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Antisense targeting in cell culture with radiolabeled DNAs —a brief review of recent progress—

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The promise of antisense targeting that any tissue with a unique genetic expression can be specifically localized with radioactivity in the living subject is the holy grail that drives this research today. If antisense targeting were to achieve even a fraction of its promise, the results could well lead a revolution in diagnostic nuclear medicine. Despite its obvious complexities, antisense targeting with radiolabeled oligomers such as DNA is making considerable progress in cell culture. As is documented in this brief review, evidence is becoming overwhelming that an antisense mechanism is probably responsible for the accumulation in tumor cells in culture of radiolabeled DNAs with base sequences antisense to target messenger RNAs (mRNAs). That an increased accumulations of these DNAs compared to control DNAs has now been seen in a substantial number of tumor cell types and mRNA targets largely eliminates any possibility of an aptameric effect being responsible for these specific accumulations. In addition, the number of antisense DNAs accumulating specifically in cells in culture has been shown to be orders of magnitude larger than that expected on the basis of steady state mRNA levels. Thus, two of the main concerns regarding antisense targeted, namely that the mechanism of localization may not be attributed to antisense and that the degree of accumulation will be impractically low for imaging, have been addressed in recent research. The remaining obstacle to successful targeting may be delivery. This review will provide a brief review of recent results, primarily from the laboratory of one of the authors (DJH), obtained in tissue culture in studies of antisense targeting and will conclude with several suggestions for future approaches.

Key words: antisense, cells, technetium-99m

INTRODUCTION

FIGURE 1 provides a simplified overview of the molecular biology involved in transcription and translation. DNA coding for the gene in question in the nucleus is unwound and transcribed by RNA polymerase into a pre-messenger RNA that carries all the information of the gene in what by definition is the "sense" sequence. The packaging of the pre-mRNA includes 5′ capping and 3′ adenylation as well as the removal of introns. The mature mRNA is then allowed to diffuse out of the nucleus into the cytoplasm. Once in the cytoplasm, it may survive minutes to hours but possible not before it is translated one or more times at the site of a ribosome into the protein for which the gene in question codes.[1–3] The objective of antisense targeting is to develop method whereby DNA and other chemical forms of oligomers with a complementary base sequence (i.e. "antisense") can effectively locate and hybridize to its targeted mRNA. Once targeted, the oligomer may act as a drug in preventing translation (i.e. antisense chemotherapy) or, if radiolabeled, may be used to image radioactivity in target tissue (i.e. antisense imaging).

Antisense targeting was first considered in the early 1970's as a novel approach to rational drug development. Whereas traditional drugs act on the proteins that are the final products of gene expression, for example to inhibit

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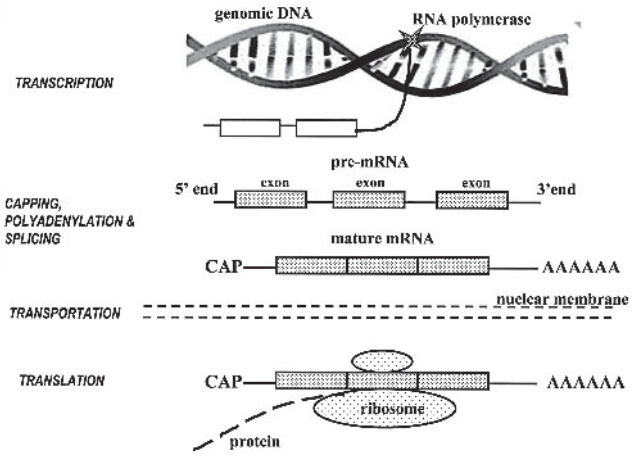

Fig. 1 Stylized depiction of transcription and translation, grossly oversimplified for clarity (adapted from Crook ST[3]).

