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Emerging Frontiers in Therapeutics of Diffuse Large B Cell Lymphoma: Epigenetics and B Cell Receptor Signaling

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ABSTRACT

This review discusses the impact of gene expression profiling and sequencing discoveries on new therapeutic strategies in Non-Hodgkin Lymphomas, particularly Diffuse Large B cell Lymphoma. Alterations in oncogenes, over-active signaling pathways down-stream of the B cell receptor, and epigenetic gene mutations will be described. We will also review new targeting strategies aimed at each of these aspects of cell biology encompassing BCL2, BTK, PKCβ, PI3K/mTOR and HDAC inhibition. Specific new drugs in clinical trials and early trial results are included as well.

Keywords: BCR Signaling; Epigenetics

1. Introduction

Non Hodgkin Lymphomas (NHL) are the 7th leading cause of cancer death in the United States [1]. Aggressive B subtypes of NHL include Diffuse Large B cell Lymphoma (DLBCL), Mantle Cell Lymphoma (MCL), and Burkitts Lymphoma (BL). Of these, DLBCL is the most common with approximately 30,000 new cases each year and accounting for nearly 40% of all Non Hodgkin Lymphomas [1]. Although the majority of cases are curable with traditional anthracycline based regimens, up to 40% of patients fail to achieve a remission or eventually relapse. This variability in response highlights the underlying biological heterogeneity of DLBCL. In the last decade, several new technologies have been developed for investigating lymphoma and other tumors. In combination with known molecular techniques, the application of gene expression profiling (GEP), and next generation sequencing have created a paradigm shift in our understanding of DLBCL.

In a seminal publication, using a customized Lymphochip array, new molecular subtypes of DLBCL were identified: Germinal Center B cell (GCB) type, and the activated B cell (ABC) type resembling activated peripheral blood B cells [2]. Subsequently, Primary Mediastinal (PMBL) was identified as a DLBCL type with a better prognosis [3]. These three molecular subtypes have different pathogenetic mechanisms with corresponding prognostic implications despite similar clinical features and risk stratifications by International Prognostic Index (IPI) [4,5]. It was noted that the PMBL group had favorable clinical features and prognosis while the ABC subtype had the worst prognosis and response to anthracycline based chemotherapy: CHOP (Cyclophosphamide, Hydroxydaunorubicin/Adriamycin, Oncovin/Vincristine, Prednisone) [3,6]. This has been further validated by several groups of investigators that used GEP to demonstrate the clinical significance of the GCB versus ABC distinction in R-CHOP (Rituxan-CHOP) treated patients [5-7]. Further GEP and sequencing data have led to the development of several new therapeutic strategies that attempt to target the different subtypes of DLBCL.

2. Targeting Oncogenes

MYC and BCL2 are well characterized oncogenes in Lymphoma, which impact proliferation and evasion of apoptosis respectively. MYC translocations are detected in B-cell lymphoma, unclassifiable with features intermediate between DLBCL and BL as well as 10% of DLBCL. These usually involve non-Immunoglobulin translocation partners [8]. Many of these lymphomas are "Double Hit" containing MYC translocations and translocations or other abnormalities of BCL2 and/or BCL6. BCL2 is translocated with the Immunoglobulin Heavy chain gene in approximately 15% of DLBCL; Most of these cases are of the GCB subtype (35%) [9]. Developing effective therapeutic strategies that target MYC has

been elusive. Recently, it has been recognized that acetyl lysine bromodomains on known MYC coactivator proteins regulate its transcription [10]. Therefore, targeting bromodomains is an attractive option. JQ1 is a novel small molecule bromodomain inhibitor that downregulates MYC transcription and transcription of MYC dependent genes.When JQ1 was tested in multiple myeloma cell lines, it decreased proliferation and caused cell cycle arrest [10]. JQ1 has been shown to decrease MYC and IL7 receptor in high-risk Acute Lymphoblastic Leukemia cell lines [11]. G-quadruplexes are secondary DNA structures resulting from DNA folding. It has recently been shown that MYC can form a G-Quadruplex. Quarfloxin is a fluroquinolone derivative. It has been chemically modified so that it interacts with and stabilizes G-quadruplexes [12]. It is currently in phase I clinical trials in advanced solid tumors and lymphoma [13].

