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COMMENTARY

ANTISENSE AGENTS IN PHARMACOLOGY

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"Antisense" in the pharmacologic context refers to the use of chemical or biological agents which interact with cellular nucleic acid targets in a sequence specific fashion. This interaction connotes a disruption in the proper transfer of genetic information within the target cell. This disruption may be reversible or irreversible. An "antisense" agent may be distinguished from other nucleic acid interacting drugs which recognize specific sequences in a nucleic acid species (e.g. netropsin and DNA) by the length of sequence recognized and by the nature of the drug:nucleic acid interaction. For an antisense agent, the interaction should statistically define a unique target. In addition, the interaction of the antisense agent with the target nucleic acid is generally considered to be based upon the primary sequence of the target, not upon the *base pair* sequence of the target. The antisense agent may be in the form of an endogenously expressed gene in an antisense configuration (i.e. inverted so as to produce an RNA transcript which is complementary to the normal transcript), encoded by a genetically engineered vector which transcribes a homologue of the target nucleic acid. the antisense agent may also be composed of RNA or DNA microinjected into the target cell, or nucleic acid analogs directly administered to cell culture medium.

Antisense agents have been under investigation for approximately 20 years. Within the last few years, largely as a result of developments in synthetic nucleic acid chemistry, compounds which can selectively attenuate gene expression in cell culture and perhaps *in vivo* have been synthesized. The makeup of these compounds and their behavior and mechanism in biological systems are currently an active area of research in many laboratories and are undergoing a rapid evolution. This commentary is designed to evaluate the applications and potential applications of antisense agents for the pharmacologist. The issues of mechanism of the biologic response mediated by antisense oligodeoxyribonucleotides (ODNs*) and evaluation of an antisense response are discussed. In addition, some practical applications are presented with the intent of suggesting the best means whereby pharmacologists may exploit current technology for their own interests. Within the last 2 years several conferences have been held to discuss developments, and a number of reviews are available which summarize studies by various laboratories using either synthetic antisense agents as "drugs" or genetically engineered minigenes which produce antisense RNAs. The reader is referred to several recent reviews which describe the structures and properties of synthetic antisense agents as well as results employing the genetic engineering approach [1–4].

For *in vivo* pharmacologic application, the current best prospects for antisense agents are likely to be nucleic acid analogs. Compounds that have been synthesized have modifications of the phosphodiester backbone of DNA and/or the anomeric conformation at the C-1 position of the sugar residues, and may be covalently coupled to other moieties which can cleave or intercalate duplex nucleic acids. There are now a variety of these compounds, many of which have desirable properties such as being stable *in vivo*, entering cells and either binding tightly to a target, thus interfering with function, or instigating regional degradation or both [1–4]. Genetically engineered antisense delivered by vector or microinjection is generally not suitable for pharmacologic application, as saturation of target sites *in vivo* is not feasible. However, there are applications of artificial antisense genes for experimental pharmacology which currently may offer advantages over oligonucleotides and oligonucleotide analogs. Instances where there is a question of what role an enzyme plays in the action of a drug or even of the suitability of a potential drug target should be highly amenable to application of genetically engineered antisense. It is generally much easier by standard molecular biological techniques to construct an antisense vector which will express antisense RNA and, as a result, attenuate gene expression than to synthesize a chemical compound which specifically and efficiently binds to a protein target. Success with this approach has been achieved in numerous systems and appears to depend predominantly upon the ability to express antisense:sense at a high level. It should be pointed out that rarely has complete ablation of a target been demonstrated with antisense RNA. Thus, genetic antisense is unlikely to be suitable in cases where even a low level of gene product can sufficiently meet the cellular demands of a particular metabolic pathway.

