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Antisense oligonucleotides: Efficient synthesis of 2′-*O*-methoxyethyl phosphorothioate oligonucleotides using 4,5-dicyanoimidazole. Are these oligonucleotides comparable to those synthesized using 1*H*-tetrazole as coupling activator?

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Abstract—Multiple 2′-O-methoxyethyl modified phosphorothioate oligonucleotides of 18–20-mer in length were synthesized at various scales using 4,5-dicyanoimidazole (DCI) as coupling activator. Extensive synthetic, analytical (using ion-pair LC–MS), and in vivo pharmacological, toxicological studies showed that oligonucleotides made with DCI and 1*H*-tetrazole are chemically and biologically equivalent. This extensive study will help the oligonucleotide therapeutic industry to move from using a potentially explosive activator (1*H*-tetrazole) to a safe activator (DCI).

1. Introduction

Oligonucleotides are finding widespread utility in various applications in diagnostics and molecular biology and as therapeutic agents.[1–4] In recent years, DNA and RNA analogues, especially phosphorothioate oligonucleotides in which one nonbridging oxygen atom of the internucleotide phosphate group is replaced by a sulfur atom, have emerged as potential drugs for treatment of a variety of diseases through antisense mechanism of action. To further increase the therapeutic value of these phosphorothioate drugs, several sugar, backbone, and base modifications are being investigated and 2′-O-methoxyethyl (MOE) sugar modified oligonucleotide chimera has been selected[5] in our laboratories (at Isis Pharmaceuticals) and multiple drugs are in various stages of human clinical trials against a variety of diseases.[6] Automated synthesis of PS-oligonucleotides is performed on a solid support (derivatized on cross-linked polystyrene), using commercially available phosphoramidites as the starting materials. A typical synthesis cycle involves four chemical reactions that are separated by rinsing steps designed to remove excess reagents. The four steps are:

(a) Acid-mediated removal of DMT-protecting group to liberate the 5′-hydroxyl group of support-bound oligonucleotide.
(b) Elongation of oligonucleotide chain by coupling of a protected nucleoside phosphoramidite in the presence of a weak acid.
(c) Stabilizing the phosphite triester [P(III) species] intermediate to form a phosphorothioate triester [P(V) species] by incorporation of a sulfur atom by oxidative sulfurization.
(d) Blocking of uncoupled 5′-hydroxyl groups to prevent elongation of failure sequences.

Repetition of this synthesis cycle allows the assembly of PS-oligonucleotides on scales ranging from nanomoles to almost up to a mole. Cleavage and separation of the oligonucleotide from the solid-support matrix, deprotection steps, purification by reversed-phase or anion exchange HPLC, and isolation of the final active pharmaceutical ingredient (API) as amorphous powder complete the synthesis of these macro-molecular biopolymers. Despite recent advances in preparative oligonucleotide chemistry, the development of improved methods for efficient synthesis of therapeutic grade oligonucleotides remains an area of active interest.[7]

Keywords: Oligonucleotide synthesis; Activator; Dicyanoimidazole; Phosphorothioate; Antisense; 1*H*-Tetrazole.
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As part of this overall objective we were interested in investigating the coupling (or elongation) step of the synthesis. Successful synthesis depends, inter alia, on this coupling reaction being fast and nearly quantitative, without serious side reactions. Activation of phosphoramidites is usually achieved by reaction with 1*H*-tetrazole. While this activator is an efficient and well-accepted standard for small-scale synthesis, it is not desirable for use in large-scale synthesis for therapeutic application. 1*H*-Tetrazole has the potential to explode if melted and, in addition, is indirectly expensive due to security concerns. Department of transportation (DOT) has banned any air-shipment and only ground movement is allowed. The possibility of dry tetrazole residues forcefully decomposing in heated equipment is real. Thus, invention of new activation reagents which are equally effective as tetrazole, inexpensive, and safe to use is highly desirable.

Various activators have been reported in the literature to be efficient, safe, and economical.[8] However, many of them have not been investigated from a therapeutic viewpoint viz. using appropriate analytical tools and asking the right questions to look for potential process-related impurities (PPRIs). Here, at Isis Pharmaceuticals, we use state-of-the-art ion-pair LC–MS for analyzing the quality of the synthesized drug. Low levels of side reaction by-products that are well resolved based on mass difference could be identified and quantitated thereby making inferences right and conclusions easy. Thus, a thorough screening of activators has resulted in 4,5-dicyanoimidazole (DCI) as the activator of choice. This activator is inexpensive, safe, not moisture sensitive, and highly effective in solid-supported synthesis of second-generation 2′-O-methoxyethyl modified phosphorothioate oligonucleotides.

Several oligonucleotide-based drugs are being evaluated in clinic and many others will be entering human trials in next several years for treating many diseases. Currently, 1*H*-tetrazole is the standard activator of choice and many drugs synthesized using this activator have moved into advanced stages of clinical evaluation. Due to the above-mentioned safety concerns among other reasons, we and others in this field are planning to switch to DCI as an alternative and preferred replacement. It is important that we show all evidences (chemical and biological) that drugs made with both activators are similar.[8a] Hence, this extensive study was undertaken. We show here for the first time that phosphorothioate oligonucleotides made with 1*H*-tetrazole and DCI are chemically and biologically equivalent.

2. Results and discussion

A class of process-related species that is observed in PS-oligonucleotides is a group of oligonucleotides that are identical to the main product, except they contain one less or more nucleotide. The shorter chains are termed deletionmers or $(n-1)$-mers and longer ones are termed $(n+1)$-mers. The deletionmers could be missing a base internally (internal deletionmers) or at a terminal (terminal deletionmers). PAGE and capillary gel electrophoresis (CGE) have been used for quantitative measurement of these undesired side products. Ion-pair high-performance liquid chromatography/mass spectroscopy (IP-LC–MS) technique is a specific, accurate, and sensitive means of quantitating oligonucleotides containing $(n-1)$- and $(n+1)$-mers within a matrix of PS-oligonucleotides. Besides overall content, low levels of individual component of $(n-1)$-mers and $(n+1)$-mers could be quantitated. It has been observed that the first coupling contributes significantly more to the $(n-1)$-mer population than the subsequent coupling steps. Efficient coupling using a good activator and capping steps are essential for using reversed-phase HPLC for preparative scale to obtain high recovery yield while retaining high quality. Removal of $(n-1)$-mer- and $(n+1)$-mer-containing oligonucleotide on preparative scale using anion exchange chromatographic technology is possible but leads to significant yield loss. Non-chromatographic efforts at eliminating/minimizing these low levels of process-related species without compromising on yield have so far not been practical and economical.[9] Based on our extensive experience, presence of these low levels of deletionmers does not influence the biological activity of the phosphorothioate parent drug.

Multiple 2′-O-methoxyethyl modified phosphorothioate oligonucleotide chimera was synthesized at various scales (Table 1) using DCI as activator. In each case, the crude oligonucleotide was analyzed by ion-pair LC–MS before purification was performed. Comparable high quality and yield were obtained in each of the oligonucleotides investigated. As an example, Figure 1 shows the ion-pair LC–MS analysis of crude material of sequence (**1**) (Table 1) synthesized using DCI and 1*H*-tetrazole as control. Table 2 summarizes the results between the two activators investigated for sequence (**1**).

