Targeting aggressive B-cell lymphomas with cell-penetrating peptides

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Abstract

DLBCL (diffuse large B-cell lymphoma) is the most common subtype of non-Hodgkin’s lymphoma. Current therapy for patients includes chemotherapy and monoclonal antibodies. Although oncogene-targeted therapy is dramatically successful for patients with certain kinds of leukemias, there are no such agents yet for DLBCL. One reason for this is that several key oncogenes involved in DLBCL pathogenesis are transcription factors, which are difficult to therapeutically target with small molecules. Recent advances in the structural and functional characterization of DLBCL oncogenes have facilitated design of CPPs (cell-penetrating peptides) with potent inhibitory effects on DLBCL and other aggressive lymphomas. CPPs targeting the Bcl (B-cell lymphoma)-6, Bcl-2, Myc and NF-κB (nuclear factor κB) oncogenic pathways, among others, could improve efficacy and reduce toxicity of anti-lymphoma therapy. Another barrier towards effective therapy in DLBCL is its profound molecular heterogeneity. Combinatorial administration of oncogene-targeted CPPs based on the molecular profiles of individual patient tumors could allow individualized targeted therapy regimens to be developed.

Therapeutic targeting of transcription factors in aggressive B-cell lymphomas

Mutations of transcription factor proto-oncogenes are a common feature of aggressive B-cell lymphomas [1]. A majority of aggressive lymphomas are of a subtype called DLBCL (diffuse large B-cell lymphoma) [1]. The locus most frequently involved in genetic lesions in DLBCL encodes a transcriptional repressor protein called Bcl-6 (B-cell lymphoma 6) [2]. Bcl-6 translocations and cis regulatory element point mutations result in its constitutive expression, which was demonstrated to be a causative factor in the development of DLBCL [2–4]. Other examples of proto-oncogene transcription factors involved in aggressive B-cell lymphomas include Myc and NF-κB [1,5].

The current treatment of DLBCL is based entirely on cytotoxic agents and/or monoclonal antibodies that target B-cells non-specifically [1]. Approx. 50–60% of patients can be cured in this way, with further improvements possible when careful risk stratification is employed [1]. Recent advances in lymphoma biochemistry and biology raise the possibility of designing drugs that block critical lymphoma oncogenes directly to provide more effective and less toxic therapeutic options. However, transcription factors such as Bcl-6 function by recruiting chromatin-modifying complexes through protein interactions, instead of through direct enzymatic modifications of substrate. Blockade of protein interactions, which can be quite large and convoluted, is not well suited to the traditional small-molecule approach [6,7]. Therefore alternative approaches may be required to target lymphomagenic transcription factors.

An additional barrier towards specific treatment lies in the molecular heterogeneity of these diseases. DLBCL is not a single entity, but rather consists of several different phenotypes, which are at least in part reflective of the oncogenes that drive each individual tumour [8,9]. Expression microarray studies have provided some insight into the complexity of DLBCL. At least two studies showed that DLBCLs can be classified into discrete subtypes [8,9]. The LLMP (Lymphoma and Leukemia Molecular Profiling Project) classified DLBCLs on the basis of whether gene expression profiles were similar to those of ABCs (activated peripheral blood B-cells) or contained genes associated with GCBs (germinal centre B-cells). An additional 40% of patients could not be classified into either group [10]. Dr Shipp and colleagues performed a comprehensive unsupervised consensus analysis of DLBCL expression profiles and found that the patients segregated into three different signatures designated BCR (B-cell receptor), OXPhos (oxidative phosphorylation) and HR (host response) [9]. The genes and patient cohorts identified by these two groups had minimal overlap, yet both classifications appear to be biologically significant. For example, ABC DLBCLs express NF-κB target genes and can be suppressed with NF-κB inhibitors [11], while BCR DLBCLs display co-ordinated regulation of Bcl-6 target genes and can be specifically suppressed by blocking Bcl-6 [12]. Moreover,
since several oncogenes may be present in a given tumour, combinatorial targeting is likely to be necessary regardless of DLBCL subtype [1]. Taken together, the twin challenges of hitting transcription factor oncogenic targets and identifying patients that are likely to respond to therapy represent a significant barrier towards development of more advanced anti-lymphoma regimens. Peptide therapeutics represent one possible route towards overcoming some of these limitations.

