Comparative inhibition of chloramphenicol acetyltransferase gene expression by antisense oligonucleotide analogues having alkyl phosphotriester, methylphosphonate and phosphorothioate linkages

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ABSTRACT

Several classes of oligonucleotide antisense compounds of sequence complementary to the start of the mRNA coding sequence for chloramphenicol acetyl transferase (CAT), including methylphosphonate, alkyltriester, and phosphorothioate analogues of DNA, have been compared to "normal" phosphodiester oligonucleotides for their ability to inhibit expression of plasmid-directed CAT gene activity in CV-1 cells. CAT gene expression was inhibited when transfection with plasmid DNA containing the gene for CAT coupled to simian virus 40 regulatory sequences (pSV2CAT) or the human immunodeficiency virus enhancer (pHIVCAT) was carried out in the presence of 30 μ M concentrations of analogue. For the oligo-methylphosphonate analogue, inhibition was dependent on both oligomer concentration and chain length. Analogues with phosphodiester linkages that alternated with either methylphosphonate, ethyl phosphotriester, or isopropyl phosphotriester linkages were less effective inhibitors, in that order. The phosphorothioate analogue was about two-times more potent than the oligo-methylphosphonate, which was in turn approximately twice as potent as the normal oligonucleotide.

INTRODUCTION

A novel class of naturally occurring biological inhibitors, antisense RNA sequences, have been described in prokaryotes ¹ and recently in eukaryotes ², and reportedly function as negative regulators of gene expression by hybridizing to their complementary target sequences. Biosynthetically generated antisense sequences have now been artificially introduced into bacteria ^{3,4}, plants ⁵ and eukaryotic cells ⁶⁻¹² and have been shown to inhibit gene expression, as well as exhibit antiviral activity ¹³⁻¹⁵ and anti-onc gene activity ¹⁶.

Recently, experiments with "normal" (phosphodiester-linked) antisense oligodeoxyribonucleotides ¹⁷⁻²³ as inhibitors have indicated that these comparatively short oligonucleotides can be taken up by cells in culture and may also be effective in producing modulation of gene expression. This would offer obvious advantages over the use of antisense RNA transcripts and allows attachment of various types of functional groups or other molecules ²⁴⁻²⁹.

The relatively short half-lives of normal oligonucleotides³⁰ in serum and in cells due to the presence of nucleases, and the low permeability of these charged molecules into normal cells, limit their potential usefulness in vivo. These limitations might be overcome by either completely replacing³¹⁻³⁸ or structurally altering the oligonucleotide sugar-phosphate backbone by means of chemical modification to introduce a nonionic moiety. Ideally, the effect of these alterations would be to simultaneously increase biological lifetime, enhance permeability into cells, and strengthen binding to polynucleotide targets under physiological conditions (low-salt, 37°C)^{32,39}. Several classes of multiply phosphate-modified oligodeoxyribonucleotides have been chemically synthesized, including oligo-alkyl phosphotriesters³⁹⁻⁴¹, oligo-methylphosphonates⁴²⁻⁴⁵, and oligo-phosphorothioates⁴⁶⁻⁴⁹ (Fig.1). As synthetic antisense compounds, these nuclease-resistant analogues^{39,50,51}, may inhibit the synthesis of specific proteins⁵²⁻⁵⁵, and are therefore potentially useful for diverse investigations of biologically related processes.

Attention has also been focused on these normal and modified oligonucleotide antisense compounds as potential antiviral agents^{4-6,9,13,15,55} in vitro and perhaps in vivo since synthesis of specific viral encoded proteins is essential to infection of cells by viruses. If synthesis of target viral proteins can be selectively inhibited without significant toxic effects on cells, progress of infection and its adverse effects can be blocked. Miller et al.⁵⁵⁻⁵⁷ have shown that oligo-methylphosphonates can specifically inhibit vesicular stomatitis virus (VSV) and herpes simplex virus, type 1 (HSV-1) replication in tissue culture, and that this inhibition is dependent on the sequence of the oligonucleotide being antisense to the mRNA. VSV replication can also be regulated by normal oligonucleotides coupled with poly(L-lysine)²⁶, and normal synthetic oligonucleotides have been shown to inhibit replication of HIV in tissue culture²².

