A cytosine analog that confers enhanced potency to antisenese oligonucleotides

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ABSTRACT Antisense technology is based on the ability to design potent, sequence-specific inhibitors. The G-clamp heterocycle modification, a cytosine analog that clamps on to guanine by forming an additional hydrogen bond, was rationally designed to enhance oligonucleotide/RNA hybrid affinity. A single, context-dependent substitution of a G-clamp heterocycle into a 15-mer phosphorothioate oligodeoxynucleotide (S-ON) targeting the cyclin-dependent kinase inhibitor, p27kip1, enhanced antisense activity as compared with a previously optimized C5-propynyl-modified p27kip1 S-ON and functionally replaced 11 C5-propynyl modifications. Dose-dependent, sequence-specific antisense inhibition was observed at nanomolar concentrations of the G-clamp S-ONs. A single nucleotide mismatch between the G-clamp S-ON and the p27kip1 mRNA reduced the potency of the antisense ON by five-fold. A 2-base-mismatch S-ON eliminated antisense activity, confirming the sequence specificity of G-clamp-modified S-ONs. The G-clamp-substituted p27kip1 S-ON activated RNase H-mediated cleavage and demonstrated increased in vitro binding affinity for its RNA target compared with conventional 15-mer S-ONs. Furthermore, incorporation of a single G-clamp modification into a previously optimized 20-mer phosphorothioate antisense S-ON targeting c-raf increased the potency of the S-ON 25-fold. The G-clamp heterocycle is a potent, mismatch-sensitive, automated synthesizer-compatible antisense S-ON modification that will have important applications in the elucidation of gene function, the validation of gene targets, and the development of more potent antisense-based pharmaceuticals.

Several methods have been developed to validate drug targets and provide insight at the molecular level of diseases. These methods include targeted deletions in mice, antibodies, aptamers, ribozymes, and antisense oligodeoxynucleotides (ONs). Effective antisense ONs have the additional option of being developed into human therapeutics.

Antisense ONs are short (7–30 nt), synthetic pieces of DNA that are rationally designed to selectively hybridize to a target RNA and interfere with the expression of the encoded protein. An effective antisense ON inhibitor must be stable to all present nucleases and demonstrate high affinity for its target RNA sequence. An effective inhibitor, in most cases, is recognized by intracellular RNase H on binding to its RNA target, and the RNA portion is degraded (1).

The first ONs tested in tissue culture were phosphodiester ONs (2). They were rapidly degraded by nucleases in culture media and, thus, showed poor antisense activity (3, 4). The phosphorothioate internucleotide linkage has circumvented this problem and has emerged as the analog of choice for creating nuclease-resistant ONs. One drawback of phosphorothioate-modified ONs is that they have decreased binding affinity for their RNA target compared with their phosphodiester-containing predecessors.

To enhance the binding affinity of phosphorothioate ONs, many ON modifications have been synthesized and tested for more potent antisense activity (reviewed in ref. 5). Identifying potent ONs is critical in developing antisense-based human therapeutics, because more potent ONs will allow for lower doses to be used. This will reduce the overall cost of therapy and alleviate the ON-related toxicities that have been observed in the clinic (reviewed in ref. 6).

Recently, we described a cytosine analog, termed G-clamp, that forms an additional hydrogen bond to guanine (7) (Fig. 1). The G-clamp base modification recognizes both the Watson-Crick and Hoogsteen faces of the complementary guanine within a helix. Binding studies demonstrated that a single G-clamp substitution within an antisense ON dramatically enhanced helical thermal stability and mismatch discrimination. However, the bioactivity of G-clamp-modified antisense ONs was unknown. For this report, we examined the sequence-context dependence, antisense activity, mismatch sensitivity, RNase H cleavage, and hybridization kinetics of a 15-mer p27kip1 phosphorothioate oligodeoxynucleotide (S-ON) containing a single G-clamp substitution. The data demonstrate that a single G-clamp substitution in a 15-mer S-ON conferred mismatch sensitivity and enhanced potency to the antisense S-ON as compared with our previous most potent multiply substituted C5-propynyl-modified S-ON. Furthermore, incorporation of a G-clamp into a previously optimized 20-mer S-ON targeting c-raf also dramatically increased the potency of the S-ON. These data demonstrate that the G-clamp modification is a highly potent, portable, and mismatch-sensitive cytosine analog.

