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Genetic and Non-Genetic Mechanisms of Resistance to BCR Signaling Inhibitors in B Cell Malignancies

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The approval of BTK and PI3K inhibitors (ibrutinib, idelalisib) represents a revolution in the therapy of B cell malignancies such as chronic lymphocytic leukemia (CLL), mantle-cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), or Waldenström's macroglobulinemia (WM). However, these "BCR inhibitors" function by interfering with B cell pathophysiology in a more complex way than anticipated, and resistance develops through multiple mechanisms. In ibrutinib treated patients, the most commonly described resistance-mechanism is a mutation in *BTK* itself, which prevents the covalent binding of ibrutinib, or a mutation in *PLCG2*, which acts to bypass the dependency on BTK at the BCR signalosome. However, additional genetic aberrations leading to resistance are being described (such as mutations in the *CARD11*, *CCND1*, *BIRC3*, *TRAF2*, *TRAF3*, *TNFAIP3*, loss of chromosomal region 6q or 8p, a gain of Toll-like receptor (TLR)/MYD88 signaling or gain of 2p chromosomal region). Furthermore, relative resistance to BTK inhibitors can be caused by non-genetic adaptive mechanisms leading to compensatory pro-survival pathway activation. For instance, PI3K/mTOR/Akt, NFkB and MAPK activation, BCL2, MYC, and XPO1 upregulation or PTEN downregulation lead to B cell survival despite BTK inhibition. Resistance could also arise from activating microenvironmental pathways such as chemokine or integrin signaling *via* CXCR4 or VLA4 upregulation, respectively. Defining these compensatory pro-survival mechanisms can help to develop novel therapeutic combinations of BTK inhibitors with other inhibitors (such as BH3-mimetic venetoclax, XPO1 inhibitor selinexor, mTOR, or MEK inhibitors). The mechanisms of resistance to PI3K inhibitors remain relatively unclear, but some studies point to MAPK signaling upregulation *via* both genetic and non-genetic changes, which could be co-targeted therapeutically. Alternatively, drugs mimicking the BTK/PI3K inhibition effect can be used to prevent adhesion and/or malignant B cell migration (chemokine and integrin inhibitors) or to block the pro-proliferative T cell signals in the microenvironment (such as IL4/STAT signaling inhibitors). Here we review the genetic and

non-genetic mechanisms of resistance and adaptation to the first generation of BTK and PI3K inhibitors (ibrutinib and idelalisib, respectively), and discuss possible combinatorial therapeutic strategies to overcome resistance or to increase clinical efficacy.

Keywords: B cell malignancies, ibrutinib, resistance, adaptation, targeted therapy, B cell receptor, BCR inhibitor

INTRODUCTION

The BCR signaling pathway plays a central role in the onset and progression of mature B cell malignancies, such as chronic lymphocytic leukemia (CLL), mantle-cell lymphoma (MCL), diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), or Waldenström's macroglobulinemia (WM). Activating mutations in the BCR signaling pathway are commonly found in DLBCL, FL, or WM (1). Though these mutations are usually missing in CLL and MCL, BCR signaling is constitutively activated and is a key player in their pathogenesis (2–5). Introducing "B cell receptor (BCR) inhibitors" in recent years has marked a revolution in treating B cell malignancies since many patients are responsive to the inhibitors of BCR-associated kinases BTK or PI3K, such as ibrutinib and idelalisib, respectively. They are now widely used as a first-line treatment or to treat relapsed/refractory diseases. However, the patient response to them varies across B cell malignancies in clinical trials as well as in real-world setting, and a large percentage of patients develop resistance or have to stop the therapy due to toxicities associated with these inhibitors' long-term use (6–14).

In this review, we summarize the genetic and non-genetic mechanisms of resistance and adaptation to the first generation of BTK and PI3K inhibitors (ibrutinib and idelalisib, respectively), and discuss possible therapeutic strategies to overcome resistance or increase clinical efficacy by using combinatorial therapeutic strategies. We also discuss the complexity of the mechanisms of action of "BCR inhibitors" and how this affects the choice of potential combinatorial therapy.

BCR SIGNALING AND ITS CROSS-TALK WITH OTHER PATHWAYS

Cell-surface immunoglobulin does not have any kinase activity itself. It is non-covalently connected to disulphide-linked heterodimers Igα and Igβ (CD79A, CD79B). After recognition and antigen binding, BCRs start to aggregate and change their conformation, which concludes in phosphorylation of tyrosine-based activation motifs (ITAMs) on Igα and Igβ's cytoplasmic domains. This phosphorylation, mediated by Src-family kinase LYN, creates a docking site for spleen tyrosine kinase (SYK) (15). The activated SYK then phosphorylates the B cell linker protein (BLNK), an adaptor protein helpful in recruiting other molecules such as Bruton tyrosine kinase (BTK). BCR stimulation also leads to phosphorylation of co-receptor CD19 and PI3K adaptor BCAP by LYN and SYK, which afterwards activates phosphoinositide 3-kinase (PI3K) leading to PIP3 generation (16, 17) (**Figure 1**).

PIP3 helps to recruit GRB2-associated-binding protein 1 (GAB1), 3-phosphoinositide-dependent protein kinase 1 (PDK1, also known as PDPK1), protein kinase B (PKB, Akt), and BTK to the plasma membrane *via* their pleckstrin homology (PH) domain. Here, Akt is phosphorylated on S473 by mTORC2 which also facilitates Akt phosphorylation on T308 by PDK1 leading to full Akt activation (18). PI3K signaling is further positively regulated by the adaptor protein GAB1, which recruits additional PI3K molecules generating more PIP3 (19, 20). On the other hand, the amount of PIP3 is negatively balanced by the activity of phosphatases such as SHIP1, SHP1, and PTEN.

