Rational Approaches to Design of Therapeutics Targeting Molecular Markers

Richard J. Klasa, Alan F. List, and Bruce D. Cheson

This paper introduces novel therapeutic strategies focusing on a molecular marker relevant to a particular hematologic malignancy. Four different approaches targeting specific molecules in unique pathways will be presented. The common theme will be rational target selection in a strategy that has reached the early phase of human clinical trial in one malignancy, but with a much broader potential applicability to the technology.

In Section I Dr. Richard Klasa presents preclinical data on the use of antisense oligonucleotides directed at the *bcl-2* gene message to specifically downregulate Bcl-2 protein expression in non-Hodgkin's lymphomas and render the cells more susceptible to the induction of apoptosis.

In Section II Dr. Alan List reviews the targeting of vascular endothelial growth factor (VEGF) and its receptor in anti-angiogenesis strategies for acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS).

In Section III Dr. Bruce Cheson describes recent progress in inhibiting cell cycle progression by selectively disrupting cyclin D1 with structurally unique compounds such as flavopiridol in mantle cell lymphoma as well as describing a new class of agents that affect proteasome degradation pathways.

I. Antisense Oligonucleotides Directed at the *Bcl-2* Gene Message in Non-Hodgkin's Lymphoma

*Richard J. Klasa, MD**

Hematologic malignancies in general and non-Hodgkin's lymphomas (NHLs) in particular are frequently associated with gain of function mutations, many characterized by balanced chromosomal translocations. Genome-wide surveys of gene expression are identifying both known and new transcripts that are overexpressed in different histological subtypes of lymphoma.[1] As we develop molecular classifications of these diseases it is assumed that a few key genes will account for the particular survival advantage conferred on malignant lymphocytes as compared to their normal counterparts. These genes and their protein products would provide rational targets for the development of therapeutic strategies to reverse this upregulation associated with the malignant phenotype.

* Division of Medical Oncology, British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver BC V52 4E6 Canada

Dr. Klasa is on the international advisory boards of Schering AG and Hoffmann-LaRoche, and is on the speakers' bureau and advisory board for Berlex Canada.

Non-Hodgkin's Lymphoma and Bcl-2

Over the past quarter century cytogenetic analysis has identified a number of reciprocal translocations that frequently occur in histologically identifiable subtypes of NHL.[2] The transposition of the *bcl-2* gene to the immunoglobulin heavy chain promoter region in the t(14;18) translocation is associated with > 90% of follicular lymphomas (FL) at diagnosis and 10% of diffuse large B-cell lymphomas (DLBCL), making it the most frequent event identified in NHL. Additionally, 50% of DLBCL overexpress the BCL-2 protein through other mechanisms, such as gene duplication, and are associated with a poorer prognosis after anthracycline-based combination chemotherapy.[3] BCL-2 is also overexpressed in mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM) and acute myelogenous leukemia (AML).[4] This same widespread pattern of distribution is also seen in a variety of solid tumors including melanoma, small cell lung carcinoma, and colon carcinoma as well as prostate and breast carcinoma, especially once the last two are hormone independent. The obvious conclusion is that BCL-2 overexpression, by whatever means, confers a fundamental advantage to malignant cells and that disruption of this overexpression might have therapeutic potential.

Bcl-2 is an anti-apoptotic member of a large family of genes involved in the regulation of programmed cell death.[5,6] Pro-apoptotic (BAX and BCL-Xs) and anti-

apoptotic (BCL-2, BCL-X_L) molecules reside within the inner mitochondrial membrane and can homo- and heterodimerize upon appropriate stimulus. These interactions control the release of substances such as cytochrome C from the mitochondria into the cytosol through the opening or closing of specific pores in the membrane, with permeability determined by the relative abundance of the different molecules. Cytochrome C is central to the activation of caspases that initiate the apoptotic process. Thus, an overabundance of BCL-2 can prevent or retard activation of the apoptotic machinery and allow survival under conditions that might otherwise be lethal to a cell (**Table 1**).

Antisense Oligonucleotide

Reverse complementary or "antisense" oligonucleotides (ASOs) are short sequences of single stranded deoxyribonucleotides complementary to the coding regions of a gene that are designed to hybridize by Watson-Crick base pairing to messenger-RNA (m-RNA) sequences and thus facilitate their degradation.[7,8] Naturally occurring antisense sequences have been identified as regulators of gene expression in a number of systems, supporting their potential for therapeutic development.[9,10] The formation of a heteroduplex of m-RNA with the DNA of the ASO engages RNaseH, an enzyme that proceeds to specifically cleave off the m-RNA moiety, destroying the message and putatively leaving the therapeutic ASO molecule able to hybridize to another message sequence.[11] This results in a reduction in the target m-RNA pool, which subsequently leads to reduction in the specific protein encoded (**Figure 1; see color page 551**). The presence of the ASO may also prevent the m-RNA from appropriately docking with the ribosomal machinery that would allow translation into a functional protein. The end result is loss of expression of that protein in the cell.

