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Commentary on Hong et al., p. 6582

Therapeutic Oligonucleotides: The Road Not Taken

Cy A. Stein and Sanjay Goel

Antisense oligonucleotide therapeutics have been in development for almost 25 years without a single U.S. Food and Drug Administration–approved product in cancer. The reasons for this absence stem, in part, from a deep lack of understanding about how to deliver these molecules to cancer cells *in vivo*. *Clin Cancer Res; 17(20); 6369–72. ©2011 AACR.*

In this issue of *Clinical Cancer Research*, Hong and colleagues (1) report on the safety and tolerability of LY2275796, a second generation antisense oligonucleotide (ASO) targeted to the eukaryotic translational initiation factor eIF-4E mRNA, a gene that has been reported to play a role in tumor initiation.

In the study, ASO maximum tolerated dose and biologic effective dose were set at 1,000 mg, because of a dose-limiting toxicity of grade 3 fatigue at the 1,200-mg dose, and target inhibition was assessed by serial tumor biopsies. At first look, the ASO seemed to achieve target silencing, but closer inspection reveals that the housekeeping genes (e.g., β-actin) were also downregulated (by 64%, compared with 80% for the target mRNA), despite *in vitro* results suggesting excellent target specificity (2). Although the target was silenced, why didn't "target inhibition" achieve "tumor inhibition" in this phase I trial? The problem, in part, is that human cancer is a multitude of processes, pathways, and "cross-talk" with profound redundancy. Cells can often "bypass" the inhibited pathway with minimal detriment to their malignant potential (3). LY2275796, therefore, fails in part because the preclinical models in which it was evaluated do not accurately recapitulate human clinical cancer. Mice and their xenografts, it has often been suggested, are not men.

Nevertheless, as the authors suggest, combining gene-targeted with cytotoxic therapy is clearly the way forward. We strongly agree, but although this point has been understood for decades, we still do not have a single U.S. Food and Drug Administration–approved ASO therapeutic agent in cancer. What has happened? The answer is that each clinical cancer trial of an ASO, in addition to its own unique problems, suffers from the problems shared by all ASO therapeutics, including RNAi. Unfortunately, despite the passage of nearly 25 years for ASOs, our level of understanding about fundamental processes that govern *in vivo* efficacy of therapeutic ASOs, particularly in cancer, is almost nil.

DNA is negatively charged, and in an 18-mer antisense molecule such as, for example, the anti–Bcl-2 ASO oblimersen (4), there are 17 negative charges. Substitution of a sulfur atom for an oxygen atom at each phosphorus atom, forming a phosphorothioate ASO, the type of ASO employed in virtually all cancer clinical trials, maintains the negative charge. However, the melting temperature (T_m) of the duplex formed between the target mRNA and a phosphorothioate antisense ASO will almost always be significantly depressed (5). In addition, whereas first generation phosphorothioate ASOs (those without any additional chemical modifications) have been believed to be exonuclease resistant, they are probably insufficiently so for *in vivo* gene silencing. [The use of 2′-methoxyethoxyoligoribonucleotide gapmers as done by Hong and colleagues (1) both greatly enhances nuclease resistance and increases T_m.] The combination of insufficient *in vivo* nuclease resistance and diminution of T_m after phosphorothioate substitution has probably done much to vitiate the efficacy of phosphorothioate ASOs in earlier clinical trials in cancer. These problems were not apparent in experiments done in tissue culture, because the ASOs were delivered into cells by lipofection, which provided extremely high nuclear concentrations. Further, data from *in vivo* experiments were often interpreted as resulting from antisense gene silencing when, in fact, they resulted from CpG sequence motifs in the phosphorothioate ASO binding to TLR9 receptors on mouse plasmacytoid dendritic cells, with the resulting "cytokine storm" leading to inhibition of tumor growth (6).