The demonstrated⁵⁵⁻⁵⁷ ability of nonionic oligo-methylphosphonates to produce sequence-specific inhibition of viral replication in tissue culture, combined with their apparently passive uptake^{50,58-60} by cells, stability to nucleases⁵⁰, and increased permeability into cells due to absence of negative charges on the phosphate groups, make these and related derivatives particularly attractive candidates as potential antiviral agents. The difficulty of obtaining suitable quantities of oligo-methylphosphonates by manual synthetic procedures introduced by Miller and coworkers⁴³⁻⁴⁵ led us

Figure 1. General structure of classes of backbone modified oligonucleotide analogues.

to develop ⁶¹ a chemically improved automated approach that is a simple extension of the Caruthers method ⁶². We have already reported synthetic routes to nonionic isopropyl ^{41b} and ethyl phosphotriester ^{41a,48} analogues, as well as phosphorothioates ⁴⁶⁻⁴⁹ which are isoelectronic with DNA. These synthetic capabilities enabled us to compare, in data presented below, the effectiveness of chemically modified oligonucleotides of identical sequence on inhibition of expression. In contrast to the study of the effects of oligonucleotides on the complex process of viral replication, we chose to utilize a eukaryotic tissue culture transfection assay of plasmid containing the gene for expression of the bacterial enzyme chloramphenicol acetyltransferase (CAT) coupled to viral (SV 40) regulatory sequences ⁶³⁻⁶⁵ as a model in vitro system for gauging the relative effectiveness of these compounds in the regulation of expression of a specific protein.

For comparison, in some experiments, we have also substituted a region of the HIV LTR containing the HIV enhancer ^{66,67} into the enhancerless CAT plasmid pA₁₀CAT₂ ⁶⁵ in place of the SV 40 enhancer. Since CAT is not

normally a component of eukaryotic cells⁶³ it is not required for any cellular function or cell viability, and there is presumably little interaction between its expression and that of other cellular proteins to complicate interpretation of the data. Furthermore, the results of the assay are readily quantifiable and the gene product is encoded in a defined genetic vector that lends itself to genetic manipulation for molecular biology studies. Previous experiments by Weintraub and coworkers^{7,8} had indicated that the CAT system was amenable to inhibition by antisense RNA. We report here the ability of a series of backbone-modified antisense oligonucleotides to inhibit the expression of CAT activity in comparison with inhibition by a normal phosphodiester oligonucleotide of identical sequence.

MATERIALS AND METHODS

Preparation of Modified Oligonucleotides and T_m Measurements.

All compounds were prepared by synthesis on an Applied Biosystems Model 380B DNA synthesizer and were purified by HPLC using either published procedures or those which will be reported elsewhere^{41,47,61}. Yields per coupling step were $\geq 97\%$. Size homogeneity was verified by either 5' end-labeling or UV-shadowing and polyacrylamide gel electrophoresis⁵³. Extinction coefficients were assumed to be approximately equal to the sum of the values for the purine (14,000) and pyrimidine (7,000) residues. Previously reported⁶⁸ methods were used to determine T_m values.

Apparent Uptake of Oligo-Methylphosphonates by CV-1 Cells

The free 5' OH group of the oligo-methylphosphonate was ³²P-end labeled⁶⁹ using [γ -³²P]ATP, 3000 Ci/mmol, (New England Nuclear), and T4 polynucleotide kinase (Bethesda Research Laboratories), and separated from unincorporated label by electrophoresis on 15% polyacrylamide gels containing 7 M urea⁵³. Brief autoradiography (15 min exposure) of wet gels permitted location of labeled oligomers. The region of the gel corresponding to the labeled oligomers was excised, ground, and extracted 3-times with 0.5 ml triethylammonium acetate buffer (0.1 M, pH 7) by vortexing followed by centrifugation. The supernatant was passed through a C18 Sep-Pak cartridge (Waters-Millipore) and the labeled product was eluted with buffer containing 30% (v/v) acetonitrile. The resultant samples were dried and resuspended in water. Apparent uptake by CV-1 cells was measured by a method described by Hoppe⁷⁰; briefly, cells were incubated with labeled oligo-methylphosphonate (6.5×10^6 cpm) at 37°C for varying lengths of time and duplicate 100 μ l aliquots containing approximately 10^7 cells were removed at specific times,

layered on the surface of 500 μ l of pre-chilled silicone oil (Versilube F 50, G.E. Silicone Products Division, Waterford N.Y.), (pre-chilled to -20°C in a Sarstedt microcentrifuge tube), and then centrifuged for 30 seconds in an Eppendorf centrifuge at ambient temperature. The bottom of the tube, which contains the cell pellet, was removed using dog toenail clippers, briefly inverted on absorbant paper to drain, and then transferred to a scintillation vial and counted.