MATERIALS AND METHODS

Cell Culture. African green monkey kidney cells (CV-1 cells, American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal calf serum. The T24 bladder carcinoma cell line was purchased from American Type Culture Collection and were cultured according to the recommended conditions.

S-ON Synthesis, Purification, and Sequences. S-ONs were synthesized by the H-phosphonate method on an automated synthesizer (model 8750, MilliGen/Biosearch) by using standard chemistry on controlled pore glass support (8). The nucleoside analogs were prepared as described (7, 9, 10). Sequences of the c-raf S-ONs are GS4204 (the same as ISIS 5132) 5’-TCC CGC CTG TGA CAT GCA TT (11), GS4206 (a

Abbreviations: ON, oligodeoxynucleotide; S-ON, phosphorothioate-ON; P13K, phosphoinoside 3-kinase.

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4-nt mismatch; *italics* denote the mismatch sites) 5′-TCC CGC CTA CTT CAT GCA TT, GS5628 (the G-clamp-modified equivalent to ISIS 5132; X indicates the G-clamp heterocycle) 5′-TCX CGC CTG TGA CAT GCA TT, and GS6206 (the G-clamp mismatch to GS5628, X indicates the G-clamp heterocycle and *italics* denote the mismatch sites) 5′-TCG CXC CTG AGT CAT GCA TT. C denotes 2′-deoxyxycytidine only in the c-raf series and 2′-deoxy-5-methylcytidine in the p27\textsuperscript{kip1} series.

**Hybridization and Dissociation Rates of S-ON/RNA Complexes.** The relative kinetics of hybridization and dissociation of S-ON/RNA complexes in *vitro* was performed as described by using \[^32\text{P}\]labeled RNA complementary to the p27\textsuperscript{kip1} antisense S-ONs (12, 13). The amount of probe hybridized to the antisense S-ON at each time point was quantitated by using 32P-labeled RNA complementary to the p27 kip1 messenger at this site is 5′-GGC GCA GGA GAG CCA (human sequences 306–320). The p27\textsuperscript{kip1} mutant sequence 5′-GCC GCA GGA GAG CCA (the *italicized* nucleotide denotes the mismatch base at 312).

**Plasmids.** The wild-type human p27\textsuperscript{kip1} plasmid was previously described (19). The mutant p27\textsuperscript{kip1} plasmid containing a thymidine substitution for the guanine at position 312 (GenBank accession no. U10906) was constructed by using primers and PCR to create a 1-bp mismatch as described (19) and was confirmed by sequencing. The sequence of the wild-type p27\textsuperscript{kip1} at this site is 5′-GGC GCA GGA GAG CCA (human sequences 306–320). The p27\textsuperscript{kip1} mutant sequence 5′-GCC GCA GGA GAG CCA (the *italicized* nucleotide denotes the mismatch base at 312).

**Immunoblot Analysis.** Cellular extracts were prepared as described (16, 20, 21). Electrophoretic and transfer conditions have been described (16, 20, 21). The p27\textsuperscript{kip1} mAb was used at a 1:1,000 dilution (Transduction Laboratories, Lexington, KY), and phosphoinositide 3-kinase (PI3K) p85 subunit polyclonal antibody was used at 1:30,000 dilution (Upstate Biotechnology Inc, Lake Placid, NY). Blots were developed by using horseradish peroxidase-conjugated goat anti-rabbit and/or anti-mouse antibodies (1:5,000 or 1:1,000, respectively; Zymed) and the enhanced chemiluminescence (ECL) Western blotting detection reagents (as per instructions, Amersham Pharmacia). Membranes were exposed to X-Omat AR film (Eastman Kodak). The immunoblots were quantitated by using a Storm 860 PhosphorImager (Molecular Dynamics) and ECL\textsuperscript{+} Western blotting detection reagents (Amersham Pharmacia) or by densitometry. All samples were normalized to P13K or p34cdc2 to control for variations in protein loading or transfer to the immunoblot membrane.
RESULTS