Potential targets for antisense agents are: genomic

* Abbreviations: ODNs, oligodeoxyribonucleotides; M-ODNs, methyl phosphonate oligoribonucleosides; S-ODNs, phosphorothioate oligodeoxyribonucleotides; α-ODN, α-anomeric DNA; PCNA, proliferating cell nuclear antigen or cyclin; TPI, triose phosphate isomerase (EC 5.3.1.1); and hTr, human transferrin receptor.

DNA integrity; transcription; and RNA processing, stability, transport, splicing and translation. While the possibility of exploiting these targets has been demonstrated *in vitro*, the rigorous demonstration of mechanism *in vivo* has only been shown for a few. Neither failure nor success *in vitro* is necessarily a good indicator of activity in whole cell systems, and even less so in an animal or human. Examples of activity against one gene product often do not translate into activity against a second gene product or to a different cell culture system. Presently, there are very few reliable tenets concerning requirements for successful application of antisense agents.

An antisense agent interacts with a target as any other drug, the major difference being the target is one or more nucleic acid species which carries a defined base sequence. Thus, two or more antisense compounds could be directed against the same molecule of mRNA but bind to different sequences within that target molecule, much as an enzyme may have multiple binding sites with different affinities for substrate and product analogs. Because binding sites in nucleic acids (e.g. mRNA or DNA) are defined by the primary structure (i.e. sequence), the number of potential binding sites is on a grand scale compared to enzyme targets. This inherent feature of an extremely large array of potential targets within any particular gene or gene product is a major potential advantage of employing an antisense agent for pharmacologic purposes. If one considers that the majority of currently used drugs interact with protein based receptors of one type or another, then the cascade mechanism of gene expression illustrates another advantage of an antisense compound. If a drug which interacts with a protein and an antisense drug which interacts with a nucleic acid species coding for that protein both bind irreversibly to their respective targets (and all considerations of stability, distribution and cell entry are set aside), the antisense compound would be expected to be at least several hundred- or thousand-fold more effective. This derives from the fact that one gene may code for hundreds of pre-mRNAs which are processed to mRNAs, each of which may then be translated to several hundred protein molecules. Thus, one antisense drug specifically interacting with one gene could, in effect, prevent the synthesis of a large number of drug receptors. However, it should be noted that even if the expression of a particular gene is shut off, the previously existing cellular end product of that gene expression is still available for cellular functions. In instances where the target is expressed in a proliferating cell (e.g. tumor, hematopoietic tissue), the phenotypic effect may be delayed until cell replication has decreased the cellular target level below a threshold. In cell types which do not proliferate (e.g. central nervous system neurons), several target half-lives may be required until this threshold is reached. These considerations suggest that for rapid and effective pharmacologic gain, the combination of an antisense agent with a drug which interacts with the end product of the gene in question may be the most effective.

Antisense drugs may be less susceptible to drug resistance mechanisms which render conventional receptor-oriented drugs ineffective. A point mutation can alter the amino acid sequence of a receptor and lead to drug-resistance. However, this type of mutant would still be susceptible to antisense agents, as a single base substitution may not be sufficient to prevent binding of an antisense agent. Additionally, it is much easier to modify an antisense compound determined by permutations of four monomeric constituents than to redesign a drug based upon the altered three dimensional structure of a drug receptor. The flexibility and fidelity of DNA synthesizers allow alterations in the sequence of an antisense compound in a facile manner.

Antisense provides an extremely simple framework with which to achieve selectivity, since selectivity is nucleic acid sequence dependent. Antiviral therapy may be the broadest horizon for the application of antisense agents, while as a treatment of viral infection antisense agents would probably not be curative. However, in cases of latent viral infection (e.g. herpes or papilloma viral infections), the maintenance of latency is a real possibility, as is the prevention of infection under conditions where factors associated with the high risk of infection are known (e.g. HIV). There is also potential application in the treatment of other pernicious infectious diseases (e.g. trypanosomiasis, malaria) where the infectious organism presents a vastly different genomic sequence which should translate into a large variety of "antisense receptors."

