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Antisense therapy for cancer

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1. Introduction

Antisense oligonucleotides (AO) are short (typically 13–20 bases in length) sequences of single-stranded DNA or RNA complementary to expressed genes and are chemically modified to protect them from enzymatic breakdown. They are designed to hybridise by Watson–Crick base pairing with mRNA transcripts encoding proteins of interest and in this way 'silence' the expression of that particular gene and subsequently its protein product. The earliest attempt to inhibit gene expression using AO was reported in 1978. The Rous sarcoma virus was downregulated when exposed to a 13-base oligonucleotide complementary to the 3′reiterated terminal sequences in a chick embryo fibroblast. Technology was developed in the early 1980s for automated synthesis of oligonucleotides with appropriate modifications of the backbones to protect against nuclease digestion and breakdown and greatly prolonging the duration of effect. With the great economy of scale, it has opened up the prospect of harnessing 'gene silencing', by synthetic oligonucleotides as a therapeutic tool for cancer. However, during the development of AO therapy, there have been a number of design and development problems to overcome.

1.1. Specificity of antisense oligonucleotides

mRNA has a three-dimensional tertiary structure that essentially enables the molecule to fold back onto itself. The result is that only certain areas with the mRNA molecule are accessible to AO. Any AO outside these accessible sites will not be functional due to their inherent inability to bind to the mRNA. In order to identify accessible sites, there are two essentially empirical approaches that can be used. The most elegant has been developed by Prof. Southern and involves the use of DNA 'chip' technology. AOs are directly synthesised onto a glass slide with a complete representation of consecutive nucleotide sequences singly or in groups of 2–20. The target RNA is labelled with radioactive material and hybridised to the 'chip'. The area of strongest radioactive signal represents the areas of optimal AO binding and defines the accessible sites in the tertiary mRNA molecule. The second method uses random libraries of AOs and looks for sites of mRNA cleavage with the RNAseH enzyme. Prior to these approaches for enzyme selection, many AOs were selected randomly and many never demonstrated effective downregulation of the target protein. Essentially, these were ineffective AOs of no therapeutic use. It cannot be overstressed how important it is to select an AO that has the capacity to bind strongly to the target mRNA. Without this ability, the effect of the AO cannot be attributed to an antisense action. Such molecules have contributed to the early scepticism concerning antisense therapy.

1.2. Metabolism and pharmacokinetics

The backbone of AO need to be modified from the oxygen (phosphorodiester) atom to an alternative such as the sulphur atom phosphorothioate (PS) in order to confer resistance to nuclease degradation. There are now a number of modifications although only the PS modification has been used extensively in human clinical studies.

The PS modification does cause some non-specific inhibition of cell growth and non-sequence dependent toxicities at higher doses, due in part to the strong protein binding related to the sulphur molecules.

The pharmacokinetics of PS molecules have been extensively studied. They are strongly protein-bound with a large volume of distribution. They can be

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administered as infusions subcutaneously or intravenously. Thirty percent of the dose is excreted in the urine within 24 h; however, there are detectable levels in most tissues in excess of 48 h, with only 15–50% degradation. Blood and plasma clearance has a terminal half life in excess of 40–60 h. The use of slow-release pumps will prolong the bioavailability of the molecule and increase steady-state levels. The PS molecules are found at their highest levels within the liver, kidney, bone marrow, skeletal muscle and skin; however, there is no significant crossing of the blood–brain barrier.

Toxicities are in general minimal below a dose of 6 mg/kg/day in humans for PS molecules; however, above this dose, toxicites related to the phosphorothioate backbone are seen and include reversible thrombocytopaenia and mild hyperglycaemia. Mild skin irritation may also be seen with subcutaneous infusions at the site of injection. Second-generation backbone chemistries may, in part, reduce these toxicities.

1.3. How effective is antisense?

There have been a number of false dawns with AOs in part due to the recognised inadequacies already described (nuclease degradation, chemistry, purification, cost, delivery and cellular bioavailability) and poor sequence specificity. The latter has probably been one of the major shortcomings. However, additional non-specific sequence effects can also occur. For example, a succession of four guanosines in the AO molecule may result in the formation of higher order structures that will inhibit cell proliferation, while certain CpG motifs will result in B-cell and NK-cell stimulation with the release of cytokines such as TNF and interferon.