The Potency of the G-Clamp Modification Is Context-Dependent. The G-clamp modification was singly substituted at every possible cytosine within the p27kip1 S-ON and tested for antisense activity (Fig. 2A). The antisense ON/cytofectin mixture was incubated with CV-1 cells for 24 h, and protein extracts were then prepared from the cells, immunoblotted, and analyzed for p27kip1 protein levels. As shown in Fig. 2B, the bioactivity of the G-clamp-modified p27kip1 S-ONs depended on the sequence context. Results from six of the seven G-clamp-modified p27kip1 S-ONs are shown. G-clamp modification of the 3′ cytosine (ON7) demonstrated poor antisense activity at 30 nM (data not shown). Comparison of the antisense activity of the six G-clamp-modified S-ONs demonstrated that ON4 was the most potent. At 10 nM, ON4 inhibited p27kip1 protein levels by 84% compared with the GS3815 cytofectin-only control (Fig. 2B, lanes 1 and 10). The rank-order potency of the six G-clamp S-ONs was ON4 > ON6 > ON3 = ON2 > ON5 > ON1. Similar rank-order potency of the S-ONs was obtained when the G-clamp S-ONs were tested by microinjection into the nuclei of CV-1 cells (data not shown). These results indicate that the antisense activity of the S-ONs depended on the sequence context of the G-clamp modification rather than variations in S-ON delivery to CV-1 cells by using GS3815 cytofectin.

G-Clamp Modified Phosphorothioate ONs Are Potent Antisense Inhibitors. The antisense activity of the most potent G-clamp-modified S-ON targeting p27kip1 (ON4) was evaluated and compared with the 5-methylcytosine control, a phenoxazine-modified, and a multiply substituted C5-propynyl-modified sequence after transfection of the individual antisense S-ONs into CV-1 cells. Structures of the base analogs are shown in Fig. 1 (see Table 2 for sequences). Dose-dependent antisense activity was seen for the C5-propynyl, phenoxazine-, and G-clamp-modified p27kip1 S-ONs (Fig. 3, lanes 2–4 and 8–13). The control S-ON failed to demonstrate any antisense activity under the conditions tested (Fig. 3, lanes 5–7). At 30 nM, the G-clamp antisense S-ON inhibited p27kip1 expression by 96% as compared with the lipid control (Fig. 3, compare lane 1 to 13), consistent with results shown in Fig. 2B. The IC50 for the G-clamp p27kip1 S-ON is between 3 and 5 nM (Fig. 3, compare lanes 11–13 with lane 1). The phenoxazine S-ON, the tricyclic core of the G-clamp, decreased p27kip1 levels by 79% at 30 nM, whereas 30 nM of our most potent C5-propynyl p27kip1 S-ON, which has 11 C5-propynyl-modified bases, decreased p27kip1 levels by 67% consistent with previous results (19, 22). Comparison of the antisense activities of S-ONs indicated that the G-clamp S-ON is 3-fold more potent than the multiply substituted C5-propynyl S-ON targeting p27kip1 (Fig. 3, compare p27kip1 levels in lanes 4 and 12). These data demonstrate that a single G-clamp modification can functionally replace 11 C5 propyne modifications in the 15-mer S-ON targeting p27kip1 and confers enhanced antisense potency.

G-Clamp S-ONs Are Sequence-Selective Inhibitors. To demonstrate that the inhibition of p27kip1 by G-clamp S-ONs was a sequence-specific antisense effect, a 2-nt mismatch G-clamp S-ON was synthesized, transfected into CV-1 cells using GS3815 cytofectin, and analyzed for its effect on p27kip1 expression. The 2-nt mismatch S-ON was constructed by switching the G-clamp base with a nearby thymidine base (5′-TGG TCT XCT TGC GCC; X indicates the position of the G-clamp modification and italics indicates mismatch position). The overall base composition of the G-clamp antisense and mismatch S-ONs was identical. The 2-nt mismatch S-ON, when normalized to the internal PI3K control, had no effect on p27kip1 expression at 90 nM, an S-ON concentration that is 20- to 30-fold higher than the IC50 for the perfectly matched S-ON. The p27kip1 antisense G-clamp S-ON demonstrated nearly complete inhibition of p27kip1 even when titrated from 90 to 10 nM (Fig. 4, lanes 3–5). These data indicate that the G-clamp S-ON-mediated inhibition of p27kip1 is due to a sequence-dependent antisense mechanism of action.

