2′-O-[2-[(N,N-dimethylamino)oxy]ethyl]-modified oligonucleotides inhibit expression of mRNA in vitro and in vivo

Thazha P. Prakash, Joseph F. Johnston, Mark J. Graham, Thomas P. Condon and Muthiah Manoharan*

Isis Pharmaceuticals Inc., 2292 Faraday Avenue, Carlsbad, CA 92008, USA

ABSTRACT

Synthesis and antisense activity of oligonucleotides modified with 2′-O-[2-[(N,N-dimethylamino)oxy]ethyl] (2′-O-DMAOE) are described. The 2′-O-DMAOE-modified oligonucleotides showed superior metabolic stability in mice. The phosphorothioate oligonucleotide ‘gapmers’, with 2′-O-DMAOE-modified nucleoside residues at the ends and 2′-deoxy nucleosides residues in the central region, showed dose-dependent inhibition of mRNA expression in cell culture for two targets. ‘Gapmer’ oligonucleotides have one or two 2′-O-modified regions and a 2′-deoxyligonucleotide phosphorothioate region that allows RNase H digestion of target mRNA. To determine the in vivo potency and efficacy, BalbC mice were treated with 2′-O-DMAOE gapmers and a dose-dependent reduction in the targeted C-raf mRNA expression was observed. Oligonucleotides with 2′-O-DMAOE modifications throughout the sequences reduced the intercellular adhesion molecule-1 (ICAM-1) protein expression very efficiently in HUVEC cells with an IC50 of 1.8 nM. The inhibition of ICAM-1 protein expression by these uniformly modified 2′-O-DMAOE oligonucleotides may be due to selective interference with the formation of the translational initiation complex. These results demonstrate that 2′-O-DMAOE-modified oligonucleotides are useful for antisense-based therapeutics when either RNase H-dependent or RNase H-independent target reduction mechanisms are employed.

INTRODUCTION

Antisense oligonucleotides have proven effective in inhibiting a number of viral and cellular gene products, both in vitro and in vivo (1–5). The activity of antisense oligonucleotides depends upon many factors, including binding affinity to target RNA, nuclease resistance, and cellular absorption and modulate the protein binding of oligonucleotides (4,6). To optimize activity, structural analogs of nucleic acids with modified heterocycle, sugar and phosphodiester backbone moieties have been synthesized (6,7–12). Some of the most successful analogs resulted from modification of the sugar at the 2′ position (6,11). The 2′-O-modified oligonucleotides used with the ‘gapmer’ technology (13) have emerged as the leading second generation candidates for clinical applications. ‘Gapmer’ oligonucleotides have one or two 2′-O-modified regions and a 2′-deoxyligonucleotide phosphorothioate region that allows RNase H digestion of the targeted mRNA.

Among the various 2′-O-modified oligonucleotides reported in the literature, the 2′-O-(2-methoxyethyl)-modified oligonucleotides (2′-O-MOE) offer a 2°C increase in melting temperature (Tm) per modification as a diester (2′-O-MOE/P=P=O) compared with the 2′-deoxyporphosphorothioate (2′-H/H/P=S) compounds (14). This modification with a phosphodiester linkage exhibits resistance to snake venom phosphodiesterase (measured as the half-life of disappearance of the full-length oligonucleotide, t1/2) at approximately the same level as a 2′-deoxyligonucleotide phosphorothioate, the first generation antisense drug. There are several 2′-O-MOE antisense oligonucleotides in clinical trials (15). We recently reported the synthesis of 2′-O-[2-[(N,N-dimethylamino)oxy]ethyl] (2′-O-DMAOE) (Fig. 1), a new carbohydrate modification that exhibits high binding affinity towards target RNA and high nuclease resistance in a snake venom phosphodiesterase assay (16). Recently, we published a convenient synthetic strategy for the synthesis of 2′-O-DMAOE-modified nucleoside phosphoramidites (Fig. 2) useful for all bases (17). Here, we report the in vivo nuclease resistance and ability to inhibit target mRNA expression in vitro and in vivo of 2′-O-DMAOE oligonucleotides. We also report the efficacy of uniformly modified 2′-O-DMAOE oligonucleotides in interfering with the formation of translational initiation complex of the translational machinery (15). A comparative analysis of in vitro and in vivo antisense initiation of 2′-O-DMAOE oligonucleotides with that of 2′-O-MOE oligonucleotides are also presented in this report.