PIP3 is also needed for optimal BTK activation, since it helps to translocate BTK to the cell membrane and *via* the interaction with its PH domain, it allows the activation of BTK's kinase activity (21). For full BTK activation after the recruitment to the cell membrane, phosphorylation at two sites is needed. Firstly, BTK gets phosphorylated by SYK or LYN at tyrosine Y551, which then leads to autophosphorylation at Y223 (22, 23). Fully activated BTK phosphorylates phospholipase Cγ2 (PLCγ2). PLCγ2 hydrolyses PIP2 into secondary messengers inositol triphosphate (IP3), which controls intracellular Ca^{2+} levels, and diacylglycerol (DAG) which, *via* protein kinase Cβ (PKCβ) activation, induces cRaf-MEK-Erk pathway activation. PKCβ also activates CARD11, which then forms a complex with MALT1 and BCL10 to activate TAK1 (24). Afterwards, TAK1 phosphorylates IKKβ which initiates the NFκB pathway (25). Apart from this, PKCβ plays a role in negative feedback regulation of BCR signaling by removing BTK from the plasma membrane by phosphorylating BTK on S180 (26). Non-redundant negative regulation is also mediated by LYN kinase, since mouse B cells with LYN knockout have a surprisingly stronger BCR signaling suggesting that LYN has a specific role in negatively regulating the pathway (27). BCR signaling propensity is also affected by levels of cell-surface molecules that act as docking sites for positive or negative BCR pathway regulators, which include molecules such as CD19, CD22, and CD32. Recently, we have shown that a notorious therapeutic target in B cell malignancies, CD20, is also a positive BCR signaling regulator (28). When CD20 is silenced, response to BCR stimulation is weaker, as underscored by the lower phosphorylation of BCR-associated kinases and impaired calcium flux (29, 30). Moreover, an additional layer of regulation involves small non-coding RNAs (microRNAs) that influence both the positive and negative regulation of BCR signaling propensity (20, 31–38).

BCR signaling is activated in the lymphatic tissue microenvironment and is closely intertwined with the pathways responsible for the cell homing and adhesion (5). BCR activation affects adhesion *via* integrin VLA4 formed by

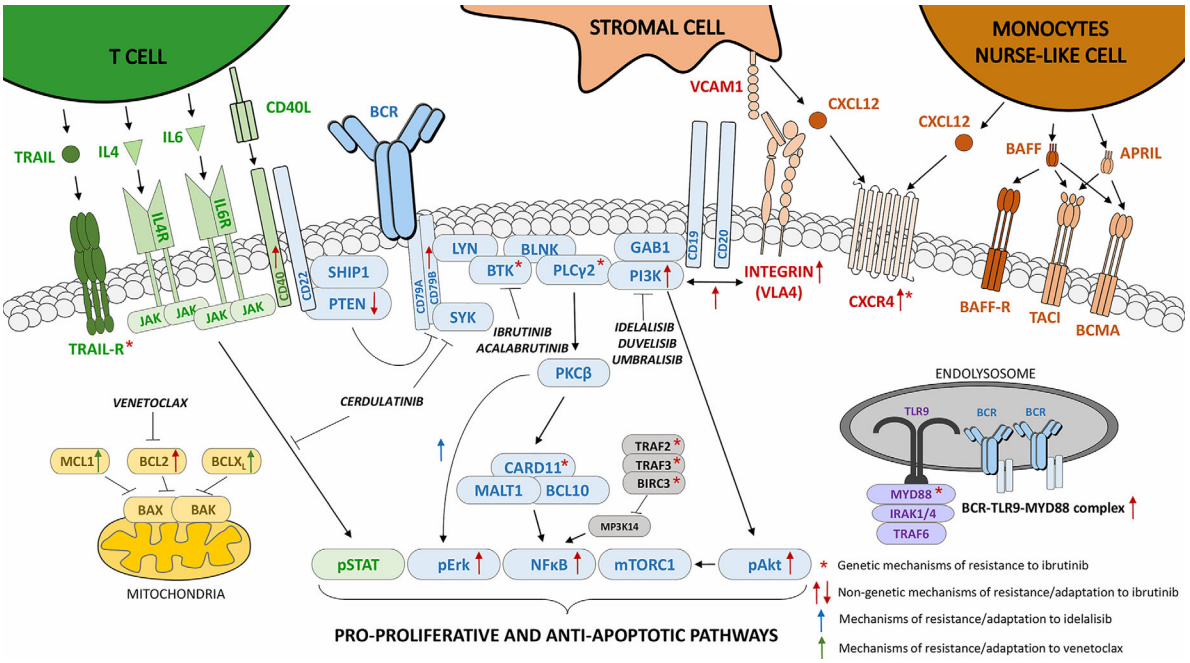

FIGURE 1 | The genetic and non-genetic mechanisms of resistance to BTK or PI3K inhibition in B cell malignancies. The "*" indicates genetic mechanisms of resistance to ibrutinib (BTK inhibitor), the red arrows indicate non-genetic mechanisms of resistance/adaptation to ibrutinib, the blue arrows indicate mechanisms of resistance/adaptation to idelalisib (PI3K inhibitor), and the green arrows indicate mechanisms of resistance/adaptation to venetoclax (BH3-mimetic [BCL2 inhibition]).

CD49d and CD29 (integrin β1); together BCR and VLA4 provide B lymphocytes with adhesion and enhanced signaling (39). CD49d activation causes SYK phosphorylation and, on the other hand, BCR stimulation leads to VLA4 activation (40–42). BCR stimulation also increases chemotaxis towards chemokines such as CXCL12 produced in the microenvironment. Binding of CXCL12 to its receptor CXCR4 activates PI3K, MAPK, and STAT3, and leads to actin polymerization and cell migration (43–45). In CLL, cell-surface IgM levels and BCR signaling is increased by the IL4 produced by T cells which also activates the JAK1-3/STAT6 pathway and upregulates the levels of anti-apoptotic proteins from BCL2 family, resulting in partial malignant B cell protection from the effects of "BCR inhibitors" (46, 47). The importance of the microenvironment can be well illustrated in CLL, where malignant B cells are dependent on constant re-circulation between the peripheral blood and lymph nodes, where they are supported by pro-survival signals from mesenchymal stromal cells, monocyte-derived nurse-like cells, and T lymphocytes (29, 43, 48–50). The supportive stromal cells produce not only chemoattractants CXCL12 and CXCL13 but also BAFF, APRIL, CD31, and plexin B1 which protect CLL cells from spontaneous and induced apoptosis by activating BCR and NFκB signaling (43, 49, 51, 52). Kinases of the BCR pathway BTK and PI3Kδ together with JAK are also involved in T cell dependent proliferation induced by CD40L and IL21, which can be inhibited by ibrutinib, idelalisib or JAK inhibitor (53).