In the ultracomplex world of phosphorothioate ASOs, oblimersen is an outlier, because its T_m with its target Bcl-2 mRNA (codons 1–6) is, for unclear reasons, significantly higher than predicted. However, oblimersen contains 2 CpG motifs and is highly immunostimulatory (6). Although earlier work suggested that Bcl-2 was an important target in melanoma, later work challenged this idea

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(7), and it is difficult to understand how silencing of this gene can meaningfully chemosensitize a virulent tumor with such extensive redundant signaling pathways (8). Nevertheless, a small phase II trial done in combination with dacarbazine (DTIC) was successful (9), and thus, the GM301 trial was launched (4). This nonblind, randomized

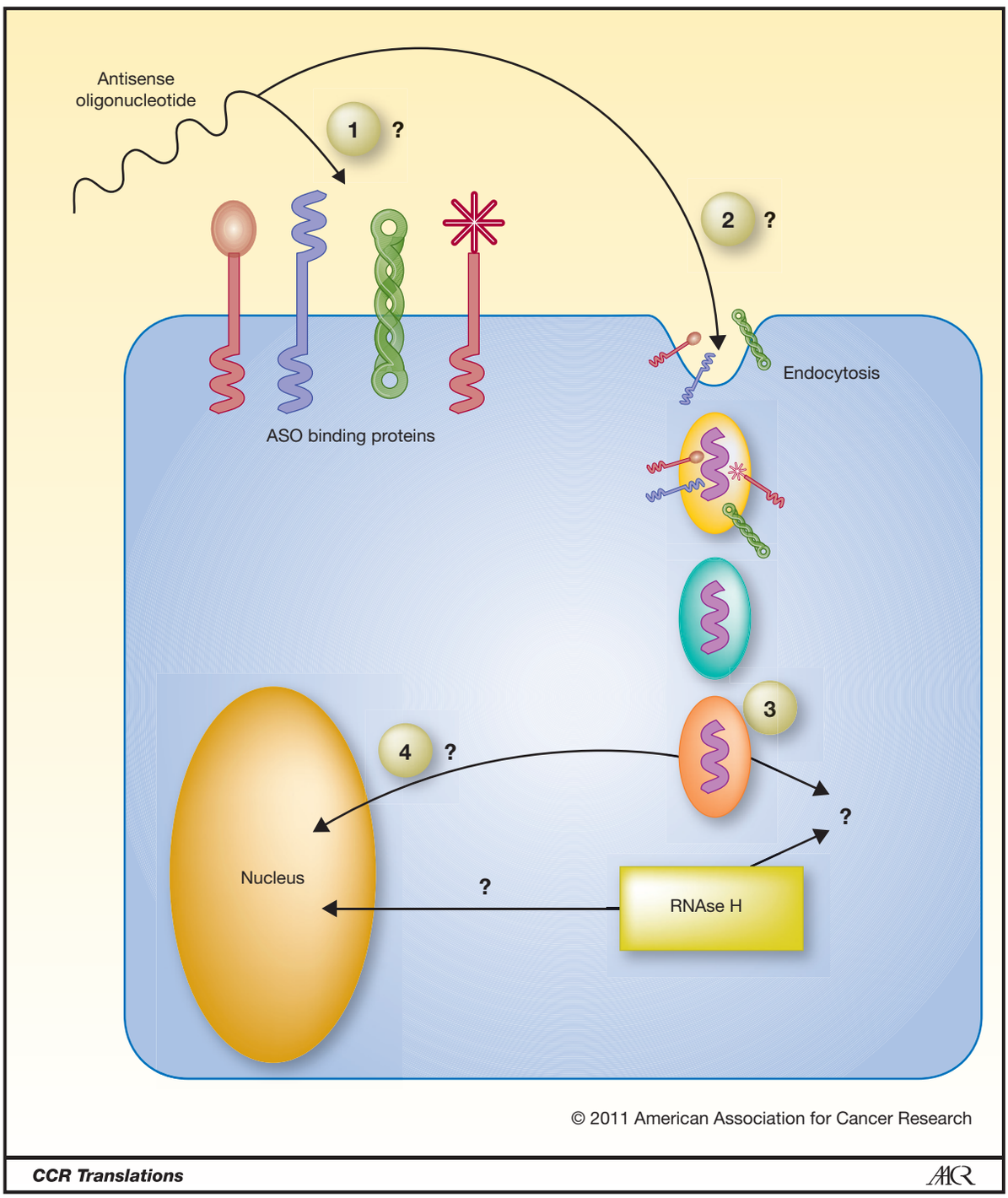

Figure 1. Features of ASO delivery to cells are not well understood. 1, ASOs bind to cell surface proteins. 2, cell surface proteins are internalized into endosomes with variable rates and mechanisms, depending on cell types. 3, hydrophobic ASO probably transits the endosomal membrane, although how this is accomplished is not understood. 4, location of target mRNA cleavage (nucleus or cytoplasm) is uncertain, although RNAse H seems to be the enzyme responsible for mRNA cleavage.

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trial in 775 patients compared oblimersen + DTIC versus DTIC alone. Patients were also prestratified by baseline lactate dehydrogenase (LDH). A continuous improvement in overall survival (OS) was observed in the patients receiving oblimersen as a function of baseline LDH. Patients with LDH $\leq$ 0.8 times the upper limit of normal (ULN) showed the greatest benefit in OS, and those with LDH $\geq$ 1.1 times the ULN showed no difference in OS (10). On the basis of these results, a randomized, phase III trial of oblimersen + DTIC versus DTIC alone was done in 300 patients with LDH $\leq$ 0.8 times the ULN (GM307). Here, however, no difference in OS was observed. Thus ended clinical trials of oblimersen, which had also failed in myeloma and whose development in chronic lymphocytic leukemia was also halted.

Why were the data in the earlier trial not reproduced? The probable reason was that the GM301 trial was not blind, unlike GM307, and patients received an average of 5 cycles of DTIC (a minimally active antimelanoma drug) in GM307 (versus 3.2 cycles in the GM301 study). This difference was sufficient to eliminate the observed increase in OS in the low LDH population in GM301.

