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Novel disease targets and management approaches for diffuse large B-cell lymphoma

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Abstract

Diffuse large B-cell lymphoma (DLBCL) responds well to treatment with CHOP and the R-CHOP regimen, but a subset of patients still fail to achieve complete or durable responses. Recent advances in gene expression profiling have led to the identification of three different subtypes of DLBCL, and confirmed that patients with the activated B-cell (ABC) disease subtype are less likely to respond well to CHOP-based regimens than those with germinal centre B-cell-type (GCB) disease. This discovery could herald the use of gene expression profiling to aid treatment decisions in DLBCL, and help identify the most effective management strategies for patients. Treatment options for patients with relapsed or refractory DLBCL are limited and several novel agents are being developed to address this unmet clinical need. Novel agents developed to treat plasma cell disorders such as multiple myeloma have shown promising activity in patients with NHL. Indeed, the immunomodulatory agent lenalidomide and the proteasome inhibitors bortezomib and carfilzomib, as single agents or in combination with chemotherapy, have already demonstrated promising activity in patients with the ABC subtype of DLBCL. One should not be complacent however when applying these agents to new disease types, because dose and drug scheduling can have marked effects on the responses achieved with investigational agents. As more targeted agents are developed, the timing of administration with other agents in clinical trials will become increasingly important to ensure maximal efficacy while minimizing side effects.

Keywords

Diffuse large B-cell lymphoma; Bcl-2; novel agents; gene expression profiling

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Introduction

Diffuse large B-cell lymphomas (DLBCL) are aggressive B-cell disorders and are the most common lymphoid neoplasms in adults [1]. As with all non-Hodgkin lymphomas (NHLs), accurate pathological diagnosis of DLBCL is important because of the morphologic, clinical, and genetic heterogeneity of the disease [1]. Immunophenotypic analysis is used to distinguish DLBCL from other lymphomas, and typical DLBCL cells express B-cell markers such as CD19, CD20, and CD79a, and are CD3-negative. Advances in gene expression profiling have enabled DLBCL to be subclassified into germinal center B (GCB) cell, activated peripheral blood B-cell (ABC) subtypes, and primary mediastinal B-cell lymphoma (PMBL) [2–5]. Patients with the GCB subtype (CD10+ or BCL6+ and MUM1–) have better survival than the ABC subtype (CD10- and BCL-6- or Mum1+), with standard cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy [5]. Over-expression of Bcl-2 might also have implications for clinical decision making in DLBCL. Although nearly all follicular lymphomas (FL) express elevated levels of Bcl-2 as a consequence of the t(14;18) translocation, most DLBCL cases also have abnormal Bcl-2 expression, which may be due to t(14;18), amplification or putatively due to over-expression of NFkB [1]. This discovery has led to the investigation of Bcl-2 as a potential therapeutic target in DLBCL.

Standard therapeutic approaches for DLBCL differ based on disease staging (localized or advanced disease) using the Ann Arbor criteria. Patients who do not have adverse risk factors, including elevated lactate dehydrogenase (LDH) levels, advanced stage II disease, age > 60 years, and/or ECOG performance status 2, generally have a favorable prognosis [6,7]. Patients with limited-stage DLBCL usually have long-term responses to doxorubicin-based chemotherapy ($\pm$ radiotherapy). In particular, the CHOP regimen followed by radiotherapy has been shown to achieve ~80% overall survival after 5 years of follow-up [8]. Addition of the anti-CD20 monoclonal antibody rituximab to CHOP (R-CHOP) has further improved the outcome in these patients [9].

R-CHOP (standard or dose dense) has also improved the outcome for patients with stage II bulky or stage III–IV DLBCL [10,11]. DA-EPOCH-R (dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin and rituximab) is effective for patients with advanced disease and has also been shown to overcome certain adverse risk factors, such as high proliferation rate [12]. Alternative therapies are available for patients with relapsed or refractory DLBCL, such as autologous and allogeneic stem cell transplantation. Investigational agents such as proteasome inhibitors and immunomodulatory agents are also being explored for patients with DLBCL with limited treatment options, and their potential applications will be discussed in this manuscript.

Bcl-2 as a therapeutic target

The Bcl-2 family of proteins regulates apoptosis and is a potential target for therapeutic intervention in several malignancies. Bcl-2 regulates the permeability of mitochondrial membranes and can make tumor cells resistant to multiple death stimuli [13,14]. There are three classes of proteins in the Bcl-2 family, which are categorized according to the

conservation of their Bcl-2 homology (BH1–4) domains: multidomain anti-apoptotic proteins (Bcl-2, Bcl-xl, Mcl-1, Bcl-w, and Bfl-1/A1); multidomain proapoptotic proteins (BAX and BAK), and BH3-only proapoptotic proteins (BID, BAD, BIM, PUMA, NOXA, HRK, BMF, and NBK/BIK) [13,14]. Several small molecules that work at the level of aberrant DNA transcription or mRNA translation are also being developed.