ASOs of 16-24 bases in length provide target specificity while shorter or longer sequences can result in random hybridization within the transcript repertoire. Selecting the target areas within a messenger RNA must ultimately take into account its tertiary structure, which will determine the accessibility of an area for hybridization. These target areas are defined in oligonucleotide arrays where the entire antisense sequence to an m-RNA is displayed in overlapping segments on a slide. The intensity of hybridization of the labeled message determines the candidate therapeutic ASOs.[12] Screening of oligonucleotide libraries has also identified RNA sites that are most accessible to hybridization and correlated these sites with protein downregulation and biological function.[13,14] More empirically, the first 6 codons of the open reading frame downstream of the AUG start site have repeatedly been found to be accessible to hybridization and have been chosen for initial development of ASOs against a number of genes.

As organisms have developed a sophisticated system for dealing with rogue strands of DNA both inside and outside the cell, the development of therapeutic molecules required chemical modifications to confer nuclease resistance and a favorable pharmacokinetic profile.[15,16] Substitutions in the phosphodiester linkage of the bases in the ASO backbone has yielded a number of molecules now in clinical development with phosphorothioates being the most widely studied first generation molecules (**Figure 2**). The sulfur substitution yields an ASO that is nuclease resistant and capable of entering the cell. It demonstrates good hybridization kinetics and has little in the way of non-sequence-dependent effects or toxicities at concentrations required to downregulate the target message. Additionally, although in tissue culture a delivery system such as cationic lipid is required for efficient intracellular penetration of these highly charged molecules, in vivo ASOs have been shown to be active in free form, possibly due to interaction with blood lipoproteins.[17,18]

The correlation of a biologic effect with the specific downregulation of target message and protein in vivo has been a major focus of the development of ASOs. However, ASOs can be very potent immune stimulators, by virtue of unmethylated CpG motifs presented in the context of certain flanking sequences, and therapeutic

Table 1. Properties of BCL-2.

- Oncogenic protein
- Anti-apoptotic
- Mitochondrial, endoplasmic reticulum, nuclear membrane localizations
- Homotypic and heterotypic dimerization within family
- Membrane channel/pore function
- Cytochrome C release from mitochondria via BCL-2 family channels regulates cell fate under stress

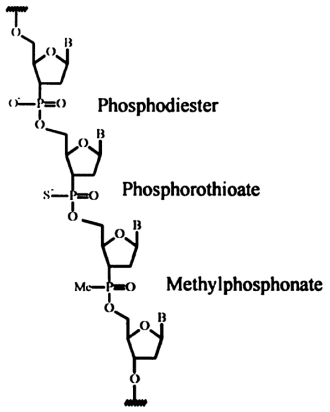

Figure 2. Phosphodiester substitutions in first generation antisense oligonucleotides.

Antisense (G3139)	(AS)	5′ tct ccc agc gtg agc cat
Reverse Polarity (G3622)	(RP)	5′ tac cga gtg cga ccc tct
Mismatch (G4126)	(MM)	5′ tct ccc agc atg tgc cat

Figure 3. Oligodeoxynucleotides targeting Bcl-2.

activity could in part be attributed to nonspecific systemic immune effects rather then to a specific ASO/mRNA interaction.[19-22] Additionally, structures such as guanosine quartets can demonstrate sequence specific but non-antisense biological activity in vitro.[23,24] Experimental designs therefore strive, by the use of appropriate control oligonucleotides (sense, missense, one and two base mismatch mis-sense) and various strains of immunodeficient animals, to isolate effects that can be attributed to the specific downregulation of target message and protein (**Figure 3**).

ASOs directed at *bcl-2*

An 18-mer phosphorothiolated oligonucleotide, G3139, directed against the first six codons of the open reading frame of the *bcl-2* gene message has been developed by Genta Pharmaceuticals (Figure 3). Studies of G3139 utilizing the BCL-2 overexpressing lymphoma cell lines DoHH-2 and SU-DHL-4 in vitro have shown downregulation of message and protein expression.[25] Tumor xenograft models in SCID mice have demonstrated therapeutic activity that is specific when compared to control animals as well as animals treated with reverse polarity sense, 2-base mismatch mis-sense and non-sense oligonucleotides.[25,26] Extensive pharmakokinetic as well as toxicity studies have been performed identifying a dose range with a good therapeutic index.[15,16] These findings supported the development of clinical trials using G3139 alone as treatment for BCL-2 overexpressing follicular lymphomas.

