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B cell signalling pathways—New targets for precision medicine in chronic lymphocytic leukaemia

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Abstract

The B cell receptor (BCR) is a master regulator of B cells, controlling cellular processes such as proliferation, migration and survival. Cell signalling downstream of the BCR is aberrantly activated in the B cell malignancy chronic lymphocytic leukaemia (CLL), supporting the pathophysiology of the disease. This insight has led to development and approval of small molecule inhibitors that target components of the BCR pathway. These advances have greatly improved the management of CLL, but the disease remains incurable. This may partly be explained by the inter-patient heterogeneity of the disease, also when it comes to treatment responses. Precision medicine is therefore required to optimize treatment and move towards a cure. Here, we discuss how the introduction of BCR signalling inhibitors has facilitated the development of functional in vitro assays to guide clinical treatment decisions on use of the same therapeutic agents in individual patients. The cellular responses to these agents can be analysed in high-throughput assays such as dynamic BH3 profiling, phospho flow experiments and drug sensitivity screens to identify predictive biomarkers. This progress exemplifies the positive synergy between basal and translational research needed to optimize patient care.

1 | B CELL RECEPTOR SIGNALLING IN NORMAL B CELLS

B cells play a key role in the adaptive immune response, which is the second line of defence against non-self pathogens. B cells form and mature in the bone marrow, and then move to the lymphatic system where they circulate the body. Here, the B cells recognize foreign antigen through their B cell receptor (BCR), leading to maturation of the B cell into either a memory B cell or an effector (plasma) B cell. The BCR is composed of two identical covalently linked immunoglobulin heavy (IgH) chains and a pair of identical immunoglobulin light (IgL) chains connected by disulphide bonds. The variable domains of the IgH and IgL chains result from gene rearrangements at the pro-B (IgH) and pre-B (IgL) cell stages and define the antigen specificity of the BCR.[1] Memory B cells express the same membrane-bound antibody as the parent B cell, while effector B cells secrete it as soluble antibodies. The BCR is anchored to the cell membrane through its transmembrane domain, which is tightly associated with a heterodimer of Igα (CD79A) and Igβ (CD79B) (Figure 1).[2] These membrane-bound proteins each have a cytoplasmic tail that harbours two conserved tyrosine residues as part of their immunoreceptor tyrosine-based activation motif (ITAM), an important signalling component of the BCR.[3,4] Phosphorylation of these ITAM tyrosine residues by the SRC family kinases Lck/Yes-related novel protein tyrosine kinase (LYN) and FYN as well as B-lymphoid kinase (BLK, Tec

family) or spleen tyrosine kinase (SYK) is the initial step of signal transduction from the BCR to the nucleus.[5,6]

BCR signalling can be initiated by two different mechanisms: antigen-induced and antigen-independent (tonic) BCR activation.[7] BCR stimulation by antigen results in BCR aggregation, ITAM phosphorylation, and consequently recruitment of SRC-homology 2 (SH2)-domain containing proteins, most often SYK, to the BCR.[8,9] SYK is then activated by SRC kinases and autophosphorylation,[10] and, together with LYN, phosphorylates the adaptor proteins CD19, B cell adaptor for phosphatidylinositol 3-kinase (BCAP), and B cell linker protein (BLNK). CD19 and BCAP recruit phosphatidylinositol-3 kinase (PI3K) to the plasma membrane. BLNK, together with PI3K, activate Bruton's tyrosine kinase (BTK) and its downstream target phospholipase Cγ2 (PLCγ2).[9] This BCR signalosome generates a wide variety of downstream effects, including activation of the PI3K-AKT-mTOR pathway and the RAS-RAF-MEK-ERK pathway.[11] BCR signalling is essential for normal immune function and for survival and proliferation of the B cells.[12] B cells that lack a functional BCR rapidly undergo cell death.[13]

The second mechanism by which BCR signalling can be induced is by tonic BCR activation, a process which is independent of ligand engagement.[14] Several mechanisms have been proposed to account for the initiation and regulation of tonic signalling. These include the self-aggregation of BCR molecules, an altered balance between constitutively active protein tyrosine kinases and protein tyrosine phosphatases, or hijacking of the BCR by the B cell activating factor of the tumour necrosis factor (TNF) family (BAFF) receptor.[15-17]

The critical role of BCR signalling in normal B cell development renders it as no surprise that BCR signalling also supports survival and growth of malignant B cells.[18,19] In this review, the focus will be on BCR signalling in chronic lymphocytic leukaemia (CLL), a disease which, due to its relatively indolent nature, has allowed detailed investigation of the tumour cells and their signalling responses over time.[20]

2 | CHRONIC LYMPHOCYTIC LEUKAEMIA

CLL is the most common form of leukaemia in western countries, with an incidence rate of 5.82/100 000 inhabitants in the United States.[21] It is a disease of the elderly, with a median age at diagnosis of 72 years, and more male than female patients are affected (1.7:1).[22] CLL is characterized by clonal expansion and accumulation of mature $CD5^+$ B cells in the peripheral blood, bone marrow and lymphoid tissues.[23] Survival of the CLL cells relies on signals from the tumour microenvironment,[24-26] which is composed of cellular components such as monocyte-derived nurse-like cells (NLC),[27] T cells[28] and mesenchymal stromal cells.[29]