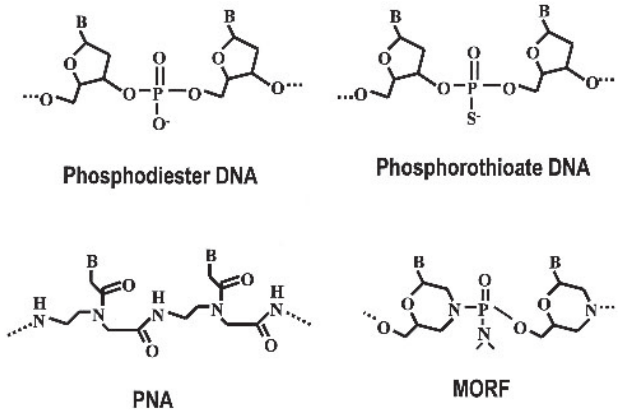

Fig. 2 A short segment of four oligomers showing the chemical structures of phosphodiester and phosphorothioate deoxyribosenucleic acid (DNA), peptide nucleic acid (PNA) and phosphodiamidate morpholino (MORF).

a protein critical to cell proliferation in cancer, antisense drugs act at an earlier stage by inhibiting the translation to proteins of the mRNA. Thanks to the human genome project and genomics in general, considerably more is now known about the mRNA base sequences for a variety of genes in humans, animals and plants-much more than is generally understood about the structure of their proteins. In principal then, it should be easy to accomplish the goal of interfering with a large variety gene expression at the mRNA level with antisense drugs. Unfortunately, antisense technology is proving to be much more complicated and challenging than was expected. Nevertheless, antisense drug development continues to progress with one drug already approved by the US FDA (Vitravene, Isis) and another in phase III trials and expected to receive approval as the first antisense drug for intravenous administration (Genasense, Genta). While the recent attention focused on small interfering RNAs may have temporarily dampened enthusiasm for antisense drug therapy,[4] if only a small number of the approximately 20 antisense drugs in late-stage development successfully enter the marketplace, the momentum should continue apace.[5]

The focus of this review is not chemotherapy with antisense drugs but antisense targeting with radioactivity of cancer cells in cell culture in the hope of validating targeting by this mechanism. These studies may be viewed as preliminary to the eventual development of nuclear medicine targeting *in vivo* with radioactivity of tumor and other tissues in connection with antisense nuclear medicine imaging. Because the number of studies with radiolabeled antisense DNAs and other oligomers remains limited in cell culture (and almost nonexistent in animals), this review will, of necessity, primarily describe results from the laboratory of one of us (DJH).

While the pioneer efforts in antisense targeting in tissue culture reported encouraging evidence of localization,[6] continuing progress in antisense target required assurances that localization could best be explained by an antisense mechanism. However, since the ultimate objective is *in vivo* imaging, it was first necessary to demonstrate that the process of radiolabeling with photon emitting radionuclides such as ^{99m}Tc do not adversely influence the targeting properties of the antisense DNA. Thus the first goal of several subsequent investigations was to determine whether chemically modifying DNA to facilitate radiolabeling with ^{99m}Tc has an important influence either on the hybridization properties or cell accumulation properties of DNA.[7]

Figure 2 presents the chemical structure of the native phosphodiester DNA, the phosphorothioate DNA and peptide nucleic acid (PNA) and phosphodiamidate morpholino (MORF), two DNA analogues. Since the native phosphodiester DNA is almost universally viewed as too unstable for *in vivo* use, virtually all clinical trials of antisense drugs are currently being conducted with the phosphorothioate variation in which a sulfur replaces one of the non-bonding oxygens in the backbone. Accordingly, the early studies of Zhang et al. primarily used a phosphorothioate DNA 18 bases in length and with a base sequence antisense to the mRNA of RIα of PKA. This mRNA is thought to be over expressed in all tumors.[8] The DNAs (antisense and controls) were each purchased with a primary amine attached via a six-member alkyl chain to the 5′ end to facilitate conjugation with chelators for ^{99m}Tc radiolabeling. Figure 3 presents the structures of the three bifunctional chelators used to evaluate the influence of different methods of labeling DNAs with ^{99m}Tc-the cyclic anhydride of DTPA and the *N*-hydroxysuccinimide esters of MAG_3 and HYNIC. The coligand used with HYNIC was always tricine.