But would a successful GM307 have constituted that elusive *in vivo* proof of principle? The answer in our opinion is no. Even ignoring the issue of whether Bcl-2 is a target in melanoma, the question of whether sufficient ASO enters melanoma cells or any other cancer cell clinically to reproducibly and robustly silence any target has never been answered. This contention is also true of LY2275796 in this trial, which seems to silence its target and downregulate housekeeping genes with similar potency and, thus, must be suspected of accomplishing both here nonspecifically. Despite huge expenditure on a vast array of delivery strategies, carrier molecules, etc., all of which suffer from cost issues, toxicity, poor delivery to tumors, or a combination, the foremost technologic hurdle blocking clinical progress for therapeutic ASOs (antisense and siRNA) in cancer is delivery. ASOs administered to patients in phase I and II trials can be found at high concentration in liver, kidney, and the small intestine, but whether sufficient concentrations for gene silencing are found in tumors and, critically, intracellularly is unknown. Further, optimal concentrations may differ dramatically depending on tumor type, ASO chemistry, route of administration, and dose schedule, none of which is usually optimized rationally for a cancer indication. In human tumors, what factors determine the rate of delivery of ASOs to cells? What factors determine the rate at which ASOs enter or leave endosomes, where they reside in cells? Where in the cell does antisense silencing occur, and how do polar ASOs transit hydrophobic endosomal membranes? All of these questions are unanswered (Fig. 1).

We know that for some newer modified ASO gapmers that produce nuclease resistance and increased T_m (e.g., those containing 3′ and 5′ locked nucleic acids), no carriers are required, at least *in vitro*, for gene silencing (11). However, it is unclear whether silencing can be produced in human tumors with these ASOs, despite some unpublished successes in human tumor xenograft models in mice. This is the road not taken in oligonucleotide therapeutics for cancer: In the search for the most rapid path to the market, the difficult, time-consuming, and costly work of meticulously studying the delivery process has never been undertaken. And for this lack of knowledge, the field of oligonucleotide therapeutics, despite the occasional glimmer of hope described by Hong and colleagues (1), continues to pay the price in its lack of clinical activity in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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