Disposition and Metabolism of GSK2251052 in Humans: A Novel Boron-Containing Antibiotic

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ABSTRACT

(S)-3-(Aminomethyl)-7-(3-hydroxypropoxy)-1-hydroxy-1,3-dihydro-2,1-benzoxaborole (GSK2251052) is a novel boron-containing antibiotic that has been in development for the treatment of serious Gram-negative infections. In this study, six healthy adult male subjects received a single i.v. dose of [14C]GSK2251052, 1500 mg infused over 1 hour. Blood, urine, and feces were collected over an extended period of 14 days, and accelerator mass spectrometry was used to quantify low levels of radioactivity in plasma at later time points to supplement the less-sensitive liquid scintillation counting technique. An excellent mass balance recovery was demonstrated that radioactivity was moderately associated with the blood cellular components, and together with GSK2251052, both were highly distributed into tissues. The parent compound had a much shorter half-life than total radioactivity in plasma, approximately 11.6 hours compared with 96 hours. GSK2251052 and its major metabolite M3, which resulted from oxidation of the propanol side chain to the corresponding carboxylic acid, comprised the majority of the plasma radioactivity, 37 and 53% of the area under the plasma versus time concentration curve from time zero to infinity, respectively. Additionally, M3 was eliminated renally, and was demonstrated to be responsible for the long plasma radioactivity elimination half-life. A combination of in vitro metabolism experiments and a pharmacokinetic study in monkeys with the inhibitor 4-methylpyrazole provided strong evidence that alcohol dehydrogenase, potentially in association with aldehyde dehydrogenase, is the primary enzyme involved in the formation of the M3 metabolite.

Introduction

GSK2251052 [(S)-3-(aminomethyl)-7-(3-hydroxypropoxy)-1-hydroxy-1,3-dihydro-2,1-benzoxaborole hydrochloride], also known as AN3365, is an antibacterial compound that has been in development for the treatment of serious Gram-negative infections. Leucyl tRNA synthetase (LeuRS) is an essential bacterial enzyme that catalyzes the coupling of the amino acid leucine onto its corresponding leucine transfer ribonucleic acid (tRNA\textsubscript{Leu}), which is used by the ribosome for protein synthesis. Inhibition of LeuRS prevents protein synthesis and stops growth of the bacteria. Through the unique chemical binding properties of the boron atom, GSK2251052 binds to the editing active site of bacterial LeuRS and forms a boron adduct with the 3’ terminus of tRNA\textsubscript{Leu} which locks the tRNA\textsubscript{Leu} to LeuRS in an unproductive state.

GSK2251052 has good in vitro activity against Enterobacteriaceae and Pseudomonas aeruginosa, and was not affected by any of the tested resistance mechanisms, including major efflux pumps, extended-spectrum β-lactamase, Klebsiella pneumoniae carbapenemase, and class C β-lactamase, which are known to be important contributors to the resistance of Gram-negative bacteria (Schweizer, 2012). It is acknowledged that there is a critical need for new antibiotics to treat Gram-negative bacterial infections (Spellberg et al., 2008), and GSK2251052 is one of several new compounds being developed that target protein synthesis as a mechanism of action (Sutcliffe, 2011).
In this article, we describe the pharmacokinetics, metabolism, and elimination of [14C]GSK2251052 in humans following a single i.v. administration, which was one of the intended therapeutic routes of administration. Sampling of blood, urine, and feces allowed the evaluation of mass balance, route(s) of elimination (renal or metabolic), identification of primary metabolites, and a comparison of the exposure and half-lives of both parent compound and total radioactivity, which included metabolites. Additionally, the enzyme(s) responsible for the metabolism of GSK2251052 was investigated by conducting in vitro experiments and a nonclinical pharmacokinetic study.

