If a pulse of drug is given, a small percentage of cells remain, which repopulates the flask. This new population of cells is then retreated with the next pulse (18, 19). Alternatively a low-level of drug is present continuously, the cells adapt to growing in the presence of the drug and then the dose of drug is slowly increased (25).

It is well known that tumors are heterogeneous (57–59). Consequently, the cancer cell lines derived from tumors are also heterogeneous. For example, breast tumors from patients who are BRCA1/2 carriers have been shown to be heterogeneous, where not all cells have lost the second BRCA1/2 allele (60). Selection with chemotherapy agents therefore often result in the isolation of a cell population that already exists in the culture. Indeed, this has been demonstrated for many drug-resistant models, particularly in projects, which examine cancer stem cells (CSCs). CSCs are thought to be responsible for tumor regeneration after

chemotherapy. Drug-resistant cell lines are often enriched for markers of stem cells. The stem-cell marker CD133 was found to be enriched in a panel of cisplatin-resistant lung cancer cell lines, with a 5-fold increase in both A549CisR and MORCisR, and a 12-fold increase in H460CisR cells (61).

There are other physical methods of separation available to select different populations from a cell line such as limited dilution or cell sorting by flow cytometry. This can isolate cells which may be more resistant to chemotherapy than other populations within the same cell line (62). The advantage of clonal populations as drug-resistant models for is that there is no drug treatment is required and the resulting model is more stable. The disadvantages however are that many clones must be established and there is no guarantee that the clonal populations derived will display any difference in drug resistance.

Clonal populations can be established by limited dilution. This relies on the ability of the cells to grow independently of each other, and as such may not be suitable for all cell lines. It involves seeding cells at a very low density to result in one cell per well of a 96-well plate. Once the cells grow to confluency, they can be tested as a clonal population. Another method to obtain clonal populations is cloning rings (62). Standard toxicity testing on the clonal populations generated will show whether they display an inherent resistance to the agent of interest.

A combination of drug treatment and cloning of cells has also been used to produce resistant models. KB-8-5-11 colchicine-resistant cells were developed from parental KB-3-1 cells by selecting clones after three stepwise increases in colchicine drug treatment (24). Clones 8, 5, and 11 were the successful clones picked each round of the selection strategy.

Cloning can also be used to investigate heterogeneity within a developed drug-resistant model. A human colon cancer cell line (LoVo) was treated with cisplatin using a continuous exposure ranging from 0.005 to 20.0 μg/mL over 20 months in culture (63). At the end of the treatment, two morphologically distinct subpopulations were observed; these were then cloned by limiting dilution. The subclones showed different patterns of cross resistance to chemotherapy agents. The first clone overexpressed the ABC efflux transporter P-glycoprotein (P-gp) and the other clone did not. Heterogeneity was also seen in cisplatin-resistant models developed from a human pancreatic cancer cell line with a mutation in DNA repair protein BRCA2. Fourteen cisplatin-resistant clones were obtained. In 7 of 14 clones, the functionality of BRCA2 had been restored by secondary mutations, the remaining clones still had a non-functional BRCA2 protein (64).

RISK-REDUCTION STRATEGIES: COMPARATIVE SELECTION

It is reasonably common to have difficulties developing resistance or to produce drug-treated daughter cell lines which have not increased in resistance relative to the parental cell line. Selection strategies which do not produce drug resistance are interesting from a clinical perspective as this is what we want to achieve for cancer patients. Unfortunately, failures to develop drug resistance are generally not reported in the literature.

As a risk-reduction strategy, a comparative selection strategy should be performed, where selection of multiple cell lines or multiple chemotherapeutic agents are performed in parallel in the laboratory. **Figure 2A** shows a strategy where two parental cell lines are each treated with two chemotherapy agents, producing four different daughter cell lines. **Figure 2B** shows one parental cell line being treated with two chemotherapy agents, in two different doses or intervals producing four different daughter cell lines. By using a comparative development strategy, it is hoped that at least one of them will successfully produce a stable drug-resistant model. It can be interesting to observe which strategies produced resistance and which did not. These may be useful observations for the clinical treatment of cancer.

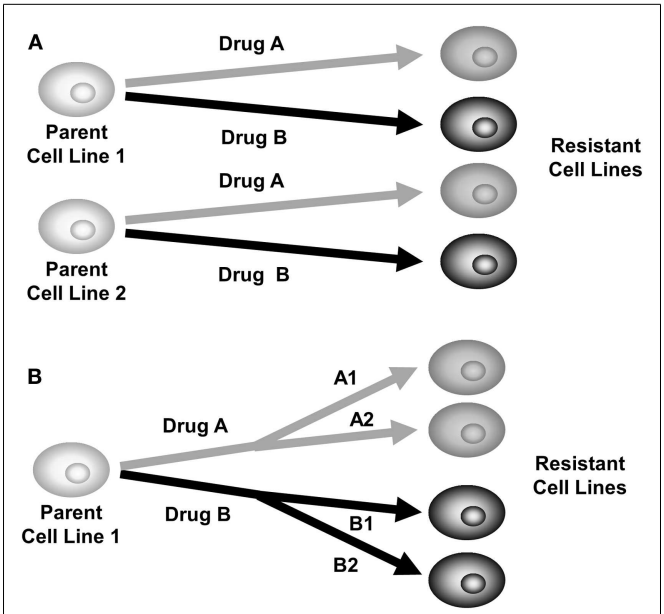

FIGURE 2 | Comparative selection of drug-resistant cell lines. **(A)** Plan for selection of two parent cell lines with two different drugs to produce four drug-resistant daughter cell lines. **(B)** Plan for selection of one parent cell line with two drugs at different doses or treatment intervals, producing four drug-resistant daughter cell lines.

An example of a comparative selection strategy used H69 SCLC cells treated with cisplatin or oxaliplatin for two time periods, 2 h or 4 days. The 4-day pulse selection produced more stable resistance than the 2-h pulse (19) (**Table 2**). Had only the 2-h pulsed treatment been tested in this model drug resistance would have not been developed. The comparative nature of the selection also led to the finding that oxaliplatin resistance developed faster than cisplatin resistance in H69 cells (19).

A large study by Tegze et al. aimed to develop 40 drug-resistant models from MCF-7 and MDA-MB-231 breast cancer cells using doxorubicin and paclitaxel as selecting agents. They succeeded in making 29 drug-resistant models, 10 doxorubicin and 4 paclitaxel-resistant MCF-7 cell lines, and 6 doxorubicin and 9 paclitaxel-resistant MDA-MB-231 cell lines. From this study it appears that paclitaxel resistance was easier to develop in MDA-MB-231 (ER-negative) cells and doxorubicin resistance was easier to develop in MCF-7 (ER-positive) cells.

A study by our group (18) developed resistance to paclitaxel and carboplatin in large cell lung cancer cell lines (H1299 and H460). Cells at low confluence in 75cm^2 flasks were exposed

to 50 μg/mL carboplatin and 150 ng/mL (H1299) or 50 ng/mL (H460) paclitaxel for 4 h. After this period, the drug was removed and the flasks were rinsed and fed with fresh complete media. The cells were then grown in drug-free media for 6 days, replenishing the media every 2–3 days. This was repeated once a week for 10 weeks. **Table 4** shows the IC_{50}s of the platinum and taxane-resistant cell lines to a variety of chemotherapy agents. Resistance in the carboplatin-selected cells (1.5–2.3-fold) was considerably less than the resistance obtained in the paclitaxel-selected cells (2.4–4.4-fold). Selected cell lines show no obvious cross resistance pattern except within families of drugs, e.g., paclitaxel and docetaxel; carboplatin and cisplatin. The TAX-selected cells were also found to be resistant to vincristine, which is unsurprising since both agents affect microtubules. Both carboplatin-selected cell lines had a modest but statistically significant increased resistance to paclitaxel. The different patterns of resistance in cell lines selected under similar conditions show the complicated nature of multiple-drug resistance. For example, H1299-cpt became sensitive to vincristine, while after identical drug treatment, H460-cpt developed significant resistance to vincristine (2.9-fold). Overall, the low-level resistance (two- to five-fold) observed in these selected cell lines may be more clinically relevant to study than higher levels of resistance and this study highlights the importance to studying mechanisms in multiple models to identify relevant pathways.

If a selection strategy fails to develop resistance, the treatment conditions can be altered in an attempt to produce higher levels of resistance. If the cells are growing very well after drug treatment, consider dose escalation. In some cases this may push the dose used above clinically relevant levels but it will increase the chance of resistance developing. Alternatively, the length of time the cells are exposed to drug can be increased or a pulsed-selection strategy could be converted to a continuous selection strategy. This may make the model less clinically relevant but may produce resistance that can be studied in the laboratory.

Table 4 | Fold resistance of H1299 and H460 resistant variants compared with their parental cell lines.

Chemotherapeutic agent	H1299-cpt	H1299-txl	H460-cpt	H460-txl
Carboplatin	2.0**	1.7***	2.3*	0.8*
Cisplatin	1.5*	1.5	1.6	0.7
5-FU	1.0	1.8**	0.9	1.1
VP-16	1.4**	1.1	0.9	1
Vincristine	0.8*	2.3*	2.9***	2.5
Adriamycin	0.9	1	1	0.9
Paclitaxel	1.2*	4.4***	1.6***	2.4***
Docetaxel	0.6	2.5***	2.3	2.8***

**p-Value <0.05; **p-value <0.01; ***p-value <0.005.*

CASE STUDIES OF DRUG-RESISTANT CELL LINES

The following section presents two case studies of drug-resistant cell lines developed in our laboratory, the reasons that selection conditions were chosen and the drug resistance outcomes of the developed cell lines. A case study using continuous selection is presented for lapatinib in breast cancer. A case study using pulsed selection is presented for temozolomide-resistant melanoma.

LAPATINIB-RESISTANT BREAST CANCER CELLS – CONTINUOUS SELECTION

Of the published models of acquired lapatinib resistance there is very little commonality in the procedures used to condition the cells, in either the concentrations of lapatinib used or in the determination of resistance status (**Table 5**) (65–72). For instance, the procedures used to develop models of acquired lapatinib resistance

Table 5 | Published cell line models of acquired lapatinib resistance, the method and concentration used to condition the cells and the proposed mechanism of lapatinib resistance.

Parent cell line	Conditioning method	Lapatinib concentration	Profiling technique	Resistance mechanism	Reference
BT474	Single cell cloning	5 μM[a]	Affymetrix array	Upregulation of ER signaling	(65)
BT474, SKBR3	Single cell cloning	5 μM[a]	Affymetrix array	Activation of RelA	(66)
SUM190	Continuous exposure	(0.25–2.5 μM)	Immunoblotting	Overexpression of XIAP	(67)
BT474	Single cell cloning	3 μM[a]	phospho-tyrosine immunoblotting	Overexpression of AXL	(68)
HCT116	Continuous exposure	10 μM[a]	Immunoblotting	Increased expression of MCL-1	(69)
HCC1954, BT474	Continuous exposure	(0.1–1 μM)	Immunoblotting	Increased expression of β1-integrin	(70)
SKBR3, MDA-MB-361, UACC893, BT474, HCC1954, SUM190	Continuous exposure	Increasing concentration up to 1 or 2 μM	Phospho-proteomic profiling	Increased SRC kinase activity	(71)
BT474, UACC812	Continuous exposure	(0.1–1 μM)	Immunoblotting	Upregulation of ER signaling	(72)

[a] *Denotes greater than peak-plasma concentration (2.5 μM).*

included a single cell cloning technique (65, 68) fixed dose conditioning (69) and dose escalation conditioning (67, 71, 72). There was significant variation in the concentrations of lapatinib used to condition the cells; many studies began with a low dose of lapatinib (e.g., 100 nM) which was dose-escalated to upwards of 2 μM. Fixed concentration conditioning was performed with concentrations of lapatinib ranging from 3 to 10 μM. The length of conditioning required to achieve resistance varied from study to study with the majority of studies taking ~12 weeks to achieve resistance, whereas other studies took up to 1 year to achieve resistance. Another variation in different models of lapatinib resistance was the definition of lapatinib resistance. Most of the studies defined their conditioned cell lines as resistant based on their ability to grow in the presence of the concentration of lapatinib used to condition the cells, only one study used an IC_{50} method while a number of studies did not quantify the level of resistance. In contrast to the previously published models of acquired lapatinib resistance, the resistant models developed by us use a relatively low dose of lapatinib relative to the IC_{50} of the resulting cell line. To our knowledge our model of acquired lapatinib resistance, HCC1954-L are the first to show that extended exposure to low dose lapatinib results in significant lapatinib resistance, with resulting lapatinib IC_{50} values significantly higher than the concentration used for conditioning.

HCC1954 cells overexpress HER2 (73) and therefore represent a cell line model of HER2-positive breast cancer. Lapatinib is a tyrosine kinase inhibitor that targets the intracellular domain of HER2 and EGFR and is approved for the treatment of HER2-positive breast cancer (74, 75). HCC1954 are moderately sensitive to lapatinib with an IC_{50} of 0.43 ± 0.03 μM (**Figure 3A**). Lapatinib is administered to cancer patients orally with a dose of 1000–1250 mg given daily (76). The median peak-plasma concentration of lapatinib reported in patients receiving 1200 mg lapatinib (once daily) was 1.2 μg/mL (2.1 μM) and the median steady-state trough concentration was 0.3 μg/mL (0.5 μM), with a range of 0.2–0.5 μg/mL (77). Therefore a continuous selection strategy is clinically relevant for lapatinib. To optimize the dose of drug used for selection, a lapatinib dose response assay was performed in order to determine the concentration of lapatinib which would result in 70% growth inhibition over a 4-day treatment. Treatment of HCC1954 cells with 1 μM lapatinib inhibited the growth of the cells by $71.5 \pm 1.2\%$ compared to untreated controls ($p = 0.004$) (**Figure 3B**). Therefore a selection strategy of continuous exposure of HCC1954 to 1 μM lapatinib was initiated with the media replenished every 4 days with fresh drug. The selection strategy was conducted in duplicate with "A" and "B" flasks as a backup in case there were problems with one flask.

HCC1954 cells were seeded into two flasks; 1×10^6 cells per $75cm^2$ flask. One flask was left untreated but was passaged alongside the treatment flask and named HCC1954-par, the cells treated with 1 μM lapatinib were named HCC1954-L. It is important to passage the untreated parental cells alongside the treated cells as a control as continuous cell culture can result in alterations in cellular characteristics, including drug resistance. The morphology of both cell lines and the sensitivity of the cell lines to lapatinib were monitored throughout the selection. After 3 months of treatment, the morphology of HCC1954-L was not altered (**Figure 3C**). For all cytotoxicity assays the HCC1954-L cells were grown in drug-free media for 5 days prior to testing. The lapatinib IC_{50} value for the HCC1954-par cells was 0.42 ± 0.01 μM, which is similar to the original HCC1954 cells. The lapatinib IC_{50} value for the HCC1954-L cells was 0.75 ± 0.07 μM (**Figure 3D**). This represents 1.8-fold increase in resistance to lapatinib. At this stage of the treatment process the lapatinib IC_{50} of HCC1954-L cells had not yet exceeded the treatment dose however they had begun to actively proliferate in the presence of lapatinib. The concentration of lapatinib was therefore increased from 1 to 1.25 μM and conditioning continued with this concentration for a further 3 months.

After 6 months of lapatinib conditioning, the sensitivity of the cells was again tested. Both the "A" and "B" flasks of HCC1954-L cells developed equivalent amounts of resistance, and the "As" were chosen for all subsequent experiments and the "Bs" frozen as a backup. The lapatinib IC_{50} value for the HCC1954-par cells was 0.42 ± 0.02 μM whereas the lapatinib IC_{50} for HCC1954-L cells was 2.67 ± 0.08 μM ($p = 0.01$) (**Figure 3E**). This represents 6.1-fold increase in resistance to lapatinib. HCC1954-L cells were deemed to be resistant to lapatinib as the lapatinib IC_{50} was above the 1 μM threshold for lapatinib sensitivity (78). The resistant cells also exhibited distinct morphological alterations compared to the parental cell line. These differences were indicated by more distinct colony boundaries and a flatter cell shape (**Figure 3F**).

In order to assess the stability of acquired resistance in the HCC1954-L cell line, sensitivity to lapatinib was assessed after freezing and thawing and following drug withdrawal. To establish a reliable cell line model of lapatinib resistance the phenotype must be stable when the cell line is frozen and re-thawed. To assess this, frozen stocks of the HCC1954-par and HCC1954-L cells were prepared in fetal calf serum containing 5% DMSO. After a minimum of 48 h in liquid nitrogen the frozen stocks were thawed and the viability of the stocks assessed by microscopy. The cells were then passaged a minimum of 3 times before lapatinib sensitivity assays were repeated (**Figure 3G**). The lapatinib IC_{50} was 0.44 ± 0.02 μM in the parental cells while the lapatinib IC_{50} in HCC1954-L cells was 2.73 ± 0.05 μM. This indicates that the HCC1954-L cells retain their resistant phenotype following a freeze/thaw cycle.

In order to access the long-term stability of the resistant phenotype, drug withdrawal assays were performed. Lapatinib was removed from the HCC1954-L cells and the sensitivity of the cells to lapatinib was tested at 4-week intervals for a period of 12 weeks, the results at each interval are illustrated in (**Figure 3G**). Following 12 weeks growth in the absence of lapatinib the lapatinib IC_{50} of the parental cells was 0.43 ± 0.05 μM while the lapatinib IC_{50} of HCC1954-L cells was 2.63 ± 0.16 μM. There was no significant difference between the initial lapatinib IC50 for either the parental or resistant cell line and the lapatinib IC50 for the cell lines after 12 weeks growth in the absence of lapatinib (**Figure 3H**).

Therefore, we successfully established a stable cell line of acquired lapatinib resistance (HCC1954-L) induced by long-term continuous treatment with sub-peak-plasma concentrations of lapatinib. The mechanisms of acquired resistance to lapatinib are being investigated in this model using proteomics and genomic techniques.

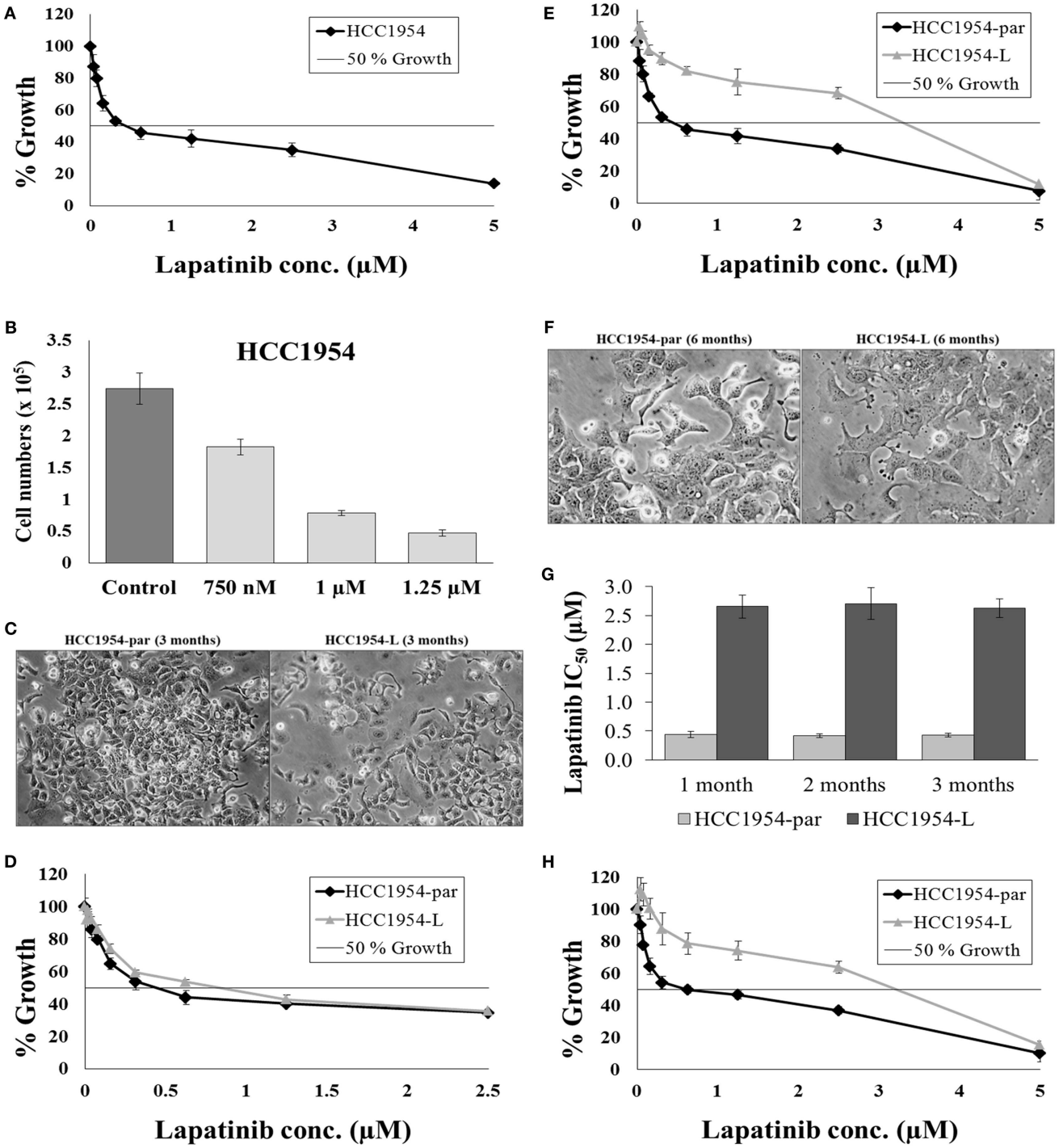

FIGURE 3 | Continuous selection of HCC1954 with lapatinib. **(A)** Proliferation of HCC1954 cells following a 5-day treatment with lapatinib. **(B)** HCC1954 cells treated with varying concentrations of lapatinib over a 4-day period. Cell counts were performed using ViaCount reagent and Guava Software and expressed relative to control untreated cells. **(C)** Images of HCC1954-par and HCC1954-L cells after 3 months of lapatinib conditioning at 100× magnification. **(D)** After 3 months conditioning with 1 μM lapatinib, the proliferation of HCC1954-par and HCC1954-L cells was measured. **(E)** After 6 months conditioning with lapatinib, the proliferation of HCC1954-par and HCC1954-L was assessed. **(F)** Images of HCC1954-par and HCC1954-L cells after 6 months of lapatinib conditioning at 200× magnification. **(G)** Sensitivity of HCC1954-par and HCC1954-L cells to lapatinib following a freeze-thaw cycle. Growth is expressed relative to untreated control cells. **(H)** Lapatinib IC_{50} values for HCC1954-par and HCC1954-L cells following 1, 2, and 3 months growth in the absence of lapatinib. All growth rates and IC_{50}s were calculated following a 5-day lapatinib treatment. Growth is expressed relative to untreated control cells. All error bars represent the standard deviation of triplicate experiments. Student's *t*-test was performed to determine significant differences: $*p < 0.05$; $**p < 0.01$.

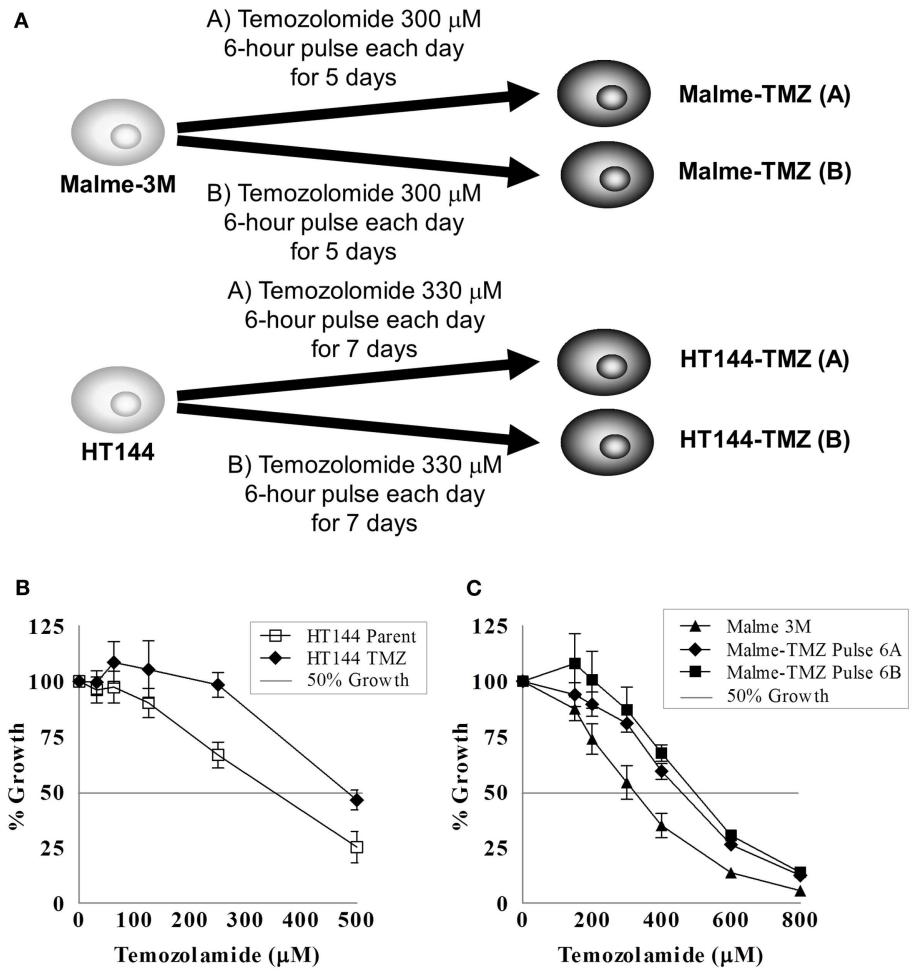

FIGURE 4 | Pulse selection of Malme-3M and HT144 with temozolomide. **(A)** Selection strategy of Malme-3M and HT144, each treatment of temozolomide was performed in duplicate. Effect of temozolomide in Malme-3M and HT144 and temozolomide "pulse selected" resistant variants. **(B)** Malme-TMZ(A) and Maleme-TMZ(B) vs. Malme-3M cells **(C)**. HT144-TMZ vs. HT144 cells. Error bars represent the standard deviation of triplicate assays.

TEMOZOLOMIDE-RESISTANT MELANOMA CELL LINES – PULSED SELECTION

Temozolomide is frequently used to treat metastatic melanoma. No dosing schedule of temozolomide has been clinically proven to be more effective than a single administration of temozolomide (79); however current treatments favor a 5-day treatment schedule (80). We found that the IC_{50} concentrations of temozolomide were in the high micromolar range in melanoma cell lines. Previous studies in two melanoma cell lines demonstrated temozolomide IC_{50} concentrations of ~800 μM (81), which is consistent with the values observed in our cell line panel of six melanoma cell lines (temozolomide IC_{50} ranged from 250 to 800 μM). However, in the clinical setting plasma levels of temozolomide only reach concentrations approaching 80 μM (82) The half-life of temozolomide is <2 h (83), which would reduce the efficacy of the drug in patients and may also explain the high IC_{50} values observed *in vitro*. A pulsed selection of drug was chosen to mimic these pharmacokinetic properties of temozolomide.

Malme-3M or HT144 melanoma cell lines were seeded at a density of 2.5×10^4 cells in a 75 cm^2 flask. The entire selection strategy was conducted in duplicate; two flasks of each cell line were set up for untreated control flasks and two for temozolomide selection (**Figure 4A**). Cells were allowed to attach for 24 h prior to treatment with chemotherapy. For Malme-3M cells, after each treatment cells were allowed to grow until confluent, then trypsinised and reseeded at a density of 2.5×10^4 cells per flask for the next round of selection. For HT144 cells, cells were grown in the flask for the 5 days of their treatment, then left to grow to confluence. After cells recovered they were trypsinised reseeded at 2.5×10^4 cells per flask for the next round of selection.

The Malme-3M cells were pulse treated with 300 μM temozolomide for 6 h and then the drug containing medium removed and replaced with fresh drug-free medium. This single pulse treatment was repeated six times. HT144 cells were treated for 6 h daily with 330 μM temozolomide for 5 days. After the five daily treatments the drug was removed and replaced with fresh drug-free media.

This treatment was repeated four times. This treatment schedule was used to replicate that of the clinical setting, where temozolomide is administered daily for 5 days, followed by a period of no treatment (84). The pulse selection strategy used in these cells allowed us to compare differences between the clinical daily administration and the lab based pulse selection to observe if either regimen resulted in increased levels of resistance acquired.

The IC_{50} for temozolomide in Malme-3M parent cells is $306 \pm 29\,\mu M$. Malme-TMZ(A) and Malme-TMZ(B) display significantly increased IC_{50}s for temozolomide of $440 \pm 21\,\mu M$ [1.44-fold increase ($p = 0.004$)] and $515 \pm 45\,\mu M$ [1.68-fold increase ($p = 0.04$)] (**Figure 4B**). The IC_{50} for temozolomide in HT144 cells is $338 \pm 25\,\mu M$. In HT144-TMZ(A), the pulse-selected variant of HT144, the IC_{50} increased to $490 \pm 15\,\mu M$, which represents a 1.45-fold increase in resistance to TMZ ($p = 0.002$) (**Figure 4C**). HT144(B) did not develop significant resistance to temozolomide, and so was not used in further studies.