**Biochemical mechanism of action of Bcl-6**

Bcl-6 is a member of the BTB (bric-a-brac, tramtrack, broad complex)/POZ (pox virus, zinc finger) zinc-finger family of transcription factors. The ability of Bcl-6 to mediate transcriptional silencing is contingent on its recruitment of co-repressor proteins through its different protein interaction motifs. The BTB domain of Bcl-6 has autonomous repression activity and can bind the SMRT (silencing mediator of retinoid and thyroid receptors), NCoR (nuclear receptor co-repressor) and BCoR (Bcl-6 co-repressor) co-repressors [13,14]. The Bcl-6 BTB domain forms an obligate homodimer [15]. An extensive and somewhat tortuous groove is formed at the interface between Bcl-6 BTB monomers [15]. Amino acid side chains projecting into this groove provide the context for binding of an unstructured 18-residue fragment (or BBD (Bcl-6-binding domain)) from the SMRT, NCoR and BCoR co-repressors [15]. The protein interaction actually requires three distinct polypeptides, i.e. two Bcl-6 BTB monomers plus the co-repressor fragment, to form a complex. The side chains necessary for high-affinity interaction with the BBD are specific to Bcl-6 so that the BBD cannot interact with other proteins in the BTB family [15]. When fused to the Tat (transactivator of transcription) protein transduction domain, exogenously administered BBD peptide could bind to the Bcl-6 lateral groove in the nuclei of lymphoma cells and exclude SMRT and NCoR from Bcl-6 repression complexes formed on the natural target genes of Bcl-6 [16]. Disruption of the Bcl-6–SMRT–NCoR complex by Tat–BBD (hereon called BPI for Bcl-6 peptide inhibitor) resulted in chromatin remodelling from a repressed to an active configuration, followed by transcriptional activation of the respective genes [16].

In addition to the lateral groove, the Bcl-6 BTB domain contains a more conserved repression-associated motif that has been described as a ‘charged pocket’. This motif contains negatively and positively charged residues and is formed by the BTB monomer interface at a site distinct from the lateral groove [17]. The BTB charged pocket may also contribute to the recruitment of co-repressors and, in the case of the PLZF (promyelocytic leukaemia zinc finger) BTB transcriptional repressor, is required for interaction with SMRT and NCoR [17]. In Bcl-6, the biochemical function of the charged pocket remains unknown. A screen for Bcl-6–BTB domain-binding aptamers identified a peptide that could inhibit Bcl-6 repressor activity without affecting the interaction with NCoR and SMRT [18]. In contrast with BPI, the aptamer appeared to bind to residues surrounding the charged pocket and not the lateral groove [18]. The charged pocket may therefore also be a druggable site for Bcl-6 CPPs (cell-penetrating peptides). Finally, since the Bcl-6 BTB domain must homodimerize to be functional, it is possible that peptides that mimic the α-helical bundles which form the monomer interface could disrupt Bcl-6 activity. Challenges to this approach include the high degree of conservation of the monomer interface between BTB proteins that could limit specificity and the difficulty for exogenous peptides to access the dimer interface.

**CPP targeting of Bcl-6 in DLBCLs**

CPPs are an effective means of delivering therapeutic payloads to target cancer cells. Like other cell types, B-cell lymphoma cells readily internalize CPPs, allowing the biological effects of Bcl-6 and other inhibitors to be determined. BPI could readily induce cell death and cell cycle arrest in Bcl-6–positive DLBCL cells both in vitro and in vivo [16,19]. DLBCL survival is therefore dependent on Bcl-6 BTB domain lateral groove co-repressor docking, demonstrating that the Bcl-6 is a bona fide therapeutic target. Bcl-6 was also shown to mediate differentiation blockade in lymphoma cells, yet BPI did not induce differentiation [16], suggesting that Bcl-6 mediates differentiation through a different biochemical mechanism. Accordingly, Bcl-6 could bind the MTA3 co-repressor through a BTB-independent interaction site [20] and MTA3 co-operated with Bcl-6 to mediate the differentiation blockade. We found that MTA3 depletion has no effect on survival of DLBCL cells [21]. Therefore peptide blockade of distinct co-repressor-binding sites could harness different biochemical mechanisms that control the specific Bcl-6-regulated oncogenic pathways. Presumably, development of peptide inhibitors of MTA3 might induce differentiation of DLBCLs, which could be a desirable therapeutic goal.

