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CLINICAL STUDIES OF ANTISENSE THERAPY IN CANCER

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1. ABSTRACT

The ability to target and inhibit individual gene expression with antisense oligonucleotides has shown promising activity in preclinical cancer models. Recent clinical studies have tested antisense compounds directed against seven cancer related genes including p53, bcl-2, c-raf, H-ras, protein kinase C-alpha, and protein kinase A. Class specific effects of the phosphorothioate backbone common to the first generation of antisense compounds have dominated the side effects of these oligonucleotides. Inhibition of target gene expression has been modest at most, and clinical activity has been primarily anecdotal. Combinations of the antisense compounds with chemotherapy and second-generation oligonucleotides offer promise that these agents might become a standard part of future cancer therapy.

2. INTRODUCTION

The ability to target and inhibit the expression of individual genes with a high degree of specificity distinguishes antisense oligonucleotide therapy from other treatments in development. Moreover, the unraveling of the molecular events that lead to malignancy and the identification of key genes involved in that process have provided investigators with a number of targets for cancer therapy. The preclinical development of several antisense compounds targeting cancer-related genes has proceeded relatively rapidly. Many have shown convincing *in vitro* reduction in target gene expression and promising activity against a wide variety of tumors. The clinical testing of the most promising oligonucleotides has taken place over the last several years, though, until recently, few studies have reached completion. Current studies involve antisense oligonucleotides targeted to p53, bcl-2, c-raf, H-ras, protein kinase C-alpha, and protein kinase A. Most studies have tested first generation oligonucleotide compounds that contain a phosphorothioate backbone, which accounts for the great similarity in toxicity profiles. The purpose of this review is to summarize the current clinical experience with antisense oligonucleotides in cancer therapy.

3. CLINICAL STUDIES

3.1. p53

The p53 gene plays a critical role in the cellular response to DNA damage, controlling the branch point of cell cycle arrest, DNA repair or apoptosis. Mutations in this gene are found in the majority of human cancers and often lead to *increased* levels of p53 protein. The inhibition of p53 expression, therefore, as a form of cancer therapy is somewhat counterintuitive. OL(1)p53 is a 20 base phosphorothioate oligonucleotide directed against exon 10 of p53 mRNA. In preclinical testing, the antisense inhibited growth of acute myelogenous leukemia blasts in cell culture (1). Based on this observation, and the fact that increased wild type and mutant p53 are detected in hematologic malignancies such as acute myelogenous leukemia and myelodysplastic syndrome, clinical studies were undertaken. Bishop and colleagues conducted a phase 1 study of OL(1)p53 in patients with relapsed or refractory acute myelogenous leukemia or myelodysplastic syndrome (2-3). Sixteen patients were treated at doses ranging from 0.05 to 0.25 mg/kg/hr for 10 days by continuous infusion. No specific toxicity was attributed to the oligonucleotide. Pharmacokinetics showed non-linear increases in mean steady state plasma concentrations. A maximum tolerated dose was not reached and no clinical responses were observed. *In vitro* measures of p53 expression from patient samples were not undertaken, though cell cultures from each patient were tested with OL(1)p53. The leukemic cell production of long term bone marrow cultures was inversely correlated with serum levels of the antisense oligonucleotide. The authors postulate that the optimum effect of antisense to p53 may require an additional DNA damaging event to achieve a selective tumoricidal effect of inhibiting p53 expression and plan additional therapies in combination with chemotherapy.

3.2. bcl-2

Bcl-2 is commonly over expressed in follicular non-Hodgkin's lymphoma and in a variety of other tumors including lung, breast colorectal, prostate, renal and acute