Thus, based on extensive analysis using ion-pair LC–MS, quality and yield of phosphorothioate oligonucleotides produced by 1*H*-tetrazole and 4,5-dicyanoimidazole are equivalent, in spite of the lower acidity of DCI.

Table 1. Chemical structure and sequence information of phosphorothioate oligonucleotides **1**–**6** and their respective biological target

Oligonucleotide	Sequence	Biological target
1	5′-*PS*-[*GAG*^{me}*C*^{me}*U*]-*d*(*TTG*^{me}*C*^{me}*CTT*^{me}*CTT*)-[*G*^{me}*C*^{me}*CA*^{me}*U*]	Glucagon receptor
2	5′-PS-[*G*^{me}*C*^{me}*U*^{me}*C*^{me}*C*]-d(TT^{me}C^{me}CA^{me}CTGAT)- [^{me}*C*^{me}*C*^{me}*UG*^{me}*C*]	PTP-1B
3	5′-PS-[*G*^{me}*C*^{me}*UGA*]-d(TTAGAGAGAG)-[*G*^{me}*U*^{me}*C*^{me}*C*^{me}*C*]	TNF-α
4	5′-PS-[^{me}*CAG*^{me}*C*]-d(AGCAGAGTCTTCA)-[^{me}*U*^{me}*CA*^{me}*U*]	Clusterin
5	5′-PS-[G^{me}C^{me}*U*]-d(AG^{me}C^{me}CT^{me}CTGGA-[^{me}*U*^{me}*U*^{me}*U*]	PTEN

Nucleotides in italics indicate 2′-methoxyethyl modified oligoribonucleotides. ^{me}C, 5-methyl(deoxy)cytidine; ^{me}U, 5-methyleuridine.

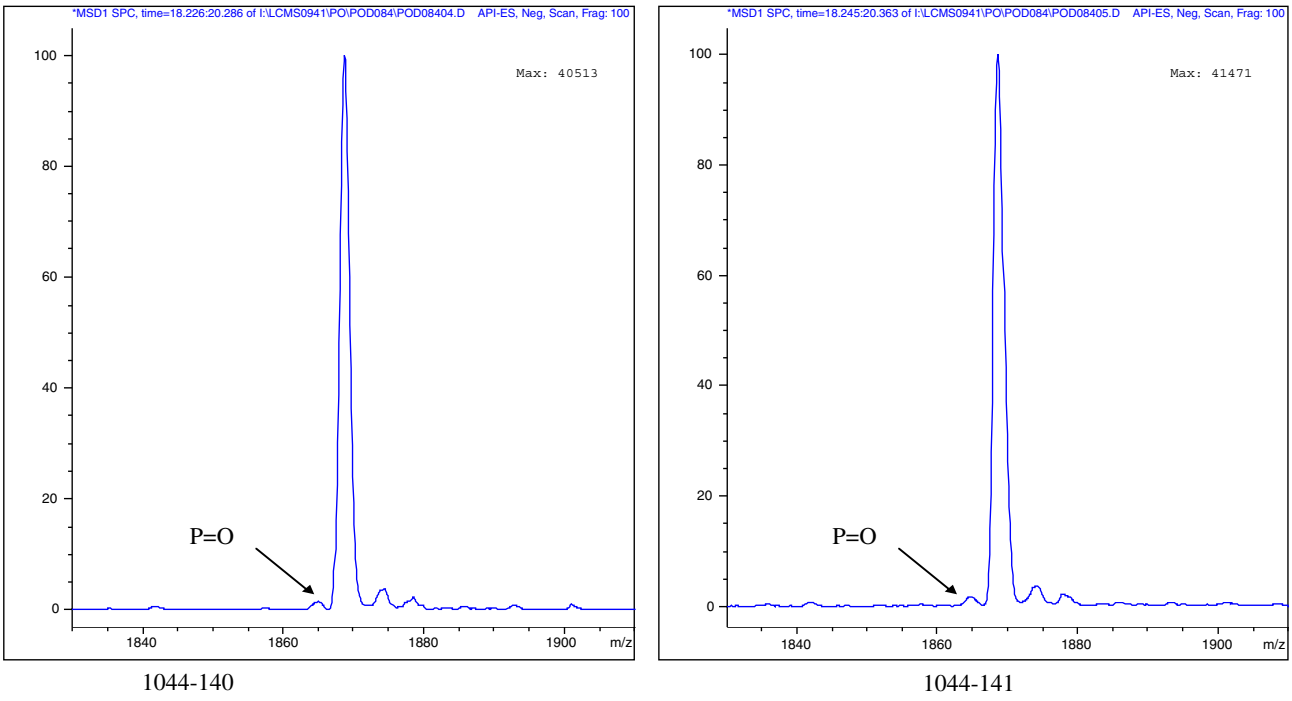


Figure 1. Ion-pair LC–MS analysis of oligonucleotide (**1**) synthesized using DCI (1044–140) and tetrazole (1044–141).

Table 2. Comparison of oligonucleotide (**1**) synthesized using different activators during coupling

Activator used for coupling	Crude yield[10] mg/μmol	Crude full length (%) (IP-LC–MS)	($n-1$)-mer (%) (IP-LC–MS)	Depurinated species (%) (IP-LC–MS)	P=O (%) (IP-LC–MS)	Purified yield (mg/μmol)
1*H*-Tetrazole	7.03	72	0.8	0.44	1.5	3.90
DCI	7.14	73	0.8	0.42	1.6	4.05

Depurinated species include $n-G$, $n-A/n-G+H_2O$, $n-A+H_2O$, and 3′-terminal phosphorothioate monoester (TPT).

3. Investigation of biological equivalence

In addition to showing chemical equivalence, biological assays can provide a sensitive test of the chemical integrity of an oligonucleotide. Sequence no. **1**, a 20-mer phosphorothioate chimera targeted against glucagon receptor (Table 1), was synthesized using 1*H*-tetrazole (ZW996-96), 4,5-dicyanoimidazole with *N*-methylimidazole (ZW996-97), and 5-ethylthiotetrazole (ETT). While using ETT as activator, two oligonucleotides were synthesized—with (ZW996-95) and without (ZW996-98) addition of *N*-methylimidazole. NMI is known to enhance the nucleophilicity of the attacking 5′-hyroxyl group on the activated phosphoramidite species. A universal control oligonucleotide [5′-PS-[$^{me}C^{me}C^{me}C^{me}U^{me}U^{me}C$]-d($^{me}C^{me}C$TGAAGGTT)-[$^{me}C^{me}C^{me}C^{me}U^{me}C^{me}C$]] (ISIS 141923) was also used for comparison. All these oligonucleotides were purified by reversed-phase HPLC, detritylated, lyophilized, analyzed by ion-pair LC–MS, and water content tested by Karl–Fischer method before evaluating in animals.

For investigation of these oligos, male six-week-old Balb/c mice were dosed twice a week for three weeks. Three doses per compound were used: 13.5, 4.5, and 1.5 mg/kg (mpk). These were labeled as H (high), M (medium), and L (low), respectively. Liver RNA was extracted for qRT-PCR analysis. Liver and spleen weights were determined. Liver, kidney, and fat tissues were snap-frozen for PK studies. Liver and kidney tissues were fixed in NBF for histology. Serum samples were prepared from all groups for serum chemistry analysis. All results are summarized in Charts 1–9.

