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Potential Use of Multiple Antisense Oligonucleotide Analogs for Cancer Prevention and Therapy

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Abstract. Antisense oligonucleotide (ASO) analogs have been used to counteract the effects of mutated genes and have been developed as therapeutic agents. Several such formulations have been subjected to clinical trials against cancer and have been passed by the FDA for clinical use. However, cancer is a complex genetic disease in which multiple mutations are known to occur for tumors to develop. Three basic stages have been delineated: 1. *Loss of cell growth control* by both oncogenes and tumor suppressor genes; 2. *Angiogenesis*, the production of capillary growth factors to allow blood supply; 3. *Metastasis* allows cancer cells to invade normal tissue. In principle, using three ASOs to down-regulate the products of the mutated genes controlling these specific processes should be possible and this could be an effective preventive method against cancer. In an alternative approach, genetic analysis of cancer cells using microarrays have shown that cassettes of genes are up- and down-regulated compared to normal tissue. Using this information ASOs could be used to down-regulate mutated genes that are up-regulated. In general ASO analog sequences could be used to target the unique pre-mRNA splice sites of selected genes in order to suppress carcinogenesis *in vitro* in selected cancer cell lines and subsequently *in vivo* in chosen mouse models. Computer programs will be used to calculate doses of a cocktail of ASOs to be administered to individual mice and ultimately patients as their tumor genetic profile changes over time.

Keywords: antisense oligonucleotide, cancer, carcinogenesis suppression, prevention, therapy

INTRODUCTION

Selective molecular recognition is the basis of all therapy and of life itself. The specific binding of a drug molecule to a protein/enzyme active site or the binding of a hormone or a natural product into a receptor are common examples. But the selective recognition by base pairing of a sequence of DNA or RNA bases are an even more stringent basis for selectivity. We propose to exploit this fact by using antisense oligonucleotide (ASO) analogs to attempt to treat or prevent cancer.

As indicated at the conclusion of a historical review of antisense oligo applications,[1] antisense should be a way to tackle complex genetic dis-

eases like cancer. In general, it is known that to enable a tumor to grow, three mutations are required[2]: 1. The suppression of *cell growth control*, i.e. loss of contact inhibition; 2. *Angiogenesis*, production of *growth factors for blood capillaries* that enable tumors to grow; 3. *Metastasis*, the ability *to invade normal tissue*. By using the appropriate oligos against splice sites in pre-mRNAs of the appropriate mutations, it might be possible to prevent cancer from developing. This would, of course, be a major project, requiring:

1. The determination of which splice sites to target in mutations in each stage of cancer development; 2. Carrying out *in vitro* studies on cancer cell lines using a combination of three (or more) oligos. This would require at least seven comparable experiments for each set of oligos and different dose regimens; 3. Eventually, with positive results *in vitro*, to extend these studies to *in vivo* experiments with suitable mouse tumor models.

Although this would be a challenging regimen, it is not without the possibility that by monitoring the genetic profile of a given cancer/tumor, it would be possible to down-regulate the mutated genes that are allowing cells to become cancerous and to prevent tumors from developing and metastasizing. Such an experimental protocol would require both the calculation of suitable proportions in a cocktail of ASO analogs with time and of course, the choice of the best ASO analog based on objective comparative criteria.[3]

The stages of carcinogenesis are more complex than indicated above. *Three main stages* can be delineated: 1. Loss of control of cell growth; 2 Production of capillary growth factors (angiogenesis), and 3. Production of tissue infiltration factors (metastasis). But in fact, each of these main stages can also be further divided into several sub-stages.[2] For example, stage 1, loss of cell growth control, can be initiated by oncogenes and down-regulation by mutation of tumor suppressor genes. Other subsidiary mutations can occur, such as loss of DNA repair mechanisms, mutations to the genes that allow the cells to evade apoptosis, and circumvention of telomere shortening and chromosomal aberrations and translocations. Stage 2 could be initiated by down and up-regulation of angiogenic signaling factors, and stage 3 by dysregulation of various cell-cell-adhesion molecules. Given this hideous degree of complexity, deciding which of these targets would be the most effective to pursue should be a matter of extensive comparative experimentation. This is why it may be preferable to *rely on the unique molecular signature of each tumor.*

SIGNIFICANCE

From its inception, the use of ASO phosphorothioate (PS) analogs have been applied in attempts to downregulate various cancer genes.[4, 5] However, the significance of this proposal is the attempt to use several oligos in a cocktail targeted against genes known to be up-regulated during carcinogenesis or found to be up-regulated during microarray genetic analysis of specific cancers. Let us say for the sake of argument that each of the three oligos is only 60% effective alone. Nevertheless, the 40% of cells unaffected by the first oligo will be 60% cured by the second oligo, that is, 16% are unaffected, and these, in turn, will be 60% cured by the third oligo that is 6.4% unaffected. In other words, using three different oligos against three different mRNA targets can, in principle achieve ca, 94% effectiveness. Of course, the eventual result will depend on how effective each oligo will actually be, and whether or not the effects will be additive, but you can see how this approach can achieve a significant amplification effect. One might also hope that the effect of two or three ASOs could be synergistic.