As mentioned previously, oligonucleotide analogs represent the most promising means for the eventual development of clinical antisense pharmacology. Apparent success in attenuating specific gene expression has been achieved with a number of analogs and various targets, but the mechanism of action in most cases has not been well defined. Initial considerations of the desirable properties of an oligonucleotide analog for antisense applications led to the design and synthesis of the first compounds with biological activity, the M-ODNs [5]. This approach circumvented what may have been the single largest problem of antisense oligonucleotides, sensitivity to nucleases. The M-ODNs are relatively stable and bind to complementary sequences of DNA or RNA in a sequence-specific fashion. However, as a result of racemization at the phosphorus of each internucleotide linkage, automated solid phase synthesis yields a collection of isomers (2^n! isomers of an oligomer with n internucleotide linkages of n+1 bases), precluding the simple preparation of pure material. Nevertheless, biological activity has been demonstrated in an assay evaluating the infectivity of cultured cells to Herpes simplex virus type 1 when co-administered or administered prior to virus infection [6]. The major drawbacks of these compounds appear to be poor product yields, length limitations resulting from poor aqueous solubility, the need for high concentrations to observe a biologic response (e.g. 100 μM), and poor generalization of biological effect to non-viral systems. There has been little success with M-ODNs, without further chemical modifications, in other biological systems or when administered subsequent to viral infection.

Remarkably, unmodified DNA oligonucleotides (ODNs) exhibit sequence-specific antisense effects in a number of cell types and against virally infected

cells. The first demonstration of a biologic effect mediated by an unmodified ODN was reported in 1978 when Zamecnik and Stephenson [7] presented evidence of suppression of the appearance of reverse transcriptase activity in RSV-infected CEF cells with a 13-mer. More recently, a number of investigators have demonstrated sequence-specific effects in non-virally infected mammalian cells, some of which are quite dramatic in their biologic consequences. These have been achieved mainly with genes encoding cell cycling or differentiation functions in cultured mammalian cell lines or primary cultures of normal human marrow or peripheral blood mononuclear cells [8–12]. Success has been observed with a variety of cell types: HL-60 cells (c-*myc*, c-*myb* target RNAs), T-lymphocytes or normal marrow mononuclear cells (c-*myc*, c-*myb* target RNAs), Balb 3T3 cells (PCNA or cyclin target RNA), and the malignant human keratinocyte cell line SCC-25 (nuclear retinoic acid receptor and cellular retinal binding protein target RNAs) [8–13]. In general, only positive results are published, and it is a common experience within the scientific community to be *unable* to achieve an antisense effect. Therefore, it is important to interpret the successful experiments for an underlying common theme. All of the referenced studies achieved success with standard ODNs. ODN lengths were in the range of 15–18 bases in all cases. In most cases, the protein product coded for by the target gene was not abolished completely, but pronounced cellular effects were still observed. In the case of HL60 cells, radiolabeled ODNs taken up by cells were intact 5 days after a single exposure, suggesting that ODN stability may be one factor in responsiveness. In one study, the actual duplex formed between the ODN and the target mRNA was detected [12], whereas in a second study activity of the antisense ODN appeared to be associated with loss of target mRNA by cellular degradation [14]. In all of these studies, the target of interest appears either to have a short half-life relative to the apparent half-life of the administered ODN, or the target mRNA is apparently degraded in response to the antisense ODN or the biological manifestation of the target gene's expression is highly sensitive to the relative level of the gene product within the cell. In the case of c-*myc*, the mRNA half-life has been determined to be less than 1 hr, while the c-*myc* protein half-life has been reported to range from 25 to 50 min [15, 16]. Both the c-*myc* mRNA and c-*myc* protein half-lives are significantly less than the measured half-life of an ODN, reported to be 24–48 hr for a 15-mer in the study by Holt *et al.* [12]. In this study, duplex formation between the administered antisense ODN and the target mRNA for c-*myc* was demonstrated. The administration of 4 μM oligomer resulted in duplex formation with approximately 30% of the endogenous c-*myc* mRNA, enough to reduce the steady-state level of c-*myc* protein 50–80% and the growth rate by 50% over 5 days. In agreement with the effectiveness of antisense ODNs on c-*myc* as relating to half-life of the mRNA and protein, c-*myb*, the other proto-oncogene demonstrating a good response to antisense ODNs, also has a short protein half-life [17]. The half-life of c-*myb* mRNA was found to vary from 22 to 30 min in non-serum stimulated MSB-1 and CEF cells, respectively, but increased significantly upon serum stimulation [18]. In contrast to the observation of duplex formation between the antisense ODN and the target mRNA, Gewirtz *et al.* [14] observed loss of the targeted c-*myb* mRNA upon treatment of normal human T-lymphocytes, interpreted as the possible mediative action of RNAse H in the antisense effect.