Transfection of Cells with Plasmid DNA

CV-1 cells were maintained in continuous culture in Dulbecco's minimal essential medium containing 10% heat inactivated fetal calf serum and split 1:10 twice weekly. One day prior to transfection cells were split and plated in 100 mm petri plates at a density of 1×10^6 cells per dish. Four hours prior to transfection cells were fed with 6 ml of fresh media containing 10% fetal calf serum and the oligonucleotide to be studied. Transfection was performed using the CaPO₄ precipitation procedure^{71,63-65} using 5 μ g of the plasmid pSV2CAT⁶³. The plasmid pA₁₀CAT₂⁶⁵ which does not contain the SV 40 enhancer was used as a control. Plasmids pSV2CAT and pA₁₀CAT₂ were kindly provided by L. Laimins. pHIVCAT was produced by cloning⁶⁹ the gel-purified Bgl II fragment (nucleotides #8629-9136 of HTLV-IIIB⁷²) from the HIV LTR (HTLV-III lambda BH-8 clone^{73,74} kindly provided by F. Wong-Staal and R. Gallo) into the Bgl II site of pA₁₀CAT₂. Twenty-four hours post-transfection, cells were refed with fresh media containing the same oligonucleotide concentration, and were then harvested 24 hours later. After removal of the media, plates were rinsed 3-times with cold phosphate buffered saline, incubated with 1 ml of buffer (0.04 M Tris HCl, pH 7.4/1 mM EDTA/0.15 M NaCl) at room temperature for 5 min, scraped from the plate and transferred to Eppendorf microcentrifuge tubes. After centrifugation for 3 min at 4°C , supernatant was discarded and pellets were frozen at -20°C . Cell pellets were assayed for chloramphenicol acetyltransferase (CAT) activity using 100 μ l aliquots of sonicated supernatant as described⁶³. TLC plates (Baker-flex Silica Gel IB, J. T. Baker) were autoradiographed and subsequently radioactive areas thus visualized were excised and counted on a scintillation counter to quantitate conversion of substrate ($[^{14}\text{C}]$ chloramphenicol, 45 Ci/mmol (New England Nuclear) to acetylated chloramphenicol product(s). Acetyl CoA was purchased from Pharmacia. "Percent conversion" was defined as the ratio of the sum of acetylated chloramphenicol species divided by the sum of all species (acetylated + unacetylated) X 100. "Percent remaining activity" was defined

as the ratio of % conversion of sample divided by % conversion of the control pSV2CAT X 100. "Percent inhibition" was defined as 100 - % remaining activity. The percentage conversion of chloramphenicol to products varied between experiments; however, the ratio of activities remained approximately the same, as previously reported ⁶⁵.

Recovery of Test Oligonucleotides from Tissue Culture Media

The spent media which contained either an oligo-methylphosphonate or an oligo-isopropylphosphotriester sample was dried in a speedvac (Savant), and the resultant residue was thoroughly mixed first with water (2-4 ml) and then with an equal volume of ethanol. After vortexing thoroughly, the mixture was allowed to stand at room temperature for 4 hr before low-speed centrifugation at room temperature and then removal of the supernatant. The supernatant was dried in a speedvac and the resultant ethanol-water (1:1 v/v)-soluble material was subjected to reversed-phase HPLC ^{41b,61} which led to ca. 50% recovery of the input oligomer.