Further therapeutic potential is suggested by in vitro experiments confirming that bcl-2 plays a major role in the response of malignant cells to various stresses which produce cellular damage, including chemotherapy (**Figure 4**).[4,27,28] Malignant cell lines transfected with a *bcl-2* gene with resultant overexpression of the protein product demonstrated increased resistance to various chemotherapeutic agents.[29-32] Additionally, cell lines overexpressing BCL-2 were rendered more sensitive to killing by chemotherapeutic agents when either antisense oligonucleotides directed at the *bcl-2* message were introduced into the culture or the cells were transfected with a vector bearing the antisense sequence.[33,34] This has been correlated with a demonstration of downregulation of bcl-2 expression. With this as background, we set out to test the in vivo combination of ASOs targeting *bcl-2* with a cytotoxic agent commonly used in the treatment of lymphoma.

ASOs to *bcl-2* and Chemotherapy

Escalating doses of G3139, cyclophosphamide and the combination of both agents were evaluated in severe combined immunodeficiency (SCID) mice bearing a systemic human DoHH2 lymphoma xenograft.[35] Experiments confirmed that G3139 was able to downregulate BCL-2 expression in vitro and that treatment with G3139 alone resulted in prolongation of median survival and cure of some animals. (**Figure 5**, left panel). This effect was dose and schedule dependent with no long-term survivors seen when a dose of 5mg/kg was given daily for 14 consecutive days as opposed to > 40% when the dose was increased to 12.5 mg/kg on the same daily schedule or either 5 or 12.5 mg/kg were administered for 14 treat-

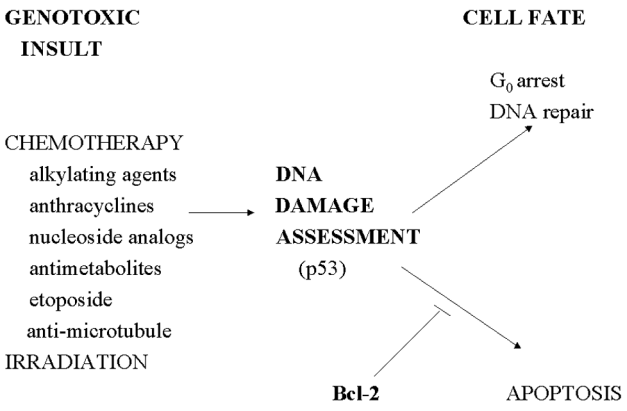

Figure 4. Targeting Bcl-2 may promote apoptosis following chemotherapy and irradiation.

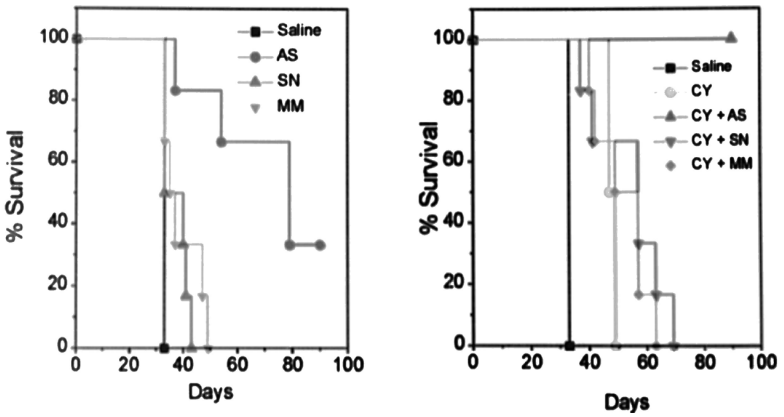

Figure 5. Survival of cohorts of 6 mice treated with oligonucleotides alone (left panel) or with cyclophosphamide (CY) (right panel).

All six surviving animals treated with cyclophosphamide and G3139 sacrificed at 90 days with no molecular evidence of disease detected.