One such investigational agent is oblimersen (G3139), a Bcl-2 antisense oligodeoxynucleotide. It exerts its anticancer activity by inhibiting Bcl-2 mRNA, down-regulating Bcl-2 protein synthesis, and inducing tumor cell apoptosis in sensitive cells. In a lymphoma SCID mouse model, oblimersen and rituximab were shown to be synergistic when oblimersen was administered 2 days prior to each rituximab dose. Combination treatment resulted in improved disease control and longer survival than treatment with either agent alone or controls [15]. When this investigational treatment regimen was evaluated in a phase II trial of 46 patients with previously treated B-cell NHL, the overall response rate (ORR) was 42% (10 complete responses) and the combination was well tolerated [15]. The greatest treatment benefit was seen in patients with indolent NHL (ORR was 60%, including 8 complete responses, for 20 patients with indolent lymphoma), although responses were limited in the seven patients with recurrent DLBCL (ORR was 28%, no complete responses).

Obatoclax mesylate (GX15–070) is also being investigated in patients with advanced-stage chronic lymphocytic leukemia (CLL), a disease which expresses high levels of Bcl-2 proteins that confer resistance to apoptosis. This small molecule has been shown to overcome Bcl-2-, Bcl-xl-, Bcl-w-, and Mcl-1-mediated resistance to BAX or BAK (pan-Bcl-2 inhibitor). In rituximab-sensitive and -resistant cell lines, obatoclax induced dose-dependent cell death and decreased the rate of DNA synthesis [16]. Similar responses were seen in a Phase I, open-label, dose-escalation study, in which 26 patients with B-cell CLL who had been previously treated with standard systemic chemotherapy received up to eight cycles of obatoclax administered every 3 weeks (1-hour infusion: 3.5–14.0 mg/m²; 3-h infusion: 20–40 mg/m²) [17]. Dose-limiting toxicities were somnolence, euphoria and ataxia and the maximum tolerated dose was 28 mg/m² over 3 h every 3 weeks. One patient achieved a partial response, and patients with anemia ($n = 3$) or thrombocytopenia ($n = 4$) experienced improvements in hemoglobin and platelet counts, respectively. Circulating lymphocyte counts were reduced in 18 patients. Activation of BAX and BAK was demonstrated in peripheral blood mononuclear cells and induction of apoptosis was shown to be related to overall drug exposure as monitored by plasma concentration of oligonucleosomal DNA/histone complexes. Further studies with this agent are being conducted to evaluate its activity as part of combination therapy.

Other Bcl-2 inhibitors are being developed as potential treatments for aggressive B-cell lymphomas. ABT-737 and its oral counterpart, ABT-263, have similar Bcl-2 inhibition profiles and have demonstrated *in vitro* activity against CLL [18,19] and NHL [20] cell lines. These agents have also been shown to synergize with proteasome inhibition [20]. ABT-263 is perhaps the most clinically attractive agent because of its oral bioavailability. In animal models, ABT-737 disrupts Bcl-2/Bcl-xl interaction with pro-apoptotic proteins, leading to the initiation of apoptosis within 2 h posttreatment. In xenograft models of

aggressive B-cell lymphoma and multiple myeloma, ABT-737 has been shown to enhance the efficacy of standard therapeutic regimens even when it exhibits modest or no single agent activity [19].

Two phase I monotherapy studies are being conducted to evaluate the pharmacokinetics, safety, and preliminary efficacy of ABT-263 in patients with relapsed or refractory lymphoid malignancies (study M06-814, 3+3 Fibonacci design) and CLL (study M06-873, continuous reassessment method) [21]. Patients received ABT-263 10–440 mg (M06-814) or 10–250 mg (M06-873) on days 1–14 of a 21-day dosing cycle. To date, 72 patients have enrolled in the ongoing studies ($n = 53$, 14/21-day dosing; $n = 19$, 21/21-day dosing), and 43 heavily pretreated patients with CLL and small lymphocytic leukemia (SLL) have been treated with ABT-263 ($n = 27$, 14/21 day dosing; $n = 16$, 21/21 day dosing). ABT-263 exposure increased proportional to dose from 10 to 440 mg with a $t_{1/2}$ of 18 h. Median progression-free survival has not yet been reached (median time on study: 260 days). Among the 43 patients with CLL/SLL, four had radiographically confirmed partial response (PRs) (99, 92, 72, and 64% reduction in lymphadenopathy) and five had unconfirmed regression in lymph node size at 72, 67, 55, 53 and 51%. In addition, nine patients maintained 50% decrease in circulating absolute lymphocyte count for 2 months. Of the 43 patients with lymphoma, 10 were fludarabine and/or alemtuzumab refractory. ABT-263 exhibited a favorable pharmacokinetic and safety profile and demonstrated antitumor activity in relapsed/refractory CLL/SLL. Dose-dependent thrombocytopenia as a result of activity against Bcl-xl was observed. Further studies are being conducted to identify the optimal dose and schedule for phase II trials and continuous 21/21-day dosing (21-day cycle) following a lead-in dose is being explored in the 275 mg (M06-814) and 300 mg (M06-873) cohorts from both studies.