Materials and Methods

Chemicals and Reagents

[14C]GSK2251052 hydrochloride (specific activity of 0.00865 μCi/mg, stated radiochemical purity of 99.4%) (Fig. 1), GSK2251052 hydrochloride salt, [2H2]GSK2251052, and [13C]M3 ([S]-3-(aminomethyl)-7-(carboxethoxy)-1-hydroxy-1,3-dihydro-2,1-benzoxaborole) were supplied by GlaxoSmithKline Active Pharmaceutical Ingredient (API) Chemistry and Analysis (Stevenage, UK). Metabolite M3 was supplied by GlaxoSmithKline API Chemistry and Analysis (Upper Merion, PA). Scintillation cocktails, Ultima Gold and Ultima-Flu M, and Deepwell LumaPlate 96-well plates were obtained from PerkinElmer (Boston, MA). For in vitro experiments, all subcellular fractions (cynomolgus monkey and human) were supplied by Xenotech (Lenexa, KS). All human subcellular fractions were supplied pooled of mixed gender, and the monkey fractions were supplied pooled from male animals.

Formulated Drug

GlaxoSmithKline supplied nonsterile GSK2251052 hydrochloride salt powder containing [14C]GSK2251052 hydrochloride, and the clinical site prepared sterile GSK2251052 containing [14C]GSK2251052 solution for infusion. Powder was dissolved in sterile water for injection to a concentration of 125 mg/ml and was sterilized via filtration. Then 12 ml of solution, equivalent to 1500 mg of GSK2251052, was diluted to 250 ml with 0.9% NaCl injection prior to infusion.

Synthesis of Metabolite M3

The synthesis of metabolite M3 is depicted in Fig. 2. GSK2251052 (hydrochloride) was neutralized with di-isopropylethylamine, and then the corresponding free base was treated with benzyl bromide in methanol, in the presence of potassium carbonate, to give the desired dibenzylamino protected compound, which was then submitted to standard oxidation conditions using chromium (VI) oxide (CrO3) in a mixture of acetic acid and acetone. The corresponding carboxylic acid was isolated as the sodium salt after basic hydrolysis. Hydrogenolysis of the N-benzyl groups using 5% palladium on carbon as the catalyst in a mixture of tetrahydrofuran and aqueous HCl produced the expected metabolite M3 as the hydrochloride salt.

Clinical Mass Balance Study

A phase I, open-label, nonrandomized, single-dose, single-center, mass balance study was conducted to investigate the recovery, excretion, and pharmacokinetics of GSK2251052 after i.v. administration. The study (NCI 01475695) was conducted according to principles of good clinical practice, applicable regulatory requirements, and the Declaration of Helsinki. The in-life portion of this study was conducted at PRA International, Stationsweg 163, 9471 GP (Zuidlaren, The Netherlands). Following Independent Ethics Committee approval by the Stichting Beoordelings Ethiek Biomedisch Onderzoek (Assen, The Netherlands) and collection of written informed consent, all subjects underwent an initial screening assessment within 30 days prior to the first dose. The screening included a medical history, physical examination, and clinical laboratory tests. Exclusion criteria included regular use of tobacco or nicotine-containing products (within 6 months prior to screening), a positive drug or alcohol test, recent participation in another research trial with an investigational product (i.e., within 30 days prior to screening), participation in a clinical trial involving a 14C-labeled compound (within the last 12 months), and any pre-existing conditions that would interfere with normal gastrointestinal anatomy, motility, or hepatic or renal function which could interfere with the absorption, metabolism, and/or excretion of the study drug. Use of vitamins, dietary and herbal supplements, antacids, any prescription drugs, or grapefruit-containing products within 7 days prior to the start of the dosing through to the follow-up visit was prohibited. Six healthy adult male volunteers with a mean age of 42.2 years (S.D. 8.28 years), mean body weight of 83.1 kg (S.D. 3.41 kg), and mean body mass index of 25.5 kg/m2 (S.D. 1.38 kg/m2) were enrolled in this study. Five subjects were Caucasian and one subject was of Arabic/North-African heritage.

The radiolabeled dose was calculated in accordance with the 1990 Recommendations of the International Commission on Radiologic Protection (ICRP Publication 60; http://www.icrp.org/publication.asp?id=ICRP%20Publication%20 2060) as implemented in the 1999 Ionizing Radiations Regulations. The calculation was based on [14C]GSK2251052 data obtained from a quantitative tissue distribution study conducted in rats. It was determined that, to comply with the International Commission on Radiological Protection guidance limit of 100 μSv (microsievert), the maximum activity would be 0.69 MBq (18.8 μCi). To ensure that this limit was not exceeded, a target dose of 15 μCi was chosen. All subjects enrolled who met eligibility criteria were fasted at least 10 hours prior to receiving a single radiolabeled i.v. dose of 1500 mg of [14C]GSK2251052 (15 μCi, 0.56 MBq) infused at a constant rate over 1 hour. Following dosing, serial and intermittent whole-blood (including blood for plasma), urine, and fecal samples were collected for a minimum of 336 hours (14 days) postdose for study assessments (recovery, excretion, and pharmacokinetic). Safety was monitored throughout the study.