3.1. Histopathology studies

Panels of liver and kidney tissues from mice dosed with different oligonucleotides were microscopically reviewed. No significant abnormality was observed and all antisense oligonucleotides appear to be well tolerated in both organs (Table 3).

3.2. RNase H initial rate determination on the duplex formed with oligos made with different activators

3.2.1. ^{32}P labeling of oligoribonucleotides. The oligonucleotide sense strand was 5′-end labeled with ^{32}P using [γ-^{32}P] ATP, T4 polynucleotide kinase, and standard procedures.[11] The labeled RNA was purified by electrophoresis on 12% denaturing PAGE.[12] The specific

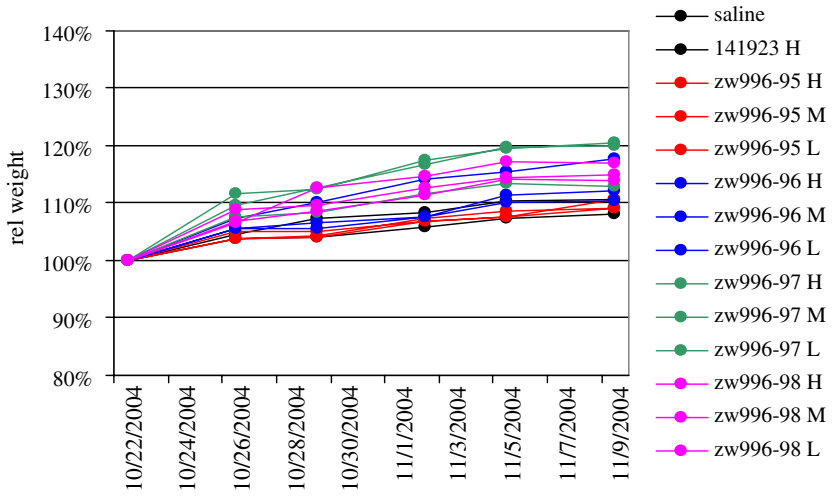


Chart 1. Comparison of body weights between different oligonucleotides (**1**) investigated.

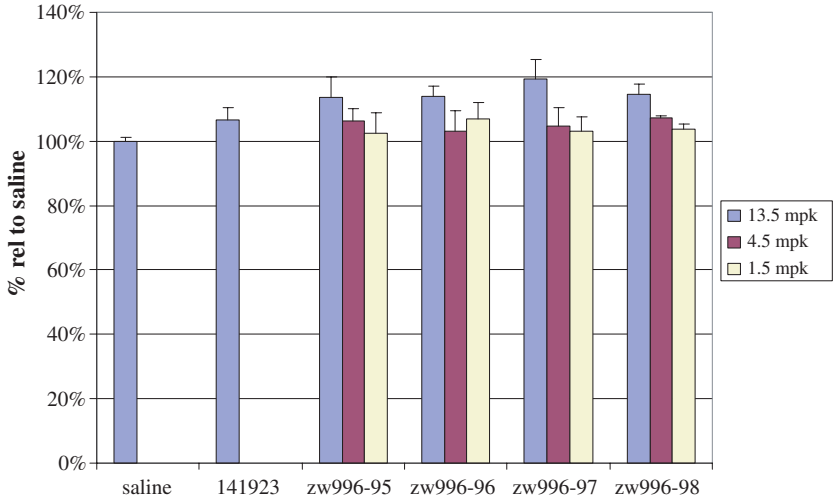


Chart 2. Comparison of liver weights between different oligonucleotides investigated.

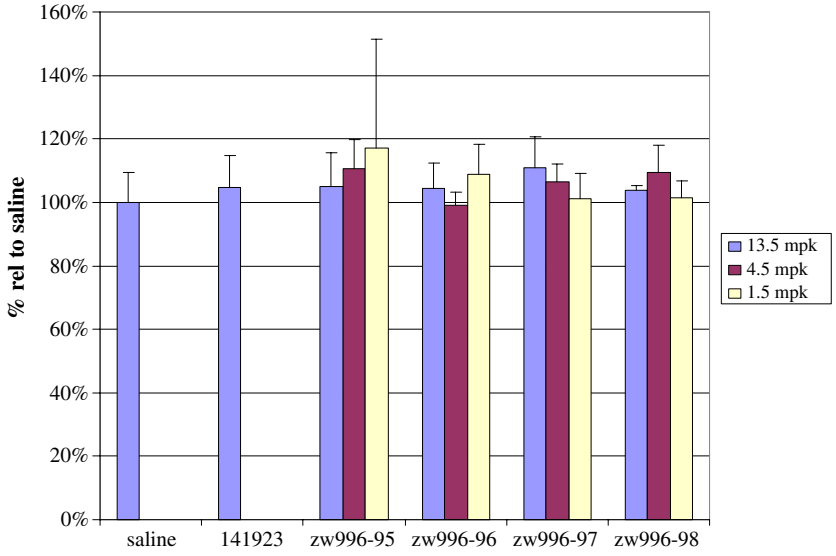


Chart 3. Comparison of spleen weights between different oligonucleotides investigated.

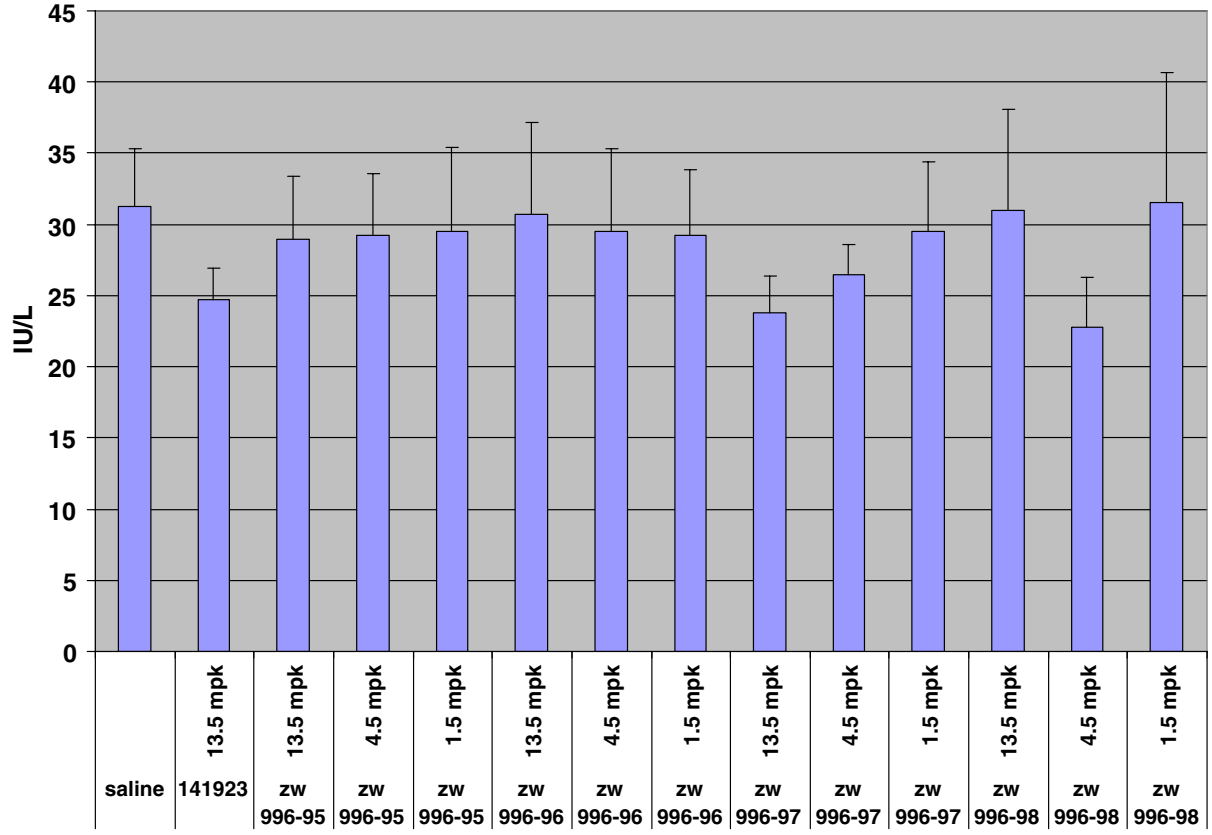


Chart 4. Comparison of alanine transaminase (ALT) between different oligonucleotides investigated.