*To whom correspondence should be addressed. Tel: +1 617 252 0700 ext. 2319; Fax: +1 617 252 011; Email: mmanoharan@alnylam.com
Present address:
M.Manoharan, Alnylam Pharmaceuticals, 790 Memorial Drive, Suite 202, Cambridge, MA 02139, USA.

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temperature until complete removal of the trityl group, as monitored by HPLC analysis. The oligonucleotides were then desalted by HPLC to yield 2' modified oligonucleotides in 30–40% isolated yield calculated based on the loading of the 3'-base to solid support (18). The oligonucleotides were characterized by electrospray mass spectroscopy and their purity was assessed by HPLC (Table 2) and capillary gel electrophoresis (CGE).

**In vivo nuclease stability assay**

The 2' O-MOE gapmer oligonucleotides 5 and 6 (Table 1) and 2' O-DMAOE gapmer oligonucleotides 7 and 8 (Table 1) were administered to BalbC mice by i.p. injection at a 50 mg/kg dose. Oligonucleotide concentrations were calculated from the oligonucleotide absorbance at 260 nm and extinction coefficients estimated according to Puglisi and Tinoco (19). After 24 h, mice were killed and the oligonucleotides were isolated from liver, kidney and spleen as described (20). Samples were analyzed using a Beckman PA/CE System Gold 5010 capillary electrophoresis system with detection at 260 nm. Samples were loaded electrokinetically by application of 5–10 kV for 5–30 s, depending on the relative sample concentration. Separations were achieved operating at 10–15 kV constant voltages for between 6 and 10 min at 50°C. The percentage of full-length oligonucleotides in each organ was determined by integration of the peak area corresponding to the full-length oligonucleotide relative to the T<sub>27</sub> phosphorothioate internal standard at 200 nM.

**Inhibition of protein kinase C-α (PKC-α) mRNA expression in vitro by 2'O-DMAOE gapmer oligonucleotides**

PKC-α mRNA expression in C127 cells was evaluated as described (21,22). Muraine C127 mammary epithelial cells were obtained from American Type Culture Collection. C127 cells were grown in T-75 flasks in Dulbecco’s modified Eagle’s medium containing 1 g of glucose per liter (DMEM; Gibco/BRL), and 10% fetal bovine serum (FBS; HyClone) until 70–80% confluent. Cells were washed twice in 10 ml of DMEM. Then, 5 ml of DMEM containing N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride/dioleoyl phosphatidylethanolamine (DOTMA/DOPE) solution (Lipofectin; final concentration, 20 µg/ml; Gibco/BRL) was added. Oligonucleotides 9 and 10 were then added to the required concentration. The cells were incubated at 37°C for 4 h, washed once with DMEM/10% FBS to remove the DOTMA/DOPE solution, and then allowed to recover for 72 h. Total mRNA was isolated from tissue culture cells by lysis in 4 M guanidinium isothiocyanate followed by cesium chloride gradient centrifugation (23). RNA (20–40 µg) was resolved in 1.2% agarose gels containing 1.1% formaldehyde and transferred to nylon membranes. The blots were then hybridized with bovine PKC-α cDNA (American Type Culture Collection) as described (24). Probe hybridized to mRNA transcript was visualized and quantitated with a PhosphorImager (Molecular Dynamics). PKC-α mRNA is expressed as two transcripts, 8.5 and 4.0 kb. The blots were then stripped of radioactivity by boiling and reprobed with a 32P-labeled glycerol-3-phosphate dehydrogenase (G3PDH) probe (Clontech) to confirm equal loading.
Quantitation of ICAM-1 protein expression in HUVEC cells was carried out as described (25). HUVEC cells were purchased from Clonetics Corp. (San Diego, CA) and cultured in EBM medium (HyClone) supplemented with 10% fetal bovine serum. Cells used for experiments were from passages two to 10 at 80–90% confluence. Cells were washed three times with Opti-MEM (Life Technologies, Inc.) pre-warmed to 37°C. Oligonucleotides 11–14 (Table 1) were premixed with 10 μg/ml Lipofectin (Life Technologies, Inc.) in opti-MEM, serially diluted to the desired concentrations and applied to the washed cells. Basal and untreated (no oligonucleotides) control cells were also treated with Lipofectin. Cells were incubated for 4 h at 37°C, at which time the medium was removed and replaced with standard growth medium with or without 5 ng/ml IL-1β and incubated at 37°C overnight. Cells were removed from the plate surfaces by brief trypsinization with 0.25% trypsin in PBS. Trypsin activity was quenched with a solution of 25% bovine serum albumin and 0.2% sodium azide in PBS (+Mg/Ca). Cells were pelleted by centrifugation (1000 r.p.m.; Beckman GPR centrifuge), resuspended in PBS, and stained with 3 μl/10⁵ cells of the ICAM-1-specific antibody, CD54-PE (Becton Dickinson) and 0.1 μg of the control antibody, IgG2b-PE (Pharmingen). Antibodies were incubated with the cells for 30 min at 4°C in the dark, under gentle agitation. Cells were washed by centrifugation procedures and then resuspended in 0.3 ml of FacsFlow buffer (Becton Dickinson) at 0.5% formaldehyde (Polysciences). Expression of cell surface ICAM-1 was then determined by flow cytometry using a Becton Dickinson FACScan. The percentage of the control antibody, IgG2b-PE, was used to confirm equal loading.