Venous blood samples were collected into tubes containing K3 EDTA as the anticoagulant. All collection times listed are from the start of the 1-hour infusion. Blood samples (6 ml) for the pharmacokinetic analysis of blood plasma radioactivity were collected predose and at 0.5, 1, 1.083, 1.25, 1.5, 2, 3, 4, 5, 6, 8, 12, 16, 24, 36, 48, 60, 72, 96, 120, 144, 168, 192, 240, 288, and 336 hours. Blood samples (2 ml) to prepare plasma for the pharmacokinetic analysis of GSK2251052 and metabolite M3 were collected predose and at 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 36, 48, and 72 hours. Additional blood samples (4 ml) were collected at 1, 6, and 12 hours to prepare plasma, which was acidified with equal volumes of 50 mM citrate buffer (pH 4.0) and used to examine the stability of a hypothetical acyl glucuronide metabolite of M3. Blood samples (15 ml) to provide plasma for metabolite profiling were collected predose and at 1, 6, 12, 24, 48, 96, 144, and 336 hours. The 96-, 144-, and 336-hour plasma samples were subsequently used for the pharmacokinetic analysis of GSK2251052 and M3. Urine was collected predose and at 0–6, 6–12, and 12–24 hours, and thereafter at 24-hour intervals through 336 hours, while feces were collected predose and at 24-hour intervals through 336 hours.

Quantification of Radioactivity

Radioactivity in blood, plasma, urine, and feces was quantified at PRA International by liquid scintillation counting (LSC) using a Packard 3100TR liquid scintillation counter (Packard Instrument Company, Downers Grove, IL) with low-level count mode. Aliquots of plasma (0.25–1 ml) and urine (1 ml) were mixed directly with appropriate amounts of Ultima Gold scintillation cocktail prior to analysis. Whole blood (0.5 ml) was incubated with 1 ml of Solvable (PerkinElmer) for 1 hour at 60°C, and cooled prior to the addition of 0.1 M EDTA (100 μl). The samples were decolorized by the addition of

![Fig. 1. Chemical structure of [14C]GSK2251052 showing location of the radiolabel.](image-url)
Stage 1

$$\begin{align*}
\text{iPr}_{2}\text{EtNH} & \xrightarrow{\text{H}_{2}} \text{O} \text{B} \text{OH} \text{HCl} \\
\text{GSK2251052} & \xrightarrow{\text{IPr}_{2}\text{EtNH}} \text{O} \text{B} \text{OH} \\
\text{K}_{2}\text{CO}_{3} & \xrightarrow{\text{MeOH}} \text{MeOH} \\
\text{NBN}_{2} & \xrightarrow{\text{HCl}} \text{NH}_{2}\text{HCl}
\end{align*}$$

Stage 2

$$\begin{align*}
\text{iPr}_{2}\text{EtNH} & \xrightarrow{\text{iPr}_{2}\text{EtNH}} \text{O} \text{B} \text{OH} \text{HCl} \\
\text{GSK2251052} & \xrightarrow{\text{IPr}_{2}\text{EtNH}} \text{O} \text{B} \text{OH} \\
\text{K}_{2}\text{CO}_{3} & \xrightarrow{\text{MeOH}} \text{MeOH} \\
\text{NBN}_{2} & \xrightarrow{\text{HCl}} \text{NH}_{2}\text{HCl}
\end{align*}$$

Stage 3

$$\begin{align*}
\text{H}_{2} & \xrightarrow{\text{H}_{2}} \text{O} \text{B} \text{OH} \\
5\% \text{Pd/C} & \xrightarrow{\text{Pd/C}} \text{MeOH} \\
aq \text{HCl} & \xrightarrow{\text{aq HCl}} \text{aq HCl}
\end{align*}$$