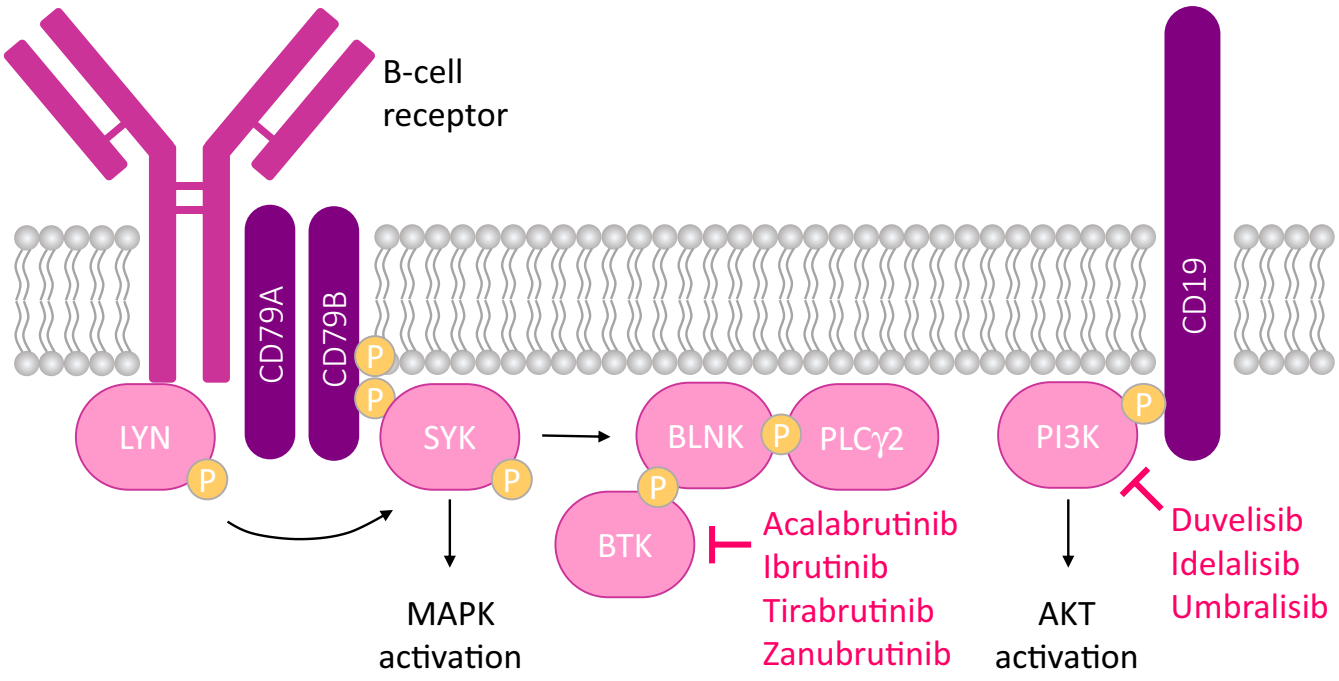

FIGURE 1 BCR signalling and targeted therapies against BTK and PI3K. The BCR is associated with CD79A and CD79B co-receptors via its transmembrane domain. The CD79A/B ITAMs are phosphorylated by proximal kinases including LYN and SYK, and SYK is activated by SRC kinases and autophosphorylation. Activated SYK triggers the formation of the BCR signalosome, which includes proteins such as BLNK, BTK, PLCγ2 and PI3K. The co-receptor CD19 regulates activation of PI3K. The MAPK and PI3K pathways are activated downstream of the BCR signalosome. Targeted therapies can interfere with the BCR signalling axis. Inhibitors of BTK and PI3K are indicated in red

TABLE 1 Approved and investigational BTK and PI3K inhibitors[a]

PI3K inhibitor	Target specificity	Approved by	Indications
Duvelisib	p110δ, p110γ	FDA	CLL, FL, SLL
Idelalisib	p110δ	EMA, FDA	CLL, FL, SLL
Umbralisib	p110δ	Not approved	—
BTK inhibitor	**Binding modality to target**	**Approved by**	**Indications**
Acalabrutinib	Irreversible	FDA	CLL, MCL, SLL
Ibrutinib	Irreversible	EMA, FDA	CLL, MCL, MZL, SLL, WM
Tirabrutinib	Irreversible	Not approved	—
Zanubrutinib	Irreversible	FDA	MCL

Abbreviations: CLL, chronic lymphocytic leukaemia; EMA, European Medicines Agency; FDA, Food and Drug Administration; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; SLL, small lymphocytic leukaemia; WM, Waldenström's macroglobulinaemia.

[a]As of June, 2020.

Several lines of evidence support the significant role of BCR signalling in the pathophysiology of CLL. Zeta chain–associated protein kinase 70 kDa (ZAP-70, SYK family) is associated with BCR signalling and is a negative prognostic factor in CLL.[30-32] Similarly, secretion of the T cell attracting chemokines CCL3 and CCL4 correlates with ZAP-70 expression and responsiveness of the CLL clone to BCR stimulation, and strongly predicts CLL prognosis and time to treatment.[33,34] More importantly, the structure of the BCR itself is among the parameters included in the international prognostic index for patients with CLL (CLL-IPI).[35] The disease is classified as either mutated or unmutated based on the degree of somatic hypermutation within the BCR antigen-binding site (immunoglobulin heavy chain variable region gene; IGVH) present in the CLL cells. A cut-off at 98% IGHV sequence homology to the germline sequence is used for the classification.[36] Although CLL is a heterogeneous disease with high variation in disease course among patients, mutated CLL is typically associated with a more indolent disease progression and better overall survival, whereas unmutated CLL shows a more aggressive course with shorter time to treatment and shorter survival.[37,38] CLL cells with an unmutated phenotype are in general more responsive to BCR stimulation than mutated CLL cells, in particular in inducing PI3K signalling.[39,40]

Evidence exists for both tonic- and antigen-induced BCR activation in CLL. Tonic BCR signalling is supported by studies on primary CLL cells showing constitutive activation of BCR pathway components including LYN,[41] SYK,[42] ERK1/2[43,44] and STAT3.[39] On the other hand, CLL cells express restricted sets of antigen receptors[36] giving rise to subsets of cases with almost identical (stereotyped) complementarity-determining region 3 (CDR3) sequences.[45,46] This suggests that a particular antigen-binding site may be critical during CLL pathogenesis. CLL cells thus appear to depend on both constitutive and induced BCR signalling that direct cell growth and survival.