Peptide inhibitors have also helped to dissect the molecular heterogeneity of DLBCL and identify DLBCLs that are biologically dependent on Bcl-6. Specifically, Bcl-6 expression or translocation does not predict biological dependence on Bcl-6 [16]. Microarray-based classifications show that Bcl-6 is more often expressed in the GCB compared with ABC categories of the COO (cell-of-origin), or the BCR compared with OxPhos categories of the CCC (comprehensive consensus cluster) [9]. Bcl-6 translocations are less common in GCBs than ABCs [8], but more common in BCR compared with OxPhos. In order to determine whether it is possible to identify Bcl-6-dependent lymphomas on the basis of co-ordinated expression of Bcl-6 target genes, a recent study performed gene set enrichment analysis of a Bcl-6 target gene list using expression microarray datasets [12]. Bcl-6 target genes were co-ordinately regulated in the BCR subtype of DLBCL, but not in the other subtypes identified by the CCC and COO. BCR–DLBCL cells were universally sensitive to BPI, while non-BCR cells were resistant [12], indicating that this subtype of DLBCL represents Bcl-6-dependent disease. BCR–DLBCL patients would
Therapeutic targeting of oncogenic pathways in DLBCL

The p53 tumour suppressor has long been considered to be a therapeutic target, since its downstream pathways induce cell death and growth arrest in many tumour cells. A peptide containing a C-terminal fragment of p53 was shown to enhance p53-mediated transactivation of its target genes and can even rescue the activity of certain cancer-associated mutant forms of p53 [28–31]. Up to 49% of DLBCLs express p53, even in the absence of p53 mutation (L. Cerchietti, M.E. Figueroa, R. Shaknovich and A. Melnick, unpublished work). P53 responses are apparently blunted in DLBCLs as well as in Burkitt’s lymphoma cells, at least in part due to the actions of Bcl-6 (L. Cerchietti, M.E. Figueroa, R. Shaknovich and A. Melnick, unpublished work) [33]. Tat–p53C' peptide could trigger p53 responses, leading to growth arrest and cell death in Burkitt’s lymphoma cells [34]. A d-amino acid retro-inverso peptide version of Tat–p53C' was more potent and readily killed lymphoma cells in vitro and in vivo in a peritoneal lymphomatosis model [34]. Many B-cell lymphomas express the CXCR4 chemokine receptor. Addition of the DV3 ligand for CXCR4 to Tat–p53C' enhanced further its anti-lymphoma activity [35]. Use of retro-inverso D-peptides with ligands targeted to lymphoma cells and addition of sequences such as the influenza virus fusogenic motif that facilitates cationic peptide escape from macropinosomes [36] could yield powerful anti-lymphoma activity.

Constitutive NF-κB signalling contributes to the malignant phenotype in several types of lymphoma including the ABC subtype of DLBCLs, primary mediastinal B-cell lymphomas and Hodgkin’s disease [5]. NF-κB pathway activation occurs mainly through phosphorylation by the IKK (inhibitor of NF-κB kinase) complex, which consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit [IKKγ or NEMO (NF-κB essential modifier)]. ABC, but not GCB DLBCL cells were responsive to IKK inhibitors in vitro [11]. In most cases, the mechanism of constitutive NF-κB activation in DLBCLs is not clear. Rarely, translocations involving the NF-κB2 locus or the Bcl-10 and MALT1 (mucosa-associated lymphoid tissue 1) proteins (upstream activators of the IKK complex) have been identified in DLBCL [37–40]. A CPP containing the Antennapedia protein transduction domain and the minimal sequence of IKKβ that binds to NEMO could disrupt formation of the IKK

CPP targeting of B-cell lymphoma oncogenes (Figure 1)

Inhibiting peptides have also been designed and tested against other lymphoma oncogenes. It has long been recognized that EBV (Epstein–Barr virus) plays an important role in the pathogenesis of Burkitt’s lymphoma and in lymphomas of immunocompromised patients [22]. A number of viral transcription factors and signal transduction mediators are implicated in fostering malignant transformation of lymphocytes [22]. EBV can enter several different stages of latency featuring expression of specific sets of viral genes. In the so-called ‘type III’ EBV latency, the EBNA (Epstein–Barr nuclear antigen) 2 transcription factor and EBNA3A/EBNA3B/EBNA3C proteins are expressed together with several other viral components. Type III latency is associated with lymphomas of immunocompromised patients [22]. Two different Tat–EBV peptides could disrupt the proliferative and survival effects of the EBV latency III program [23,24]. EBNA2 can mimic the function of the Notch transcriptional activator by interacting with the CBF1 (CCAAT-binding factor 1) protein [23,25]. A Tat–EBNA2 fusion construct containing the EBNA2 minimal CBF1-binding domain could disrupt this interaction resulting in down-regulation of viral gene products, blockade of proliferation and suppression of colony formation in infected B-cells [23]. The EBNA3C protein contributes to lymphoma cell proliferation at least in part by interacting with the SCFSkp2 protein and modulating its effects on cyclin A and p27 [26,27]. A Tat-fusion peptide containing the EBNA3C Skp2-interaction domain could block this interaction and inhibit proliferation and colony formation of EBV-infected B-cells [24]. Moreover, Tat–EBNA3C also inhibited the outgrowth of lymphoma cells from a patient with EBV-positive post-transplant lymphoma [24]. Disruption of the EBV latency III lymphomagenesis programme through peptide interference holds promise as a therapeutic strategy in the EBV-positive lymphomas of immunocompromised patients.