M3

iPr$_2$EtNH: Di-isopropylethylamine  
BnBr: Benzyl Bromide  
K$_2$CO$_3$: Potassium Carbonate  
MeOH: Methanol  
CrO$_3$: Chromium (VI) Oxide  
AcOH: Acetic Acid  
Pd/C: Palladium on Carbon  
aq HCl: aqueous Hydrochloric Acid

Accelerator Mass Spectrometry Analyses

Initially, plasma samples were analyzed for radioactivity using liquid scintillation counting as described previously. Samples in which concentrations were determined to be below the lower limit of quantification (LLQ: 870 ng equivalents/ml, 20 dpm/ml) were reanalyzed by accelerator mass spectrometry (AMS; LLQ 2.0 ng equivalents/ml, −0.05 dpm/ml) at GlaxoSmithKline (Ware, Hertfordshire, UK). Analysis by AMS requires conversion of samples via a two-step process of oxidation to CO$_2$ and then reduction to graphite (Vogel, 1992). The AMS provides an isotope ratio of $^{14}$C/$^{12}$C from which $^{14}$C per milligram of carbon is derived (Klody et al., 2005). A carbon content of control plasma of 4.38%, previously established using a Costech Carbon Analyzer (Valencia, CA), was used as the basis for the carbon content of all plasma samples analyzed. Untreated plasma sample aliquots (60 μl) were dried together with copper oxide, sealed into evacuated quartz tubes, and heated at 900°C for 2 hours. The CO$_2$ thus formed was cryogenically transferred into evacuated tubes containing zinc powder, titanium hydride, and cobalt catalyst, and the tubes were sealed. The reduction tubes were heated to 500°C for 4 hours, followed by 550°C for 6 hours to complete the graphitization process. Carbon as graphite, deposited on the cobalt, was pressed into aluminum cathodes and analyzed by AMS on a National Electrostatics Corp. (Middleton, WI) 250-kV single-stage accelerator mass spectrometer (Young et al., 2008). Control samples, including Australian National University (Acton, ACT, Australia) sugar and sodium benzoate, were processed with the analytical run. Instrument standards of pooled Australian National University and synthetic graphite were used to normalize the data and check suitability of instrumental background, respectively. The data from the AMS and carbon content analyses were combined to provide radiocarbon levels for each sample, with background subtraction of predose plasma concentration data as appropriate.

Sample Preparation for Metabolite Profiling

Urine. Urine was pooled across sampling times (0–120 hours) on a total sample weight basis to produce a representative pool for each subject containing ≥90% of the radioactivity excreted in the urine (90.2% of the dose). Additionally, 120–336-hour urine samples from each subject were pooled in the same manner, and equal volumes of each pool were combined to produce a single composite sample. The pooled urine was centrifuged at −21,000g for 10 minutes, and a portion of each supernatant (500–1500 μl) was profiled using high-performance liquid chromatography (HPLC) with radiochemical detection. Triplicate-weighed aliquots of urine (200–2000 μl) were assayed using LSC before and after centrifugation to determine the recovery of radioactivity.

Feces. Feces were pooled across sampling times (0–120 hours) on a total sample weight basis to produce a representative pool for each subject containing ≥90% of the radioactivity excreted in the feces (6.0% of the dose). Due to the low levels of radioactivity in fecal samples, two sets of fecal homogenates were extracted in parallel. One set was used to monitor the recovery of radioactivity, and the second set was used for profiling. The homogenates (−1 g) were extracted twice by the addition of 4 volumes of acetonitrile:methanol:water:formic acid (50%:25%:25%:1%, v/v/v/v) followed by sonication for 20 minutes. Following each extraction, samples were centrifuged at 1620g for 10 minutes at 25°C, and the supernatants were combined in tared tubes. The total weights of the supernatants were determined, and triplicate-weighed aliquots (100–1500 μl) were assayed using LSC to determine the recovery of radioactivity. The supernatants were evaporated to dryness under a stream of nitrogen and reconstituted in 500 μl of water:methanol:water:formic acid (90:10:10:v/v/v) before they were sonicated and centrifuged at 1620g for 10 minutes. The supernatants were transferred to microcentrifuge tubes and centrifuged at 21,000g for 5 minutes. To increase the recovery of radioactivity, the residual pellets were rinsed with 100 μl of water. Following centrifugation, the rinses were combined with the corresponding extracts. The total weights of the
extracts were determined, and triplicate-weighed portions (10–100 µl) were analyzed using LSC to determine the recovery of radioactivity upon reconstitution. Portions (400 µl) of each fecal extract were profiled using HPLC with radiochemical detection.

