Metabolism, pharmacokinetics, tissue distribution, and stability studies of the prodrug analog of an anti-HBV dinucleoside phosphorothioate

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Non-standard abbreviations:

SMNH: Small molecule nucleic acid hybrids
CYP: Cytochrome P$_{450}$
ODN: Oligonucleotides
PS-ODN: Phosphorothioate oligonucleotides
CPG: Controlled-pore-glass
si-RNA: Small Interfering RNA
SGF: Simulated gastric fluid; SIF: Simulated intestinal fluid
DMT-: 5' - O - 4,4-dimethoxytriphenylmethyl-
dA: 2'-Deoxyadenosine; T: Thymidine
CAP: Capping reagent
TpsT: 3',5'-linked thymidine -thymidine phosphorothioate
3'-dApsT: 3',5'-linked deoxyadenosine-thymidine phosphorothioate-5'
Abstract

The alkoxycarbonyloxy dinucleotide prodrug $R_p, S_p$-2 is an orally bioavailable anti-HBV agent. The compound is efficiently metabolized to the active dinucleoside phosphorothioate $R_p, S_p$-1 by human liver microsomes and S9 fraction without Cytochrome P450-mediated oxidation or conjugation. The conversion of $R_p, S_p$-2 to $R_p, S_p$-1 appears to be mediated by liver esterases and occurs in a stereospecific manner and is consistent with our earlier reported studies of serum-mediated hydrolytic conversion of $R_p, S_p$-2 to $R_p, S_p$-1. However, further metabolism of $R_p, S_p$-1 does not occur. The presence of a minor metabolite, the de-sulfurized product 10 was noted. The prodrug $R_p, S_p$-2 was quite stable in simulated gastric fluid (SGF), where as the active $R_p, S_p$-1 had a half-life < 15 min. In simulated intestinal fluid (SIF), the prodrug 2 was fully converted to 1 in about 3 h whereas 1 remained stable. In order to ascertain the tissue distribution of the prodrug 2 in rats, the synthesis of 35S-labeled $R_p, S_p$-2 was undertaken. Tissue distribution studies of orally-, and i.v.-administered of radiolabeled $^{35}$S-[2] demonstrated that the radioactivity concentrates in the liver, with the highest liver/plasma ratio in the i.v. group at 1 h being 3.89 (females), and in the p.o. group at 1 h being 2.86 (males). The preferential distribution of the dinucleotide 1 and its prodrug 2 into liver may be attributed to the presence of nucleoside phosphorothioate backbone since phosphorothioate oligonucleotides (PS-ODNs) also reveal similar tissue distribution profile upon i.v. administration.
Introduction

Nucleotides are among the most important ligands for a number of intracellular and extracellular proteins involved in biochemical reactions and cell signaling (Saito et al., 2006). Additionally, nucleic acid–protein interactions constitute essential events in a variety of cellular processes (Ollis and White, 1987; Sanger, 1983). Therefore, rationally designed agonists and antagonists of specific nucleotide-, and nucleic acid–protein interactions should have broad therapeutic applications against multiple diseases (Ellington and Conrad, 1995).

“Small molecule nucleic acid hybrids” (SMNH), which are 2 to 6 nucleotides long represent a new class of small molecule chemical entities that can be rationally designed to target key nucleotide-, and nucleic acid–protein interactions involved in a disease process. Indeed, recent reports suggest that mono-, di-, tri-, and short-chain oligonucleotides possess significant and diverse biological activities that can be exploited for therapeutic applications (Iyer et al., 2004; Hannoush et al., 2004; Wagner et al., 1996).

In previous studies, we have shown that in general, SMNH class of molecules has unique metabolic and pharmacokinetic properties that are distinct from traditional small molecule drugs (Iyer et al., 2004a; Iyer et al., 2006). Thus, in contrast to the hydrophobic small molecule compounds, which are subject to Cytochrome P_{450} (CYP)-mediated Phase I oxidative processes and Phase II conjugation reactions, SMNH analogs, are not metabolized by CYP enzyme systems. Furthermore, in in vivo studies, we have reported that a representative dinucleoside phosphorothioate 1 does not seem to be metabolized by Phase I or Phase II processes and is mostly eliminated as intact compound (Iyer et al., 2006). Lack of CYP metabolism of SMNH molecules is a unique pharmaceutical attribute that confers distinct advantages in that such molecules are not subject to “first pass effect”, and they can be combined with different classes of drugs without the potential for drug-drug interactions (Gibaldi et al., 1971). Indeed, in the context of antiviral therapeutics, a cocktail of drugs each with unique mechanisms of action that
can act synergistically with minimal toxicity is particularly important for the treatment of chronic infections (Perillo, 2004). Consequently, the development of SMNH analogs as antiviral agents is of great interest.

SMNH compounds are small molecule counterparts of the more widely studied class of long-chain phosphorothioate oligonucleotides (PS-ODN). Extensive ADME studies of PS-ODN reveal a pattern in which following i.v. administration, they are: (a) rapidly cleared from plasma after absorption, (b) preferentially distributed to liver, kidney, spleen, and bone marrow, (c) metabolized by exo-, and endo-nucleases into shorter oligonucleotide fragments, and (d) excreted primarily through kidneys (Geary, 2009; Peng et al., 2001). Studies of duplexed synthetic oligonucleotides as small interfering RNA (siRNA) (Hannon, 2002; Tuschl, 2001) show a similar ADME trend although the overall pharmacokinetic and tissue distribution profile is dependent on the extent of chemical modification and the conjugating moieties (Li and Liang, 2010; Van De Water et al., 2006). It was therefore of interest to evaluate the ADME properties of SMNH compounds.