Overall, there is crosstalk between the BCR, chemokine signaling and cell adhesion pathways. Therefore, the success of "BCR inhibitors" lies not only in inhibiting the BCR pathway itself but also in inhibiting other processes. In CLL and some lymphomas, BTK/PI3K inhibition results in malignant B cells egressing from the lymph nodes, causing transient lymphocytosis in patients (8, 54, 55).

MECHANISTIC EFFECTS OF IBRUTINIB ACTION

Ibrutinib is an orally administered small-molecule inhibitor targeting BTK. It binds to BTK covalently, selectively, and irreversibly, inhibiting its phosphorylation and enzymatic activity. BTK is an important kinase in BCR signaling needed for B cells to properly develop (56, 57). Inhibiting it with ibrutinib leads to a loss of pro-survival signals from BCR activation by ligands, and also impairs the "tonic" BCR signals that sustain B cell survival. BTK inhibition decreases cell proliferation as well as interferes with the activation of downstream molecules in BCR pathway such as PLCγ2, Akt and Erk irrespective of BCR stimulation (58–61). As BTK is not only involved in BCR signaling (see above), ibrutinib also disrupts CXCR4 internalization, impairs migration toward CXCL12 and also indirectly decreases total BTK levels (62). Ibrutinib further disrupts signaling from CXCR5 and integrins, molecules that allow B lymphocyte migration and adhesion

(63, 64). Altogether, ibrutinib inhibits BCR stimulation, B cell proliferation, and migration toward homing chemokines such as CXCL12 and CXCL13. It also blocks BCR-dependent CCL3 and CCL4 chemokine release in CLL and decreases CCL4, CCL22, and CXCL13 levels in the serum of ibrutinib-treated MCL patients (54, 61). As mentioned above, inhibiting the adhesion and homing capacity causes transient lymphocytosis in CLL and MCL patients (54, 55). In most of the patients, this resolves within 8 months after starting therapy (55). CLL cells together with non-malignant immune cells after ibrutinib treatment are of a quiescent phenotype as shown by the expression of the genes involved in senescence and/or cell quiescence (30, 55, 65). Apart from the mentioned mechanisms of ibrutinib action, it also affects the microRNAs' expression, resulting in higher levels of several tumor suppressors and inhibition of cell proliferation (66, 67).

Ibrutinib has been approved for therapy of CLL, MCL, WM, and marginal zone lymphoma (MZL). Though it is a potent drug, not all patients are responsive to ibrutinib and a significant number of them acquire resistance to the treatment or discontinue the therapy due to toxicities that are most likely caused by ibrutinib off-target inhibition of molecules such as BLK, JAK3, EGFR, and several TFK members (for a list of off-targets of ibrutinib and other BTK inhibitors see **Supplementary Table 1**). In the following sections, we will summarize the genetic mechanisms of ibrutinib resistance, the non-genetic mechanisms of adaptation/resistance by activating compensatory pro-survival pathways and describe possible solutions to different types of ibrutinib resistance (**Figure 1**).

GENETIC MECHANISMS OF IBRUTINIB RESISTANCE

Genetic mechanisms of primary or acquired resistance to ibrutinib have been widely studied and recurrent mutations associated with resistance have been described in B cell malignancies (**Table 1**, **Figure 1**). Whole-exome sequencing revealed mutations in BCR-involved proteins BTK and PLCγ2 in ~80% of CLL patients with acquired resistance to ibrutinib (7, 96), however, some studies have reported a much lower frequency of these mutations (97, 98). The most common mutation in *BTK* is a C481S point mutation which interferes with the binding of ibrutinib to BTK (7, 68). Other mutations in the *BTK* gene were also found in ibrutinib-resistant patients and have been suggested to affect either ibrutinib binding to BTK or

TABLE 1 | Recurrent mutations in ibrutinib-resistant patients and possible therapeutic strategies to overcome them.

Mutated gene/aberration	Disease	Mechanism	Possible therapeutic strategy	Ref.
BTK	CLL, MCL, WM, MZL	reversible binding of ibrutinib	third-generation BTK inhibitors, PROTAC-BTK, inhibitors of LYN and SYK	(7, 68–77)
PLCG2	CLL, MCL, WM, MZL	BTK-independent activation	inhibitors of RAC2, LYN, and SYK	(7, 68–71, 78, 79)
CARD11	CLL, MCL, WM, DLBCL, FL	↑ NFκB	proteasome or MALT1 inhibitor	(12, 71, 80–83)
BIRC3, TRAF2, TRAF3	MCL	↑ NFκB	MP3K14 inhibitor	(84, 85)
CCND1	MCL	cell cycle progression	unknown	(86)
CDKN2A and *MTAP* co-deletion	MCL	cell cycle progression	PRMT5 inhibitor	(87)
SMARCA2, *SMARCA4*, *ARID2*	MCL	disruption of SWI-SNF complex; ↑ BCL_{XL}	BCL_{XL} inhibitor	(88)
$MYD88^{mt}/CD79B^{wt}$	DLBCL	MYD88-dependent and BCR-independent subtype	SYK or STAT3 inhibitor	(9, 89, 90)
KLHL14	DLBCL	↑ MYD88-TLR9-BCR super-complex	inhibition of BCR-dependent NFκB activation/mTOR inhibitors	(91)
TNFAIP3	DLBCL	↑ NFκB	unknown	(82)
2p+	CLL	↑XPO1	XPO1 inhibition (selinexor)	(92)
Del 8p	CLL	Loss of *TRAIL-R*, insensitivity to TRAIL-induced apoptosis	unknown; possibly venetoclax	(93)
Del 6q	WM	↑ MYD88/NFκB, loss of regulators of apoptosis	unknown	(94, 95)
Del 8p	WM	↑ TLR/MYD88, loss of *DOK2*, *BLK* and *TNFRSF10A/B*	unknown	(94)

↑ represents pathway/gene activation or upregulation.
"Del" stands for deletion of a chromosomal region.