Inhibition of retinol-binding protein I and human nuclear retinoic acid receptor α gene expression by antisense ODNs has also been successful when assayed for biological as well as biochemical effect. The half-life of each protein appears short (1 to 1.5 hr), in keeping with the above observations of achieving successful results. However, the decrease in the level of translation products for the two target mRNAs was high, exceeding 90% for the retinoic acid receptor and 70–92% for the retinol-binding protein and a consequent decrease of clonality of more than 83% [13]. It is significant that the estimated critical sustained level of receptors required for clonal growth is about 20–25% that of untreated cells in this system [13]. This threshold of suppression for a biological effect was also observed when antisense ODNs targeted to cyclin or PCNA were used [8]. In this latter study, 18-mers were able to completely suppress DNA synthesis and mitosis in exponentially growing BALB 3T3 cells even though average cellular levels of PCNA (as measured by immunofluorescence) only decreased to about 40% control values.

Again, in all these successful studies the half-life of the target mRNA was short compared to the probable half-life of the antisense ODN and/or there is a critical threshold below which the amount of gene product being targeted does not mediate the biological effect in question. These are the properties of the target gene system which are likely to render ODNs successful antisense agents. Since many of the genes which control proliferation, differentiation and hormone response are short-lived, there is a wide assortment of available targets for antisense ODNs for pharmacologists who study these systems. If ODNs are ineffective, the reason is likely to be high nuclease activity of target cells or serum, secondary structure of the target mRNA, or inability to reduce the target nucleic acid species sufficiently. One alternative to using ODNs is to resort to phosphorothioate analogs of DNA (S-ODNs) which tend to be longer lived within cells. Potential drawbacks include availability of these compounds and non-specific cell toxicity. Due to the general difficulties in obtaining oligonucleotide analogs and a lack of knowledge of how these compounds function (see below), genetic antisense constructs probably remain the most reliable alternative for the experimentalist.

The aforementioned discussion of nucleic acid properties which render them suitable targets for antisense ODNs has not addressed the mechanism of antisense ODN action. It appears that ODNs and some ODN analogs (e.g. S-ODNs) may work by one or more mechanisms: (a) hybrid arrest of translation (or arrest of other required functions such as splicing or nuclear/cytoplasmic transport) (b) by a "killer" mechanism where the ODN:RNA hybrid duplex

region serves as a substrate for RNAse H (which degrades the target RNA region bound to the ODN), or (c) other cellular systems for mRNA degradation (also "killer" mechanisms). Examples exist of hybrid arrest and for a "killer" mechanism.

