

New modalities in oncology: antisense oligonucleotides

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New and innovative cancer treatments are appearing at an astonishing rate. Although the array of new approaches seems breathtaking, most can be grouped into a few categories based on underlying purpose. For example, with our greater understanding of the genetic defects that predispose to malignant cell growth has come the idea of therapeutic agents targeted to a single mutant protein. This approach can take the form of very specific kinase inhibitors (e.g., STI571 or Gleevec) or can attempt to actually manipulate the expression level of the target protein. This paper discusses the technology of antisense oligonucleotides as an example of the latter approach.

THEORY OF ANTISENSE TECHNOLOGY

During gene transcription, the duplex strand of DNA becomes partially uncoiled and the 2 complementary strands, "sense" and "antisense," separate. The antisense strand of DNA is utilized as a template to generate messenger RNA (mRNA) that is translated into protein product in the cytoplasm of the cell. This cytoplasmic mRNA is in the "sense" orientation, and so translation can be prevented by complementary base-pair binding of "antisense" oligonucleotides as short as 18 bases long (1), either by steric blocking of translation or by destruction of the bound mRNA via RNase-H (Figure 1). Thus, by selecting appropriate "antisense" nucleotide sequences, it is possible to inhibit the expression of virtually any gene product in a cell without directly affecting other cellular components.

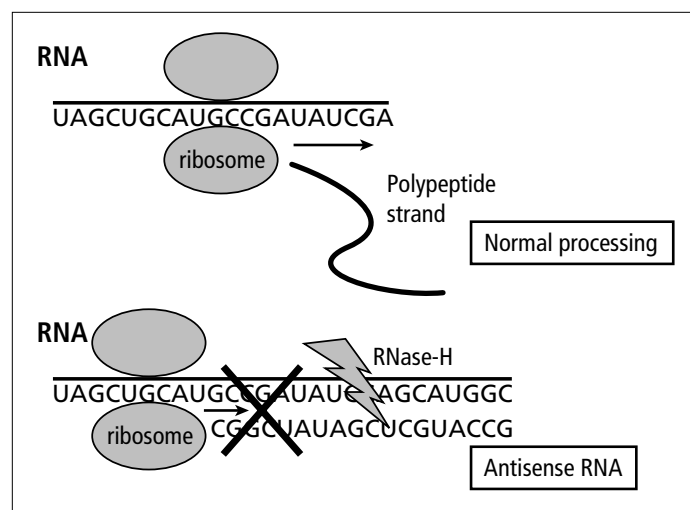


Figure 1. Antisense oligonucleotides inhibit transcription of mRNA.

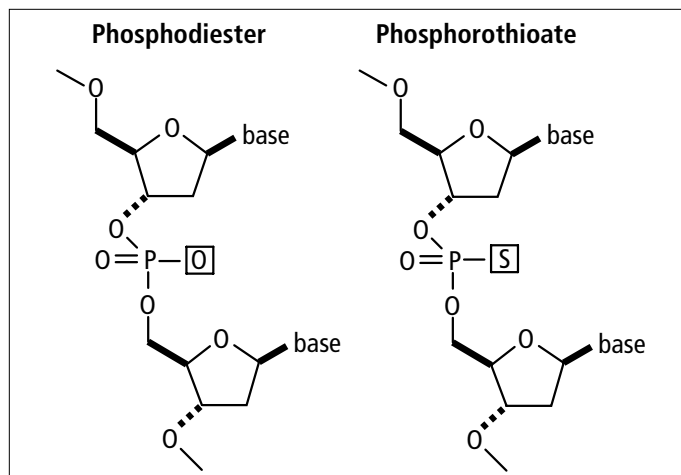


Figure 2. Creation of phosphorothioate backbone.