During drug selection of cell lines, cells can acquire altered sensitivity to other chemotherapeutic drugs. The two temozolomide-selected cell lines from each parent cell line with the highest levels of resistance [Malme-TMZ(B) and HT144(A)] were tested with four drugs to examine the chemosensitivity between the parent and the resistant cell lines (**Table 6**). The melanoma cell line HT144 and the temozolomide-selected variant HT144-TMZ display similar sensitivity to cisplatin and epirubicin whilst the resistant cell line is significantly more sensitive to mitoxantrone ($p = 0.02$). Malme-3M and the pulse-selected cell line Malme-TMZ have similar IC_{50}s for EPI and mitoxantrone. Malme-TMZ is significantly more resistant to cisplatin ($p = 0.001$) and both HT144-TMZ and Malme-TMZ are significantly more resistant to docetaxel than the parent cell lines Malme-3M and HT144 ($p = 0.02$; $p = 0.02$), although the IC_{50} values are still in the very low nanomolar range.

Two temozolomide-resistant cell lines [Malme-TMZ(B) and HT144(A)] were established using two different selection methods. Duplicate selection proved useful in this selection strategy as one of the variants HT144-TMZ(B) did not develop resistance. Although the level of resistance induced was relatively low, these two cell lines provide unique clinically relevant models to study acquired temozolomide resistance in melanoma (85). The temozolomide-resistant variants were cross resistant to cisplatin. As temozolomide and cisplatin are both DNA damaging agents, there may be common mechanisms of resistance to the DNA damage induced by these agents.

Table 6 | Fold resistance of HT144 and Malme-3M resistant variants compared with their parental cell lines.

Chemotherapeutic agent	HT144-TMZ	Malme-TMZ
Cisplatin (nM)	1.4	2.0*
Epirubicin (nM)	1.3	0.8
Mitoxantrone (nM)	0.2*	1.3
Docetaxel (nM)	1.4*	1.2*

**Indicates a p-value <0.05 as calculated by Student's t-test.*

MAINTAINING DRUG-RESISTANT CELL LINES FOR RESEARCH

Once resistance has been established with the selection strategy the stability of the resistance needs to be determined. One important test of the stability of the model is the recovery of the drug-resistant phenotype from the frozen stocks. If the phenotype is lost or resistance is significantly lower on freeze thaw then the model will not be practical to use in the laboratory. If the resistance is not stable on freeze thaw then the drug-resistant cells need to be treated for longer, possibly with a higher dose of chemotherapeutic.

The long-term stability of resistance also needs to be examined. Resistant cell models that have been selected by continuous exposure to drug should be grown for several months to determine if the resistance phenotype remains present. Some cell lines may be completely stably resistant (**Figure 5A**) and are grown in the absence of chemotherapeutic, such as DLKP-A or IGROVCDDP (21, 25). Regular monitoring by cytotoxicity assay is required to make sure that the resistance phenotype of the cell lines persists.

Alternatively, the cells can be grown continuously in chemotherapeutic, either at the dose used in selection or a lower maintenance dose. This may be if the cells are not stably resistant on removal of the chemotherapeutic or if the researchers wish to ensure consistency of experiments. KB-CP20 cisplatin-resistant cells which were selected with increasing concentrations of cisplatin up to $20\,\mu g/mL$ over a period of 6 months. The

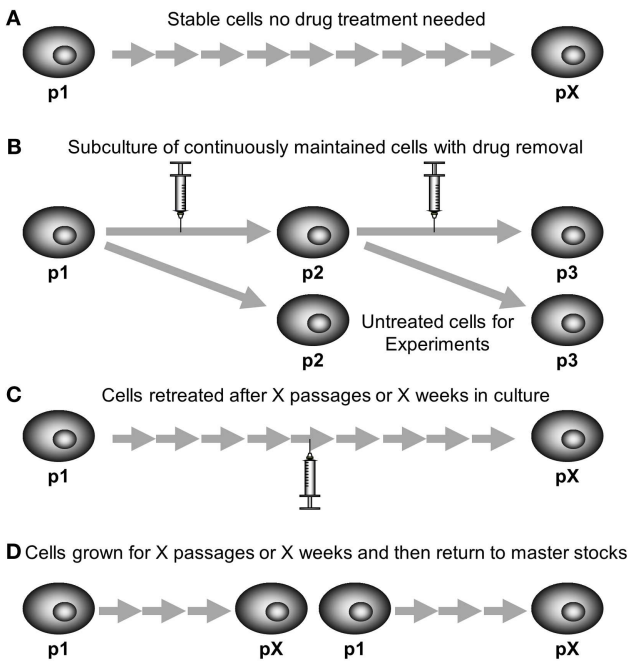

FIGURE 5 | Maintaining drug-resistant cell lines in cell culture. **(A)** Stable cell lines require no drug treatment to maintain their resistant phenotype. **(B)** Some cell lines are grown continually in the presence of chemotherapy, chemotherapy needs to be removed for one subculture prior to using for experiments. **(C)** Some models are repeat pulse treated after a certain number of passages or weeks in culture once their resistant phenotype begins to fade. **(D)** Some models are discarded and new cells grown from master stocks after a certain number of passages or weeks in culture once their resistant phenotype begins to fade.

resistance was then maintained in media containing 5 μg/mL cisplatin (22). KB-5-8-11 colchicine-resistant cells are also maintained in 100 ng/mL of colchicine (24). Resistant cell models which are maintained in chemotherapy drug need to be grown in drug-free media for a passage prior to conducting experiments (86). This is so that the drug-free controls of experiments are cells not exposed to drug, rather than cells grown in the maintenance dose of chemotherapy. **Figure 5B** shows a subculture schematic for this technique. Another approach to maintaining stability is growing cells in drug-free media but using a pulse treatment at regular intervals (**Figure 5C**). This can be used even if the cell model was originally developed by continuous exposure. The CEM/E25 and CEM/E1000 epirubicin-resistant variants of CCRF-CEM leukemia cells were established by continuous exposure then grown without drug and resistance was maintained by repeat pulse treatment every 6 weeks with the selecting doses of epirubicin 25 and 1000 ng/mL, respectively (15, 16).

Resistant models that are selected by pulse selection are often less stable than their continuously selected counterparts. However, IGROVCDDP is a stably resistant cell line established by pulse selection using dose escalation (21). Pulse-selected cell lines which lose their resistant phenotype can also be maintained by re-treatment with the selecting dose (**Figure 5C**) such as K562/DNR resistant leukemia cells (17). Alternatively, instead of repeating the pulse treatment, resistant cells can be grown for a certain number of weeks or passages and then new stocks are defrosted of an earlier passage with the resistant phenotype present (**Figure 5D**). H69CIS200 and H69OX400 cisplatin and oxaliplatin-resistant cells were 1.5–2-fold resistant to platinums for 5–6 weeks in drug-free culture and then the resistance phenotype faded over the next 6–8 weeks in culture (20). This technique is also often used with resistant models regardless of selection strategy to ensure consistency, so that cells within a limited range of passage numbers are used for all experiments.

A REPRODUCIBLE EXPERIMENT?

Many mechanisms of resistance exist for each chemotherapy drug. The chemotherapeutic drug cisplatin has been studied in drug-resistant cell models for many years and mechanisms of resistance include decreased accumulation of drug, inactivation by glutathione and increased DNA repair (87). These mechanisms need not all occur in the same drug-resistant model. Over time more common mechanisms will be identified by their occurrence in many drug-resistant models.

A comparative selection strategy could involve parallel selections of the same parental cell line with the same chemotherapy agent, under the same treatment conditions. Similar or different mechanisms could develop in these independent treatments. This is the randomness of natural selection. A study by Tegze et al. developed multiple drug-resistant cell lines from MCF-7 and MDA-MB-231 breast cancer cells (88). The parent cell lines were split and new cell lines were generated in parallel by treatment with gradually increasing concentration of doxorubicin or paclitaxel. The study aimed to produce 10 resistant sublines for each agent in each parent cell line. Using a continuous treatment strategy they produced 29 resistant models over an 18-month period. There were 10 doxorubicin and 4 paclitaxel-resistant MCF-7 cell lines and 6 doxorubicin and 9 paclitaxel-resistant MDA-MB-231 cell lines. The fold resistance values compared to the parental cell lines show up to 46- and 28-fold resistance to doxorubicin and paclitaxel, respectively. The cell lines turned out to be highly heterogeneous for the mechanisms of drug resistance present, and in general only a few mechanisms are activated in one cell line to achieve drug resistance. Of note, the expression of P-gp did not correlate with resistance in the cell line models, despite the development of models with two P-gp substrates. This suggests that in some of the models P-gp was activated early in the selection process and became a dominant mechanism, in others this did not occur.

Two models of cisplatin resistance were developed from H69 SCLC cells in the same research group in successive years (19, 89). These models were developed independently rather than in parallel. H69-CP and H69CIS200 were developed with 100 or 200 ng/mL of cisplatin, respectively. Both cell models were two- to four-fold resistant to cisplatin, and had decreased expression of p21 which may increase the cell's ability to progress through the cell cycle in the presence of DNA damage. Both the H69-CP and H69CIS200 cells showed no decrease in cellular cisplatin accumulation. However, the H69-CP cells have increased levels of cellular glutathione and are cross resistant to radiation whereas the H69CIS200 cells have neither of these changes.

The cell line IGROV-1 has been used to develop cisplatin drug-resistant models by many research groups. IGROVCDDP cisplatin-resistant cells have an unusual resistant phenotype; they are cross resistant to paclitaxel as they overexpress P-gp (90). It is unusual but not unprecedented to see a model of acquired cisplatin resistance overexpress P-gp (63, 91–94). This most likely represents a generalized stress response to long-term cisplatin treatment as cisplatin is not a P-gp substrate (95). IGROVCDDP cells do not have increased total cellular glutathione but the way glutathione is recycled within and from outside the cell is enhanced, increased enzyme activity of glutathione reductase and gamma-glutamyltransferase 1 (GGT1) was present (90). In contrast, IGROV-1/Pt0.5 and IGROV-1/Pt1, platinum-resistant cell lines are sensitive to P-gp substrates, have increased cellular glutathione and decreased GGT1 (96) which is the reverse pattern to that seen in the IGROVCDDP platinum/taxane-resistant cells. However, it should be noted, that different research groups can of course have different sub clones of a parent cell line and this can be a factor for the differences in the resistant models produced.

These examples demonstrate that the same cell line, treated with the same chemotherapy agent leads to the development of a heterogeneous range of drug-resistant models. Therefore, the development of drug-resistant models should be regarded as a process rather than an experiment that can be repeated in biological triplicate. If parallel models of the same treatment are produced the heterogeneity between drug-resistant cell lines should be examined with interest rather than dismissed as a non-reproducible experiment.

CONCLUSION

We have provided a detailed guide to the decision-making process for the development and ongoing maintenance of drug-resistant cancer cell lines. There is no one right way to make drug-resistant

cell lines. The case studies from our laboratories highlight how we have successfully developed models in a variety of ways for use in research projects.

METHODS

CELL CULTURE

H1299, H460, HCC1954, Malme-3M, OVCAR8, UPN251, and cells and their drug-resistant variants were grown in antibiotic and chemotherapy-free RPMI (Sigma #R8758). HT144 cells and their resistant variants were grown in antibiotic and chemotherapy-free McCoys 5A medium (Sigma). HT144, HCC1954, Malme-3M, OVCAR8, and UPN251 and their resistant variants were supplemented with 10% FCS (Lonza, Belgium). H1299, H460, and their resistant variants were supplemented with 5% FCS. All cell lines were maintained in a humidified atmosphere with 5% CO2 at 37°C. All cultures were tested routinely and were *mycoplasma*-free.

GROWTH CURVES FOR OPTIMIZATION OF SELECTION DOSES

OVCAR8 or UPN251 cells were plated in duplicate into 6-well plates at a cell density of 1×10^4 cells/mL in 1 mL media. A control plate was set up separately with duplicate wells. On day 2 1 mL of media with drug was added to all plates excluding the control, which received drug-free media to the same volume. On day 5 media was changed on all plates and replaced with drug-free media. The control plate and one drugged plate were taken down and cell counted. Cell counts for the control were compared to the drug treatment. A percentage cell survival was calculated in order to see the effects of drug treatment on cell growth/survival.

$$\text{Percentage cell survival} = \frac{\text{Average cell number of drugged cells}}{\text{Average control cell number}} \times 100$$

Over subsequent days one plate for each drug dose was observed under a light microscope to see when normal growth had returned. When cells were deemed to have returned to confluence this plate was cell counted to confirm recovery. Percentage cell survival will now be above or climbing to 100%. The time taken for cells to resume growth and return to confluence was recorded.

CYTOTOXICITY ASSAYS

To determine the cytotoxicity of chemotherapy drugs, cell growth/viability was measured using an acid phosphatase assay; $1.5–3 \times 10^3$ cells were seeded in flat-bottomed 96-well plates and incubated overnight prior to addition of drug. Chemotherapeutics were obtained from St Vincent's University Hospital, Dublin, Ireland. Lapatinib was purchased from Sequoia. Temozolomide was obtained from the National Cancer Institute. Other inhibitors and modulators were obtained from Sigma. Drug-free controls were included in each assay. Plates were incubated for a further 5 (HCC1954, Malme-3M and HT144) or 7 days (H1299 and H460) at 37°C in a humidified atmosphere with 5% CO_2 and cell viability was determined using an acid phosphatase assay (97). Growth of drug-treated cells was calculated relative to control untreated cells in biological triplicate.

STATISTICS

All experiments were performed at minimum in triplicate. Two-sample, two-tailed Student's *t*-tests were used to determine significant differences using $p < 0.05$ as a cut off.

AUTHOR CONTRIBUTIONS

Britta Stordal conceived the need for a review of methods of development of drug-resistant cell lines and wrote the manuscript. Martina McDermott, Alex J. Eustace, Steven Busschots, Laura Breen contributed their expertise in drug-resistant cell line development, contributed data for case studies and assisted with locating references, and drafting the manuscript. Norma O'Donovan, John Crown and Martin Clynes mentored the development of the drug-resistant cell lines used in the case studies, as well as contributing their expertise in drug-resistant cell line development. All authors approved the final version of the manuscript.

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Small and innovative molecules as new strategy to revert MDR

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Multidrug resistance (MDR) is a complex phenomenon principally due to the overexpression of some transmembrane proteins belonging to the ATP binding cassette (ABC) transporter family. Among these transporters, P-glycoprotein (P-gp) is mostly involved in MDR and its overexpression is the major cause of cancer therapy failure. The classical approach used to overcome MDR is the co-administration of a P-gp inhibitor and the classic antineoplastic drugs, although the results were often unsatisfactory. Different classes of P-gp ligands have been developed and, among them, Tariquidar has been extensively studied both *in vitro* and *in vivo*. Although Tariquidar has been considered for several years as the lead compound for the development of P-gp inhibitors, recent studies demonstrated it to be a substrate and inhibitor, in a dose-dependent manner. Moreover, Tariquidar structure–activity relationship studies were difficult to carry out because of the complexity of the structure that does not allow establishing the role of each moiety for P-gp activity. For this purpose, SMALL molecules bearing different scaffolds such as tetralin, biphenyl, arylthiazole, furoxane, furazan have been developed. Many of these ligands have been tested both in *in vitro* assays and in *in vivo* PET studies. These preliminary evaluations lead to obtain a library of P-gp interacting agents useful to conjugate chemotherapeutic agents displaying reduced pharmacological activity and appropriate small molecules. These molecules could get over the limits due to the antineoplastic-P-gp inhibitor co-administration since pharmacokinetic and pharmacodynamic profiles are related to a dual innovative drug.

Keywords: MDR, P-gp, dual effect, multitarget drugs, MDR reverting activity

INTRODUCTION

Human ATP binding cassette (ABC) transporters belong to a family of 49 genes classified in seven subfamilies (A–G) (1, 2).

Some of these transporters are involved in multidrug resistance (MDR) such as ABC-B1 (P-glycoprotein, P-gp), ABC-G2 (breast cancer resistance protein, BCRP), and ABC-C1-6 (MDR associated proteins, MRP1-6) (3).

Multidrug resistance is a complex phenomenon that limits the efficacy of chemotherapeutic treatment. Some tumors are intrinsically resistant to pharmacological therapy, while others, initially sensitive to chemotherapy, become resistant during the treatment. Resistance to anticancer drugs is due to several factors such as pharmacokinetic, tumor micro-environmental changes, or cancer cell-specific factors that occur at different levels:

– increased drug efflux or decreased drug influx;
– drug inactivation;
– drug target modification;
– apoptosis evasion.

The first of these mechanisms is mediated by plasma membrane transporters such as P-gp.

Several strategies were suggested for reversing MDR and, among them, the co-administration of anticancer drugs with an ABC transporter inhibitor has been proposed to improve the bioavailability of chemotherapeutic agents (4, 5).

Among MDR pumps, P-gp is one of the most studied because of the broadest substrate specificity and the widest tissues and organs distribution such as liver, intestine, brain, and kidneys (6). This transporter actively effluxes several compounds from cells and, being overexpressed in tumor cells exerting a significant effect on the bioavailability, distribution, and activity of many drugs, especially those used in the cancer treatment (7).

P-glycoprotein is a 170-kDa phosphorylated glycoprotein encoded by MDR1 gene. Structurally, P-gp contains 12 transmembrane helices organized in 2 membrane spanning domains (MSDs), each containing 6 transmembrane helices and 2 nucleotide-binding domains (NBDs) responsible for ATP binding (3, 8) (**Figure 1**).

This protein uses ATP hydrolysis as the energy source for the translocation of several structurally unrelated molecules (9). This suggests the presence of different binding sites (10, 11). Indeed, four distinct interacting binding sites have been identified in P-gp structure (**Figure 2**). Sites I–II are assigned for the binding of substrates, site III is for the modulators, and site IV binds the inhibitors. It has been hypothesized that the binding site of inhibitors is folded to inhibit the ATP binding and so the pump, although binds the substrate, cannot extrude it. The four binding

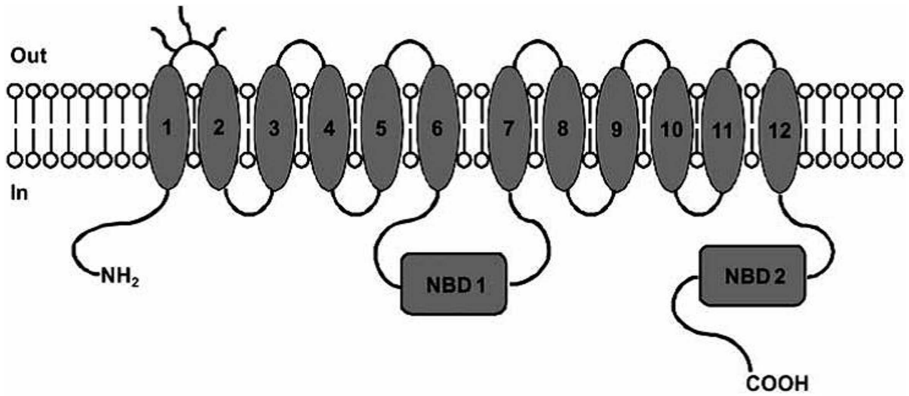

FIGURE 1 | P-gp structure: MSDs and NBDs. Picture reported in Ref. (6).

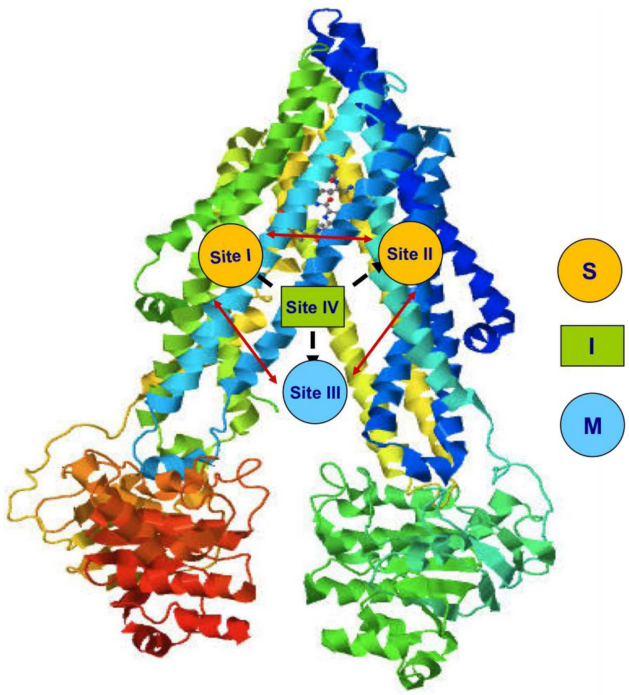

FIGURE 2 | P-gp binding sites (S: substrate, M: modulator, I: inhibitor).

sites are able to allosterically communicate in a negative heterotropic manner and the binding to one of these sites switches the other sites to a low-affinity conformation (9).

Several models have been proposed for P-gp efflux: (1) pore, (2) flippase, (3) hydrophobic vacuum cleaner, and (4) two-cylinder engine (12, 13).

In the pore model, drugs binding P-gp to the cytosol are transported out of cells through a channel created by protein.

In the flippase model, P-gp links the drugs that are transported from the inner to the outer compartment of the plasma membrane against a concentration gradient.

In the hydrophobic vacuum cleaner model, molecules, recognized by P-gp in the lipid bilayer, enter into the protein from the membranous site and exit through the central cavity.

In the two-cylinder engine model, it has been hypothesized that P-gp contains two drug-binding sites, in which each half-transporter has its own drug carrier (14).

The translocation mechanism of P-gp was blocked by inhibitors activity (12).

Indeed, the initial step of the translocation process is the binding of drugs to an high-affinity site and simultaneously the binding of ATP to the NBDs. Drug and ATP binding are coupled to the ATP hydrolysis and two ATP molecules are needed for the turnover; the first molecule is responsible for drug translocation and the second is needed to set the transporter in the basal state (**Figure 3**).

Compounds interacting with P-gp have been classified into three categories: substrates, inhibitors, and modulators (**Figure 4**).

Substrates are molecules that are actively transported by the protein and therefore have a higher concentration outside the cell with respect to the cytosol (10).

High substrate concentration causes a block of the pump by saturating the substrate-binding sites and in literature, this finding led to a mistake in terms of intrinsic P-gp-interacting mechanism (10).

Modulators modify substrate-binding site through a negative allosteric mechanism. Imaging studies with radiotracers demonstrated that modulators are able to alter the substrate-binding site in a non-competitive manner, modifying the maximal receptor density (B_{max}) but not the dissociation equilibrium constant (K_d). Therefore, it suggests that allosteric communication between substrate- and modulator-binding sites exists (10).

Inhibitors block the translocation activity of P-gp by interfering with the ATP binding to NBD. However, although different mechanisms, substrates, modulators, and inhibitors could exert the same final biological effect restoring cell sensitivity to chemotherapeutic agents.

BIOLOGICAL ASSAYS

The characterization of P-gp-interacting mechanism of drugs is an important task in the development of P-gp ligands and it is performed by specific biological *in vitro* assays (15) (**Figure 5**).

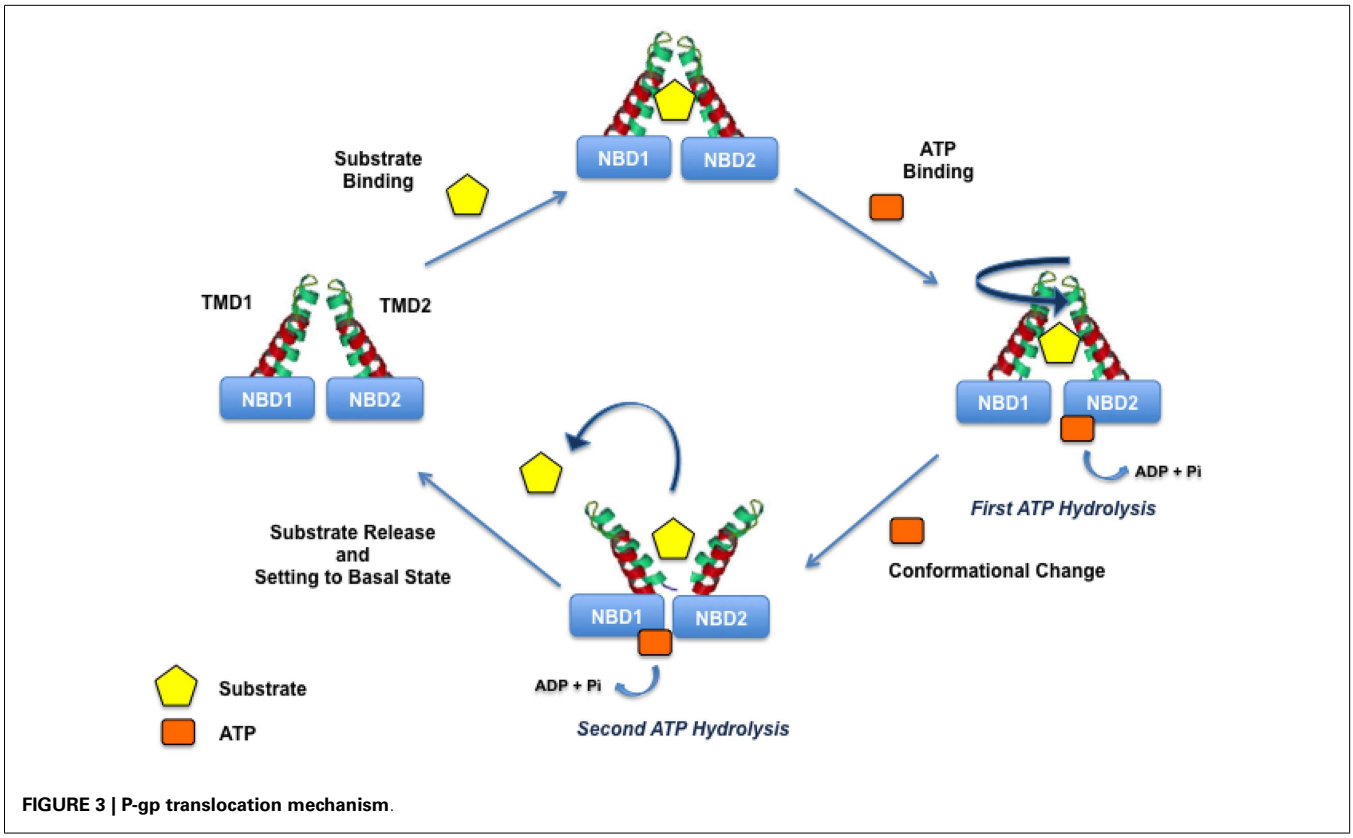

FIGURE 3 | P-gp translocation mechanism.

A wide range of methodologies has been used to characterize the P-gp interaction. These methods employ intact cells or purified protein and a combination of different approaches is often required to identify the mechanism of interaction.

The identification of the P-gp-interacting mechanism is performed by the combination of three biological assays:

- determination of the apparent permeability (P_{app});
- ATP cell depletion;
- inhibition of the P-gp-mediated transport of a fluorescent probe (Calcein or Rhodamine);
- everted gut sac model.

APPARENT PERMEABILITY DETERMINATION

Apparent permeability (P_{app}) is a pharmacokinetic parameter that is determined in Caco-2 cells system, a cell monolayer model suitable for the study of the passive and active transport through the biological membranes. Indeed, in this system the Basolateral–Apical flux (B → A), representative of passive diffusion, and Apical–Basolateral flux (A → B), representative of active P-gp-modulated transport, are determined.

The BA/AB ratio is useful to identify P-gp inhibitors (BA/AB <2), P-gp substrates (BA/AB from 18 to 20), or P-gp modulators (BA/AB ranging from 2 to 18).

ATP CELL DEPLETION

This assay, performed in Caco-2 cell monolayer and in Madin–Darby Canine Kidney cells (MDCK) overexpressing P-gp, permits to establish if the compound is able to deplete ATP. Substrates activate ATPase whereas inhibitors are not transported unchanging the ATP cell level.

INHIBITION OF CALCEIN-AM TRANSPORT

This assay is useful to determine the potency (EC_{50}) of P-gp ligands and is performed in MDCK cells, stably transfected for P-gp overexpression (MDCK-MDR1). The assay is carried out using a non-fluorescent prodrug, the acetoxymethyl ester of calcein (calcein-AM), which is a P-gp substrate. In the presence of a P-gp modulator, calcein-AM diffuses into the cytosol where it is hydrolyzed to the fluorescent dye calcein, that is not a P-gp substrate and since hydrophilic, it cannot diffuse through the membrane (17). In this assay also Rhodamine may be employed as a probe although calcein-AM is more useful because Rhodamine displays good cell permeability and therefore, its fluorescence determination at stationary state is more complex than calcein-AM.

THE EVERTED GUT SAC MODEL

This assay is an *ex vivo* method to study the P-gp-mediated intestinal absorption of drugs and their interactions with CYP450 enzymes (18, 19). This double information (the effect of P-gp-mediated transport and CYP450-metabolizing activity) is obtained since the everted gut sac assay is performed on isolated rat ileum where CYP450 enzymes and P-gp are present.

This combined study is needed because inhibitors and substrates may display overlapping activities toward CYP450 enzymes and the P-gp pump (20).