Surface plasmon resonance was used to measure the association rate constants (the dissociation rates were too slow for reliable determination by the instrument in use at the time) for hybridization to the phosphorothioate DNA

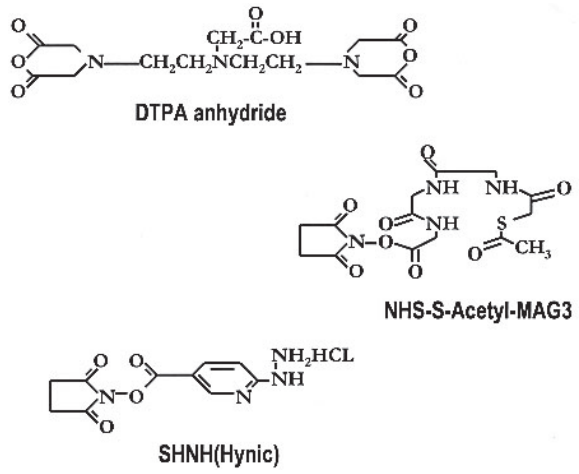

Fig. 3 The chemical structures of DTPA cyclic anhydride, NHS-MAG_3 and SHNH (HYNIC) bifunctional chelators whereby oligomers have been radiolabeled with ^{99m}Tc.

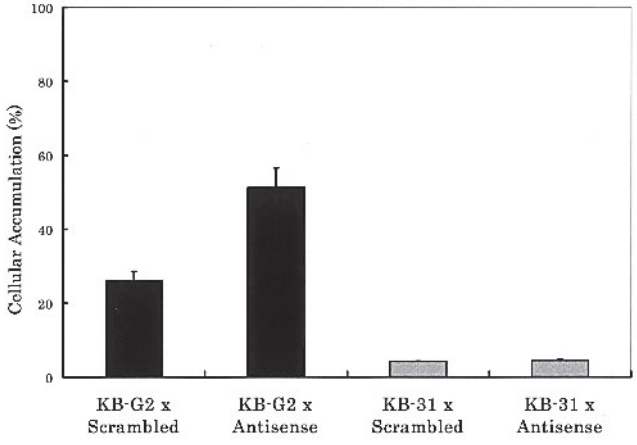

Fig. 4 An example of the results obtained in cell culture showing statistically significant increased accumulations of ^{99m}Tc-labeled antisense DNA compared to control scrambled DNA in KB-G2 MDR++ cells overexpressing the Pgp target mRNA compared to KB-31 MDR+ control cells (Nakamura K et al., unpublished data 2003). Error bars show one standard deviation.

in its native form (except for the addition of the biotin group on the 5′ end whereby the sense DNA was immobilized for the measurement) of the native DNA, the amine/linker/DNA and the chelator/amide/linker/DNA where the chelator was either DTPA, MAG_3 or HYNIC. All DNA were uniformly modified phosphorothiolates. No significant differences were observed in association rate constant for any chemical form of DNA. The results of measuring melting temperatures for three of these combinations led to the same conclusion.[2] Thus, the methods under consideration for radiolabeling could be used with assurances that hybridization affinities would not be significantly altered as a result of the chemical modifications.

While the hybridization affinities were independent of

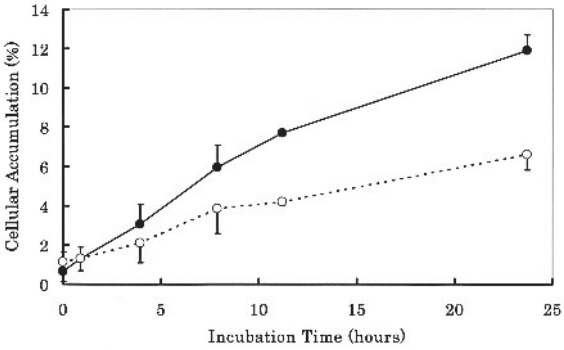

Fig. 5 Another example in cell culture of the statistically significantly higher accumulations of antisense DNAs in cancer cells compared to control DNAs, in this case obtained in ACHN cells with a ^{35}S radiolabeled antisense DNA against the RIα (Zhang Y et al., unpublished data 2003). Closed circles show antisense DNA accumulations, open circles show control sense DNA accumulations. Error bars show one standard deviation.