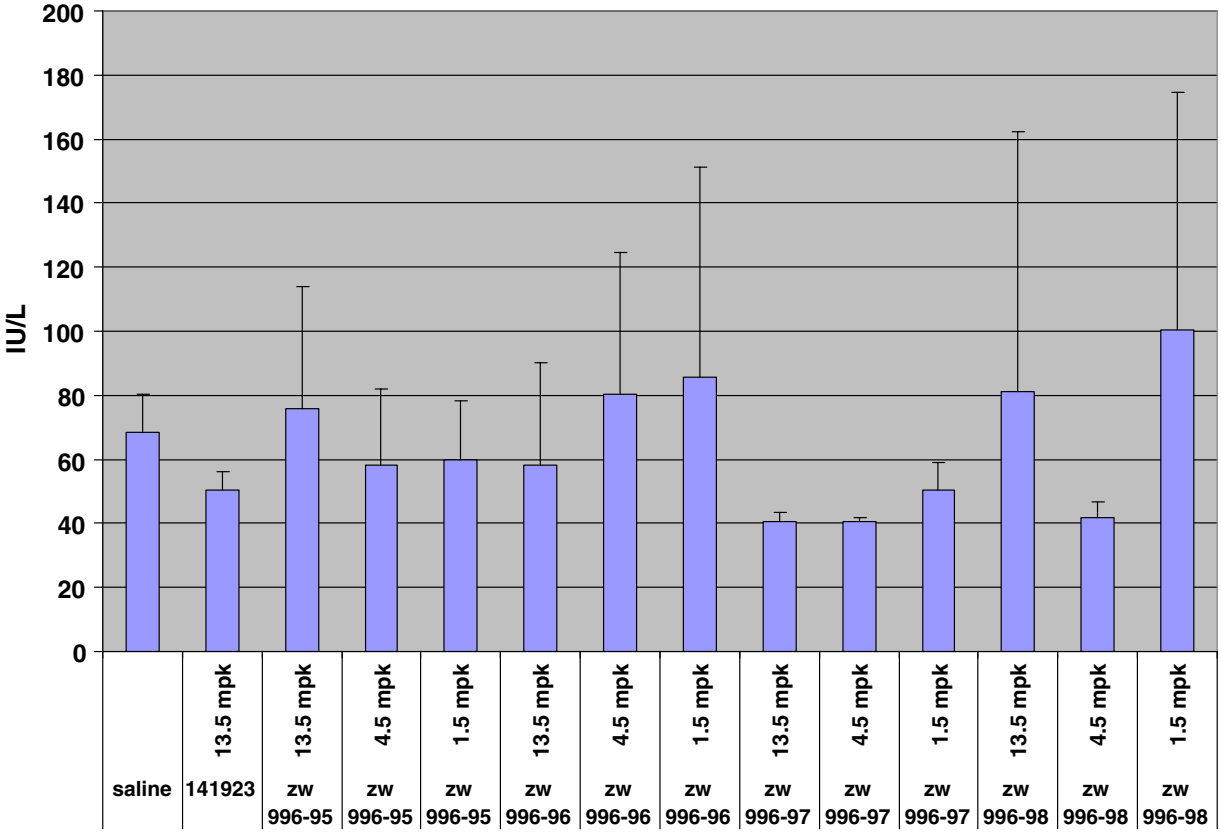


Chart 5. Comparison of aspartate transaminase (AST) between different oligonucleotides investigated.

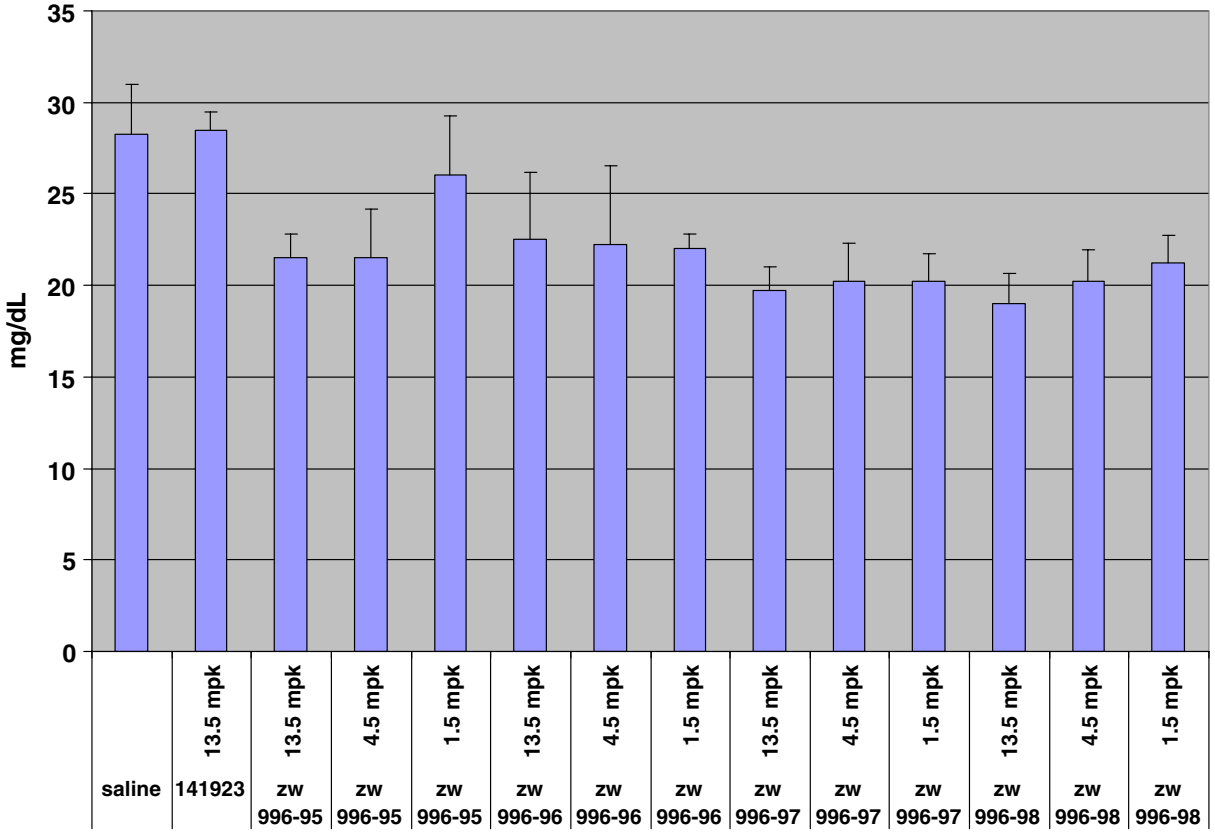


Chart 6. Comparison of blood urea nitrogen (BUN) between different oligonucleotides investigated.