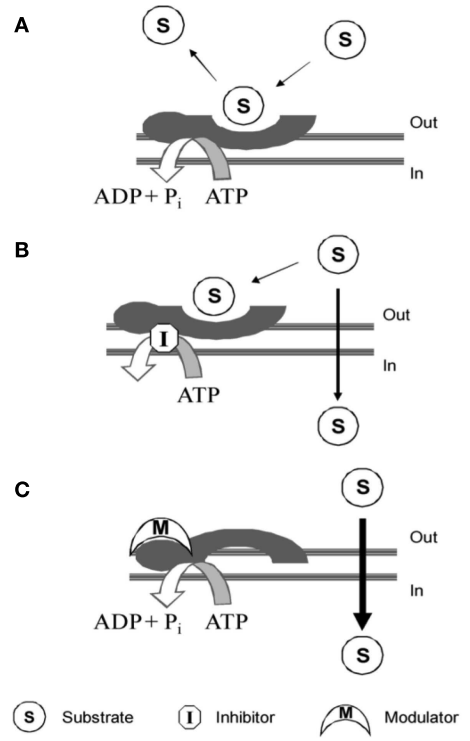

FIGURE 4 | P-gp interacting mechanism of: (A) substrate; (B) inhibitor; (C) modulator. Picture reported in *Journal of Medicinal Chemistry* (2010) 53:1883–97.

In this method, the transport of a known P-gp radiolabeled or fluorescent substrate, in the absence and presence of a P-gp-interacting agent, is evaluated. The flux of a P-gp substrate such as Rhodamine 125, from serosal to mucosal compartment and *vice versa*, is represented by the efflux ($k'2$) and influx rate constants ($k'1$), respectively. These determinations are carried out in the presence of a P-gp-interacting agent to determine $k''2$ and $k''1$, the efflux and influx constants of the tested substrate after P-gp interaction.

P-gp SUBSTRATES, MODULATORS, INHIBITORS

The most important studied P-gp ligands are classified in three different categories.

P-gp SUBSTRATES

This class is the most extensively studied and Verapamil and *N*-desmethyl-loperamide (**Chart 1**) are to date the gold standard of this class of compounds.

Verapamil, a calcium channel blocker, was found to reverse MDR (21) and it has become the reference compound for developing other P-gp substrates. It saturates the pump at high doses and therefore, it is a potential ligand for reversing MDR in co-administration with antineoplastic agent for different types of cancer (21, 22). However, verapamil cannot be employed because of toxic cardiovascular side effects. Despite this, the radiolabeled compound, ^{11}C-verapamil, has been developed to visualize P-gp function and to date, it is considered to be the reference substrate for imaging P-gp activity (23). However, verapamil is quickly metabolized by CYP450 enzymes giving radiometabolites, some of which are themselves P-gp substrates (24).

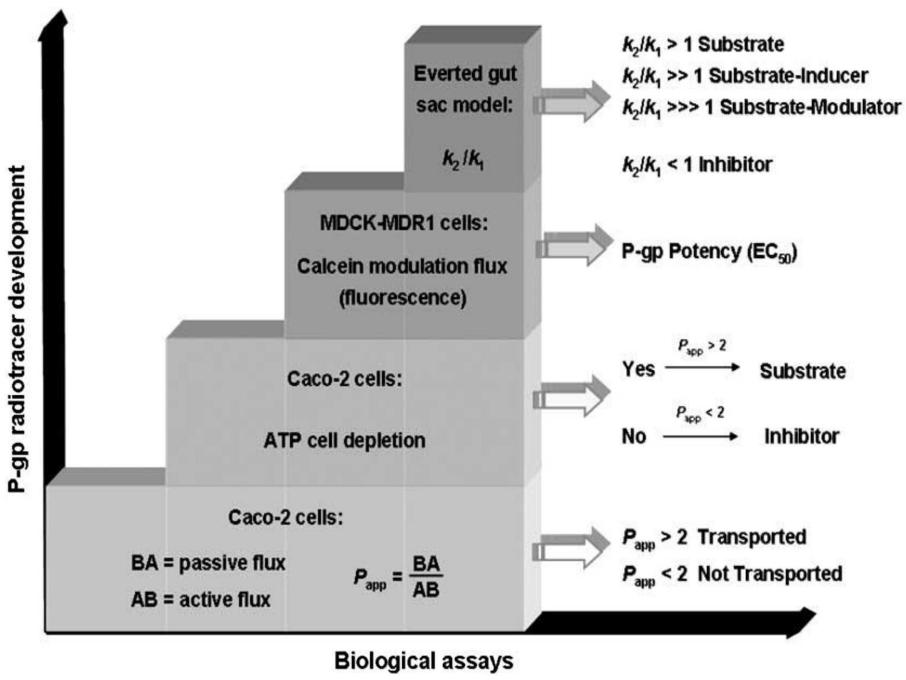

FIGURE 5 | Characterization of P-gp ligands (k_1: influx constant; k_2: efflux constant). Picture reported in Ref. (16).

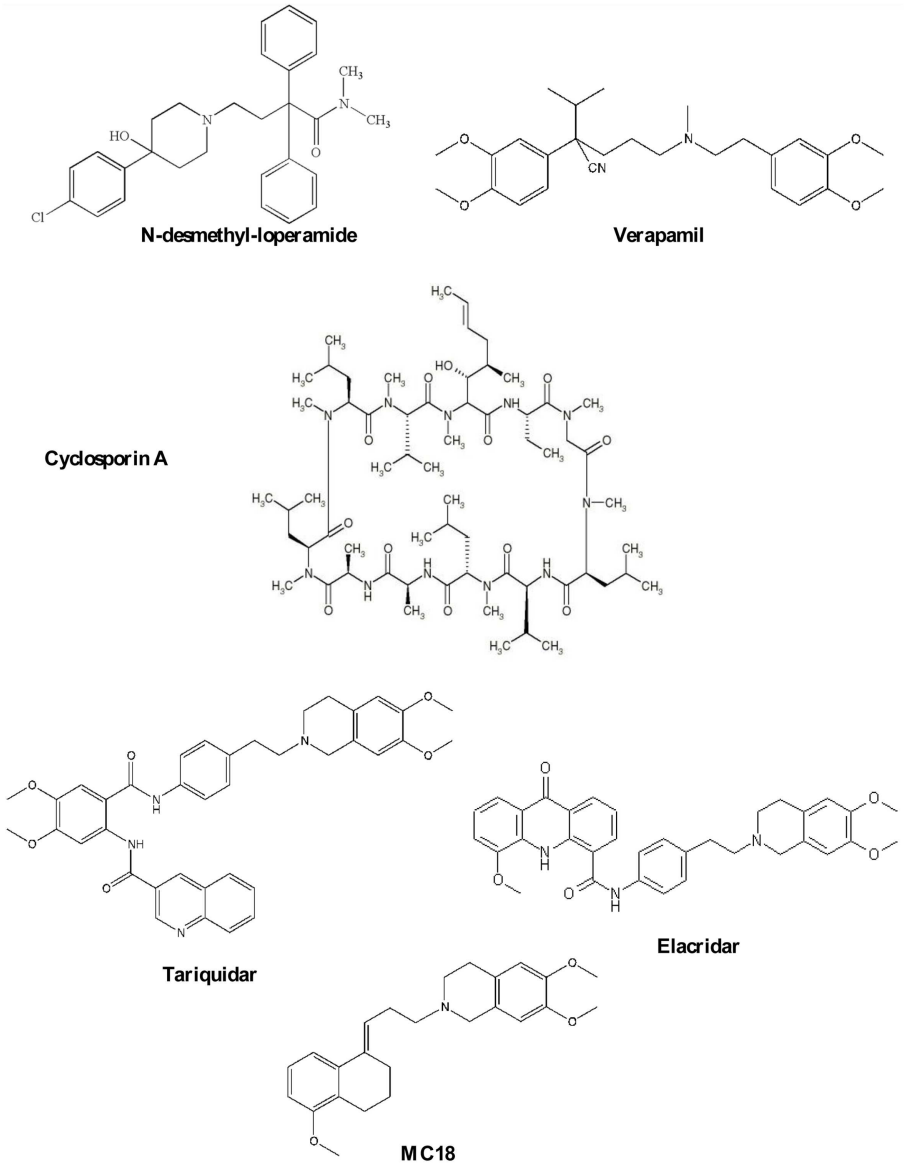

CHART 1 | P-gp ligands.

N-desmethyl-loperamide (dLop) is the major metabolite of Loperamide and at low concentrations it acts as substrate while at high concentrations, as reported for verapamil, dLop saturates the pump (25). Also, dLop has been radiolabeled and used for imaging P-gp *in vivo* by PET analysis (26).

P-gp MODULATORS

Cyclosporin A (CsA, **Chart 1**), an immunosuppressant agent, is a P-gp modulator, widely used *in vitro* as a tool to study MDR because it restores the cell concentration of chemotherapeutic agents. In imaging studies, the co-administration of CsA with a radiolabeled P-gp substrate (27) has been performed to visualize the P-gp activity because it increases radiotracer cell uptake by modulating the P-gp-binding sites. However, CsA treatment enhances the uptake of the radioligand in all regions where P-gp is present including targeted and non-targeted tissues (2–15, 17–30).

P-gp INHIBITORS

Elacridar (**Chart 1**) is a dual P-gp/BCRP ligand and can be orally administered. It was tested in combination with doxorubicin in patients with advanced solid tumors (31). At the recommended dose of doxorubicin, a pharmacologic hematologic toxicity was observed, mainly consisting of leukocytopenia and granulocytopenia.

Moreover, Elacridar was co-administrated with topotecan (32), a P-gp and BCRP substrate (Phase I) with unsatisfactory results (33). ^{11}C-Elacridar is tested *in vivo* to evaluate the overexpression of P-gp and BCRP in human colon adenocarcinoma (33, 34).

Tariquidar (**Chart 1**), an anthranilic derivative, is the most potent P-gp ligand in nanomolar range. It has been co-administrated in clinical trials with chemotherapeutic agents for restoring the efficacy of therapy (35–38). Results were quite unsatisfactory because of poor selectivity against other ABC transporters that are not involved in MDR. Tariquidar has been evaluated *in vivo* for diagnosing breast tumors in animal model using (R)-^{11}C-verapamil (35, 39, 40).

Recently, the suitability of ^{11}C-tariquidar and ^{11}C-elacridar for visualizing cerebral P-gp expression in healthy human subjects, in analogy to a previous preclinical study (16, 35), was investigated. However, ^{11}C-tariquidar and ^{11}C-elacridar displayed a "substrate-like *in vivo* behavior"; in particular, they are dual P-gp/BCRP substrates and these findings disagreed with *in vitro* results (41).

(*E*)-6,7-Dimethoxy-2-[3-(5-methoxy-3,4-dihydronaphthalen-1(2*H*)-ylidene)propyl]-1,2,3,4-tetrahydroisoquinoline, better known as MC18 (**Chart 1**), is a small molecule bearing tetralin moiety (42). To date, ^{11}C-MC18 is the first P-gp inhibitor studied *in vivo* in PET studies. It displayed fourfold higher uptake in the target organs compared with ^{11}C-tariquidar and ^{11}C-elacridar (43).

STRATEGIES TO REVERT MDR

The pivotal role of P-gp in MDR has stimulated the development of P-gp ligands able to reverse the resistance to a wide number of drugs. Hence, the need to design potent and selective P-gp inhibitors stimulated the development of small molecules on which structure–activity relationship (SAR) studies could be easily and better performed. The development of these compounds is depicted in **Figure 6** and it is based on the synthesis of bioisosteres obtained through subsequent lead optimization studies.

SMALL LIBRARIES FROM VERSATILE SCAFFOLDS

Tetralin derivatives

The *lead compound* of this class is MC18 (**Figure 6A**) ($EC_{50} = 1.50\,\mu M$), bearing an (*E*)-double bond, a potent P-gp inhibitor (42). When the double bond shifts into the tetralin ring, the ligands are less potent than MC18 and are P-gp substrates. Moreover, the presence and the position of methoxy substituent on tetralin nucleus are important in terms of the potency and intrinsic activity. The saturated derivative, MC266 ($EC_{50} = 6.35\,\mu M$), was the best P-gp substrate in this class. Therefore, the partial conformational restriction of spacer is involved in the P-gp-interacting mechanism. These two lead compounds, inhibitor and substrate respectively, have been ^{11}C-radiolabeled and tested *in vivo* PET studies leading to significant and coherent results in comparison with the *in vitro* data previously reported (43).

Biphenyl and naphthyl derivatives

The conformational restriction of MC18 seems to be a requirement for improving P-gp-inhibitory activity. In order to evaluate this statement, the restriction of the spacer linking the non-basic moiety was tested in a series of molecules bearing two different fragments: 1,4-biphenyl and 2-naphthyl moieties (**Figure 6A**) (44).

In the biphenyl series, 4-biphenyl derivatives displayed the best activity in P-gp-inhibitory activity and among these compounds, the best result was obtained for MC70 ($EC_{50} = 0.69\,\mu M$). In recent years, MC70 was extensively studied in order to confirm its P-gp-interacting activity considering its role to enhance the chemotherapeutic agent when co-administrated (45).

Although tetralin and biphenyl derivatives displayed high P-gp activity, they were active toward other ABC transporters such as BCRP and MRP1.

Aryloxazole and arylthiazole derivatives

Aryloxazole and arylthiazole derivatives (**Figure 6A**) were designed as cycloisosters to improve the P-gp-inhibitory activity and selectivity. The results demonstrated that aryloxazole and arylthiazole derivatives, designed as cycloisosteres of biphenyl derivative MC70, were found to be less potent than the reference compound in inhibiting P-gp (46). Indeed, these compounds were screened by SAR studies toward BCRP and MRP1 giving interesting structural determinants for these pumps as depicted (**Figure 7**). Finally, it was found that both aryloxazoles and arylthiazoles were P-gp substrates.

Furthermore, the aryl fragments were replaced by a naphthyl nucleus and three heteronuclei (oxazole, thiazole, and furyl) have been evaluated (47). The obtained results showed that the replacement of aryl nucleus with naphthyl moiety lead to obtain compounds with three different activity profiles:

1. P-glycoprotein inhibitors: unsubstituted oxazoles or bearing −F and −OH on the naphthyl fragment;
2. Unambiguous substrates (48): oxazole bearing $-OCH_3$ on the naphthyl fragment and thiazole bearing −Br and −OCH3 on the naphthyl fragment.
3. Ambiguous substrates (48): oxazole bearing Br on the naphthyl fragment and unsubstituted thiazole or bearing Br on the naphthyl fragment.

Finally, all furyl derivatives were ambiguous substrates.

Galloyl-based derivatives

The 3,4,5-trihydroxybenzoyl and 3,4- and 3,5-dihydroxybenzoyl fragments (**Figure 6A**) have been employed as scaffolds for a set of ligands that are representative of pharmacophoric nucleus of tariquidar (49).

For this purpose, compounds have been divided into four different structural series that present:

1. variation at R,
2. variation at R1,
3. polyhydroxy derivatives,
4. pyrogallol 1-methyl ethers.

The benzamides of the first set showed good P-gp-inhibitory activity (IC_{50} ranging from 20 to 1.4 μM). These outcomes led to deepen the study with two approaches:

1. keeping fixed the 3,4,5-trimethoxy-*N*-(2-nitrophenyl)benzamide scaffold and introducing a series of R1 substituents in the 4-position of the aniline moiety;
2. desmethylating one or more methoxy groups belonging to A ring.

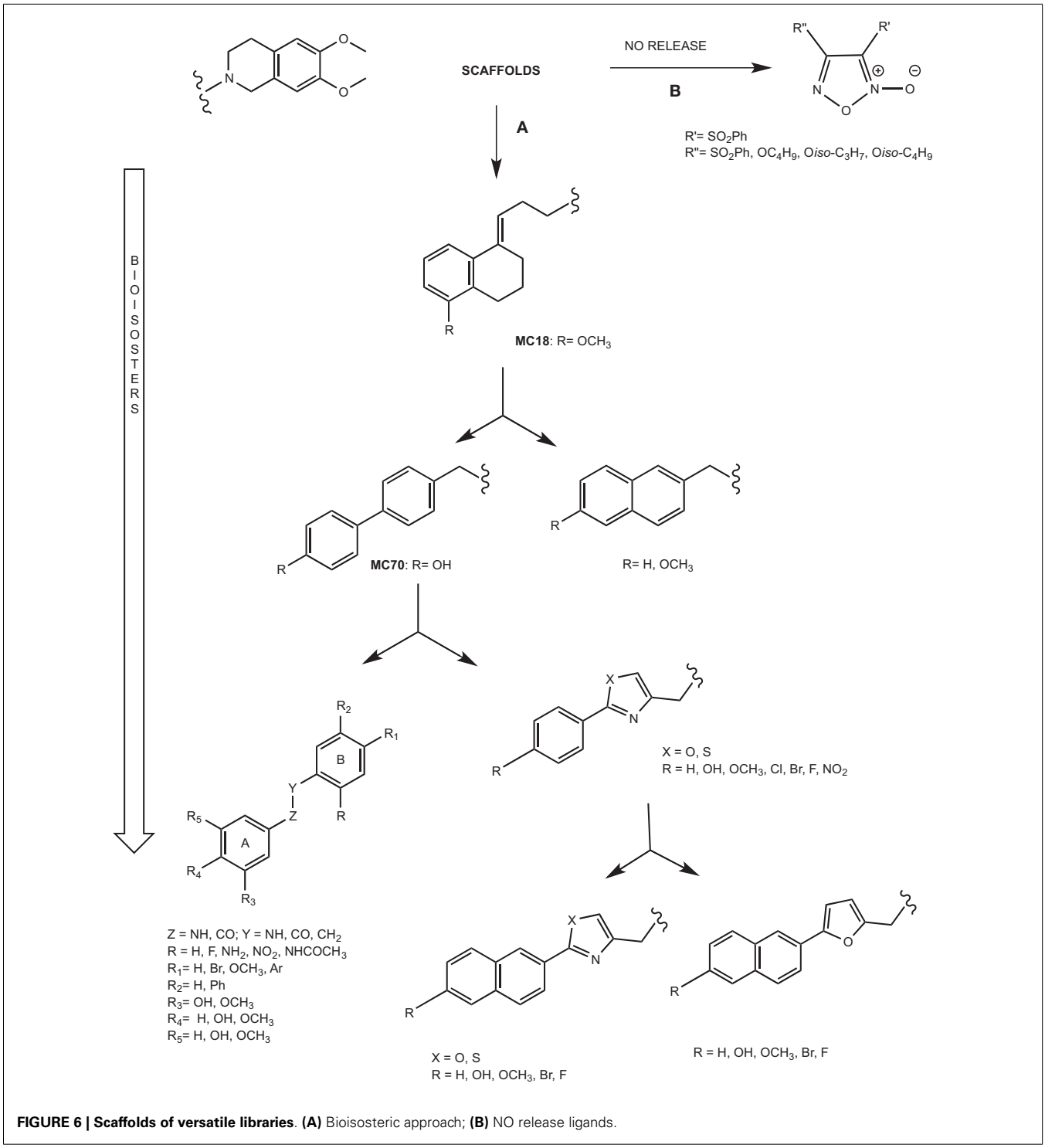

FIGURE 6 | Scaffolds of versatile libraries. **(A)** Bioisosteric approach; **(B)** NO release ligands.

The evaluation of data suggested that, with the exception of the 4-bromo and 4-methoxy congeners, all compounds of this second set were potent and selective P-gp inhibitors. In particular, molecules bearing R = NO_2 and in R1 H or benzo[1,3]dioxol-5-yl displayed submicromolar activity.

The screening of the gallamide derivatives indicated a moderate inhibitory potency for P-gp, independently with respect to the number and position of phenolic groups.

In the last series, all the pyrogallol-1-monomethyl ether derivatives showed moderate P-gp-inhibitory activity.

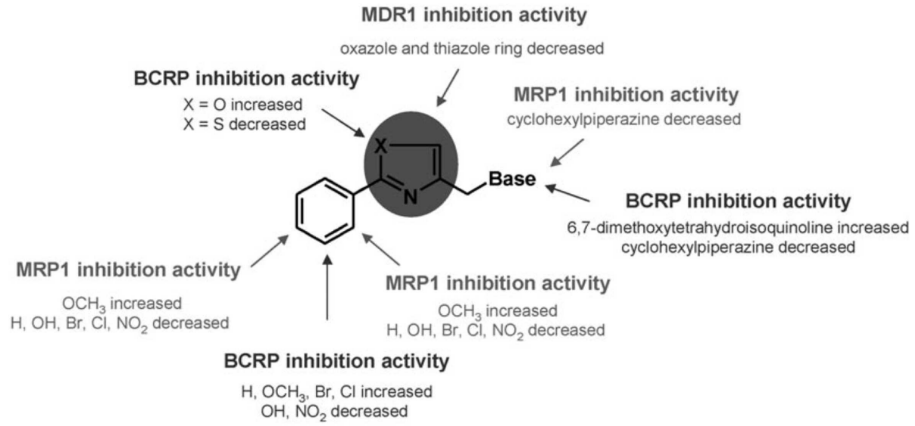

FIGURE 7 | SAR studies of aryloxazole and arylthiazole derivatives. Picture reported in Ref. (47).

Taking into account that the most important inhibitory activity changes were mainly concerned with the structural modifications on B ring, the role of the amide function was evaluated by testing the corresponding anilide and amine. The amine was equipotent with respect to the other tested compounds toward P-gp, despite the drastic change in terms of planarity and conformational flexibility. By contrast, the anilide showed no inhibitory activity toward P-gp. The 3,4,5-trimethoxyamide derivatives displayed moderate inhibitory activity toward P-gp.

Furoxan derivatives

Another strategy to reverse MDR is the nitration of a tyrosine present in TM6 domain of P-gp (50). It was reported that furoxans are able to produce *in situ* NO interacting with a thiol group. In fact, furoxans (**Figure 6B**) have been developed on the base of their properties to induce NO release and this pharmacological effect is the mechanism of coronary dilators (51–53). Moreover, the correlation between a decreased NO synthesis and MDR onset in doxorubicin-sensitive and doxorubicin-resistant cells has been widely reported (54). For this reason, a series of furoxan derivatives was designed and tested in activity and selectivity toward ABC transporters. Firstly, diphenylfuroxan derivatives and 3- and 4-phenylfuroxan isomer pairs, bearing different substituents with stereo-selective and lipophilic properties, were evaluated. In particular, the compounds having electron-withdrawing substituent and high lipophilic group such as phenylsulfonyl, displayed the best P-gp activity. The evaluation of phenylsulfonylfuroxan isomer pairs, bis(phenylsulfonyl)furoxan derivatives, and 3-phenylsulfonyl substituted furoxans, bearing alkoxy groups at position 4, displayed that 3-phenylsulfonyl substituted furoxans were found to be P-gp inhibitors, and the 4-substituted ligands showed the best activity and selectivity. Indeed, the best results were obtained both for 3,4-diphenylsulfonyl derivative ($EC_{50} = 3.0\,\mu M$) and for alkoxy derivatives such as *n*-Butoxy, *iso*-Propoxy, and *iso*-Butoxy ($EC_{50} = 2.26$, 2.15, and $2.23\,\mu M$, respectively) (55).

DUAL EFFECT DRUGS

Although the co-administration of a P-gp inhibitor and an antineoplastic agent could be considered a potential strategy to revert MDR, to date this approach was not clinically available because of pharmacokinetic limitations, in particular different apparent permeability, bioavailability, and metabolism. For this reason, others approaches were taken into account in the recent past:

– use of P-gp-targeted antibodies (56);
– encapsulation of anticancer drugs in liposomes (57);
– nanospheres able to circumvent MDR (58).

New innovative approaches to revert MDR could be: the development of molecules having a dual effect, potent cancer cell-killing agent and P-gp activity/expression inhibitor, and the collateral sensitivity (CS).

For example, some taxanes inhibited P-gp activity employing Rhodamine 123 as a fluorescent dye (59). These compounds could disclose new perspectives because they not only acted as cytotoxic agents but also inhibited the activity of P-gp efflux pump.

In another study (60), starting from Indirubin (a traditional Chinese medicine), the most potent derivative, PH II-7, has been evaluated. This compound showed antitumor activity inducing itself apoptosis and S phase cell cycle arrest, and in the meantime, it is not a P-gp substrate and so, high cell concentration of this compound was detected. The confocal microscopy displayed that this compound was not P-gp effluxed and significantly increased Adriamycin and Vincristine effect by reversing MDR.

MULTITARGET DRUGS

Another strategy could be the design of multitarget drugs bearing scaffolds depicted in **Figure 6**, having antitumor and P-gp inhibitory activities. An example is the hybridization of the NO-donor furoxan scaffold with the anilinopyrimidine moiety present in Gefitinib, leading to phenylsulfonylfuroxan-anilinopyrimidine derivative (**Figure 8**). This compound displayed epidermal growth factor receptor (EGFR) inhibitory activity in the treatment of non-small-cell lung cancer (NSCLC) (61). It induced apoptosis in

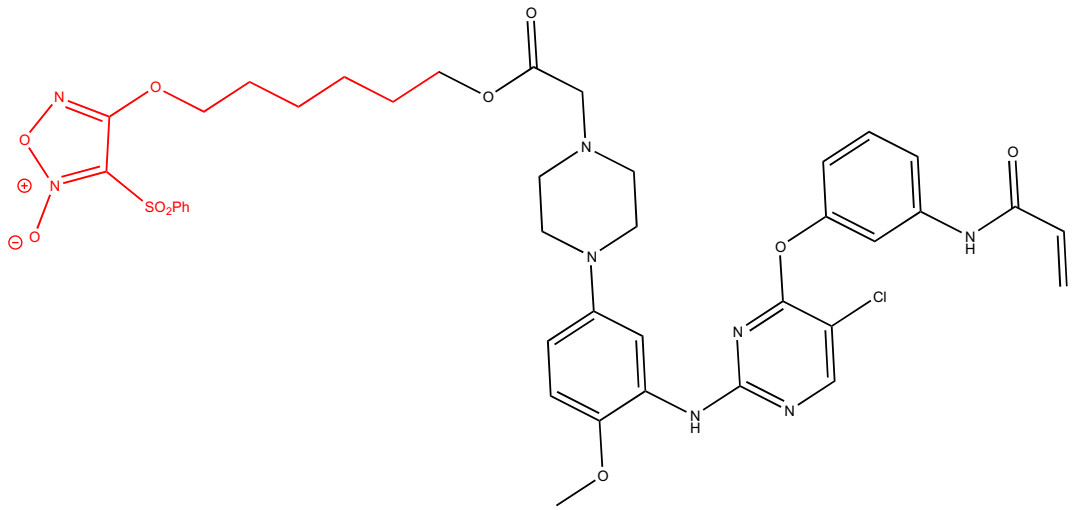

FIGURE 8 | NO-donor furoxan moiety linked to Gefitinib-like scaffold.

H1975 and HCC827 cells, inhibited EGFR downstream signaling in H1975 cells, and suppressed the nuclear factor-κB activation in H1975 cells. Furthermore, it released high levels of NO in H1975 cells but not in normal human cells, inducing apoptosis, inhibiting metastasis, and sensitizing tumor cells to chemotherapy by the inhibition of drug efflux transporters (51, 62). In this study, the two nuclei, furoxan and anilinopyrimidine, were separately evaluated for their effects. The activity showed by the two nuclei separately was lower than that exerted by the linked molecule. These results suggest that the antiproliferative activity of the compound might be attributed to the synergic effects of anilinopyrimidine and NO-donor moieties.

In another study, a class of 4-substituted methoxybenzoylarylthiazoles (SMART, **Figure 9**) was evaluated (63). These compounds exhibited great potency *in vitro* and broad spectrum cellular cytotoxicity. The *in vitro* and *in vivo* evaluation of the anticancer properties of three SMART compounds demonstrated that they potently bound to the colchicine-binding site in tubulin, inhibited tubulin polymerization, arrested cancer cells in G2/M phase, and induced apoptosis. Moreover, these compounds were able to overcome MDR since they were found equally cytotoxic in a parent cell line (OVCAR-8) and in a MDR-positive cell line (NCI/ADR-RES).

These findings demonstrated that some scaffolds such as furoxan and arylthiazole, already reported in our library as P-gp ligands, could be considered as the starting point to develop multitarget drugs. In these studies, the chemotherapeutic moiety is linked to furoxan (NO donor useful to revert MDR) or arylthiazole fragment (P-gp modulator), and the final effect was more potent that the single effect expected from each drug.

COLLATERAL SENSITIVITY

An alternative potential approach to treat drug-resistant tumors is the CS where several compounds selectively kill MDR cells without affecting the non-resistant parental cells (64).

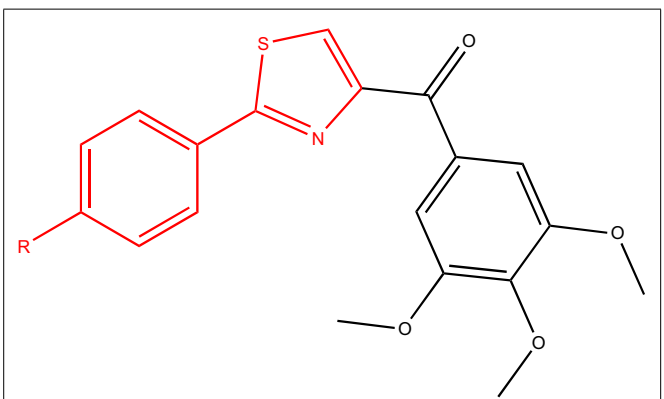

FIGURE 9 | SMART ligand.

Different hypotheses have been proposed to better explain this mechanism:

- production of reactive oxygen species (ROS);
- energetic level changes;
- extrusion of essential endogenous substrates for cell survival;
- perturbation of cell membranes.