DEVELOPMENT OF ANTISENSE OLIGOMERS AS THERAPEUTIC AGENTS

To make the jump from fascinating laboratory technique to clinical therapeutic agent requires overcoming several potential hurdles. The most obvious of these is the nearly immediate degradation of normal phosphodiester-linked nucleotides by the exonucleases abundant both in serum and within cells. To achieve meaningful cellular exposure, therefore, all antisense approaches require protective modifications to the oligomer nucleotides, the most common being substitution of a sulfur for the nonbridging oxygen in the nucleotide linkage to give a phosphorothioate backbone (2) (Figure 2).

Phosphorothioate-linked nucleotides have the advantage that, because of their charge-to-mass ratio, they are able to cross cell membranes more easily than phosphodiesters but still retain the capability to activate RNase-H enzymes. In fact, the physical and chemical properties of phosphorothioate oligonucleotides are independent of their specific sequences and are the primary determinants of the pharmacokinetic behavior of the molecules, so that preclinical data generated from one compound translate, in part, to the other compounds. In addition, the pharmacokinetic behavior of the phosphorothioate nucleotides is generally

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consistent across species and shows an initial rapid clearance from the plasma due to tissue distribution and (to a lesser extent) exonuclease action (3). In contrast, intact oligonucleotide residing in the tissues is slowly metabolized and exhibits slow overall clearance from tissues, with a half-life of approximately 1 to 2 days in rodents and >3 days in monkeys (4). Therefore, these compounds will accumulate in tissue with repeated administration and so may (theoretically) lead to chronic toxic effects. Note, however, that the tissue distribution is saturable so that, at some threshold infusion dose, plasma values can increase markedly. Since acute toxicities are probably related to peak plasma concentrations, while subchronic and chronic effects are related to the area under the curve (AUC) and tissue concentrations, different dosing regimens present somewhat different potential problems.

ACUTE TOXICITIES

Native DNA binds to several proteins, so it is perhaps not surprising that phosphorothioate oligonucleotides do as well, often with higher capacity, albeit lower affinity. In fact, specific protein binding is most likely responsible for the few acute toxicities observed in the initial development of antisense oligonucleotides as systemic therapeutic agents. However, since oligomer binding to protein is reversible and predominantly ionic, and since there is rapid plasma clearance of these oligomers (see above), toxicities due to protein binding are, for the most part, transient.

The one significant exception to this is also the most potentially serious of the acute toxicities: activation of the complement cascade. This arises because the complement regulatory protein factor H is a DNA-binding protein (5) and so also binds to phosphorothioate oligonucleotides. The alternative complement pathway can be considered as constitutively active but held in check by a number of regulatory proteins, including factor H. Activation of the alternative pathway can occur when factor H levels are sufficiently decreased by depletion or sequestration due to binding to phosphorothioate oligonucleotides in high concentration. Once activated, the vascular permeability engendered by complement products can result in volume depletion, pulmonary edema, and hypotension, even if the initial insult was transient. Complement activation was first observed in monkeys (indeed, the only animal model in which it has occurred) when several monkeys treated with short bursts of high doses of antisense oligonucleotide developed an anaphylactic-like reaction with hypotension. Notably, no episodes occurred with slower infusion rates (6).

A second area of concern with the systemic infusion of antisense oligomers occurs because antisense oligonucleotides can also bind to factor Xase (7), leading to prolongation of the activated partial thromboplastin time (aPTT). Again, this effect is clearly dose dependent but, in contrast to the situation with complement activation, is indeed transient, due to the rapid plasma clearance of the oligomer. Protamine sulfate rapidly reverses the inhibition (8) and so could be used for treatment of adverse events due to this effect.