and chronic leukemias (4). The bcl-2 protein inhibits apoptosis and may play a significant role in the development of malignancies such as lymphomas and in the response to cytotoxic chemotherapy or radiation. Thus, inhibiting bcl-2 alone or simultaneously with cytotoxic agents may have therapeutic effects. Several studies using the bcl-2 antisense produced by Genta (G3139) are underway either as single agents or in combination with chemotherapy. G3139 is an 18 base phosphorothioate antisense oligonucleotide complementary to the first six codons of the bcl-2 mRNA. The antisense had shown the ability to inhibit bcl-2 expression *in vitro* and to eradicate tumors in mouse models with lymphoma xenografts. In a phase 1 study of G3139, Waters and colleagues treated patients via daily subcutaneous infusion for 14 days at doses ranging from 4.6 mg/m^2 to 198.8 mg/m^2 per day (5-6). Twenty-one patients with non-Hodgkin's lymphoma of any histologic grade with immunohistochemical evidence of over expressed bcl-2 were treated in eight cohorts. The maximum tolerated dose was 147.2 mg/m^2/day with dose limiting toxicities consisting of thrombocytopenia, fatigue and fever. Plasma levels of G3139 were equivalent to active *in vivo* levels among those receiving doses above 36.8 mg/m^2/day. Of 20 evaluable patients, one complete response lasting 30+ months and one minor response was noted. Bcl-2 protein expression was assessed in tumor samples by FACS analysis in 13 patients, and was reduced in five. The study showed that the antisense could reduce expression of the target bcl-2 gene and had some indication of clinical activity after a single short course of administration. Toxicity was assessed only with one cycle of therapy, so the toxicity, as well as potential efficacy, of longer courses of treatment remains to be determined.

The same oligonucleotide has been tested in a variety of solid tumors. Morris and colleagues treated 32 patients, most of whom had prostate cancer, with doses of G3139 ranging from 0.6 to 5.3 mg/kg/day (7). Patients received 14 days of antisense by continuous infusion followed by four weeks of observation. Up to three cycles were administered. Thus far, no dose limiting toxicity was recognized although grade 3 rash, fatigue and leukopenia were noted. Five patients had assessments of peripheral blood bcl-2 expression. Declines of up to 50% were observed in two of the patients. Three patients have also received weekly paclitaxel (100 mg/m^2 on weeks 2 and 3) at doses of G3139 of 4.1 mg/kg/day or greater (8). The combination was based on preclinical data suggesting a synergistic effect of G3139 and taxanes. The only additional toxicity attributed to the addition was grade 2 mucositis. The study is currently ongoing.

In vivo xenograft models of melanoma have suggested that G3139 could enhance tumor cell apoptosis and decrease growth of melanoma cells particularly when administered with dacarbazine (9). Based on this observation, Jansen and colleagues started a phase 1 study using escalating doses of G3139 ranging from 0.6 to 5.3 mg/kg/day for 14 days by continuous infusion combined with dacarbazine at 200 mg/m^2/day for five days (10). Cycles were continued after a two week rest. Nine patients with advanced melanoma have been treated thus far without significant toxicity related to either G3139 or dacarbazine. Pharmacokinetics showed a dose dependent rise in G3139. Five of seven patients have had stabilization of disease for 2 to 9+ months, while two patients have had significant reductions in metastatic sites of disease. Bcl-2 expression in tumor samples was diminished to 25-50% of baseline values in all but one patient who was treated at the lowest dose level. The activity of the combination and reduction in target gene expression in this ongoing study are promising though a larger number of patients need to be treated.

3.3. c-myb

C-myb encodes a transcription factor that is preferentially expressed in hematopoietic cells (11). Constitutive expression of c-myb has been shown to inhibit the differentiation of murine erythroleukemia cells *in vitro*. Hence, inhibition of c-myb may play a role in inhibiting leukemic cells. Lynx has developed a 24 base pair phosphorothioate oligodeoxynucleotide (LR-3001) that targets codons 2-9 of c-myb. *In vivo* animal modes have show good antitumor activity against a leukemia mouse model. One clinical study is evaluating the ability of LR-3001 to purge CD34+ marrow cells in patients with chronic myelogenous leukemia undergoing autologous transplantation (12). A second is assessing the safety of LR-3001 when given via seven days of continuous intravenous infusion among patients with chronic myelogenous leukemia and acute myeloid leukemia (13). Results from these studies are still awaited.

