Metabolism of GTI-2040, a Phosphorothioate Oligonucleotide Antisense, Using Ion-pair Reversed Phase High Performance Liquid Chromatography (HPLC) Coupled With Electrospray Ion-trap Mass Spectrometry

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ABSTRACT

GTI-2040 is a 20-mer phosphorothioate oligonucleotide, which is complementary to the messenger ribonucleic acid (mRNA) of the R2 subunit of ribonucleotide reductase. This study characterized both the *in vivo* and *in vitro* metabolism of GTI-2040. A highly specific ion-pair reversed-phase electrospray ionization (IP-RP-ESI) liquid chromatography-mass spectrometry (LC-MS) method was used for the identification of GTI-2040 and metabolites from a variety of biological samples including exonuclease enzyme solutions, plasma, urine, mouse liver/kidney homogenates, and human liver microsomes. Progressively chain-shortened metabolites truncated from the 3' terminal of GTI-2040 were detected in all of the evaluated biological samples. GTI-2040 was found to be a good substrate for 3' but not 5' exonuclease. While the pattern of *n*-1 chain-shortened 3'-exonucleolytic degradation was similar in the mouse liver and kidney homogenates, the latter was found to contain a larger number of shortenmers, the kidneys appeared to possess higher enzymatic reactivity toward GTI-2040. Thus, metabolism of GTI-2040 was found to occur in a variety of biological samples, mainly mediated by the 3' exonuclease.

KEYWORDS: Metabolism, phosphorothioate oligonucleotides, GTI-2040, liquid chromatography/mass spectrometry

INTRODUCTION

Phosphorothioate oligonucleotides (PS-ODNs) have been extensively investigated in recent years as antisense drugs for the treatment of a variety of diseases including viral infections, inflammatory disorders, and cancers.¹⁻⁵ With the

substitution of one of the non-bridge oxygen atoms by sulfur, PS-ODNs exhibit improved resistance to nuclease, enhanced antisense activity, and favorable pharmacokinetic properties, which may contribute to their therapeutic potential.^{1,6} Despite their chemical stability, the *in vitro* and *in vivo* metabolism of PS-ODNs was still observed. Most of the metabolic studies on oligonucleotides have been performed using radiolabeled oligonucleotides, capillary gel electrophoresis (CGE) and high-performance liquid chromatography (HPLC).⁷⁻¹⁰ CGE separates oligonucleotides based on the chain length and is sensitive to nanomolar levels. However, many of these methods are not specific and they provide no structural information. Recently, several novel ion-pair reversed-phase (IP-RP) HPLC methods, coupled with electrospray ion-trap mass spectrometry (ESI MS) have been published, which are useful in identification of metabolites of antisense oligonucleotides (AS-ODNs).^{2,11,12} These methods not only permit separation of AS-ODNs at single-nucleotide resolution but also largely suppress cation adduct formation, thus providing a more accurate mass measurement.^{13,14} The molecular weights of the metabolites can be determined via the deconvolution technique, and the sequence as well as the identity of metabolites can be confirmed from the fragmentation pattern generated by the tandem mass spectra.^{2,11,15} Thus, the *in vitro* and *in vivo* metabolism of several PS-ODNs such as G3139 and ISIS 2302 have been characterized using liquid chromatography-tandem mass spectrometry (LC-MS/MS).^{2,16-18}

Major metabolites of PS-ODNs isolated from plasma and tissues following the systemic administrations of the parent drugs in mice, rats, and monkeys have been characterized as the 3' chain-shortened products, presumably formed by the action of 3' exonuclease.¹⁹⁻²² Minor 5' chain-shortened metabolites and metabolites probably formed by endonuclease were also observed.^{2,16,23,24} *In vitro* PS-ODN metabolism studies have also been performed in other biological matrices such as tissue culture medium, serum, urine, human and animal plasma, and solutions containing purified bacterial enzymes.^{22,25-28} Although as a class of compound, the metabolism profiles among PS-ODNs are similar, sequence- and

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length-dependency may exist, and these differences may affect their pharmacokinetic (PK) properties and pharmacodynamic (PD) responses.²⁻⁵ The extrapolation from metabolism of a known PS-ODN to that of a new PS-ODN may not be accurate.

GTI-2040 is a phosphorothioate oligonucleotide targeting the R2 subunit of ribonucleotide reductase, a crucial enzyme in DNA synthesis.²⁹ GTI-2040 is currently under phase 1 clinical evaluations for the treatment of solid tumors and acute myeloid leukemia (AML) as a single agent or in combination therapy.³⁰ Until now, no metabolism study of GTI-2040 has been reported. Because of the use of chromatographic separation and mass spectral filtering, herein, a highly specific IP-RP-HPLC/ESI MS was employed to investigate the in vitro and in vivo metabolism of GTI-2040 in a variety of biological samples, including plasma from treated patients, urine from treated rats, solutions containing pure enzyme, mouse liver/kidney homogenates, and human liver microsomes.