The first hypothesis takes into account that several CS agents are substrates of P-gp, stimulating ATPase activity and in the meantime, the substrate extrusion from the plasma membrane into the extracellular environment (65). Once back in the extracellular environment, substrates repeat this cycle and P-gp performs a process known as futile cycling to increase the ATP hydrolysis inducing oxidative stress. MDR cells initiate apoptosis when ROS levels overcome a certain limit. Two reported ligands inducing CS are siramesine and the P-gp substrate and

σ_2 agonist 9-[4-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)butyl]-9*H*-carbazole that generate more ROS in the MCF7/Adr than in the MCF7 cell line (66).

Another hypothesis is that P-gp-expressing cells are more sensitive to changes in energy utilization. Indeed, several compounds that interfere with cellular metabolic pathways, such as glycolysis or oxidative phosphorylation, have been identified as MDR-selective agents. The glycolysis antimetabolite 2-deoxy-D-glucose (2-DG) seems to confirm this finding. Indeed, 2-DG activates apoptosis and selectively kills numerous MDR cell lines compared to drug-sensitive parental lines.

The extrusion hypothesis asserts that CS agents mediate cytotoxicity by stimulating, sensitizing, or facilitating the extrusion of endogenous essential components. This phenomenon is not reported in P-gp-expressing cells but it may be the case for MPR1-mediated CS.

Moreover, several CS agents alter membrane biophysical properties (67). Indeed, they induce membrane perturbation in P-gp-expressing cell lines, leading to the hypothesis that changes in membrane structure and fluidity contribute to CS (68). Pentazocine and verapamil are reported to reduce membrane fluidity in the colchicine-resistant B30 cell line.

CONCLUSION

To date the co-administration of a chemotherapeutic drug with a P-gp inhibitor, the encapsulation of anticancer drugs in liposomes, and the nanosphere formulation to reverse MDR failed for several reasons. This review aims to disclose new strategies in the design of multitarget drugs useful toward MDR. Interesting approaches are: (i) the development of drugs bearing a unique moiety responsible for the anticancer effect and MDR reversing activity; (ii) the design of molecules bearing different pharmacophores for multitarget activity; (iii) the evaluation of CS agents.

In the present review, we overviewed our P-gp ligands library to suggest new scaffolds that could be used to design multitarget drugs in accordance with the approaches already reported.

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ABCG2 is not able to catalyze glutathione efflux and does not contribute to GSH-dependent collateral sensitivity

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ABCG2 is a key human ATP-binding cassette (ABC) transporter mediating cancer cell chemoresistance. In the case of ABCC1, another multidrug transporter, earlier findings documented that certain modulators greatly increase ABCC1-mediated glutathione (GSH) efflux and, upon depletion of intracellular GSH, induce "collateral sensitivity" leading to the apoptosis of multidrug resistant cells. Recently, it has been suggested that ABCG2 may mediate an active GSH transport. In order to explore if ABCG2-overexpressing cells may be similarly targeted, we first looked for the effects of ABCG2 expression on cellular GSH levels, and for an ABCG2-dependent GSH transport in HEK293 and MCF7 cells. We found that, while ABCG2 overexpression altered intracellular GSH levels in these transfected or drug-selected cells, ABCG2 inhibitors or transport modulators did not influence GSH efflux. We then performed direct measurements of drug-stimulated ATPase activity and ^{3}H-GSH transport in inside-out membrane vesicles of human ABC transporter-overexpressing Sf9 insect cells. Our results indicate that ABCG2-ATPase is not modulated by GSH and, in contrast to ABCC1, ABCG2 does not catalyze any significant GSH transport. Our data suggest no direct interaction between the ABCG2 transporter and GSH, although a long-term modulation of cellular GSH by ABCG2 cannot be excluded.

Keywords: breast cancer resistance protein ABCG2, collateral sensitivity, glutathione efflux, intracellular glutathione depletion, multidrug resistance protein ABCC1, selective apoptosis, modulators

INTRODUCTION

The development of multidrug resistance (MDR) constitutes a major issue in cancer treatment. Overexpression of the three human ATP-binding cassette (ABC) transporters, ABCB1 (P-glycoprotein/P-gp; Juliano and Ling, 1976), ABCC1 (multidrug resistance protein 1/MRP1; Cole et al., 1992), and ABCG2 (breast cancer resistance protein, BCRP; Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999) has been proposed as one of the main causes of the MDR phenotype in resistant cancer cells. These proteins use ATP hydrolysis as energy source to catalyze the efflux of multiple structurally and functionally diverse chemotherapeutics from cancer cells.

Research has mainly focused on inhibitors development, in order to block this efflux mechanism and then restore chemotherapeutics efficacy. Unfortunately, scientists had to face clinical failures of third-generation ABCB1 inhibitors optimized *in vitro*, such as Zosuquidar (Cripe et al., 2010) or valspodar (Kolitz et al., 2010). In addition to improving clinical trials conducted with efflux inhibitors (Robey et al., 2010), alternative strategies to overcome the MDR phenotype need to be explored. Recently, a new strategy, so called collateral sensitivity (CS), characterized by hypersensitivity to small molecules triggering a preferential cytotoxicity, has been studied (Szakacs et al., 2006; Hall et al., 2009), and four different mechanisms have been hypothesized to underlie the hypersensitivity of ABCB1- and ABCC1-overexpressing cancer cells. CS agents may (i) produce reactive oxygen species by depleting intracellular ATP; (ii) exploit energetic sensitivities caused by ATP depletion; (iii) induce the extrusion of vital endogenous substrates; or (iv) perturb the plasma membrane (Pluchino et al., 2012).

A screening study identified two compounds as potential ABCG2-related CS agents in HEK293 transfected cells, one of them (NSC103054) directly interacting with the transporter (Deeken et al., 2009), and very recently an ABCG2 inhibitor (NP-1250) was reported to induce CS in mitoxantrone-selected MCF7 cancer cells (Ito et al., 2013). Although a mechanism based on extracellular vesicles photodestruction have been shown for another ABCG2-dependent CS (Goler-Baron and Assaraf, 2012), no direct mechanisms have yet been demonstrated; however, these different studies indicate that CS agents, specific for ABCG2, could be developed.

Reduced glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is a tripeptide ubiquitously expressed in cells and involved in many

Abbreviations: ABC, ATP-binding cassette; BCRP (ABCG2), breast cancer resistance protein; CS, collateral sensitivity; 2′,5′-DHC, 2′,5′-dihydroxychalcone; DNP-SG, *S*-(2,4-dinitrophenyl)glutathione; GSH, reduced glutathione; MDR, multidrug resistance; MRP1 (ABCC1), multidrug resistance protein 1; P-gp (ABCB1), P-glycoprotein

signaling pathways. It has been shown that ABCC1-overexpressing cells were hypersensitive to verapamil through a sharp GSH depletion due to an ABCC1-mediated efflux (Trompier et al., 2004). This phenomenon was further investigated in order to target resistant cancer cells in the frame of a new strategy to overcome the MDR phenotype in cancer (Barattin et al., 2010; Genoux-Bastide et al., 2011). ABCC1 is also known to transport oxidized glutathione disulfide (GSSG) which is however present in low amounts (Keppler et al., 1997). Based on our experience with ABCC1-specific CS and on recent reports in which ABCG2 was proposed as a new GSH transporter (Brechbuhl et al., 2009, 2010) we aimed at developing new ABCG2-specific modulators able to induce ABCG2-mediated GSH extrusion in order to induce a drastic intracellular GSH depletion leading to cell death.

In this study, we focused on searching inducers of ABCG2-dependent depletion of intracellular GSH among known death inducers of ABCC1-overexpressing cells, such as verapamil and xanthones (Genoux-Bastide et al., 2011), or known ABCG2 inhibitors (Ahmed-Belkacem et al., 2005; Valdameri et al., 2012). To ascertain the direct role of ABCG2 in GSH efflux, we measured direct transport of radioactive GSH in membrane vesicles.

MATERIALS AND METHODS

COMPOUNDS

Verapamil, Ko143, apigenin, ATP, chrysin, ditiothreitol (DTT), galangin, 5,5′-dithiobis(2-nitrobenzoic acid; DTNB), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), quercetin, glutathione (GSH), glutathione reductase, and bicinchoninic acid (BCA) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Acivicin was purchased from CliniSciences (Montrouge, France). ^{3}H-methotrexate and ^{3}H-GSH were purchased from Moravek Biochemicals and PerkinElmer, respectively. All other tested compounds were kindly provided by Prof. Ahcène Boumendjel (UJF Grenoble, France) and prepared as previously described (Genoux-Bastide et al., 2011; Valdameri et al., 2012). Tested compounds were dissolved in DMSO and stored at −20°C; they were warmed to 25°C and diluted in Dulbecco's modified Eagle's medium (DMEM) just before use (0.5% DMSO final concentration).

CELL CULTURE

The cell lines were kindly provided by Drs Susan Bates and Robert Robey, NCI, Bethesda, MD, USA. The selected human breast cancer cell line (MCF7-MX100) and the human fibroblast HEK293 cell line transfected with either *ABCG2* (HEK-*ABCG2*) or the empty vector (HEK-pcDNA3.1) were prepared as respectively reported (Honjo et al., 2001; Robey et al., 2003). The HEK293 and MCF7 cells were maintained in DMEM (high glucose, PAA) and in Roswell Park Memorial Institute medium (RPMI-1640, PAA) respectively, supplemented with 10% fetal bovine serum (FBS, PAA), 1% penicillin/streptomycin (PAA) and with 0.75 mg/ml G418 (for HEK-pcDNA3.1 and HEK-*ABCG2* cells) or 100 nM mitoxantrone (for MCF7-MX100 cells). Cells were cultured at 37°C, 5% CO_2 in a humid atmosphere. Sf9 insect cells were cultured at 27°C in TNM-FH insect medium supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml)–streptomycin (100 μg/ml; Sigma Aldrich, Hungary).

INTRACELLULAR GLUTATHIONE ASSAY

HEK293 and MCF7 cells were seeded in 96-well plates at respective densities of 1×10^4 and 2×10^4 cells/well. After 24 h in culture, cells were exposed to the different compounds during 6 or 24 h under normal culture conditions. They were then washed with 200 μl PBS 1X (PAA), stirred during 1 h at 4°C with 100 μl of 10 mM HCl and freezed at −20°C overnight, to be lysed. The intracellular total glutathione (reduced GSH and oxidized GSSG) was measured using the method described by Tietze (1969) as modified by Anderson (1985). About 70 μl of the lysate were used to measure intracellular total glutathione and 20 μl for protein quantitation, both being performed in 96-well plates. Total glutathione was assessed by adding 100 μl of a reaction buffer containing 266 μM NADPH, *GSH reductase* at 10 U/ml and 555 μM DTNB, and the absorbance was read at 412 nm in a microplate reader (PowerWave 340, Biotek) every 30 s during 2 min. The slope for each sample and glutathione standard range was determined to quantify sample glutathione. Protein quantitation was performed using the BCA assay. The results were expressed in nmol glutathione/mg protein and intracellular total glutathione percentages were calculated using the 0 μM samples as 100%.

EXTRACELLULAR GLUTATHIONE ASSAY

HEK293 cells were seeded in 24-well plates at a density of 1.5×10^5 cells/well. After 24 h in culture, cells were co-treated with the compound and 0.5 mM acivicin (to block GSH degradation out of the cells) during the 24-h incubation time. Supernatants were collected and cells were washed with 200 μl PBS 1× and treated as for intracellular total glutathione measurement. About 70 μl of the supernatant were used to assess total extracellular glutathione, and protein titration was performed with cell lysate, by the same method as described for intracellular glutathione measurement.

CELL PROLIFERATION AS DETERMINED BY MTT ASSAY

The MTT colorimetric assay, as previously described (Mosmann, 1983), was used to assess the sensitivity of cells to compounds toxicity. HEK293 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h under normal culture conditions, cells were treated with compounds at increasing concentrations. After 72-h incubation under normal culture conditions a 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium Bromide (MTT) solution was added (0.5 mg/ml final concentration) in wells, and cells were incubated for 4 h at 37°C. Thereafter, supernatants were carefully withdrawn and 100 μl/well of the buffer ethanol/DMSO (50/50, v/v) were added to solubilize the reduced formazan dye under stirring. Absorbance at 570 and 690 nm were determined by using a microplate reader (PowerWave 340, Biotek). Results were expressed as the difference between OD_{570} and OD_{690}; cell survival percentage was calculated using 0 μM sample OD as 100%.

MEMBRANE PREPARATION

For obtaining membrane vesicles insect cells were infected with recombinant baculoviruses containing the cDNA of wtABCG2 or ABCG2-K86M (Ozvegy-Laczka et al., 2005) or of ABCC1 (Bakos

et al., 1996). Membrane preparation and cholesterol enrichment of ABCG2-containing membranes was then performed as described earlier (Ozvegy et al., 2001; Telbisz et al., 2007).

ATPase ACTIVITY ASSAY

The ATP hydrolytic activity of ABCG2 has been determined as described in Ozvegy et al. (2001) and Telbisz et al. (2007). When the effect of GSH was investigated a minor modification in the assay buffer was introduced. About 10 mM DTT was used instead of 2 mM to prevent the oxidation of GSH.

^{3}H-METHOTREXATE AND ^{3}H-GSH TRANSPORT ASSAY

Sf9 membrane vesicles containing 90 μg protein were incubated in the presence or absence of 4 mM MgATP (or 4 mM MgATP + 1 μM Ko143 or 4 μM MK571) in a buffer containing 40 mM 3-(*N*-morpholino) propanesulfonic acid–Tris (pH 7.0), 56 mM KCl, 6 mM $MgCl_2$, and 10 mM DTT, in a final volume of 140 μl, at 37°C for 5–10 min as indicated on the figure legends. The measurement was started by the addition of 50 μM [^{3}H]methotrexate (Moravek Biochemicals) or 0.1–1 mM ^{3}H-GSH and carried out as described earlier (Ozvegy-Laczka et al., 2005).

STATISTICAL ANALYSIS

Statistic *t*-test analyses were performed using the SigmaPlot 12 software with $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

INTRACELLULAR GLUTATHIONE CONCENTRATION IN ABCG2-OVEREXPRESSING CELLS

In order to determine the influence of ABCG2 on cellular glutathione levels, we used two different cell lines overexpressing this transporter. The high level of ABCG2 expression and functionality, through ability to transport a number of substrate drugs, were previously described, in both transfected HEK-ABCG2 cells (Robey et al., 2003) and drug-selected MCF7-MX100 cancer cells (Honjo et al., 2001). Moreover, we performed western blot analyses which revealed that all cell lines did not express the ABCC1 protein (data not shown). The intracellular concentration of total glutathione (free GSH + oxidized GSSG) appeared to be significantly modulated by the presence of overexpressed ABCG2 (**Figure 1**). The glutathione level was lower in ABCG2-transfected HEK293 cells by comparison to the same cells transfected by the pcDNA3.1 empty vector (100 ± 8 versus 130 ± 11 nmol glutathione/mg protein). Interestingly, in drug-selected MCF7 cancer cells, which also overexpress ABCG2, the intracellular glutathione content was significantly higher than in the parental MCF7 cells (154 ± 7 versus 125 ± 10 nmol glutathione/mg protein). These data may indicate a long-term modulation of glutathione levels in various ABCG2-overexpressing cell types. Since total glutathione is known to be essentially constituted of free GSH and low amounts of oxidized GSSG, we measured both components separately in the different cell lines, upon incubation with 2-vinylpiridine behaving as a thiol scavenger. In all cases, the remaining oxidized GSSG was too low to be detected (not shown here), indicating no evidence of any change in the ratio between reduced and oxidized forms of glutathione.

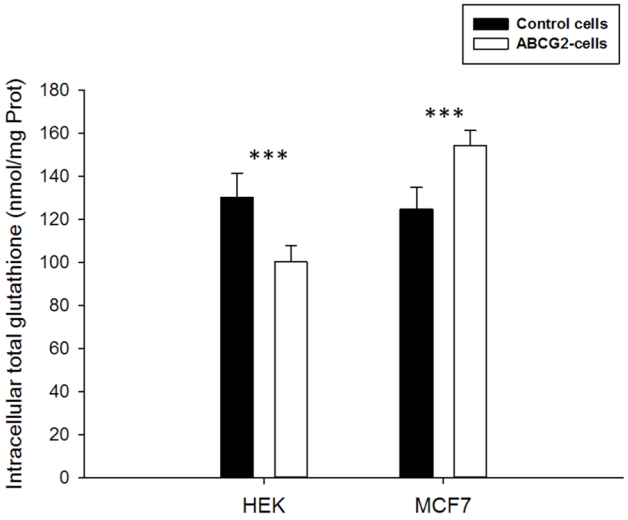

FIGURE 1 | Basal total intracellular glutathione levels in HEK293 or MCF7 cells. The values of either ABCG2-overexpressing cells (white bars) or control cells (black bars) represent means ± SD corresponding to at least three independent experiments performed in triplicates. The differences observed between both cell line pairs were significantly different. *t*-test analysis: $^{***}p < 0.001$.

INABILITY OF MODULATORS TO STIMULATE AN ACTIVE ABCG2-MEDIATED GLUTATHIONE EFFLUX

Since the 2′,5′-DHC chalcone was reported to stimulate ABCG2-dependent GSH efflux (Brechbuhl et al., 2010), the effects produced by addition of 2′,5′-DHC at increasing concentrations (up to 40 μM) were analyzed here on the intracellular glutathione levels of both transfected and drug-selected cells. A weak concentration-dependent decrease appeared in ABCG2-transfected cells after 6-h incubation with 2′,5′-DHC (**Figure 2A**), but not after 24-h incubation where an increase in intracellular glutathione content was observed in both cell lines (**Figure 2B**). By contrast, in drug-selected MCF7 cells, no decrease in glutathione content appeared after 6-h incubation (**Figure 2C**); a significant difference in glutathione level was observed after 24-h incubation, which was however essentially due to a higher increase in control cells than in ABCG2-overexpressing cells (**Figure 2D**). The extracellular glutathione content increased after 24-h incubation of ABCG2-transfected cells with increasing 2′,5′-DHC concentrations (around 40% at 10 μM), but the increase was at least as high in control cells indicating that it was not dependent on ABCG2 (**Figure 2E**).

We then studied the effects of verapamil which is known to strongly stimulate GSH efflux in ABCC1-overexpressing cells, leading to a fast and massive intracellular glutathione depletion able to trigger apoptosis (Trompier et al., 2004; Perrotton et al., 2007). A significant decrease of intracellular glutathione was indeed observed in ABCG2-transfected cells with increasing verapamil concentrations, up to 40 μM, which was 25–30% higher than in control HEK293 cells (**Figure 3A**). However, no decrease in glutathione content was observed under the same conditions with the ABCG2-overexpressing drug-selected cells,

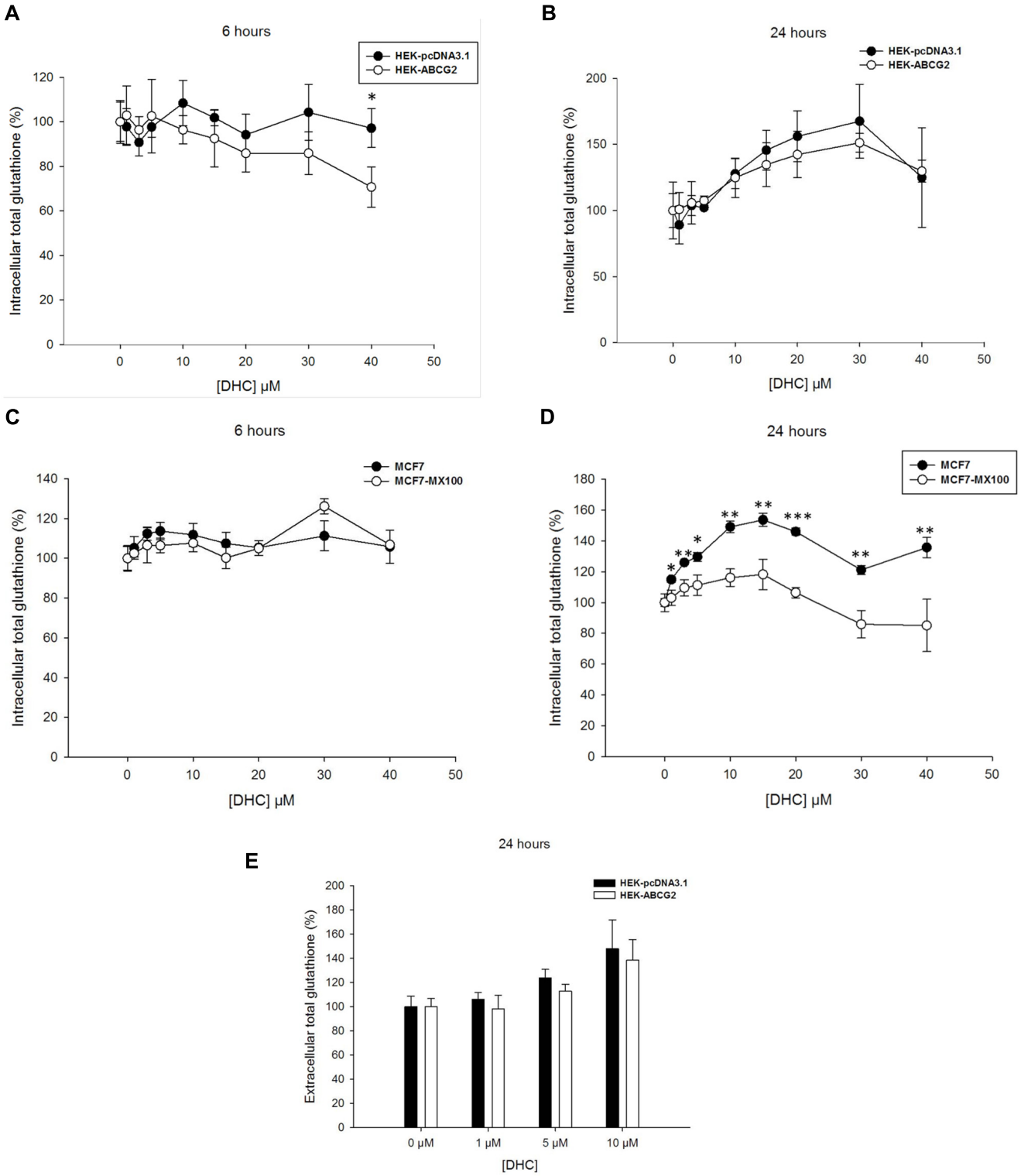

FIGURE 2 | Effects of 2′,5′-DHC increasing concentrations on total intracellular and extracellular glutathione levels. 2′,5′-DHC did not induce intracellular GSH depletion in ABCG2 cells (white circles) by comparison to control cells (black circles) in either HEK293 transfected cells during 6 **(A)** or 24 h **(B)**, or MCF7 cancer cells during 6 **(C)** or 24 h **(D)**. Moreover, there was no net ABCG2-dependent increase in extracellular GSH **(E)** induced by 2′,5′-DHC when comparing HEK-ABCG2 (white bars) and HEK-pcDNA3.1 (black bars) cells after 24-h incubation. The values represent means ± SD corresponding to at least two independent experiments performed in triplicates. Only the differences observed in **(D)**, between MCF7 and MCF7-MX100 cell lines at 24 h, were significant. *t*-test analysis: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

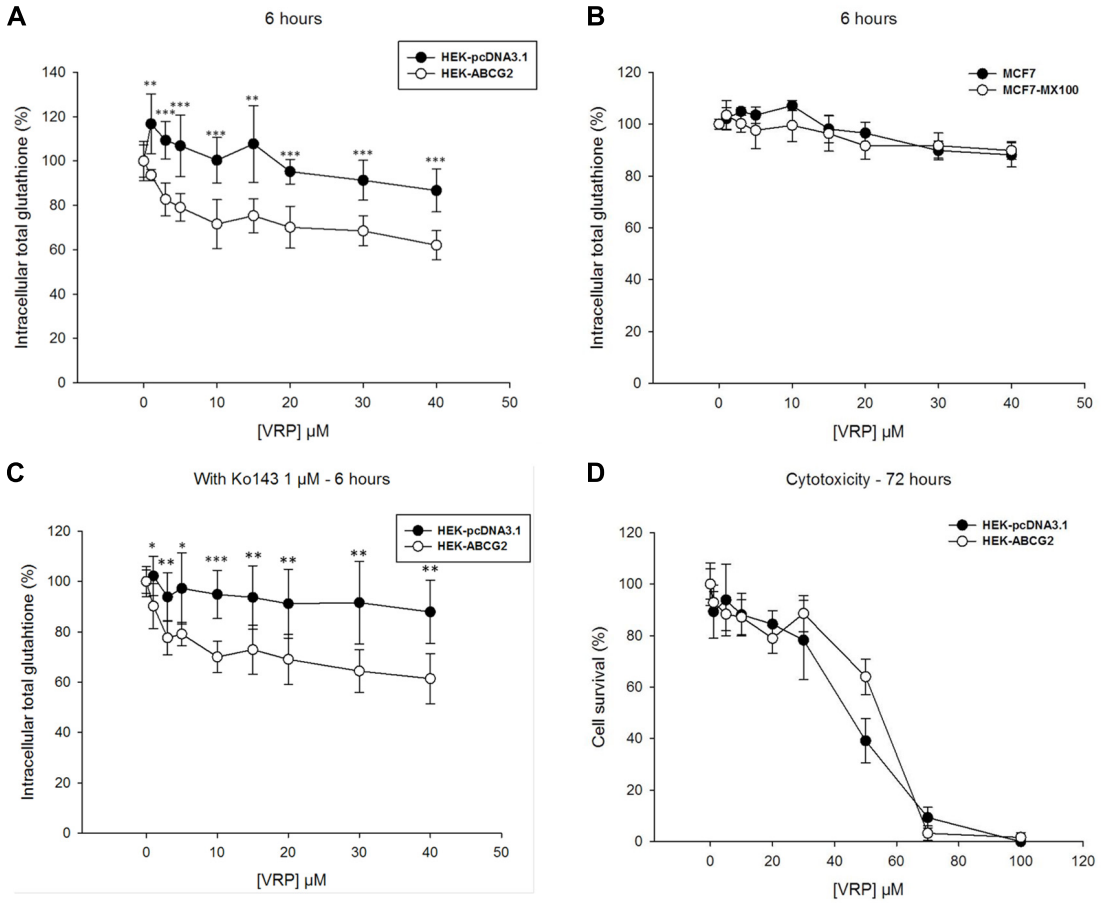

FIGURE 3 | Effects of verapamil on intracellular glutathione depletion and cells survival. Verapamil induced a significant intracellular GSH depletion in HEK293 transfected cells during 6-h incubation **(A)**, but not in the MCF7 selected cells **(B)** when comparing ABCG2-overexpressing cells (white circles) to control cells (black circles). This weak effect was not inhibited by Ko143 **(C)**, the difference in intracellular glutathione remaining unchanged. It was not either correlated to any ABCG2-specific collateral sensitivity in MTT cell survival assays **(D)** with ABCG2-overexpressing cells (white circles) and control cells (black circles). The values represent means ± SD corresponding to at least two independent experiments performed in triplicates. *t*-test analysis: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

which behaved similarly to control MCF7 cells (**Figure 3B**). The ABCG2-related decrease of intracellular glutathione was therefore further characterized in the presence of Ko143, a potent and specific inhibitor of ABCG2 transport activity. **Figure 3C** shows no significant alteration by comparison to **Figure 3A**, therefore indicating that such a decrease in intracellular glutathione was not dependent on ABCG2 activity. This was further confirmed by the absence of any CS toward verapamil cytotoxicity, as determined by MTT assays, since the ABCG2-transfected cells were not more sensitive than the control cells (**Figure 3D**).

Finally, two other series of compounds were investigated for their ability to modify the intracellular glutathione level. The first series included xanthones (X8, 9, 10, 18, 22, 23) known to induce, similarly as verapamil, a strong depletion in intracellular glutathione in ABCC1-overexpressing cells (Genoux-Bastide et al., 2011), and the second series contained chalcones (C27, 37, 38, 40; Valdameri et al., 2012) and 6-prenylchrysin (6-Pc; Ahmed-Belkacem et al., 2005) known as ABCG2 inhibitors. **Figure 4** shows that some xanthones induced a significant decrease in intracellular glutathione, up to around 30% for X8 and X9 and 20% for X23, similarly to the effect observed with verapamil in **Figure 3A**. By contrast, the ABCG2 inhibitory chalcones, except for C27, and 6-prenylchrysin did not induce any decrease of intracellular glutathione in ABCG2-transfected cells.