the labeling method, both the tumor cell accumulations in cell culture and the biodistributions in normal mice were significantly affected. After about 4 h of incubation with ACHN renal adenocarcinoma cells, the difference in cell accumulation became significant and increased with incubation time in the order DTPA > HYNIC < MAG_3.[7] The biodistributions in normal mice at 4 h were also different with HYNIC showing the highest liver, kidneys and spleen levels.[7] These patterns bear some similarity to that shown for two peptides radiolabeled with the same three chelators.[9] Thus, while the amine-derivitized DNAs could be successfully radiolabeled with ^{99m}Tc using each of three chelators and that in each case, the radiolabel was stable and there was no influence on hybridization properties, there were profound differences in the pharmacokinetics of the radiolabel in normal mice and profound differences in cellular uptake. Commonly, MAG_3 was used in that laboratory for most subsequent studies for convenience.

Having shown that oligomers may be successfully radiolabeled with imageable radionuclides, the goal of subsequent studies would then be to establish to the greatest extent possible that localization in cell culture was due to an antisense mechanism. While a direct demonstration of an antisense mechanism remains elusive even in cell culture, indirect evidence of this mechanism is now common. If increased accumulations are observed for a variety of DNAs, radiolabeled by different methods, with antisense base sequence against different mRNA targets and in different cell types, compared to control DNAs with irrelevant base sequences (e.g. sense, random, scrambled), then an aptameric mechanism for the increased accumulations may be largely excluded. Since oligomers with different base sequences will show differences in behavior, a large number of such studies with positive accumulations compared to controls will be

necessary to exclude aptameric effects as a plausible mechanism. A statistically significant increased accumulation of radioactivity has been observed in LS174T colon cancer cells and ACHN kidney cancer cells with endogenously labeled ^{35}S as well as ^{99m}Tc-radiolabeled uniform phosphorothioate MAG_3-DNA against the RIα mRNA of PKA compared to sense and random DNA controls but not in HC2 cells with irrelevant murine RIα mRNA.[10] An increased accumulation of radioactivity in MDA-MB-231 breast cancer cells with ^{99m}Tc labeled HYNIC-DNA antisense to the c-myc oncogene compared to the sense DNA control has also been observed.[11] Recently a statistically significant increased accumulation was also observed in targeting of multidrug resistance mRNA in epidermal carcinoma cells KB-G2 and KB-31 with ^{99m}Tc labeled antisense MAG_3-DNA compared to the scrambled DNA control.[12] Figure 4 provides but one example of the results obtained in cell culture, in this case showing statistically significant increased accumulations of ^{99m}Tc-labeled antisense DNA compared to control scrambled DNA in KB-G2 MDR++ cells over expressing the Pgp target mRNA compared to KB-31 MDR+ control cells. Should an antisense DNA assume a particular configuration that encourages increased cellular accumulation by an aptameric effect, this would be extremely unlikely to be the case for each of the large variety of base sequences, labeling methods, mRNA targets and cell types described in the above studies.

Another test of specific mechanism of localization is to demonstrate that accumulation decreases with increasing dosage as binding becomes saturated. When ACHN cells were incubated with ^{99m}Tc-labeled antisense DNA with increasing concentrations of unlabeled antisense DNA in the range 7 to 100 nM and compared to the accumulations of ^{99m}Tc-labeled sense DNA with increasing concentrations of unlabeled sense DNA in the same range, the difference in antisense compared to sense DNA accumulations decreased and became statistically insignificant as the concentration increased.[10] A similar behavior has recently been observed for ^{99m}Tc-labeled antisense and control DNAs in the KB-G2 and KB-31 cells (Nakamura, unpublished observations, 2003).

Even taken together, these observations do not constitute proof of an antisense effect but they do strongly suggest that the observed accumulation of radioactivity in cells exposed to antisense DNA under the conditions of these investigation is probably due, at least in part, to an antisense mechanism.