MATERIALS AND METHODS

Drugs and Chemicals

GTI-2040, a 20-mer PS-ODN with the sequence of 5'-GGC TAA ATC GCT CCA CCA AG-3', was provided by the National Cancer Institute (National Institutes of Health, Bethesda, MD) and used without further purification. Putative 3' end (3' N-1, 3' N-2, 3' N-3, heretofore GTI-2040, is omitted) and 5' end (5' N-1, 5' N-2, 5' N-3) metabolites of GTI-2040, from which 1 to 3 nucleotides were deleted from the 3' or 5' end, respectively, were all purchased from Integrated DNA Technologies (Coralville, IA). The purity and identity of each oligomer were verified by HPLC-UV-mass spectrometry (model: LCQ, ThermoFinnigan, San Jose, CA). Phosphodiesterase I (EC 3.1.4.1), a 3' to 5'-exonuclease, from snake (*Crotalus adamanteus*) venom phosphodiesterase (SVP), was obtained from USB (Cleveland, OH). Phosphodiesterase II (EC 3.1.16.1), a 5' to 3'-exonuclease, from bovine spleen was purchased from Sigma-Aldrich Co (St Louis, MO). Pooled human liver microsomes from 18 individuals (10 male and 8 female donors) were obtained as a stock emulsion (20 mg of protein/mL in 250 mM sucrose) from BD Biosciences (Woburn, MA). HPLC-grade methanol, triethylamine (TEA, 99.5%) triethylammonium bicarbonate (TEAB) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.8%) were purchased from Sigma-Aldrich Co. HPLC-grade water was generated by an E-pure water purification system (Barnstead, Dubuque, IA).

Instrumentation

LC-MS/MS analysis was performed on a ThermoFinnigan LCQ ion-trap mass spectrometer coupled with a Shimadzu

HPLC system (Shimadzu, Kyoto, Japan) and SPD-M10A photo diode array (PDA) detector (Shimadzu). The Shimadzu HPLC system consisted of 2 LC-10AT *vp* pumps and a SIL-10AD *vp* autosampler.

HPLC and LC-MS Conditions

Previously reported HPLC and LC/MS conditions¹⁷ were used in the separation and identification of GTI-2040 and its chain-shortened metabolites. In brief, the separation was accomplished on a 2.5- μ m Waters Xterra MS18 column (50 $\times$ 2.1 mm) coupled to a MSC18 10 $\times$ 2.1 mm guard column (Waters Corp, Milford, MA). The mobile phase was prepared as Mobile phase A consisting of 100 mM HFIP buffered to pH 8.3 by 8.4 mM TEA and Mobile phase B consisting of 100 mM HFIP and 8.6 mM TEA (pH 8.3) in methanol (50:50, vol/vol). Gradient elution was used for the oligomer separation at a flow rate of 0.2 mL/min. The elution was initiated with 30% Mobile phase B followed by a linear increase to 45% in 30 minutes, returning to 30% B in 2 minutes, and maintaining as such for 8 minutes before the next run. Column temperature was set to 50°C throughout the analysis using a column heater (Keystone, Woburn, MA). Autosampler temperature was kept at 4°C throughout the sample run. The PDA was operated to give the spectra from 200 to 600 nm. The LC-MS was operated with an ESI source in the negative ion mode. The LC effluent was introduced into the ion source without split. The electrospray high voltage was set at 2.0 to 2.2 kV, the tube lens was offset to 31 V, and the inlet capillary was heated to 190°C. The LCQ ion-trap mass spectrometer was operated with a background helium pressure of 1.75×10^{-3} torr, a typical electrospray needle voltage of 4.5 kV, a sheath gas flow of 80 (arbitrary unit), and an auxiliary nitrogen gas flow of 30 (arbitrary unit). Triple play mode (full scan, zoom scan, and MS/MS)^{17,31,32} was used for the identification of major metabolites of GTI-2040. Full mass scan was in the range of 600 to 2000 d, zoom scan was based on the most intense peak from the full scan mass spectrum, and data-dependent tandem MS/MS was collected from the most abundant ion. Collision energy was set to 25% to 35% with isolation width of 3.0 d. The mass spectrometer was tuned to its optimum sensitivity of charge states from $[M-3H]^{3-}$ to $[M-7H]^{7-}$ by infusion of either GTI-2040 or 3'N-1 through 3'N-3. All of the operations were controlled by the ThermoFinnigan Xcaliber software on a Windows NT 4.0 system.

Sample Preparation by Solid Phase Extraction

Samples containing GTI-2040 and metabolites were thawed and centrifuged at 1000g for 5 minutes. One milliliter of the supernatant was then mixed with 2 mL of 0.1 M TEAB and allowed to stand at room temperature for 30 minutes for ion-pair formation between the oligonucleotides

and TEA. GTI-2040 and related metabolites were extracted and isolated on an Oasis HLB cartridge packed with 60 mg material (Waters Corp). The extraction cartridges were preconditioned with 1 mL acetonitrile followed by 1 mL of 0.1 M TEAB (pH 8.0). Plasma or urine samples mixed with 0.1 M TEAB, or 0.1 M TEAB reconstituted tissue homogenate or microsomal extract were loaded onto the solid phase column. The proteins and salts were removed by sequential washing with 3 mL of 0.1 M TEAB, 3 mL of distilled water, and 3 mL of 10% acetonitrile in 0.1 M TEAB by gravity flow. Then, the ODNs were eluted with 3 mL 50% acetonitrile, and the eluant was evaporated to dryness under N₂. The residue was reconstituted with 150 µL of Mobile phase A, and 50 µL aliquot was analyzed by LC-MS.