Saline=control animals ; AS=antisense oligonucleotide G3139 directed at *bcl-2* ; SN=reverse sequence sense control ; MM= 2-base mismatch control.

ments on alternate days (28-day schedule). Similarly, cyclophosphamide treatment alone resulted in no long-term survivors at lower doses but was able to cure animals at high doses. The addition of G3139 to low dose cyclophosphamide resulted in the cure of the majority of animals (Figure 5, right panel). The interaction between the two agents does show dose-response correlations; for the two low doses of cyclophosphamide tested, increasing the dose of G3139 from 2.5 to 5 mg/kg resulted in longer median survivals and overall increase in long-term survivors. A rather striking result was achieved when a completely ineffective dose of cyclophosphamide (15 mg/kg, median survival 36 days and no long-term survivors) was combined with a modestly effective dose of G3139 (2.5 mg/kg, 61 day median survival and 16% long-term survivors) to produce a 72-day median survival and 50% long-term survivors. Mice sacrificed at 90 days showed no histological evidence of any disease in all tissues analyzed, including immunoperoxidase staining for BCL-2, or molecular detection of *bcl-2*, by PCR. This suggests that chemotherapy at very modest doses could be made much more effective without increasing toxicity when combined with an antisense oligonucleotide. Such an increase in the efficacy of currently available agents could significantly alter the prognosis of a large number of moderately chemotherapy sensitive human tumors, resulting in longer median survivals and increasing the potential for cure.

The model has direct relevance to the clinical situation faced in NHL, where patients typically present with a chemotherapy-sensitive tumor at diagnosis that regresses only to recur within months to years post-treatment. The DoHH2 cell line was derived from a follicular lymphoma carrying a t(14;18), which results in *bcl-2* gene overexpression. The aggressive nature of the disease in this model is, however, more suggestive of a transformation to a higher-grade histology, a common event in follicular lymphoma. Indeed, a recent re-exploration of the molecular and cytogenetic features of the cell line, using more sensitive detection techniques, has revealed a second translocation involving the c-myc oncogene with a resultant derivative chromosome 8 carrying t(8;14;18).[36] We have recently described this phenomenon of double translocation of both bcl-2 and c-myc in a subset of patients with small non-cleaved cell (Burkitt-like) lymphoma, which represents a very aggressive form of the disease.[37]

Clinical Studies

G3139 has been studied as a single agent in a phase 1 trial in heavily pretreated (median of 4 prior regimens) patients with relapsed NHL.[38,39] Twenty-one patients with follicular (9), small lymphocytic (8), diffuse large B-cell (3) or mantle cell (1) lymphomas that expressed BCL-2 were treated at 8 dose levels ranging from 4.6 to 195.8 mg/m^2/day by continuous subcutaneous infusion for 14 consecutive days. No significant toxicity was seen up to doses of 110 mg/m^2/day. One complete and 2 minor responses as well as 9 disease stabilizations were seen in this heavily pretreated group. BCL-2 protein was decreased in 7 out of 16 samples examined, including 2 from accessible tumor sites and 5 samples of peripheral blood or marrow mononuclear cells.

One study combining a chemotherapeutic agent with G3139 has been reported in metastatic melanoma,[40] and studies are ongoing in a number of other solid tumors (melanoma, prostate carcinoma) and hematological malignancies (myeloma, chronic lymphocytic leukemia, and acute myeloid leukemia). A phase 1 study at our institution in relapsed follicular lymphomas combining escalating doses of both cyclophosphamide and G3139 has not identified any unexpected toxicity. The last patient enrolled has received cyclophosphamide 750 mg/m^2 with 2.3 mg/kg/day of G3139 by continuous intravenous infusion for 14 consecutive days (**Table 2**).

Conclusions

The identification of overexpression or aberrant expression of genes that result in a gain of function, through genome wide surveys of cells and tissues in varying states, will provide unprecedented insight into the biology of hematological malignancies. Specific down-regulation of such overexpression with antisense oligonucleotides allows disruption of single gene function at the messenger RNA and protein level and the study of downstream events in the involved molecular pathways both in vitro and in vivo. Genes that are critical to the differential growth and survival advantage enjoyed by malignant cells are being identified and are logical therapeutic targets. The development of ASOs directed at the *bcl-2* gene provides a model by which a systemic therapy for a metastatic disease has been taken from the laboratory through preclinical studies to early phase clinical trials, building on knowledge of this particular gene's role in cellular apoptosis. Combining multiple antisense

Table 2. Phase I study of G3139 and cyclophosphamide in relapsed follicular lymphoma.

Level	G3139 mg/kg/day CIVI (day 1 to 14)	Cyclophosphamide mg/m^2/IV (day 8)
1	0.6	250 done
2	1.2	250 done
3	2.3	250 done
4	2.3	500 done
5	2.3	750 ongoing
6	3.1	1000
7	5.0	1000

strategies with other therapeutic modalities has the potential to increase the specificity of the treatments available to our patients, thus improving their efficacy and reducing toxicity.