Chemically modified antisense oligonucleotides (Szymkowski, 1996; Dias and Stein, 2002) as “hybrid”, or “gapmers” have been reported to be orally bioavailable in vivo (Agrawal et al., 1995; Tillman et al., 2008), although the extent of pharmaceutical bioavailability and mechanisms of absorption are not known. In contrast, in vitro studies using Caco-2 cell lines and in vivo studies in rats revealed that none of the SMNH analogs have potential for oral bioavailability, presumably due to the negative charge(s) on their backbone that impedes their passive diffusion across the cellular lipid bilayer (Iyer et al., 2006; Iyer et al., 2004 a; Coughlin et al., 2010).

As part of further development of the SMNH compounds, we have undertaken preclinical studies to develop the anti-HBV SMNH analog 1 as an orally bioavailable compound. The analog 1 was not stable in SGF, due to its susceptibility to acid-catalyzed depurination, but was stable in
SIF. An ideal SMNH analog is required to be stable in the GI tract and therefore the development of a prodrug strategy for oral delivery appeared logical.

We have reported the synthesis and evaluation of various orally bioavailable prodrug derivatives of 1 (Padmanabhan et al., 2006). These analogs are designed to mask the negative charge on the backbone of the dinucleotide, and to improve their hydrophobicity to facilitate passive diffusion through cell membrane. As a part of the continuing development of a prodrug analog 2, we evaluated its Absorption, Distribution, Metabolism, and Elimination (ADME) properties, its stability in SGF and SIF, and oral bioavailability. The results are presented here.

Materials and Methods

Materials for Chemical synthesis: DMT-\(N^{Bz}\)-dA and DMT-T, DMT-U\(\_2\)-OMe, were obtained from Reliable Biopharmaceuticals, (St. Louis, MO) and used as such. T-phosphoramidite monomer was obtained from Rasayan Chemicals, CA. Anhydrous pyridine, triethylamine, and dimethylformamide, obtained from Sigma-Aldrich, Milwaukee, WI, were freshly distilled from CaH\(_2\) prior to use. Other reagents such as 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC), \(N,N\)-dimethylaminopyridine, ethylthiotetrazole, dichloroacetic acid and triethylamine were obtained from Sigma-Aldrich, Milwaukee, WI and used as received. Controlled pore glass support (CPG) was obtained from Prime Synthesis, Inc (Aston, PA). Cap A and Cap B were obtained from American International Chemicals (Natick, MA).

Synthesis of the dinucleotide 1. The dinucleotide 1 was synthesized on a multimillimol scale using dA-loaded-CPG support in conjunction with solid-phase phosphoramidite chemistry (Beaucage and Iyer, 1992) (Fig. 1). For the synthesis, we employed a specially fabricated LOTUS reactor\(^\circledast\) as previously described (Iyer et al., 2005a). Nucleoside-loaded support (112 g, 89 mmol) prepared as described (Padmanabhan et al., 2005;) was detritylated using dichloroacetic acid (DCA) in dichloromethane (DCM) (2.5%, DCA/DCM, 3 X 400 ml) with DCM
washes (3 x 400 ml) between each DCA/DCM treatment and the support was subsequently 

washed thoroughly with DCM (5 X 400 ml) and later with acetonitrile (low water, < 30 ppm). 

Detritylated nucleoside was coupled with 2'-O-methyluridine phosphoramidite (5 eq) in the 

presence of 5-ethylthiotetrazole (ETT, 0.4 M, 10 eq) in anhydrous acetonitrile under argon. After 

acetonitrile washings, sulfurization was carried out using 3H-benzodithiole-3-one-1,1-dioxide 

reagent (5 eq) (Iyer et al., 1990a; Iyer et al., 1990b ) to give the fully protected CPG-bound 

dinucleotide. The 5'-trityl group was removed using 2.5% DCA-DCM (3 X 400 ml) and the CPG 

washed with DCM (5 X 400 ml), acetonitrile (3 X 400 ml) and finally with water (3 X 500 ml). The 

dinucleotide 1 was released from the support by deprotection and cleavage using NH\textsubscript{4}OH (200 

ml, 28-30%) at ~30 °C under orbital shaking overnight. The ammoniacal solution was acidified 

with glacial acetic acid to pH 6 under cooling followed by filtration to give crude 1, which was 

purified by three-stage preparative HPLC as below.

Desalting of the dinucleotide 1 solution was carried out in a 500 ml stainless steel 

column. Initially, the column was equilibrated with NH\textsubscript{4}OAC (0.1 M pH 7.0, at a flow rate of 20 

ml/min) followed by loading of dinucleotide 1 solution. The dinucleotide 1 was eluted from the 

column using a gradient of acetonitrile and water. The desalted dinucleotide 1 solution was 

concentrated to 20% of volume in vacuo, diluted with water and subjected to ion-exchange 

chromatography using Toyopearl Super Q 650M resin. Following equilibration of the column with 

NaOAc (0.1 M), 1 was loaded on to the column, which was washed with three column volumes 

of NaOAc (0.1 M, pH 7.0). The dinucleotide 1 was eluted with NaOAc (0.1 M) and NaCl (1 M) 

buffer and further purified by reversed-phase HPLC on a C-18 column. The sodium salt of 1 was 

eluted with 20:80, acetonitrile:water. The eluent was lyophilized to obtain the dinucleotide 1 as a 

white fluffy solid.