Despite some of these non-specific effects, it is becoming clear that an AO that shows specific binding to the target mRNA, cleaves it in the presence of RNAseH and causes a reduction in the target protein, is an effective AO for therapy.

1.4. Antisense targets for cancer

There are a host of targets that have been investigated for anticancer therapy, some of which have been taken to the clinic (see Table 1). These include Bcl-2, NF-KB (p65), c-myb, c-myc, ERBB2, jun, fos, Ha-ras, Ki-ras, DNA methyltransferase, PKA, C-raf kinase, mdm2 and PKC-α. The assumption is that these will be key molecules in the process of tumorigenesis. The aim is to alter the tumour-cell kinetics, to reduce tumour-cell growth or sensitise to chemotherapeutic agents. Much of the therapeutic approach to antisense therapy is well illustrated by the Bcl-2 AO.

1.5. Bcl-2 as an antisense target

Many malignant cells have an inherent resistance to chemotherapy due to the abnormal expression of the antiapoptosis genes, of which Bcl-2 is a prominent member. It has been the aim of Bcl-2 antisense oligonucleotide (AS) therapy to reduce Bcl-2 protein in order to overcome this resistance. Lymphoma associated with the t(14;18) translocation (classically follicular lymphoma) and deregulated expression of the Bcl-2 gene, conferring chemoresistance and subsequent protection against cell death, is a logical target for AO. It is the presence of increased Bcl-2 levels that confers a worse prognosis by preventing apoptosis. This occurs at the level of the mitochondria, by preventing the loss of membrane potential and susequent activation of caspases (predominantly caspase 3). Blocking Bcl-2 production should bring about the death of the cell by reversing this process. Non-sequence-related effects have caused much scepticism following initial enthusiasm for antisense therapy; however, an increased understanding of these additional oligonucleotide properties has led to a number of emerging therapeutic AO molecules for clinical use, working through a true antisense downregulation of the target gene and protein. This includes Bcl-2 AO (G3139 now known as 'Gena-

Table 1
Antisense oligonucleotides in clinical trials for oncology

Target	Tumor	Route	Phase	Sponsor
c-myb (LR-3001)	CML	IV	1	Lynx
bcl-2 (G3139)	NHL	SC/IV	2	Genta
	Leukaemia	(CLL/AML)	2	
	Prostate		2	
	Melanoma		3	
	Myeloma		2	
PKA (GEM 231)	Solid tumours	IV	1	Hybridon
Ha-ras (ISIS 2503)	Solid tumours	IV	1	Isis
PKC-α (ISIS 3521)	Solid tumours	IV	2	Isis
c-raf kinase (ISIS 5132)	Solid tumours	IV	2	Isis/Novartis

sense' developed in collaboration with Genta, USA, an antisenses research company), with phase I–III studies commenced in the field of cancer.

2. Bcl-2 antisense oligonucleotide G3139: preclinical studies

2.1. Target selection

Sequences targeted to the RNA loop structures show logerithmically greater hybridisation than those in the areas flanking the loop [1]. The initiating codon of mRNA, the AUG start site, is the area from which protein synthesis is initiated. AO targeted to contain complementary sequences to this area are most frequently used in antisense experiments [2,3]. Such a site was targeted for the Bcl-2 AO and proved useful, as determined by its ability to bind specifically to Bcl-2 RNA in a cell-free system, downregulate Bcl-2 protein and induce apoptosis in lymphoma cell lines. Oligonucleotide length is important. Two useful AO of 18 (G3139) and 20 bases (G3854) were initially studied, and both showed good intracellular internalisation. The shorter oligonucleotide was developed further for clinical use. Changing the linking molecule with the PS [4], where sulphur atoms replace oxygen, was accomplished to confers resistant to nuclease enzymes. To date, the *in vivo* phase I and II human studies have all been based on PS molecules and this is the molecule of choice for current AO anticancer research.