3.4. c-raf

C-raf-1 is a serine/threonine protein kinase and acts downstream of Ras in the MAP kinase signal transduction pathway. Mutations in ras or raf genes resulting in over expression or aberrant expression of these genes have been identified in many human cancers. ISIS Pharmaceuticals has developed a 20 base pair oligonucleotide (ISIS 5132) with a phosphorothioate backbone designed to hybridize to the 3' untranslated sequences of c-raf-1 mRNA. Several phase 1 studies have been conducted with the oligonucleotide in patients with refractory solid tumors. Three studies with ISIS 5132 conducted thus far have varied the scheduling of administration.

Stevenson and colleagues reported final results of a phase 1 study of ISIS 5132 administered by 2-hour intravenous infusions three times a week for three consecutive weeks (14). Doses were increased from 0.5 mg/kg to 6.0 mg/kg in nine cohorts. Thirty-one patients with refractory tumors were treated. Transient low grade fevers and fatigue were observed in many patients. There was a trend towards increased activated complement component C3a that was not associated with clinical signs or symptoms. Even though a maximum tolerated dose was not defined, the dose escalation was stopped at 6.0 mg/kg since the maximum plasma concentrations of oligonucleotide approached that associated with complement activation in primates. Pharmacokinetics were predictable as the AUC increased linearly with dose. One patient with metastatic colon cancer had a 20% shrinkage

of disease lasting for 8 cycles. Of note, peripheral blood cell expression of c-raf-1 decreased by 90% and paralleled the reduction in tumor size. A second patient with renal cell carcinoma had stable disease for 10 courses of ISIS 5132 before progression. Inhibition of gene expression was tested by RT-PCR methods in patients treated at doses greater than 2.5 mg/kg. Thirteen of 14 patients showed a reduction to a median of 42% of initial values within 48 hours of dosing. The reduction occurred at all dose levels, and did not appear to have a dose response relationship. The important findings in this experience were the reduction in mRNA from peripheral blood samples among most patients and the stabilization of disease in two patients. However, the reduction in target gene expression was variable and decreased only by about 50%. As suggested in the patients with clinical responses, it may be necessary to achieve greater inhibition of target to see increased therapeutic effect.

ISIS 5132 was also administered via 21 days of continuous infusion (15). The advantages of the continuous infusion are in ease of administration and in potentially greater inhibition of target gene expression. Patients received doses ranging from 0.5 to 5.0 mg/kg/ day over 21 days followed by a 7 day rest. Thirty-four patients with advanced cancer were treated. Grade 4 thrombocytopenia was seen in two patients, one patient at 1.0 mg/kg/day that was associated with sepsis, and a second treated at 5.0 mg/kg/day. Two other patients had grade 3 thrombocytopenia, one of which was related to sepsis. One patient had grade 4 fever with grade 3 hypotension, and two patients had grade 3 fatigue. Preliminary pharmacokinetic analysis showed non-linear increases in mean steady state concentration at doses up to 4.0 mg/kg. Tumor response was seen in one patient with ovarian cancer at 3.0 mg/kg/day. She had a 97% decrease in CA-125 from 1490 to 47 U/ml during treatment for 7+ cycles. A second patient had stable renal cell cancer for 9 months before progression.

A third schedule of ISIS 5132 tested the oligonucleotide in a weekly infusion (16). Patients with advanced solid tumors or lymphoma received weekly infusions of ISIS 5132 given over 24 hours. Doses were increased from 6 to 30 mg/kg/week. Nineteen patients were treated thus far. The most common toxicity was low grade fever in 15 patients. One patient experienced grade 3 fever at 18 mg/kg. More serious toxicities occurred at 30 mg/kg and included a Coombs positive hemolytic anemia and renal failure, possibly related to tumor progression. At doses of 18 mg/kg/week, complement activation was seen in increasing levels of C3a, Bb and aPTT. However, there was no clinically significant sequelae or progressive increase with repeated dosing. Transient thrombocytopenia was observed in 9 of 18 patients at all dose levels, and did not appear to be dose dependent. No objective tumor responses were observed.