**Synthesis of 2.** The prodrug 2 was prepared by the S-alkylation of the dinucleotide 1 

with iodomethyl isopropyl carbonate (3). The iodo-compound 3 was prepared as described
before (Padmanabhan et al., 2006). The product 3 was characterized by \(^1\)H-NMR, \(^{13}\)C-NMR and MS. \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) ppm 1.2 (6H, s), 4.8 (1H, m), 5.8 (2H, s); \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\) ppm, 154, 76, 36, and 22; MS, ESI(+), 244.

The S-alkylation protocol was essentially as described (Coughlin et al., 2010). Following HPLC purification, 2 was obtained as a lyophilized powder as an \(R_p, Sp\) diastereomeric mixture (~ 55:45, 52%).

**Spectral data of \(R_p,Sp\)-2:** \(^1\)H-NMR (CD\(_3\)OD), \(\delta\) 1.16 (s, 3H), 1.24 (s, 3H), 2.49 (m, 1H), 2.89 (m, 1H), 3.45 (s, 3H), 3.75 (s, 2H), 4.21 (m, 1H), 4.3 (d, 1H), 4.45 (m, 2H), 4.77 (m, 1H), 4.85 (m, 1H), 5.15 (m, 1H), 5.42 (d, 1h), 5.44 (d, 1H), 5.71 (d, 1H), 6.01 (d, 1H), 6.46 (t, 1H), 7.98 (d, 1H), 8.21 (s, 1H), 8.29 (d, 1H) ppm; \(^{13}\)C-NMR (CD\(_3\)OD), \(\delta\) 22.62, 40.68, 59.34, 59.42, 61.59, 61.84, 67.81, 67.92, 69.03, 72.35, 74.79, 76.78, 83.09, 83.14, 83.44, 85.22, 85.99, 86.11, 86.61, 103.39, 103.47, 120.52, 120.72, 141.09, 141.22, 142.28, 150.61, 152.39, 152.47, 154.00, 154.68, 154.93, 157.39, 165.06 ppm; \(^{31}\)P–NMR (CD\(_3\)OD), \(\delta\) 27.7, 28.6 ppm; MS, m/z, 703.6.

**Synthesis of radiolabeled compounds**

**Synthesis of \(^{35}\)S-[2].** The dinucleoside phosphorothioate ester \(^{35}\)S-[2] (34.2 mg, total activity 4.44 mCi, 91.81 uCi/umol) was prepared by radiosyntheses (Fig. 1, A-B). The \(^{35}\)S-label was installed on the phosphorothioate backbone using \(^{35}\)S-3H-benzodithiole-3-one-1,1-dioxide (Iyer et al., 1994). \(^{35}\)S-[2] had a radiochemical purity of > 98% as assessed by radio-HPLC (Supplemental Fig. 1). Detailed methods of radiosynthesis and analysis are reported elsewhere (Rhee et al., 2012). All materials were stored at –20 °C until ready to use.

**In vitro metabolism and stability studies of 2**

Metabolism studies using liver microsomes and S9 fractions were carried out as described (Dalvie et al., 2009). **Materials.** Pooled human liver microsomes, S9 from liver, \(\beta\)-nicotinamide adenine dinucleotide phosphate (NADPH), alamethicin, uridine 5’-diphosphogluconic acid (UDPGA), adenosine 3’-phosphate 5’-phosphosulfate lithium salt
hydrate, S-(5′-adenosyl)-L-methionine iodide, acetyl coenzyme A sodium salt, *pepsin* and *pancreatin* were purchased from Sigma-Aldrich.

The metabolite samples were analyzed by reversed-phase HPLC using Waters instrument equipped with 600E Controller, PDA 996 detector, Waters 717 Auto sampler with Millennium software. A Waters Nova-Pak C18 4 um (3.9 x 300 mm) column operating on a gradient of 100% A (0.1 M ammonium acetate) to 100% B (0.1 M ammonium acetate: acetonitrile (20 : 80) with a flow rate of 0.5 ml/min over 41 min was employed.

LC/MS analysis of the samples was performed on an Agilent 1100 Series HPLC with UV detection and MSD system. For the LC analysis of the samples, a Phenomenex Luna C18 column (5 μM, 4.6 X 250 mm) operating at 25 °C was employed. Other chromatographic conditions were: Injection volume 50 μl; elution gradient, 5 to 50% acetonitrile in 0.1 M NH₄OAc over 45 min; flow rate, 0.5 ml/min. In the mass spectrum, the molecular mass signals represented [M + H] and/or [M + Na] ions. The MS instrument was an electro-spray ionization, ion-trap mass spectrometer. The mass spectra were obtained in full ion-scan mode with a range between 100 to 1200 m/z. Other parameters were: resolution 13,000 m/z/sec, dry temp. 350 °C, capillary ramp range 1500 to 4500 V, nebulizer pressure 60 psi, dry gas flow 10 L/min, trap target 30,000, and max. accum. time 100 ms. Instrument calibration was verified using seven compounds of known MW between 118 and 2722 Da. The predicted chemical formulae and calculated accurate mass were obtained from Chem Draw Std 8.0 (CambridgeSoft Corporation, Cambridge, MA) based on the proposed metabolic pathways and putative structures.

**In vitro metabolism of 2 using liver microsomes.** A stock solution of prodrug 2 was prepared in de-ionized water at 1 mM concentration and diluted further to make a 10 μM solution. The incubation mixture contained liver microsomes (1 mg/ml), NADPH (1.3 mM), UDPGA (5 mM), Alamethicin (10 μg/ml) in 1 ml of 1 X PBS buffer, at 10 μM final concentration
of 2. The liver microsomes were treated with alamethicin in an ice-bath for 15 min prior to use. NADPH and UDPGA were added and the incubate maintained at 37 °C in a shaking water bath. Samples were taken out at 1, 4, 6, and 8 h and quenched with acetonitrile (2 ml). The resulting precipitate was removed by centrifugation for 10 min, supernatant lyophilized and reconstituted in 200 μl water for HPLC analysis. The metabolite samples were also independently evaluated by LC/MS analysis.