3 | BCR INHIBITORS IN CLL

Not all CLL patients require treatment. Little beneficial effect of therapy has been reported for patients with indolent disease,[47,48] and therefore, a watch-and-wait approach is recommended. However, treatment may be indicated for patients with active or symptomatic disease, or with advanced Binet or Rai stages. Traditionally, chemotherapy, later in combination with immunotherapy, has been the standard of care.[49] For physically fit patients younger than 65 years, who typically present with a mutated IGVH gene, chemoimmunotherapy with fludarabine, cyclophosphamide and the anti-CD20 antibody rituximab (FCR) remains standard of care as it may have curative potential.[22] The less toxic combination of bendamustine and rituximab (BR) is administered to patients older than 65 years.

The observation that BCR signalling is aberrantly activated in CLL led to development of small molecule inhibitors targeting components of the BCR pathway (Figure 1). Approval of the first targeted therapies for treatment of CLL in 2014, by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), completely changed the disease management. The CLL community now seems to be moving towards a chemotherapy-free treatment regimen. Approved and investigational BCR inhibitors relevant for CLL are discussed below (Table 1). Interestingly, the game-changing BCR inhibitors were identified based on a functional approach examining cell signalling, and not by genetics, which traditionally has dominated precision medicine.

3.1 | PI3K inhibitors

PI3Ks constitute a family of enzymes that regulate a diverse set of cellular processes, including proliferation, differentiation, survival and intracellular trafficking. The PI3K signalling pathway is one of the most frequently mutated in

TABLE 2 Selected ongoing and completed clinical trials with targeted therapies in CLL

Agent	Class inhibitor	Study	Study phase	R/R-TN	IGHV status (n of mutated/n of unmutated)	TP53/del17p (number of patients)
Venetoclax + OB vs OB + Chl	BCL-2	NCT02242942	3	TN	159/244	32 (TP53) 31 (del17)
Venetoclax	BCL-2	NCT01889186	2	R/R	30/7	60 (TP53) 106 (del17p)
Venetoclax	BCL-2	NCT02141282	2	R/R	67/50	29 (TP53) 42 (del17)
Duvelisib (R/R vs TN)	PI3K	NCT01476657	1	R/R-TN		
Duvelisib vs Ofatumumab	PI3K	NCT02004522	3	R/R	177/142	49
Duvelisib + FCR	PI3K	NCT02158091	1/2	TN	nr	nr
Duvelisib + Venetoclax	PI3K/BCL-2	NCT03534323	1/2	R/R		
Idelalisib	PI3K	NCT00710528	1	R/R	5/54	13
Idelalisib + BR vs Placebo + BR	PI3K	NCT01569295	3	R/R	70/346	137
Idelalisib + Ofatumumab vs Ofatumumab	PI3K	NCT01659021	3	R/R	56/205	103
Idelalisib + R vs Placebo + R	PI3K	NCT01539512	3	R/R	52/168	87
Idelalisib + Tafasitamab vs Venetoclax + Tafasitamab	PI3K/BCL-2	NCT02639910	2	R/R	nr	nr
Umbralisib + Ibrutinib	PI3K/BTK	NCT02268851	1	R/R	8/13	4 (TP53) 4 (del17p)
Acalabrutinib vs Acalabrutinib + OB vs OB + Chl	BTK	NCT02475681	3	TN	nr	nr
Acalabrutinib vs Ibrutinib	BTK	NCT02477696	3	R/R		
Acalabrutinib + Venetoclax(+/−)OB vs FCR vs BR	BTK/BCL-2	NCT03836261	3	TN		0
Acalabrutinib + Venetoclax + OB	BTK/BCL-2	NCT03580928	2	TN	14/23	10
Ibrutinib	BTK	NCT01744691	2	R/R	97/19	145
Ibrutinib (R/R vs TN)	BTK	NCT01105247	1/2	R/R-TN	38-94	36
Ibrutinib vs Chl	BTK	NCT01722487	3	TN	151/118	nr
Ibrutinib vs Ibrutinib + R vs BR	BTK	NCT01886872	3	TN	nr	nr
Ibrutinib vs Ofatumumab	BTK	NCT01578707	3	R/R	208/182	127 (del17)
Ibrutinib vs R	BTK	NCT01973387	3	R/R	49/98	36
Ibrutinib + BR vs Placebo + BR	BTK	NCT01611090	3	R/R	101/418	0
Ibrutinib + BR	BTK	NCT01292135	1	R/R	nr	7
Ibrutinib + OB vs Chl + OB	BTK	NCT02264574	3	TN	106/123	41
Tirabrutinib	BTK	NCT02457559	1	R/R	nr	nr
Tirabrutinib + Idelalisib(+/−)OB	BTK/PI3K	NCT02968563	2	R/R	nr	nr
Tirabrutinib vs Tirabrutinib + Idelalisib vs Tirabrutinib + Entospletinib	BTK/PI3K/SYK	NCT02457598	1b	R/R	nr	12
Tirabrutinib + Entospletinib vs Tirabrutinib + Entospletinib + OB	BTK/SYK	NCT02983617	2	R/R	nr	nr
Zanubrutinib	BTK	NCT02343120	1/2	R/R-TN	14 UM-CLL	18
Zanubrutinib vs Ibrutinib	BTK	NCT03734016	3	R/R		

Abbreviations: B, bendamustine; BCL-2, B cell lymphoma 2; BTK, Bruton's tyrosine kinase; Chl, chlorambucil; CR, complete response; FCR, fludarabine-cyclophosphamide-rituximab; nr, not reported; OB, obinutuzumab; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PI3K, phosphatidylinositol 3-kinase; R, rituximab; R/R, relapsed/refractory; SYK, spleen tyrosine kinase; TN, treatment naïve.