BLNK binding to BTK, altogether leading to PLCγ2 activation even in the presence of ibrutinib (93, 99–101) (**Supplementary Table 2**). *PLCG2*, a gene coding for PLCγ2, seems to mostly harbor gain-of-function mutations when PLCγ2 can be activated by RAC2 or SYK and LYN without BTK (7, 78, 79, 100) (**Supplementary Table 2**). As mutations in *BTK* and *PLCG2* occur early before relapse on ibrutinib, they may serve as a biomarker to indicate a need to change the therapy before disease progression (102). They can also co-occur, which may bring complications to solving the resistance with next-generation BTK inhibitors (103). Apart from the mentioned mutations, resistance to ibrutinib in CLL has also been associated with chromosomal aberrations such as 8p deletion and gain in the 2p region (92, 93). The 8p region contains a gene for TRAIL receptor and its deletion results in insensitivity to TRAIL-induced cell death (93). On the other hand, 2p gain causes exportin-1 overexpression (XPO1), regulating the transport of proteins between the nucleus and cytoplasm (92). Additionally, CLL patients that progress or develop Richter's transformation on ibrutinib recurrently harbor mutations of tumor-suppressor *TP53*, splicing factor *SF3B1*, or NFκB pathway regulator *CARD11*, however, whether these genetic aberrations may directly impact response to ibrutinib during Richter's transformation remains unclear (80). Though, despite good initial response in most of CLL patients, it has been shown that mutations in *TP53* are responsible for a worse prognosis in a long-term ibrutinib treatment and they also partially protect CLL cells *in vitro* from ibrutinib-induced apoptosis and inhibition of proliferation (104–107). This might be related to the recently described role of p53 in the negative regulation of BCR signalling (31, 32).

Mutations in *BTK* and *PLCG2* have also been found in MCL and WM patients with acquired ibrutinib resistance as well as in one MZL patient (69–71). List of the most common *BTK* and *PLCG2* mutations is provided in the **Supplementary Table 2**. Though CLL cells on ibrutinib have a decreased NFκB binding to DNA elements, activating an alternative NFκB pathway by genetic changes is another mechanism responsible for ibrutinib resistance, mostly in MCL (65). This is caused by mutations in *BIRC3*, *TRAF2*, or *TRAF3*, whose absence leads to MP3K14 enzyme stabilization and constitutive activation of alternative NFκB pathway (**Figure 1**) (84, 85, 108, 109). Recurrent mutations in MCL patients who have relapsed on ibrutinib have also been found in *CARD11*, a protein responsible for BCR-induced NFκB activation, or in *CCND1*, a cyclin that promotes G1-S cell cycle progression (24, 81, 86). *CDKN2A* and *MTAP* co-deletion was also observed in ibrutinib-resistant MCL tumors (87). Additionally, loss and/or mutations in the SWI-SNF chromatin remodeling complex lead to the upregulation of anti-apoptotic BCL_{XL} and cause a primary or acquired resistance to the combination of ibrutinib and venetoclax in MCL (88).

In Waldenström's macroglobulinemia (WM), mutations in *CARD11* also lead to ibrutinib resistance, and in WM patients the ibrutinib resistance may be accompanied by 6q and 8p chromosome region deletions that expand from pre-existing clones or emerge during treatment (71, 94). These chromosomal regions contain important signaling pathway regulators. The 6q region loss involves negative regulators of MYD88/NFκB (*TNFAIP3, HIVEP2, TRAF3IP2, IRAK1BP1*), an inhibitor of BTK (*IBTK*), and regulators of apoptosis (*FOXO3, BCLAF1, PERP*). The genes deleted on 8p include *DOK2*, a TLR/MYD88 signaling inhibitor, *BLK*, another target of ibrutinib that is important for B cell proliferation and differentiation, and *TNFRSF10A/B*, a gene encoding for TRAIL receptor (94, 95). Common mutations in WM are WHIM-like mutations in *CXCR4* and L265P mutations in *MYD88*, a mediator of Toll-like receptor signaling (110). In WM, $MYD88^{L265P}$ activates NFκB *via* BTK and IRAK1/4, making these cells sensitive to ibrutinib (110, 111). WHIM-like mutations in *CXCR4* are responsible for impaired CXCR4 internalization upon stimulation and result in constant Akt and Erk activation. However, mutations in *MYD88*, which occur in 90% of WM patients, seem to have a more profound effect on the WM cell survival than $CXCR4^{WHIM}$, as MYD88 inhibition could not be rescued by the $CXCR4^{WHIM}$ mutation (112). These facts might explain why, even though WHIM-like mutations promote ibrutinib resistance *in vitro*, $MYD88^{wt}$/$CXCR4^{wt}$ patients have a lower response rate to ibrutinib therapy than $MYD88^{L265P}$/$CXCR4^{wt}$ or $MYD88^{L265P}$/$CXCR4^{WHIM}$ patients (13, 113).

In DLBCL, ibrutinib seems to be more effective in patients with an activated B cell-like DLBCL (ABC-DLBCL) subtype rather than in patients with germinal center B cell-like DLBCL (GC-DLBCL) due to constitutively active BCR signaling in ABC-DLBCL. However, even amongst ABC-DLBCL, complete or partial response was only detected in 37% of patients (9, 82), and a phase III clinical trial confirmed the benefit of adding ibrutinib to R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) therapy only in younger patients with non-GC-DLBCL (114). As for resistance, it has been shown that DLBCL patients carrying mutations in *MYD88* and simultaneously having wild-type *CD79B* are primarily resistant to ibrutinib. As other combinations ($MYD88^{mt}$/$CD79B^{mt}$, $MYD88^{wt}$/$CD79B^{mt}$, $MYD88^{wt}$/$MYD88^{wt}$) are sensitive to ibrutinib, there is a possibility of an MYD88-dependent but BCR-independent ABC-DLBCL subtype (9). These findings might be explained by the formation of a multiprotein super-complex consisting of MYD88, TLR9 and BCR. It activates the NFκB pathway and is found in ABC-DLBCL tumors that respond to ibrutinib (**Figure 1**) (115). On the other hand, cells with inactivated *KLHL14*, a negative BCR component regulator often mutated in DLBCL, induce the NFκB pathway by activating the MYD88-TLR9-BCR super-complex, which partially protects them from ibrutinib-induced cell death (91). The role of knockout of the *KLHL14* tumor suppressor was demonstrated in rescuing the ABC-DLBCL cell line from apoptosis when IgM and CD79A were knocked down, which is a manipulation normally lethal to the DLBCL cells (91). Lastly, the above mentioned mutations in *CARD11* and inactivating mutations in *TNFAIP3*, a negative NFκB regulator, were also found in DLBCL patients not responding to ibrutinib treatment (82).