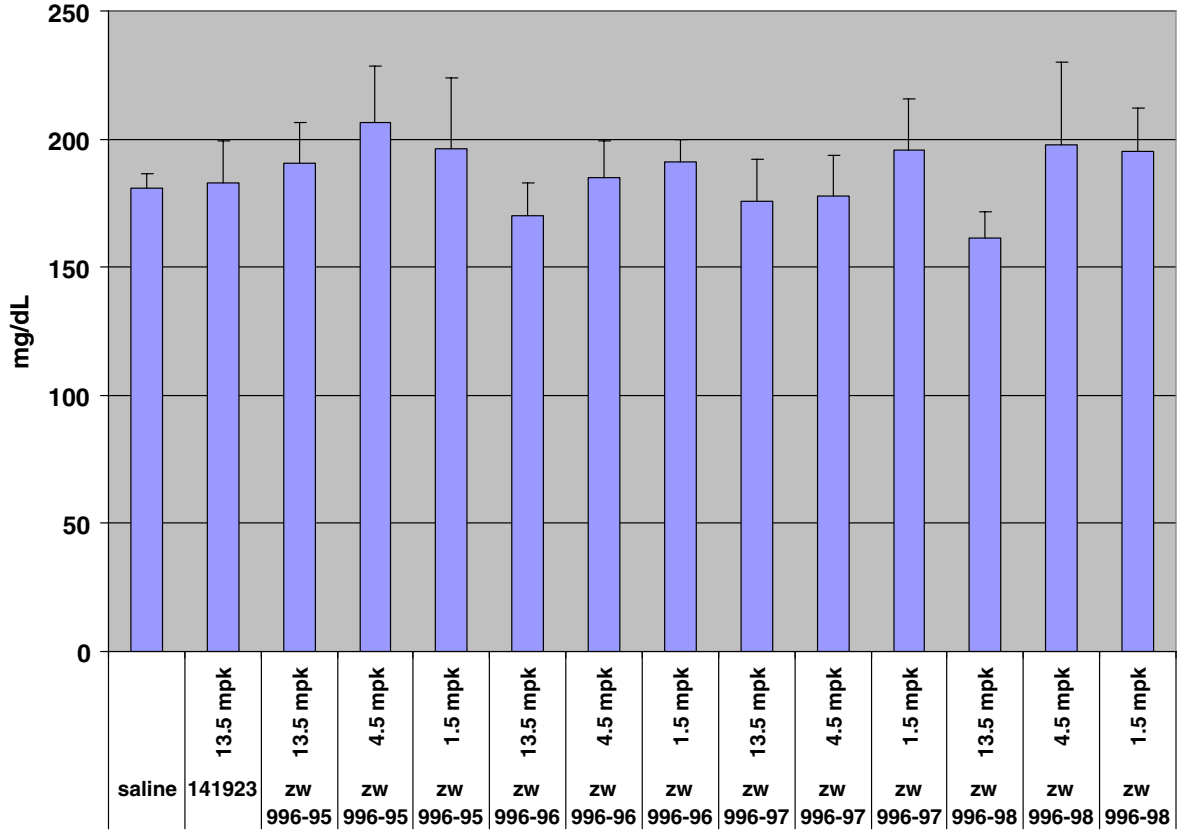


Chart 7. Comparison of glucose levels between different oligonucleotides investigated.

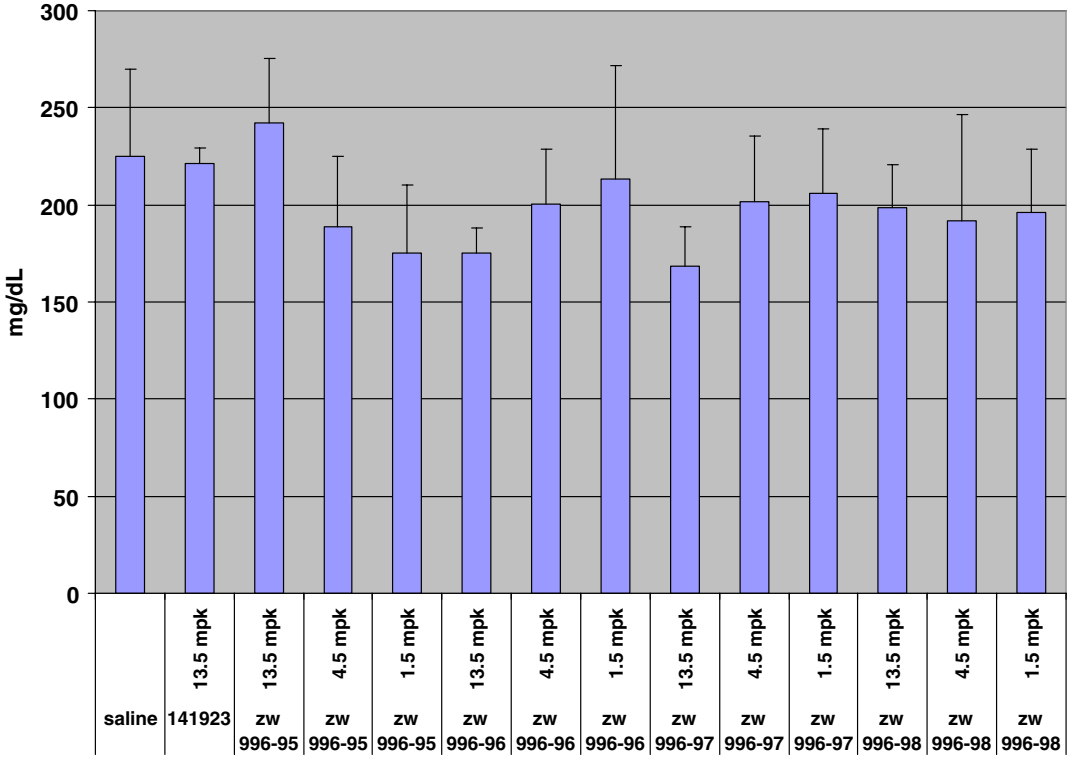


Chart 8. Comparison of triglyceride levels between different oligonucleotides investigated.