**Metabolism of 2 in S9 fractions from human liver.** The prodrug 2 (10 μM) was incubated with a mix containing S9 fraction (pooled from human) (1 mg/ml), NADPH (1.3 mM) UDPGA (5 mM), alamethicin (10 μg/ml) in 1 ml of 1 X PBS buffer. S-(5′-adenosyl)-L-methionine iodide (0.1 mM), adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (0.1 mM) and acetyl CoA (1 mM).

The S9 fraction was treated with alamethicin on ice for 15 min prior to use. Incubations were carried out at 37 °C in a shaking water bath. Samples were removed at 1, 4, and 6 h and quenched with 2 ml of acetonitrile. After 60 min at room temperature, the resulting precipitate was removed by centrifugation. The supernatant was lyophilized, reconstituted in 200 μl water for HPLC analysis. The lyophilized samples were also independently evaluated by LC/MS analysis.

**Stability studies of 2 in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).** SGF and SIF were prepared as per USP, 1993. Briefly, SGF was prepared by dissolving NaCl (2.0 g), purified pepsin (3.2 g, from porcine stomach mucosa), HCl (36%, 7 ml) and diluting with water to 1 lit. This test solution had a pH 1.2. For the preparation of SIF, monobasic potassium phosphate (6.8 g) was dissolved in of water (250 ml), NaOH (0.2 N, 77 ml) and H₂O (500 ml) were added. Pancreatrin (10 g) was added, mixed and solution adjusted to pH 6.8 with 0.2 N HCl and finally diluted with water to 1 lit.
A stock solution of 2 was prepared (2 mg in 100 μl DMSO). To 10 μl of stock solution, 200 μl of either SGF or SIF was added and incubated at 37 °C. At different time points (15 min, 30 min, 1 h, and 3 h) aliquots were drawn and quenched with acetonitrile. The pH of SGF sample was adjusted to 7.0 using ammonium acetate buffer. The precipitate was removed by centrifugation. The supernatant was lyophilized and the residue reconstituted in 200 μl water for HPLC analysis as before.

**Tissue-distribution and metabolism of ^35^S-[2]**

Tissue distribution and metabolism studies were carried out using Sprague-Dawley rats obtained from Harlan (Livermore, CA) and Charles River Laboratories (Hollister, CA). A total of 18 animals (9 males and 9 females), 8 to 13 weeks old, weighing 245 to 303 g were used. Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). General procedures for animal care and housing were in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Standards Incorporated in 9 CFR Part 3, 1991. Animals destined for the 24 h sacrifice were housed in glass metabolism cages to allow for urine and feces collection. Light cycle was 12 h light/12 h dark; temperature range was 65° to 72°F, and the number of ventilations was 10 room volumes per hour, with no re-circulation of air. Purina Certified Rodent Chow #5002 ad libitum was used. Water (Purified, Reverse Osmosis) was provided ad libitum.

The prodrug ^35^S-[2] was administered by bolus intravenous (i.v.) via tail vein, or oral gavage (p.o.) at a single dose. The dosing volume employed was 5 ml/kg and the experimental duration was 24 h. Evaluation parameters were: radioactivity levels in blood, tissues, carcass, and excreta. For i.v. administration, the vehicle employed was a mixture of 25% polyethylene glycol 400 (PEG-400): 75% sterile saline for Injection (0.9% sodium chloride for Injection, USP) and that for p.o. administration was 0.05 M citric acid buffer, pH 2.5.
**Formulations:** Intravenous and oral dose formulation(s) were prepared by adding the appropriate amount of 2 in the vehicle to achieve the target concentration and specific activity. \(^{35}\text{S}-[2]\) was added to the unlabeled drug formulation (under reduced light conditions) to give the correct final drug and radioactivity concentration. Dose formulations were prepared fresh at room temperature on the day of administration and used within 2 h.

**Experimental Procedures (In-Life Evaluations):** Mortality/Morbidity evaluations were carried out at least once daily and animals were examined for clinical signs related to the pharmacology and toxicology of the test article, gross motor and behavioral activity, and observable changes in appearance. Urine and feces were collected ~ 1, ~4, and ~24 h post-dose. Blood (~200 μl to ~300 μl) was collected from the retro-orbital sinus under 60% CO\(_2/40%\) O\(_2\) anesthesia, just prior to necropsy, into tubes containing EDTA in wet ice. To generate plasma, samples were centrifuged within 15 min of collection at 1750 X g for 6-8 min. Plasma and whole blood were transferred to cryovials and stored at –20 ºC for storage until analysis for radioactivity.

All animals were euthanized at their scheduled sacrifice with an overdose of sodium pentobarbital administered i.p. at 1, 4, or 24 h. Liver, lung, kidney, heart, brain, spleen, and tail were collected and stored frozen at approximately -20°C. The tissues were analyzed for radioactivity by liquid scintillation counting after homogenization and treatment with a tissue solubilizer. Total radioactivity (dpm) was determined and calculated as a concentration (μg-equiv/ml or g) and as a % of the administered dose.