These three phase 1 studies of ISIS 5132 have shown generally similar toxicities of fatigue, fever, and varying thrombocytopenia or complement activation. Constitutional symptoms were more severe and frequent with once weekly infusions than with other schedules and were likely related to peak level effects. The overall side effects are similar to that reported with other oligonucleotides and likely related to class effects of the phosphorothioate oligonucleotide rather than sequence specific effects. Ease of administration favors the 21 day continuous infusion schedule, though information is lacking in regard to differences in efficacy of inhibiting the target gene expression. Additional studies of ISIS 5132 include a phase 1 study in combination with 5FU and leucovorin (17) and planned phase 2 studies of that combination in colon cancer.

3.5. H-ras

The ras gene family encodes a family of GTP-binding proteins that play a pivotal role in regulation of cell proliferation and cell death. Activation of ras genes has been associated with tumorigenesis and enhanced proliferation of tumors. Many activating mutations in ras in human tumors make this an attractive target. ISIS 2503 is a phosphorothioate oligodeoxynucleotide that hybridizes to the translation initiation region of human H-ras mRNA. *In vitro* and *in vivo* studies show inhibition of H-ras mRNA and protein expression as well as the ability to inhibit the growth of a variety of xenograft tumors. Two studies have been conducted thus far with ISIS 2503 in patients with solid tumors. As with the previous anti-ras studies, these phase 1 studies varied in the scheduling of administration.

Gordon and colleagues reported results of ISIS 2503 administered via 24 hour intravenous infusions weekly for three weeks (18). Nineteen patients, mostly with colon cancer, have been treated at 3 to 24 mg/kg per dose. Three patients developed a hemolytic uremic syndrome during the first infusion. In two cases, the toxicity was self limited and resolved in 3-7 days. The third episode resulted in acute renal failure requiring dialysis. Since two episodes occurred at the 24 mg/kg level, the maximum tolerated dose was determined to be 18 mg/kg. Other toxicities include mild fevers and grade 2 allergic rashes. One grade 3 thrombocytopenia was seen coincident with reversible renal insufficiency. No clinical evidence of complement activation was observed. One patient with melanoma experienced a minor response (< 50% shrinkage) in a liver metastasis lasting 27 weeks. Pharmacokinetics showed linear and dose related increases in serum concentrations. Peripheral blood mononuclear cells are currently being conducted for analysis of H-ras expression.

ISIS 2503 was also administered via 14 day continuous infusion followed by a seven day rest (19). Twenty-two patients with advanced cancers received 1.0 to 10.0 mg/kg/day. Toxicity included grade 2 fevers in two patients at 10.0 mg/kg and grade 2 fatigue in three patients at 4.5 or 10.0 mg/kg. Grade 2 thrombocytopenia was seen in one patient at 2.0 mg/kg, though this was associated with bacteremia. Pharmacokinetics showed dose related increases in steady state levels of antisense. No objective responses were seen although four patients had stable disease for 6-10 cycles of treatment. H-ras mRNA levels from peripheral blood lymphocytes were measured by

Northern analysis. No consistent effect was seen on mRNA levels in five patients treated at doses of 1.0 or 2.0 mg/kg/day. Although a maximum tolerated dose was not defined, a dose of 6.0 mg/kg/day was chosen for future studies.

3.6. Protein kinase C

Antisense to PKC-alpha is the third antisense oligonucleotide developed by ISIS Pharmaceuticals for cancer treatment. PKC is an attractive target in cancer therapy as it is involved in the signaling pathway that controls cellular proliferation, and perturbations of PKC expression have been implicated in the growth and progression of some human tumors. Moreover, non-specific inhibitors of PKC such as UCN 01 are in clinical trials and have demonstrated evidence of antitumor activity. ISIS 3521 is a phosphorothioate oligonucleotide, 20 nucleotides in length. It targets the 3' untranslated portion of human PKC-alpha mRNA and demonstrates substantial *in vitro* inhibition of mRNA as well as anti tumor effect in human xenograft models. The studies conducted thus far consist of phase 1 studies that vary the scheduling of administration and several studies in which the antisense is combined with chemotherapy.