Oblimersen, an antisense nucleotide, was one of the first agents targeting BCL2 in development. It was tested in clinical trials in several solid tumors and hematologic malignancies. Early phase clinical data were promising in CLL but phase III data have been unconvincing, and its future development remains uncertain [14]. Obatoclax was developed as a small molecule with affinity to BCL-2, BCL-XL, and MCL-1. Interestingly, obatoclax did not cause thrombocytopenia which has served as an *in vivo* marker of pharmacodynamics of BCL-XL inhibition [15] Obatoclax has shown modest clinical activity in a phase I combination with fludarabine and rituximab in relapsed CLL with partial response(PR) of 54% with no complete remissions [16]. In Small cell lung cancer, while early phase data indicated responses, a randomized phase II clinical trial in combination with chemotherapy did not show a statistically significant benefit in overall response rate (ORR) [17]. ABT-263 and ABT-199 are the next generation of BCL2 inhibitors. ABT263 has been very active in clinical trials with good response rates but thrombocytopenia has been a dose limiting toxicity. Preclinical data have shown reproducible thrombocytopenia caused by mitochondrial membrane permeabilization in platelets thought to be mediated by BCLXL, and leading to apoptosis and clearance by liver and spleen macrophages [18]. This is also suggested by relative resistance of younger platelets to ABT 263 that have higher levels of circulating BCLXL. This has been mitigated by the development of ABT 199. A key feature of this drug is that it lacks the high affinity binding to BCL-XL. This decreases the amount of thrombocytopenia allowing for higher dosing and correspondingly better response rates [19]. It is currently undergoing phase I trials in CLL and NHL. Additional agents in preclinical development include Sabutoclax which is a novel pan pro survival BCL2 family protein inhibitor currently in preclinical development [20]. These agents are summarized in **Table 1**.

2.1. Targeting B Cell Receptor Signaling

Recently in both Chroniclymphocytic leukemia (CLL) and particularly the ABC-DLBCL subtype, Chronic active B cell receptor (BCR) signaling has been recently identified as an important pathway. While it was known that CARD11 mutations are present in 10% of ABC-DLBCL, the oncogenic drivers associated with wildtype CARD11 were unknown. Through RNAi screen data, BCR signaling and Bruton's Tyrosine Kinase (BTK) were identified as key components of survival in ABC-DLBCL [24]. Germline mutations in BTK are associated with X-linked Agammaglobulinemia. It has a variable phenotype characterized by recurrent bacterial infections in affected males in the first two years of life [25]. More than 600 different mutations in BTK have been reported, and two thirds of mutations are premature stop codons, splice defects, or frameshift mutations that interfere with translational processing of the BTK transcript [26].

BCR signaling is mediated by CD79A and CD79B. When an antigen occupies BCR, Srcfamily kinases phosphorylate tyrosines in the ITAM motifs of CD79A and CD79B [27]. This activates spleen tyrosine kinase (SYK) by which in turn initiates a signaling cascade that involves BTK, phospholipase Cγ, and protein kinase C β (PKCβ). BTK forms a complex with B-cell linker (BLNK) and Phospholipase Cγ leading to the activation of multiple pathways including Nuclear factor-kappaB (NF-κB), Phosphatidylinositol 3-kinase (PI3K), Extracellular signal-regulated kinase (ERK), Mitogen activated protein kinase (MAP) and Nuclear factor of activated T cells (NFAT) [27]. This process is distinct from tonic BCR that promotes cell survival in mature B lymphocytes in mouse models; in mouse cells, when the BCR was conditionally ablated, all mature B cells died over a course of 2 weeks [28]. Implication of BTK in chronic active BCR signaling, and discovery of targeted BTK inhibitors has led to impressive clinical data. Recently, in an interim analysis of a multicenter, Open-Label, Phase 2 study of PCI32765 (targeted inhibitor to BTK) in ABC-DLBCL, the ORR in relapsed refractory DLBCL was 40%. 60% responses occurred in ABC-DLBCL tumors with CD79B mutations but responses were also seen in 37% wildtype CD79B suggesting that responses to PCI-32765 do not require a BCR mutation [29].

PKCβ is a serine threonine kinase expressed by normal and malignant B lymphocytes. It is required for BCR survival signals including activation of NF-κB; it phosphorylates CARMA1 via IKK/TAK1. Enzastaurin is an acyclic bisindolylmaleimide that was initially developed as an adenosine triphosphate-competitive, selective inhibitor of PKCβ [30]. The compound also modulates the PI3K/AKT pathway in selected tumor models. In preclinical studies, Enzastaurin induced apoptosis and inhi-

Table 1. Targeting oncogenes in lymphoma.