The total radioactivity in plasma, presented as μg-equiv/ml, was also evaluated using WinNonlin Professional (v 5.2), non-compartmental approaches with sparse sampling feature to determine the elimination half-life (t\(_{1/2}\)), time to maximum plasma concentration (T\(_{\text{max}}\)), observed maximum plasma concentration (C\(_{\text{max}}\)) and area under the plasma concentration time profile (AUC). C\(_{\text{max}}\) and AUC were presented as the mean ± standard error of the data.
Results

Synthesis of compounds. The prodrug \( R_p,S_p \)-2 was prepared by chemoselective \( S \)-alkylation of the dinucleotide \( 1 \) (Fig. 1). For our studies, we employed the \( \sim 55:45 \), \( R_p:S_p \) mixture of \( 1 \), which was synthesized in large-scale using nucleoside-loaded CPG support in conjunction with solid-phase phosphoramidite chemistry, using a specially fabricated LOTUS reactor\( ^{®} \). Briefly, the \( \mathrm{dA} \)-linked CPG support was prepared using our recently developed ultrafast functionalization and loading process for solid supports. Coupling of the \( 2' \)-OMe uridine phosphoramidite to the \( \mathrm{dA} \)-loaded support was achieved using a solution of 5-ethylthiotetrazole as a coupling reagent. The sulfurization of the internucleotidic dinucleoside phosphite coupled product \( 5 \) was carried out using \( 3H-1,2\)-benzodithiole-3-one-1,1,1-dioxide. Following processing, chromatographic purification, and lyophilization, the sodium salt of \( R_p,S_p \)-1 (\( \sim 60:40 \) mixture) was obtained, which was characterized by \( ^{31}\text{P} \)-, and \( ^{1}\text{H} \)-NMR and was >96% pure.

The chemoselective \( S \)-alkylation of \( R_p,S_p \)-1 with iodomethyl isopropyl carbonate gave the prodrug \( R_p,S_p \)-2. In turn, the iodomethyl carbonate was prepared by halogen exchange reaction with the corresponding chloro-compound as described previously (Padmanabhan et al., 2006). Following HPLC purification, and lyophilization, \( 2 \) was obtained in overall isolated yields of 50 to 52% from \( 1 \). \( ^{31}\text{P} \)-NMR of \( 2 \) showed two peaks representing a \( \sim 55:45 \) ratio of the \( R_p:S_p \) isomers and the mass spectrum was consistent with the expected molecular ion of 703 for \( 2 \).

In vitro metabolic studies of \( 2 \) using liver microsomes. The metabolism protocols followed were essentially as described by others (Dalvie et al., 2009), with slight modifications. Thus, exposure of the prodrug \( R_p,S_p \)-2 to liver microsomes up to 8 h resulted in its stereospecific conversion to the dinucleotide \( R_p,S_p \)-1 (Fig. 2). Indeed, the LC/MS evaluation of the microsomal incubate revealed that the major product(s) of metabolism was the \( R_p,S_p \)-dinucleotide \( 1 \) (Figs. 3, 4). A minor amount of the desulfurized product \( 10 \) (< 5%) was also detected as determined by MS analysis (m/e, 571) (Fig. 5). A few minor metabolites (<10%)
were also seen (Fig. 2). However, the identity of these metabolites could not be firmly established based on molecular ion. There was no evidence for any quantifiable formation of the de-aminated analog 9 (the inosine analog corresponding to 1 that could be formed by the action of *adenosine deaminase*). The absence of 9 was additionally confirmed by independent synthesis and co-chromatography along with the metabolite incubate. None of the other predicted metabolites (Fig. 6) such as *O*-dealkylated ribonucleoside analog of 2 (i.e., 10) or that of 1 and the 8-oxo-deoxyadenosine analog of 1 (i.e., 11) and 2 (i.e., 12) were apparent based upon molecular ion analysis in LC/MS. Our curiosity in the formation of 11 and 12 was triggered by the reported susceptibility of the 8-position of purine nucleosides to oxidation (Ahmad and Mond, 1985). Also, there was no apparent formation of 5’-phosphorylated derivatives of 1 or other phase II conjugation products. In all cases, in the microsomal metabolism, both *R*<sub>p</sub>- and *S*<sub>p</sub>-isomers of prodrug 2 underwent stereospecific conversion to the active 1 with only minor amounts of desulfurized product (10) [corresponding to 1] being observed (Fig. 5). It was also gratifying to find that there appears to be no significant rate differences in the conversion of the individual *R*<sub>p</sub>- and *S*<sub>p</sub>-isomers of 2 to *R*<sub>p</sub>- and *S*<sub>p</sub>-isomers of 1 respectively. These results are consistent with serum-mediated bioreversibility studies previously reported by us (Coughlin et al., 2010).