II. Targeting Angiogenesis in Hematologic Malignancies

*Alan F. List, MD**

The seminal observations that the growth and metastatic potential of solid tumors is dependent upon the formation of new blood vessels triggered an enormous expansion in angiogenic research that has yielded novel therapeutics targeting an array of angiogenic molecules. Investigations of the relevance of angiogenesis in hematologic malignancies are still at an early stage, but accumulating evidence indicates that the angiogenic profile of many hematologic malignancies is distinct from that of solid tumors. As progeny of a common endothelial and hematopoietic stem cell, hematologic malignancies may elaborate and respond to angiogenic factors in a paracrine or autocrine fashion, contributing to tumor cell survival and expansion, adhesion, bone resorption and immune suppression.

Angiogenesis

Blood vessel development is characterized by two distinct biologic processes, vasculogenesis, and angiogenesis. *Vasculogenesis*, which is largely restricted to embryonic development, involves de novo endothelial cell differentiation from mesodermal precursors as the prerequisite for coordinated blood vessel generation.[1] Angiogenesis, the process of new blood vessel formation from preexisting vessels, is responsible for the generation of neovasculature in adult life, and occurs physiologically during wound healing and within female reproductive organs during the menstrual cycle, as well as in pathologic conditions such as proliferative retinopathy, arthritic synovium, and human malignancies.[2] Angiogenesis is a multistep process that includes both activation and resolution phases. The *activation phase* is responsible for the sequential events of basement membrane degradation, endothelial cell proliferation and migration, and capillary lumen formation. The *resolution phase* is responsible for the maturation and stabilization of the newly formed microvasculature through the recruitment of pericytes, promotion of basement membrane reconstitution, and subsequent extinction of the endothelial cell mitogenic response. A large number of pro-angiogenic molecules and endogenous angiogenesis inhibitors that coordinate the angiogenic response have been delineated (**Table 3**).[3] Vascular endothelial growth factor (VEGF), first identified in 1989 and later isolated from the HL-60 myeloid leukemia cell line, is a critical regulator of vascular development that is responsible for activation of endothelial cell proliferation during vasculogenesis and the direction of capillary sprouting during angiogenesis.[4] Indeed, gene inactivation studies indicate that VEGF is essential to the neoplastic angiogenic response.[5]

* Arizona Cancer Center, 1515 N Campbell, Room 3945, PO Box 245004, Tucson AZ 85774

VEGF and Receptor Tyrosine Kinases

The VEGF-A molecule is a disulfide-linked homodimer represented by five different isoforms generated by alternate exon splicing of gene message.[6] The corresponding VEGF monomers range in size from 121 to 206 amino acids, with smaller molecules (i.e., 121, 145, and 165 amino acids) representing the secreted and diffusable isoforms, whereas the larger proteins (189, 206 amino acids) are sequestered by heparin sulfate residues present on cell surfaces or within the extracellular matrix.[7,8] Although all isoforms are biologically active, the $VEGF_{165}$ isoform predominates and is recognized as a more potent and bioavailable endothelial cell mitogen.[9,10] Recent investigations indicate that the VEGF family is composed of five members in addition to the prototype, VEGF-A, including VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF (placental growth factor).[6] Within the arterial wall, VEGF is produced by smooth muscle cells in response to oxidative stress and other stimuli.[11] Autocrine production of VEGF and corresponding receptor upregulation is also demonstrable in endothelial cells in response to hypoxia, nitric oxide, VEGF deprivation and other cellular stresses.[12,13]

Trophic response to the VEGF family members is directed by selective interaction with structurally homologous type III receptor tyrosine kinases (RTKs), including VEGFR-1, originally termed *fms*-like tyrosine kinase (*FLT*-1),[14] VEGFR-2 or kinase insert domain-containing receptor (KDR)/fetal liver kinase-1 (*flk*-1),[15] and the recently characterized VEGFR-3 or *FLT*-4 receptor (**Figure 6**).[16] Each of these receptors contains seven extracellular immunoglobulin homology domains that create the ligand-binding site, a single short transmembrane-spanning sequence, and a cytoplasmic tail that contains the tyrosine kinase domain akin to that of the *c-kit* and PDGF-receptors.[17,18] The external ligand-binding component of VEGFR-3 differs from the other VEGF receptors in that the fifth immunoglobulin domain is cleaved during receptor processing to yield covalent, disulfide-linked subunits.[19] In adults, VEGFR-1 and VEGFR-2 expression is limited to the vascular endothelium, monocytes (VEGFR-1), and primitive hematopoietic precursors (VEGFR-2), whereas VEGFR-3 is re-

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