Identification of GTI-2040 Metabolites in Plasma From AML Patients

Patients with AML were treated with GTI-2040 at 5 mg/kg/d as a continuous intravenous infusion (CIVI) for a total of 144 hours. Blood was drawn during and after infusion. Plasma was separated by centrifugation at 1400g for 20 minutes. GTI-2040 and metabolites were isolated from the plasma samples using a solid phase extraction (SPE) procedure as described in the previous section and then analyzed by LC-MS. PK study showed that GTI-2040 steady-state concentrations (C_{ss}) were achieved within 4 hours and remained so until the end of CIVI (data not shown). Patients samples used in metabolism identification were obtained during the steady-state.

This phase I clinical trial and all related clinical PK studies were performed under appropriate protocols as approved by the Institutional Review Board (IRB) of The Ohio State University.

In Vitro Enzymatic Reaction

To prepare 10 U/mL stock solutions of phosphodiesterase I (SVP) and phosphodiesterase II, each of the lyophilized enzymes was reconstituted in Tris-salts buffer:glycerol (50:50, vol/vol) solution containing 110 mM Tris-HCl, pH 8.9, 110 mM NaCl, 15 mM MgCl₂, and stored at -20°C before use. The reaction solution containing 5 µM GTI-2040 and 0.5 U/mL SVP or phosphodiesterase II was prepared in a buffer composed of 100 mM HFIP titrated to pH 8.3 by 8.4 mM TEA (mobile phase A). Initially, the incubation was performed following dilution with the initial buffer; however, the resolution and sensitivity of the peaks were compromised, especially for the LC-MS analysis, due to the presence of high salt. The use of HFIP/TEA buffer provided a better resolution and retained the presence of the same number of peaks. Thereafter, all enzymatic incuba-

tions were performed in this buffer. After incubation at 37°C with gentle shaking for 10 hours, the reaction was stopped by freezing, and the mixture was stored at -80°C until analysis. For analysis, the sample was thawed at 4°C, and an aliquot was analyzed by LC-MS without further sample preparation.

In Vitro Incubation of GTI-2040 With Fresh Human Blood

An appropriate aliquot of GTI-2040 stock solution was added into 1 mL fresh human blood drawn from a donor within 1 hour to a final concentration of 5 µM. The blood sample was then incubated at 37°C for 24 hours with frequent inverted mixing. The whole blood was then centrifuged at 1400g for 10 minutes, and the plasma was separated. GTI-2040 and its metabolites were isolated from plasma by SPE as described before. The extract was then analyzed by LC-MS for metabolite identification.

In Vitro Incubation of GTI-2040 With Mouse Tissue Homogenates

The liver and kidneys were excised from mice following CO₂ anesthesia and were frozen in liquid N₂ immediately after rinsing in phosphate buffered saline solution (PBS)

Figure 1. Ion-pair reversed phase high performance liquid chromatography (IP-RP-HPLC)-UV chromatogram (A) and liquid chromatography-mass spectrometry (LC-MS) total ion chromatogram (TIC) (B) of standards of GTI-2040, 3' N-1, 3' N-2, and 3' N-3. UV was set at 260 nm.

Figure 2. Total ion chromatogram (TIC) and the full scan mass spectra of GTI-2040 and major metabolites (M1-M5) from the plasma extract of a patient given GTI-2040 for 72 hours continuous IV infusion. GTI-2040 and metabolites were extracted from plasma using solid-phase extraction (SPE) and were detected by an ion-pair reversed-phase electrospray ionization liquid chromatography-mass spectrometry (RP ESI LC-MS) method. Oligonucleotides mass spectra are characterized as an ion envelope containing multiple ion peaks possessing different charge state, which can be determined by zoom scan. (A) shows the TIC of GTI-2040 and M1 to M5 metabolites; (B) shows the mass spectrum of M1, the putative 3'N-1 metabolite with retention time (RT) of 14.8, which contains an ion envelope including $[M-6H]^{6-}$, $[M-5H]^{5-}$ and $[M-4H]^{4-}$ ions; (C) shows the mass spectrum of M2, the putative 3'N-2 metabolite at RT 14.2 minutes, which contains the most abundant ion of $[M-3H]^{3-}$; and (D) shows the mass spectrum of M3, the putative 3'N-3 metabolite at RT 12.9 minutes containing the most abundant ion of $[M-3H]^{3-}$.

Figure 3. The electrospray ionization liquid chromatography-mass spectrometry (ESI LC-MS) full scan mass spectrum (A) and the corresponding deconvoluted mass spectrum (B) of GTI-2040, which shows its molecular weight.

and weighing. Liver or kidney homogenate was prepared with an addition of 1:10 (wt/vol) ice-cold nuclease buffer (100 mM Tris-HCl, 1 mM magnesium chloride, pH 8.0) and homogenized on ice with a tissue homogenizer (VIRTIS, Gardiner, NY). An appropriate aliquot of GTI-2040 stock solution was added into 1 mL of liver or kidney homogenate to a final concentration of 5 μ M. The homogenates then were incubated at 37°C with gentle shaking and frequent, inversed mixing. After 10 hours incubation, 0.5 mL of 3 mg/mL proteinase K in buffer containing Tris-HCl (pH 8.0), 10 mM EDTA was added into the mixture, and the incubation was continued for 8 hours to digest tissue components.