AT-101 is an orally-active, pan-Bcl-2 inhibitor (including Bcl-2, Bcl-xl, Bcl-w, and Mcl-1) and is being investigated in early-stage testing. This agent induces apoptosis by operating as a BH3 mimetic and an independent upregulator of Noxa and Puma. By blocking the binding of Bcl-2 family members with proapoptotic proteins and upregulating specific proapoptotic factors, AT-101 lowers the threshold for cancer cells to undergo apoptosis in various tumor types. In preclinical models, AT-101 has been shown to synergize with conventional chemotherapy in models of DLBCL and mantle cell lymphoma (MCL) [22]. One phase II study has reported results in 23 patients with untreated FL, and although treatment was well tolerated, the combination of AT-101 and rituximab achieved a response rate following induction of 26% (95% CI = 10.2–48.4), with 4% complete response (CR); the best ORR was 70% (95% CI = 47.1–86.8), with a 35% CR [23]. A randomized trial will be required to definitively determine the level of activity of AT-101 and rituximab in patients with lymphoma.

Historically, Bcl-2 expression has been shown to correlate with poor survival in patients with DLBCL [24], particularly the ABC disease subtype [3]. Recent studies suggest that although Bcl-2 expression does not predict outcome in all patients with DLBCL, it is associated with poor progression-free and event-free survival in patients with CD-10-negative disease [25]. These findings highlight the importance of prognostic markers when selecting treatment for DLBCL, and Bcl-2 remains a relevant predictor of outcome.

Application of novel agents in diffuse large B-cell lymphoma

The investigation of regimens containing novel agents with less toxicity than conventional management strategies might represent a future treatment option for patients with DLBCL who are not eligible for high-dose chemotherapy [26]. This could be of particular importance amid suggestions that more DLBCL resistant to or recurring after upfront use of rituximab-based immunochemotherapy regimens comprise a more resistant tumor phenotype. Several novel agents are being evaluated in patients with relapsed/refractory DLBCL, including immunomodulatory agents and the proteasome inhibitor bortezomib.

Role of immunomodulatory agents

Novel thalidomide derivatives (IMiDs) exert their effects via activation of the innate or adoptive immune system, modification of the cytokine micro-environment in the tumor bed, or by the inhibition of angiogenesis. Initially developed to treat multiple myeloma, these agents have demonstrated synergistic effects in combination with rituximab when evaluated in animal models of lymphoma [27]. In one study, the immunomodulatory agents lenalidomide (CC-5013) and pomalidomide (CC-4047) demonstrated the potential to increase the antitumor effects of rituximab against B-cell lymphomas and lead to the further investigation of lenalidomide and pomalidomide (Actimid®) in this setting. Addition of pomalidomide to rituximab increased median survival in mice from 38 days to 74 days ($p = 0.002$), compared with 21 days for the placebo group. The survival effects were similar, but less pronounced with lenalidomide (45 days for rituximab alone vs. 58 days for rituximab + lenalidomide; $p = 0.167$).

In a study by Reddy *et al.*, the IMiDs lenalidomide and pomalidomide increased the recruitment of natural killer cells to subcutaneous lymphoma sites in mice. This response was achieved via stimulation of dendritic cells and modification of the cytokine micro-environment associated with an increase in Mip-1, TNF- α , and interferon- γ , and they augmented rituximab-associated, antibody-dependent cellular cytotoxicity [28]. In B-cell lymphoma models, the IMiDs demonstrated antiangiogenic effects suggesting potential activity in combination with rituximab in patients with relapsed/refractory B-cell lymphoma. These *in vivo* studies lead to the initiation of a phase II, single-arm, multicenter trial evaluating the safety and efficacy of lenalidomide monotherapy in patients with relapsed or refractory aggressive NHL ($n = 49$). Patients were treated with lenalidomide 25 mg once daily on days 1–21, every 28 days, for 52 weeks, until disease progression or intolerance [29]. Approximately half of patients had DLBCL (53%), and patients were heavily pretreated (four prior treatment regimens for NHL). Objective response rate was 35%, including 12% CR/unconfirmed CR and responses were achieved in aggressive histologic subtypes (ORR 19% in 26 patients with DLBCL). Median duration of response was 6.2 months, and median progression-free survival (PFS) was 4.0 months. The most common grade 4 adverse events were neutropenia (8.2%) and thrombocytopenia (8.2%); the most common grade 3 adverse events were neutropenia (24.5%), leukopenia (14.3%), and thrombocytopenia (12.2%). The results showed that lenalidomide monotherapy is active in relapsed or refractory aggressive NHL, with manageable side effects.