Process	Target	Small Molecule Inhibitor	Development/ Response	Focal Adverse Event	Reference
Proliferation	MYC	JQ1:BET Bromodomain Inhibitor	Preclinical: Multiple myeloma All	N/A	[10,11]
		Quarfloxcin	Ongoing Phase I	N/A	[13]
		Oblimersen	Phase I NHL: 1/21 CR	Thrombocytopenia	[21]
		Obatoclax	Phase I CLL in combination with Fludarabine/ Rituximab: 54% PR.	Reversible mental status changes.	[22]
Apoptosis	BCL2	ABT 263	Phase 1: Relapsed Refractory Lymphoid Malignancies: 10/46 PR Relapsed Refractory CLL: 31% PR	Thrombocytopenia Myelosuppression	[19, 22]
		ABT 199	Ongoing first in human Phase CLL/NHL	N/A	[23]
		Sabutoclax	Preclinical: CML patient samples	N/A	[20]

bited the proliferation of DLBCL cell lines and xenografts at low micro molar doses [30]. In a randomized phase II trial, Enzastaurin in combination with Rituxan-CHOP (R-CHOP) was associated with an increased progression free survival (PFS) and complete response (CR) rates [31]. It is currently in phase III trials: PRELUDE in DLBCL [32]. Fostamatinib is a SYK inhibitor that is orally bioavailable. In preclinical experiments, target inhibition of SYK abrogated BCR signaling and induced apoptosis. When SYK was inhibited by R406 and R788 (Fostamatinib), it was associated with tumor regression [33]. This led to a phase I/II trial of Fostamatinib in DLBCL. At a phase II dose of 200mg twice daily, the drug was well tolerated but ORR of 23.5% was noted with one only 1 CR [34]. The future of this drug in DLBCL remains uncertain.

Another, downstream pathway activated by BCR is the PI3K/AKT (Protein Kinase B)/mTOR (Mammalian target of Rapamycin) [35]. It is clinically significant in lymphoma and CLL.mTOR is serine-threonine kinase that is present in two distinct complexes: mTORC1 and mTORC2. mTORC2 phosphorylates AKT, SGK1(serum and glucocorticoid-regulated kinase), PKC, and mTORC1 mediates cell growth and proliferation via the eIF4E (Eukaryotic translation initiation factor 4E) binding proteins [36]. Everolimus is a rapamycin analog approved by the FDA for Renal cell carcinoma. Everolimusinduced a G1 arrest in DLBCL cell lines. Further, increased cytotoxicity was observed in rituximab sensitive cell lines [37]. It was evaluated in a phase II trial of 77 patients NHL and median age of 70. The predominant NHL subtypes enrolled on the trial included DLBCL, MCL, and Grade III Follicular lymphoma (FL). The ORR was 30% with majority being PRs and three CRs. Grade III toxicities included myelosuppression, hyperglycemia, and respiratory Infections [38].

P110α, p110β, p110δ and p110γ mediate the activation of the PI3K pathway via cell surface receptors. Of these, the p110δ isoform is highly expressed inhematopoetic cells, and required for cells to respond to BCR [39]. CAL-101/GS1101 was identified as a potent and selective inhibitor of p110δ. It blocked constitutive PI3K signaling resulting in decreased phosphorylation of AKT and induction of apoptosis [40]. Finally, it has been shown that activated BCR also results in tyrosine phosphorylation of JAK-STAT via LYN. An *in vitro* phosphorylation assay demonstrated that LYN directly phosphorylates STAT3 [41]. Ruxolitinib is an oral selective inhibitor of JAK1 and JAK2 that is FDA approved for the treatment of Myelofibrosis. It is currently in a multicenter phase II study in DLBCL [42]. These agents are summarized in **Table 2**.

2.2. Targeting Epigenetics

Post translational modification of histones via epigenetic mechanisms is critical for maintaining integrity of chromatin and gene expression. Recently, several studies have implicated epigenetic mechanisms in the development of DLBCL. In a recent study that initially sequenced genomic DNA of 14 NHL and paired normal tissues, 717

Table 2. Targeting the bcr signaling pathway in lymphoma.