Median follow up (months)	CR (%)	ORR (%)	PFS (median in months)	PFS (% at 2 y)	OS (median in months)	OS (% at 2 y)	Reference
28.1	49.5/23.1	84.7/71.3	Not reached	88.2/64.1	Not reached	91.8/93.3	(Fischer K et al, 2019[125])
12.1	17	79.4	Not reached	72 (est. 1 y)	Not reached	86.7 (est. 1 y)	(Stilgenbauer S et al, 2016[126])
14	9	65	24.7	75 (est. 1 y)	nr	91 (est. 1)	(Jones JA et al, 2018[127])
	4/0	56/83	15.7/not reached				(Flinn IW et al, 2018a)[58]
22.4	0.6/0.6	73.8/45.3	13.3/9.9	60/39 (1 y)	nr	nr	(Flinn IW et al, 2018b[128])
nr	nr	nr	nr	nr	nr	nr	
nr	nr	72	15.8	nr	Not reached	nr	(Brown JR et al, 2014[53]
14	3/0	70.0/45.5	20.8/11.1	nr	Not reached/31.6	79/71 (1 y)	(Zelenetz AD et al, 2017[129])
16.1	1.1/0	75.3/18.4	16.4/8.0	nr	Not reached	nr	(Jones JA et al, 2017[130])
3.8	0/0	81/13	Not reached/5.5	93/46	Not reached	92/80 (1 y)	(Furman RR et al, 2014)[54]
nr	n.r	90.9/76.9	nr	nr	nr	nr	
26	27	90	Not reached	90	Not reached	95	(Davids MS et al, 2019)[61]
29	nr	85/94/79	Not reached/not reached/22.6	82/90/34 (30 mo)	Not reached	94/95/90 (30 mo)	(Abstract; Sharman JP et al, 2019)[75]
8	25	100	nr	nr	nr	nr	(Abstract; Lampson BL et al, 2019[131])
11.5	8	83	not reached	63	not reached	75	(O'Brien S et al, 2016)[132]
60	10/29	89/87	51/(not reached)	44/92 (5 y)	not reached	60/92 (5 y)	(O'Brien S et al, 2018)[133]
18.4	4/2	82.4/35.3	(not reached)/18.9	90/52 (18 mo)	nr	98/85	(Burger JA et al, 2015)[72]
30	7/12/26	93/94/75	(not reached)/(not reached)/43	87/88/74	nr	90/94/95	
44	nr	42.6/4.1	(not reached)/8.1	59/3 (3 y)	not reached	74/65 (3 y)	(Byrd JC et al, 2019)[134]
17.8	3.8/0	53.8/7.4	not reached/8.3	74/11.9 (18 mo)	not reached/26.1	nr	(Huang et al, 2018)[135]
17	10/3	83/68	not reached/13.3	79/24 (18 mo)	not reached	nr	(Chanan-Khan A et al, 2016)[136]
37.3	40.0	93.3	not reached	78.6	nr	nr	(Brown JR et al, 2015[137])
31.3	19/8	88/73	(not reached)/19	79/31 (30 mo)	not reached	86/85 (30 mo)	(Moreno C et al, 2019)[138]
32.5	nr	96	38.5	nr	44.9	n.r	(Walter HS et al, 2017)[78]
nr	nr	nr	nr	nr	nr	nr	
15/34/30	7/7/10	83/93/100	not reached/32/ not reached	nr	nr	nr	(Danilov AV et al, 2020)[139]
nr	0/6.7	100/90	nr	nr	nr	nr	
13.7	2.6	96.2	not reached	100 (est. 1 y)	nr	nr	(Tam CS et al, 2019)[76]
							(Hillmen et al, 2020)[77]