EXPERIMENTAL CONSIDERATIONS

Literature Analysis: For 40 years, ASO analogs have been employed in downregulating cancer genes in experimental research and as therapeutics ("genetic medicines") in cancer therapy. We conducted a series of literature searches using various search engines using the term "antisense oligonucleotide" combined with various other terms of interest. The results are displayed in **Table 1**.

Table 1. Literature Searches for Antisense Oligonucleotide[1]

Plus	Scifinder-n[2]	WoS[3]	PubMed[4]
Cell line	6,146	443	1,112
Oncogene	1,957	247	755
Angiogenesis	1,016	222	133
Metastasis	1,403	195	129
Tumor suppressor gene	97	135	13
DNA repair gene	6	0	0
Cell line and oncogene	774	48	280
In vivo	2,953	1,332	862

[1] All searches of phrases were included within quotation marks.
[2] Scifinder-n is the chemical search engine of the ACS.
[3] Web of Science is the search engine used for Life Sciences.
[4] PubMed is the biomedical search engine of the NLM.

Notably, we were both surprised and pleased at the large number of hits we obtained. This means two things, first of all, the very large number of such studies provides, in effect, a *proof of concept* for this approach, given that so many different ASOs have been used in so many different cancers, both *in vitro* and *in vivo*. But, secondly, it means that to analyze these results and obtain the necessary information about cancer cell lines studied, genes targeted and ASOs used, will be a challenging task. Of course, there will be significant overlap between the three search engines used: Scifinder-n for the chemical literature, Web of Science for the life sciences literature, and PubMed for the medical literature. We need to find an automatic means of "mining" this data from the thousands of search results. It will be one of our first steps in this project to find a means to do this, perhaps using an AI-based data mining program.

Target Sequences in Cancer Cell Lines: Once we have carried out an analysis of all the literature searches and removed duplicates, we will need to discover how many cell lines and genes have been targeted successfully by which ASO sequences and if some have been used repeatedly.

These should be the ASOs we can use in our studies to attempt to obtain increased efficacy with more than one oligo

Combinations of ASO Analogs: In each cancer cell line used, we will target those genes that are involved in various aspects of carcinogenesis and in angiogenesis and /or metastasis if available. We will use combinations of two or three oligos targeted against different genes, and for each set of three oligos, this would involve seven combinations (1, 2, 3, 1-2, 1-3, 2-3, 1-2-3). Once we have established a pattern of relative effectiveness, we would go on to the use of human tumors in nude mice with the same or similar combinations of ASOs.

Types of ASO: We intend to have the ASO synthesized almost certainly as the phosphorothioate (PS) analogs that seem to have been the most successful type. However, there are competing claims by the users of various analogs,[1], and we hope soon to have the results of comparative molecular dynamics studies[6] of the duplexation of the most commonly used analogs,[3], which hopefully can guide us as to which chemically modified analog is preferable*.

INNOVATION

The approach outlined in our proposal has the potential to become an effective novel therapeutic approach to cancer as well as a method of cancer prevention. The very large number of studies published that show the efficacy of ASOs *in vitro* and *in vivo* against cancer genes provides in effect a *proof of concept* as a basis for these studies. Also, there have been quite a number of such ASOs approved by the FDA for clinical use.[7, 1]

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* Recently Cy A. Stein published an article entitled "Phosphorothioates and Me" (Nucl. Acid Therap. https://doi.org/10.1089/nat.2024.0032), which unusually has no references! In this he states: "I began to work in the oligonucleotide field during the first week of March 1987, when as a medical oncology fellow, I joined a laboratory at the NIH, which had recently commenced studies with phosphorothioate (PS) oligonucleotides (oligos) to silence HIV gene expression." That laboratory was in fact mine, officially The Section of Biophysical Pharmacology of the Clinical Pharmacology Lab. NCI. I provide the key citations here. Our prior work led to the initial publication: Matsukura, M., Shinozuka, K., Zon, G., Mitsuya, H., Reitz, M., Cohen, J. S., and Broder, S. Phosphorothioate Analogs of Oligodeoxynucleotides as Novel Inhibitors of Replication and Cytopathic Effect of Human Immunodeficiency Virus, *Proc. Natl. Acad Sci. USA. 84:* 7706-7710, 1987. His research project led to the subsequent publication: Stein, C. A., Subasinghe, C., Shinozuka, K., and Cohen, J. S. Physicochemical Properties of Phosphorothioate Oligodeoxynucleotides, *Nucl. Acids Res. 16:* 3209-3221, 1988. He subsequently had 20 co-authored publications from my laboratory (3 of them reviews), some with other collaborators.