Evidence for participation of RNAse H in the antisense effect *in vivo* derives mainly from work performed in *Xenopus* oocytes which are rich in RNAse H [19]. It was shown that microinjected antisense ODNs can mediate site-directed degradation of target mRNAs [19]. Results from *in vitro* translation experiments which employ rabbit reticulocyte lysates indicate that rabbit reticulocytes lysates contain RNAse H activity on a sporadic basis and, depending upon the reticulocyte preparation, either mechanism may be active [20–23]. M-ODNs and α-ODNs cannot serve as RNAse H substrates when forming duplexes with RNA [23, 24]. This has been considered as evidence favoring an RNAse H mediated mechanism since the M-ODNs are relatively stable compounds which bind in a sequence specific fashion and the α-ODNs bind to complementary nucleic acid targets more tightly than the naturally occurring β-anomers examined using *in vitro* assays, yet neither exhibit strong antisense activity [23, 24]. Additionally, S-ODNs, which do exhibit *in vivo* activity against a variety of targets form excellent substrates for RNAse H when bound to complementary sequences of RNA [25]. It is now generally assumed that RNAse H can play an important role in the activity of antisense oligonucleotides in mammalian cells although rigorous proof is lacking. Where examined in mammalian cells, target mRNA has not, as yet, been demonstrated to be degraded as would be expected for an RNAse H mechanism or, when degraded, the demonstration of site-specific degradation (which would occur by an RNAse H mediated mechanism by definition) has not been established [8, 12]. The general observations that the initiation site for protein synthesis and the 5′ noncoding region are susceptible targets for antisense (ODNs or antisense RNA) whereas the coding regions or more 3′ regions of the mRNA are not, argue against the necessity of RNAse H intervention *in vivo*. This is because RNAse H mediates an efficient effect regardless of the antisense ODN binding site on the mRNA (provided the site is within the protein coding region) [19, 20, 22, 23]. Other cellular mechanisms of mRNA turnover which may contribute to, or are the major mode of antisense action against some targets have not been studied.

It is clear that the primary and/or secondary structure of some mRNAs plays a critical role in their stability. Key examples from mammalian systems exist for the triose phosphate isomerase (TPI) mRNA and the human transferrin receptor (hTr) mRNA [26, 27]. In the case of the TPI, mRNA alterations in the primary or secondary structure in the 5′ two-thirds of the coding sequence destabilize the mRNA [26]. In the case of the hTr mRNA, a stem-loop structure in the 3′ noncoding region of the molecule governs mRNA stability modulated by a trans-acting factor [27]. The possibility that disruption of secondary structure and not necessarily RNAse H can also mediate the effect of an antisense ODN should be investigated as in some cases this could be used to target a refractory mRNA molecule. It is also possible that this information could be used to stabilize a mRNA, enhancing the growth-promoting activity resulting from the expression of these genes. For example, there is an AU-rich motif, found in the 3′ noncoding region of many growth factor related mRNAs, which codes for mRNA instability [28]. The implication is that the 3′ ends of mRNAs may even be preferred targets to destabilize or *stabilize* a target message, if the biochemistry of that message's turnover dictates. It has been demonstrated, in one case, that the 3′ end of a target mRNA may be an effective one for an antisense effect [29].

For the pharmacologist who has a desire to make use of antisense agents for their own purposes, the best approach will depend upon the target properties (if known) and, of course, the resources available. If the target RNA is short-lived, the best option seems to be ODNs targeted at or around the initation site for protein synthesis. If this fails (with repeated additions to cell culture into the range of 30 μM), the best alternative may be either to attempt synthesis of S-ODN analogs or to clone and transfect with an antisense expressing plasmid. ODNs can be obtained from nucleic acid synthesis facilities available at most research institutes or universities. Both ODNs and S-ODNs are available commercially. In addition, other analogs are becoming available on a commercial basis (e.g. α-ODNs and ODNs linked to intercalating agents), which may be an alternative for some investigators with deep pockets. Unfortunately, the selectivities of most analogs has not been well established in a biologic context. It is obvious some may have non-specific effects which may render the manifestation of the antisense effect difficult to interpret *in vivo*. There are no guarantees of success; even if the approach taken is the cellular expression of an antisense gene, these may be thwarted by the presence of an RNA : RNA "unwindase" activity [30, 31].

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