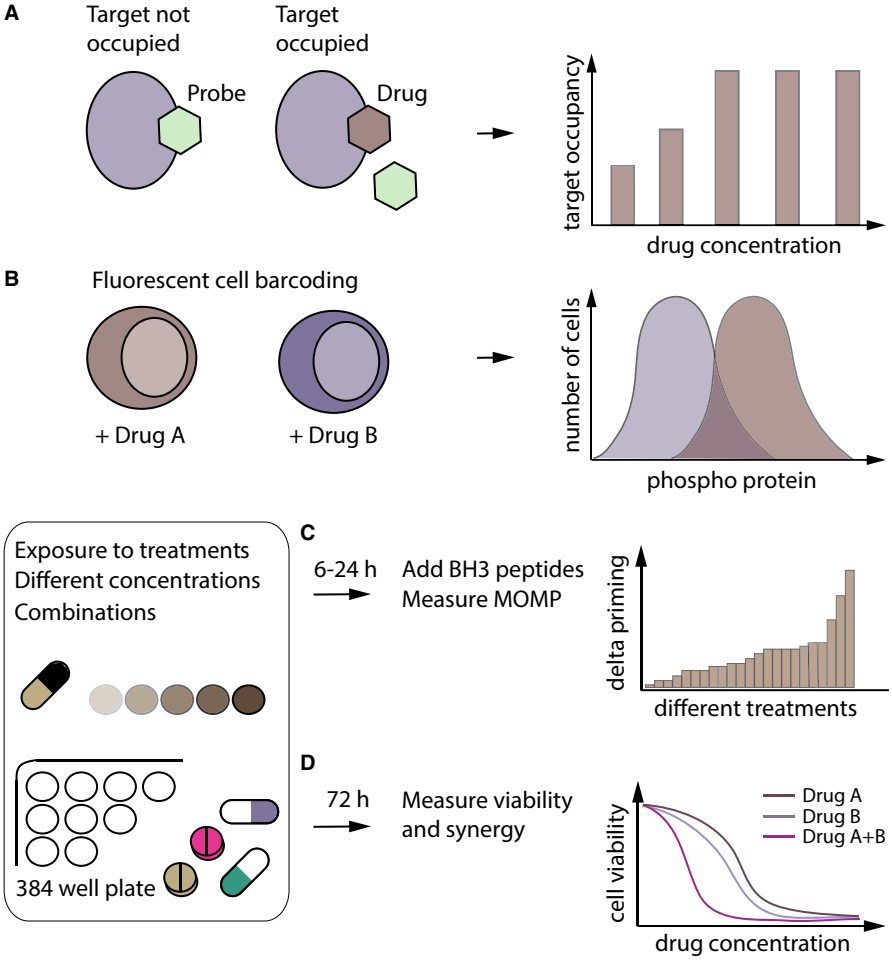

FIGURE 2 Functional approaches to precision medicine. A, An example of how to measure target engagement is shown. The non-occupied binding site of the targeted agent can be labelled with a probe (left). The ratio of probe-labelled target to total target can then be quantified to measure target occupancy at different concentrations of the drug (right). B, Phospho flow with fluorescent cell barcoding. Cells are treated with different drugs followed by fixation and fluorescent cell barcoding (left). The samples are then combined, permeabilized and stained with phospho-specific antibodies for simultaneous analysis by FACS (right). C, Dynamic BH3 profiling. Cells are treated with different drugs and drug combinations in 384-well plate format for 6-24 h. The cells are then permeabilized and incubated with BH3 peptides for 30-90 min. MOMP (mitochondrial outer-membrane permeabilization) is indicated by induced loss of cytochrome c, which can be detected by flow cytometry (for soluble cells) or microscopy (for adherent cells). High level of MOMP (delta priming; right) indicates that the respective drug is effective at inducing proapoptotic signalling. D, In vitro drug sensitivity assessment. Cells are treated with different drugs and drug combinations in 384-well plate format for 72 h. Cell viability is then measured by luminescence assays or microscopy, and synergy can be assessed for the drug combinations (right)

cancer.[50] Four classes of PI3Ks exist. Class 1 PI3Ks are heterodimers consisting of a p85 regulatory subunit and a p110 catalytic subunit, each of which exists as different isoforms. The catalytic subunits p110α and β are expressed ubiquitously, while p110γ and δ primarily are expressed in leucocytes.[51] The latter subunits were therefore recognized as attractive therapeutic targets in haematological malignancies (Figure 1).

Interest in developing p110δ inhibitors came after it was discovered that mice lacking this isoform did not develop mature B cells.[52] The first drug to obtain FDA approval was the specific p110δ inhibitor idelalisib for relapsed CLL patients who had received at least two prior therapies (Table 1). In the first phase 1 clinical trial (NCT00710528) with heavily pretreated patients and high-risk patients, an overall response rate of 72% and a 15.8-month median progression-free survival were reported (Table 2). 20% of the patients had a grade ≥ 3 pneumonia, and only 1 patient had a grade ≥ 3 alanine transaminase (ALT) or aspartate transaminase (AST) increase.[53] A phase 3 study (NCT01539512) comparing idelalisib and rituximab with placebo and rituximab in relapsed patients who could not undergo chemotherapy showed a markedly improved overall survival at 12 months (92% vs 80%) and overall response (81% vs 13%; Table 2). Serious adverse events occurred in 40% of the patients in the idelalisib arm and 35%

in the placebo arm.[54] These studies showed promising results with acceptable toxicity and idelalisib was therefore moved into first-line clinical trials. However, while idelalisib was highly active also in treatment naïve patients, serious adverse autoimmune events were frequent; 79% of the patients had ALT or AST elevation and 54% experienced transaminitis grade 3 or higher.[55] Therefore, all clinical trials on previously untreated patients were terminated in March 2016,[56] and idelalisib is not given to treatment naïve and younger patients today. Some of the adverse autoimmune effects of idelalisib may be due to inhibition of regulatory T cells.[57]

Duvelisib, a dual inhibitor of both the p110δ and p110γ isoforms (Table 1), received FDA approval in September 2018 for refractory or relapsed CLL patients who have received at least two prior therapies. A phase I study (NCT01476657) showed an overall response rate (ORR) of 56% for patients with relapsed/refractory CLL and 83% for previously untreated CLL patients (Table 2).[58] Colitis and transaminitis were frequently observed in this study as well, with treatment naïve patients being at higher risk.[58] Duvelisib is now being tested in combination with the B cell lymphoma-2 (BCL-2) inhibitor venetoclax for relapsed CLL patients (NCT03534323) (Table 2). Duvelisib has shown synergy with venetoclax in vitro, and duvelisib treatment is associated with changes in apoptotic regulators that may sensitize the CLL cells to venetoclax treatment.[59]

Several additional PI3K inhibitors are currently under investigation in clinical trials.[56] Umbralisib is a next-generation PI3K inhibitor selective for the p110δ isoform (Table 1). In a recent phase 1 study (NCT01767766), this agent showed fewer autoimmune side effects as a monotherapy compared with the PI3K inhibitors idelalisib and duvelisib.[60] In a recent phase 1 study with 21 relapsed/refractory CLL patients (NCT02268851), umbralisib was combined with another drug targeting the BCR pathway, the BTK inhibitor ibrutinib. The combination was well tolerated, and the overall response was 90% with a 2-year overall survival of 95% (Table 2).[61] This is promising for the use of this drug in future combination regimens.