To rigorously test the sequence specificity of the G-clamp-modified S-ON, the antisense binding site in the p27kip1 expression vector was mutated at a single base pair and microinjected with the G-clamp S-ON that targeted wild-type p27kip1 (ON4). The mutation in the p27kip1 expression vector changed the complementary guanosine in the transcribed mRNA, which hybridized to the G-clamp modification, to a uracil. Although the G-to-U substitution resulted in an amino
Fig. 4. G-clamp-modified S-ONs demonstrate sequence-specific inhibition of p27kip1. Immuno blot analysis of PI3K and p27kip1 in CV-1 cells transfected with 2.5 μg/ml GS3815 cytofectin complexed in the absence (lane 1) or presence of a range of concentrations of the G-clamp-modified S-ON, ON4, (lanes 2–5), or 2-nt mismatch G-clamp-modified S-ONs (lanes 6–9). The sequence for the 2-nt G-clamp p27kip1 mismatch S-ON is 5′-TGG CTC XCT TGC GCC, where the X indicates the position of the G-clamp substitution and italics denotes the mismatch sites. The G-clamp-modified p27kip1 antisense S-ON inhibited p27kip1 by 98.8% at 90 nM, 95.4% at 30 nM, 81.0% at 10 nM, and 26.7% at 3 nM when normalized to PI3K and compared with p27kip1 protein levels in the control lane (lane 1, GS3815 cytofectin only). No effects on p27kip1 levels were seen in extracts treated with the 2-nt mismatch S-ON. At 90, 30, 10, and 3 nM of the mismatch S-ON, p27kip1 was expressed at 112, 107, 119, and 100%, respectively, of the control lane.

Acid change in p27kip1, expression of the mutant p27kip1 was unaffected as compared with the wild-type p27kip1 expression after microinjection (data not shown). The mutant or the wild-type p27 plasmid was microinjected with the G-clamp S-ON-targeting p27kip1. A plasmid that expressed β-galactosidase was coinjected as an internal control and marker for injected cells. Eighteen hours later, the cells were fixed, immunostained, and the β-galactosidase-positive cells were analyzed for p27kip1 expression. Microinjection of the two p27kip1 plasmids with the C5-propynyl-modified p27kip1 anti-sense S-ON was also done for comparison. Both modified p27kip1 S-ONs inhibited expression of the wild-type p27kip1 plasmid (Table 1). A consistent with the transfection results shown in Fig. 3. No effect on β-galactosidase expression was observed. A 5-fold reduction in antisense activity was observed for the G-clamp S-ON when targeting the mutant p27kip1 (Table 1, ON4, compare IC50 of 0.05 to 0.25 μM). In contrast, the bioactivity of the C5-propynyl S-ON was essentially unaffected by a single mismatch in the p27kip1 target sequence. IC50 for wild-type p27kip1 was 0.20 μM, whereas the IC50 for the mutant plasmid was 0.25 μM. These results confirm that the G-clamp p27kip1 S-ON functions by an antisense mechanism of action and further demonstrate that the G-clamp S-ON is a potent, sequence-specific, and target-selective antisense inhibitor.

Hybridization Properties of G-Clamp-Modified S-ONs. A gel-shift assay (see Materials and Methods) was used to investigate the rates of association and dissociation of the four modified p27kip1 S-ONs (G-clamp, unmodified, phenoxazine, and the multiply substituted C5-propynyl S-ONs) previously characterized for antisense potency in cell culture. As shown in Table 2, the G-clamp S-ON bound to its RNA target 1.7-fold faster than the unmodified S-ON and 1.9-fold faster than the phenoxazine S-ON; in contrast, the G-clamp bound 1.5-fold slower relative to the multiply-substituted C5-propynyl S-ON.

Analysis of the dissociation rates of all of the modified S-ONs indicated no dramatic difference. The unmodified S-ON/RNA duplex, which showed the most rapid dissociation, showed 47% dissociation after 27 h, whereas the C5-propynyl S-ON and the G-clamp S-ON/RNA complexes were dissociated 28% and 36%, respectively. The relative rates of dissociation 1.0 for the unmodified S-ON/RNA duplex, 0.77 for the G clamp, and 0.60 for the C5-propynyl S-ON.

Overall the G-clamp-modified S-ON has a relative affinity for its RNA target that is 2.2-fold greater than the unmodified S-ON and 2.7-fold greater than the phenoxazine S-ON. These modest effects correlate with the increased potency observed for the G-clamp S-ON as shown in Fig. 3. However, the multiply substituted C5-propynyl S-ON had the highest affinity, yet the G-clamp S-ON demonstrated the most potent antisense inhibition of p27kip1.