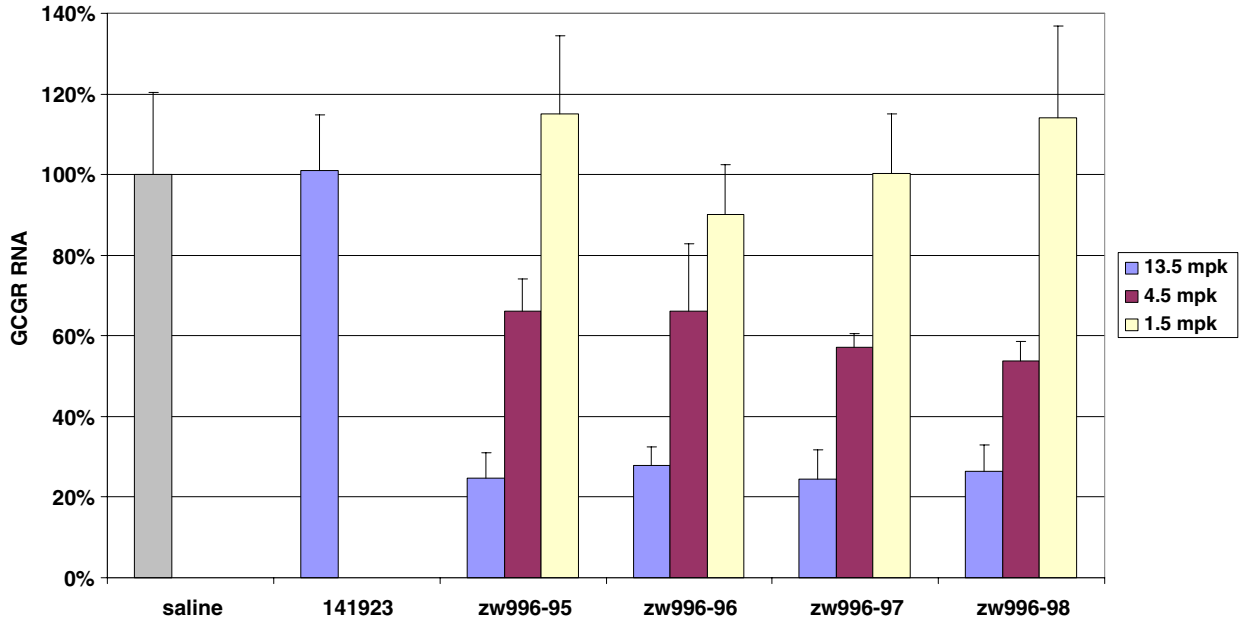


Chart 9. Comparison of liver GCGR RNA between different oligonucleotides investigated.

activity of labeled oligonucleotide was approximately 6000 cpm/fmol.

3.3. Determination of initial rates

Hybridization reactions were prepared in 120 μl of reaction buffer [20 mM Tris–HCl, pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, and 0.1 mM β-mercaptoethanol] containing 100 nM antisense phosphorothioate oligonucleotide (ISIS 180475), 50 nM sense oligoribonucleotide, and 100,000 cpm of ^{32}P labeled sense oligoribonucleotide. Reactions were heated at 90 °C for 5 min and cooled to 37 °C prior to adding $MgCl_2$. Hybridization reactions were incubated overnight at 37 °C. Hybrids were digested with 0.5 ng human RNase H1 at 37 °C.[11] Digestion reactions were analyzed at specific time points in 3 M urea and

Table 3. Histopathology studies in mice

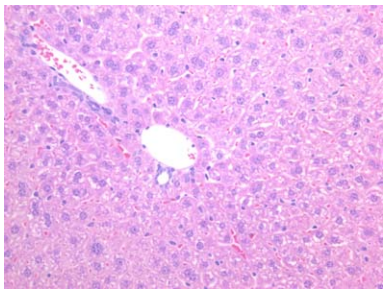
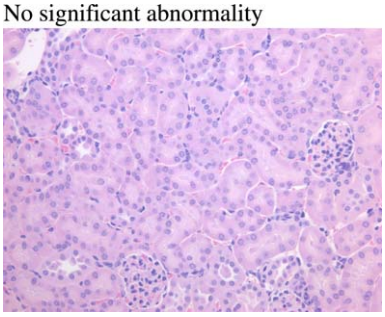
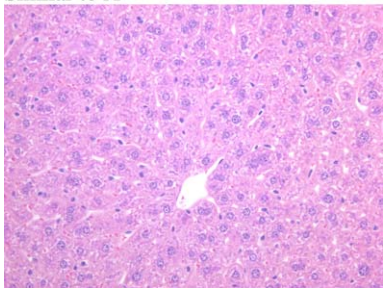
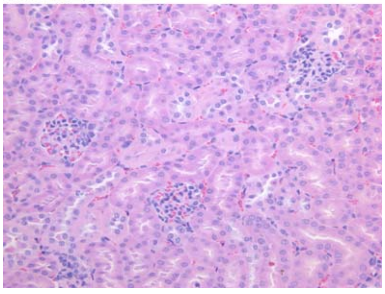
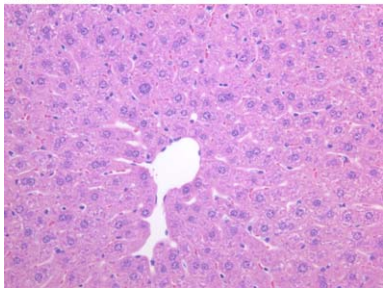
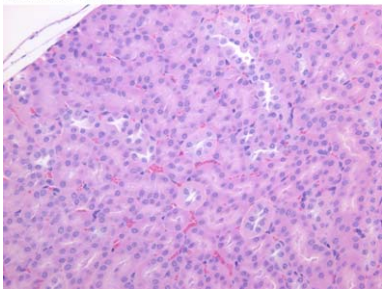
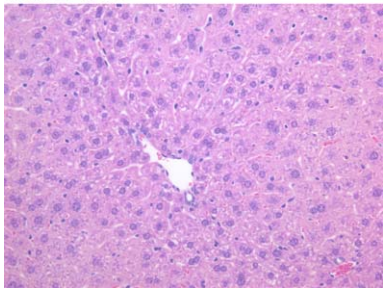
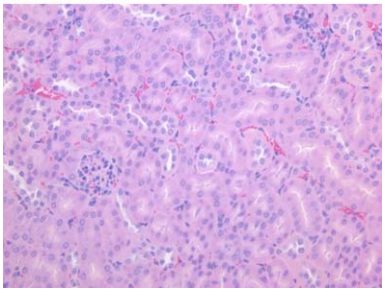
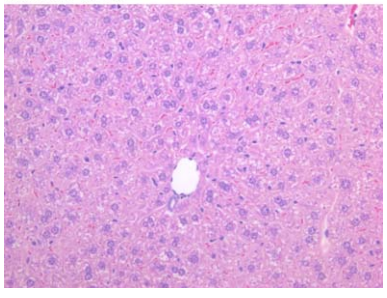
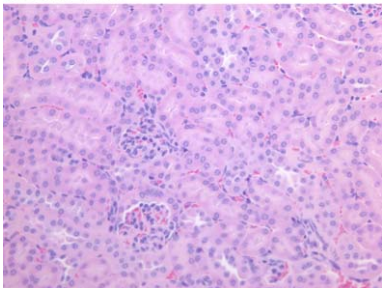
Group	Treatment	Target	Time pts	Positive histopath findings mouse liver	Positive histopath findings mouse kidney
A	Saline				No significant abnormality 
B	141923 13.5mpk			Similar to A 	Similar to A 
C	Zw996-95 135mpk	mGCGR		Similar to A 	Similar to A 
D	Zw996-95 4.5mpk			Similar to A 	Similar to A 
E	Zw996-95 1.5mpk			Similar to A 	Similar to A 

Table 3 (*continued*)