### Inhibition of C-raf mRNA expression in BalbC mouse treated with 2'-O-DMOE gapmer oligonucleotides

Female BALB/c mice (6 weeks old; Harlan Sprague–Dawley, Indianapolis, IN) were housed three to a cage under conditions meeting National Institute of Health regulations (26). Oligonucleotides 5–8 were administered in 0.9% NaCl, i.p. at indicated dose levels once daily for 3 days and tissues were harvested for analysis. Total mRNA was extracted from mouse liver by rapid homogenization of the tissue in 4 M guanidinium isothiocyanate followed by centrifugation over a cesium chloride gradient. RNAs (20–40 μg) were resolved in 1.2% agarose gels containing 1.1% formaldehyde (Polysciences). Expression of cell surface ICAM-1 was then determined by flow cytometry using a Becton Dickinson FACScan. The percentage of the control antibody, IgG2b-PE, was used to confirm equal loading.

### RESULTS AND DISCUSSION

#### Nuclease stability of modified oligonucleotides in vivo

The in vivo metabolic stability of antisense drugs is crucial for the desired pharmacological effect. The naturally occurring oligonucleotide phosphodiesters are rapidly degraded in biological fluids by nucleases (15). Chemical modifications at the backbone, sugar and/or base have been shown to stabilize oligonucleotides to enzymatic digestion with a 1/2...
The first generation antisense drug on the market is a DNA analog where one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by sulfur. This chemical modification made antisense drugs stable to nucleases when compared with natural DNA. Further improvement in metabolic stability was achieved by modification at the 2’ position of the sugar (6). In order to evaluate the effect of 2’-O-DMAOE modification on in vivo metabolic stability of the oligonucleotides, we have synthesized a 2’-O-DMAOE oligonucleotide with phosphorothioate backbone chemistry (PS, 7) and with mixed backbone chemistry (PO/PS, 8). These 2’-O-DMAOE oligonucleotides were compared to oligonucleotides 5 and 6 with 2’-O-MOE modifications for metabolic stability in mice.

The oligonucleotides 5–8 were administered to BalbC mice by i.p. injection at a 50 mg/kg dose. After 24 h, mice were killed and the oligonucleotides were isolated from liver, kidney, and spleen. The percentage of full-length oligonucleotides in each organ (Fig. 3) was determined by CGE analysis. After 24 h, 80–100% of full-length phosphorothioate oligonucleotides 5 and 7 were isolated from these organs, whereas only 25–40% of mixed backbone oligonucleotides 6 and 8 was isolated. The role of 2’-O-DMAOE modification in enhancing the stability of oligonucleotides against exonuclease-mediated degradation has been reported (16,17). The observed in vivo metabolic stability of oligonucleotides with 2’-O-DMAOE modifications is consistent with the reported in vitro metabolic stability of 2’-O-DMAOE-modified oligonucleotides. These data suggest that antisense drugs with 2’-O-DMAOE modifications would be available for pharmacological action longer than 24 h. This also implies that antisense drugs with 2’-O-DMAOE modifications will exhibit pharmacology with a lesser number of doses compared with first generation antisense drugs.

Effect of 2’-O-DMAOE-modified oligonucleotides on PKC-α mRNA expression in C127 cells

In order to evaluate the efficacy of inhibition of gene expression via RNase H digestion of target mRNA, a 2’-O-DMAOE gapmer oligonucleotide 10, targeting human serine/threonine-specific PKC-α expression (21,22) was synthesized.