2.2. Sequence-specificity for Bcl-2 antisense

The aim for antisense researchers is to show that an AO targeted against a gene downregulates that gene in a sequence-specific manner with no other effects. It follows that all antisense experiments must be interpreted with adequate reference to control parameters. Primarily, it is essential to investigate the possibility of the AO having a non-sequence-specific effect, which would negate its use for a true 'gene silencing' strategy. Whatever the mechanism, the important consideration in assessing antisense effects is to establish sequence specificity of the AO against control oligomers, and to demonstrate a decrease in the amount of protein produced by the gene targeted. This has been demonstrated for G3139 and G3854 using both sense and nonsense controls. Downregulation of the Bcl-2 protein was demonstrated at 72 h onwards in lymphoma cells with the t(14;18) translocation with antisense oligonucleotides, but not the control sense and nonsense oligonucleotides [5]. The result is in keeping with the Bcl-2 protein having a relatively long half-life. The effect was specific to cell lines dependent on Bcl-2 for survival. No non-specific antiproliferative effects were seen [5].

2.3. Bcl-2 AO pharmacokinetics and toxicities

There is some knowledge about the pharmacokinetics of PS oligonucleotides with intravenous (i.v.), or intraperitoneal (i.p.) infusions [6,7]. Degradation is not marked (15–50%) up to 48 h, when detectable levels in the tissues are still present. Excretion occurs in the urine with 30% loss within 24 h. Plasma clearance by both routes is biphasic with an initial half-life of 15–25 min and a second half-life, related in part to protein binding, of 20–40 h, representing elimination from the body. We have confirmed similar finding with subcutaneous delivery of Bcl-2 antisense (G3139) in mice [8]. G3139 has a high-volume distribution due to high protein binding, particularly to albumin. Organ distribution varies with good uptake into bone marrow, liver, spleen, kidney and lymphatics (haematological sites predominantly), lesser uptake into other tissues and minimal uptake into the central nervous system [8]. Subcutaneous AO infusions prolongs the bioavailability of the molecule and increase steady-state levels [8,9].

Minimal toxicities, at a dose up to 10 mg/kg/day for 2 weeks, have been administered as an i.v. continuous infusion, avoiding the potential i.v. bolus toxicities and possibly providing greater bioavailability [8]. Those associated with bolus administration are rapid peripheral vasodilatation and a transient anticoagulant effect. Thioate chemistry effects are rapidly reversible and include mild thrombocytopaenia (dose-dependent) and mild hyperglycaemia (non-dose-dependent) requiring no treatment.

3. Preclinical Bcl-2 AO therapy in lymphoma

The systemic use of AO has its attractions where it might be hoped to have a specific antitumour effect while avoiding the many non-specific toxicites caused by chemotheraputic substances. It is this approach that has been most beneficial in lymphoma. The Bcl-2 oncogene has been implicated in the oncogenicity of a wide variety of haematological malignancies and cancers, including melanoma, breast, lung and bowel carcinomas. Bcl-2 protein directly prolongs cellular survival by blocking programmed cell death. Approaches to downregulate the protein by antisense therapy have been pursued as an antitumour strategy. Initial attempts to manipulate the Bcl-2 gene with antisense DNA consisted of transfecting a Bcl-2 antisense sequence in a plasmid into a human T-cell lymphoma cell line [10]. Transfection with a combination of Bcl-2 and C-MYC sense plasmids markedly enhanced the tumorigenicity of this cell line in a nude mouse model. The Bcl-2 antisense gene reduced survival of the cell line following growth factor deprivation. When Bcl-2 AO was substituted for the antisense gene a similar effect was observed, with reduced

expression of Bcl-2 protein [11]. In follicular lymphoma cell lines with high and deregulated Bcl-2 protein levels due to the t(14;18) translocation, Bcl-2 AO directed at the open reading frame of Bcl-2, compared with control sense and nonsense oligonucleotides gave specific downregulation of Bcl-2 protein with a subsequent induction of apoptosis [5]. The AO has little or no effect on the viability of cell lines not expressing high levels of Bcl-2, suggesting that the AO is specifically targeting cells with high levels of Bcl-2 expression. Down-regulating Bcl-2 in a cell that is heavily dependent on Bcl-2 expression for its survival advantage appears to commit the cell to an apoptotic death even when Bcl-2 is subsequently upregulated again, suggesting an irreversible process. It has been demonstrated that the AO is exerting its effect by induction of apoptotic cell death, as indicated by DNA fragmentation and characteristic apoptotic bodies. Similar sequence-specific effects are seen with Bcl-2 AO on human leukaemia cell lines with high Bcl-2 expression [10,11].