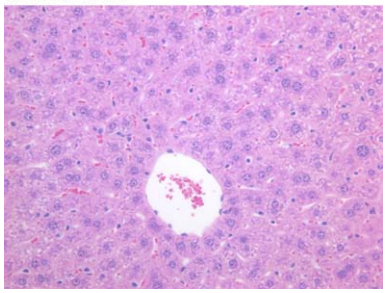
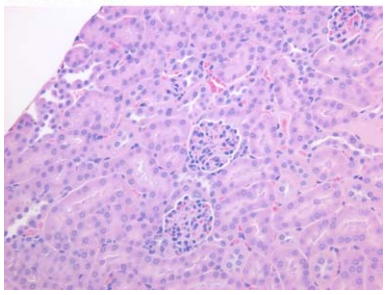
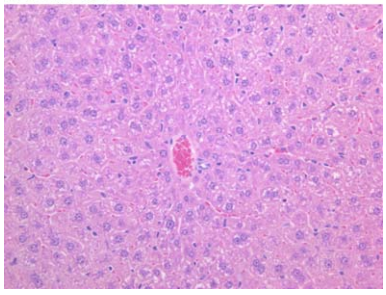
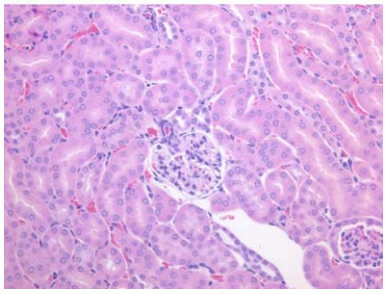
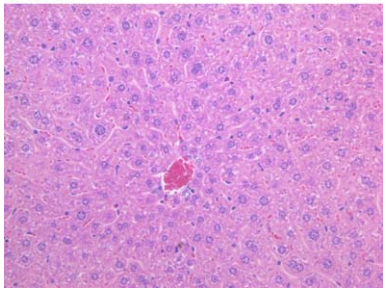
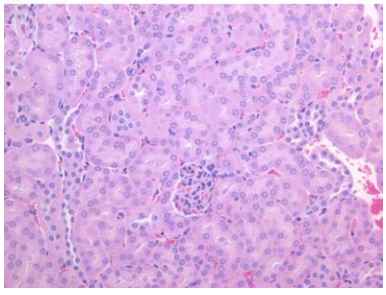
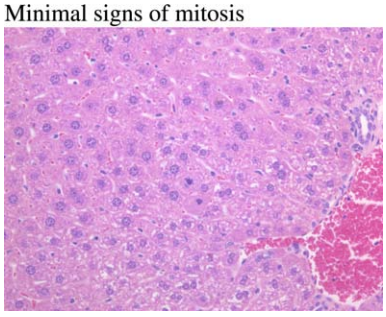
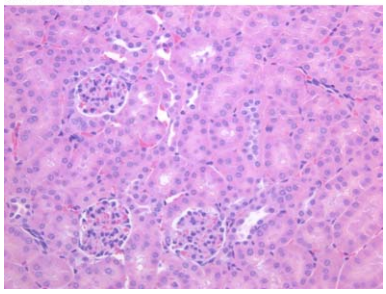
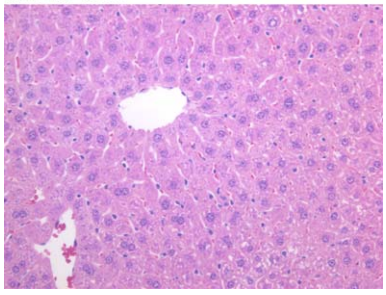
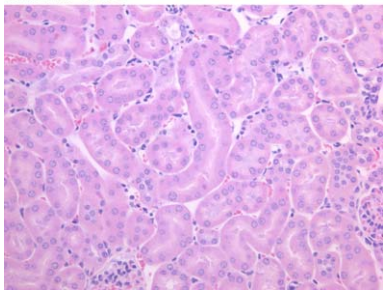
Group	Treatment	Target	Time pts	Positive histopath findings mouse liver	Positive histopath findings mouse kidney
F	Zw996-96 135mpk	mGCGR		Similar to A 	Similar to A 
G	Zw996-96 4.5mpk			Similar to A 	Similar to A 
H	Zw996-96 1.5mpk			Similar to A 	Similar to A 
I	Zw996-97 135mpk	mGCGR		Minimal signs of mitosis 	Similar to A 
J	Zw996-97 4.5mpk			Similar to A 	Similar to A 

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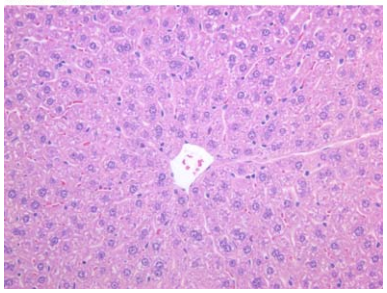
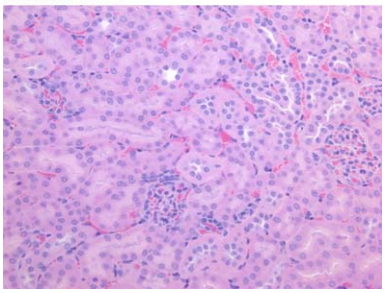
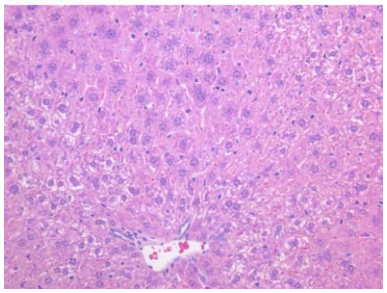
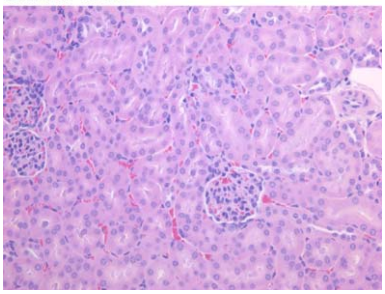
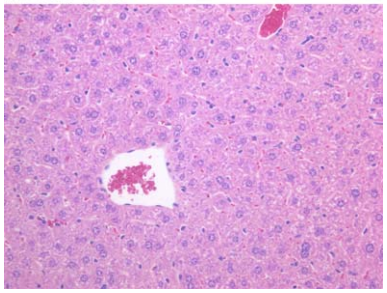
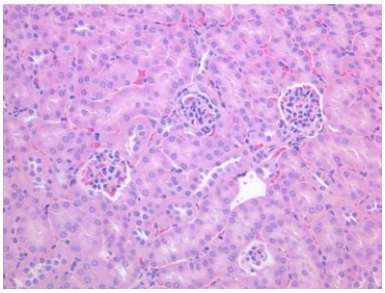
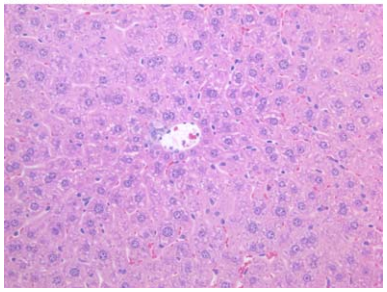
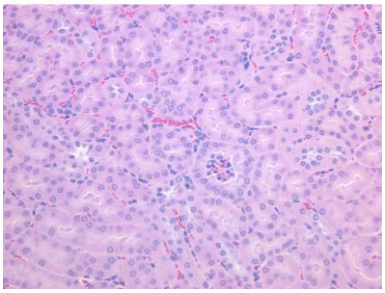
Table 3 (*continued*)

Group	Treatment	Target	Time pts	Positive histopath findings mouse liver	Positive histopath findings mouse kidney
K	Zw996-97 1.5mpk			Similar to A 	Similar to A 
L	Zw996-98 135mpk	mGCGR		Similar to A 	Similar to A 
M	Zw996-98 4.5mpk			Similar to A 	Similar to A 
N	Zw996-98 1.5mpk			Similar to A 	Similar to A 

20 nM EDTA. Samples were analyzed by trichloroacetic acid assay.[13] The concentration of substrate converted to product was plotted as a function of time. The initial cleavage rate (V_o) was obtained from the slope (pM converted substrate per minute) of best-fit line derived from $\geqslant 5$ data points within the linear portion (<10% of the total reaction) of the plot.[14] The errors reported were based on three trials and are shown in Table 4.