The genetic mechanisms of resistance, especially the *BTK* mutations that develop during ibrutinib therapy, are a clear indication of the on-target effects of the utilized small molecular inhibitors. The other mutations in the BCR signaling pathway members such as *PLCG2* or *CARD11* also demonstrate that malignant B cells try to gain or lose gene activity in order to overcome the inhibition of the key BCR-associated kinase (**Table 1**). However, it has been shown that cancer cells can also utilize non-genetic mechanisms to bypass the inhibition of a key pathway (116–120). In the following section we will review these potential mechanisms for ibrutinib/BTK-inhibition based therapy, and suggest the implications for combinatorial therapeutic strategies (**Table 2**).

NON-GENETIC MECHANISMS OF ADAPTATION TO IBRUTINIB

Patients resistant to ibrutinib that harbor the above-mentioned mutations often have these mutations subclonally in a relatively small fraction of surviving malignant B cells. Malignant non-mutated cells that co-exist with BTK^{C418S} mutated cells might be protected by the mutated cells though this has only been conceptually demonstrated in $MYD88^{mt}$ WM and ABC-DLBCL. Mutated cells showed Erk1/2 activation, which led to pro-survival chemokine release and protection of BTK^{wt} cells (147). Even if this could explain the survival of the non-mutated sub-populations of cells, there are still ibrutinib-resistant patients (>20% of ibrutinib-resistant patients in CLL) that do not show any genetic mutations responsible for the resistance (96). This, together with slow cell apoptosis during ibrutinib therapy, suggest that there are non-genetic mechanisms of resistance and cells are able to partially adapt to ibrutinib and BTK inhibition (**Table 2**, **Figure 1**). Collectively, it seems that malignant B cells activate BTK-independent compensatory survival pathways under ibrutinib treatment, mainly PI3K/mTOR/Akt pathway and adhesion. Activating the NFκB pathway also induces some degree of ibrutinib resistance. This was described mainly by genetic mechanisms as mentioned before, but the non-canonical NFκB pathway together with MAPK signaling can also be activated and thus protect the cells from ibrutinib in MCL cells by activating CD40 (148, 149).

Activating the PI3K pathway is a well-known mechanism that rescues BCR deficient mature B cells from apoptosis (150, 151). It is therefore not surprising that more and more studies see a similar mechanism in malignant B cells by which they overcome BTK inhibition. Activated Akt has been observed in ibrutinib-resistant CLL and DLBCL cell lines, together with downregulated FoxO3a and PTEN levels (**Figure 1**) (121). Activating Akt/MAPK *via* CD79B overexpression has been sufficient to induce ibrutinib resistance in primary ABC-DLBCL (152). Ibrutinib also synergizes with CRISPR-Cas9 knockout of PI3Kδ (91). PI3K pathway activation was observed in B cell lymphoma patient-derived xenograft models with acquired resistance to ibrutinib and the growth of these tumors was blocked by combination of ibrutinib and idelalisib (122).

However, activation of PI3K/mTOR/Akt signaling after ibrutinib has been best described in MCL. Activated Akt and Erk levels, but not BTK, correlate with the response to ibrutinib in MCL cell lines and furthermore, ibrutinib-responsive patients have dephosphorylated Akt as opposed to non-responsive patients (69, 153). Ibrutinib in MCL strengthens cell adhesion *via* integrin dimer VLA4 formed by β1-integrin and CD49d and activates the PI3K/Akt pathway in this way (**Figure 1**). Zhao *et al*. described this phenomenon by showing that ibrutinib-resistant cells have a higher β1-integrin expression that helps to form an ILK-Rictor complex that activates a pro-survival mTORC2/Akt pathway. This was disrupted by ibrutinib in combination with dual mTOR1/2 inhibitor AZD8055 or dual PI3K-mTOR1/2 inhibitor BEZ235 (123). Here, it is also worth mentioning that ibrutinib-resistant MCL samples upregulate the mTOR signaling pathway (as well as genes involved in cell cycle regulation and MYC targets) compared to ibrutinib-sensitive cells (87). A similar compensatory survival mechanism was seen by Guan and colleagues who demonstrated that stromal cells protect MCL cells from ibrutinib-induced death *via* their interaction with VLA4. A combination of ibrutinib with VLA4 blockage or with an inhibitor of PI3K catalytic p110α subunit disrupted the interaction and overcame the resistance (124).

In CLL, the cells lose the ability to adhere to fibronectin almost completely and partly to stromal cells when treated with ibrutinib *in vitro* for a short period of time (63, 154). Interestingly though, BCR stimulation activates VLA4 in CLL cells exposed to ibrutinib for an extended time *via* a BTK-independent

TABLE 2 | Non-genetic mechanisms of resistance/adaptation to ibrutinib and possible therapeutic strategies to overcome them.

Mechanism of resistance/adaptation	Disease	Possible therapeutic strategy	Ref.
↑ PI3K-Akt pathway	CLL, MCL, DLBCL	PI3K, mTOR, or XPO1 inhibitor	(92, 115, 121–132)
↑ JAK-STAT	CLL	Dual SYK/JAK-STAT inhibitor (Cerdulatinib)	(133)
↑ MYC	MCL	HSP90 inhibitor	(134)
↑ MAPK pathway	CLL, MCL, DLBCL	MEK inhibitor	(125, 135, 136)
↑ BCL2	CLL, DLBCL	BCL2 inhibitor (venetoclax)	(30, 125, 129, 137–143)
Metabolic reprogramming	CLL, MCL	OXPHOS inhibitor, inhibitor of fatty acid oxidation	(87, 144)
Integrin-mediated protection	CLL, MCL	VLA4 inhibition (FAK inhibitor)	(45, 145)
Resistant cancer stem cells	MCL	Wnt pathway inhibitor	(146)