Using the *in vivo* lymphoma model from the same cell line [5], an effect against tumour growth *in vivo* [5,12] was also possible with a 2-week infusion at 100 μg daily achieving a plasma level of approximately 0.1 μM. Both G3139 and G3854 Bcl-2 AO showed similar efficacy. G3139 was chosen to pursue an *in vivo* human study being shorter by two bases. Sixty mice with lymphoma were treated with Bcl-2 AO G3139 (sequence 5′-tctcccagcgtgcgccat-3 supplied by Genta, USA) and showed almost complete abolition of lymphoma in 50 (83%) of the mice; however, disease was still present in the remaining mice (17%) . Extention of the treatment to 3 weeks, at the same dose, showed complete eradication of lymphoma in all animals, even at a PCR level. A dose response was also established and, at a dose of 300 μg/day for 2 weeks, complete disease eradication was observed [9]. No abolition of lymphoma was seen in any other group of controls which produced a similar pattern of disease to untreated mice. The experiments were also repeated in NOD/SCID mice which have no NK, B or T cell activity, and similar efficacy was observed. This is of importance as it has been postulated that some AO may work through induction of interferon or tumour necrosis factor released by NK or B cells [13]. As efficacy was seen in mice incapable of producing these cytokines, it provides further evidence for a direct Bcl-2 AS effect. These results suggest that duration of treatment may be as important as the dosage, and certainly considerably less antisense is required, to give complete disease eradication, with a 3-week infusion compared to 2. Having undergone rigorous efficacy, pharmacokinetic and toxicity studies to complete preclinical evaluation in partnership with Genta (an antisense research company, San Diego, USA), these studies have now been extended into a phase I study for lymphoma patients with high Bcl-2 expression and who have failed at least two therapy regimens (taking place at The Royal Marsden Hospital, Sutton, UK).

4. A phase I Bcl-2 antisense for lymphoma

Low-grade and follicular non-Hodgkin's lymphomas are essentially incurable. They have an indolent course, initially chemosensitive, but eventually relapsing with chemoresistant disease. The median survival in the region 10 years from diagnosis. In the later stages of the disease, these tumours develop chemoresistance and often transform to a more aggressive histological appearance. Intermediate/high grade (aggressive, diffuse) non-Hodgkin's lymphoma provide a better chance of long-term survival in the region of 40% with combination chemotherapy such as CHOP (Cyclophosphamide, Adriamycin, Vincristine, Prednisolone) but 60% will die of their disease having initially responded to therapy. Bcl-2 expression appears to be a major factor in therapy failure. As already stated, overexpression of the Bcl-2 gene results in resistance to programmed cell death (apoptosis) [14] leading to chemoresistance [15]. Two recent studies have examined the prognostic significance of Bcl-2 overexpression in patients with diffuse, large B cell lymphoma. In both these studies, multivariate analyses confirmed the importance of Bcl-2 expression as an independent prognostic marker [16,17]. In low-grade, follicular non-Hodgkin's lymphoma, Bcl-2 overexpression is seen in virtually all the tumours. High levels of the Bcl-2 protein expression occur for a number of reasons, one of these being a t(14;18) translocation. However, the prognostic significance in both low-grade and more aggressive lymphomas does not lie in the specific translocation but in the overexpression of Bcl-2 [17–19]. Therapies based on the molecular biology of the disease offer an alternative approach which may improve both response and survival. The preclinical data with Genasense (G3139 Bcl-2 AO) suggested a logical group of patients with relapsing lymphoma and high Bcl-2 expression, for a phase I study to examine the toxicities of such a treatment [5]. In addition, this group of patients could give some indication of antilymphoma activity.

5. Design of the trial

Patients eligible for the study were required to have B-cell non-Hodgkin's lymphoma of any grade that demonstrated immunohistochemically to overexpress the Bcl-2 protein in a lymph node biopsy. Patients were also required to have relapsing disease following completion of at least two forms of conventional treatment (including chlorambucil and fludarabine), a life expectancy of more than 12 weeks, normal renal and liver function, a white blood count greater than 3×10^9/l and

a platelet count in excess of 100×10^9/l. Toxicity assessment was scored according to the common toxicity criteria (CTC) (NCI, 1988). This included areas of concern pinpointed in *in vitro* and animal studies.