One addition observation from these cell culture studies relates to the number of antisense DNAs accumulating specifically per cell. Figure 5 presents another of many examples of the statistically significantly higher accumulations of antisense DNAs in cancer cells compared to control DNAs. This particular figure shows the increased accumulations of ^{35}S-labeled DNAs in ACHN cells. From cell culture results such as this using the number of cells per well and the known specific activity of the radiolabeled DNAs, it may be calculated that the specific accumulation (i.e. antisense minus control DNA) is approximately 10^{5-6} antisense DNA molecules per cell after 10–24 hrs of incubation (and corresponds to an increase in concentration of antisense oligomers from nM in the media to mM concentration in the cytoplasm). These specific accumulations are many orders of magnitude greater than the steady-state target mRNA concentrations that are usually assumed to be in the range of 1–1000 copies per cell.[10] Furthermore, these results are in general agreement with the saturation cell studies described above. Saturation is occurring at DNA concentrations of about 50 nM. This concentration in 10^5 cells/m*l* per well corresponds to about 10^8 DNA/cell. Since about 1% of this is incorporated, it may be calculated that about 10^6 DNAs have accumulated per cell at saturation.

While calculations based on other published results confirm that accumulations far in excess of assumed steady state mRNA levels have been observed in cell culture,[6] not all investigations have shown this to be the case.[13] Others have even failed to observe increased accumulations of antisense DNAs.[13,14] These unfavorable results may be due to the use of concentrations of DNA in the 0.1–1 μM range. The results of Zhang et al. suggest that increased accumulations of antisense DNAs may not be in evidence if the DNA concentration exceeds about 50 nM during incubation. Recently, an 11-fold higher cellular accumulation of an ^{111}In-labeled peptide nucleic acid (PNA) oligomer (conjugated with the transduction peptide PTD-4) was observed in culture compared to a control nonsense sequence in Raji cells that over express the bcl-2 mRNA and higher accumulations were observed in this cell type compared to U937 cells that do not over express the target mRNA. This increase was calculated to correspond to accumulations of more than 2000 antisense molecules per cell.[15]

The positive results described above showing high levels of DNA accumulating in cancer cells in culture by what is likely to be an antisense mechanism are particularly relevant since estimates of tissue counting rates achievable *in vivo* that are based only on steady state mRNA concentrations would suggest that antisense imaging may be unfeasible in many instances.[2] The impression that antisense imaging may not be achievable has been reinforced by the general absence of positive results thus far in the limited number of published studies of *in vivo* antisense targeting.[15,16] Recently positive images were reported in mouse xenografts in which the target mRNA could be upregulated by epidermal growth factor.[17] Superior image were obtained following ^{111}In-labeled DNA administration in mice bearing the tumor following upregulation with EGF. However, since no significant differences were observed between the antisense DNA and its random control, the improvement in imaging with upregulation may not be due to an anti-

sense effect. Possibly the intratumor administration of EGF has altered tumor properties to explain the increased accumulations (Recently, several *in vitro* and/or *in vivo* studies have been reported in which control cells have been used.[18–20] While useful in other ways, the results of these investigations shed little light on whether accumulations were by an antisense mechanism since control oligomers were not used). To our knowledge, a positive image in a tumored animal by intravenous administration that may be reasonably attributed to antisense targeting has not yet been reported. In a pioneer study, Dewanjee et al. reported surprisingly positive results in a mouse tumor model using an antisense DNA of unspecified sequence complementary to a sequence within the initiation codon site of the c-myc oncogene mRNA.[21] However, these favorable results have yet to be reproduced.

DISCUSSION

A fair evaluation of studies in cell culture with antisense oligomers permits the conclusion that thus far results have generally been positive. Accumulations have been observed by what appears to be an antisense mechanism and that the magnitude of these accumulations suggest that *in vivo* imaging may be practical, at least in certain situations. Nevertheless, definitively positive images by an antisense effect have yet to be reported. Among the factors that may best explain these failures is insufficient delivery of antisense DNA into tumor cells. While in culture cellular accumulations are at levels that would provide adequate nuclear medicine images, the barriers to *in vivo* cellular accumulations will certain reduce these levels considerable. Another factor contributing to the lack of success thus far in antisense imaging may be nonspecific accumulations of radiolabeled DNAs in non target tissues leading to high background radioactivity. Because of the enormous complexity of antisense targeting, unlimited approaches could be considered to address these factors. Three approaches are suggested herein as reasonable and deserving of attention: 1) use of carriers to increase cellular accumulations; 2) different chemical forms of antisense oligomers; and 3) selection of mRNA targets with high turnover rates.