G-Clamp S-ON Are RNase H-Competent. The lack of correlation between the affinity of the G-clamp S-ON and its enhanced antisense activity relative to the C5-propynyl S-ON suggested that the rate of RNase H cleavage of the target RNA may account for the increased potency of the G-clamp p27kip1 antisense S-ON. To directly test this possibility, the same four modified S-ONs as above were tested and compared in their ability to activate RNase H cleavage of the target RNA. The S-ON/[32P]RNA heteroduplexes were incubated with HeLa nuclear extracts (a source of human RNase H) for various times, and the cleavage products were analyzed. Reaction times were adjusted so that >70% of the S-ON/RNA heteroduplex was intact after RNase H cleavage. These conditions allowed fine mapping of the cleavage site. The results indicate that the RNA portion of the G-clamp S-ON/RNA heteroduplex was efficiently cleaved by RNase H. No differences in the cleavage pattern were observed for the unmodified S-ON and the G-clamp S-ON (Table 2, ON10 and ON4, respectively), suggesting the single G-clamp modification in the major groove of the helix was easily accommodated by RNase H.

Analysis of the percent cleavage showed that the G-clamp S-ON/RNA duplex was cleaved 3.5-fold more rapidly than the C5-propynyl S-ON/RNA duplex (Table 2, compare ON4 and ON8). This difference in RNase H cleavage rate may, in part, account for the increased potency observed for the p27kip1 G-clamp S-ON as compared with our previous most potent C5-propynyl p27kip1 S-ON.

A Single G-Clamp Modification Enhanced the Potency of a Previously Optimized Antisense S-ON. To highlight the portability and potency of the G-clamp modification, a single G-clamp substitution was incorporated into a previously optimized antisense S-ON targeting c-raf (5′-CTG CGC TCG TGA CAT GCA TT; X = G-clamp) and tested for antisense activity. The first-generation c-raf antisense S-ON (5′-TCC CGC TCG TGA CAT GCA TT) was identified by Monia et al. (11, 23) as the most potent S-ON out of a screen of 34 distinct S-ONs spanning the entire c-raf gene. This 20-mer c-raf antisense S-ON is currently being tested in phase II trials for the treatment of cancer (reviewed in ref. 6).
The antisense S-ONs and their corresponding 4-nt mismatch S-ONs were delivered to T24 bladder carcinoma cells by using 2.5 μg/ml GS3815 cytofectin and a range of S-ON concentrations. Based on earlier results (Fig. 3), the G-clamp S-ONs were tested at S-ON concentrations 10-fold lower than the unmodified S-ONs. Forty-eight hours later, protein extracts were prepared, separated by using SDS-PAGE, and analyzed for c-raf protein levels. The cyclin-dependent kinase (cdec2) was used as an internal control.

An antisense-specific decrease in c-raf levels was observed for both the G-clamp S-ON and the unmodified S-ON (Fig. 5) under all conditions tested. At 10 nM, the G-clamp c-raf S-ON specifically reduced c-raf protein levels by 63%, whereas the 4-nt G-clamp mismatch had no effect on c-raf levels. A similar 62% decrease in c-raf levels (Fig. 5, lane 2) was observed with the unmodified c-raf S-ON at a 25-fold higher S-ON concentration (250 nM) relative to the singly substituted G-clamp S-ON. These antisense results with the unmodified c-raf S-ONs are consistent with previous reports (11, 24). Our data suggest that incorporation of a single G-clamp modification, even into a previously optimized antisense S-ON, can dramatically enhance the potency of an antisense S-ON.

**DISCUSSION**

The G-clamp modification is a remarkably potent heterocycle modification. A single G-clamp enhanced the potency of a S-ON relative to 11 C5-propynyl modifications yet retains the sequence selectivity of an unmodified base. For most S-ON modifications, there is a tradeoff between increased potency and sequence selectivity. For example, the fully C5-propynyl-modified p27kip1 is a potent antisense inhibitor (Fig. 3) and shows high-affinity for its RNA target, yet it is insensitive to a single nucleotide mismatch, as shown in Table 1. The G-clamp p27kip1 S-ON also is a potent inhibitor of p27kip1, more potent than the C5-propynyl p27kip1 S-ON, and, yet, it demonstrated a 5-fold reduction in antisense activity when a single nucleotide mismatch existed between the G-clamp modified p27kip1 S-ON and its RNA target. A similar 5-fold reduction in activity was observed previously by using an unmodified 17-nt S-ON that selectively targeted a mutant ras gene containing a single base change at codon 12 (25). The sequence selectivity of the G-clamp p27kip1 S-ON might be further increased by optimizing the length of the S-ON.