The final results of a phase 1 study of ISIS 3521 were recently reported (20). Investigators administered the antisense over 21 days by continuous intravenous infusion followed by a 7 day rest. Doses were increased from 0.5 to 3.0 mg/kg/day. Twenty-one patients with relapsed or refractory cancers were treated. Mild grade thrombocytopenia, grade 2, was seen in two patients at doses ranging from 0.5 to 1.5 mg/kg/day. A third patient had grade 3 thrombocytopenia at 2.0 mg/kg/day on day 7 of infusion. In all three cases, platelet counts returned to normal by the time of the next cycle. Dose limiting toxicities consisted of grade 3 fatigue and thrombocytopenia at a dose of 3.0 mg/kg/day. Four patients had grade 3 fatigue, where they spent greater than 50% of the day in bed. One patient had grade 4 thrombocytopenia and a second had grade 2 thrombocytopenia with grade 4 bleeding. There was no clear effect of ISIS 3521 on aPTT or complement activation. Pharmacokinetic measurements showed rapid plasma clearance and dose-dependent steady state concentrations of ISIS 3521. Three of four patients with ovarian cancer had evidence of tumor response. One patient had a rapidly growing clear cell carcinoma that shrank by >50%, with a partial response lasting 11 months. Two other patients with elevated CA 125 experienced a decline in tumor markers of 40% and 76% lasting 5 and 7 months respectively. Inhibition of the target gene expression was not examined. The activity of ISIS 3521 in this phase 1 study has led to an ongoing phase 2 study in ovarian cancer.

Other schedules of ISIS 3521 have been examined. Nemunaitis and colleagues reported results of thrice weekly infusions, each given over 2 hours, repeated for three of every four weeks (21). Doses of ISIS 3521 were escalated from 0.15 to 6.0 mg/kg/day. Toxicities included grade 2 and grade 3 thrombocytopenia at 5.0 mg/kg/day, and grade 3 fatigue and grade 3 nausea. Transient, asymptomatic elevation of aPTT and C3a occurred in a dose-related fashion. Dose escalation was halted at 6.0 mg/kg/day because peak plasma levels approached a level associated with complement activation in primates. Pharmacokinetics showed dose-related but non-linear increases in maximum plasma concentrations and area under the curve measurements, suggesting saturable organ distribution. Two patients with non-Hodgkin's lymphoma experienced partial responses lasting at least 6 and 14 months.

An ongoing study of ISIS 3521 is exploring a weekly infusion of oligonucleotide. Doses ranged from 6 to 24 mg/kg and were given over 24 hours weekly. Eleven patients were treated thus far (22). Grade 3 toxicities have included fevers and bleeding at 18 mg/kg, and chills at 24 mg/kg. Transient activation of complement split products C3a and Bb were seen particularly at doses = 18 mg/kg. It appears that this schedule, as with ISIS 5132, results in greater constitutional symptoms and complement activation.

The findings from these series of phase 1 studies of ISIS 3521 were similar to that of the anti-raf compound. Toxicities were likely related to class specific effects with schedule dependent variability. Anti-tumor activity was also observed, though in a small minority of patients.

Several *in vivo* studies of cancer xenograft models have shown an additive effect of ISIS 3521 and a variety of chemotherapy agents. Two ongoing studies are exploring the safety of ISIS in combination with chemotherapy. Preliminary results of ISIS 3521 with 5 FU were recently reported (23). ISIS 3521 was administered over 21 days by continuous infusion starting at 1.0 mg/kg/day, half of the maximum tolerated dose established in other studies. 5 FU and leucovorin were given at standard doses (425 mg/m^2 and 20 mg/m^2 respectively) on days 1-4 of each 28 day cycle. Preliminary results did not show a pharmacokinetic interaction between the antisense and chemotherapy agents and no unexpected toxicity at the maximum tolerated dose of ISIS 3521 at 2.0 mg/kg/day. Antitumor activity was observed with the combination, but it will take a randomized study to determine the additional effect of the antisense itself.