The Genasense (Genta Incorporated, USA) was administered for 2 weeks subcutaneously on the abdomen as a continuous infusion using a portable syringe driver. This directly mimicks the *in vivo* animal model. Subcutaneous infusions of PS oligonucleotides may cause some infusion-site inflammation. The infusion sites were therefore changed when early signs of inflammation were observed. Toxicity was monitored for the initial 48 h of treatment as an inpatient but thereafter as an outpatient. A single 2-week course of treatment was given. If there was evidence of response, a second course was considered. The starting dose was 4.6 mg/m^2/day which was well below the 1/10th LD_{1O} in mice. The dose was increased by 100% within each patient cohort unless grade 2 or greater toxicity was observed according to the European Organisation for Research and Treatment of Cancer (EORTC) schema. The maximum tolerated dose was defined as the dose which causes grade 3 or 4 toxicity in 50% or more patients. Response was evaluated from CT scan performed pretreatment, at weeks 2 (end of infusion) and 6, and classified using the World Health Organisation (WHO) criteria. A complete response (CR) was defined as disappearance of all disease and a partial response (PR) 50% or greater reduction in the bidimensional product of measurable disease. Greater than 25% increase in the bidimensional product of measurable disease was defined as progressive disease (PD) and all other states were defined as stable disease (SD). Additional parameters of lymphoma activity were serum LDH and the numbers of circulating lymphoma cells identified morphologically changes in excess of 20% were considered relevant).

Samples of blood, bone marrow and fine-needle aspirates of lymph nodes were collected at the start of weeks 0, 2 and 6. Mononuclear cells, freshly separated by Ficoll–Isopaque centrifugation (Lymphoprep; Nycomed, Norway), were suspended in 10% DMSO and stored in liquid nitrogen. At the time of analysis, the samples were fixed in 70% ethanol, incubated with an antibody to the Bcl-2 protein (DAKO, clone 124) followed by further incubation with an anti-IgG FITC second antibody. Bcl-2 protein levels were determined by flow cytometric analysis of gated lymphocytes. Within this population, Bcl-2-negative and -positive cells were identified. The mean value and standard deviation were determined by gating on the Bcl-2 population. All samples from a single patient were labelled simultaneously under the same conditions. Controls for non-specific protein expression (patient 3 onwards) were determined by flow cytometry using incubation with FITC conjugated HLA-A,B,C antibody (Scrotec).

A portion of the lymph node biopsy from patient 6 was disaggregated and 10^7 cells injected i.p. in to three non-obese diabetic, severe combined immunodeficient (NOD/SCID) mice within 24 h of the biopsy. Each mouse was fitted with a subcutaneous osmotic AZLET pump which delivers a 2-week infusion. This consisted of either 0.9% saline, a control reverse sense ollgonucleotide (0.4 mg/kg/day sequence 5′-taccgcgtgcgaccctct-3) or antisense (0.4 mg/kg/day Anticode G3139 sequence) starting on day 1 for 14 days. The proportion of lymphoma cells in the spleen, liver and bone marrow was determined using flow cytometry to identify cells dual labelling for CD45 (conunon leucocyte antigen) and CD 19 (B cell lymphoma marker).

6. Results of the phase I Bcl-2 antisense study

21 patients have been entered into the trial over a 3-year period [20,21] and it has now been completed. All had been extensively pretreated and had relapsing disease satisfying the entry criteria for the study. The patients all had stage IV disease.

6.1. Tolerable toxicity profile

The main aim of a phase I study is to determine the dose-limiting toxicity in this case for G3139 [20,21]. The toxicites seen have essentially been minimal as reported for the first 9 patients where 100% dose increments (up to 2 mg/kg/day for patients 7, 8 and 9) were achieved as planned [20,21]. The dose was further increased to a dose slightly in excess of 6 mg/kg/day and, at this dose, toxicity was seen in the form of throbocytopaenia and essentially prevented further increases. The course of treatment did not need to be curtailed due to the thrombocytopaenia. A reduction in the increment dose below this level (aprroximately 5 mg/kg/day) for a further 3 patients did not result in toxicity. In the few patients at the higher dose, Bcl-2 oligonucleotide concentration has been measured in the region of 1 μM, although individual variability is seen. A plateau level has not been reached as rapidly (up to 2 weeks), unlike the mice where a constant state is reached within 4 days [8]. Interferon and cytokine levels were monitored during the treatment and showed no rise. It is of note that the non-toxic dose is currently well within the therapeutic range observed in the animal models.