NO DETECTABLE INTERACTION BETWEEN GSH AND ABCG2 IN EITHER ATPase OR TRANSPORT ASSAY

We previously demonstrated that the baculovirus-insect cell heterologous expression system is a useful tool for the detection of interactions between a given test compound and ABCG2 (Szakács et al., 2008). Briefly, compounds modifying the ATP hydrolytic activity of ABCG2 interact with the transporter, and can be either transported substrates or inhibitors of the protein. In order to define whether GSH is able to interact with ABCG2, we have tested its effect in the ATPase assay using cholesterol-loaded Sf9 vesicles ensuring higher ABCG2 activity. We found that the ATPase activity of ABCG2 was not affected by GSH addition up to 10 mM, by contrast to a transported substrate such as 1 µM quercetin which

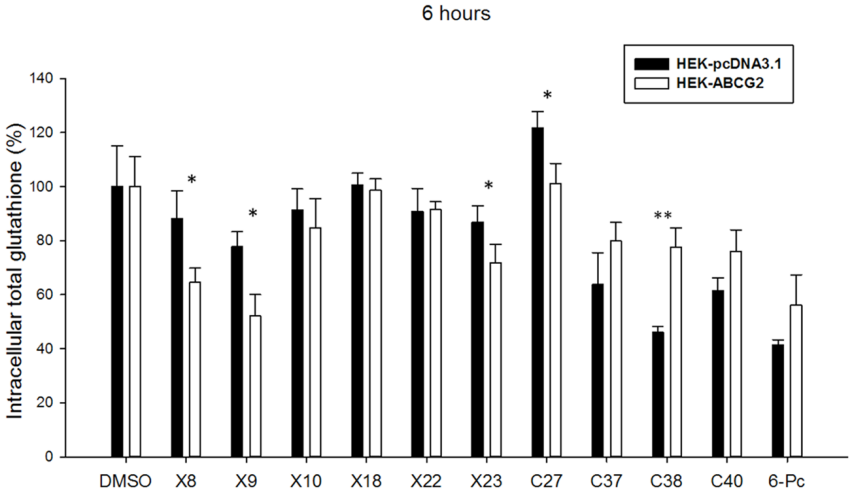

FIGURE 4 | Effects of other modulators on ABGC2-specific intracellular glutathione depletion. Screening of Xanthones (Genoux-Bastide et al., 2011), Chalcones (Valdameri et al., 2012), and 6-Prenylchrysin (Ahmed-Belkacem et al., 2005) reveal a significant ABCG2-dependent intracellular glutathione depletion in 6-h incubation for some compounds, as indicated. The values represent means ± SD corresponding to at least two independent experiments performed in triplicates. *t*-test analysis: $*p < 0.05$, $**p < 0.01$.

stimulated twofold the basal ATPase activity, and the ABCG2-specific inhibitor Ko143 which fully inhibited (**Figures 5A,B**). GSH did not alter the quercetin-stimulated ATPase activity either. Moreover, no effect was produced by the glutathione-conjugate *S*-(2,4-dinitrophenyl)glutathione (DNP-SG; **Figure 5B**) known to be actively transported by ABCC1 (Leier et al., 1994).

As the ATPase assay did not give any proof of interaction between GSH and ABCG2, we investigated the ability of GSH to modify the transport of ^{3}H-methotrexate. As shown in **Figure 6**, the ABCG2-mediated transport of tritiated methotrexate was not significantly inhibited by GSH addition, up to a 10 mM concentration, by difference with 1 μM Ko143 leading to the low background level observed with inactive mutant ABCG2. This contrasts with the reported prevention by 10 μM methotrexate against the increased extracellular GSH level observed in transformed yeast expressing human ABCG2 (Brechbuhl et al., 2010).

INABILITY OF ABCG2 TO CATALYZE AN ACTIVE TRANSPORT OF GSH

Finally, we measured the direct transport of ^{3}H-GSH into ABCG2-containing membrane vesicles. We found that, in contrast to ABCC1 serving as a positive control, no direct, ATP-dependent and specific inhibitor-sensitive, transport of tritiated GSH by ABCG2 could be detected in insect-cell membrane vesicles (**Figure 7**). Any ABCG2-mediated GSH transport could not be either determined at other ^{3}H-GSH concentrations (0.1 or 1 mM, data not shown).

DISCUSSION

The key results of this paper strongly suggest that human ABCG2 is unable to transport GSH. This has been demonstrated by direct measurement of ATP-dependent tritiated GSH uptake in inverted vesicles of insect-cell membranes overexpressing human ABCG2. In contrast, human ABCC1 catalyzed a high level of ATP-dependent and MK571-sensitive GSH transport under the same conditions. There was a low level of GSH accumulation in the presence of ABCG2 observed without ATP, which was also observed in the presence of the selective ABCG2 inhibitor Ko143 (Allen et al., 2002), or when the catalytically inactive K86M ABCG2 mutant was expressed. Thus, this background GSH binding could not be attributed to any ABCG2-mediated active transport.

This result is fully consistent with the lack of effect of GSH, even at high concentrations, on both basal and quercetin-stimulated ABCG2-ATPase activity of the insect cell membrane vesicles. Indeed, transported substrates such as prazosin, quercetin, or nilotinib (Telbisz et al., 2012) strongly stimulate the basal ATPase activity, then enhancing "coupled" ATPase activity. Our present results also show the lack of any effect by DNP-SG on the ABCG2 transporter, suggesting that glutathione conjugates are not transported by ABCG2. This is in contrast to various compounds conjugated with either sulfate (Suzuki et al., 2003) or glucuronate (Chen et al., 2003), whereas DNP-SG is actively transported by ABCC1 (Leier et al., 1994). The lack of ABCG2-mediated GSH transport is also consistent with the lack of any antagonism by GSH addition against ABCG2-mediated tritiated-methotrexate transport in inverted vesicles. These results, however disagree with the methotrexate-induced inhibition of GSH efflux reported in transformed yeast cells, expressing human ABCG2 (Brechbuhl et al., 2010).

Our results from experiments using membrane vesicles are quite consistent with those obtained with either transfected or drug-selected ABCG2-overexpressing cells where we did not observe any sharp and rapid decrease of intracellular GSH stimulable by modulators (such as 2′,5′-DHC, verapamil, or xanthones), or alterable by ABCG2 inactivation (such as using the potent Ko143 inhibitor). In addition, there was no inverse correlation between the observed decrease of intracellular GSH and increase of extracellular GSH, as also noticed in other drug-selected cancer cells overexpressing ABCG2 (Brechbuhl et al.,

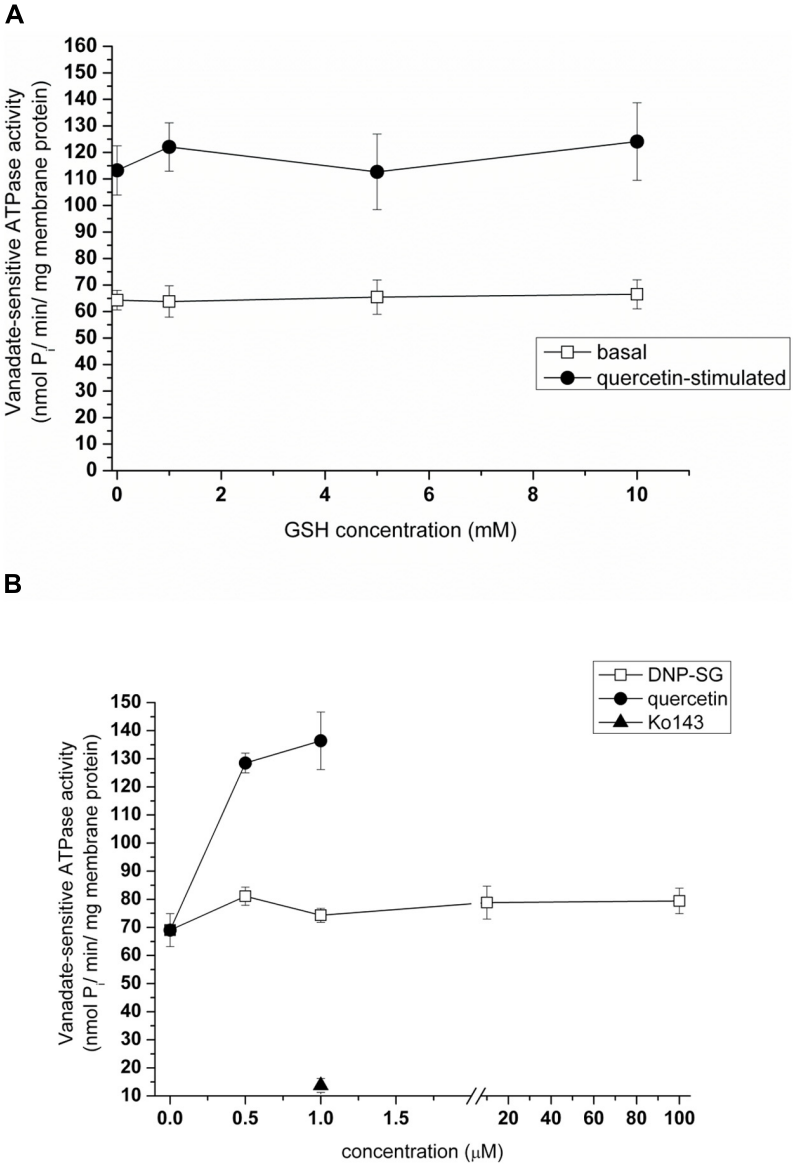

FIGURE 5 | Effects of GSH and DNP-SG on the ATP hydrolytic activity of ABCG2. Sodium orthovanadate-dependent ATP cleavage by wtABCG2 in 2 mM cholesterol-loaded Sf9 membrane vesicles was determined in the presence of increasing concentrations of GSH **(A)** both in the absence ("basal") and in the presence of a known transported substrate (1 μM quercetin, "quercetin-stimulated") without producing any significant effect. It was also assayed with increasing concentrations of DNP-SG or quercetin or with 1 μM Ko143 **(B)**. Data points represent the average ± SD values of two independent measurements.

2010). This contrasts with the strong effects clearly observed with ABCC1-overexpressing cells (Trompier et al., 2004; Perrotton et al., 2007; Barattin et al., 2010; Genoux-Bastide et al., 2011).

Nevertheless, the intracellular total glutathione concentration appeared to be modulated by overexpressed ABCG2 since, for unknown reasons possibly resulting from different signaling pathways, glutathione was lower in HEK293 transfected cells and higher in the drug-selected MCF7 cancer cells by comparison to their respective control cells. In addition, a significant decrease of intracellular glutathione was actually observed, either in some cases with 2′,5′-DHC, as previously reported (Brechbuhl et al., 2010), or with known ABCC1 modulators such as verapamil and xanthones. Such a decrease of intracellular glutathione however displayed special characteristics, such as being slow, requiring at least 6–24 h incubation, and not depending on ABCG2 activity since it was not altered by Ko143 inhibition. These results are more likely compatible with the induction of associated signaling pathways, leading to changes in intracellular GSH, than with a direct GSH transport.

ABCG2 is known to be regulated by a number of signaling pathways including NF-KB (Shen et al., 2010), RAR/RXR (Hessel and Lampen, 2010), hedgehog (Singh et al., 2011),

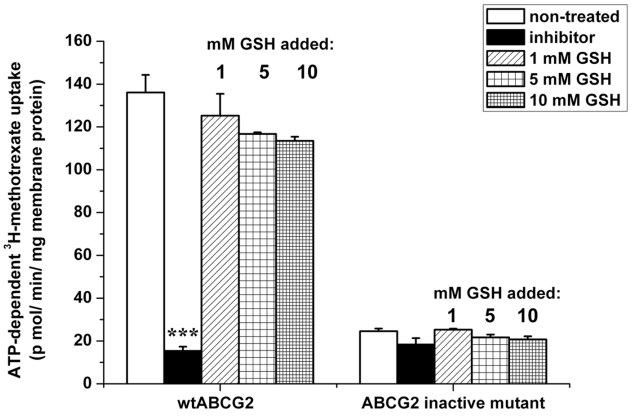

FIGURE 6 | Effect of GSH on the transport of methotrexate by ABCG2. ATP-dependent transport of ^{3}H-methotrexate in 2 mM cholesterol-loaded insect-cell membranes expressing ABCG2 (either wild-type or the inactive K86M mutant) was measured for 10 min at 37°C. Transport was determined in the absence or presence of an ABCG2-specific inhibitor (1 μM Ko143) or 1–10 mM GSH. Bars represent the average ± SD values of at least two measurements. *t*-test analysis: ***$p < 0.001$.

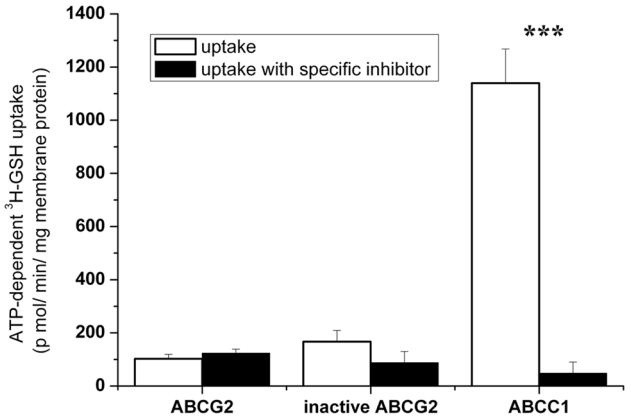

FIGURE 7 | Investigation of the transport of ^{3}H-GSH into wtABCG2-containing insect vesicles. Uptake of 500 μM ^{3}H-GSH was measured in Sf9 vesicles expressing ABCC1, wtABCG2, or inactive ABCG2, for 5 min at 37°C. ATP-dependent transport with or without the ABCC1- or ABCG2-specific inhibitor, 4 μM MK-571 or 1 μM Ko143 respectively, is shown. Bars represent the average of three independent measurements ± SD values. *t*-test analysis: ***$p < 0.001$.

P13K/AKT (Nakanishi and Ross, 2012), JNK1/c-jun (Zhu et al., 2012), HER2 and EGFR/HER1 (Gilani et al., 2012), and ERK1/2 (de Boussac et al., 2012). Some signaling pathways, such as notch (Battacharya et al., 2007), CXCL12-CXCR4 (Katoh and Katoh, 2010), Oct4-TCL1-AKT (Wang et al., 2010), PTEN/P13K/Akt (Li et al., 2011), β-catenin/Tcf (Usongo and Farookhi, 2012), AhR (Dubrovska et al., 2012), and HIF-2α with TGF-β/Smad2 (Cui et al., 2013) are related to the ABCG2 status as a marker of stem cells or stem-like cancer cells. Nrf2, a critical transcription factor that regulates antioxidants, detoxification enzymes, and drug efflux proteins in response to oxidative stress (Hong et al., 2010; Singh et al., 2010; Chen et al., 2012; Zhang et al., 2012; Ishikawa et al., 2013; Shelton and Jaiswal, 2013), may provide a link between cellular GSH homeostasis and ABCG2 expression. Whatever the mechanism(s) involved, changes in intracellular GSH are evidently too slow and too low to induce a sufficient cellular GSH depletion susceptible to trigger CS-induced cell apoptosis, as observed for ABCC1. It is still an important question if, and how, an ABCG2-dependent CS can effectively be produced. Indeed, the few known examples report very low selectivity ratio values, limited to 2.5–3 (Deeken et al., 2009), by comparison with the values, at least one order of magnitude higher, reported for both ABCB1- (Ludwig et al., 2006; Hall et al., 2009; Türk et al., 2009; Pluchino et al., 2012) and ABCC1- (Trompier et al., 2004; Barattin et al., 2010; Genoux-Bastide et al., 2011) overexpressing cells. Such a difference may be at least partly related to the complex involvement of ABCG2 in many signaling pathways. Further identification and characterization of mechanisms directly connecting ABCG2 to CS-associated apoptosis and signaling are mandatory for establishing a new therapeutic strategy, selectively targeting and eliminating resistant cancer cells.

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Is redox signaling a feasible target for overcoming multidrug resistance in cancer chemotherapy?

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Under physiological conditions, a balance between oxidants and antioxidants exists. Reactive oxygen species (ROS), are continuously generated by aerobic cells and eliminated through scavenging systems to maintain redox homeostasis. The two main sources of ROS are mitochondria and the NADPH oxidases family, but ROS are produced also by the cytochrome P450 system, xanthine oxidase and nitric oxide synthase (Holmstrom and Finkel, 2014). Because of ROS reactivity toward lipids, proteins and DNA, spatial and temporal regulatory strategies exist to regulate their intracellular levels. Excessive ROS levels are controlled by specific intracellular enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin reductase, and glutathione S-transferase (Glasauer and Chandel, 2014).

Cells aim to maintain a redox homeostasis: low levels of ROS, which are locally produced, can be potent mitogens and are required for various biological processes such as cell survival, growth and proliferation, angiogenesis, gene expression (Finkel, 2012). In contrast, changes in redox balance result in oxidative stress and aberrant cell signaling. Many studies have shown the critical role of detoxifying enzymes and antioxidant proteins in modulating the correct balance between apoptosis and carcinogenesis. Firstly, higher ROS levels could play a causal role in cancer development and progression by inducing DNA mutations, genomic instability, aberrant pro-tumorigenic signaling. After that, cancer cells adapt to oxidative stress and counteract the potential toxic effects of ROS to promote cell proliferation, survival and metabolic adaptation to the tumor microenvironment: sustained cell proliferation and mitogenic signaling (Weinberg and Chandel, 2009), increased cell survival and disruption of cell death signaling (Clerkin et al., 2008), epithelial to mesenchymal transition, metastasis (Nishikawa, 2008) and angiogenesis (Ushio-Fukai and Nakamura, 2008). Therefore, cancer cells are dependent on maintaining high enough ROS levels (redox imbalance) and an altered redox environment that allow for protumorigenic cell signaling without inducing cell death (Glasauer and Chandel, 2014).

Despite new discoveries and some clinical successes, the major obstacle to the effective treatment of human cancer is still the development of multidrug resistance (MDR) (Simon and Schindler, 1994). The mechanisms involved are complex and multifactorial (Baird and Kaye, 2003), but it is now accepted that classical redox transcription factors (NF-kB, HIF, p53, PI3K, AP-1) are involved in the development of MDR. Both carcinogenesis and MDR are frequently associated with an increased oxidative stress and activation of redox metabolism: this could affect the efficacy of cancer treatments by multiple mechanisms, including apoptosis, angiogenesis, metastasis, inflammatory reaction, and chemosensitivity (Morrow et al., 2006; Kuo, 2009). As a result, to balance oxidative stress, cancer cells increase their antioxidant capacity: according to our experience, for example, glutathione (GSH) plays a pivotal role in MDR development.

Besides classical redox pathways many studies recently focused on other redox-sensitive factors. Nuclear factor-erythroid 2 related factor 2 (Nrf2), via its binding to antioxidant response element (ARE), regulates the expression of cytoprotective genes: classical antioxidant enzymes including SOD and catalase, phase 2 detoxifying enzymes, and stress response proteins such as heme oxygenase 1 (Kaspar et al., 2009). In quiescent conditions, Nrf2 is anchored in the cytoplasm to Kelch-like ECH-associated protein 1 (KEAP-1), an adaptor protein which facilitates the Nrf2 ubiquitination and proteasomal degradation. Nrf2 nuclear accumulation is mainly mediated by KEAP-1-dependent turnover: its thiol-modification has long been associated to a primary response to ROS production (Dinkova-Kostova et al., 2002). Owing to its cytoprotective functions, Nrf2 has been traditionally studied in the field of chemoprevention; however, its overexpression or hyperactivation may participate in tumorigenesis of a wide number of solid cancers and leukemias (Nioi and Nguyen, 2007; Shibata et al., 2008; Homma et al., 2009). Moreover, Nrf2 activity is connected with oncogenic kinase pathways, structural proteins, hormonal regulation, other transcription factors, and epigenetic enzymes involved in the pathogenesis of various tumors (Gañán-Gómez et al., 2013). In addition to protecting cells from ROS, Nrf2 seems to play a direct role in MDR acquisition in many cancer types. Recent studies suggested a dark side of Nrf2

pathway by showing that high level of Nrf2 can promote cancer formation and contribute to chemoresistance (Hayes and McMahon, 2006; Lau et al., 2008; Wang et al., 2008; Kensler and Wakabayashi, 2010; Gañán-Gómez et al., 2013). For example, a greater nuclear accumulation of Nrf2 leads to constitutive overexpression of ARE-containing genes including drug efflux pumps, which facilitate the development of resistance (Meijerman et al., 2008). The expression of Nrf2 in cancer cells is increased during acquired resistance to doxorubicin and tamoxifen in ovarian and breast cancer cells (Kim et al., 2008; Kaspar et al., 2009). In addition, stable overexpression of Nrf2 or its upregulation by tert-butylhydroquinone resulted in enhanced resistance of cancer cells to some chemotherapeutic agents (Wang et al., 2008). High expression of Nrf2 and its target genes in MCF-7 and MDA-MB-231 mammospheres compared to corresponding adherent cells is associated with increased resistance to taxol and anchorage-independent growth (Wu et al., 2014). Moreover, transport activities of several MDR-associated proteins (MRPs) are regulated by GSH availability, and γ-glutamylcysteine synthetase (GCS) is the rate-limiting enzyme for its *de novo* biosynthesis. Transcriptional regulation of γ-GCS and MRP1 expression is mediated by an ARE that contains a consensus sequence for Nrf2; so, co-regulation of γ-GCS and MRP1 would facilitate the efflux activity (Glasauer and Chandel, 2014).

APE-1/Ref-1 (Apurinic-apyrimidinic endonuclease 1/Redox Factor 1) is a multifunctional protein with both DNA repair and transcriptional regulatory activities by facilitating DNA binding of numerous transcription factors involved in cancer promotion and progression, (AP-1, NF-κB, HIF, CREB, p53) (Luo et al., 2008). APE-1 requirement for cellular survival and its frequent overexpression in tumor cells strongly suggests a fundamental role in preventing cell death and controlling proliferation (Tell et al., 2005). Elevated APE-1 levels have been found in ovarian, cervical, prostate cancers, rhabdomyosarcoma and germ cell tumors (GCTs) correlating with the tumors radiosensitivity (Evans et al., 2000). Furthermore, immunohistochemistry in sections of GCTs from patients with testicular cancer of various histologies revealed high levels of APE-1 expression, suggesting a relation with their relative resistance to therapy (Robertson et al., 2001). Other evidences revealed that APE-1 contributes to alkylating agent resistance (Silber et al., 2002) or radioresistance in human glioma cells (Naidu et al., 2010), promotes resistance to radiation plus chemotherapy in medulloblastoma and primitive neuroectodermal tumors and in pediatric ependymomas (Bobola et al., 2011). Moreover, APE-1, preferably in the acetylated form, stably interacts with Y-box-binding protein 1 and enhances its binding to the Y-box element, leading to the activation of the *MDR1* gene. Indeed, a systematic increase in both APE-1 and MDR1 expression was observed in non-small-cell lung cancer tissue samples (Chattopadhyay et al., 2008).

Forkhead box O (FoxO) proteins are a family of transcription factors that, regulated by several stimuli, modulate genes involved in differentiation, proliferation, survival, apoptosis, migration and DNA repair (Dansen and Burgering, 2008; Yang and Hung, 2009). Upon exposure to an oxidative stress, FoxOs can lead to apoptosis or adaptive responses, depending on the entity of the stress. FoxO proteins have an important role in regulating cellular antioxidant defenses through the induction of genes encoding Mn-SOD and catalase; therefore, loss of FoxO function could contribute to increase the cellular ROS levels, eventually leading to DNA damage (Dansen and Burgering, 2008). FoxOs are deregulated in several tumors including breast and prostate cancers, glioblastoma, rhabdomyosarcoma, and leukemia (Myatt and Lam, 2007). During tumor development, the inhibition of FoxO3 transcriptional activity promotes cell transformation, cancer progression, and angiogenesis (Yang and Hung, 2009). Therefore, FoxOs inactivation seems to be an important step in carcinogenesis and increasing their activity could represent a therapeutic strategy (Myatt and Lam, 2007; Yang and Hung, 2009). Additionally, under continuous stress FoxOs could also induce the expression of important genes for drug efflux and antioxidant defense: the same molecules are responsible for not only the initial therapeutic response to cancer drugs, but also the subsequent acquisition of drug resistance (Zhang et al., 2011; Gomes et al., 2013). Sustained FoxO activation may promote MDR and cell survival: FoxO3 and FoxO1 induce MDR1 expression respectively in K562 leukemic cells and adriamycin-resistant breast cancer cells (Han et al., 2008; Yang and Hung, 2009). In addition, the proximal promoter region of the human *MRP2* gene contains four putative FoxO binding sites, and its transcription was stimulated by FoxO1 overexpression in MCF-7 cells (Choi et al., 2013). FoxO1 expression was distinctively upregulated in paclitaxel resistant cell line and enhanced by exposure to paclitaxel with subcellular translocation; in addition, FoxO1 overexpression was frequently observed in cancer tissue samples from chemoresistant patients (Goto and Takano, 2009). Paradoxically, cytostatic and cytotoxic effects of a diverse spectrum of anti-cancer drugs, such as paclitaxel, doxorubicin, lapatinib, gefitinib, imatinib, and cisplatin, are mediated through the FoxO3 activation and/or the inhibition of its direct target FOXM1. Moreover, there are also studies in which cisplatin-resistant cells had decreased levels of FoxO3 expression and were more sensitive to the anticancer agent mithramycin than their parental cells: FoxO3 knockdown increased cell proliferation and resistance to cisplatin (Shiota et al., 2010). However, deregulation of FoxOs has been recently found also in leukemia, where active FoxOs maintain leukemia stem cells and stimulate drug resistance genes, contributing to leukemogenesis (Zhu, 2014).

Several approaches have been undertaken to combat MDR. In the light of these findings, modulation of cellular redox levels could have important implications for the development of potential anticancer therapies. Several reports have demonstrated that Nrf2 silencing in cancer cells could decrease cell proliferation and enhance sensitivity to chemotherapeutic agents in lung, gallbladder, and ovarian tumors (Meijerman et al., 2008; Singh et al., 2008). Very recently, brusatol, an inhibitor of the Nrf2 pathway, was discovered to suppress Nrf2 level and its target genes, enhancing intracellular ROS, sensitizing MCF-7 and MDA-MB-231 mammosphere cells to taxol and reducing anchorage-independent growth

(Wu et al., 2014). Reducing the APE-1 amount in cancer using RNA interference and antisense oligonucleotide technology sensitizes tumor cells to a variety of chemotherapeutic agents. For example treatment of a human pancreatic cancer cell line (Panc-1) with antisense oligonucleotides to APE-1 resulted in a dramatic increase in gemcitabine sensitivity (Lau et al., 2004). Therefore, selective APE-1 activity inhibition could have potential therapeutical significance and be a promising avenue to develop novel cancer treatments (Jiang et al., 2008; Bapat et al., 2009). APE-1 may be a useful target for modifying radiation tolerance: the inhibitors lucanthone and CRT004876 were employed, the former a thioxanthene previously under clinical evaluation as a radiosensitizer for brain tumors and the latter a more specific inhibitor (Naidu et al., 2010); knockdown of APE-1 gene expression may significantly sensitize pancreatic cancer cells to radiotherapy (Chen et al., 2013). Finally, some studies demonstrated that Bcl-2 could directly interact with APE-1 via its BH domains: gossypol, a Bcl-2 homology 3 (BH3)-mimetic agent binds to the BH3 domain of Bcl-2 family members and inhibits the repair activity and the redox function of APE-1 (Qian et al., 2014). Because of its pivotal role in drug sensitivity as well as resistance, the complex of FoxO could be a viable strategy for cancer treatment and drug resistance overcoming, while in cancer patients might also help to predict and monitor their clinical response to chemotherapy.

Although in the past antioxidants were seen as tumor suppressors, recent research uncovered the "dark side of antioxidants" (Wang et al., 2008; Sayin et al., 2014), which are used by cancer cells to promote survival and growth. The dependence of cancer cells on ROS homeostasis may represent the cancer cell's "Achilles Heel" and could be potentially exploited to target them therapeutically: pro-oxidant cancer therapy can affect the different ROS production and redox regulation between normal and cancer cells. At last, recent discoveries about Nrf2, APE-1, FoxO and their potential contribution in the development, maintenance and evolution of MDR in cancer, open a novel therapeutic window for cancer treatment. High levels of ROS can be toxic to cancer cells and potentially induce cell death via oxidative stress while sparing normal cells. Therein, redox modulators could be promising tools in MDR cancer prevention and treatment; nevertheless, because of the complexity underlying drug resistance, it will be necessary to do careful antioxidant profiling of tumor cells to identify clinically relevant therapeutic targets.

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Nanomaterials for reversion of multidrug resistance in cancer: a new hope for an old idea?

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Cancer is a major cause of mortality in the modern world, with more than 10 million new cases every year. This outline is expected to rise in the next few decades since the majority (59%) of people diagnosed with cancer is aged over 65. In fact, around one in three people will be diagnosed with cancer throughout their lifetime (Siegel et al., 2012; Bosetti et al., 2013).

The foundation of cancer treatment is surgery, chemotherapy, radiation, antibody-blocking therapy, or a combination of these therapies (Hanahan and Weinberg, 2000). Still, many clinical chemotherapeutic and radiotherapeutic regimes are not exceptionally effective, due to multidrug resistance mechanisms, depending on the patient and the type of tumor. Therefore, there is an urgent need for more effective and valuable cancer therapeutics, in order to reduce the impact of the chemotherapeutic agents on the healthy tissues by creating more selective systems toward the cancerous cells (Alison, 2001; Perez-Tomas, 2006).

Multi-drug resistance (MDR) in cancer refers to the capacity of cancer cells to survive or become resistant from treatment of a wide variety of drugs. Cancer chemotherapy has become progressively sophisticated within the last years; however there are not any cancer therapies 100% effective against disseminated cancer. This is in fact a major problem once approximately 70% of patients do not respond to initial chemotherapy and the five-year survival rate for these patients is a low 10–30%. Relapse is also frequent (Diseases, 2000).

Mechanisms of MDR include decreased uptake of drugs, reduced intracellular drug concentration by activation of the efflux transporters, modifications in cellular pathways by altering cell cycle checkpoints, increased metabolism of drugs, induced emergency response genes to impair apoptotic pathways and altered DNA repair mechanisms (Gottesman, 2002). P-glycoprotein (P-gp) is the best known membrane transporter used in MDR and has been first described in the late 1970s (Juliano and Ling, 1976). Since then, the phenomenon of cancer drug resistance became a hotspot of cancer research (Gottesman, 2002; Ullah, 2008).