↑ *represents pathway/gene activation or upregulation.*

manner involving PI3K. Also, higher CD49d levels in patients prevent ibrutinib-induced lymphocytosis and cause a lower nodal response. This translates into a shorter progression-free survival in ibrutinib treated patients (42). Analogically, it has been described that ibrutinib induces CXCR4 expression on cell-surface, which might translate to a paradoxically increased responsiveness of these cells to chemokine ligands, however, it is likely that the response to chemokines in this context is not completely physiological since BTK is involved in the chemokine pathway (30, 155). Time to progression can also be predicted by cell-surface IgM levels prior to ibrutinib treatment, suggesting another non-genetic mechanism of ibrutinib resistance in CLL, potentially similar to the CD79B overexpression in DLBCL (**Figure 1**) (152, 156). In CLL, the mechanism might be explained by BCR signaling bypassing BTK upon BCR crosslinking as ibrutinib is not able to properly inhibit Ca^{2+} mobilization and Erk1/2 phosphorylation when surface IgM levels are high (156). Moreover, cell-surface IgM levels rise during ibrutinib treatment in CLL patients, although this might depend on the time since the start of therapy (30, 157).

Activating the MAPK pathway might also be one of the compensatory mechanisms for BTK inhibition, as noted in a recent study. Upregulation of the genes involved in MAPK was observed by Forestieri et al. in residual CLL cells after ibrutinib treatment and in addition, acquired mutations in *BRAF*, *NRAS*, and *KRAS* were found in a fraction of patients (135). This might explain the observed synergy between MEK inhibitors with "BCR inhibitors" in B cell lymphomas (125, 136).

Besides the described pathways, other ibrutinib resistance mechanisms are also possible and fit the biology of B cells. It has been shown that MYC acts as a key downstream BCR effector, and its over-expression can rescue the absence of BCR activity in some B cells (158, 159). Indeed, upregulation of MYC has been observed in ibrutinib-resistant MCL cell lines and this resistance can be reversed by inhibiting HSP90 (134). Protection from ibrutinib can also be provided by cells in the microenvironment (133, 160, 161). On the other hand, CLL cells resistant to BTK inhibition recover the ability to produce and respond to IL4 and require less T cell help for growth (162). Lymphoma relapse can potentially also arise from cancer stem cells described in MCL and FL. Their quiescent phenotype, together with high ABC-transporter activity gives them general drug-resistant properties (163–166). Indeed, the MCL-initiating cells were found to be resistant to ibrutinib and could be eliminated by inhibiting Wnt signaling pathway whose genes were overexpressed in these cancer stem cells (146).

TARGETING IBRUTINIB RESISTANCE

There are several potential ways to overcome ibrutinib resistance such as i) in cases with specific *BTK* mutations using third generation BTK inhibitors which do not target C481, or PROTAC mediated BTK degradation, ii) using different molecular targets once a patient is resistant to ibrutinib, or iii) preventing the resistance by a more rapid B cell elimination that would lower the chance of developing resistance or activating compensatory survival pathways (**Figure 1**, **Tables 1** and **2**). Additionally, time-limited or more selective treatment would likely lower toxicities in patients as up to 40% of CLL patients discontinue ibrutinib therapy, which is caused mostly by the toxicities (11, 167).

Unfortunately, second-generation BTK inhibitors such as acalabrutinib, zanubrutinib, or tirabrutinib are not able to overcome the resistance caused by BTK^{C481S} since they bind to the same protein region as ibrutinib. Their advantage is their higher selectivity with less off-targets (**Supplementary Table 1**) and lesser toxicities than ibrutinib, making them more feasible for patients intolerant to ibrutinib (168–172). Acalabrutinib has recently been approved for CLL and MCL and zanubrutinib for MCL (171, 173). The solution to BTK^{C481S} mutations could be the use of non-covalent third-generation BTK inhibitors that are able to inhibit the kinase's activity independently of C481S (**Supplementary Table 1**). BTK inhibitors such as fenebrutinib (GDC-0853), LOXO-305, or vecabrutinib are currently in the early phases of clinical testing even for patients with BTK^{C481S} (72–74). ARQ 531 has shown better efficacy than ibrutinib in murine models resembling Richter transformation, targets CLL not only with *BTK* but also *PLCG2* mutations and has off-target activity against kinases in Erk signaling and kinases in the Src family (75). The off-targets of various BTK inhibitors are summarized in the **Supplementary Table 1**. A different but also promising therapeutic strategy is provided by PROTAC which degrades its target with E3 ligase. PROTAC-induced BTK degradation is highly selective and effective in treating BTK^{C481S} ibrutinib-resistant mouse models (76, 77). CLL cells with R665W and L845F mutations in *PLCG2* are sensitive to RAC2 or SYK and LYN inhibition (78, 79). Inhibiting RAC2 or its binding partner VAV1 is synergistic with ibrutinib also in DLBCL just like inhibiting STAT3 or SYK together with ibrutinib in *MYD88* mutated DLBCL (89–91).

Another approach would be combining ibrutinib with compensatory survival pathway inhibitors such as PI3K/mTOR/Akt or NFκB (**Table 2**). As mentioned before, ibrutinib synergizes with PI3K/mTOR/Akt pathway inhibitors in MCL, CLL and DLBCL (121–126). A combination of ibrutinib and umbralisib, a next-generation PI3Kδ inhibitor, was studied in a clinical trial in CLL and MCL patients with promising results (127). It has been shown that ibrutinib increases CLL-cell sensitivity to mTOR inhibitors as well as proteasome and PLK1 inhibitors (128). Targeting mTOR combined with ibrutinib was also suggested in ABC-DLBCL by Phelan et al. as mTORC1/2 inhibitor AZD2014 further attenuates formation of MYD88/TLR9/BCR super-complex when compared to ibrutinib alone (115). CC-115, a dual mTOR/DNA-dependent protein kinase inhibitor, is now in a clinical trial and is able to revert CD40-mediated resistance to venetoclax and also inhibits BCR signaling in CLL patients with acquired idelalisib resistance (174). Inhibiting the PI3K/mTOR/Akt pathway has been shown to be successful also in *in vitro* drug screening in DLBCL, where PI3K inhibitors synergized with ibrutinib (129). The same study, confirmed by others, proved a synergy between