Drug	Target	Disease	Reference
PCI-32765/Ibrutinib	BTK	ABC-DLBCL	[29]
Enzaustaurin	PKCβ	DLBCL	[30-32]
Fostamatinib	SYK	DLBCL	[34]
Everolimus	mTOR	DLBCL	[38]
GS1101	P110δ	CLL, Indolent NHL	[40]
Ruxolitinib	JAK-STAT	DLBCL	[42]

coding single nucleotide variants were identified affecting 651 genes. These 14 NHL cases were then reanalyzed with another 113 samples including 83 DLBCL with RNA sequencing; the tumor and matched normal DNA from these cases were re-sequenced to confirm 109 genes with multiple somatic mutations. It was remarkable that genes with roles for histone modification were frequent targets for somatic mutations in DLBCL [43]. MLL2 (Myeloid/Lymphoid or mixed lineage leukemia gene) showed the most significant number of nonsense single nucleotide variants including 37% nonsense mutations, 46% read frame altering indels, 8% point mutations at splice sites, and 9% non-synonymous amino acid substitutions. MLL2 was mutated in 59% DLBCL cell lines, 32% patient samples and in none of the paired normal tissues. It is one of the six human H3K4 specific methyl transferases. Tri methylated H3K4 (H3K4me3) is epigenetically associated with promoters of actively transcribed genes. These mutations are likely inactivating since both alleles of the genes were affected by the mutations resulting in loss of MLL2 activity. MLL2 mutations were distributed amongst both ABC and GCB-DLBCL subtypes [43].

Histone acetylation is moderated by Histone acetyltransferases (HATs); it isan epigenetic mechanism for keeping chromatin in an open, transcription ready state. Histone Deacetylases (HDAC) keep the chromatin in adeactelyated repressive state. MEF2 (Mycocyte enhancer factor-2) proteins are a family of transcription factors that can act as transcriptional coactivators or corepressors of HAT and HDAC. Under normal intracellular calcium levels, MEF2 is bound by type IIa HDACs [44]. Increased cytoplasmic calcium displaces the bound HDAC allowing for competitive binding of the HATs CREB-binding protein (CREBBP) and E1A binding protein p300 (EP300). This enables transcription of MEF2 target genes by acetylation of lysine residues on Histone H3 (H3K27). Somatic mutations of MEF2 that involved non synonymous single nucleotide variantsare unique to FL and GCB-DLBCL [43,45,46]. Mutations in HATs principally CREBBP are one of the most frequent mutations in DLBCL found in >30% of cases [45]. Thus, HDAC inhibition is a promising clinical strategy in DLBCL.

Several HDAC inhibitors are in clinical trials for DLBCL. Panobinostat is currently undergoing phase II trials as a single agent or in combination with rituximab or Everolimus in DLBCL [47,48]. Vorinostat was well tolerated as a single agent in DLBCL but had suboptimal responses. It is currently being evaluated in combination with cytotoxic therapy in multiple trials [49]. The next generation HDAC inhibitors in early clinical development include Abexinostat, Bellinostat, and Rocilinostat (**Figure 1**). In preclinical studies with CLL patient samples, Abexinostat resulted in cell death with physiologically achievable IC50. It was also found to be synergistic with Bortezomib. Increase in Histone H3 acetylation and P21 protein, a known cyclin dependent kinase inhibitor up regulated by histone acetylation were also noted [50]. Abexinostatwas well tolerated in patients with relapsed FL, and MCL. While the median PFS in MCL was 4 months, at a median follow up of 10.3 months the PFS was 86% in FL patients [51]. A co-operative group trial evaluated the efficacy of Bellinostat in relapsed refractory DLBCL patients with up to 5 prior chemotherapy regimens. In this trial, 2 PRs were observed at 5 and 13 months after registration [52]. Rocilinostat is currently in preclinical development, and it is synergistic with Bortezomib in both DLBCL and Multiple Myeloma cell lines [53,54].

BCL6 is an oncogene also involved with HAT repression and is frequently altered in DLBCL through translocations and other mechanisms involving the 3q27 locus. The protein is typically expressed in the GCB subtype of DLBCL and is a potent therapeutic target in DLBCL. A recombinant 120 amino acid peptide RI-BPI containing the SMRTdomain inhibited transcriptional repressor activity of BCL6 and had potent in vitro lymphoma effects [55]. It was also noted that BCL6 repressed the expression of EP300 and its cofactor HLA-B associated transcript 3(BAT3). RI-BPI induced expression of p300 and BAT3, resulting in acetylation of p300 targets including p53 and Heat shock protein (hsp) 90. Induction of p300 and BAT3 was required for the antilymphoma effects of RI-BPI, since specific blockade of either protein rescued human DLBCL cell lines from the BCL6 inhibitor (**Figure 1**) [56]. RI-BPI has recently shown promising preclinical synergy with small molecule inhibitors of BCL2, NEDD8 activating enzyme and Bortezomib [57].