CHRONIC TOXICITIES

The kidney is the primary site of oligonucleotide distribution in both rodents and nonhuman primates and thus is the potential target organ for toxicity in these species. Although tissue

concentrations of oligonucleotide in the liver are not as high as in the kidney, the total amount accumulated is larger, probably reflecting the difference in size of the 2 organs (4). Liver toxicity in animals is mostly manifested by increases in transaminase levels. Other organs such as spleen, bone and bone marrow, and lymph nodes also accumulate oligonucleotide to varying degrees. The relationship between dose and tissue exposure varies among tissues but, particularly in the kidney and liver, isn't linear with dose. This nonlinear uptake by the 2 biggest accumulators of oligonucleotides probably explains the nonlinear plasma pharmacokinetics, i.e., as the liver and kidney become saturated, the plasma AUC begins to increase markedly (4). Interestingly, although the plasma AUC following subcutaneous administration is about half that following intravenous administration, oligonucleotide accumulation in tissue is generally independent of the route of administration, with the exception of draining lymph nodes and organs that are highly dependent upon the plasma concentration, e.g., intestine or skeletal muscle (4). This may have particular significance for tumors that are predominantly found in local lymph nodes, such as melanoma.

TRIALS AT THE MARY CROWLEY MEDICAL RESEARCH CENTER

At the Mary Crowley Medical Research Center (MCMRC), we have completed 3 phase I trials involving targeting by antisense molecules to protein kinase C- α (PKC- α), *C-raf*, and *H-ras* and are currently finishing a fourth trial with an antisense molecule to Bcl-2. Tumor cell growth is often discontinuous, so that at any one time a large proportion of tumor cells are quiescent. Therefore, exposure of the tumor to an inhibiting agent must, theoretically, be prolonged, and all 4 studies were designed to provide sustained administration of the oligonucleotide. Further, the toxicities caused by the phosphorothioate backbone of these antisense oligonucleotides occurred at threshold levels that are sometimes exceeded by intermittent bolus injections, a problem avoided by lengthening the infusion times.

ISIS 3521, an antisense oligomer to PKC- α

A variety of hormones and growth factors directly or indirectly activate one of the isozymes of PKC. Specifically, PKC- α is often implicated in malignant transformation and proliferation. For example, PKC- α expression is elevated in human breast cancers (9), and inhibition of PKC- α suppresses the growth of a variety of tumor cell lines.

ISIS 3521 is a 20-mer phosphorothioate oligodeoxynucleotide that hybridizes to the 3'-untranslated region of human PKC- α mRNA. Treatment with ISIS 3521 results in reduction in PKC- α mRNA expression in several tumor cell lines with a median inhibitory concentration of 50 to 100 nM. In a phase I study, we treated 36 patients with advanced cancer at doses ranging from 0.15 to 6 mg/kg/day for 3 days per week for 3 weeks every 4 weeks (10). Treatment-related toxicities included mild to moderate nausea, fever, and fatigue. There were only transient increases in either aPTT or complement C3a values in a few patients, but none were associated with clinical events.

Ten patients showed at least stabilization of disease at the 2-month assessment, but the most significant responses were seen in 2 patients with non-Hodgkin's lymphoma who completed 17 and 9 cycles of therapy and achieved complete responses. These

responses have proven to be long lasting, implying that for some lymphomas, even relatively short periods of inhibition of signaling pathways can be effective in inhibiting tumor dynamics.

ISIS 2503, an antisense oligonucleotide to H-ras

Ras genes (3 are known, H-, K-, and N-*ras*) encode small, 21-kd G proteins that mediate signals from many common growth-factor receptors. G protein stimulation of a growth factor signal pathway is usually rapidly dampened. However, if one of the 3 *ras* genes is mutated at a position critical for GTPase activity (usually codons 12, 13, and 61), the encoded G protein continuously signals the cellular growth pathway, predisposing to malignant transformation.

Inhibition of this abnormal Ras activity should then have significant effects as an anticancer therapy and, interestingly, even a less-than-complete reduction of *ras* expression can still lead to reversal of a malignant phenotype (11). ISIS 2503 is a phosphorothioate 2'-oligodeoxyribonucleotide, 20 nucleotides long, designed to hybridize to a sequence in the initiation translation region of human H-*ras* mRNA. In cell culture, ISIS 2503 specifically reduces the expression of H-*ras* mRNA and protein and inhibits cell proliferation (12).