After centrifugation, the supernatant was collected and was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0, Invitrogen, Carlsbad, CA) to remove both proteins and lipids. Under this condition, oligonucleotides remained in the aqueous phase, while proteins and lipids stayed in the organic phase. The aqueous fraction was evaporated to dryness. The residue was reconstituted with 3 mL of 0.1 M TEAB followed by SPE as described in the Sample Preparation by Solid Phase Extraction section.

In Vitro Incubation of GTI-2040 With Pooled Human Liver Microsomes

The reaction suspension was prepared in 1 mL nuclease buffer containing 5 μ M of GTI-2040 and diluted human liver microsomes (0.5 mg of protein/mL). The reaction was performed at 37°C and incubated for 10 hours in a water bath with gentle shaking. At the end of incubation, 0.5 mM EDTA was added to stop the reaction. The mixture was then centrifuged at 1400g for 10 minutes, and the supernatant was separated and stored at -80°C until analysis. GTI-2040 and its metabolites were isolated by SPE and analyzed by LC-MS.

Urinary Excretion of GTI-2040 and Metabolites in the Rat

Two female Sprague-Dawley rats weighing ~340 to 380 g were purchased from Harlan (Indianapolis, IN). GTI-2040 in sterile saline was injected into the tail vein at a single dose of 20 mg/kg. Each rat was housed in a metabolism cage and urine samples were collected at intervals of 0 to 7, 7 to 12, and 12 to 24 hours after dosing. The total urine volume was measured, and the urine was immediately frozen at -80°C until analysis. Following SPE, identification of urinary metabolites was accomplished by LC-MS. The animal study was performed under a protocol that adhered to the “Principle of Laboratory Animal Care” by the National Institutes of Health and was approved by The Ohio State University and the Institute and Laboratory Animal Care and Use Committee.

Table 1. Sequences and Molecular Weights of GTI-2040 and Its Metabolites in Human Plasma*

Identity	Sequence (from 5' to 3')	Observed Mw	Theoretical Mw	MS Spectra (charge)
GTI-2040	GGCTAAATCGCTCCACCAAG	6375.5	6375.23	1061.5 (6-) 1274.5 (5-) 1592.9 (4-)
3'N-1	GGCTAAATCGCTCCACCAA	6031.1	6030.9	860.5 (5-) 1004.2 (6-) 1205.3 (5-)
3'N-2	GGCTAAATCGCTCCACCA	5699.4	5701.6	1898.8 (3-)
3'N-3	GGCTAAATCGCTCCACC	5370.2	5372.3	1789.7 (3-)
3'N-4	GGCTAAATCGCTCCAC	5064.9	5066.8	1688.3 (3-)
3'N-5	GGCTAAATCGCTCC	4758.3	4761.7	1586.1 (3-)

*Mw indicates molecular weight; MS, mass spectrometry.

Table 2. Assignment of Fragment Ions Obtained From the MS² Spectrum Following the Collision-induced Dissociation on the Ion at m/z 1004.0 ([M-6H]⁻⁶) From 3' N-1 Standard and M1 Peak*

m/z	Ion assignment	Sequence (from 5' to 3')
346.0	w ₁ ⁻¹	A
675.1	w ₂ ⁻¹	AA
981.5	w ₃ ⁻¹	CAA
1285.1	w ₄ ⁻¹	CCAA
802.6	w ₅ ⁻²	ACCAA
959.1	w ₆ ⁻²	CACCAA
1271.6	w ₈ ⁻²	TCCACCAA
1165.8	w ₁₁ ⁻³	CGCTCCACCAA
1272.4	w ₁₂ ⁻³	TCGCTCCACCAA
1036.3	w ₁₃ ⁻⁴	ATCGCTCCACCAA
894.7	w ₁₄ ⁻⁵	AATCGCTCCACCAA
960.5	w ₁₅ ⁻⁵	AAATCGCTCCACCAA
853.6	w ₁₆ ⁻⁶	TAAATCGCTCCACCAA
1019.4	w ₁₉ ⁻⁶	GGCTAAATCGCTCCACCAA
442.1	a ₁ -B ₁ ⁻¹	G
787.1	a ₂ -B ₂ ⁻¹	GG
1412.1	a ₄ -B ₄ ⁻¹	GGCT
1741.2	a ₅ -B ₅ ⁻²	GGCTA
689.4	a ₆ -B ₆ ⁻³	GGCTAA
798.0	a ₇ -B ₇ ⁻³	GGCTAAA
1007.4	a ₉ -B ₉ ⁻³	GGCTAAATC
1122.4	a ₁₀ -B ₁₀ ⁻³	GGCTAAATCG
1224.1	a ₁₁ -B ₁₁ ⁻³	GGCTAAATCGC
920.1	a ₁₄ -B ₁₄ ⁻⁵	GGCTAAATCGCTCC
872.24	a ₁₆ -B ₁₆ ⁻⁶	GGCTAAATCGCTCCAC

*MS indicates mass spectrometry.

Data Analysis

Raw spectra were processed with Xcalibur (Version 1.2, ThermoFinnigan). Molecular weights of the oligonucleotides were obtained by automatic deconvolution using the BioMass program (Xcalibur 2.1). The Simple Oligonucleotide Sequencer software (SOS), (SOS Version 1.10, Jef Rozenski, University of Utah, Salt Lake City, UT) was used to derive the oligonucleotide sequences from product ion mass spectra.