The efficacy and safety of lenalidomide in patients with relapsed or refractory aggressive NHL was evaluated in the phase II, open-label, multicenter NHL-003 study [30]. Results from the subset of 73 patients with DLBCL showed an ORR of 29% with a CR rate of 4%. Median duration of response was 7.0 months at last follow-up. As reported in the study by Wiernik *et al.*, the most common grade 3 or 4 adverse events observed were neutropenia (31%) and thrombocytopenia (15%).

In a retrospective study of 19 patients with DLBCL (13 with pure DLBCL and 6 with composite histologies) who were treated with lenalidomide at the Roswell Park Cancer Institute in the USA, differences were noted in the response rates between GCB and ABC disease subtypes (unpublished data). ORR was 11% for patients with the GCB phenotype compared with 78% for the ABC phenotype ($p = 0.011$). Progression-free survival was also significantly prolonged for patients with the ABC subtype (2.4 vs. 11.2 months; $p = 0.008$). These data suggest that patients with the ABC disease subtype are likely to have the most durable response to lenalidomide therapy. Based on the findings from these studies, a phase III trial evaluating the effects of lenalidomide in patients with relapsed/refractory DLBCL is planned, and will stratify patients according to immunohistochemistry (IHC) disease subtype.

Role of the proteasome inhibitor bortezomib

Advances in gene expression profiling are helping to identify specific molecular features of lymphomas that regulate their responsiveness to chemotherapy and improve our understanding of the fundamental differences between the three disease subtypes in DLBCL [2,31]. Indeed, insights obtained from gene expression profiling studies have identified over-expression of nuclear factor- κ B (NF- κ B) as a potential therapeutic target in the ABC subtype of DLBCL, which has an inferior outcome with conventional chemotherapy compared to the GCB subtype [4,32]. Unlike GCB, both ABC DLBCL and PMBL are characterized by the constitutive activation of the NF- κ B pathway, and small molecule inhibitors of I κ B kinase have demonstrated selective toxicity *in vitro* in ABC subtypes [33].

The novel agent bortezomib, better known for its activity against multiple myeloma and MCL, is being investigated as a potential treatment for DLBCL because it blocks degradation of phosphorylated I κ B α , thereby inhibiting NF- κ B activity [34,35]. The results from a study conducted by Dunleavy *et al.* suggest that bortezomib can enhance the activity of chemotherapy in ABC type DLBCL [36]. The study involved 49 patients with relapsed or refractory DLBCL who had previously received doxorubicin-based therapy. The study was divided into two arms: in the first arm (Part A), patients received bortezomib alone and if they progressed on treatment or had very aggressive disease when they were accrued onto the study, they received bortezomib in combination with DA-EPOCH chemotherapy. While the study demonstrated that bortezomib as a single agent was ineffective in relapsed/refractory DLBCL, interestingly patients with ABC DLBCL preferentially benefited (compared to patients with GCB DLBCL) from the addition of bortezomib to doxorubicin-based treatment (Table I). These results confirm that molecular profiling can provide information that could be used to optimize (and personalize) chemotherapeutic and other therapeutic strategies.

An ongoing phase II trial in untreated patients with DLBCL is also looking at the role of proteasome inhibition in combination with immuno-chemotherapy. Leonard *et al.* have reported results in 35 patients with previously untreated disease who received CHOP-21 + rituximab (375 mg/m² per cycle) plus bortezomib (0.7, 1.0, or 1.3 mg/m²) on days 1 and 4 of each cycle [37]. Treatment was generally well tolerated, with the most frequently reported adverse event being peripheral neuropathy (55%). The ORR was 90% with a 68% CR/CRu rate; the Kaplan–Meier estimate of PFS at 2 years was 72%. Correlation of outcome with disease subtype is ongoing (by using immunohistochemistry to predict subtype), and results suggest that bortezomib might enhance the activity of chemotherapy in ABC, but not GBC DLBCL.