The left-hand panel gives examples of important oncogenes and tumour suppressors involved in DLBCL are indicated with stars. Proteins that have been targeted with CPPs are in black boxes. Arrows indicate induction and bars indicate inhibition. The right-hand panel indicates whether involvement of oncogenes is tilted more towards proliferation or survival. See the text for further explanation. Note that (i) the connection between Myc and Bcl-6 denotes the recent discovery that these two oncogenes, which are often co-expressed in DLBCL can interact and enhance each other’s oncogenic potential [49], (ii) Bcl-6 can directly repress p53 as well as attenuate its activity by repressing ATR (ataxia telangiectasia mutated- and Rad3-related) and Chk1 (checkpoint kinase 1) [33,50].
complex, block activation of NF-κB via TNFα (tumour necrosis factor α) signal transduction at high micromolar concentrations and attenuate inflammatory reactions in vivo [41]. Peptides containing the NEMO C-terminal coiled coil or leucine zipper motifs fused to the Antennapedia protein transduction domain could block NEMO oligomerization, and more potently disrupt NF-κB activity and kill tumour cells at low micromolar concentrations [42]. Peptides that disrupt the IKK complex or block NF-κB functions are potential therapeutic agents in DLBCLs with constitutive NF-κB signalling.

The Myc oncogene is frequently expressed in DLCLB and Burkitt’s lymphomas, often associated with t(8;14) translocation [43]. The oncogenic effects of Myc are at least in part dependent on its activity as a transcription factor. Myc appears to bind to thousands of loci and regulates an extensive network of gene products involved in cell cycle, metabolism and other processes [44]. The transcriptional function of Myc is dependent on its dimerization with the Max protein and transcriptional co-activators such as TTRAP (transactivation/transformation-associated protein), Ini-1 and histone acetyltransferases [45]. Therapeutic peptides designed to disrupt Myc interactions with any of these proteins could impair or modulate its activity. For example, Tat-fusion peptides incorporating the helix 1 motif of Myc could disrupt its activity by blocking interaction with Max [46]. Retro-inverso Myc helix 1 peptides have anti-proliferative activity in breast cancer cells [46] and might also be expected to have anti-lymphoma activity.

Finally, although not a transcription factor, the Bcl-2 oncogene is often overexpressed through translocations and other mechanisms in several types of B-cell lymphomas including DLBCL [1]. A t(14;18) translocation places Bcl-2 under the immunoglobulin heavy chain promoter, resulting in its constitutive expression [1]. This translocation is a hallmark of follicular cell lymphomas, but also occurs in DLBCL [1]. Bcl-2 is a critical antagonist of the intrinsic pathway of apoptosis, culminating in mitochondrial pore formation by the Bax and Bak proteins [47]. Bcl-2 in turn can be inhibited by several members of BH3 (Bcl-2 homology domain 3)-only proteins including Bid and others [47]. A BH3 α-helix stabilized by hydrocarbon stapling could readily penetrate B-cells and induce apoptosis of B-cell leukaemia cells in vitro and in vivo [48].

Taked together, B-cell lymphomas are a target-rich environment for CPP therapeutic agents that block critical protein interactions that are involved in maintaining the growth and survival of lymphoma cells. Since many of these oncogenes are co-expressed in B-cell lymphomas [1], it is logical to postulate that combinatorial peptide targeting tailored according to the presence of such oncogenes could yield markedly superior anti-lymphoma activity. Along these lines, we have found that sequential administration of BPI followed by Tat-p53C’ synergizes a p53 response with enhanced lymphoma cell killing (L. Cerchietti, M.E. Figueroa, R. Shaknovich and A. Melnick, unpublished work). Timing of administration of each peptide is critical, since lymphoma cells acquire maximal sensitivity to p53 peptides 24 h after Bcl-6 blockade. It is reasonable to expect that careful sequencing will be essential to maximally harness the power of other combinatorial targeting strategies as well. Since several of these peptides (such as BPI and Tat-p53C’) function by restoring cellular checkpoint mechanisms, it is likely that they would potentiate or synergize with chemotherapy drugs. Clinical translation of therapeutic CPPs could therefore improve efficacy and reduce toxicity of existing anti-lymphoma treatment regimens.

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References