EZH2 is the catalytic subunit of the PRC2 (Polycomb repressive complex 2) that enables methylation of histone H2 on lysine 27 (H3K27), decreasing gene expression and inhibiting transcription of dependent genes. EZH2 overexpression correlates with poor prognosis in Prostate adenocarcinoma and Renal cell carcinoma. Somatic mutations have been recently identified in the SET domain of EZH2 occurring in FL and up to 22% GCB-DLBCL. A mutation that results in the replacement of tyrosine to

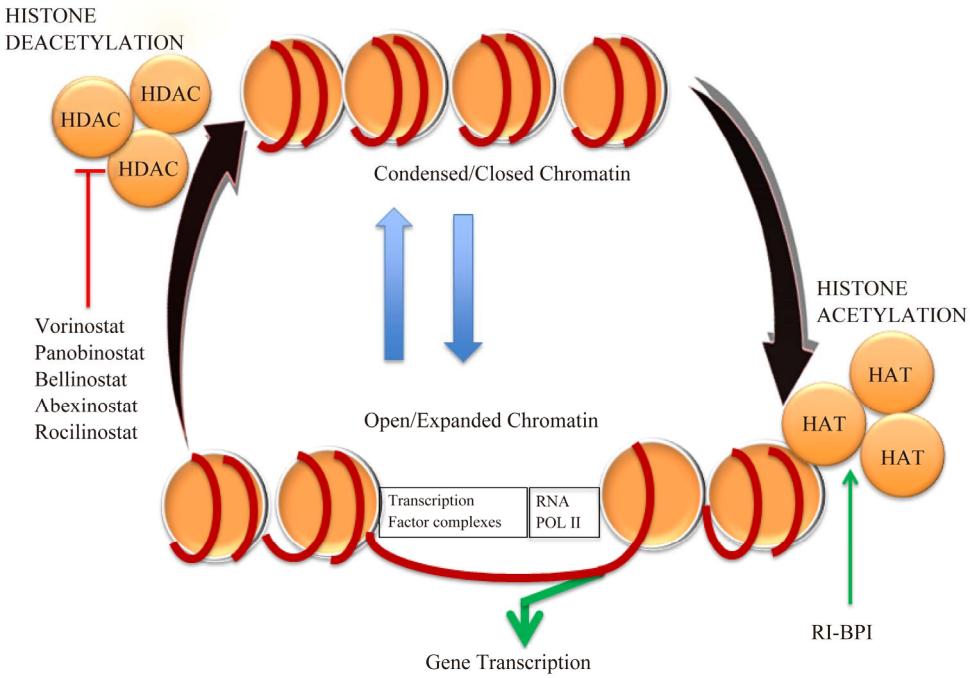

Figure 1. When the histones are deacetylated by Histone Deacetylases (HDAC), the chromatin is condensed into a closed structure, and expression of target genes usually tumor suppressors is repressed. Histone acetylation by Histone Acetyl transferases (HATs) permits transcription of target genes.

histidine on exome 15: Tyr641 was associated with increased H3K27 tri-methylation(H3K27me3) [58]. EZH2 inhibitors are currently in preclinical development; GSK-126 has been shown to decrease global H3K27me3 levels and reactivate silenced PRC2 target genes thus inhibiting the proliferation of EZH2 mutant DLBCL cell lines. It also markedly inhibited the growth of EZH2 mutant DLBCL xenografts [58].

In summary, genomic technologies including gene expression profiling and next generation sequencing are providing astonishing insights into the biology of lymphoma. It is apparent that multiple pathways are altered and that lymphomas frequently use more than one strategy to their advantage. In fact, many different genetic and epigenetic changes are present in any one case. Subclones can also exist with yet additional changes reflecting ongoing tumor heterogeneity [59]. In DLBCL, abnormalities of MYC, BCL2, the BCR and its downstream effector molecules, as well as epigenetic modifying genes are all frequently altered. New drugs are being designed to specifically target these abnormalities. As single agents, these new drugs are currently under evaluation with early promising results. Combinations of such targeted therapies hold the potential of even more effective therapy.

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