Other investigational and novel agents in diffuse large B-cell lymphoma

In addition to the investigational treatment strategies already outlined, many other agents targeting different signaling pathways are being explored (Table II).

MEK inhibitors

Inhibitors of the MEK/ERK signaling pathway might have applications in lymphoma management. The oncogene MCT-1 is amplified in T-cell lymphoid and NHL cell lines and subsets of primary DLBCL cells exhibit elevated levels of the MCT-1 protein [38,39]. Lymphoid cell lines overexpressing MCT-1 exhibit increased growth rates and appear to be protected against apoptosis. In addition, the MCT-1 gene might control activation of survival pathways and therefore direct inhibition of MEK, ERK or MCT-1 are interesting targets in DLBCL [40]. The most clinically advanced MEK inhibitor is AZD6244, which has shown tumor suppressive activity in preclinical cancer models including melanoma, pancreatic, colon, lung, and breast cancers. This agent is being investigated in phase II clinical trials and a protocol for evaluation of AZD6244 in patients with DLBCL is under development.

PARP inhibitors

Poly(ADP-ribosyl)ation is a post-translational modification that affects signal transduction. The enzyme responsible for the majority of poly(ADP-ribose) polymerization in cells, PARP-1, not only promotes DNA repair but also mediates a caspase-independent form of apoptosis in response to stressors such as irradiation. PARP-1 is also associated with increased NF- κ B levels and tumor resistance. Macro-PARPs are part of a large family of PARP-like proteins also called B-aggressive lymphoma proteins (BAL1, 2a/2b, 3, or PARP-9, PARP-14, and PARP-15). PARP-14 mediates regulation of gene expression and lymphocyte physiology involving IL-4 and influences pathologic processes involving B lymphocytes [41]. Therefore, there is a rationale for evaluating PARP inhibitors as potential treatments for B-cell lymphomas.

PARP inhibitors, such as BSI-201 and olaparib (AZD2281), are typically investigated in patients with solid tumors that are known to have faulty BRCA genes (specifically breast, ovarian and prostate cancers). However, ABT-888 is currently being evaluated in early clinical trials in patients with solid tumors and lymphoid malignancies, and two trials in patients with NHL have been approved by the National Cancer Institute in the USA.

ABT-888 is an orally bioavailable PARP-1 and -2 inhibitor with chemosensitizing and antitumor activities. It inhibits DNA repair and potentiates the cytotoxicity of DNA-damaging agents.

Mammalian target of rapamycin inhibitors

The phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway integrates signals from multiple receptor tyrosine kinases and regulates many cellular processes, including proliferation, growth and survival. In lymphoma cells this pathway has been shown to be upregulated [42]. Perhaps the most well-known members of this drug class are temsirolimus and everolimus, which are licensed for use in patients with renal cell carcinoma. Both these agents have been investigated in patients with NHL.

Four studies have been conducted with temsirolimus in patients with MCL and NHL [43–46]. In one phase III trial ($n = 162$), single-agent temsirolimus was associated with significant improvements in PFS and a trend toward longer OS compared with the investigators' preferred therapies in patients with relapsed MCL [44]. Similarly, in a trial of 82 patients with relapsed NHL, temsirolimus 25 mg i.v. administered weekly achieved an ORR of 35% with 25 patients maintaining stable disease [45]. Everolimus also has activity in patients with relapsed aggressive NHL. Results from a trial conducted at the Mayo Clinic and Dana Farber Cancer Institute (MC048G) showed that everolimus 10 mg/day achieved an ORR of 35% and 29% for DLBCL and MCL, respectively.

Another mTOR inhibitor with potential in lymphoid malignancies is AZD8055. This agent is being evaluated in a phase I/II, open-label, multicenter trial in patients with advanced solid tumors, lymphomas, and endometrial cancers.

Histone deacetylase inhibitors

One of the most rapidly progressing areas of research into hematological malignancies has been in the development of small molecule inhibitors of histone deacetylase (HDAC) enzymes [47]. Reversible acetylation mediated by HDAC influences a range of physiological processes, many of which are affected by cancer. HDAC inhibitors are potent antiproliferative agents, which cause cell-cycle arrest, apoptosis, cell differentiation and in some cases autophagy.

HDAC inhibitors are potent antiproliferative agents with relatively few effects on normal tissues, but the mechanism by which this antitumor activity is mediated remains unclear. A large number of clinical trials are ongoing in both solid tumors and hematological malignancies using a variety of HDAC inhibitors. In particular, the efficacy and safety of belinostat, entinostat and vorinostat are being investigated in DLBCL and other lymphomas. Perhaps their most important applications will be to produce additive or synergistic effects in combination with other agents [48,49].