In a phase I study, we administered a 2-week continuous infusion of ISIS 2503 to 22 heavily pretreated patients with solid malignancies (13). Doses were increased with each cohort of patients and ascended from 1.0 mg/kg body weight to 10 mg/kg body weight of ISIS 2503. Intravenous infusion was continuously administered for 14 consecutive days followed by 1 week of rest (for a total 21-day cycle). Toxicity was minimal in most respects: only a few grade 3 toxicities, and no grade 4 toxicities, were seen. The major side effect was fatigue, which became prominent at the highest dose levels, although this did not represent a true dose-limiting toxicity. At the doses employed in this study, we saw neither significant activation of the complement system nor prolonged aPTTs clearly attributable to ISIS 2503.

No patients achieved a complete or partial response, although 4 patients did have stabilization of disease lasting 2 months or longer. Two of these were prolonged, and the most interesting was seen in a patient with pancreatic cancer treated at 8 mg/kg who had stable disease for 9 cycles. Of note, 90% of pancreatic tumors display some sort of *ras* mutation, although most of these involve K-*ras* (14).

In this study, we also looked at H-*ras* mRNA levels in peripheral blood mononuclear cells in 8 patients. Seven of the 8 demonstrated decreases in mRNA levels with infusion of ISIS 2503, although these decreases generally were modest, with only 2 patients showing a decrease below 50% of pretreatment levels. No correlation was seen between the dose of ISIS 2503 and subsequent decrease in mRNA levels.

ISIS 5132, an antisense oligonucleotide to C-raf

Activated *ras* recruits *raf* kinase to the cell membrane, where it is phosphorylated by membrane-bound tyrosine kinases (15). *Raf* kinases (3 are presently known, A-, B-, and C-*raf*) are serine/threonine kinases that serve as central regulators of mitogen-activated protein kinase (MAPK) and MAPK kinase (MEK). MAP kinases interrelate with a number of substrates and are involved in transformation by most oncogenes (16). Inhibition

of *raf*-associated pathway activity has antiproliferative effects on malignant cells and also seems to correlate with the sensitivity of malignant cells to treatment, with elevated levels of C-*raf* conferring resistance to radiotherapy (17) and paclitaxel (18, 19). This appears to be a specific effect; similar sensitivity is not seen in similar malignant cells treated with *cis*-diamminedichloroplatinum (20).

ISIS 5132, a phosphorothioate 2'-oligodeoxynucleotide 20 nucleotides in length designed to hybridize to the 3'-untranslated sequence of the C-*raf* kinase mRNA, specifically reduced the expression of C-*raf* kinase mRNA in human cancer cell lines and demonstrated antitumor activity, particular in cell lines containing *ras* mutations (21).

We performed a phase I study utilizing a continuous intravenous infusion of ISIS 5132 administered for 21 days every 4 weeks to 34 patients with a variety of solid tumors that were refractory to standard therapy (22). The dose of ISIS 5132 was increased in sequential cohorts of patients as toxicity allowed. As in the other studies, toxicity was modest in all respects. Again, we found no relationship between ISIS 5132 infusion and activation of the complement system. There were also no consistent elevations of aPTT related to any dose level. Again, 2 patients had stabilization of disease for 9 and 10 months with tumors (renal cell and pancreatic carcinoma, respectively) that normally have relatively aggressive courses. The most striking response, however, was in a patient with ovarian carcinoma who had a 97% decrease in CA-125 levels in response to the antisense infusion.

G3139, an antisense oligonucleotide to Bcl-2

In contrast to the molecules already described, which are active in the growth-stimulating pathways, Bcl-2 is an inhibitor of programmed cell death (i.e., apoptosis). Therefore, overexpression of Bcl-2 results in abnormal cell accumulation, and such overexpression is common in several tumors, particularly non-Hodgkin's lymphomas. An antisense oligonucleotide targeted to the open reading frame of Bcl-2 mRNA (G3139) causes specific down-regulation of Bcl-2 expression and leads to increased apoptosis in both cultured malignant cells and animal models.