Similar to the serum-mediated conversion of 2 to 1 in the case of microsomes also, liver esterases appear to be the major metabolizing enzymes involved in the hydrolytic conversion of 2 to 1. Mechanistically, it appears that the formation of 1 from 2 occurs by nucleophilic attack of the serine hydroxyl group of the esterase on the carbonyl carbon of 2 to give 11, followed by the intramolecular attack of the incipient oxyanion on the phosphoryl group to give the cyclic intermediate 12 (Fig. 7). The transient species 12 could reorganize to give the trigonal bipyramidal intermediates 13 and 14 that could interconvert by pseudorotation (Westheimer, 1968). Although in the intermediate 14, the S-acyloxyalkyl group is favorably poised to depart...
from an apical direction [that could cause the formation of desulfurized product 10], its formation is presumably disallowed because of the energetics involved in the reorganization of the initially formed enzyme-substrate complex 12. Consequently, the hydrolytic pathway is directed to occur via 13 to yield the desired 1 with minimal formation of 10. Interestingly, it appears that the 2'-OMe substituent in the dinucleotide structure facilitates hydrolysis of the ester group in each of the isomers with almost equal ease. We hypothesize that the 2'-OMe substitution in the furanose ring of $R_p$, $S_p$-2 favors a $C_{3\text{-endo}}$ vs a $C_{2\text{-endo}}$ conformation that might optimally orient the ester group in both isomers for enzyme-mediated nucleophilic attack with equal ease. In our earlier bioreversibility studies of $R_p$, $S_p$-2 using serum, we have found that the individual isomers are stereospecifically converted to $R_p$, $S_p$-1 at almost equal rates (Coughlin et al., 2010). However, the bioreversion of the corresponding S-alkyl derivatives of the dinucleotides TpsT, and dApsT (both of which lack the 2'-OMe substituent), occurs with significant rate differences between $R_p$ and $S_p$ isomers (Padmanabhan et al., 2006; Coughlin et al., 2010). These results suggest that subtle conformational effects are in play in the enzyme-mediated hydrolysis of 2. The facile bioconversion of 2 to 1 in liver and plasma is also consistent with broad substrate specificity of the ubiquitous esterase enzymes.

**In vitro metabolism of 2 by S9 fractions.** Similar to liver microsomal studies, exposure of the prodrug 2 to S9 fractions also resulted in stereospecific conversion of 2 to the dinucleotide 1 (Supplemental Fig. 2). Evaluation of the incubate by LC/MS revealed that besides the major product 1, minor amounts of the desulfurized product 10 (< 5%) were also formed. A few minor metabolites were also observed. The pattern of metabolites was similar to that observed with liver microsomes. There was no apparent evidence of any phase II conjugative reactions of 2 or that of the initially formed active metabolite 1.

**Stability studies of 2 in SGF and SIF.** An important requirement for oral bioavailability of the prodrug 2 is its stability in gastric fluid. Given the known susceptibility of the dinucleotide 1
to acid-catalyzed decomposition, it was not known whether 2 would have adequate stability
gastric fluid. Therefore, we evaluated the stability of 2 and the dinucleotide 1 in SGF and SIF by
HPLC analysis of aliquots at different time points. Whereas the dinucleotide 1 decomposed
rapidly in SGF (t_{1/2} < 15 min), we were gratified to find that the prodrug 2 displayed high stability
in SGF with t_{1/2} > 3 h (Fig. 8). In SIF, 2 was almost completely converted to 1 in ~ 3 hour where
as 1 was stable in SIF (Supplemental Fig. 3). Our stability data on prodrug 2 is consistent with
that reported for SATE pronucleotides (Shafiee et al., 2001). However, the mechanistic rationale
for the greater stability of 2 in SGF compared to 1 is not established as yet.

Tissue Distribution and excretion of ^{35}S-[2]. Each rat was administered ^{35}S-[2], 10
mg/kg, by either the i.v. or p.o. route. The i.v. dose of radioactivity was 15.5 ± 0.64 μCi (males)
and 14.2 ± 0.15 μCi (females). The p.o. dose of radioactivity was 50.1 ± 0.91 μCi (males) and
48.0 ± 2.02 μCi (females). The total recovery of radioactivity determined at 24 h after i.v.
administration of ^{35}S-[2] was 83.7 ± 0.32% and 84.9 ± 0.36%, for male and female rats and
after oral administration was 83.0 ± 3.59% and 80.4 ± 6.79 %, for male and female rats,
respectively.

Table 1 summarizes the excretion of radioactivity after i.v. and oral dosing of ^{35}S-[2].
Following i.v. administration of ^{35}S-[2], approximately 20% of the dose was excreted in the urine
in the first hour. In both males and females, the major route of excretion after i.v. dosing was in
the urine (55-60% of the radioactive dose) while about 10-20% of the dose was excreted in the
feces in 24 h. Oral administration of ^{35}S-[2] resulted in 35-40% of the dose eliminated in the
urine in 24 h. In male rats, a similar fraction of the dose was excreted in the feces, while female
rats excreted a much smaller fraction in the feces, ~3% in 24 h.

A larger fraction of the total dose of radioactivity was found in the carcass of females in
the oral group (and the intestines were observed to contain more fecal material), suggesting that
excretion by this route is probably similar in male and female rats, but that females had not yet
eliminated feces during the study period. Comparing the same time points, the highest fraction of the dose was present in liver > blood ≈ plasma > kidney > lung, after both i.v. and p.o. administration. Brain, spleen, and heart had 0.5% or less of the dose at all time points and for both i.v. and p.o. routes of administration. **Supplemental Figures 4 and 5** are graphical representations of the time course of the presence of radioactivity in the principal tissues in male and female rats respectively. After i.v. administration, the concentration of radioactivity in liver and kidney was higher than that of the plasma levels at the three time points 1, 4, and 24 h, whereas lung had a similar concentration of radioactivity compared to that of plasma. After oral administration of $^{35}$S-[2], the highest concentration of radioactivity was observed at 4 h post dose. Based on the concentration ratios of tissue/plasma, radioactivity from $^{35}$S-[2] tended to concentrate in the liver and kidney following both i.v. or p.o. administration (Fig. 9). The time of peak concentration after a p.o. dose was 4 h for plasma, blood and tissues. Tissue distribution evaluation demonstrated that the radioactivity concentrates in the liver, with the highest liver/plasma ratio in the i.v. group at 1 h, 3.89 (females) and in the p.o. group at 1 h, 2.86 (males).