ibrutinib and inhibition of IRAK4, a mediator for TLR and NFκB activation whose targeting is studied not only in DLBCL but in CLL as well (129, 175, 176). Furthermore, the synergy with ibrutinib was also seen in combination with selinexor, an XPO1 inhibitor, although this seems to be regulated again *via* the PI3K/mTOR/Akt pathway as selinexor restores a nuclear abundance of FoxO3a and PTEN after ibrutinib treatment, resulting in the inhibition of PI3K/mTOR/Akt signaling activation (**Figure 1**) (121, 129). Absence of tumor suppressors FoxO3a and PTEN in the nucleus could be an explanation for ibrutinib resistance in 2p+ CLL patients who overexpress *XPO1* and why selinexor and next-generation XPO1 inhibitors seem to be efficient in preclinical CLL and MCL models where it reduces NFκB binding to DNA (92, 130–132). Another molecule that could be targeted together with BTK is the MP3K14 enzyme, a member of an alternative NFκB pathway constitutively activated in ibrutinib-resistant MCL patients due to mutations in *BIRC3*, *TRAF2*, or *TRAF3* (**Figure 1**) (84, 85).

An attractive strategy to increase the treatment efficacy is combining drugs that are already approved in therapy. Recently, ibrutinib has been shown to be efficient with venetoclax, a BH3 mimetic that inhibits anti-apoptotic molecule BCL2 and is approved for CLL treatment (125, 137, 138, 177). This combination is rational as ibrutinib induces BCL2 expression and on the other hand, decreases anti-apoptotic MCL1 levels, which can be a cause of venetoclax resistance (**Figure 1**) (30, 139–141). Higher BCL2 levels have also been found in DLBCL patients with poorer response to ibrutinib therapy (142). Furthermore, ibrutinib inhibits malignant cell proliferation while venetoclax targets preferentially resting subpopulations, potentially explaining the synergy of these two drugs in B cell malignancies (129, 143). Combining ibrutinib with fludarabine, a purine analog commonly used together with cyclophosphamide and anti-CD20 monoclonal antibody rituximab to treat CLL patients, has been shown to be synergic *ex vivo* (125). Ibrutinib with immune modulator lenalidomide and rituximab is under investigation in DLBCL and MCL but has not been successful in CLL (178–181). A profoundly studied possibility for therapy is combining "BCR inhibitors" with widely-used anti-CD20 monoclonal antibodies (28). However, adding rituximab to ibrutinib did not bring any clinical benefit and this is likely due to ibrutinib downregulating CD20 levels and/or interfering with effector cell functions (28, 30, 182, 183). Furthermore, ibrutinib has been shown to negatively regulate anti-CD20 induced apoptosis in MCL cell lines (184). A combination of ibrutinib with a more efficient anti-CD20 antibody, obinutuzumab, is now approved for CLL therapy. However, the real benefit of obinutuzumab still remains unclear as the control arm of the clinical trial was chlorambucil with obinutuzumab (185). In the ELEVATE clinical trial, acalabrutinib or acalabrutinib plus obinutuzumab were both superior to chlorambucil plus obinutuzumab (170). Even though it is not yet clear whether this combination reduces the occurrence of acquired BTK inhibition resistance, it is true that re-distributing malignant cells to the peripheral blood makes malignant cells more susceptible to monoclonal antibodies (145, 186). An interesting option is ibrutinib combined with anti-ROR1 monoclonal antibody in CLL, which is expected to have a great specificity for malignant B cells, and ROR1 levels are not reduced during ibrutinib therapy (187). Promising results have also been obtained from a fixed-duration treatment with venetoclax and obinutuzumab in CLL (188). Therapy nowadays aims for a time-limited treatment setting as it would lower the selection pressure, leave shorter time for cells to compensate for inhibition of targeted pathway, and reduce toxicities in patients.

It has been reported that patients who relapse or are intolerant to one kinase-inhibitor benefit from a change to different small-molecule inhibitors rather than chemotherapy (189). Novel therapeutic targets and drugs are therefore being investigated. Promising results have been obtained by targeting different players in BCR signaling such as PKCβ or MALT1 whose inhibition is effective in ABC-DLBCL, MCL, and naïve as well as ibrutinib-resistant CLL (190–193). Cerdulatinib, a dual SYK/JAK-STAT inhibitor, targets BCR signaling and is also able to overcome microenvironmental protection and blocks proliferation in ibrutinib-resistant primary CLL samples and BTK^{C481S} lymphoma cell lines (**Figure 1**) (133). Directly targeting microenvironmental interactions, migration and adhesion could also have potential use in therapy of ibrutinib-resistant patients *via* the use of natalizumab or CXCR4 inhibitor plerixafor (45, 145). Lastly, malignant cells of ibrutinib-resistant CLL and MCL patients show metabolic reprogramming, which has also been suggested as a possible therapeutic target (87, 144).

EFFECTS OF IDELALISIB AND MECHANISMS OF RESISTANCE

Another molecule in BCR signaling widely therapeutically targeted in B cell malignancies is PI3K. Activated PI3K/Akt axis in B cell malignancies and its signaling pathway's role in cell survival makes it an attractive therapeutic target (3, 151, 194, 195). PI3K exists in four catalytic isoforms: p110α, p110β, p110γ, and p110δ. p110α (PI3Kα) and p110δ (PI3Kδ) are both needed for "tonic" (antigen-independent) BCR signaling, while only p110δ is needed for antigen-induced BCR signaling (196). The PI3Kδ isoform is targeted by widely-used idelalisib, which has been approved for the treatment of CLL, FL, and non-Hodgkin lymphomas. Idelalisib not only thwarts the PI3K/Akt/mTOR pathway but also inhibits cell migration towards chemokines and adhesion to stromal cells, which, just like with ibrutinib, leads to an initial increase in the number of lymphocytes in the peripheral blood caused by lymphocyte migration out of the tissues in CLL. This is accompanied by a reduction in Akt phosphorylation and other downstream effectors as well as by apoptosis induction (8, 197–199). Unfortunately, due to serious adverse effects and infections, it has been suggested to primarily give idelalisib to CLL patients with progression on ibrutinib or indolent NHL patients with progression on two prior therapies (14, 200, 201). As already mentioned, promising results have recently been seen in a clinical trial of a combination of ibrutinib and a next-generation dual PI3Kδ/CK1ϵ inhibitor, umbralisib (127). Furthermore, the importance of the p110γ subunit is now