Hedgehog inhibitors

Hedgehog-mediated signaling promotes the growth of solid tumors, but is also important for the development of hematological malignancies [50]. Hedgehog ligands secreted by bone

marrow, nodal and splenic stromal cells, function as survival factors for malignant lymphoma and plasmacytoma cells derived from transgenic E mu-myc mice or isolated from humans with these malignancies [51]. Hedgehog pathway inhibition in lymphoma induces apoptosis through downregulation of Bcl-2 and, *in vivo*, inhibits expansion of mouse lymphoma cells and reduces tumor mass in mice with fully developed disease. Furthermore, patients with DLBCL with high expression of the ATP-binding cassette (ABC)G2 (a downstream target of sonic hedgehog signaling) showed reduced overall survival and failure-free survival compared with patients whose tumors had low or no expression of ABCG2 [52]. This suggests that sonic hedgehog signaling proteins and ABCG2 are aberrantly expressed and that ABCG2 expression has prognostic implications in DLBCL. Thus, hedgehog signaling dysregulation might be involved in the pathogenesis of DLBCL.

One hedgehog inhibitor, GDC-0449, has shown promising results in patients with metastatic basal cell carcinoma. GDC-0449 is currently being evaluated in a pivotal trial in advanced basal cell carcinoma, and in phase II trials in metastatic colorectal cancer and advanced ovarian cancer. Further trials are planned in medulloblastoma, small cell lung cancer and pancreatic cancer in association with the NCI, and the molecule is expected to be tested in other indications, including acute myeloid leukemia (AML) and multiple myeloma (MM).

Targeted therapies (anti-CD19)

CD19 is a transmembrane glycoprotein that regulates B-cell receptor signaling in conjunction with CD21 and CD81. CD19 is overexpressed in most NHLs and leukemias. The success of chemotherapy + rituximab in NHL has clearly demonstrated the utility of immunotherapies in B-cell disorders. Although CD19 cell surface expression is lower than CD20, it begins earlier and persists longer through B-cell maturation [53]. Consequently, the spectrum of lymphoid malignancies expressing CD19 is broader and the potential application of anti-CD19 therapy is more widespread than anti-CD20 therapy.

Several anti-CD19 antibodies are at various stages of clinical development. The most advanced agent is blinatumomab, which is undergoing phase I and II testing in acute lymphoblastic leukemia (ALL) and NHL, respectively. The results from an ongoing phase I trial of blinatumomab in relapsed NHL showed dose-dependent activity in MCL, FL, and CLL with responses observed in 11 out of 27 patients treated at doses of 0.015 mg/m² per day and higher (five CRs and six PRs) [54]. At the 0.060 mg/m² per day dose level, seven out of seven patients had objective responses. Besides one relapse after 14 months, no treatment failure had been observed for responders at dose levels of 0.030 and 0.060 mg/m² per day. Five patients at these dose levels had ongoing responses in excess of 6 months. Partial remissions converted into CRs in two patients 4 weeks after end of infusion, suggesting either a reduction in lesion size due to efflux of a previously expanded T-cell pool or prolonged T-cell activity. Two other anti-CD19 targeted therapies are undergoing trials in humans (SAR3419 and MDX-1342), and two others are in preclinical testing (Table II) suggesting that this approach is feasible and could have major implications for the treatment of relapsed/refractory NHL.

The range of anticancer agents undergoing preclinical and clinical testing is vast and has significant implications for the treatment of DLBCL and other types of NHL. Identifying

combinations of current and investigational agents that have improved efficacy in resistant disease subtypes, and that have acceptable tolerability profiles will be a major challenge. In addition, clinical trials of single agents might not give clear indications of true efficacy, and study designs and endpoints may need to be adjusted to allow for the activity of these agents when used in combination with conventional chemotherapy.

Dosing schedule considerations for clinical trial design

Many compounds show activity when evaluated *in vitro* and *in vivo* within the laboratory setting. However, translating these data into safe and convenient dosing schedules for human testing while demonstrating potential efficacy, can be complex. This can be illustrated using examples of drug candidates that performed well *in vitro*, but were less obviously effective when tested *in vivo* and in patients.