Despite several of the new novel therapies having great effects in CLL, acquired resistance to therapy remains an issue. Understanding what mechanisms underlie resistance, and development of rational drug combination regimens have been suggested to be important for preventing resistance.[62] Mechanisms of resistance to PI3K inhibitors remain unknown; however, targeting alternative molecules such as BTK or BCL-2 in addition to PI3K could be potential bypass strategies.[62] Over-activation of the PI3K/AKT/mTOR pathway might cause resistance to venetoclax treatment, and prevention of this over-activation by combining venetoclax with a PI3K inhibitor is therefore a rational combination strategy.[63]

3.2 | BTK inhibitors

BTK is a cytoplasmic protein tyrosine kinase that belongs to the tyrosine kinase expressed in hepatocellular carcinoma (Tec) family of non-receptor tyrosine kinases (TFKs) (Figure 1).[64] In 1952, the phenotype of Btk deficiency was first described in a boy who presented with recurrent bacterial infections due to deficiency in humoral immunity.[65] This severe primary immunodeficiency was named X-linked agammaglobulinaemia (XLA). In 1993, the causative gene of XLA, Btk, was first identified and isolated.[66] BTK is expressed in all haematopoietic cells except T lymphocytes and plasma cells.[67] BTK regulates cellular processes including survival, proliferation and migration. In addition, BTK is critical to B cell motility and homing,[68] explaining the trafficking of lymphocytes between lymph node and blood, and the lymphocytosis observed in response to treatment with BTK inhibitors.[69]

BTK inhibitors are classified as either irreversible or reversible, referring to their binding to the target. Indeed, most of the small molecule targeted drugs for cancer therapy are irreversible binders (Table 1). These are considered the most potent, but cancer cells acquiring resistance are an increasing issue.[70] Ibrutinib was the first-in-class BTK inhibitor and is an irreversibly acting and orally bioavailable drug. Currently, ibrutinib as a single agent is one of the options as a first-line treatment for CLL patients. When compared to treatment with the anti-CD20 monoclonal antibody ofatumumab in previously untreated patients (NCT01578707), ibrutinib showed significantly improved progression-free survival and 12-month overall survival rate (90% in ibrutinib arm vs 81% in ofatumumab arm) (Table 2).[71] In a randomized phase 3 trial (RESONATE-2, NCT01722487), ibrutinib also showed superiority over the chemotherapeutic drug chlorambucil in previously untreated CLL or small lymphocytic lymphoma (SLL) patients (Table 2).[72] In general, patients tolerate ibrutinib treatment well. In a 5-year follow-up study, most of the adverse events reported were either grade 1 or 2. Of the grade 3 events reported, infections (9.3%) and atrial fibrillation (5.8%) were most common, but also diarrhoea (3.5%), rash (2.3%) and arthritis (2.3%) were reported.[73] Many of the side effects are believed to be due to off-target effects, and this provides a rationale for developing novel, more selective BTK inhibitors.

Of the novel BTK inhibitors developed so far, acalabrutinib is at the most advanced stage and is approved by the FDA for treatment of adults with CLL. Acalabrutinib is an irreversible inhibitor of BTK, reported to be more selective and to have less off-target effects when compared to ibrutinib.[74] This could mean higher efficacy and fewer side effects. Acalabrutinib is currently tested in two different phase 3 clinical trials for patients with previously

untreated CLL. One is combining acalabrutinib with venetoclax and obinutuzumab compared to chemoimmunotherapy (NCT03836261) (Table 2). The second study, Elevate-TN, is comparing acalabrutinib alone and in combination with obinutuzumab with chemoimmunotherapy (NCT02475681) (Table 2). Interim results from the Elevate-TN study estimates the 30-month progression-free survival to be 90% in the acalabrutinib + obinutuzumab arm, 82% with acalabrutinib as a single agent and 34% in the obinutuzumab and chlorambucil arm.[75] Acalabrutinib is now being compared head-to-head with ibrutinib in a phase 3 study (NCT02477696) (Table 2). Combining targeted therapies with either other targeted therapies, chemotherapy or chemoimmunotherapy is appealing because of the possibility of achieving deeper and long-standing remissions.[62]

Zanubrutinib is another potent and highly selective, irreversible next-generation BTK inhibitor, approved by the FDA for treatment of mantle cell lymphoma (MCL) (Table 1). So far, it has shown promising effects on activity and safety (NCT02343120) (Table 2).[76] A phase 3 study is currently recruiting 600 patients to compare the overall response rates of zanubrutinib versus ibrutinib in patients with relapsed or refractory CLL or SLL (NCT03734016) (Table 2).[77] The selective and irreversible BTK inhibitor tirabrutinib (ONO/GS-4059) (Table 1) has continued to show promising results in a long-term follow-up of relapsed/refractory CLL patients in a phase 1 clinical study (NCT02457559) (Table 2).[78] Estimated median PFS was 38.5 months, median overall survival was 44.9 months and the treatment continued to be well tolerated. Because of high efficacy and minimal toxicity, tirabrutinib is now in phase 2 clinical trials (Table 2). One study assesses the effect of tirabrutinib combined with entospletinib and obinutuzumab in relapsed/refractory CLL patients (NCT02983617). Interim results at week 25 showed an overall response rate of 100% in the tirabrutinib and entospletinib arm and 90% in the tirabrutinib, entospletinib and obinutuzumab arm (Table 2). A second phase 2 clinical trial evaluates the effect of tirabrutinib in combination with idelalisib and obinutuzumab in relapsed or refractory CLL patients (NCT02968563) (Table 2). Results from this study are awaited.

Targeted therapies have revolutionized CLL treatment over the last few years. PI3K inhibitors have great potential in CLL treatment and novel PI3K inhibitors are under development and are in different stages of clinical testing. The hope is that some of these drugs can reduce the toxicity associated with idelalisib, and that novel combinations including PI3K inhibitors can increase their efficacy and tolerability. BTK inhibitors have altogether showed positive results in the treatment of CLL, with high efficacy and tolerability also in the elderly patients. Next-generation BTK inhibitors are now becoming available to patients that experiences off-target side effects or resistance to ibrutinib (Table 1). Combining BTK inhibitors with other drugs may also become important to overcome the resistance issues seen with ibrutinib.