Clinical activity was confirmed in a trial at the Royal Marsden Hospital, where a daily subcutaneous infusion of G3139 was administered for 2 weeks to 21 patients who had Bcl-2-positive relapsed non-Hodgkin's lymphoma (23). The daily dose of Bcl-2 antisense was increased incrementally to over 110 mg/m², with the dose-limiting toxicity being thrombocytopenia, fever, and hypotension. One patient had a complete response, and 2 patients had partial responses as determined by computed tomography scans. Bcl-2 levels could be measured by flow cytometry in 16 patients and were reduced in 7. In subsequent trials in the USA, G3139 has also shown activity in multiple myeloma and melanoma. At the MCMRC, we are currently concluding a trial of G3139 in chronic lymphocytic leukemia (CLL). To be enrolled in the trial, patients must have CLL, have been treated previously with at least one regimen containing fludarabine, and require further treatment. Because CLL seemed unusually sensitive to G3139 in previous trials of multiple subtypes of lymphoma, the current phase I trial was developed to determine an appropriate dose in this particular disease.

UNRESOLVED ISSUES

There are 2 primary difficulties to be resolved before antisense oligonucleotides can be regarded as a major addition to cancer therapy. The most practical is simply determination of the best method for drug delivery. Cellular uptake of oligonucleotide is limited and seems to vary among cell types. Such varied uptake is probably also why efforts to measure mRNA suppression by assaying peripheral blood lymphocytes produces such uneven results; normal lymphocytes take up antisense oligonucleotides very poorly. Various formulations, including liposomal carriers, have been tried without convincing results. Direct injection of the antisense oligonucleotides delivers the highest tumor concentrations but, of course, is of limited utility in treating systemic malignancy. Interestingly, gut epithelial cells are fairly avid in antisense oligonucleotide uptake, so oral formulations are possible and may represent the best hope for prolonged administration.

This finding is important because the second unresolved issue with antisense approaches concerns the fact that the targeted oncogene is forever present in tumor cells but may be active only sporadically. That is, since most solid tumor cells are quiescent much of the time, growth activity may not coincide with delivery of the antisense oligonucleotide. Alternatively, if pro-oncogenic signaling continues past the time of antisense oligonucleotide administration, then tumor growth is just delayed. Both of these considerations imply that longer durations of antisense oligonucleotide exposure are better which, again, leads back to the question of optimal drug delivery. The one exception to these considerations seems to be non-Hodgkin's lymphoma, in which prolonged responses have been seen with at least 2 different antisense oligonucleotides. The reasons for this are not clear.

CONCLUSIONS

Antisense oligonucleotides represent a new and potentially exciting type of molecular therapeutic agent that allows targeting of specific pro-oncogenic proteins. Despite theoretical concerns, initial trials show antisense oligonucleotides to have only limited toxicity. By and large, however, efficacy in solid tumors other than lymphoma has been limited, suggesting that the molecules are cytostatic rather than cytotoxic and that the primary role for many of the antisense oligonucleotides will be in combination with chemotherapy or radiation therapy. Still to be determined is the best method of antisense oligonucleotide delivery.