Bcl-2 inhibitors provide an ideal example of this problem. In principle, Bcl-2 inhibitors should increase the efficacy of conventional chemotherapy; however, route and timing of administration can lead to unexpected results when applied in the clinical setting. The ability of a combination of bortezomib and oblimersen to sensitize human lymphoma cells to cyclophosphamide was investigated by O'Connor *et al.* [55]. Cytotoxicity assays were used to determine whether there were any additive or synergistic effects from combining bortezomib, oblimersen, and cyclophosphamide. Then different dosing schedules of the combination were tested *in vivo* using a SCID mouse model of human lymphoma. Results showed that the effects of bortezomib and oblimersen were additive and markedly enhanced the efficacy of cyclophosphamide, but that simultaneous administration of all three drugs resulted in compromised treatment outcomes. However, animals treated with the triple combination in a schedule-dependent manner (48 h of pre-exposure to oblimersen prior to bortezomib and cyclophosphamide) demonstrated complete tumor regression (histologically confirmed by marked necrosis and caspase-3 activation).

Similar studies have been conducted with the Bcl-2 inhibitor ABT-737 evaluating the effects of simultaneous administration, with both 24 and 48 h of pre-exposure prior to administration of a proteasome inhibitor. When used as single agents in the same concentrations as in the combination therapies, ABT-737 and proteasome inhibitors modestly decreased mitochondrial membrane potential. In contrast, the combination of drugs strongly induced mitochondrial membrane depolarization and induced apoptosis [20]. Analysis of different dosing schedules highlighted differences in the efficacy of different regimens in SCID mouse models of MCL. Indeed when bortezomib was administered as 0.5 mg/kg on day 1 and at 0.75 mg/kg on days 5 and 10 in combination with ABT-737, tumor responses were more pronounced than other regimens delivering the same total does of bortezomib.

The same group published findings from investigations conducted using AT-101 (a BH3 mimetic) [23]. *In vitro*, AT-101 exhibited concentration- and time-dependent cytotoxicity against lymphoma and multiple myeloma cell lines, and enhanced the activity of cytotoxic agents. In MCL cell lines, AT-101 was synergistic with carfilzomib, etoposide, doxorubicin, and cyclophosphamide. When AT-101 was administered sequentially with cyclophosphamide, but not simultaneously, the drugs acted synergistically in a cell line of

transformed large B-cell lymphoma. Furthermore, addition of AT-101 to cyclophosphamide and rituximab in a SCID mouse model of drug-resistant B-cell lymphoma enhanced the efficacy of conventional therapy, but only when given in a schedule-dependent manner.

The efficacy of the investigational antifolate pralatrexate is also affected by dose scheduling. Pralatrexate has been shown to have greater potential activity against NHL than methotrexate [56], and in combination with gemcitabine is expected to show similar synergistic effects to sequential administration of methotrexate and cytarabine with reduced toxicity. In a study conducted by Toner *et al.*, the combination of pralatrexate and gemcitabine was superior to methotrexate and cytarabine combinations, but showed schedule dependency [57]. *In vivo*, complete remission of tumors was only achieved in animals receiving pralatrexate followed by gemcitabine. Based on these findings and the positive results of a phase I study with pralatrexate conducted in patients with cutaneous T-cell lymphoma [57,58], a phase I/IIa study evaluating varying dosing schedules of pralatrexate and gemcitabine in patients with relapsed or refractory lymphoproliferative malignancies was initiated. Interim results have shown that the combination is feasible (approximate response rate of 21%) with acceptable toxicity when administered every 2 weeks. The maximum tolerated dose of each drug was increased by 50% when given on the same day compared with dosing on sequential days. Phase II trials evaluating same-day (pralatrexate 10 mg/kg and gemcitabine 400 mg/kg) and sequential (pralatrexate 15 mg/kg and gemcitabine 600 mg/kg) dosing with the 2-weekly schedule are planned.

The results of these studies show the utility of preclinical animal models in understanding the pharmacology of investigational agents, but suggest that they cannot be used to predict efficacy in humans. They also highlight the need to explore different dosing schedules, which is becoming increasingly important given the number of targeted agents that are being developed and are currently under investigation.

Discussion

DLBCL is the most common type of aggressive NHL, and the type and success of treatment used depends entirely upon molecular subclassification and clinical prognostic indices. Gene expression studies have identified at least three subtypes of DLBCL and provided some insight into why certain subtypes may have a worse outcome. Expression of Bcl-2 is a marker of reduced survival in the ABC subtype, whereas the presence of Bcl-6 (a marker of GCB type DLBCL) predicts a favorable response to CHOP-based therapy. Such knowledge of prognostic markers is essential to assist with optimizing treatment strategies for patients who are likely to respond, and for identifying patients who might benefit from enrolment in clinical trials of regimens incorporating novel agents, or for those whose disease is likely to be refractory to, or relapse after, initial treatment.