Despite of the discovery of multiple new gene/protein expression signatures or factors associated with drug resistance by high throughput "-omics" technologies, none of these findings has been useful in producing efficient and specific diagnostic assays or for improvement of updated chemosensitizers. Clinical success has also been limited due to issues regarding safety, once one of the most common strategies against MDR is the development of ATP-binding cassette (ABC) transporter inhibitors, which are poorly effective and specific, increasing the toxicity associated with chemotherapy (Lage, 2008).

Nanotechnology and nanomaterials in particular, are expected to provide a range of devices to treat cancer as their sizes are well matched in size to biologic molecules and structures found inside living cells (Conde et al., 2012).

The development of nanoscale devices and structures has provided major breakthroughs in monitoring and fighting cancer (Qian et al., 2008; Ren et al., 2012; Conde et al., 2013). Cancer nanotechnology offers a wealth of safety and innovative tools to treat and diagnose cancer, such as multifunctional, targeted devices capable of bypassing crucial biological barriers and to deliver multiple therapeutic agents directly to cancer cells and adjacent tissues around tumor microenvironment (Sanvicens and Marco, 2008).

Nanoparticles (NPs) are usually produced to deliver and enhance the drug concentration inside the cancer cells, using both active and passive targeting. (NPs) are excellent tumor-targeting vehicles because of the unique inherent property of solid tumors. Numerous tumors present with defective vasculature and poor lymphatic drainage, due to their rapid growth, resulting in an enhanced permeability and retention (EPR) effect. This effect allows (NPs) to accumulate preferably at the tumor site. Once the tumor is directly connected to the main blood circulation system, multifunctional (NPs) may exploit several characteristics of the newly formed vasculature and efficiently target tumors (Conde et al., 2012; Schroeder et al., 2012). This effect constitutes one of the major advantages of (NPs) against MDR mechanisms. In fact, lipid (NPs) and nanocapsules, polymeric (NPs), metal (NPs), dendrimers and liposomes have been reported to circumvent drug resistance (Dong and Mumper, 2010) (**Figure 1**).

The most common nanomaterials to use against (P-gp) and ABC transporters resistance are non-ionic surfactants (i.e., poly(ethylene glycol), Tween 80® and Pluronics®) that usually form hydrogel bonds with the protein to escape from the recognition and therefore increase the uptake of the nanoformulated drug (Gao et al., 2012). The ABC transporters are also expressed in normal cells and so it

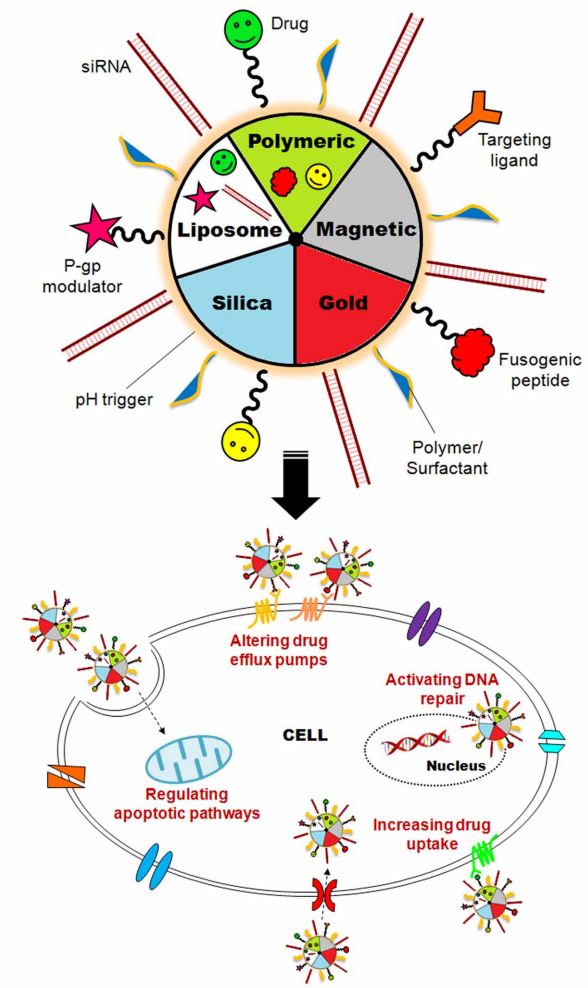

FIGURE 1 | Nanotechnology against multidrug resistance. Smart nanomaterials (i.e., liposomes, polymeric, magnetic, silica, and gold nanoparticles functionalized with MDR inhibitors and targeting moieties) can be used to circumvent essential MDR mechanisms, by increasing drug efflux and metabolism and activating DNA repair and apoptotic pathways.

is necessary to inhibit these drug efflux transporters in tumor tissues preferentially, while minimizing the inhibition in normal tissues (Patel et al., 2013).

Some of these nanomaterials have been used to overcome drug efflux by drug transporters, increasing the drug retention effect in cancer cells. These systems comprise the nanoformultation (specially with liposomes, nanodiamonds, mesoporous silica nanoparticles) of siRNAs and drugs like doxorubicin (DOX), used to hamper cancer progression, inhibiting the drug detoxification, by suppressing cell defense mechanisms, activating apoptosis and DNA repair (Minko et al., 1999; Chen et al., 2009, 2010; Chow et al., 2011).

Other drugs like vincristine, verapamil, cyclosporin, paclitaxel, oxaliplatin, cisplatin, and curcumin have been delivered using polymeric (NPs) and nanoemulsions made of surfactants (Soma et al., 2000; Devalapally et al., 2007; van Vlerken et al., 2007; Abu Lila et al., 2009; Ganta and Amiji, 2009; Song et al., 2009; Yadav et al., 2009; Aryal et al., 2010).

This combinatorial delivery of multiple drugs and MDR inhibitors opens up a huge amount of schemes to target the comprehensive mechanisms of MDR especially via the EPR effect, and at the same time it lifts up some important concerns about this approach.

Although most of these studies reported a co-delivery of multiple agents in the same nanoparticle such as siRNAs, chemotherapeutic drugs, antibodies and other MDR inhibitors, these formulations are far from achieving excellent results. This happens once the majority of drugs such as paclitaxel are hydrophobic and the MDR inhibitors like siRNAs anti-MDR associated genes are hydrophilic. This fact impairs one of the most amazing characteristic of the nanoparticle systems that is the high loading capability in the same formulation. Another problem with the loading of multiple drugs in the same nanoformulation is the molar ratios between molecules that are often incomparable and sometimes very difficult to control and quantify. This is a key element in dosage optimization and maximization of the combinatorial effects along with the nanoformulated composites (Creixell and Peppas, 2012; Gao et al., 2012).

Moreover, the interaction between chemotherapeutic drugs and MDR inhibitors (i.e., siRNAs) may occur when using coadministration. In fact, some of the drugs can strongly influence endosomal escape and siRNA recognition by the RNA Induced Silencing Complex (RISC), which are crucial obstacles/limitations for translation of RNAi into clinical setting (Lee et al., 2013). This mutual interactions between drugs and siRNAs need to be further explored and optimized.

This raises another challenge, which is the endosomal escaping of the nanoformulated drugs. Upon endocytosis the NPs-drugs usually go to the lysosomal and endosomal compartments, facing a strong acidic and enzymatic environment (Hu and Zhang, 2009). A biomolecule that bypasses the endosome would greatly increase the therapeutic effect. For example, a fusogenic peptide would promote endosomal escape by a pH-responsive mechanism in which the peptide becomes protonated at the acidic pH of the endosome, destabilizing the endosomal membrane when the protonated peptide fuses with it, enabling the delivery of the siRNAs to the cytoplasm without suffering degradation. Poly(ethylene imine) (PEI) is a cationic polymer and is also used as carrier for siRNA due to its high siRNA binding capacity and its proton sponge effect for endosomal escape (Creixell and Peppas, 2012).

However, some effective nanoparticle formulations (in particular liposomes and micelles) have been reported using the endocytic uptake of drug to avoid MDR (Thierry et al., 1993; Huwyler et al., 2002; Rapoport et al., 2002). This occurs since formulated/encapsulated drugs are more effective than free drugs, as sometimes they have more cytotoxicity to resistant cancer cells and/or improved internalization yields (Huwyler et al., 2002; Gabizon et al., 2003).

An alternative to the use of siRNA-NPs are the antisense oligodeoxynucleotides nanoformulations, once are more stable in biological matrices and have more resistance to enzymatic degradation (Conde et al., 2010; Wang et al., 2010).

In addition of active targeting, the long circulating half-life and drug release kinetics are important aspects of an effective strategy to combat MDR. In fact, the rate of drug release from the NP decides their therapeutic effectiveness. When fast drug release may lead to their lost while in blood stream circulation, a slow kinetic may predispose cancer cells to more resistance once they may not compete with the drug efflux pumps (Hu and Zhang, 2009).

To increase life-time, long circulating-NPs are usually coated with poly(ethylene glycol) (PEG), which has the ability to circulate for a prolonged period of time to allow efficient target of a particular cell/tissue/organ and retards the uptake by the macrophages and monocytes, from the reticuloendothelial system (RES) (Kommareddy et al., 2005; Uchida et al., 2005).

Concerning the stimuli-responsive drug release, pH-sensitive triggers are the most common strategies once tumoral tissues are well known for presenting acidic conditions, as well as the lysosomal and endosomal compartments, which represent a strong advantage for selective drug release. Several nanoformulations of pH-triggered drug release have been reported, especially those using pH-sensitive liposomes (Fattal et al., 2004), polymeric (NPs) (Roux et al., 2002), micelles (Lee et al., 2008) and hydrogels (Griset et al., 2009). Generally, (NPs) are coated with biomolecules (i.e., phosphatidylethanolamine, poly(ethylene glycol), poly(L-histidine), poly(L-lactic acid), polymethacrylates, poly(amidoamine)) that, when protonated, destabilize the formulation (that become leaky in acidic environment) leading to the accelerated/controlled intracellular drug release. This kinetic release overwhelms the (P-gp) drug efflux pumps (Hu and Zhang, 2009).

Although the use of organic (NPs) such as liposomes, lipids, micelles, and polymeric (NPs) constitute the major strategy to deliver high amounts of drugs and MDR inhibitors, the use of inorganic (NPs) to reverse MDR in cancer has also been reported.

The most frequent systems usually combine silica (Rigby, 2007; Chen et al., 2009), magnetic (Cheng et al., 2011; Singh et al., 2011; Klostergaard and Seeney, 2012) or gold (NPs) (Dreaden et al., 2012; Tomuleasa et al., 2012) with specific drugs and siRNAs. The silica (NPs) are ideal candidates for the loading of large amounts of drugs and other components, due to high surface area to volume ratio and large pore volume. The magnetic (NPs) allow for physical/magnetic enhancement of the passive mechanisms for the accumulation of magnetic-responsive (NPs) into tumor tissue, leading to increase cellular uptake. Gold (NPs) also have shape/size-dependent optoelectronic properties and the endosomal-based route for gold nanoparticle cellular uptake is one of the main advantages for overcome MDR (Ayers and Nasti, 2012; Creixell and Peppas, 2012).

As described above, an extensive knowledge has been gathered in the emerging field of nanomaterials to overcome multidrug resistance mechanisms. Many of the drugs available to circumvent MDR were in the past unavailable to target this mechanism due to low solubility and/or stability. Nanomaterials made these drugs a possible strategy to target MDR. However, many anti-cancer drugs have never been used in nanomedicine (Dong and Mumper, 2010). Moreover, nanomaterials have also provided an effective platform to deliver high loads of drugs (thus lower chances of resistance) in a specific and controlled way (using pH-responsive and stimuli-sensitive), with surface-modified to improve circulation time, preventing the uptake by the RES. Another advantage of using nanomaterials for MDR regression is the drastic reduction of the IC(50) value for most of the nanoformulated drugs, reducing the clinical doses of the conventional chemotherapeutic agents, which show high levels of cytotoxicity. This allows the expansion of the anticancer therapeutic window.

However, many challenges involved in biocompatibility and specificity of nanomaterials against MDR need to be further investigated. Other crucial issues embrace the fact that agents that can reduce MDR *in vitro*, are sometimes useless in patients, probably because some drugs get entrapped in circulation by serum proteins, for example. In addition, new nanoparticle-based targeting moieties such as antibodies, minibodies and peptides should be explored as an additive effect of EPR.

Moreover, it is important to realize that cancer cells need to lose their chemoprotective features mediated by MDR genes, at the same time the chemotherapy-sensitive non-cancerous cells (i.e., bone marrow stem cells) need to be protected from the effects of chemotherapeutic agents. In fact, the destruction of these cells constitutes the single most important dose-limiting toxicity factor in cancer therapy. If we could repopulate the blood system with chemoresistant blood cells the patients could receive higher doses of anticancer agents than could be given normally.

This raises the important issue of the specificity and toxicity of nanomaterials as one of the major obstacles to treat multidrug-resistant tumors. Therefore, any nanoformulation that target MDR must do so in a way that is tumoral specific, in order not to affect the normal function of healthy cells. This may be achieved using, for example, effective targeting ligands combined with valuable pro-drugs.

Some of the described drug nanoformulations here are now in human clinical trials. So it is therefore predictable that nanomaterials against MDR will eventually become commonplace in the oncology clinic in the near future.

AUTHOR CONTRIBUTIONS

João Conde conceptualized the manuscript and wrote the draft. Pedro V. Baptista and Jesús M. de la Fuente revised the manuscript. All authors contributed in the revision process. All authors read and approved the final manuscript.

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ABC transporters in CSCs membranes as a novel target for treating tumor relapse

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CSCs are responsible for the high rate of recurrence and chemoresistance of different types of cancer. The current antineoplastic agents able to inhibit bulk replicating cancer cells and radiation treatment are not efficacious toward CSCs since this subpopulation has several intrinsic mechanisms of resistance. Among these mechanisms, the expression of ATP-Binding Cassette (ABC) transporters family and the activation of different signaling pathways (such as Wnt/β-catenin signaling, Hedgehog, Notch, Akt/PKB) are reported. Therefore, considering ABC transporters expression on CSCs membranes, compounds able to modulate MDR could induce cytotoxicity in these cells disclosing an exciting and alternative strategy for targeting CSCs in tumor therapy. The next challenge in the cure of cancer relapse may be a *multimodal strategy*, an approach where specific CSCs targeting drugs exert simultaneously the ability to circumvent tumor drug resistance (ABC transporters modulation) and cytotoxic activity toward CSCs and the corresponding differentiated tumor cells. The efficacy of suggested multimodal strategy could be probed by using several scaffolds active toward MDR pumps on CSCs isolated by tumor specimens.

Keywords: CSCs, MDR, TICs markers, HTS, P-gp

INTRODUCTION

The emerging concept about tumorigenesis is that cancer lesions are organized in a hierarchy of heterogeneous cell populations displaying different biological properties and tumorigenic potentials (Lorico and Rappa, 2011). Among these populations, only a small portion, the cancer stem cell or tumor-initiating cell (CSC/TIC) subpopulation is able to induce tumor formation and growth, leading to differentiated cells, the bulk of the tumor. In 1977 Hamburger and Salmon suggested the "*CSC theory*" by hypothesizing that cancer originates from uncommon cells, CSCs, showing pluripotency and self-renewal (Hamburger and Salmon, 1977; Boman and Wicha, 2008). In 1997 Lapidot and coworkers identified CSCs in leukemia on the basis of cell-surface-markers expression (Lapidot et al., 1994). The discovery of CSCs in leukemia and in several solid tumors, such as breast carcinoma (Al-Hajj et al., 2003; Hemmati et al., 2003; Singh et al., 2003; Carla et al., 2005; Fang et al., 2005; Xin et al., 2005; Lawson et al., 2007; Li et al., 2007; Ricci-Vitiani et al., 2007), was the proof of the *CSC theory* in cancer development.

CSC/TICs are characterized by the following properties: (a) production of all types of cells in a tumor, including CSC/TICs and non-CSC/TICs; (b) unlimited self-renewal and division capacity; (c) quiescence or slow proliferation, and (d) resistance to conventional antineoplastic therapy (Clevers, 2011). Moreover, recent studies have demonstrated that CSC/TIC phenotypes such as self-renewal and pluripotency are acquired by the activation of oncogenic genes or the inactivation of tumor suppressor genes (Baccelli and Trumpp, 2012). Since CSCs are characterized by specific surface markers, this subpopulation of cells can be isolated from mixed tumorigenic and non-tumorigenic tumor cells, by different methods of immune selection. CD44 targeting is used in the treatment of acute myeloid leukemia (AML) (Jin et al., 2006), CD24 targeting is for the treatment of colon and pancreatic cancer (Sagiv et al., 2008) and CD133 is targeted for the treatment of hepatocellular and gastric cancer (Smith et al., 2008). The current antineoplastic agents, able to inhibit bulk replicating cancer cells, and radiation treatment are not efficacious toward CSCs and, therefore, targeting these cells could be an helpful strategy for eradicating tumors more efficiently.

However, CSCs possess several intrinsic mechanisms of resistance to current chemotherapeutic drugs. Among these mechanisms, the expression of ATP-Binding Cassette (ABC) transporters family (An and Ongkeko, 2009; Calcagno et al., 2010; Fuchs et al., 2010; Clevers, 2011; Moitra et al., 2011; Pietras, 2011) and the activation of different signaling pathways such as Wnt/β-catenin signaling (Teng et al., 2010; Yeung et al., 2010; Takebe et al., 2011; Janikowa and Skarda, 2012), Hedgehog (Hh), Notch (Kobune et al., 2009; Wang et al., 2009; Zhao et al., 2009; Takebe et al., 2011; Janikowa and Skarda, 2012; Jiang et al., 2012), Akt/PKB, ATR/CHK1 survival pathways (Ma et al., 2008; Korkaya et al., 2009; Jiang et al., 2012) and constitutive activation of NF-κB are reported (Zhou et al., 2008; Liu et al., 2010).

The first of these mechanisms is the overexpression of ABC transporters such as P-glycoprotein (P-gp), Breast Cancer

Resistance Protein (BCRP), and Multidrug Resistance-associated Proteins (MRPs) that use the energy of ATP hydrolysis to extrude compounds out of cells (Colabufo et al., 2010). These proteins are overexpressed in several tumors and, since responsible for drug efflux, are the main cause of MultiDrug Resistance (MDR) (Colabufo et al., 2010). Among these transporters, P-gp is the mostly studied and is localized in the luminal membrane of endothelial cells constituting the Blood-Brain Barrier (BBB), Blood-CerebroSpinal Fluid Barrier (B-CSF), and Blood-Testis Barrier (BTB); thus P-gp exerts a protective function in our body (Pharm et al., 2008; Colabufo et al., 2010). BCRP as P-gp monomer, is considered a "half-transporter" and it effluxes the same P-gp substrates (Pharm et al., 2008; Colabufo et al., 2010). MRPs differ from P-gp for the presence of an additional and specific five transmembrane domain and it efflux organic ions with high molecular weight (Pharm et al., 2008; Colabufo et al., 2010).

Since several antineoplastic drugs are substrates of ABC transporters, one of the strategy to reverse MDR is the use of inhibitors toward these pumps and their co-administration with antineoplastic agents (Perez-Tomas, 2006; Teodori et al., 2006; Gimenez-Bonafe et al., 2008). However, when antineoplastic drugs and P-gp inhibitors are co-administrated, an increased toxicity has been observed because, at the same time, the protective role of P-gp is abolished (Coley, 2010).

Over the last decade, our research group has developed P-gp ligands with different scaffolds displaying different P-gp intrinsic activities (Colabufo et al., 2008a,b, 2009, 2013; Contino et al., 2012, 2013a,b; Nesi et al., 2014). Therefore, considering ABC transporters expression on CSCs membranes, compounds able to modulate MDR activity could induce cytotoxicity in these cells disclosing an exciting and alternative strategy for targeting CSCs in tumors therapy.

CSCs BIOMARKERS

CSCs display several cell surface markers and their detection is useful for the identification of CSCs in tumors. In addition, the development of antibodies or antibody constructs toward these markers is also suggested as a therapeutic strategy to target CSCs (Kwon and Shin, 2013).

CD133 AND ALDH1

CD133 and ALDH1 have been identified as markers of TICs in primary human ovarian tumors (Landen et al., 2010). CD133 has been also identified as surface marker to characterize adult stem/progenitor cells in human thyroid glands (Thomas et al., 2006). ALDH1 and CD133 are also markers for the identification of CSCs in colorectal carcinoma (CRC) (Zhou et al., 2014). CD133, together with CD44, is a specific cell-surface marker of prostate cancer (Wang et al., 2013a).

CD44, CD117, CD24

The hyaluronic acid (HA) receptor CD44 and the stem cell factor receptor CD117 are specific surface markers of ovarian CSC/TICs (Zhang et al., 2008). CD44+CD117+ cells isolated from human ovarian adenocarcinomas represent a subpopulation with an ovarian tumor-initiating capacity, that, injected in mice, induce original tumors from which they are derived (Zhang et al., 2008). CD24+ cells, isolated from patient tumor specimens, are enriched in ovarian CSC/TICs and have stem-like properties, such as chemoresistance, self-renewal and differentiation (Gao et al., 2010).

METHODS FOR THE ISOLATION OF CSCs

Fluorescence-Activated Cell Sorting (FACS) is a magnetic cell separation method used to isolate CSCs based on the expression of specific cell surface markers, such as CD24, CD44, and CD133 (Wright et al., 2008; Takaishi et al., 2009). The detection can be performed with specific antibodies in flow cytometry, competitive ELISA (cELISA) or magnetic beads (Dobbin and Landen, 2013). Xia and coworkers have developed humanized antibodies toward the surface marker CD133 that was detected by cELISA (Xia et al., 2013). However the limitation of surface markers recognition is that the results can be ascribed to the specific studied population. Therefore, a second method, represented by the detection of the activity of a specific protein such as the membrane pump ABCG2 or ALDH1A1 enzyme, seems to be more useful than markers recognition alone. The first technique uses the DNA-binding dye Hoechst 33342, originally developed for bone marrow cells (Goodell et al., 1996). This fluorescent dye, used to identify a Hoechst-negative CSC "side population" (Kondo et al., 2004), has been successfully employed to isolate stem cells from solid tissues such as skeletal muscle, lung, liver, testis, kidney, skin, mammary gland and brain (Challen and Little, 2006). This method allows to select the cells displaying an increase in the Hoechst 33342 efflux, ABCG2-mediated, from the nucleus. The limitation of this method is due to the dye toxicity (Siemann and Keng, 1986; Erba et al., 1988).

The high ALDH activity described for CSCs suggested also this enzyme as a probe to isolate these cells. Aldefluor, an ALDH1 substrate, initially used for the isolation of hematopoietic stem cells by FACS (Storms et al., 1999; Cheung et al., 2007) and for CSCs separation in tumor tissue and cancer cell lines (Moreb, 2008; Jiang et al., 2009), has been successfully used for ALDH1 activity detection of CSCs in lung (Jiang et al., 2009), prostate (Li et al., 2010), breast (Charafe-Jauffret et al., 2010), colon (Huang et al., 2009a), and bladder (Su et al., 2010). Aldefluor contains the ALDH1 substrate BODIPY-aminoacetaldehyde (BAAA), that is converted into the fluorescent metabolite BODIPY-aminoacetate (BAA) by ALDH1 (Storms et al., 1999). BAA is then retained in living cells, because of its charge and also because Aldefluor inhibits the multidrug-resistance transporters. Therefore, Aldefluor-treated cells, with high ALDH1 activity, display high fluorescence and can be isolated by FACS into two subpopulations: ALDH-hi and ALDH-low (Moreb, 2008). This method has been validated for some cancers but it has not been widely employed because of some limitations due to cells handling that can induce stress, disrupt gene expression, and lead to altered cell physiology (Orfao and Ruiz-Arguelles, 1996). To overcome these limitations, the use of Adelfluor staining has been assessed in adherent cell cultures, that resulted in the attached-cell Aldefluor method (ACAM). ACAM displays several advantages since single-cell imaging of physiological processes in CSCs is easier in monolayers than in cells suspension or tumorsphere cultures that are usually used for

growing CSCs (Fael Al-Mayhani et al., 2009; Pollard et al., 2009; Hirschhaeuser et al., 2010).

CSCs have also been usually sorted into SP cells using UV excitation ($\lambda = 350$ nm) but the limitation of this method was the cell damage caused by UV radiation exposure. Tomiyasu et al. reported a new method in which CSCs have been sorted by a violet laser ($\lambda = 407$ nm) to avoid DNA damage (Tomiyasu et al., 2014).

Another method to select CSCs is the use of molecular probes, termed aptamers, that are produced by the iterative *in vitro* selection process SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers are short single-stranded oligonucleotides of DNA or RNA sequences that fold into unique secondary and tertiary structures that selectively bind the target proteins and other small molecules with high affinity and selectivity. They can be synthesized *in vitro* and therefore do not require animal or bacterial hosts for production. In recent years, in order to improve aptamers qualities and reducing time and cost of production, a number of new SELEX variants have been performed and among them, cell-SELEX uses whole living cells as target to select DNA aptamers (Shangguan et al., 2006; Sefah et al., 2010). Aptamers have many appealing features: low molecular weight, easy chemical modifications, low toxicity and immunogenicity, long shelf-life, high affinity (K_d value is in the picomolar to nanomolar range) and high specificity. They can be used in diagnosis, for purification processes, in drug discovery and in therapy (Proske et al., 2005). In particular, the therapeutic perspectives involve the targeting of chemotherapeutic agents, nanoparticles, drug-encapsulated liposomes and radioactive materials on cell specific biomarkers. Aptamers bind their targets with high affinity and specificity discriminating very closely related targets. The anti-theophylline aptamer is able to distinguish theophylline from caffeine, and the anti L-arginine RNA aptamer binds more strongly L-arginine than D-arginine (Jenison et al., 1994; Geiger et al., 1996). Aptamers are also useful in drug delivery strategy: 2′-fluoro- RNA aptamers have effect toward the highly expressed prostate specific membrane antigene (PSMA) (Lupold et al., 2002) and Docetaxel-encapsulated nanoparticles functionalized with a specific aptamer (A10) target PSMA expressing cells (Farokhzad et al., 2006). Another interesting example is AS1411 aptamer that recognizes Nucleolin (NCL), a multifactorial protein involved in many cellular pathways of cancer. AS1411 has been functionalized with cisplatin-liposome and evaluated in MCF-7 (NCL positive) and LNCaP (NCL negative) cell lines. The cytotoxic effects were higher in MCF-7 cells as compared to LNCaP cells (Cao et al., 2009). Moreover, specific aptamers are used in several assays to isolate, enrich and identify CSCs and for the characterization of specific markers for CSCs such as aptamers toward HA domain of CD4, RNA aptamers targeting CD133, a cell surface glycoprotein considered a universal marker of normal hematopoietic and organ-specific stem cells and aptamers identifying brain CSCs (Griguer et al., 2008; Kim et al., 2013).

STRATEGIES TO ERADICATE CSCs

CSCs are responsible for tumor re-growth and potentially resistant to antitumor therapies. Therefore, the development of strategies that can act effectively against this subpopulation of cells has been envisaged, such as (**Figure 1**):

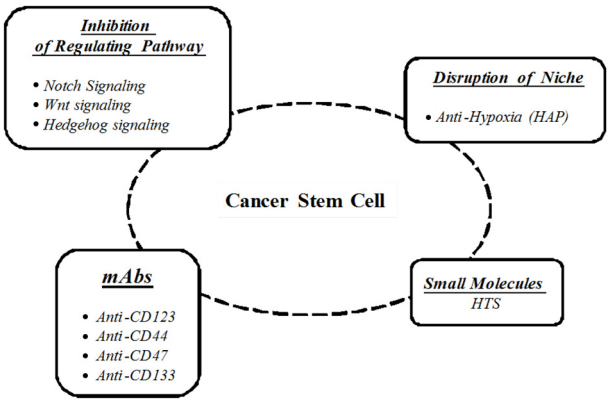

FIGURE 1 | Cancer stem cells targeting strategies.

- Immunological therapies;
- Evaluation of genes and pathways pivotal in CSCs regulation;
- High-throughput screening (HTS) of potential inhibitory compounds;
- Regulation of tumor microenvironment.

IMMUNOLOGICAL THERAPIES

Since current cancer therapies fail to eradicate CSCs, causing cancer recurrence and progression, the use of monoclonal antibodies (mAbs) and antibody constructs selective for CSCs is a novel cancer therapeutic strategy. Several studies have been performed on cell surface markers that are associated with CSCs. These markers have been suggested as targets for antibodies, for antibody-drug conjugates (ADCs) in immunological therapies to overcome the side effects and the limitations of current cancer chemotherapies (Naujokat, 2014). Several mAbs, directed toward specific surface markers (Anti-CD123, Anti-CD44, Anti-CD47, Anti-CD133) or toward protein of CSCs (Anti-Notch1 and Anti-Frizzled) have been reported (Naujokat, 2014). Antibody constructs can be classified as bispecific antibodies (BsAb), targeting two different surface markers (such as BsAb-CD123 and -CD3) or as trispecific antibodies when they target three different markers (such as CD123, CD33, CD16) (Naujokat, 2014). The use of mAbs or mAbs constructs had high efficacy in tumor xenografts mice and in some clinical trials and since these markers or proteins are expressed in CSCs, they can be specifically targeted by mAbs or constructs without collateral tissue damage. Moreover, it is also possible to conjugate antineoplastic drugs to mAbs leading to ADCs. As example, Lou et al. have developed a dual-targeting drug delivery system displaying an outer layer of polymers and traditional anti-cancer drugs such as doxorubicin dispersed in the polymer (Lou et al., 2012). The internal part is represented by the ADCs since includes mAbs directed to the specific marker of CSCs (such as anti-CD133 mAb or anti-CD44 mAb), highly cytotoxic drugs (such as calicheamicin) and linkers. The inner ADCs can be released from the delivery system in tumor cells where CSCs are recognized by the specific mAb of the system and ADCs are

endocytosed. Some ADCs have displayed good antitumor effects and have entered preclinical trials (Iyer and Kadambi, 2011).