For comparison, we synthesized the previously characterized 2’-O-MOE gapmer 9 of the same sequence. The serine/threonine-specific PKC family members regulate a variety of cellular responses, including proliferation, differentiation and apoptosis (27,28). The oligonucleotide 10 reduced the expression of PKC-α mRNA in human C127 cells (Fig. 4) with an IC_{50} of 33.4 nM (Table 3) and was 2.4-fold more potent than the 2’-O-MOE gapmer 9 (IC_{50} 80.6 nM, Table 3). This increase in potency correlates well with the superior binding demonstrated by the 2’-O-DMAOE-modified oligonucleotides (16,17).

Effect of 2’-O-DMAOE-modified oligonucleotides on inhibition of the ICAM-1 translation initiation complex in HUVEC cells

2’-O-MOE-modified oligonucleotides 11 and 13 have been shown to selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translational initiation complex in HUVEC cells (25). The inhibition of protein expression by these oligonucleotides is due to selective interference with the formation of 80 S translational initiation complexes. We have synthesized fully modified 2’-O-DMAOE oligonucleotides 12 and 14 (Table 1) and compared their activity with 2’-O-MOE oligonucleotides 11 and 13 (Table 1). The oligonucleotides were analyzed for inhibition of IL-1β-stimulated ICAM-1 protein expression in HUVEC cells. These antisense oligonucleotides exhibited dose-dependent reduction of ICAM-1 protein expression very
are representative of two independent experiments.

Figure 5. Dose-response characteristics for the reduction in ICAM-1 protein expression in HUVEC cells treated with oligonucleotides (2'-O-MOE), (2'-O-DMAOE), (2'-O-MOE) and (2'-O-DMAOE). Results are comparable with the 2'-O-MOE-modified oligonucleotides. These data suggest that 2'-O-DMAOE-modified oligonucleotides could be potentially useful as agents to inhibit translation by steric blockade.

Inhibition of C-raf mRNA expression in mouse liver after systemic administration of phosphorothioate 2'-O-DMAOE-modified oligonucleotides

The phosphorothioate oligonucleotide (Table 1) was previously characterized as a potent inhibitor of C-raf kinase expression (29). In the current study, the same sequence with 2'-O-MOE and 2'-O-DMAOE gapmer constructs (5–8) (Table 1) were used to target C-raf mRNA in female Balb C mice. The female Balb/C mice were administered oligonucleotides at 3, 10, 25 and 50 mg/kg, once daily for 3 days. The mice were killed and the tissue was harvested for analysis. Total mRNA was isolated. The mRNA levels were quantitated by northern blot and PhosphorImager analysis. The phosphorothioate 2'-O-DMAOE gapmer oligonucleotide (7) was a potent inhibitor of C-raf mRNA in mouse liver (Fig. 6), with activity comparable to the 2'-O-MOE gapmer oligonucleotide (5) (Fig. 6). The oligonucleotides 6 and 8 with mixed backbone chemistry were not effective in inhibition of C-raf mRNA expression in vivo. It has been reported that 2'-O-MOE modification combined with phosphodiester backbone exhibited 10-fold more rapid plasma clearance compared with the corresponding oligonucleotide phosphorothioates (30). The lack of activity with mixed backbone gapmer oligonucleotides (6 and 8) may be attributed to their rapid plasma clearance, and as a result, poor tissue distribution of these oligonucleotides.

In conclusion, oligonucleotides with 2'-O-DMAOE modifications were synthesized with phosphorothioates (PS) and mixed backbone (PO/PS) chemistries. The uniformly modified 2'-O-DMAOE oligonucleotides were also synthesized in good yield. The 2'-O-DMAOE modification improved the in vivo metabolic stability of the oligonucleotides compared with first generation antisense oligonucleotides. The 2'-O-DMAOE-modified gapmers were 2-fold more potent than 2'-O-MOE oligonucleotides in inhibition of gene expression in vitro. The uniformly modified 2'-O-DMAOE oligonucleotide inhibited ICAM-1 protein expression in vitro, presumably by interfering with the formation of the translational initiation complex at a level comparable with the 2'-O-MOE. The 2'-O-DMAOE gapmer oligonucleotide phosphorothioates designed to inhibit C-raf expression in mice showed very good potency, again comparable with the 2'-O-MOE gapmer. In summary, we have demonstrated the efficacy of the 2'-O-DMAOE-modified oligonucleotides in reducing the targeted message in vitro and in vivo. The 2'-O-DMAOE-modified oligonucleotides support inhibition of gene expression by the RNase H-mediated mechanism and by interfering with the translational initiation machinery. Our study demonstrates that 2'-O-DMAOE-modified oligonucleotides should be valuable for inhibition of gene expression using either an RNase H-dependent or RNase H-independent mechanism.

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