4 | BCR INHIBITORS MAY FACILITATE IMPLEMENTATION OF FUNCTIONAL PRECISION MEDICINE

Despite the recent therapeutic advances, CLL is still considered incurable. The disease is characterized by large interpatient heterogeneity in both pathologic features and clinical outcome, requiring personalized management approaches. The novel targeted therapies are effective, but only in a subgroup of patients, and the administration is currently indefinite and based on incomplete patient stratification. While gene mutations have been identified in CLL, these are not yet actionable.[79] A complementary approach to genomics is therefore needed to introduce precision medicine and improve patient care. We suggest that functional precision medicine, that is the use of functional assays to identify predictive biomarkers, should be considered. Targeted therapies are especially compatible with such high-throughput drug response analyses. The increased clinical relevance of these compounds may therefore accelerate the implementation of functional precision medicine in the clinic. Functional assays have several applications. They are used for drug discovery purposes, that is to identify new candidate drugs. They can serve as companion diagnostics, that is to determine if a certain drug is suitable for a specific patient. And they can be used to guide personalized medicine by screening large numbers of drugs for a single patient. Below, we discuss some of the assays that are currently used in preclinical investigations for the ex vivo determination of responses to cancer treatments, with a focus on application to haematological diseases.

4.1 | Measurement of target engagement or target response

To what degree a targeted agent binds to its molecular target may correlate with patient response to that drug. For example, ibrutinib inactivates BTK by irreversibly binding to the amino acid residue Cys481.[80,81] The irreversible binding once it has occurred would take the target:drug complex out of the equilibrium with free target and free drug and would mean that it may be possible to dose drug lower or more intermittently and yet achieve the same target occupancy. BTK occupancy in peripheral blood mononuclear cells (PBMCs) reflects drug activity (Figure 2A). While the

recommended daily dose of ibrutinib is 420 mg, a pilot study showed that the dose can be reduced step-wise to 140 mg/d and still occupy almost all BTK molecules in tumour cells from CLL patients.[82] A reduction in dose could reduce the inhibition of off-target proteins and consequently lower toxicities. It would be of interest to apply these tests to patient samples before start of treatment to determine the optimal dose for the individual patient. To the best of our knowledge, such studies have not been performed on BTK inhibitors. However, examples of predictive tests exist for other targets in other cancers.[83] It is well known that melanoma patients with BRAF mutation have elevated activation of MAPK and that the successful inhibition of this pathway with BRAF or MEK inhibitors correlates with response to therapy. To test the efficacy of the pathway inhibitors, protein phosphorylation can be measured by applying a kinase substrate peptide microarray over which the lysed patient sample is dispensed.[84] When measuring kinase activity in melanoma patient samples, no difference in substrate phosphorylation was observed between major genotypes such as mutations in BRAF, NRAS, cyclin-dependent kinase inhibitor 2A (CDKN2A) or TP53. Differences could only be observed when the lysates were exposed to the BRAF inhibitor vemurafenib ex vivo.[85] This illustrates the value of functional data in patient stratification.

4.2 | Cell signalling analysis by phospho flow

Analysis of several pathways simultaneously may enable better response predictions than analysis of only one pathway. Phospho flow is a powerful technique for this purpose, based on flow cytometry that measures protein phosphorylation events at the cellular level. This feature distinguishes the method from other antibody-based approaches such as Western blots, enzyme-linked immunosorbent assay (ELISA), protein array, and reverse phase protein array (RPPA).[86] The method is commonly combined with fluorescent cell barcoding, meaning that each cell sample is stained with a unique set of fluorescent dyes, that is a barcode, to allow for combination and simultaneous analysis of control and test samples (Figure 2B).[87] Advantages of barcoding include reduced antibody consumption, increased data robustness and enhanced speed of acquisition. Application of phospho flow has provided valuable information about baseline and drug-induced cell signalling in several haematological malignancies, including CLL,[39,43,88,89] acute myeloid leukaemia (AML)[90-92] and non-Hodgkin lymphomas,[93] as well as in normal haematologic subsets present among the malignant cells.[94] The method gives highly reproducible results, and cryopreservation does not affect signalling responses in B lymphocytes.[95] By performing high-throughput phospho flow experiments in a 96-well plate format, changes in phosphorylation level of 35 proteins in response to short (20 minutes) treatment with the BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib was systematically analysed in CLL cells.[89] Results showed that the targeted agents primarily affect proteins in the BCR signalosome and in the PI3K pathway. In the same study, a method to investigate synergy between ibrutinib and venetoclax, traditionally applied to cell viability data,[89,96] was adapted to signalling-response data. This combination of ibrutinib and venetoclax has been used with success in clinical studies on CLL patients.[97-99] Based on analyses of phospho flow data, it was shown that synergy between the two drugs can be achieved at doses that are much lower than the recommended daily dose of both ibrutinib and venetoclax. The potency of the targeted agents is supported by retrospective studies on CLL patients that have been treated with reduced doses of ibrutinib or venetoclax in order to limit toxicities.[100-105] These studies show that the effect of the treatment is not compromised by lowering the dose, indicating that further dose adjustment studies are warranted.

4.3 | BCL-2 homology domain (BH3) profiling

Proteins of the BCL-2 family control the intrinsic apoptosis pathway by regulating mitochondrial outer-membrane permeabilization (MOMP).[106] The BCL-2 family includes both inhibitors and inducers of apoptosis, and, essentially, when the proapoptotic proteins overwhelm the antiapoptotic proteins, MOMP is initialized. While BH3 profiling measures how close the mitochondria are to a threshold of apoptosis, dynamic BH3 profiling estimates the effect an applied drug has on moving the mitochondria closer to this threshold (Figure 2C). BH3 profiling can also identify antiapoptotic vulnerability. The method was useful in the clinical development of venetoclax in CLL and AML as it helped identify these diseases as largely BCL-2 dependent.[107,108] The assays are performed by titrating BH3 peptides derived from the α-helical BH3 domains of proapoptotic BCL-2 family proteins and testing how much is required to overwhelm the antiapoptotic proteins and thus induce MOMP.[109,110] For cells that are highly primed for apoptosis, little BH3 peptide is required to induce MOMP, and vice versa. Dynamic BH3 profiling can be used in high-throughput analyses of drug sensitivity. The assays are performed in 384-well plate format, and the cells are exposed to drugs for 6-24 hours. Apoptotic priming is then measured by BH3 profiling. A drug-induced increase in BH3 peptide-induced MOMP indicates induction of proapoptotic signalling. Several studies have shown that early measurement of drug-induced proapoptotic signalling predicts the in vivo response to the drug.[111-115]