6.1.1. Haematological toxicity

The evaluation of haematological toxicities in lymphoma with bone marrow disease (commonly present in progressive low-grade disease) is not easy as it is required to determine the contribution from the disease versus the contribution of the AO. Taking this into

consideration, there was no antisense-related haematological toxicity. However, patient 8 developed grade 3 leucopaenia and grade 2 thrombocytopaenia associated with a haemophilus influenzae chest infection at the start of the 2-week treatment infusion. The leucopaenia and thrombocytopaenia resolved while the antisense infusion continued following treatment with i.v. antibiotics, suggesting a non-oligonucleotide effect. Patient 9 developed grade 2 thrombocytopaenia at the end of the 2-week drug infusion and, in addition, marked (up to 31%) eosinophilia were observed. Subsequently, at week 6, bone marrow infiltration and progressive disease in lymph nodes was observed. The thrombocytopaenia and eosinophilia resolved on subsequent treatment with chemotherapy indicating these effects were more likely to be due to advancing lymphoma than the antisense oligonucleotide. Lymphopaenia was present in 4 patients (patients 3, 7, 8 and 9) pretreatment and did not worsen with therapy. The anaemia observed (in patients 2, 5 and 9) was not dose related and appeared to be equated with advancing bone marrow infiltration. One of the other areas of concern, when evaluating toxicity, was that of potential T cell alterations brought about by Bcl-2 downregulation in T cell development. The CD4:CD8 was carefully monitored and no significant alterations were seen. No abnormalities in coagulation or clotting factors was observed. An additional area of concern was that of stem-cell suppression, although a role for Bcl-2 has not been demonstrated in the stem cell. No evidence of treatment-related aplasia has occurred in any of the patients, although bone marrow biopsies have been regularly examined in each patient. The dose-limiting toxicity has been that of a readily reversible thrombocytopaenia. This occurs towards the final few days of the 2-week infusion only at the highest doses, but has not been associated with clinical bleeding or bruising. The platelet count starts to rise within days of completing the infusion and the bone marrow contains adequate numbers of megakaryocytes. This could be due to increased peripheral destruction or a direct toxic effect on the megakaryocyte. It is becoming clear that this is an effect due to the PS chemistry and not Bcl-2 downregulation as it has also been observed in subsequent phase I studies against other gene targets when the dose of oligonucleotide approaches 6 mg/kg/day. It may possibly be avoided in the future by second-generation backbone chemistries for the oligonucleotides; however, it must also be noted that this dose is well above that required for theraputic efficacy and downregulation of the target RNA and protein.

6.1.2. Non-haematological toxicity

The G3139 has been well tolerated and the non-haematological toxicities are shown in [20,21]. Of those seen, they can be ascribed primarily to the PS chemistry and not to the effects of Bcl-2 downregulation. All patients had a transient rise in non-fasting blood glucose levels, observed within 24 h of starting the infusion. None exceeded 12 mmol/l and blood glucose levels were not dose related and returned to normal after stopping the therapy. No intervention was required. Similar findings have been documented in another PS human trials targeting P53 [22]. Some of the patients developed an infection, none of which could be directly attributed to the antisense therapy. The other significant toxicity directly related to antisense therapy was a local skin reaction surrounding the infusion site. In 19 patients, this simply required resiting the line site every 3–4 days. However, 2 patients suffered a local inflammatory reaction which became unacceptably painful about 12 h after starting treatment. A skin biopsy from the inflamed area demonstrated increased infiltration of T lymphocytes. Despite several site changes and dilution of drug concentration by 50%, the inflammation persisted and treatment could not to be continued. 2 patients treated subsequently at these dose levels and 3 with a 100% dose increment did not experience the same degree of reaction. Patients in i.v. phase I studies with G3139 have not experiencied inflammatory or allergic reactions to the oligonucleotide. It is possible that the skin response is due to a local high concentration of the oligonucleotide at the site of injection leading to inflammation.