RESULTS

Sequence Selection and Apparent Uptake of Oligo-Methylphosphonate by CV-1 Cells

Using the published nucleotide sequence for the CAT gene ^{77,78}, we selected a 21-base sequence (5'-AUGGAGAAAAAUCACUGGA-3') that begins with the AUG which codes for the initial methionine as a likely ⁵⁵ target for inhibition of translation by antisense oligonucleotides. An oligo-methylphosphonate with the complementary sequence 5'-TCCAGTGATTTTTTCTCCAT-3' was therefore synthesized having a 5'-terminal phosphodiester linkage for solubility and labeling purposes ⁴⁵. Although

Figure 2. Time course of apparent uptake of a 5'-monovalent 21-mer oligo-methylphosphonate by CV-1 cells. Points are the average of duplicate determinations.

oligo-methylphosphonates had previously been shown to be taken up by several cell lines^{50,54,55,57,58}, it was important to examine the ability of CV-1 cells, which are readily transfected by pSV2CAT with resulting expression of CAT activity, to take up these compounds. In addition, the uptake of oligomers longer than 9- to 12-mers⁵⁰ had not been heretofore studied. The oligo-methylphosphonate was end-labeled with ³²P, purified from unincorporated label, and analyzed for apparent uptake by the CV-1 cells. As shown in Fig. 2 there was a time-dependent increase in label associated with the pelleted CV-1 cells over a period of 2 hr. This suggested that these cells were able to take up the labeled oligonucleotide, although membrane association without entry into the cell could not be excluded with this protocol. To ensure the presence of an adequate amount of oligomer in the cells at the time of transfection, oligomer samples were added to the media when the cells were fed 4 hr prior to transfection.

Effect on Inhibition of Expression of CAT Activity by Variation of Chain Length of Oligo-Methylphosphonate

Preliminary experiments indicated that the aforementioned 21-mer produced some inhibition of CAT expression and had no apparent effect on viability of the cells. Furthermore, the oligomer could be purified from spent media by means of HPLC, and could be reintroduced into the cells to produce inhibition, which indicated that it was either not metabolized or only slowly metabolized by the CV-1 cells. The relatively long period of time required for uptake of the 21-mer oligo-methylphosphonate led us to investigate whether shorter oligomers might be taken up more efficiently and whether they might prove to be superior inhibitors. Consequently, a series of 5'-truncated, 5'-monovalent oligo-methylphosphonates comprised of 9-, 12-, and 15-mers were examined in addition to the full-length 21-mer for their ability to inhibit CAT

TABLE I
Relationship Between Oligo-Methylphosphonate Chain Length
and Inhibitory Effect on CAT Gene Expression

Chain Length of Oligomer ¹	% Remaining Activity ²
(None)	100)
9 mer	92
12 mer	40
15 mer	35
21 mer	60

¹Oligomer concentration was 30 μ M.

²See Methods section for definition

Figure 3. Autoradiogram showing inhibition of expression of CAT activity by 5'-monovalent oligo-methylphosphonates of different chain length. The upper two rows of spots represent the two monoacetylated forms of chloramphenicol, which migrate faster than the unmodified substrate, seen in the bottom row. Samples represent, from left to right, assay of extracts of cells transfected in the presence of no oligomer, 30 μ M 9-mer, 30 μ M 12-mer, 30 μ M 15-mer, and 30 μ M 21-mer oligo-methylphosphonate.

expression. After transfection with pSV2CAT in the presence of a given oligomer, cells were harvested and CAT activity was assayed. Fig. 3 shows an autoradiogram derived from such an experiment. The oligomers apparently decreased the conversion of chloramphenicol to its acetylated forms, and the 15-mer appeared to be the most effective chain length tested. This was confirmed by obtaining a quantitative measure of the degree of inhibition by use of counts determined for spots excised from a TLC plate (TABLE I). In accord with the visual observations of Fig. 3, the most effective oligomer that was examined was the 15-mer, which produced 65% inhibition compared to the control with no oligomer added. Interestingly, there was substantial loss of ability to inhibit when chain length was increased to a 21-mer, even though the extent of hybridization would be expected to increase. The high hydrophobicity of the 21-mer noted during its preparation, in comparison with the shorter oligomers, may have led to a lower effective concentration and thus contributed to its less potent inhibition, as could structural features of the mRNA target²¹.

Effect of Concentration of Oligomer on Inhibition

Experiments were performed to assess the ability of different concentrations of the aforementioned oligo-methylphosphonate 15-mer to inhibit expression of CAT activity. Since oligodeoxyribonucleotides have been shown to have antisense activity in various *in vitro* test systems¹⁷⁻²⁶,

TABLE II
Concentration Dependence of Inhibitory Effects
of Different Types of Oligomers

Oligomer Type	Concentration, μM	% Remaining Activity ²
(None	—	100)
Normal phosphodiester	5	100
	10	79
	20	77
	30	66
Oligo-methylphosphonate	5	91
	10	70
	20	54
	30	35
Oligo-phosphorothioate	10	35
	30	16

¹Transfections were performed in the presence of 15-mers of the indicated oligomer.