1. Milligan JF, Jones RJ, Froehler BC, Matteucci MD. Development of antisense therapeutics. Implications for cancer gene therapy. *Ann N Y Acad Sci* 1994; 716:228-241.
2. Agrawal S, Tamsamani J, Galbraith W, Tang J. Pharmacokinetics of antisense oligonucleotides. *Clin Pharmacokinet* 1995;28:7-16.
3. Leeds JM, Graham MJ, Truong L, Cummins LL. Quantitation of phosphorothioate oligonucleotides in human plasma. *Anal Biochem* 1996;235:36-43.
4. Geary RS, Leeds JM, Henry SP, Monteith DK, Levin AA. Antisense oligonucleotide inhibitors for the treatment of cancer: 1. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des* 1997;12:383-393.
5. Gardner WD, White PJ, Hoch SO. Identification of a major human serum DNA-binding protein as beta 1H of the alternative pathway of complement activation. *Biochem Biophys Res Commun* 1980;94:61-67.
6. Galbraith WM, Hobson WC, Giclas PC, Schechter PJ, Agrawal S. Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey. *Antisense Res Dev* 1994;4:201-206.
7. Sheehan JP, Lan HC. Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. *Blood* 1998;92:1617-1625.
8. Henry SP, Monteith D, Levin AA. Antisense oligonucleotide inhibitors for the treatment of cancer: 2. Toxicological properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des* 1997;12:395-408.
9. O'Brian C, Vogel VG, Singletary SE, Ward NE. Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res* 1989;49:3215-3217.
10. Nemunaitis J, Holmlund JT, Kraynak M, Richards D, Bruce J, Ognoskie N, Kwoh TJ, Geary R, Dorr A, Von Hoff D, Eckhardt SG. Phase I evaluation of ISIS 3521, an antisense oligodeoxynucleotide to protein kinase C-alpha, in patients with advanced cancer. *J Clin Oncol* 1999;17:3586-3595.
11. Aoki K, Yoshida T, Matsumoto N, Ide H, Sugimura T, Terada M. Suppression of Ki-ras p21 levels leading to growth inhibition of pancreatic cancer cell lines with Ki-ras mutation but not those without Ki-ras mutation. *Mol Carcinog* 1997;20:251-258.
12. Chen G, Oh S, Monia BP, Stacey DW. Antisense oligonucleotides demonstrate a dominant role of c-Ki-RAS proteins in regulating the proliferation of diploid human fibroblasts. *J Biol Chem* 1996;271:28259-28265.
13. Cunningham CC, Holmlund JT, Geary RS, Kwoh TJ, Dorr A, Johnston JF, Monia B, Nemunaitis J. A phase I trial of H-ras antisense oligonucleotide ISIS 2503 administered as a continuous intravenous infusion in patients with advanced carcinoma. *Cancer* 2001;92:1265-1271.
14. Friess H, Kleeff J, Korc M, Buchler MW. Molecular aspects of pancreatic cancer and future perspectives. *Dig Surg* 1999;16:281-290.
15. Marais R, Light Y, Paterson HF, Marshall CJ. Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *EMBO J* 1995;14:3136-3145.
16. Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp UR. The ins and outs of Raf kinases. *Trends Biochem Sci* 1994;19:474-480.
17. Riva C, Lavieille JP, Reyt E, Brambilla E, Lunardi J, Brambilla C. Differential c-myc, c-jun, c-raf and p53 expression in squamous cell carcinoma of the head and neck: implication in drug and radioresistance. *Eur J Cancer B Oral Oncol* 1995;31B(6):384-391.
18. Britten RA, Perdue S, Opoku J, Craighead P. Paclitaxel is preferentially cytotoxic to human cervical tumor cells with low Raf-1 kinase activity: implications for paclitaxel-based chemoradiation regimens. *Radiother Oncol* 1998;48:329-334.
19. Rasouli-Nia A, Liu D, Perdue S, Britten RA. High Raf-1 kinase activity protects human tumor cells against paclitaxel-induced cytotoxicity. *Clin Cancer Res* 1998;4:1111-1116.
20. Warenus HM, Seabra LA, Maw P. Sensitivity to cis-diamminedichloroplatinum in human cancer cells is related to expression of cyclin D1 but not c-raf-1 protein. *Int J Cancer* 1996;67:224-231.
21. *Investigator's Brochure ISIS 5132 (CGP 69846A)*. Isis Pharmaceuticals, Inc, 1998.
22. Cunningham CC, Holmlund JT, Schiller JH, Geary RS, Kwoh TJ, Dorr A, Nemunaitis J. A phase I trial of c-Raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin Cancer Res* 2000;6:1626-1631.
23. Waters JS, Webb A, Cunningham D, Clarke PA, Raynaud F, di Stefano F, Cotter FE. Phase I clinical and pharmacokinetic study of bcl-2 antisense oligonucleotide therapy in patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2000;18:1812-1823.