PhotoChemical Internalization (PCI) is a another innovative drug delivery technology based on light-controlled cytosolic release of drugs entrapped in endosomes or lysosomes (Stratford et al., 2013).

The combination of CD133-targeting therapeutics with PCI technology, where light-activation is constrained to the tumor, has been performed to develop an immunotoxin consisting of a biotinylated anti-CD133 mAb bound to streptavidin-saporin system (Stratford et al., 2013). This combined strategy has been used in a sarcoma model system (harboring CSCs within the CD133 high population) and the results have demonstrated that PCI-based drug delivery by the CD133-receptor inhibited cells viability and growth, and the ability to form tumors *in vivo*. Since CD133 overexpression is also found in different tumors, the study performed on sarcoma model can be transferable to treatment of all solid tumors expressing CD133 (Smith et al., 2008; Waldron et al., 2011; Bostad et al., 2013).

REGULATING PATHWAYS

The inhibition of biological pathways crucial in the regulation of the renew and the maintenance of CSCs, in combination with traditional chemotherapies, is a promising strategy for the treatment of CSCs and to circumvent MDR.

Notch pathway

The Notch pathway is important in gene regulation and in cell differentiation processes. This pathway is involved in the pathogenesis of several human tumors such as ovarian cancer (Park et al., 2006). There are four members of the mammalian Notch receptor family (Notch 1–4) activated through a series of cleavage events. The mature Notch receptors are composed of two subunits generated from an initial cleavage event by furin-like convertase. Blaumueller et al. (1997) Notch signaling pathway activation occurs when a Notch receptor binds to ligands. This binding causes a receptor conformational change allowing a second cleavage by tumor necrosis-factor-alpha converting enzyme (TACE) (Brou et al., 2000). The following cleavage is carried out by preselenin (a γ-secretase) releasing Notch intracellular domain and activating target genes expression. Notch plays a significant role in ovarian CSCs regulation and in platinum resistance (Park et al., 2006; Takebe et al., 2011). Also, Notch 3 inhibitors increase the sensitivity of ovarian cancer to cisplatin, reducing the ovarian CSC population (McAuliffe et al., 2012).

Wnt pathway

The Wnt signaling pathway, regulating several processes fundamental in embryogenesis such as proliferation, differentiation, polarity, adhesion and motility, plays key roles in tumorigenesis. Indeed, the progression of several cancers is associated with specific mutations in Wnt pathway components resulting in dysregulated β-catenin-mediated gene transcription. There are two distinct pathways for transduction of Wnt signals: the "canonical" Wnt/β-catenin pathway and the "non-canonical" β-catenin-independent pathway (Clevers and Nusse, 2012). The "canonical" pathway is activated by several Wnt ligands through the binding to Frizzled (FZD) receptors and to low-density lipoprotein receptor-related proteins-5/6 (LRP5/6) co-receptors. As a result, β-catenin accumulates in the cytoplasm, translocates to the nucleus and regulates transcription of Wnt/β-catenin target genes by binding to the T-cell factor/lymphoid enhancer factor (TCF/LEF) (MacDonald et al., 2009). In the absence of Wnt signaling, β-catenin levels are regulated by a cytoplasmic destruction complex (DC). DC is a multiproteins complex composed of protein Axin, the tumor suppressor adenomatous polyposis coli protein (APC), casein kinase α (CK1α), glycogen synthase kinase 3 β (GSK3) and additional associated proteins such as the members of the PARP-family of poly-ADP-ribosylation enzymes (Tankyrases, TNKSs). Axin, the concentration-limiting component of DC, is pivotal for the stability of the β-catenin-DC. By destabilizing Axin, TNKSs directly control the levels of this protein. Therefore, TNKSs inhibition, promoting Axin stabilization, leads to β-catenin phosphorylation and degradation, attenuating Wnt signaling (Liu et al., 2002; Lee et al., 2003). For this reason, in recent years, considerable efforts have been made to identify drugs that inhibit Wnt/β-catenin signaling. Another factor exerting a fundamental role in the biogenesis of Wnt pathway is represented by a member of the Membrane-Bound O-Acyl Transferase (MBOAT) family, Porcupine (PORCN) (Tanaka et al., 2000). PORCN is an integral membrane enzyme resident in the endoplasmic reticulum, required for the lipid modification of Wnt proteins. Acylation by PORCN is essential for the proper secretion and activity of Wnt signaling and, therefore, its inhibition is an attractive therapeutic strategy in diseases with increased Wnt signaling (Chen et al., 2009).

Hedgehog pathway

The Hh pathway is involved in several developmental processes of cells: determination of cell fate, patterning, proliferation, survival, and differentiation (Varjosalo and Taipale, 2008). In mammals, three Hh proteins (Sonic Hh, Indian Hh, and Desert Hh) are derived from proteolysis of inactive precursor that contains its own autoprocessing domain. Hh acts by binding to 12 transmembrane glycoprotein components Patched (Ptch). In the absence of ligand, Ptch constitutively represses the activity of Smoothened (Smo), a 7-pass transmembrane spanning protein with homology to G-protein coupled receptors. When Hh ligand binds to Ptch, the repression of Smo is released and the expression and/or post-translational processing of the three GLI zinc-finger transcription factors, inducing the expression of several target genes, is modulated (Sasaki et al., 1999). Overactivation of Hh pathway is associated with cancer and emerging data from many human tumors, including glioblastoma, breast cancer, pancreatic cancer, and chronic myeloid leukemia (CML), have suggested that Hh signaling regulates CSCs (Liu et al., 2006; Bar et al., 2007; Feldmann et al., 2007; Zhao et al., 2009). The Hh signaling pathway interacts also with other pathways commonly activated in human cancers, such as the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Riobo et al., 2006).

PI3K/AKT pathway

PI3K/AKT is important for pluripotency maintenance of stem cells. PI3K enzymes are normally regulated by growth factors

and are useful to phosphorylate phospholipids on the plasma membrane (Hennessy et al., 2005). In addition, it has been suggested that PI3K/Akt activation pathway is required for CSCs maintenance and viability in breast cancer, prostate cancer, and brain tumor (Zhou et al., 2007; Hambardzumyan et al., 2008; Dubrovska et al., 2009). The PI3K/Akt pathway can also modulate functions of ABC transporters through various mechanisms. Inhibition of the PI3K/Akt pathway causes BCRP internalization in hematopoietic stem cells and glioma stem-like cells. Hence, the PI3K/Akt pathway can be an attractive target for cancer therapy (Mogi et al., 2003; Bleau et al., 2009).

HIGH-THROUGHPUT SCREENING (HTS)

The development of agents active toward CSCs population may be useful in the treatment of patients with recurrent cancer. HTS allows to identify potential compounds targeting specifically CSCs. In recent years, several studies have been carried out and in one of these studies the chemical library Microsource Spectrum collection of 2000 compounds has been screened to identify therapeutic agents inhibiting Glioblastome Multiforme stem cells (GSCs). This library has been selected based on the structural and biological diversity and consisted of FDA approved drugs, compounds in the late-phase clinical trials, experimental drugs and natural products. Among these, Disulfiram, a clinically approved drug for the treatment of alcoholism, was identified as a potent inhibitor of multiple patient-derived GSCs (Germain et al., 2013).

Another study has been carried out in order to identify agents able to selectively inhibit a cell-line model of breast CSCs screening over 300,000 compounds of the Molecular Libraries Small Molecules Repository (MLSMR) from the National Institute of Health (NIH). Among these compounds, a cinnamide analog displayed 20-fold selective inhibition of the breast CSC-line cell (HMLE_sh_Ecad) over the control (HMLE_sh_eGFP) (Hothi et al., 2012).

In another study, HTS of 825 potential drugs (the National Cancer Institute's "Mechanistic Set" library) toward ovarian CSCs and the subsequent identification of compounds displaying potential activity as ovarian CSC therapeutic agents is reported. Several compounds were classified as inhibitors and, among these, 5 compounds FDA-approved (dactinomycin, plicamycin, vinblastine, vincristine, and mepacrine) were identified as anticancer drugs (Mezencev et al., 2012).

TUMOR MICROENVIRONMENT

It has been proposed that CSCs are maintained by their surrounding microenvironment called "niche." Niche, important for CSCs sustaining, is composed by immune and stromal cells, blood vessels and extracellular matrix components. Biological processes, such as inflammation, hypoxia and angiogenesis, are important for CSCs, and signaling from this microenvironment is crucial for the activation of pathways involved in the maintenance of TICs functions (Cabarcas et al., 2011; Hanahan and Weinberg, 2011). Tumor hypoxia is implicated in the development of resistance to many conventional chemotherapeutic agents. It induces a decreased sensitivity to apoptotic and other cell-death signals, and increased signaling promoting angiogenesis, proliferation and systemic metastasis capacity (Feldmann, 2001; Kunz and Ibrahim, 2003). The initial response of cancer cells to hypoxia is the activation of hypoxia responsive transcription factors (HIFs) that regulate a number of genes involved in glucose metabolism, cell survival, erythropoiesis, stem cell maintenance, angiogenesis and resistance to chemotherapy and radiotherapy (Majmundar et al., 2010). Molecules like langkamide, piplartine and 3,4,5-trimethoxycinnamic acid (**Chart 1**), displaying HIF-2 inhibitory activity, may be used for targeting HIFs mediated regulation of CSCs (Bokesch et al., 2011). Another important transcription factor, involved in tumor growth, progression, and in the resistance to chemotherapy, is NFkB: this factor and HIF1α together regulate transcription of thousand genes controlling vital cellular processes (Gupta et al., 2010).

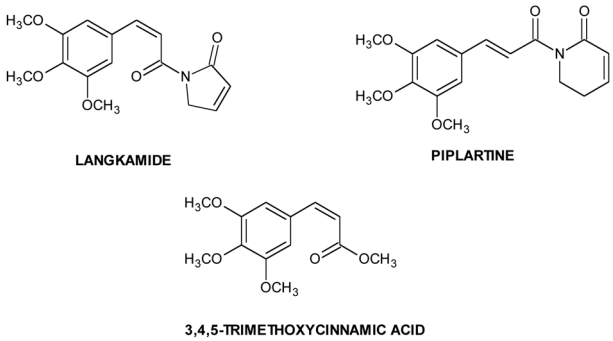

Chart 1 | Natural HIF inhibitors.

MOLECULES ACTIVE TOWARD CSCs

PORCUPINE AND TANKSs INHIBITORS

In the last years, considerable efforts have been made to identify drugs able to inhibit Wnt/β-catenin signaling, either by blocking Wnt secretion (PORCN inhibitors) or by interfering with β-catenin, by binding its transcription factor targets (TANKSs inhibitors).

Starting from four IWP ligands, additional PORCN inhibitors have been identified, that are characterized by two structural motifs of IWPs (phthalazinone and pyrimidinone moieties) interacting with PORCN and important for the activity. The lead optimization step led to a significant improvement in activity and to the identification of IWP-L6 (**Chart 2**) as a new sub-nanomolar PORCN inhibitor (Chen et al., 2009; Wang et al., 2013b). The first TANKSs inhibitor, identified by Huang et al. through a chemical genetic screen, was XAV-939 (**Chart 2**) (Huang et al., 2009b). It selectively affects β-catenin-mediated transcription, by stimulating its degradation by stabilizing Axin. By HTS, JW55, IWR-1, and WIKI4 (**Chart 2**) as specific tankyrases inhibitors have been discovered (Chen et al., 2009; James et al., 2012; Waaler et al., 2012).

HAP: HYPOXIA-ACTIVATED BIOREDUCTIVE PRODRUGS

The hypoxic microenvironment of solid tumors has attracted significant attention as target for the development of novel therapeutics for cancer treatment. A new class of drugs, the

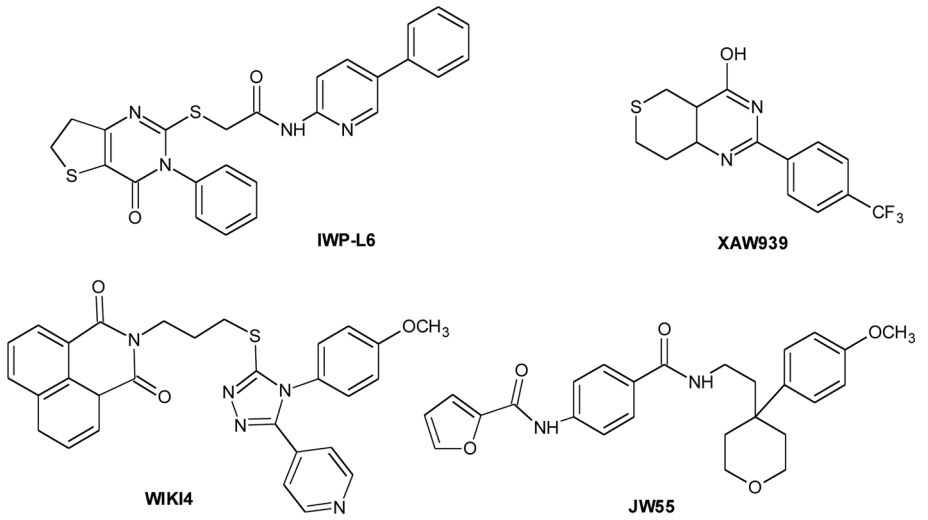

Chart 2 | Porcupine and Tankyrase inhibitors.

hypoxia activated prodrugs (HAP), has been designed for selective activation under low oxygen conditions, typical of many solid tumors. These are prodrugs that are enzymatically converted into active metabolites (effectors) by endogenous enzymes under the hypoxic conditions that prevail in tumors (Rauth et al., 1998). Taking into account the different activation profile, HAP could be classified into two groups: (1) Class I HAP (such as benzotriazine *N*-oxides tirapazamine and SN30000), activated under relatively mild hypoxia; (2) Class II HAP (such as the nitro compounds PR-104A or TH-302), activated only under extreme hypoxia (Yin et al., 2012; Foehrenbacher et al., 2013). Several hypoxia-specific prodrugs are in various stages of clinical development, although no registered agents have been used in clinical therapy. Moreover, the bioreductive compounds are classified in five classes: (1) aromatic *N*-oxides; (2) aliphatic *N*-oxides; (3) nitro(hetero)cyclic compounds; (4) quinones; (5) metal complexes.

The *N*-oxide tirapazamine (TPZ, SR4233) (**Chart 3**) has been the most extensively evaluated compound in the clinic to date. TPZ exhibited up to 300-fold higher toxicity under anoxic conditions than aerobic conditions *in vitro* (Zeman et al., 1986). TPZ undergoes one-electron reduction to generate the corresponding radical able to produce DNA breaks and lesions (Shinde et al., 2010). Although TPZ has been evaluated in a number of phase II clinical trials (Marcu and Olver, 2006), the results have not been translated into increased efficacy over conventional treatment in phase III clinical trials (Rischin et al., 2010). SN30000 (**Chart 3**) is a TPZ analog with improved pharmacokinetic properties and is presently scheduled to enter phase I clinical trials (Hicks et al., 2010).

N-oxide banoxantrone (AQ4N) (**Chart 3**) is metabolized under hypoxia giving a high affinity DNA intercalating agent, AQ4, that inhibits topoisomerase II (Patterson et al., 1994). In preclinical studies, AQ4N, combined with radio- or chemotherapy, demonstrated significant increasing activity (Patterson et al., 2000).

PR-104 (**Chart 3**), currently in phase II clinical trials, is a water-soluble phosphate ester pre-prodrug that rapidly is hydrolyzed *in vivo* to the prodrug PR-104A, a dinitrobenzamide mustard that is reduced to *p*-hydroxylamine and *p*-amine metabolites by various oxidoreductases (Singleton et al., 2009). Another promising compound, TH-302 (**Chart 3**), is a 2-nitroimidazole-based nitrogen mustard prodrug. The reduction in hypoxic cells to 2-nitroimidazole and the subsequent bromoisophosphoramide mustard affects DNA repair pathways (Meng et al., 2012). TH-302, in combination with chemotherapy, is currently in phase II and III clinical trials.

Quinone compounds (porfiromycin, RH1, and apaziquone EO9) (**Chart 3**) show greater selectivity toward hypoxic cells where their activation under hypoxia is carried out by one-electron reductases (Saunders et al., 2000; Phillips et al., 2013; Guise et al., 2014). Finally, although metal complexes could be potentially used as hypoxia-selective agents, none have been developed for clinical use so far.

MISCELLANEOUS

Several molecules, with unrelated chemical structures, are active toward CSCs. Among these, the antihelmintic niclosamide (**Chart 4**) selectively targets ovarian CSCs, and its effect is associated with the inhibition of metabolic pathways related to redox regulation (Yo et al., 2012). Metformin (**Chart 4**), an antidiabetic drug, selectively targets CSCs in multiple types of cancer including prostate, lung and breast (Hirsch et al., 2009; Shank et al., 2012) and, in combination with chemotherapeutic agents (such as doxorubicin, paclitaxel, and carboplatinum) inhibits tumor growth (Gotlieb et al., 2008). This drug also displays an anti-proliferative and pro-apoptotic effects on ovarian CSCs *in vitro* (Yasmeen et al., 2011). Retinoids, in co-administration with cis-platinum, affect ovarian CSCs, whereas carboplatinum alone is not active (Whitworth et al., 2012). Ericalyxin B, a diterpenoid isolated from *Isodon eriocalyx* displaying antitumor

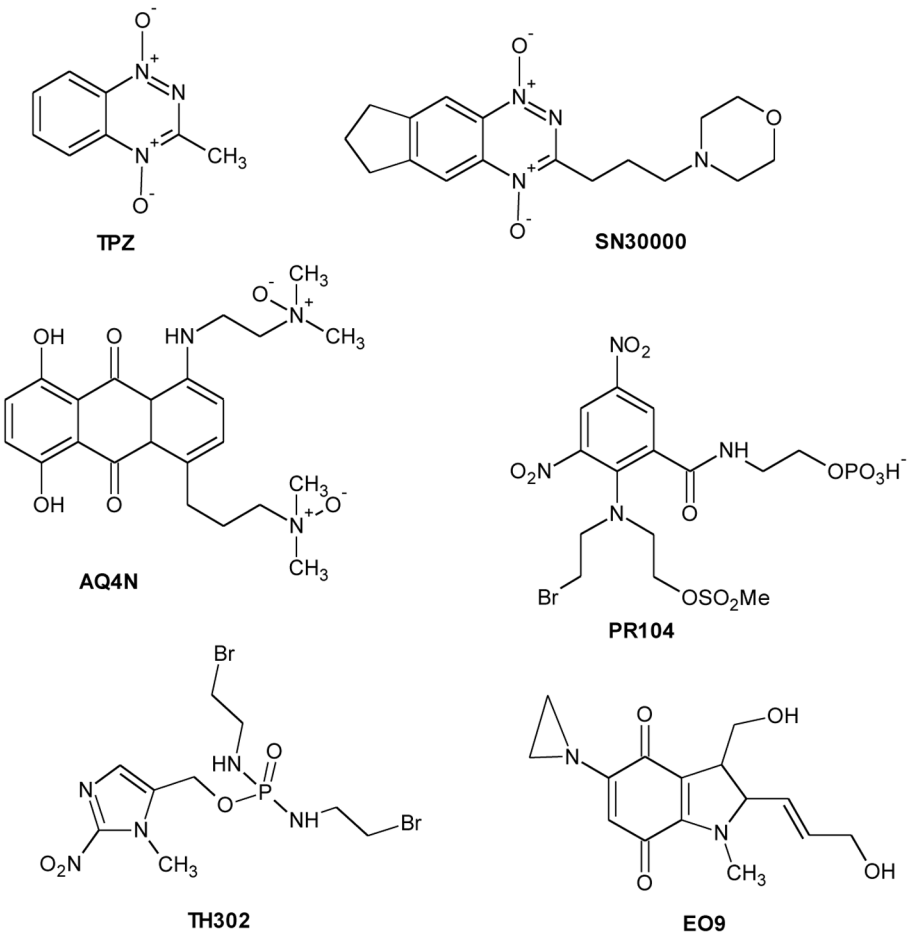

Chart 3 | Hypoxia Activated Prodrugs (HAP).

effects *via* multiple pathways, and 3-Bromopiruvate target ovarian CSCs inducing cell death (Leizer et al., 2011; Wintzell et al., 2012). Dactinomycin and Plicamycin, two FDA-approved CSCs-inhibitory compounds, are used in the treatment of several cancers including gestational trophoblastic neoplasia, Wilms' tumor, testicular cancer and hypercalcemia associated with advanced malignancy (Kennedy, 1970; Perlia et al., 1970; Lewis et al., 1972; Frei, 1974; Fraschini et al., 2005; Lee et al., 2006). Disulfiram (**Chart 4**), used in alcoholism treatment, is able to reduce cell growth and self-renewal of glioblastoma stem cells resistant to temozolomide *in vitro* by activating apoptotic pathways that modulate Bcl-2 family (Liu et al., 2012). DECA-14 (**Chart 4**), an analog of dequalinium (DECA-10), an antimicrobial agent and Rapamycin were identified as neuroblastoma (NB) TIC-selective agents. Both compounds induce CSCs death *in vitro*, reduce NB xenograft tumor weight *in vivo* and decrease self-renewal in treated tumors (Smith et al., 2010). Furthermore, NV-128 (**Chart 4**), an isoflavone derivative, induces cell death in chemoresistant CSCs population by inhibiting mitochondrial function and activating cell death pathways (Alvero et al., 2011).

CSC AND MDR: REPOSITIONING STRATEGY

The failure of cancer therapy is often due to tumor recurrence after chemotherapy because in several cancers a residual pool of CSCs remains (Dean et al., 2005). TICs that survive after therapy evolve in a population of chemoresistant cells able to sustain the growth of a more aggressive tumor. Among all the protective mechanisms for CSCs, ABC proteins overexpression is one of the mostly reported (An and Ongkeko, 2009; Calcagno et al., 2010; Fuchs et al., 2010; Clevers, 2011; Moitra et al., 2011; Pietras, 2011). There are two models useful to explain the connection between MDR and CSCs: (i) the original MDR model and (ii) the acquired MDR model (Holohan et al., 2013). The first model proposes that, after exposure to the chemotherapeutic agent, only CSCs expressing ABC transporters repopulate the tumor. The second suggests that, after chemotherapy, only CSCs survive and this population of survival cells, after mutations, originates new and more aggressive drug-resistant cell phenotypes (Holohan et al., 2013). The combination of CSCs targeting agents with novel or conventional cytotoxic drugs could lead to a potentiated effect. Flavonoids, a large family of polyphenolic molecules, modulate MDR transporters and inhibit CSCs growth (Colabufo et al.,

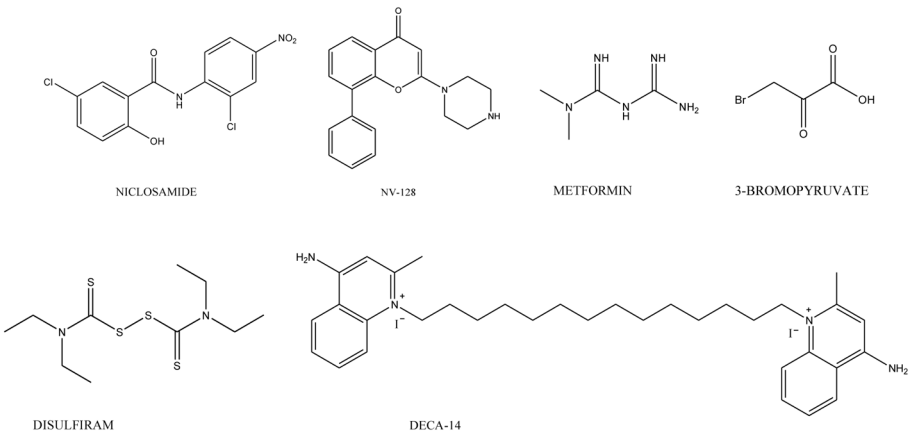

Chart 4 | Agents targeting CSCs.

2008c). Flavonoids anticancer properties are due to their antimitotic activity and to the inhibition of several enzymes. Recently, it has been suggested that flavonoids inhibit the function of ABC transporters such as P-gp, MRPs, and BCRP. Structural requirements for the interaction with these pumps are hydrophobic character and planarity. Flavonoids show low toxicity but, in the meantime, display a broad spectrum of biological activities (Colabufo et al., 2008c). Moreover, several studies have pointed out that flavonoids are able to affect CSCs. Luteolin, Casticin and 8-Bromo-7-methoxychrysin (BrMC) are active on glioma stem-like cells (Feng et al., 2012). BrMC is active on hepatocellular carcinoma (Hep-G2 cell line) where it induces apoptotic cell death by involving ROS products (Yang et al., 2010). LY294002, a PI3K specific inhibitor, blocks osteosarcoma CSCs cell cycle (G0/G1) in a dose-dependent manner inducing apoptosis by preventing phosphorylation of PKB/Akt *via* PI3K phosphorylation inhibition. Several studies show that this compound also inhibits BCRP, P-gp, and MRP1 highly expressed in stem cells. LY294002 reverses MDR in CSCs overexpressing BCRP by internalizing this pump (Imai et al., 2012). LY294002 competitively inhibits MRP1-mediated doxorubicin efflux in drug-resistant HT29RDB colon carcinoma cells. Sensitization was not restricted to doxorubicin, but it was also observed in cells treated with cisplatin, topotecan, and mitoxantron (Abdul-Ghani et al., 2006). Moreoever, LY294002 antagonizes transport activity of P-gp by reducing the degree of vincristine resistance in L1210/VCR mouse leukemic cell lines in a concentration-dependent manner (Barancík et al., 2006). However, the simultaneous inhibition of several cellular signaling pathways, transporters and channels may cause severe side effects.

Salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus*, is able to affect CSCs in gastric cancer, lung adenocarcinoma, osteosarcoma, colorectal cancer, squamous cell carcinoma (SCC) and prostate by interfering with ABC transporters and CSC crucial pathways (Dong et al., 2011). The combination of salinomycin and the antibody for the anti-human epidermal growth factor receptor 2 (anti-HER2), trastuzumab, was found more efficacious than trastuzumab single-treatment in MCF-7-derived breast CSCs and HER2-expressing breast cancer cells. In colorectal cancer and in SCC, salinomycin, but not oxaliplatin or cisplatin, is able to reduce the portion of CSCs (Basu et al., 2011; Dong et al., 2011; Tang et al., 2011; Wang, 2011; Zhi et al., 2011; Ketola et al., 2012; Oak et al., 2012). Moreover, salinomycin is active toward human AML CSCs, because it overcomes ABC transporter-mediated MDR and apoptosis resistance and inhibits P-gp/MDR1 in different cancer cells (Fuchs et al., 2010; Riccioni et al., 2010).

CONCLUSIONS AND PERSPECTIVES

Although several items about CSCs theory remain open, there is a large evidence demonstrating that CSCs are essential to initiate and maintain tumors. CSCs are involved in tumor re-growth and are resistant to conventional anti-cancer therapy because of several mechanisms. Among them, the overexpression of ABC transporters in CSCs membranes represents a novel target for eradicating tumor relapse. To date, several strategies have been employed to isolate, target CSCs and circumvent MDR: (i) the development of sensitive biomarkers and targeted-aptamers; (ii) the evaluation of CSCs microenvironment; (iii) the study of the genes and pathways involved in CSCs regulation; (iv) evaluation of known and new molecules able to affect both TIC populations and ABC transporters. Therefore, the next challenge in this field may be a *multimodal strategy*, an approach in which specific CSCs targeting drugs exert simultaneously the ability to circumvent tumor drug resistance (ABC transporters modulation) and cytotoxic activity toward CSCs and the corresponding differentiated tumor cells. The efficacy of this suggested multimodal strategy will be probed by using several scaffolds active toward MDR pumps on CSCs isolated by tumor specimens.

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P-glycoprotein mediated efflux limits substrate and drug uptake in a preclinical brain metastases of breast cancer model

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The blood–brain barrier (BBB) is a specialized vascular interface that restricts the entry of many compounds into brain. This is accomplished through the sealing of vascular endothelial cells together with tight junction proteins to prevent paracellular diffusion. In addition, the BBB has a high degree of expression of numerous efflux transporters which actively extrude compounds back into blood. However, when a metastatic lesion develops in brain the vasculature is typically compromised with increases in passive permeability (blood-tumor barrier; BTB). What is not well documented is to what degree active efflux retains function at the BTB despite the changes observed in passive permeability. In addition, there have been previous reports documenting both increased and decreased expression of P-glycoprotein (P-gp) in lesion vasculature. Herein, we simultaneously administer a passive diffusion marker (^{14}C-AIB) and a tracer subject to P-gp efflux (rhodamine 123) into a murine preclinical model of brain metastases of breast cancer. We observed that the metastatic lesions had similar expression ($p > 0.05$; $n = 756$–1214 vessels evaluated) at the BBB and the BTB. Moreover, tissue distribution of R123 was not significantly ($p > 0.05$) different between normal brain and the metastatic lesion. It is possible that the similar expression of P-gp on the BBB and the BTB contribute to this phenomenon. Additionally we observed P-gp expression at the metastatic cancer cells adjacent to the vasculature which may also contribute to reduced R123 uptake into the lesion. The data suggest that despite the disrupted integrity of the BTB, efflux mechanisms appear to be intact, and may be functionally comparable to the normal BBB. The BTB is a significant hurdle to delivering drugs to brain metastasis.