RESULTS

Identification of GTI-2040 Metabolites in Plasma From Treated Patients

A highly specific LC-MS analytical method previously developed in this laboratory for antisense G3139 and metabolites was applied to GTI-2040 and metabolites in patients' plasma.¹⁴ Representative LC-UV/MS chromatograms show separation of GTI-2040 and its 3' metabolites (Figure 1). A total ion chromatogram (TIC) and the corresponding mass spectra obtained from the extract of patients' plasma collected at 72 hours in 6-day IV infusion are shown in Figure 2. In addition to the peak of GTI-2040, 5 peaks, denoted as M1, M2, M3, M4, and M5, are found and illustrated in the TIC chromatogram (Figure 2A). GTI-2040 was identified at the peak with the retention time of 15.8 minutes and its mass spectrum is shown in Figure 3A. As shown, an ion envelope

with a distribution of multiple ion peaks possessing different charge states was found for GTI-2040, which is typical for ESI mass spectra of oligonucleotides.^{11,21,33} Major ions at m/z 1061.5, 1274.1, and 1593.1 were assigned to GTI-2040 with 6, 5, and 4 negative charges, respectively. The charged states could also be revealed by zoom scan, and the data-dependent scan provided the MS/MS information for specific ions, which are helpful in sequence determination. Based on this information, the molecular weight of GTI-2040 was readily obtained by deconvolution as shown in Figure 3B.

An ion envelope including major ions at m/z 1004.0 ([M-6H]⁻⁶), 1205.0 ([M-5H]⁻⁵) and 1506.6 ([M-4H]⁻⁴) was observed for Peak M1 (Figure 2B). Peaks M2 and M3 also showed multiple charged ions of different intensities with the most abundant ions at m/z 1898.9 ([M-3H]⁻³) for M2 peak and m/z at 1789.5 ([M-3H]⁻³) for M3 peak (Figures 2C and 2D, respectively). A similar strategy for the identification of GTI-2040 was applied to these unknown peaks. Table 1 summarizes the calculated and observed molecular weights of GTI-2040 and its metabolites. As shown, following deconvolution, the putative GTI-2040 peak yielded [M-H]⁻ at 6375.5, which matched the theoretical molecular weight of GTI-2040 with $\pm 0.004\%$ mass accuracy.

Similarly, molecular weights of M1, M2, M3, M4, and M5 were determined to be 6031.3, 5699.4, 5370.2, 5064.9, and 4758.3, respectively, which differ from the theoretical molecular weights of 3' N-1 to N-5 metabolites by 0.2, 2.2, 2.1, 1.9, and 3.4 amu and from the theoretical molecular weights of 5' N-1 to N-5 by 0.2, 16, -8.3, 5.1, and 30.9 amu, respectively. The higher mass difference for some components appeared to be associated with their lower abundance. There were smaller mass differences between the metabolites and the corresponding 3'-end deletion standards, suggesting that the metabolites resemble the 3'-cleavage products. For this class of compounds, the small mass differences are difficult to be rationalized by the combination of oxidation/reduction processes and are considered to be reasonable within the experimental error. In addition, their MS¹ mass spectra are identical to those of the corresponding 3' chain-shortened standards (Figure 2; only N-1 to N-3 are shown). Furthermore, according to the sequence of GTI-2040, the nucleotide sequences from N-2 to N-5 at the 3' end, but not the molecular weights are different from those at the 5' end. The ion envelopes of N-2 to N-5 chain-shortened metabolites from the 3' end of GTI-2040 were found to be different from those of the synthetic 5' end shortenmer standards (data not shown). Based on the similarity in the mass spectral pattern to that of the parent compound, the deconvoluted masses, their MS¹ mass spectra compared with the standards, ion-envelope information, and HPLC elution sequence, the peaks of M2 to M5 in Figure 1A were assigned as 2 to 5

Figure 4. Fragment ions assignments following collision-induced dissociation of ions at m/z 1004 from the mass spectra of (A) 3'-N-I standard and M1 peak, and from the mass spectrum of (B) 5'-N-I standard.

nucleotide-deletion metabolites from the 3' end of GTI-2040, respectively. A similar strategy for the detailed metabolism study of another antisense compound has recently been published.¹⁷

While the observed molecular weight of M1 is similar to 3'N-1 GTI-2040, it cannot be differentiated from that of 5'N-1, because the molecular weights of both 3'N-1 and 5'N-1 are identical. To distinguish between these 2 possibilities, we constructed the oligonucleotide sequences of 3'N-1 and 5'N-1 GTI-2040 based on the MS² fragment ion spectra

with the aid of the SOS software.^{15,34} MS² spectra of 3'N-1 and 5'N-1 standards were obtained using the most abundant ion [M-6H]⁻⁶ at *m/z* 1004.0 and compared with the MS² spectra of M1 on the same ion. The most diagnostic ions for the determination of the 3'→5' sequence are the w series ions and for the determination of the 5'→3' sequence are the a-B ions.^{35,36} Seven w ions and 5 a-B ions generated from MS² of M1 peak were identified and assigned (Table 2, Figure 4A). Using the same method, the w and a-B ions ladders were constructed and the sequences were aligned with the 3'N-1