²See Methods section for definition

equivalent concentrations of the normal, phosphodiester-linked, 15-mer oligodeoxynucleotide were compared with the oligo-methylphosphonate. The results, shown in TABLE II, indicate that increasing concentrations of either normal or methylphosphonate oligomer produce increasing levels of inhibition; however, the oligo-methylphosphonate is a more effective inhibitor than the normal oligomer at all concentrations tested. Similar experiments performed with the oligo-phosphorothioate analogue also resulted in concentration dependent inhibition (TABLE II). An N^3 -methylthymidine-containing

TABLE III
Effect of Oligomer Type on CAT Activity¹

Oligomer Type	% Remaining Activity ²
(None	100)
Alternating isopropyl triester	100
Normal phosphodiester	65
Alternating ethyl triester	49
Alternating methylphosphonate	40
Oligo-methylphosphonate	35
Oligo-phosphorothioate	16

¹Transfections were performed in the presence of 15-mers of the oligomer at a concentration of 30 μM .

²See Methods section for definition.

derivative of this oligo-phosphorothioate, which was specifically modified at the underlined positions: GATTTTTTCTCCAT, and was known to be less able to bind to DNA⁷⁷, showed approximately 2-fold less inhibition.

Analysis of Inhibition by Related Modified Oligonucleotides

A series of 15-mers of identical antisense sequence, but with different classes of backbone modifications, were tested at a concentration of 30 μ M for relative efficiency in inhibiting expression of CAT activity. The general structures of these compounds are presented in Fig. 1, and the specific structures are shown below. The results obtained after transfection and assay of resultant CAT activity are summarized in TABLE III.

normal phosphodiester,	$z = x = y = 5'0\text{-P}(0)(\text{O}^-)\text{-O}3'$
oligo-phosphorothioate,	$z = x = y = 5'0\text{-P}(0)(\text{S}^-)\text{-O}3'$
oligo-methylphosphonate,	$z = 5'0\text{-P}(0)(\text{O}^-)\text{-O}3'$ $x = y = 5'0\text{-P}(0)(\text{CH}_3)\text{-O}3'$
alternating-methylphosphonate,	$z = y = 5'0\text{-P}(0)(\text{O}^-)\text{-O}3'$ $x = 5'0\text{-P}(0)(\text{CH}_3)\text{-O}3'$
alternating-ethyl triester,	$z = y = 5'0\text{-P}(0)(\text{O}^-)\text{-O}3'$ $x = 5'0\text{-P}(0)(\text{OCH}_2\text{CH}_3)\text{-O}3'$
alternating-isopropyl triester,	$z = y = 5'0\text{-P}(0)(\text{O}^-)\text{-O}3'$ $x = 5'0\text{-P}(0)(\text{OCH}(\text{CH}_3)_2)\text{-O}3'$

The phosphorothioate derivative appeared to be the most effective compound in this series, producing approximately 85% inhibition at a concentration of 30 μ M. The 5'-monovalent oligo-methylphosphonate was only slightly more effective than the heptavalent "alternating" methylphosphonate, which has modified and normal phosphodiester groups positioned one after the other along the chain. The analogous alternating ethyl phosphotriester was somewhat less inhibitory, but was more effective than the normal oligonucleotide, while the alternating isopropyl phosphotriester showed little or no detectable inhibitory activity.

Effect of Enhancer-Type on Inhibition

To determine whether the above described inhibition data was enhancer-specific, we examined the effect of replacing the SV 40 enhancer with an enhancer from another virus. The HIV enhancer was ligated into

PA₁₀CAT₂ at the Bgl II site in place of the SV 40 enhancer. Experiments with pHVCAT using CAT assay conditions identical to those employed for pSV2CAT showed it to be much less efficient than pSV2CAT in stimulating the expression of CAT activity in CV-1 cells; however, this was not unexpected since enhancers are known to be tissue-specific⁶⁵. Separate additions of each of the modified oligonucleotides resulted in inhibition of CAT gene expression, indicating that gene inhibition was not enhancer specific.