Keywords: drug resistance, chemotherapy, autoradiography, fluorescence microscopy, tumor

INTRODUCTION

The successful treatment of central nervous system (CNS) tumors and metastases using chemotherapy depends on the ability of therapeutic concentrations of drug to cross the blood–brain barrier (BBB). More than 98% of potential CNS active anticancer drugs fail in preclinical work and or clinical trials because of inadequate BBB penetration (Pardridge, 2007). Clinically this results in many anticancer agents failing to substantially reduce tumor burden and or significantly prolong survival (Deeken and Loscher, 2007).

The microvasculature of the brain is a unique anatomical structure which serves as a homeostatic and regulatory barrier between the blood and the brain parenchyma (Hawkins and Davis, 2005). Specifically, endothelial cells that line the blood vessels of the brain capillaries are fused together by numerous tight junction protein complexes, which restrict blood components from passively diffusing between the cell margins to gain entry into brain. The tight junction protein complexes consist of a number of proteins such as zonula occludins, junctional adhesion molecules, and claudins which function as a unit to seal the endothelia margins. Further the outside of the brain capillary is surrounded by astrocytic foot processes and pericytes that also contribute to the restriction of paracellular diffusion (Abbott et al., 2010).

Further restricting the brain entry of a large number of drugs and drug classes are efflux transporters at the BBB. Efflux transporters are richly expressed in the brain vasculature and have been shown to restrict the accumulation of antiepliptics, antidepressants, and antipsychotics (Schinkel et al., 1995; Loscher and Potschka, 2005). Multiple efflux transporters at the BBB act to actively extrude or prevent drug accumulation into brain, these include P-glycoprotein (P-gp; ABCB1) (Schinkel et al., 1996), breast cancer resistant protein (BCRP; ABCG2; Polli et al., 2009), multidrug resistance associated proteins (MRP; ABCC1-6; Breedveld et al., 2005), and organic anion transporters (OATs; Hagenbuch and Meier, 2004).

The net effect of the anatomical and molecular features of the BBB is that to a large degree it restricts drug movement from blood into brain. But some drugs are able to penetrate the BBB. Drug and/or solute permeation across the BBB is mostly limited

to low molecular weight lipid-soluble molecules. Molecules that are large (typically >700 Da), protein bound or are hydrophilic will have difficulty crossing the BBB and accumulating in brain to a sufficient degree (Lipinski et al., 2001).

However, the vasculature within a brain tumor is different from the normal BBB. Previously it has been shown that the blood-tumor barrier (BTB) vasculature has disrupted integrity compared to the intact BBB. This disruption can allow small molecule to accumulate into lesions up to 30–100-fold more than the accumulation of the molecule in normal brain (Lockman et al., 2010; Taskar et al., 2012). While the degree of breakdown at the BTB does correlate with increases in drug uptake it is not clearly defined whether efflux pumps continue to limit drug uptake into metastatic lesions (Gallo et al., 2003). It has been previously shown that the BTB expresses P-gp (Cordon-Cardo et al., 1990); however, the expression of P-gp may be variable among different tumors types (Henson et al., 1992). In addition to P-gp expression at the BTB, many cancers have been shown to express functional P-gp *in vivo* which may restrict the cellular accumulation of chemotherapuetics.

Herein we set out to determine the expression and function of P-gp in a preclinical model of brain metastases of breast cancer using quantitative fluorescence microscopy and autoradiography. We observed that P-gp is expressed at the BTB in brain metastases at nearly similar levels to the BBB. In addition, P-gp is highly functional in limiting the lesion accumulation of the P-gp substrate, Rhodamine 123 (R123) despite significant passive permeability increases.

MATERIALS AND METHODS

CHEMICALS

R123 was purchased from Molecular Probes Invitrogen (Eugene, OR, USA). Verapamil was purchased from Sigma (St. Louis, MO, USA). Cyclosporine A was purchased from Tocris Biochemicals (St. Louis, MO, USA). ^{14}C-labeled aminoisobutyric acid (AIB) was purchased from American Radiolabelled Chemicals (St. Louis, MO, USA). All other chemicals used were of analytical grade and were used as supplied.

ANIMALS

Female NuNu mice (∼24 g; 8 weeks of age) were purchased from Charles River Laboratories (Kingston, NY, USA) and were used for all the perfusion experiments done in this study. All studies were approved by the Animal Care and Use Committee and were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

IN SITU MOUSE HEART PERFUSION TECHNIQUE

The *in situ* mouse heart perfusion technique was utilized to evaluate brain uptake of R123 (Takasato et al., 1984; Lockman et al., 2003a) Mice were anesthetized with ketamine/xylazine (100 and 8 mg/kg, respectively) and the heart exposed. Body temperature was monitored and maintained at 37°C using a heating pad attached to a feedback device (YSI Indicating Controller, Yellow Springs, OH, USA). Prior to insertion of the cannula, the right cardiac atrium was cut to prevent venous return. Cannulation of the left cardiac ventricle was done using butterfly syringe (28G) attached to a perfusion apparatus. Perfusion fluid was pumped into the left cardiac ventricle by a cannula at a constant rate of 2.5 mL/min (Dagenais et al., 2000) using a Harvard Model 944 dual channel pump (Harvard Apparatus, South Natick, MA).

The perfusion fluid consisted of HCO_3 buffered physiological saline, containing 128 mM NaCl, 24 mM $NaHCO_3$, 4.2 mM KCl, 2.4 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 0.9 mM $MgSO_4$, and 9 mM glucose (pH ∼7.35; [Na] = 154.4 mM). All solutions were filtered, oxygenated, warmed to 37° C, and adjusted to pH 7.35 prior to perfusion. To determine initial brain uptake of R123, perfusion fluid containing R123 (50 μg/mL) was infused into the systemic circulation for 30–120 s. At the end of each experiment, mice were sacrificed, and the brain was rapidly removed (<60 s) from the skull. The brain was flash frozen in isopentane (−65°C). Concentration of the fluorophore (R123) in brain was determined using fluorescent microscopy and regional permeability was expressed by the unidirectional transfer constants, K_{in} (mL/s/g) derived from Eq. 1.

QUANTIFICATION OF R123 USING FLUORESCENCE MICROSCOPY

Fluorescence was observed with an Olympus MVX10 stereomicroscope (objective: 2×, NA 0.5) with an optical zoom range from 0.63 to 12.6. The excitation and emission of R123 was obtained using a GFP filter (excitation/band pass filter of 470/40, emission/band pass filter of 525/50 and dichromatic mirror at 495 nm; Chroma Technology, Bellow Falls, VT, USA). Tissue sections of 20 μm were obtained at −23°C using a cryotome (Leica CM3050S, Leica Microsystems, Buffalo Grove, IL, USA), mounted on charged glass slides, and kept at −23°C. Data were analyzed using quantitative fluorescence microscopy and all images were obtained with 15 ms exposures, though a 2.0 objective at 4× magnification (Olympus MVX10) with a monochromatic cooled CCD scientific camera (Retiga 4000R, QImaging, Surrey, BC, Canada). Slidebook® 5 software (Intelligent Imaging Innovations, Denver, CO, USA) was utilized to determine sum intensity per gram of brain which then converted into concentration of dye per gram of brain using the brain homogenate standards. The voxel by voxel sum intensity of fluorescence for brain homogenate samples was obtained with the 2× objective. The optical zoom range was maintained at 4× for a total optical magnification of 8×. The sum intensity per gram of brain homogenate was obtained using a set exposure time of 15 ms with camera gain settings of 615. The total fluorescence intensity signal for each concentration was then plotted as a function of grams of brain which was calculated using the area in microns squared multiplied by the thickness of the brain sample (20 μm) to obtain a total brain volume that was analyzed. The brain volume (μm^3) was multiplied by the density of brain tissue (1.04 g/cm^3) as similarly reported by (Tengvar et al., 1983) to obtain a weight of brain tissue that was analyzed.

PREPARATION OF BRAIN STANDARDS

To calculate the concentration of the R123 in brain, standard curves were generated in rat brain homogenates. Briefly, 100 μL of standard solution of the dye was added to each of 500 mg of the brain and homogenized. The homogenized mass was flash frozen

in isopentane (−80°C) and sliced into 20 μm sections using a cryostat −23°C and mounted onto glass, superfrost slides. The slices were analyzed using quantitative fluorescence microscopy and the sum intensity per gram of brain homogenate was plotted against concentration of the dye.

KINETIC ANALYSIS

Unidirectional uptake transfer constants (K_{in}) were calculated from the following relationship to the linear portion of the uptake curve:

$$Q^*/C^* = K_{in}T + V_0 \quad (1)$$

where Q^* is the quantity of fluorophore (R123) in brain (μg/g) at the end of perfusion, C^* is the perfusion fluid concentration of fluorophore (μg/mL), T is the perfusion time (s) and V_0 is the extrapolated intercept ($T = 0$ s; "vascular volume" in mL/g). After determination of a perfusion time that allowed adequate amount of fluorescent marker to pass into brain and yet remained in the linear uptake zone, K_{in} was determined in single time-point experiments as:

$$K_{in} = [Q^* - V_0C^*]/C^*T]$$

(Takasato et al., 1984; Smith and Takasato, 1986).

ANTIBODY STAINING

Tissues were rehydrated in PBS and then fixed in 4% paraformaldehyde (PFA) for P-gp (Abcam, Cambridge, MA), cytokeratin (Abcam) and CD31 (BD Pharmingen, San Jose, CA), ice-cold methanol for ABCB1 (Santa Cruz Biotechnology), CD31 (BD Pharmingen). After three PBS washings (5 min), slides were blocked with 4% goat serum and 0.2% Triton-X 100 (1 h). After blocking, primary antibodies were added, followed by overnight incubation at 4°C. The next day, the slides were washed and secondary antibodies and DAPI (1 mg/mL) were added (1 h). Slides were again washed, DAKO mounting medium was added, and coverslips were applied.

RESULTS

To determine if P-gp expression is present in the vasculature of brain metastases, we analyzed the brains of tumor bearing mice using immunofluorescence staining for both P-gp and the vascular marker CD31 to quantify the amount of colocalization (**Figure 1**). There was significant expression of P-gp at the BBB and BTB (**Figure 1B**). Overall there was no difference between the fluorescent intensity of P-gp staining in the CD-31 defined regions in tumor vasculature (22.9 ± 0.4 A.U.; $n = 756$ vessels) and in the normal brain vasculature (22.6 ± 0.3 A.U.; $n = 1214$). In addition, there was positive P-gp staining that did not co-localize to the vasculature, but surrounded metastasis cells suggesting that P-gp may also be present on the metastatic cancer cells.

We measured P-gp function by the time dependent accumulation of the fluorescence P-gp substrate R123 according to previous methodology (Mittapalli et al., 2013). Using fluorescent brain standards we determined the blood to brain unidirectional transfer coefficient (K_{in}) of R123 in normal brain and in metastatic lesions by calculating the concentration of R123 divided by the concentration in the perfusate and plotted this over time (30–120 s; **Figure 2A**). We then applied a previously calculated correction to the vascular volume by perfusion of non-permeable [^{14}C]-sucrose and measuring its vascular space (0.015 ± 0.002 mL/g). We observed that the uptake of R123 was linear within the perfusion time with a K_{in} of 0.12 ± 0.03 μL/s/g. To determine if we could inhibit P-gp mediated efflux of R123, we added P-gp inhibitors verapamil and cyclosporine A (Choi and Li, 2005; Breedveld et al., 2006; Baumert and Hilgeroth, 2009) at various concentrations to the R123 perfusate in separate experiments (**Figure 2A**). Upon co-perfusion of R123 and each inhibitor, there was an increase in R123 permeability; Cyclosporin A (2.4 ± 0.5 μL/s/g); and Verapamil (2.2 ± 0.2 μL/s/g)] indicating that R123 uptake into brain is limited by the efflux function of P-gp at the BBB.

We then plotted R123's LogD (octanol/water coefficient; pH = 7.4) and observed K_{in} in comparison to known passive

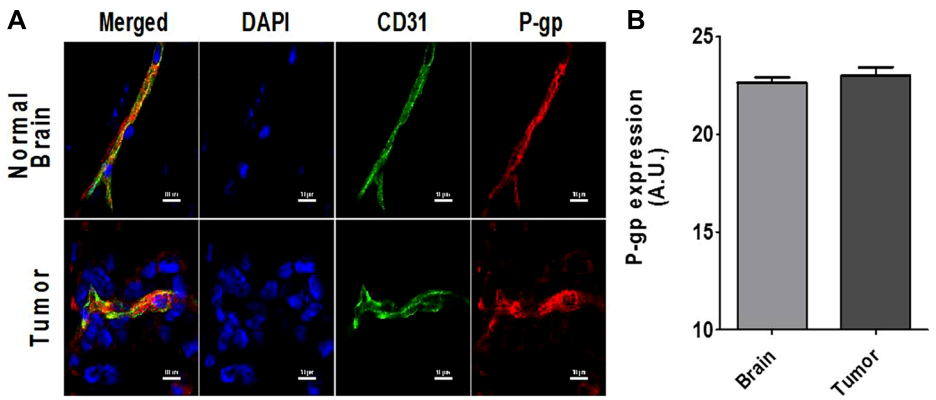

FIGURE 1 | (A) Representative images of the co-localized expression of P-gp (red) in a capillary in normal brain (top row) and a blood vessel in a 231Br brain metastasis (bottom row) are shown. Endothelial nuclei as well as the nuclei of the 231Br lesions are shown in blue (DAPI). Blood vessels (CD-31 expression) in both sections are shown in green (Alexa Fluor 488). P-gp expression is shown in red (Alexa Fluor 594). **(B)** The bar graph shows the relative P-gp expression per vessel as defined by CD31 stained regions. Mean ± SEM; BTB; $n = 756$ vessels and BBB $n = 1214$ vessels).

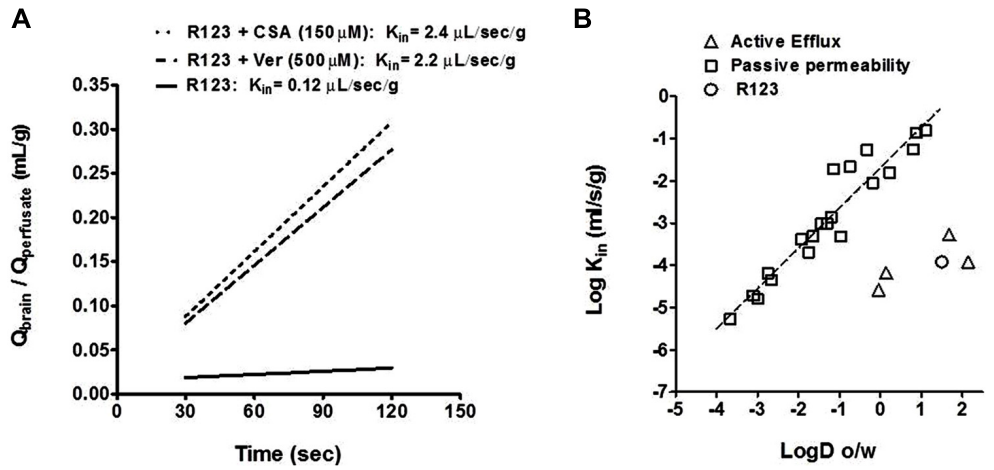

FIGURE 2 | (A) The K_{in} of R123 in the presence of the known P-gp inhibitors, cyclosporin A and verapamil, increases brain distribution (as reported by the K_{in}) by >10-fold. All data represent mean ± S.E.M for total brain; $n = 3$–5 for all groups. Statistics: one-way ANOVA; Dunnett's. **(B)** The relationship between LogD (octanol/water coefficient; pH = 7.4) and observed K_{in} is used to profile a molecule or drug's mechanism of distribution into brain. Compounds that are known to cross the BBB via passive diffusion are plotted using gray squares and those subject to efflux are plotted with gray triangles. R123 (open circle) falls below ∼3 log units the line of identity for passive permeability indicating it may be subject to efflux.

permeability compounds (Begley, 1996) and efflux substrates (Summerfield et al., 2007; **Figure 2B**). Molecules and drugs that passively diffuse into brain exhibit a linear relationship between their LogD (octanol/water coefficient) and their observed Log K_{in} while molecules which are subject to efflux will exhibit observed Log K_{in} values well below the value predicted by its LogD (**Figure 2B**). R123 has a LogD of 1.51 (Forster et al., 2012) and Log K_{in} of −3.93 (calculated from observed K_{in}; **Figure 2A**) which places R123 several orders of magnitude below a passively diffusing molecule's profile which supports the evidence of R213's restriction from brain via an efflux transporter.

To determine BTB passive permeability and whether P-gp influences R123 uptake into brain metastases of breast cancer, tumor-bearing mice were injected with ^{14}C-AIB (passive permeability tracer) which was allowed to circulate for 10 min before a 2 min R123 perfusion, which was followed by sacrifice (**Figure 3**). Autoradiography analysis of the brains revealed elevated permeability to ^{14}C-AIB (∼4.9-fold increase). The passive permeability marker tracer's uptake did not correlate ($r^2 = 0.17$ for AIB) with metastases size (**Figure 3A**). R123 uptake, however, was not different from that of normal brain on average (∼0.98-fold change) in metastatic lesions and had no correlation ($r^2 = 0.033$) to metastasis size (**Figure 3B**). R123 permeability did not correlate passive permeability changes as measured by ^{14}C-AIB ($r^2 = 0.0008$) (**Figure 3C**) accumulation suggesting that R123 remains restricted from the brain parenchyma via P-gp mediated efflux. The observed R123 K_{in} value for normal brain (BDT; brain distant to tumor) regions of metastases bearing mice ($K_{in} = 0.11 \pm 0.06 \times 10^{-1}$ μL/s/g) (**Figures 4A–C**) was consistent with previous K_{in} measurements in tumor-free mice (**Figure 1B**). And, the K_{in} of R123 in the BTB (within metastases) was 0.12 ± 0.003 μL/s/g which was not different than that of normal brain ($p > 0.05$).

DISCUSSION AND CONCLUSION

In the current study, we present data suggesting P-gp retains its efflux function at the BTB despite a disruption in the integrity of the BBB induced by the presence of a metastatic lesion. Of notable methodology, to the best of our knowledge we are the first to combine quantitative fluorescence microscopy to measure R123 P-gp mediated efflux and quantitative autoradiography to measure changes in BTB passive permeability (^{14}C-AIB) in the same brain slice. This method is able to directly shed light on two independent processes occurring at the BTB.

The utilization of R123 to evaluate P-gp function is well established (Hegmann et al., 1992). However, there is less evidence regarding R123's affinity and efflux transport to other transporters that contribute to drug restriction to brain. R123 has been reported to be subject to transport by BCRP (Doyle et al., 1998), and OCT 1 & 2 (Jouan et al., 2012), and MRP2 (Munic et al., 2011). Though studies using specific transporter inhibitors at correct concentrations show P-gp primarily transports R123 and restricts accumulation into brain (Wang et al., 1995). Moreover, the magnitude of R123 efflux by P-gp is greater than that of BCRP and MRP1 (Chopra, 2004) and therefore should represent the major pathway of active efflux transport at the BBB and BTB.

Due to the difficulty in performing the *in situ* brain perfusion in mice, we modified the *in situ* brain perfusion to a cardiac perfusion method in female Nu/Nu mice bearing brain metastases of breast cancer to characterize P-gp function *in vivo*. This method has similar advantages to the *in situ* brain perfusion method in that we may control aspects of the perfusion to determine both influx and efflux kinetics, transporter inhibition coefficients, and BTB or BBB permeability (Smith and Allen, 2003). This control helps determine accurate apparent permeability coefficients (Lockman et al., 2005a), the degree to what a substrate is efflux back into blood (Lockman et al., 2003b), inhibition constants for

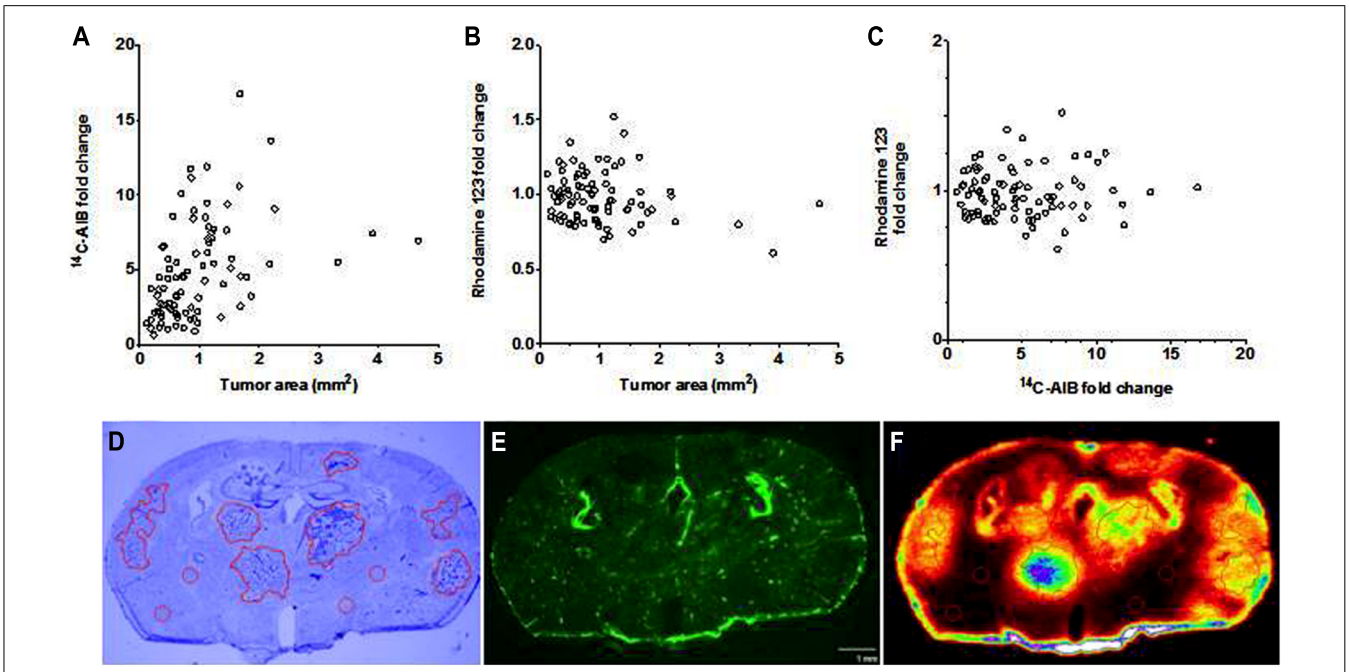

FIGURE 3 | The BTB is variably compromised for compounds entering via passive diffusion but retains P-gp mediated efflux. The passive permeability marker ^{14}C-AIB fold change in brain metastases did not correlate ($r^2 = 0.167$) with metastasis size (mm^2) **(A)**. R123 fold change in brain metastases did not correlate ($r^2 = 0.033$) with metastasis size **(B)**. There was no observed relationship ($r^2 = 0.0009$) between the fold increase in brain metastases of ^{14}C-AIB (passive permeability marker) and R123 (P-gp substrate) **(C)**. One representative brain slice showing metastases location (**D**, cresyl violet), R123 fluorescence distribution (**E**, fluorescence microscopy), and ^{14}C-AIB brain uptake (**F**, quantitative autoradiography).

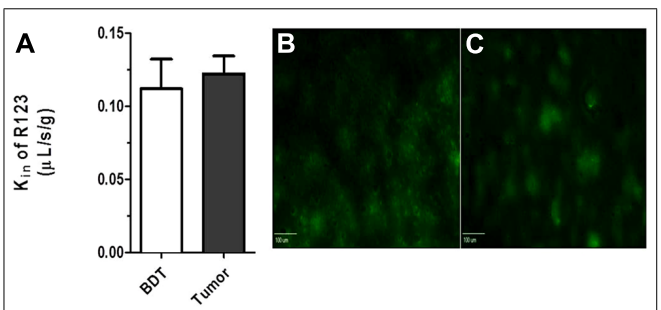

FIGURE 4 | (A) No difference ($p > 0.05$; student t-test, $n = 3$–5) was seen between R123 blood to brain transfer constant K_{in} between values measured in normal brain ($K_{in} = 0.11 \pm 0.01$ μL/s/g) and metastases ($K_{in} = 0.12 \pm 0.02$ μL/s/g). Representative R123 fluorescence images in normal brain **(B)** and within a metastasis **(C)** (scale bar = 100 μm).

transporters (Lockman et al., 2001) and a direct measurement of BBB and BTB integrity (Lockman et al., 2003a, 2004, 2005b)

Using the cardiac perfusion method, R123 accumulated in brain linearly over 2 min of perfusion time. Our observed blood to brain transfer constant (K_{in}) was ~10-fold less than what would be the calculated K_{in} based on values of similar molecules in terms of their octanol/water coefficient and molecular weight. The lower observed K_{in}, is typically seen when the compound is actively extruded by the BBB back into blood (Begley, 1996). Further confirmation that R123 is extruded by an efflux mechanism at the BBB was suggested by the significantly increased uptake of R123 from blood to brain after the addition of verapamil or cyclosporine A to the perfusate (Mittapalli et al., 2013).

Of importance to this study, the simultaneous administration of a passive permeability marker and a tracer subject to P-gp mediated efflux allowed us to measure BTB integrity and functional efflux. Both parameters have been shown to significantly impact drug uptake into metastases (Lockman et al., 2010) but have not been simultaneously measured directly in metastatic lesions. Our initial hypothesis prior to the experiment was that since we have seen increased permeability at the BTB in metastases (Lockman et al., 2010), we would also see a similar increase in R123 distribution into the lesion. However we did not observe R123 accumulation within metastases.

There are two possible explanations that may provide insight to the lack of increased R123 permeability in the lesion. First, it is known that P-gp is expressed in the vasculature of human brain tumors and metastases (Guo et al., 2010). Although, P-gp expression at the BTB has been shown to be variable among different types of tumors within the CNS (Cordon-Cardo et al., 1990; Toth et al., 1996; Tews et al., 2000) as well as different between separate intracranial metastases (Demeule et al., 2001; Lockman et al., 2010). We observed some variability of P-gp expression in the vessels of our metastases, but overall P-gp expression was not significantly different in the over 2,000 vessels we analyzed between the BTB and the BBB. Accordingly, this may be a reason why there was little overall difference in tissue accumulation of R123 between the two tissue types. Another possible explanation is that we observed tumor cells directly adjacent or proximal to

the vasculature also express P-gp, which may also contribute to the restriction of R123 in the lesions. Overall, the pattern of distribution for each tracer suggests that the BTB is disrupted yet its efflux transport mechanisms are intact and can limit brain and or tumor uptake of P-gp substrates.

This work does have translational value to human drug distribution to brain. The expression of BCRP at human BBB is ∼2 fold higher as compared to the expression levels at mouse BBB. The P-gp expression is 3 fold higher at mouse BBB as compared to the expression levels at human BBB. So BCRP still plays a major role at human BBB (Hoshi et al., 2013) suggesting P-gp plays a major functional role in the human BBB. While some studies have supported little efflux contribution for various anti-cancer drug to brain (Agarwal et al., 2011), others have demonstrated P-gp at the BBB and BTB restricts the uptake of many anti-cancer agents; such as paclitaxel, docetaxel, vemurafenib, erlotinib, axitinib, and tamoxifen (Gallo et al., 2003; Kemper et al., 2004; Wang et al., 2010; Iusuf et al., 2011; Poller et al., 2011; Mittapalli et al., 2012; Taskar et al., 2012; Agarwal et al., 2013). Attempts to modify P-gp using inhibitors have shown promise in preclinical settings (Kemper et al., 2004; Mittapalli et al., 2012; Agarwal et al., 2013).

Although we, and others, have observed variably elevated accumulations of small molecules across the BTB in brain metastases, the data herein provide evidence that P-gp retains much of its residual function. Thus, BTB function in this preclinical model may be viewed as only partially compromised and retains significant ability to impede uptake of therapeutic compounds. Given the large list of drugs, particularly anticancer agents such as paclitaxel and doxorubicin, which are subject to P-gp mediated efflux, the clinical impact of this retained function suggests the BTB remains a significant barrier in delivering chemotherapeutics into metastatic lesions.

AUTHORS' CONTRIBUTIONS

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3. Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc) Chris E. Adkins, Rajendar K. Mittapalli, Vamshi K. Manda, Tori B. Terrell, Kaci A. Bohn, Celik Yasemin, Tiffany R. Grothe, Julie A. Lockman, Paul R. Lockman
4. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis) Chris E. Adkins, Rajendar K. Mittapalli, Vamshi K. Manda, Mohamed I. Nounou, Afroz S. Mohammad, Tori B. Terrell, Julie A. Lockman, Paul R. Lockman
5. Writing, review, and/or revision of the manuscript Chris E. Adkins, Mohamed I. Nounou, Afroz S. Mohammad, Tori B. Terrell, Paul R. Lockman
6. Study supervision Paul R. Lockman

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