emerging in CLL. Its activation does not respond to BCR stimulation but increases in response to CD40L/IL4 and cells with overexpressed PI3Kγ show an enhanced cell migration towards CXCL12. A dual PI3Kδ/γ inhibitor, duvelisib, seems to have a bigger impact on cell migration than idelalisib alone and is now approved to treat CLL/SLL and FL (197). Another PI3K inhibitor, copanlisib, is approved for relapsed/refractory FL. It is a pan-class I PI3K inhibitor that inhibits all four PI3K isoforms with higher selectivity against PI3Kα and PI3Kδ (202). Its advantage lies not only in its molecular mechanism as inhibition of other PI3K isoforms can lower viability and migration of B cells, but it also seems to cause fewer adverse events in patients when compared to idelalisib (197, 203–205).

Despite idelalisib's and other PI3K inhibitors' ability to initially control the disease in some patients, a fraction of patients develop resistance. Unfortunately, unlike in ibrutinib treated patients, the mechanisms of resistance remain mostly unclear (8, 206). No recurrent mutations were found in patients progressing on idelalisib nor have they been found in a mouse model resistant to PI3Kδ inhibition (207, 208). Two studies have shown the role of MAPK signaling in resisting PI3Kδ inhibition. Firstly, Murali et al. confirmed activating mutations in MAPK pathway leading to Erk phosphorylation in patients resistant to PI3K inhibition and suggested that blocking Erk might sensitize patients to PI3K inhibitors (**Figure 1**) (209). Secondly, a non-genetic mechanism of resistance was found in PI3Kδ resistant mice, where the upregulation of insulin-like growth factor 1 receptor (IGF1R) led to MAPK signaling activation. IGF1R upregulation was caused by FoxO1 and GSK3β and the resistance was resolved by inhibiting the receptor with linsitinib (208). Initial experiments with copanlisib point to IL6 signaling as a main player in copanlisib resistance; levels of IL6 and phosphorylation of STAT5, Akt, p70S6K, and MAPK were increased in copanlisib-resistant B cell lymphoma cell lines and the resistance was reversible by JAK inhibitor (210).

Extensive research is needed in order to reveal the resistance mechanisms for PI3K inhibitors and it seems that they might be more complex than in BTK inhibition. Despite their adverse effects in patients and the emergence of BCL2 inhibitors and next-generation BTK inhibitors, the PI3K/mTOR/Akt signaling pathway plays an important role in B cell malignancy pathogenesis and its inhibition might find its therapeutic place.

DISCUSSION

Targeting BCR signaling is now a commonly used therapy strategy for B cell malignancies. Unfortunately, some patients are primarily resistant to "BCR inhibitors" or develop resistance during the course of treatment. Furthermore, the now required continuous treatment often leads to toxicities and forces a change of therapy. *BTK* and *PLCG2* mutations are the most common and best-described mechanisms of resistance to BTK inhibitor ibrutinib, although recurrent mutations in other genes or aberrations in larger chromosomal regions have been described as being responsible for the resistance across the B cell malignancies. Interestingly, malignant B cells are able to overcome BTK inhibition by non-genetic mechanisms as well. These include activation of compensatory survival pathway, such as PI3K/mTOR/Akt, NFkB, or MAPK signaling pathways. Compensatory survival is also provided by the upregulation of anti-apoptotic BCL2, MYC or adhesion involved integrins. Even though a lot is known about BTK inhibition, PI3K inhibitor resistance remains largely unclear. Several studies point to MAPK pathway activation as a compensatory mechanism to PI3K inhibition, but further research is needed in this area.

Resistance caused by *BTK* mutations can be solved by third-generation BTK inhibitors now in clinical trials or by BTK degradation by PROTAC-based compounds; mutations in *PLCG2* by combining BTK inhibition with RAC2 or SYK and LYN inhibition. Compensatory survival by upregulating other signaling pathways could be solved by combining several inhibitors as well. The synergy between blockage of BTK and the PI3K/mTOR/Akt pathway has been shown repeatedly in CLL, DLBCL and MCL. It remains largely unknown how the non-coding part of the genome influencing BCR signaling is affected by "BCR inhibitors" and if (de)regulation of these molecules could contribute to therapy resistance or be directly used as a therapeutic target (20, 31, 34, 35). Novel therapeutic targets and strategies are still being investigated and their inhibition is tested alone or in combination with "BCR inhibitors". These combinations should be supported by analyzing the responses of malignant cells to individual drugs and using multi-omics to identify possible compensatory signaling pathways to be co-targeted by small-molecule inhibitors. Furthermore, co-targeting two kinases in seemingly the same pathway can also have a synergistic effect, as observed by BTK and PI3K inhibition's synergy. Analyzing individual patient-to-patient response to "BCR inhibitors" could help to identify specific compensatory pathways and this could help to define a personalized combinatorial therapy. However, it is likely that there might be some universal responses to "BCR inhibitors" in each B cell malignancy and this could follow pre-existing mechanisms that allow B cells to survive for an extended time without antigen encounter. Single cell analysis of the response to BTK/PI3K inhibitors in multiple patients could help to understand if the response or adaptation to therapy follows a generally uniform course or if there are major intra- and inter-patient specific mechanisms. Besides transcriptomics and proteomics, research should focus on describing the cells' immunophenotypic profiles after "BCR inhibition" to identify surface molecules that could be targeted by monoclonal antibodies, since this can be of high specificity for cancer cells and low toxicity in general. The time dynamics of changes during therapy should also be studied in order to describe the mechanisms of early and long-term adaptation and potentially identify an optimal time point for adding a second drug in combination. Improving clinical efficacy of drug combinations containing "BCR inhibitors" should allow a time-limited treatment with a deep molecular response, decreased chance of resistance and limited toxicities associated with long-term therapy.

AUTHOR CONTRIBUTIONS

LO and MM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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