Table 3. Assignment of Fragment Ions Obtained From the MS² Spectrum Following the Collision-induced Dissociation on the Ion at m/z 1004.0 ([M-6H]⁻⁶) From the 5' N-1 Standard*

m/z	Ion Assignment	Sequence (from 5' to 3')
362.0	w_1^{-1}	G
691.1	w_2^{-1}	AG
1325.1	w_4^{-1}	CAAG
1630.1	w_5^{-1}	CCAAG
981.1	w_6^{-2}	ACCAAG
1284.1	w_8^{-2}	CCACCAAG
1444.1	w_9^{-2}	TCCACCAAG
1280.8	w_{12}^{-3}	CGCTCCACCAAG
1122.6	w_{14}^{-4}	TCGCTCCACCAAG
1093.5	w_{17}^{-5}	AAATCGCTCCACCAAG
1154.5	w_{18}^{-5}	TAAATCGCTCCACCAAG
1019.4	w_{19}^{-6}	GCTAAATCGCTCCACCAAG
442.1	$a_1-B_1^{-1}$	G
747.1	$a_2-B_2^{-1}$	GC
1396.1	$a_4-B_4^{-1}$	GCTA
862.1	$a_5-B_5^{-2}$	GCTAA
1026.6	$a_6-B_6^{-2}$	GCTAAA
1511.6	$a_9-B_9^{-2}$	GCTAAATCG
1215.8	$a_{11}-B_{11}^{-3}$	GCTAAATCGCT
1146.4	$a_{14}-B_{14}^{-4}$	GCTAAATCGCTCCA
1298.9	$a_{16}-B_{16}^{-4}$	GCTAAATCGCTCCACC
1381.1	$a_{17}-B_{17}^{-4}$	GCTAAATCGCTCCACCA
1170.5	$a_{18}-B_{18}^{-5}$	GCTAAATCGCTCCACCAA

*MS indicates mass spectrometry.

and 5'N-1 standards (Table 3, Figure 4B). The MS² mass spectrum of M1 was essentially identical to that of 3'N-1 but not 5'N-1, and these fragment ions are consistent with the fragmentation assignment for 3'N-1. As shown in Figure 4, MS² mass spectrum of 5'N-1 (Figure 4B) showed a distinct pattern of w ion and a -B ion series from those of 3'N-1 and M1 peaks (Figure 4A). For instance, w_2^{-1} at m/z 674.9 and w_4^{-1} at m/z 1285.1 were assigned as sequence of 3' AA and 3' AACC in 3'N-1 and M1, while the w_2^{-1} (m/z 691.1) and w_4^{-1} (m/z 691.1) ions correspond to sequence of 3' GA and 3' GAAC in 5'N-1 fragmentation, respectively. Similarly, the fragment ion of $a_5-B_5^{-2}$ (m/z 871.0) corresponding to the sequence of 5' GGCTA was identified in M1 and 3'N-1, while the $a_5-B_5^{-2}$ ion (m/z 862.6) was identified as 5'GCTAA in 5'N-1. Therefore, M1 ion was identified as 3'N-1 metabolite of GTI-2040 in plasma from AML patients.

In summary, 5 metabolites were identified in AML patients' plasma extract as progressively chain-shortened oligomers from the 3' end of GTI-2040. Minor metabolites from 5'

exonuclease or endonuclease activity may potentially exist but were not detected because of the sensitivity limitation.

Role of 3'-Exonuclease in GTI-2040 Metabolism

We then speculated that 3' exonuclease was the major enzyme responsible for GTI-2040 metabolism in vivo. The role of 3' exonuclease in GTI-2040 metabolism was examined by the in vitro enzymatic reaction, using snake venom phosphodiesterase (SVP). As shown in Figure 5A, a typical right-triangle-shaped pattern was observed in the HPLC chromatogram after 10-hour incubation of 5 μ M GTI-2040 in 100 mM HFIP/8.4 mM TEA pH 8.3 buffer Mobile Phase A (MPA) containing 0.5 U/mL SVP at 37°C. GTI-2040 and its 3'N-1, 3'N-2, and 3'N-3 shortenmers were identified by LC-MS and MS/MS, using the same strategies as described previously in the section "Identification of GTI-2040 Metabolites in Plasma from AML Patients" (Figure 5B). Under the same condition, GTI-2040 was stable in MPA without the enzyme and no interference was found in the SVP control (data not shown). In contrast, GTI-2040 exhibited resistance to the phosphodiesterase II under the same reaction condition, with almost no degradation products detected (Figure 5C). Using a very high level of phosphodiesterase II (5U/mL), GTI-2040 only showed slight degradation after 24-hour incubation (Figure 5D). It is well known that SVP is a 3' specific enzyme, which hydrolyzes 5'-mononucleotides from 3'-hydroxy terminal DNA, and phosphodiesterase II is a 5' specific enzyme, which hydrolyzes 5'-mononucleotides from 5'-hydroxy terminal DNA. Based on the above results, GTI-2040 is expected to be a good substrate for 3' exonuclease but not 5' exonuclease. Consistent with the in vivo results, the in vitro enzymatic incubation indicated the sequential nucleotide deletion from the 3' terminus in degradation of GTI-2040. Similar patterns of sequential nucleotide degradation from 3' terminus were also observed using 3'N-1 or 3'N-2 of GTI-2040 as substrate in SVP solution (Figure 6).