An ongoing study of ISIS 3521 with carboplatin and paclitaxel was recently updated (24). In this phase 1 study, patients first received fixed doses of carboplatin (AUC 6) and paclitaxel (175 mg/m^2). With cycle 2 and beyond, patients received ISIS 3521 over 14 days by continuous infusion with chemotherapy on day 4. Cycles were repeated at 21 day intervals. Doses of ISIS 3521 were escalated among patient cohorts from 1.0 to 2.0 mg/kg/day, while the dose of carboplatin was increased from an AUC of 5 to 6. Seventeen patients were treated in the phase 1 portion of this study. Pharmacokinetic evaluation did not show an interaction between antisense and either chemotherapy agent. Toxicities were similar between cycles one and two, suggesting that the antisense did not potentiate side effects. Because of interesting activity

among patients with non-small cell lung cancer, a phase 2 study of ISIS 3521 at 2.0 mg/kg/day for 14 days with carboplatin (AUC of 6) and paclitaxel (175 mg/m^2) has commenced. Thus far, 15 patients with non-small cell lung cancer have been treated. The response rate appears better than expected at 60% with 90% of patients still alive at a median follow up of 8 months. Enrollment in the phase 2 portion is continuing. If the interesting response rate and survival data are confirmed in a larger group of patients, then a randomized phase 3 study may be entertained to establish the additional contribution of the antisense oligonucleotide.

3.7. Protein kinase A

The oligonucleotides described thus far, so called first generation antisense compounds, have consisted of phosphorothioate backbone oligonucleotides. The first of the next generation of antisense compounds, GEM 231, was developed and tested by Hybridon, Inc. GEM 231 is an 18-base oligonucleotide mixed-backbone RNA/DNA hybrid that targets the RI a subunit of PKA-I. The mixed backbone oligonucleotide confers greater stability than similar first generation compounds. *In vivo*, it down regulated expression of PKA-RI alpha and demonstrated activity against human cancer xenografts alone or synergistically with chemotherapy agents. GEM 231 was tested in a phase 1 study in patients with a variety of refractory solid tumors (25). Patients received escalating doses of GEM 231 ranging from 20 to 360 mg/m^2 twice a week over two hours intravenously for eight weeks. Thirteen patients were enrolled thus far. Toxicities included grade 1-2 fever and flu-like symptoms at doses = 240 mg/m^2. No treatment related thrombocytopenia or complement activation was noted. Dose limiting toxicities consisted of transient prolongation of aPTT at 360 mg/m^2 and reversible grade 3 transaminase elevation in 3 of 3 patients at 360 mg/m^2 after 4.5 to 10 weeks of treatment. The maximum tolerated dose was therefore established at 240 mg/m^2 for four weeks. The only evidence of antitumor activity was in one patient with colon cancer treated at 360 mg/m^2 who had a slight decrement in a previously rising CEA at the end of the treatment period. Pharmacokinetics showed that maximum plasma concentrations wee linear over the dose range examined, without evidence of accumulation in the plasma with repeated doses. Further studies will evaluate the effect of the oligonucleotide on expression of the target gene in peripheral blood lymphocytes and specific tissues.

4. CONCLUSIONS

The first generation of antisense compounds consists of oligonucleotides containing phosphorothioate backbones. Class specific side effects have dominated the adverse effect profile and include transient thrombocytopenia, fatigue and fever. Complement activation or clotting abnormalities appeared particularly among schedules that resulted in high peak levels of antisense. The pharmacokinetics of these compounds have generally been linear, though several of the compounds have demonstrated non-linear kinetics at higher doses of prolonged infusions. Combinations of antisense and chemotherapy thus far have not shown unexpected pharmacokinetic interactions, and suggest that antisense-chemotherapy combinations might be administered safely. Inhibition of target genes by the antisense compounds has not been assayed in all of the clinical studies. When examined, reductions in expression in target gene expression in peripheral blood cells have been modest, at most, and variable from patient to patient. Finally, some clinical activity of the antisense alone has been demonstrated in a small number of patients, and has provided impetus for more focussed phase 2 studies.

Some of the limitations of the first generation compounds will be overcome with additional modifications such as with the Genta RNA:DNA hybrid. These newer generation compounds offer hope that current dose limiting side effects might be avoided and that greater inhibition of target genes could be achieved.

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