4.4 | In vitro drug sensitivity assessment

The ultimate way to use functional tests to guide clinical decisions would be to screen a patient's tumour cells for drug sensitivity in vitro to identify the most effective treatment for that patient (Figure 2D). Over the past decade, such high-throughput assays have been developed. The experiments are typically performed in a 384-well plate format, and the cells are commonly exposed to drugs for 72 hours. Tyner et al have assessed the sensitivity of leukaemic cells from 151 patients to a panel of 66 kinase inhibitors.[116] Based on these data, the authors were able to develop an algorithm that correctly predicted pathway dependencies. For example, cells from patients with mutant Fms-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD)-positive AML were killed by drugs that target FLT3, while cells from patients with BCR-ABL-positive chronic myeloid leukaemia (CML) were killed by drugs targeting ABL. In a proof-of-principle case, the authors showed that in vitro drug sensitivity could predict clinical response as well as development of drug resistance.

Using a similar screening assay, Wennerberg and colleagues developed an individualized systems medicine approach to optimize precision medicine.[117] Samples from 28 AML patients were exposed to 187 drugs for 72 hours before cell viability was assessed. Two patients were treated with a combination of dasatinib, sunitinib and temsirolimus in an off-label compassionate use setting based on the results from the drug sensitivity screen. Both patients responded, but the response was short-lived. The drug sensitivity data were used both to suggest the mechanism of resistance, as well as potential ways to counteract it with combinatorial therapies.

Dietrich et al have measured ex vivo drug sensitivity of 246 patient samples from various blood cancers to 63 drugs.[118] In addition, they performed analysis of genomics, transcriptomics and DNA methylation status to understand determinants of drug responses. For CLL, they found that the BCR pathway was linked to trisomy 12, an important driver of the disease. Further, they were able to classify the disease into phenotypic subgroups based on dependency of the BCR, mTOR or MEK in association with genomic features as mentioned above. In addition to trisomy 12, the mutation status of IGHV was the most important modulator of drug response. This is in agreement with clinical observations.[35] Importantly, the study showed that ex vivo drug responses were associated with disease outcome, underscoring the potential of functional assays as diagnostic tools.

Finally, a study by Schmidl and colleagues combined epigenome profiling with single-cell chemosensitivity profiling and bioinformatic data integration in order to identify pharmacologically exploitable vulnerabilities in CLL cells collected from patients before and during ibrutinib treatment.[119] By this approach, the authors identified ibrutinib-induced changes which could be used for rationally designing ibrutinib combination therapies.

Taken together, these studies demonstrate that direct assessment of drug sensitivity may give patient benefit even when knowledge about the underlying biology is missing. This approach can therefore complement genomic precision medicine, where a treatment is given based on the presence of a specific gene mutation.[120] The clinical utility of drug sensitivity-based treatment decisions is currently investigated in prospective clinical trials on leukaemia and lymphoma (ClinicalTrials.gov identifiers: NCT01620216, NCT03096821).

4.5 | Patient-derived xenografts and other mouse models

In order to elucidate personalized treatment options based on an in vivo setting, patient-derived mouse xenografts (PDXs) can serve as valuable tools.[121] The PDXs are models of cancer where human tumour material is grafted into immunodeficient or humanized mice. PDX models are used to determine the contribution of tumour heterogeneity to therapeutic responsiveness, to understand tumour evolution over time and under drug pressure, and to investigate mechanisms leading to resistance to therapy.[121] While the models are useful, they also come with challenges. The time it takes to make the PDX, perform in vivo experiments, harvest and analyse data, can be several months. This is a significant amount of time considering that the clinician and patient are waiting for the results to make a treatment decision. Experiments with PDX models are also costly compared to ex vivo functional approaches. At present, these models are therefore less efficient tools with respect to guiding clinical decisions in precision medicine.[120]

In addition to PDX models, transgenic mouse models of CLL exist.[122,123] These models are valuable tools for preclinical studies, and can provide insightful information on pathogenic mechanisms. However, as CLL is heterogeneous in nature, it is important to keep in mind that these models are limited to mimicking one state of the disease or only certain aspects of the disease. Data generated from these models should therefore be interpreted with care. Interestingly, a BTK C481S knock-in mouse model was recently generated.[124] This cysteine to serine substitution is the most common mechanism for acquired resistance to BTK inhibitors, and these mice are resistant to irreversible BTK inhibitors. This model may prove useful to identify novel therapeutic targets.[124]

Together, the available PDX and transgenic mouse models are valuable hypothesis-testing tools and provide novel insights on tumour biology and drug mechanisms. Nevertheless, their use in precision medicine, where

treatment decisions may be required with some urgency, is currently limited.

5 | CONCLUSIONS AND OUTLOOK

Signalling through the BCR is essential for B cell survival. Studies on B cell malignancies such as CLL, in which cell signalling is aberrantly regulated, have accelerated our understanding of the cellular and molecular players involved in B cell physiology and pathology. Over the past few years, small molecule inhibitors that target BCR-associated kinases have demonstrated clinical success. The BTK inhibitor ibrutinib and the PI3K inhibitor idelalisib are first-in-class drugs approved for the treatment of CLL, and have revolutionized the management of this disease. Several next-generation agents have since been developed, some of which are already approved for treatment of B cell malignancies (Table 1). Despite novel treatment strategies, we still face the challenge of treatment resistance. A more complete understanding of the in vivo mechanisms of action of these drugs are needed, as well as biomarkers that can predict response to treatment and guide precision medicine. Functional assays are valuable tools that can help reach these goals.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SSS and LK wrote the manuscript. All authors read, commented and approved the final manuscript.

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