6.2. Evidence for efficacy

Unlike chemotherapy responses, which are usually rapid and maximal within a week, the effect of Bcl-2 AO appears to be slower and more prolonged in duration, with continued reduction in lymophoma bulk in excess of 6 weeks from the end of the infusion [20,21]. The most impressive response was seen in patient 8 where no lymph node masses larger than 1 cm by 6 weeks and had achieved a response in all lymph node sites, the largest pretreatment lymph node measuring 2.5×2.0 cm. This patient remains in remission 3 years after starting treatment without having further therapy. No other patients achieved CR; however, 2 have had partial responses, a further 8 have had a durable stabilisation of disease and at least 2 of this latter group have shown symptomatic improvement. For example, within 48 h of starting treatment, patient 1 had resolution of sweats and pruritus which lasted 3 weeks. On retreatement at the same dose, this patient again had resolution of the sweats and pruritus again. Patient 8's lymphoma-related alcohol intolerance resolved such that he was able to drink alcohol for the first time in 2 years. Of the first 9 patients evaluated, 2 out of 3 patients had a reduction in their circulating lymphoma cells (patients 2 and 6), 4 out of 9 patients had a reduction in their scrum LDH levels (patients 1, 2, 6 and 8).

The full evaluation of all the patients has not yet been completed, but it is clear that there is solid evidence of therapeutic efficacy with only a 2-week infusion of the Bcl-2 antisense. For a phase I study, this represents a successful agent for further evaluation in a phase II trial to concentrate primarily on efficacy.

6.3. Bcl-2 expression

Technically and ethically, it is difficult without ready access to tumour material for the evaluation of Bcl-2 expresssion. It was felt that circulating lymphoma cells could offer this opportunity as well as fine-needle aspirates from lymph nodes when readily accessible, without potentially endangering the patients' well being. Over the treatment period and the subsequent 4 weeks, Bcl-2 levels were measured by flow cytometry in peripheral blood samples containing circulating lymphoma cells. Bcl-2 levels were specifically reduced in the lymph node aspirates for those patients where they were readily accessible.

6.4. Further treatment

8 out of the first 9 patients have gone on to receive further chemotherapy with a variety of regimens. 6 out of the 8 patients treated with further chemotherapy have achieved PR using chemotherapy for which response had been poor previously. Patient 9 was treated with the chemotherapy regimen ChlVPP (Chlorambucil, Vinblastine, Procarbazine, Prednisolone) 5 weeks after finishing antisense treatment and unusually developed complete alopecia.

6.5. Phase II and III studies with Genasense

Genasense has now been used in a number of clinical settings including phase 2 studies in combination with chemotherapy. This works on the basis that lowering the Bcl-2 protein with the Genasense leads to sensitisation of the malignant cells to chemotherapy. A number of solid tumours, including malignant melanoma, breast cancer, prostate cancer and small-cell lung cancers, have shown evidence for synergy. Genasense has recently received fast track and orphan drug designation for the treatment of myeloma, chronic lymphatic leukaemia (CLL) and acute myeloid leukaemia. At the ASH 2001 meeting, Susan O'Brien from the MD Anderson Hospital in Texas presented some preliminary patient studies with Genasense as a single agent with good efficacy at a dose of 3 mg/kg/day. Our own group presented data showing synergism between Bcl-2 antisense and Rituximab in CLL cells. Multicentre studies have now been commenced for both CLL and myeloma combining the Genasense with chemotherapy. The stage III study of Genasense with chemotherapy for malignant melanoma is nearing completion. A phase II study for mantle cell lymphoma will also commence shortly in our institution.