Both lenalidomide and bortezomib with chemotherapy have demonstrated efficacy in relapsed or refractory DLBCL; particularly the ABC subtype. This activity in the ABC subtypes, at least for bortezomib, is putatively due to its inhibitory effects on the NF- κ B signaling pathway. Both these agents appear to improve the cytotoxic effects of

chemotherapy, and lenalidomide might also further improve the efficacy of rituximab-based regimens.

Advances in gene expression technology have also facilitated the development of small molecules that target specific signaling pathways in lymphoma cells. For example, many Bcl-2 inhibitors are undergoing clinical testing in the hope that they will eventually offer expanded treatment options for patients with aggressive, advanced-stage DLBCL. HDAC inhibitors also show great potential in the treatment of lymphoma because of their selectivity, and the results of trials investigating combinations of belinostat, entinostat, and vironstat with conventional chemotherapy are eagerly awaited.

The development of immunotherapy has revolutionized the treatment of many cancers and NHL is no exception. The introduction of rituximab improved treatment outcome of patients with DLBCL, and CD19-targeted antibody therapy is now also being explored. The lower, yet more frequently expressed levels of CD19 compared with CD20 have the potential to extend the benefits of monoclonal antibody therapy to different types of cancer and further increase treatment options for patients with relapsed/refractory disease. Although tumor responses to investigational agents in patients with DLBCL have been mixed, it will be important to ensure that dosing and timing of administration of drug combinations are optimized so that the true efficacy and safety profiles of these regimens can be determined. As already observed with oblimersen and ABT-737, taking time to develop optimized dosing schedules for combination therapy incorporating targeted therapies can be crucial to observing the maximum efficacy of any combination. In particular, using sequential administration to incorporate developmental agents in existing treatment regimens seems to produce the best results. Results of trials with pralatrexate and gemcitabine exploring the efficacy and safety of various dosing and administration schedules are expected to improve on the responses and dose-limiting toxicities observed with methotrexate and cytarabine. The designs of clinical trials exploring combinations of novel agents must, however, take into consideration that such combinations can result in unanticipated toxicities that are not seen when the agents are used alone.

Our improved understanding of lymphoma biology and tumor cell signaling pathways has been instrumental in identifying a host of new targets for drug intervention in DLBCL. This in turn, has lead to the development of several new classes of agents specifically targeting the activities of tumor cells without adversely affecting normal tissue. Advances in gene expression profiling are helping to clarify which patients are likely to respond to which treatments, and to provide more tailored and effective management strategies. This is an exciting time in the evolution of DLBCL therapy, and will ultimately result in higher response rates and durable remissions for patients with difficult to treat disease subtypes.

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Table I.

Response rates with DA-EPOCH-B in patients with relapsed and refractory DLBCL.

Treatment	<i>n</i> (%)	CR	PR	ORR	<i>p</i>
All patients	44	8 (18)	7 (16)	15 (34)	–
DLBCL (<i>de novo</i>)	31 (70)	7 (23)	6 (19)	13 (42)	0.63
Molecular subtypes	27	6 (22)	6 (22)	12 (44)	
ABC	12 (44)	5 (42)	5 (42)	10 (83)	<0.001
GCB	15 (56)	1 (67)	1 (7)	2 (13)	

DLBCL, diffuse large B-cell lymphoma; CR, complete response; PR, partial response; ORR, overall response rate; ABC, activated B-cell; GCB, germinal center B.

Table II.

Investigational agents being evaluated or with potential to be evaluated in patients with DLBCL by the National Cancer Institute.

Drug class	Product name	Disease target
AKT inhibitors	MK 2206	Multiple tumor types
CDK inhibitors	SCH 727965	Multiple tumor types including mantle cell lymphoma and B-cell lymphomas
MEK inhibitors	AZD 6244	Solid tumors, Hodgkin lymphoma and DLBCL
PARP inhibitors	ABT-888	Multiple tumor types including NHL
mTOR inhibitors	Temsirolimus	Multiple tumor types including, MM, NHL and DLBCL
	AZD 8055	
	Everolimus	
HDAC inhibitors	Belinostat	Multiple tumor types including NHL and DLBCL
	Entinostat	
	Vorinostat	
Hedgehog inhibitors	GDC-0449	MM/AML/solid tumors
TRAIL	AMG 655	Pancreatic cancer
Anti CD19	Blinatumomab	NHL and ALL
	SAR3419	NHL
	MDX-1342	Rheumatoid arthritis and CLL
	XmAb5574	NHL/CLL/ALL
	SGN-19A	NHL/CLL/ALL

AKT; CDK, cyclin-dependent kinase; MEK, methyl ethyl ketone; PARP, Poly (ADP-ribose) polymerase; mTOR, mammalian target of rapamycin; HDAC, histone deacetylase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DLBCL, diffuse large B-cell lymphoma; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.