**Table 2** summarizes the pharmacokinetic parameters for total radioactivity in plasma, calculated as μg-equiv/ml. The elimination half-life ($t_{1/2}$) for radioactivity after an i.v. dose was 8 to 9 h. The observed maximum plasma concentration ($C_{\text{max}}$) of radioactivity was about 2.5 μg-equiv/ml in the oral dose group at 4 h and approximately twice that in the i.v. group at 0.25 h. The ratio of the AUC$_{po}$ to AUC$_{iv}$ was 0.50 and 0.78 for males and females, respectively.

Although individual fractions from tissues and plasma at different time points were not analyzed by radio-HPLC, based upon the metabolism and stability studies of 1 and 2, it is reasonable to assume that the radioactivity in tissues and plasma corresponds mostly to that of $^{35}$S-[1] or $^{35}$S-[2] and minimally due to $^{35}$S-containing metabolites or elemental $^{35}$S.
The studies described in this paper were undertaken as part of the preclinical development of a prodrug 2 of the dinucleoside phosphorothioate 1, a representative SMNH compound, having anti-HBV activity. Metabolic studies of \( R_p, S_p \)-2 using liver microsomes and S9 fractions show that it is stereospecifically converted to \( R_p, S_p \)-1 via hydrolysis mediated by esterases. Neither 2 nor 1 was susceptible to oxidative metabolism by CYP-, or exo-, and endo-nuclease-mediated fragmentation. Furthermore, there was no evidence of phase II conjugation reactions. The prodrug 2 was quite stable in SGF compared to the dinucleotide 1.

Tissue distribution studies of p.o.-, and i.v.-administered \(^{35}\text{S}}\)-[2] demonstrated that the radioactivity concentrates in the liver. Based on the metabolic studies, it is likely that most of the radioactivity in the liver is associated with either \(^{35}\text{S}}\)-[2] or \(^{35}\text{S}}\)-[1]. The preferential distribution of the dinucleotide 1 and its prodrug 2 into liver may be attributed to the presence of nucleoside phosphorothioate backbone since PS-ODN also reveal similar tissue distribution profile upon i.v. administration (Geary, 2009).

The dinucleoside phosphorothioate prodrug 2 is quite stable in SGF and appears to be less stable in SIF where it is converted to the active 1. In acidic pH, 2 is likely to be protonated in the ring nitrogen of adenine and consequently 2 may not be absorbed from stomach by passive diffusion. It is hence likely that oral absorption of the prodrug 2 occurs predominantly in the upper duodenal region where a significant portion of the molecule may be in the unionized form. It is not known whether nucleotide transporters in the GI tract have any role in the active transport-mediated absorption of 2 and 1 (Zheng et al., 2004; Ritzel et al., 2001).

These observations further support the hypothesis that the observed anti-HBV activity of 1 and 2 (Iyer et al., 2004a, Iyer et al., 2004b; Coughlin et al., 2010) is associated with the intact dinucleotide structure. The tissue distribution studies show that following the oral administration of 2 in rats, the compound is rapidly absorbed and is readily distributed significantly to the liver,
the target organ for HBV. The compound is converted rapidly to the active \( \text{1} \) via plasma and/or liver esterases and is concentrated in the liver. Once inside the cell, it is possible that the negatively charged \( \text{1} \) remains trapped in the intracellular compartment and is made available for sustained antiviral effect.

The metabolism, and tissue distribution studies of the prodrug \( \text{2} \) have revealed a number of interesting findings that may have implications in the ADME of the longer chain PS-ODN counterparts. As mentioned before, ADME studies of PS-ODN and oligos with different chemical modifications reveal a “class pattern” in which following i.v. administration, they are rapidly cleared from plasma after absorption, and are preferentially distributed to liver, kidney, spleen, and bone marrow. In contrast, our evaluation of ADME properties of SMNH compounds reveals that they are preferentially distributed in liver and kidney with less distribution to other tissues. Our studies suggest that distribution to tissue compartments other than liver and kidney may depend on the length and charge of the PS-ODN.

In toxicology studies, i.v. administration of PS-ODN shows a number of dose-dependent effects such as complement activation, splenomegaly, elevation of liver transaminases, lymphoid hyperplasia, immune stimulation manifested as multi-organ mixed mononuclear cell infiltrate with fibroblast proliferation, increased IgM and cytokine production etc that are charge-, and sequence-dependent, as well as, species-dependent (Henry et al., 1997; Henry et al., 1999). siRNAs are also known to induce immune-stimulatory effects via activation of Toll-like receptors 7 and 8 (Marques and Williams, 2005) that constitute one of their important off-target effects. On the other hand, 7-day, and 14-day dose-ranging toxicology and toxicokinetic studies of \( \text{2} \) do not show these toxicological characteristics (unpublished results) that are hallmarks of PS-ODN and siRNA.

PS-ODN and siRNA are primarily metabolized by \textit{exo-}, and \textit{endo-nucleases} into shorter oligonucleotide fragments, and excreted primarily through kidneys. It is possible that most of the
ODNs are metabolized eventually into much shorter fragments including dinucleotides, which are then eliminated. In this context, it is pertinent to mention that PS-ODNs, including those that carry additional sugar modifications, appear to be retained in the tissues over extended periods and slowly metabolized predominantly in liver and kidney over time. Although several reports of metabolic studies of ODN with different sequences and chemical modifications have been reported (Geary, 2009), the biological effects and fate of the shorter fragments that result from metabolism of the parent ODN remain largely unknown. Our studies show that SMNH compounds, particularly dinucleotides may represent the terminal fragments of nuclease-mediated metabolism of longer-chain ODNs.