We have found that the position the G-clamp modification within the S-ON greatly influenced the antisense potency of the G-clamp substitution. As shown in Fig. 2, the antisense activity of the singly G-clamp-substituted p27kip1 S-SONs varied after transfection into CV-1 cells. The reasons for this contextual or positional effect are at present unclear. Full elucidation of these effects will require further study. Until then, based on the limited data set, the tentative empirical conclusion is that the optimal position for a single G-clamp modification is between two pyrimidines.

In *in vitro* analysis of several modified S-ONs indicated that the G-clamp S-ON hybridized faster and formed a more stable interaction relative to the unmodified S-ON or the phenoxazine S-ON, but the differences were small. This enhanced binding affinity translated into the increased potency observed when tested by transfection or microinjection in various cell lines. However, the S-ONs with the highest *in vitro* binding affinity are not always the most potent antisense inhibitors. Previous results comparing the antisense activity of thiazole-substituted S-ONs to C5-propynyl-modified S-ONs demonstrated that, although the thiazole-modified ON showed higher affinity for RNA than the C5-propynyl S-ON, the thiazole-
substituted S-ON was a much poorer antisense inhibitor (13). Similarly, the C5-propynyl p27kips1 S-ON demonstrated a higher relative affinity for the same target RNA relative to a single substituted G-clamp p27kips1 S-ON, yet when transfected into CV-1 cells, the G-clamp S-ON was a more potent antisense inhibitor, as shown in Fig. 3. One possible explanation for the enhanced potency of G-clamp S-ONS is their ability to activate RNase H cleavage. Despite the bulkiness of the G-clamp heterocycle, the G-clamp S-ON/RNA heteroduplex was a 3-fold better substrate for RNase H cleavage relative to the C5-propynyl S-ON/RNA duplex (Table 2). Fine mapping of the RNase H cleavage site of the G-clamp S-ON/RNA hybrid indicated that the cleavage sites adjacent to the G-clamp were unaffected by the aminoxy tethering arm in the major groove (data not shown). These results highlight the unique ability of the G-clamp S-ON to hybridize in a selective fashion to its RNA target and to activate RNase H-mediated cleavage of the RNA, resulting in potent antisense-specific gene inhibition.

The in vitro binding properties and RNase H activity of the G-clamp S-ON may not be sufficient to explain the enhanced antisense potency observed. S-ON/RNA helix dissociation is known to occur at a much faster rate intracellularly relative to in vitro conditions (12). The dissociation process intracellularly is likely mediated by numerous proteins with helicase activity. The G-clamp modification could result in substantial stabilization of the S-ON/RNA duplex intracellularly by rendering the helix a poor substrate for such helicases. This hypothesis awaits further testing.

All first-generation, conventional S-ONs that have entered phase I safety studies have demonstrated safety profiles that have allowed their continued clinical development (26–29). The continued development of the G-clamp modification in antisense-based therapeutics also depends on the demonstration of acceptable safety and pharmacokinetic profiles in preclinical models. ON modifications can dramatically alter the pharmacokinetics of ONs in vivo (30). In vivo analysis of 2′-O-alkyl-modified S-ONS indicated faster plasma elimination and more rapid excretion compared with unmodified S-Ons (31), and C5-propynyl-modified S-ONS have demonstrated in vivo liver toxicities that have precluded their development as human therapeutics (W.M.F. and R.W.W., unpublished results). However, preliminary in vivo data suggests that a single G-clamp modification in a 15-mer S-ON does not alter the pharmacokinetic, toxicity, or tissue-distribution properties of the S-ON as compared with a conventional antisense S-ON when dosed as high as 50 mg/kg for 10 days in mice (Steven Davis, personal communication). These preliminary results suggest that G-clamp-modified S-ONS have the appropriate in vivo profile to continue their development as a more potent and safer second-generation, antisense-based human therapeutic.

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