In Vitro Metabolism Profiles of GTI-2040 in Tissue-Related Samples

As shown in Figure 7, the HPLC analysis of the in vitro incubation of GTI-2040 with fresh human blood displayed the same degradation pattern as that in SVP. The peaks eluted earlier than GTI-2040 were identified as the 3' chain-shortened metabolites. Thus, blood is a potential source for metabolism of GTI-2040. Not surprisingly, after incubation of GTI-2040 with mouse liver homogenate, several chain-shortened oligomers of GTI-2040 were observed. 3'N-1, 3'N-2, and 3'N-3 were identified by both HPLC-UV and LC-MS (Figures 8A and 8B). Similarly, the 3' end deleted metabolites were observed in GTI-2040 incubation with human liver microsomes (Figures 8C and 8D); both 3'N-1

Figure 5. High performance liquid chromatography (HPLC)-UV chromatogram (A) and total ion liquid chromatography-mass spectrometry (LC-MS) chromatogram (B) of GTI-2040 and metabolites after 5 μ M of GTI-2040 was incubated in snake venom phosphodiesterase (SVP, 0.5 U/mL) for 10 hours. HPLC-UV chromatogram of GTI-2040 metabolism after incubation of GTI-2040 with phosphodiesterase II (0.5 U/mL) for 10 hours (C) and with phosphodiesterase II (5 U/mL) for 24 hours (D).

and 3'N-2 were identified by both LC-UV and LC-MS systems. In mouse kidney homogenate, 3'N-1 and 3'N-2 were also identified (Figures 9A and 9B). However, in the LC-MS metabolism profiles of GTI-2040 in mouse kidney homogenate, an earlier eluting peak with a relatively high intensity was also detected, and this was identified as 3'N-5 GTI-2040, according to its deconvoluted molecular weight and the ion fragmentation pattern from its MS² spectrum. No 5'-related chain-shortened metabolites were observed in liver and kidney homogenates within the detection limit of 1 μ mol.

Urinary Metabolites

A 24-hour urine sample was collected from AML patients after receiving 5 mg/kg/d GTI-2040 as a continuous IV infusion for 144 hours. After a 250-fold enrichment by lyophilization and SPE extraction, no metabolites were detected by LC-MS. In the rat, extensive metabolism of GTI-2040 was observed in 0- to 7-hour urine following an IV bolus dose with GTI-2040 at 20 mg/kg, as shown in Figures 9C and 9D. The 3'N-1, 3'N-2, 3'N-3, 3'N-4, and 3'N-5 GTI-2040 were identified using both HPLC-UV and LC-MS.

Figure 6. High performance liquid chromatography (HPLC)-UV chromatograms of the sequential metabolism profiles of (A) GTI-2040, (B) 3'N-1, or (C) 3'N-2 after each of them was incubated in 0.5 U/mL snake venom phosphodiesterase (SVP) solution.

Some small peaks were eluted before 10 minutes, and they may be the progressively 3'N-5 chain-shortened metabolites; however, the possibility that they are the random chain shorteners generated from endonuclease still exists.

Figure 7. High performance liquid chromatography (HPLC)-UV chromatogram of GTI-2040 and metabolites after 5 μ M of GTI-2040 was incubated in human blood for 24 hours.

Figure 8. Metabolism profiles of GTI-2040 in mouse liver homogenates (A, B) and in human liver microsomes (C, D). GTI-2040 (5 μ M) was incubated in mouse liver homogenate (0.1 g/mL) for 10 hours. GTI-2040 and metabolite from mouse liver homogenate extracts were detected in high-performance liquid chromatography (HPLC)-UV chromatogram (A) and in liquid chromatography-mass spectrometry total ion chromatogram (LC-MS TIC) (B). GTI-2040 (5 μ M) was incubated in human liver microsomes (HLM) (0.5 mg of protein/mL) for 10 hours. GTI-2040 and metabolites from HLM were detected in HPLC-UV chromatogram (C) and in LC-MS TIC (D).

Concentrations of GTI-2040 and its metabolites in rat urine collected after 7 hours were too low to be detected.

DISCUSSION

Ion-pair reversed phase HPLC is an efficient method for separation of oligonucleotides. In the LC-MS system, using TEA-HFIP as an ion-pairing buffer provides a single nucleotide resolution for the phosphorothioate oligonucleotides.

Figure 9. Metabolism profiles of GTI-2040 in mouse kidneys homogenates (A, B) and in rat urinary excretion (C, D). GTI-2040 (5 μ M) was incubated in mouse kidney homogenates for 10 hours. GTI-2040 and metabolites from the kidneys homogenates were detected in high-performance liquid chromatography (HPLC)-UV chromatogram (A) and in liquid chromatography-mass spectrometry total ion chromatogram (LC-MS TIC) (B). Rat urine was collected 0 to 7 hours after an IV injection of GTI-2040 at 20 mg/kg was given to the rat. GTI-2040 and metabolites in the rat urine were detected in HPLC-UV chromatogram (C) and in LC-MS TIC (D).