Furthermore, as described before, SMNH analogs have a variety of biological activities. We have recently found (Iyer et al., 2010) that certain SMNH analogs can activate cellular cytosolic pathogen recognition receptors (PRRs) such as retinoic acid inducible gene (RIG-I) (Akira et al., 2006; Saito et al., 2007; Myong et al., 2009; Katze et al., 2002) and nucleotide oligomerization domain protein 2 (NOD2) (Sabbah et al., 2009; Ting et al., 2010) that cause stimulation of innate immune pathways, Interferon production and induction of antiviral state (Saito et al., 2007).

In conclusion, ADME studies of SMNH compounds suggest that they: (a) are not subject to CYP metabolism, and (b) can be delivered orally via a prodrug strategy. Also, the prodrug derivatization facilitates distribution into the liver - the target organ for hepatitis viruses. A greater understanding of biological activities of SMNH analogs may help to design ODNs with minimal off-target effects for application as antisense, RNAi, aptamers and immunomodulatory compounds. Further studies on the preclinical development of SMNH compounds are planned and results will be reported in due course.
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Conducted experiments: Coughlin, Padmanabhan, Pandey, Iyer, O'Loughlin.

Contributed new reagents or analytical tools: Coughlin, Pandey.

Performed data analysis: Iyer, Coughlin, Pandey, Padmanabhan, Green.

Wrote or contributed to writing of the manuscript: Iyer, Pandey, Padmanabhan, Marquis, Mirsalis.
References


Footnotes:

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Figure Legends

Fig. 1. A. Synthetic scheme depicting the solid-phase synthesis of 1; B. Synthesis of the prodrug 2 from 1.

Fig. 2. Representative HPLC profile at different time-points of aliquots from the incubation of \( R_p, S_p \)-2 with liver microsomes.

Fig. 3. Mass spectrum of the prodrug 2. Peak at 704 represents the [M+H] ion

Fig. 4. Mass spectrum of the dinucleoside phosphorothioate 1 resulting from the metabolism of 2 by liver microsomes. The peak at 588 Da represents the [M+H] ion

Fig. 5. Mass spectrum of the desulfurized product 10 resulting from the metabolism of 2 by liver microsomes. The peak at 572.1 Da represents the [M+H] ion.

Fig. 6. Predicted pathways for Phase I biotransformation of \( R_p, S_p \)-2 by liver microsomes.

Fig. 7. Proposed mechanism of bioconversion of \( R_p, S_p \)-2 to \( R_p, S_p \)-1 by liver esterases. The proposed mechanism is based on an earlier publication (Coughlin et al., 2010) and references cited therein.

Fig. 8. Representative time-course HPLC profiles of incubates of \( R_p, S_p \)-2 in SGF that demonstrates the stability of 2 in the acidic environment of stomach.

Fig. 9. Ratio of Liver to plasma radioactivity concentration following i.v. and oral administration of \( ^{35}S \)-[2] in male and female rats. Data is presented as mean values derived at each time point from nine male and nine female rats.
Table 1. Excretion data following oral and i.v. administration of $^{35}$S-[2]. Data is presented as mean and s.d. values derived at each time point from nine male and nine female rats.

<table>
<thead>
<tr>
<th>Route</th>
<th>Sex</th>
<th>Time (h)</th>
<th>Urine % of dose Mean</th>
<th>SD</th>
<th>Feces % of dose Mean</th>
<th>SD</th>
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<td>I.V.</td>
<td>M</td>
<td>0-1</td>
<td>22.83</td>
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<td>NS</td>
<td>NC</td>
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<td></td>
<td></td>
<td>1-4</td>
<td>11.33</td>
<td>6.72</td>
<td>0.01</td>
<td>NC</td>
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<tr>
<td></td>
<td></td>
<td>4-24</td>
<td>29.60</td>
<td>11.46</td>
<td>16.90</td>
<td>4.57</td>
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<tr>
<td></td>
<td></td>
<td>0-24</td>
<td>56.15</td>
<td>5.13</td>
<td>16.90</td>
<td>4.57</td>
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<tr>
<td>I.V.</td>
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<td>15.81</td>
<td>5.16</td>
<td>NS</td>
<td>NC</td>
</tr>
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<td></td>
<td></td>
<td>1-4</td>
<td>6.34</td>
<td>2.86</td>
<td>NS</td>
<td>NC</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>P.O.</td>
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<td>NS</td>
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<td>NC</td>
</tr>
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<tr>
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<td>4-24</td>
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<td>34.59</td>
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<td>36.88</td>
<td>6.26</td>
<td>2.72</td>
<td>2.34</td>
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</table>
Table 2. Pharmacokinetic parameters for total radioactivity in plasma following oral and i.v. administration of $^{35}$S-[2]. Data is presented as mean ± s.d. values derived at each time point from nine male and nine female rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>I.V. Dose</th>
<th>Oral Dose</th>
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<tbody>
<tr>
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<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>7.8</td>
<td>9.0</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (μg-equiv/ml)</td>
<td>6.51 ± 0.60</td>
<td>4.49 ± 0.16</td>
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<tr>
<td>$C_{\text{maxpo}}/C_{\text{maxiv}}$</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AUC (h-μg-equiv/ml)</td>
<td>53.58 ± 1.37</td>
<td>35.21 ± 3.23</td>
</tr>
<tr>
<td>$AUC_{\text{po}}/AUC_{\text{iv}}$</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
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Fig. 1A

Fig. 1B
Fig. 7
Fig. 9