Consider first the use of carriers to encourage cellular accumulations of antisense DNAs. Perhaps the most serious challenge facing *in vivo* applications of oligomers, whether for gene therapy, antisense chemotherapy or antisense imaging, is poor cellular accumulations. Experience suggests that DNAs or other oligomers administered "naked" are unlikely to provide sufficient target accumulations for impressive images and that some vector will be required as carrier. Cationic liposomes as carriers are convenient when used with anionic oligomers such as DNA since binding is by simple charge attraction. Increased accumulations of antisense DNA in cells in culture has been observed using cationic liposomes.[11] Unfortunately, in that study, accumulation of the control DNA was elevated in proportion such that the advantage in target/nontarget ratio to the use of the carrier was minimized. Possibly of more use are the cationic peptides sometimes called transporters because they can transport drugs efficiently into cells. One example is the Tat peptide through which the AIDS virus gains access into cells. Being cationic, the Tat peptide may also be radiolabeled with anionic DNAs by simple charge attraction but when labeled in this way, the peptide may lose its unique transporting properties.[22]

Studies of different carriers may eventually result in improved cellular accumulations for antisense oligomers but they are unlikely to address the second factor, namely high levels of nonspecific accumulations. Consider next the chemical form of antisense oligomers. Literally thousands of chemical modifications of DNA have been synthesized and tested. One of the interesting features of oligomers for *in vivo* applications is the large variation in properties imparted by the various backbone structures. Presumably this variability extents to cellular accumulations as well. However most modified DNAs are not available commercially and are therefore restricted to use by those laboratories with synthesis facilities. Two oligomers that are commercially available are PNAs and MORFs, the structures of which are shown in Figure 2. Both are charge neutral and therefore will not bind by charge attraction to cationic carriers, thus complicating their use. A recent study of antisense MORFs radiolabeled as usual with ^{99m}Tc and directed against the RIα mRNA showed minimal accumulations in ACHN cells in culture as expected for uncharged oligomers.[23] However, the antisense minus random control differential was much larger than that reported previously for DNAs and may be due in large part to more rapid efflux of the control MORF.

Thus studies of different chemical forms may eventually result in lower levels of nonspecific accumulations and, possibly, improved cellular accumulations of antisense oligomers. Another approach that may result in increased specific accumulations is to consider the mRNA target. Because the number of antisense DNAs accumulating specifically in tumor cells in culture are orders of magnitude higher than that expected from steady-state mRNA levels, it has been suggested that the antisense DNA may be acting in some manner to preserve its target mRNA.[10] If so, the mRNA turnover rate would be more important to successful targeting than the steady-state mRNA level as an increased transcription rate would increase the cellular concentration of the target mRNA if stabilized by the antisense DNA. Therefore among the criteria considered in the selection of mRNA targets may be turnover rate. If this hypothesis is correct, an increased accumulation of antisense DNA may be expected as the number of targeted mRNAs increases while accumulations in nontarget tissues would presumably remain unchanged.

CONCLUSIONS

We have attempted to provide in this review a brief overview of the current state of antisense targeting in cell culture, citing studies primarily from the laboratory of one of us (DJH) to do so. When the results of this and other laboratories are considered collectively, the conclusion is that successful *in vitro* targeting has been demonstrated. That successful *in vivo* targeting has not yet been convincingly demonstrated may therefore be attributed to unfortunate choices thus far of antisense base sequence, chemical form of oligomer, radiolabeling method, carrier, mRNA target, target tissue, etc. While the number of such factors that may require optimization for successful antisense targeting is large, the effort to achieve this optimization will be worthwhile if useful antisense targeting *in vivo* can be achieved.

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