TEA provides the counterion TEA^+ in the formation of ion-pair with the negatively charged phosphorothioate oligonucleotides.³⁷ The use of the ion-pair buffer helps to decrease the interference from sodium adducts and improves the performance of deconvolution and the mass accuracy. HFIP, being a highly volatile weak acid, enhances the ionization efficiency and increases the detection sensitivity.³⁷ In this study, we found that 100 mM HFIP combined with 8.6 mM TEA (pH 8.3) shifted the ion envelope to the low charge states and offered good ion-pair efficiency and higher MS

sensitivity, similar to our previous work with another antisense G3139.¹⁷

A careful examination of the HPLC chromatograms of the GTI-2040 metabolism profiles reveals a pattern of uneven spacing peaks, for example, 3'N-1 was closely eluted following the parent GTI-2040 (Figure 8A), while the separation between 3'N-2 and 3'N-3 was significantly wider. As reported by Gilar,¹² when cleavage of ODN metabolites occurred between CA from the 3' end, the separation seemed to be enhanced; while the separation selectivity decreased, when cleavage occurred between AG. This finding is consistent with our observations, in that the elution between 3'N-1 and the parent GTI-2040 (an AG cleavage) was closer, and that for 3'N-2 and 3'N-3 (a CA cleavage) was longer. It has been suggested that oligonucleotide sequence (nucleobase composition) affects the separation of oligonucleotide ladders.¹² The different retention behaviors of oligonucleotide ladders in ion-pair RP-HPLC may result from the relative difference in the hydrophobicity of nucleobases.^{38,39} A loss of the more hydrophobic A or T mononucleotides from the 3' end results in a pronounced, shorter retention time than loss of rather hydrophilic C and G mononucleotides.¹² Other reasons may exist (eg, the differences in polarity of nucleosides may affect their interaction with the column materials) resulting in their different elution behaviors.

PS-ODN metabolism usually generates a progressive n-1 chain-shortened pattern, typically showing a right-triangle ladder,^{21,22,27} whereas metabolism of ODNs by endonuclease would be expected to produce a profile characterized by an irregular distribution of chain-shortened metabolites, because endonuclease cleaves nucleic acids at internal sites and produces nucleotide fragments of various sizes.⁴⁰ Thus far, metabolism of PS-ODNs with endonuclease activity has not been observed,^{16,21,41} and GTI-2040 undergoes sequential metabolism consistent with other PS-ODNs. The sequential metabolism of GTI-2040 is also supported by the similar pattern of gradual hydrolysis when 3'N-1 or 3'N-2 is used as the substrate (Figure 6).

This study suggests that GTI-2040 is unstable in human blood at 37°C, and the compound was susceptible to the 3' exonuclease cleavage in circulation. However, the rapid distribution of PS-ODNs ($t_{1/2} \alpha \sim 0.5$ hour) to tissues and the slow efflux rate from tissues (days) following systemic^{8,41,42} administration suggests that its metabolism in circulation does not contribute significantly to the overall metabolism of GTI-2040 relative to other sites.

As reported in the animal studies, liver and kidneys are the 2 major organs that accumulate and metabolize PS-ODNs.^{8,27,43,44} For GTI-2040, sequential 3'-end chain-shortened metabolites were detected from both mouse liver and kidney homogenates (Figures 8A, 8B, 9A, and 9B). While the HPLC-UV chromatograms from the mouse liver homogenate

showed a typical right-triangle pattern of the metabolites ladder (Figure 8A), in the mouse kidney homogenate it appeared that more shortened oligomers were generated (Figure 9A). It has been shown in tissue biodistribution studies following the infusion of GTI-2040 in the rat and monkey that kidneys contained the highest accumulation of GTI-2040.⁴⁵ Since a previous study reported the observation of acute renal failure in mice resulting from renal cortical necrosis or acute tubular necrosis when administered with very high dose (≥ 100 mg/kg) of a 20-mer PS-ODN,⁴⁶ the high accumulation of GTI-2040 and metabolites in kidneys may potentially relate to the renal toxicity and caution of the renal side-effect in patients should be considered when a high dose of GTI-2040 is given.

In human liver microsomes, the metabolism profiles were found to be associated with 3'-end cleavage and displayed the similar pattern observed in mouse liver homogenate and in SVP as well, as shown in Figures 8C and 8D. This finding may imply that human liver microsomes retain the exonuclease activity and could be used as a human-related enzyme system in testing the metabolism of PS-ODNs.

Due to the limited sensitivity of MS, the rapid eluted metabolites with lengths shorter than 3'N-3 in tissue homogenates could not all be detected and identified by mass spectrometry. These peaks may be derived from the sequential hydrolysis by the exonuclease, but possibility of the shortenmers generated from the endonuclease activity may exist, since endonucleases cleave oligonucleotides into small fragments directly. The enzyme reaction studies showed that GTI-2040 was highly resistant to 5' exonuclease (phosphodiesterase II). Therefore, 5' degradation, if present, would be a minor degradation pathway. In fact, within the detection sensitivity of this method, we did not observe the 5'-end chain-shortened oligomers from both in vivo and in vitro studies. Oxidative products were also not found as the metabolites of GTI-2040.

CONCLUSION

Using a highly specific ion-pair reversed phase HPLC/ESI-MS, metabolism of GTI-2040 was found to be mainly mediated by the 3' exonuclease, and the progressive 3'-end chain-shortened metabolites were detected. 5'-end related degradation and oxidative products were not observed in metabolites of GTI-2040.

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