DISCUSSION

The results described herein have demonstrated that the transient expression system for CAT can be effectively utilized to compare relative effects of antisense oligonucleotide chain length, concentration, and backbone modification. These data show that a 15-mer oligo-methylphosphonate and its 5'-truncated 12-mer version produced a comparable amount of inhibition, which was better than that exhibited by either the 5'-truncated 9-mer or the 5'-extended 21-mer. The lower inhibition of the latter oligomers could be due to a lower T_m and greater hydrophobicity, respectively. Among the various types of oligomers having the same 15-base sequence, it is significant that the oligo-phosphorothioate was about 2-times more effective than either the oligo-methylphosphonate or the alternating-methylphosphonate, all of which have comparable T_m values⁷⁷ (ca. 40°C). T_m values were measured at physiological salt concentration (ca. 0.2 M NaCl) for duplexes with the complementary, unmodified oligonucleotide used as a model for the mRNA target sequence. The somewhat less effective inhibition of the alternating ethyl triester vs. alternating methylphosphonate oligomer, and the apparent inactivity of the alternating isopropyl triester, are also significant in view of the fact that the model duplexes of these compounds have similar T_m values⁷⁷ (ca. 40-42°C). This absence of an obvious correlation between inhibitory and hybridizing ability (T_m) may be due to differences in non-specific binding of the oligomer to non-nucleic acids in the cell or culture media. While Ts'o and Miller and their coworkers^{55,57} have amply demonstrated that oligo-methylphosphonates (and presumably alternating methylphosphonates of the type reported here) are capable of blocking expression by means of hybridization to RNA, we can only speculate that the same is true for the structurally similar alternating ethyl triester. Similarly, the known¹⁷⁻²³ mechanism of inhibition of unmodified oligonucleotides through a binding-induced mechanism (or combination of binding followed by RNase H digestion⁷⁸), suggests that the same

mechanism(s) apply in the case of the isoelectronic oligo-phosphorothioate. The nuclease-resistance of the latter can account for its approximately 4-fold higher potency than the unmodified oligomer. The observation that the N-CH₃ thymidine-containing phosphorothioate analogue (which does not effectively bind to its antisense normal counterpart *in vitro*⁷⁷) still produces a significant level of inhibition of CAT activity, suggests that additional studies are essential to define the mechanism of inhibition.

The expression system described herein, combined with the availability of efficient, automated syntheses, will hopefully lead to further investigations of antisense sequences to obtain effective inhibitors. For example, experiments have been described using sequences antisense to the 5' noncoding region, 5' initiation codon and adjacent coding region, 3' termination region, splice junctions, and sequences predicted by computer models to be exposed regions or hydrogen-bonded regions^{7,21,22,26,52,55,57,60}. In addition, recent experiments with normal 15-mers coupled to poly-lysine and complementary to the AUG start codon and adjacent 5' noncoding region²⁶, suggest that relatively low concentrations (in the nanomolar range) are effective at inhibiting VSV replication, compared with previous inhibition experiments by Miller and coworkers⁵⁵ requiring micromolar concentrations of oligo-methylphosphonates complementary to the 5' initiation codon and adjacent coding region⁵⁵. It will be of interest to compare the effectiveness of these poly-lysine and other derivatives in the CAT expression system where identical sequence and chain length can be compared directly.

It should be mentioned that although relative effects of different oligomers can be effectively compared in the transient expression system employed in our experiments, the apparent concentrations of oligomer required for inhibition cannot be realistically expected to accurately reflect those concentrations which might be required to inhibit in other situations. Approximately 10% of the cells are actually transfected and each of the transfected cells will contain multiple copies of CAT DNA, whereas in the typical situation *in vivo*, each cell will contain a single copy of a gene per haploid chromosome number. In cells infected by a virus, more than one copy of the viral genome is likely to be present in each cell. Izant and Weintraub^{6,7} have used sense and antisense plasmids to study the ratios of antisense:sense required for inhibition and find that ratios of 5:1 are required to produce some inhibition, and ratios of 100:1 produce substantial but still incomplete inhibition. The actual intracellular ratios involved in

our experiments are unknown. In any event, antisense oligonucleotides offer tremendous potential as regulators of specific gene function both *in vitro* and *in vivo*, and as tools to define the function of genes for which the sequence is known (e.g. oncogenes), particularly if a rational approach to designing the most effective structural class and sequence can be defined. Hopefully, assays akin to that described herein will be of use in this connection.

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