7. Conclusions

In vitro and *in vivo* studies culminating in a human phase I study with Bcl-2 antisense (Genasense) to inhibit expression of Bcl-2 have demonstrated good efficacy with low toxicity [20,21]. Tumour regressions, improvement in the laboratory parameters, symptom improvement together with downregulation of target protein expression [20,21] has also been achieved. These results, although very preliminary, certainly match a good chemotheraputic drug [20]. 3 patients had evidence of tumour shrinkage on the CT scanning with 1 patient maintaining this improvement 3 years on. The combination of this evidence supports the theory that Bcl-2 antisense oligonucleotides have antilymphoma activity [20,21]. *In vitro* studies demonstrate specific reduction in mRNA, protein expression and induction of apoptosis using Bcl-2 antisense [5]. For the first time, this study demonstrates evidence of downregulation of the target protein in humans and the parallel animal model provides evidence of a sequence-specific antilymphoma effect. It was observed that 6 out of 8 patients who were treated with chemotherapy following antisense treatment went on to achieve a partial remission. Patient 9 developed complete alopecia with ChlVPP, a treatment regimen with a documented 0.5% incidence of this side-effect [23]. It is known that Bcl-2 levels in the hair follicle are normally high [24] and Bcl-2 knock out mice develop abnormalities in hair pigmentation [25,26]. These results suggest that Bcl-2 antisense, when given prior to chemotherapy, may have a sensitising effect. Potential uses of Bcl-2 antisense extend beyond the field of lymphoma as overexpression of Bcl-2 is seen in breast, lung, melanoma, leukaemia, prostate, gastric and colorectal tumours. This is speculative as it was outside the study parameters, but is currently being addressed and may have relevance to further human trial designs. A summary of studies currently taking place with Genasense is shown in Table 1. Having shown it is possible to reduce Bcl-2 expression with Bcl-2 antisense oligonucleotide, these studies (including a phase II study for lymphoma in our institution) is aimed at using the lowered Bcl-2 antisense to increase sensitivity to chemotherapy.

An important aim of the phase I study was to establish toxicity of Bcl-2 antisense oligonucleotides. A maximum tolerated dose has been established at 4 mg/kg/day. Concerns about potential toxicity came from a number of sources. Mouse toxicity data using this compound demonstrated myocardial and liver necrosis, splenomegaly and deaths at high dosages (50 mg/kg/day). The dose levels at present are more than 10-fold

less than those at which toxicities were observed in mice. Primate studies revealed no clinical toxicity and autopsy revealed only mild injection site inflammation and non-specific pathological changes within the kidneys at doses up to 10 mg/kg/day for 2 weeks [7]. Primate studies using high doses of other PS compounds demonstrated hypotension and deaths if the drugs were give as a rapid i.v. bolus but not when given as an infusion. Furthermore, the hypotensive events were not sequence-related [27]. Another human study, using a 20-base fully phosphorothioated oligonuelcotide targeted against p53 in patients with acute myeloid leukaemia and myelodysplastic syndrome at doses up to 6 mg/kg/day as an i.v. infusion, demonstrated no compound-related toxicity [22]. There was concern that this compound might have direct a sequence-related effect on cells which express high Bcl-2 levels under normal conditions, but this has not so far been observed. The memory B cells, mature (CD4+ and CD8+) T cells, neuronal tissue and intestinal mucosa have high Bcl-2 levels. Bone marrow stem cells do not normally overexpress Bcl-2 and another member of the Bcl-2 family, Bcl-X_L, appears to be of greater importance here, so adverse effects against the stem cells were considered unlikely [28]. It also seems unlikely that neuronal tissue would be damaged as oligonucleotides do not cross the blood-brain barrier. In addition, Bcl-2 knock out mice in which both copies of the Bcl-2 gene have been deleted by recombinant DNA techniques are viable without neurological deficit, suggesting that Bcl-2 is not of prime importance to the stem cells or neuronal tissue. However, the knock out mice do subsequently develop lymphopaenia, apoptotic involution in the thymus and spleen, polycystic kidneys, grey hair in the second follicle cycle [25,26] and distorted small intestine [26]. In practice, and at the current dose levels, major Bcl-2 sequence specific toxicities do not occur.

The only significant treatment-related toxicity is almost certainly PS-related. This was an inflammatory response at the injection site. In 2 patients, this was severe enough for the treatment to be stopped. The severity of reaction, however, was not seen in other patients, even at higher doses. Transient non-fasting hyperglycaemia has also been observed during the treatment period. This had no clinical implications but has also been noted in a recently published report of antisense treatment against p53, suggesting it may be related to the PS backbone chemisty, and does not appear to be dose-related [22].

The potential uses of Bcl-2 antisense oligonucleotides are many. They include a single-agent use, treatment of minimal residual disease or *ex vivo* purging of bone marrow or peripheral stem-cell harvests. Potentially, the most important application may be to overcome chemoresistance. With care, novel therapies based on the biology of the malignant cell may be determined on a scientific basis and may help improve the treatment of patients with these diseases. Gene silencing by antisense oligonucleotides has a role to play as demonstrated in lymphomas.

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