The oligonucleotide being studied here has proven to be highly sensitive to biochemical and biological changes when chemically modified. This oligonucleotide when synthesized with different activators either performed similar or slightly better when DCI was used indicating that both the oligonucleotides are functionally equivalent.[15]

4. Conclusions

Extensive chemical, biochemical, and biological investigations have demonstrated that 2′-O-methoxyethyl modified phosphorothioate oligonucleotides synthesized using DCI and 1*H*-tetrazole as activators are equivalent.

Table 4. Comparison of oligonucleotides against initial cleavage rates

Activator used during synthesis	V_o (nM/min)	Standard deviation	Ratio to 1*H*-tetrazole
1*H*-Tetrazole	2.069	0.155	1.000
4,5-Dicyanoimidazole	2.247	0.096	1.086

Multiple oligonucleotides of good quality and yield have been synthesized at scales ranging from 100 μmol to 600 mmol to demonstrate the performance of this activator.

5. Materials and methods

DCI and 1*H*-tetrazole were purchased from American International Co. *N*-Methylimidazole (NMI) was purchased from Aldrich and used as such. Standard 5′-*O*-4,4′-dimethoxytrityl-3′-*O*-cyanoethyl-*N*,*N*′-diisopropylphosphoramidite reagents of protected deoxyribonucleosides and 2′-*O*-methoxyethylribonucleosides (benzoyl-protected dA, MOE A, dC and MOE meC, isobutyryl-protected dG, and MOE G, T, and 5meU) were purchased from Pierce, Milwaukee, WI. Phenylacetyl disulfide (PADS) was purchased from Acharya Chemicals, Dombivli, India, and dichloroacetic acid from Clariant Life Sciences, Germany. Derivatized polystyrene supports loaded with corresponding nucleoside or an universal linker molecule were obtained from GE Amersham Biosciences, Uppsala, Sweden.

5.1. Oligonucleotide synthesis

Syntheses were performed on a GE Amersham Biosciences Akta 100 DNA/RNA synthesizer closely resembling the production-scale synthesizer (OligoProcess). Either a nucleoside succinate-loaded or UnyLinker, a universal linker molecule loaded on PS200 solid support (loading 200 μmol/g), was used for evaluation.[16] The support was packed in a stainless steel column (volume 24 ml). Details of the synthesis cycle are given in Table 5. Dichloroacetic acid (10%) in toluene was used for deblocking of dimethoxytrityl (DMT) groups.[17] Extended detritylation condition (twice the column volume and contact time as the normal cycle) was used to remove the DMT group from secondary hydroxyl group of the UnyLinker molecule. Unlike dichloromethane, which gives conductivity-based or UV-based DMT yields, no conductive-based detritylation yields were obtained when toluene was used as solvent for deblocking. Standard cyanoethyl-protected phosphoramidites were used for synthesis.

4,5-Dicyanoimidazole (0.7 M) in the presence of *N*-methylimidazole (0.1 M)[8a] or 1*H*-tetrazole (0.45 M) in acetonitrile was used as activator during coupling step. Low-water acetonitrile (water content < 10 ppm) was used for preparing phosphoramidite and activator solutions.[18] 1.75 equiv of amidites (both deoxy and 2′-*O*-methoxyethylribonucleosides) and a ratio of 1:1 (v/v) of amidite to activator solution were used during coupling step. Phenylacetyl disulfide (PADS) (0.2 M in 1:1 3-picoline/CH_3CN, v/v) was used as sulfur transfer reagent.[19] Capping reagents were made to the recommended GE Amersham Biosciences' recipe: cap A: 1:4 *N*-methylimidazole/CH_3CN (v/v), cap B: 2:3:5 acetic anhydride/pyridine/CH_3CN (v/v/v). At the end of synthesis, the support-bound DMT-on oligonucleotide was treated with a solution of triethylamine and acetonitrile (1:1, v/v) for 2 h to remove acrylonitrile formed by deprotection of cyanoethyl group from phosphorothioate triester.[20] Subsequently, the solid support containing oligonucleotide was incubated with concentrated aqueous ammonium hydroxide at 55 °C for 13 h to complete cleavage from support, elimination of UnyLinker molecule to liberate 3′-hydroxy group of oligonucleotide, and deprotection of base-protecting groups.

5.2. HPLC analysis and purification of oligonucleotides

Purification of oligonucleotides by reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters Novapak C_{18} column (3.9 × 300 mm) using a Waters HPLC system (600E System Controller, 996 Photodiode Array Detector, 717 Autosampler). For analysis an acetonitrile (A)/0.1 M triethylammonium acetate gradient was used: 5–35% A from 0 to 10 min, then 35–40% A from 10 to 20 min, then 40–95% A from 20 to 25 min, flow rate = 1.0 ml/min/50% A from 8 to 9 min, 9 to 26 min at 50% flow rate = 1.0 ml/min, t_R (DMT-off) 10–11 min, t_R (DMT-on) 14–16 min. The DMT-on fraction was collected and was evaporated in vacuum, redissolved in water and the DMT group was removed as described below.

5.3. Dedimethoxytritylation

An aliquot (30 μl) was transferred into an Eppendorff tube, and acetic acid (50%, 30 μl) was added. After 30 min at room temperature, sodium acetate (2.5 M, 20 μl) was added, followed by cold ethanol (1.2 ml). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centri-

Table 5. Synthesis parameters of cycle used on GE Amersham Biosciences Akta 100 DNA/RNA synthesizer at 1 mmol scale

Step	Reagent	Volume (ml)	Time (min)
Detritylation	10% dichloroacetic acid/toluene	72 (144)	3 (6)
Coupling	Phosphoramidite (0.2 M) and DCI (0.7 M) + NMI (0.1 M) in CH_3CN	8.8, 8.8	3
Sulfurization	PADS (0.2 M) in 1:1 pyridine/CH_3CN (v/v)	43	3
Capping	Ac_2O/pyridine/CH_3CN, NMI/CH_3CN	30, 30	2.5

Volume and time in parentheses indicate the condition used for detritylation of UnyLinker molecule.

fuge, the supernatant was discarded, and the precipitate was rinsed with ethanol and dried under vacuum.

5.4. Sample preparation for mass spectrum analysis

HPLC-purified and dedimethoxytritylated oligonucleotide was dissolved in 50 μl water and ammonium acetate solution (10 M, 5 μl) and ethanol were added and vortexed. The mixture was cooled in dry ice for 20 min and after centrifugation the precipitate was isolated. This procedure was repeated two more times to convert the oligonucleotide to the ammonium form. Oligonucleotide was redissolved in water/isopropanol (1:1, 300 μl) and piperidine (10 μl) was added.

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