**Metabolite Profiling and Identification**

Urine and fecal samples were analyzed using an Agilent-1260 HPLC system (Hewlett Packard, Palo Alto, CA) and a Waters Atlantis T3 column (4.6 × 250 mm, 5 µm; Waters Corporation, Milford, MA). Mobile phase A consisted of 100 mM ammonium acetate (pH 4.5, pH adjusted with 100 mM acetic acid) and mobile phase B consisted of 100 mM ammonium acetate (pH 4.5)/methanol (10:90, v/v). The gradient was held at starting conditions (10% B) from 0 to 10 minutes and increased linearly to 70% B from 10 to 55 minutes, and then to 95% B from 55 to 55.1 minutes. The gradient was held at 95% B from 55.1 to 60 minutes and returned to starting conditions at 60.1 minutes and held for a further 5 minutes. The column was held at 95% B from 60 to 60.1 minutes and returned to starting conditions (10% B) from 0 to 10 minutes and increased linearly to 70% B from 10 to 55 minutes, and then to 95% B from 55 to 55.1 minutes. The gradient was held at 95% B from 55.1 to 60 minutes and returned to starting conditions at 60.1 minutes and held for a further 5 minutes. The column was allowed to equilibrate for an additional 10 minutes between injections. The analyses were performed at 30°C and a flow rate of 1 ml/min. The HPLC effluent was split, with 80% of the sample collected into deep-well LumaPlate-96 solid scintillant microplates (PerkinElmer; 0.2 minutes/well) and the remaining 20% directed to a Thermo Finnigan LTQ-Orbitrap Hybrid mass spectrometer equipped with an Electrospray Ionization source (Thermo Scientific, San Jose, CA) for metabolite identification. The LumaPlates, containing dimethylsulfoxide (10 µl/well) to reduce nonspecific binding of drug-related material, were dried on SPE Dry 96 Dual plate driers (Aronauto Technologies, Foster City, CA) under a stream of heated nitrogen and analyzed using a PerkinElmer TopCount NXT scintillation counter (PerkinElmer Life Sciences, Downers Grove, IL). Data from the TopCount NXT were imported into Laura software (version 3.4.11; LabLogic Systems, Inc., Sheffield, UK), and the chromatograms were manually integrated. The lower limits of radiochemical detection (defined as peak height) were determined based on the proposal by Currie (1968) for the measurement of paired radioactive activity, and calculated using counting time (30 minutes) and instrument background. Integrated peak areas of less than 3 times background are reported as <LLQ. Background was assigned for each run by selecting representative regions at the beginning and end of each chromatogram. The radioactive drug-related components in feces and urine are reported as a percentage of the administered dose, and as a percentage of the radioactivity in the matrix (to 1 decimal place). Results were corrected for the recovery of radioactivity following centrifugation (urine) or extraction and reconstitution (feces).

Metabolites were characterized using a ThermoFinnigan Orbitrap XL (Thermo Scientific) mass spectrometer in the positive mode based on accurate mass measurements from full-scan MS data [m/z (mas-to-charge ratio) 100–700 at 30,000 resolution]. Mass spectral data obtained from metabolites identified in the preclinical species (unpublished observations) were also used in the assignment of metabolite structures.

**Monkey Pharmacokinetic Study with 4-Methylpyrazole**

GSK2251052 was administered to cynomolgus monkeys (3 per sex per group) as a single constant rate i.v. infusion over 1 hour at a dose level of 35 mg/kg, either alone or 1 hour after an oral gavage administration of the alcohol dehydrogenase (ADH) inhibitor 4-methylpyrazole (4-MP) (Sigma-Aldrich, St. Louis, MO). GSK2251052 was formulated to help mg/ml as a solution in 0.9% sodium chloride injection, USP (saline), pH 5, and administered at a dose volume of 5 ml/kg/h. 4-MP was formulated as a solution at 8.75 mg/ml in purified water and administered to cynomolgus monkeys at a dose volume of 4 ml/kg. Venous blood samples (0.5 ml) for the plasma pharmacokinetic analysis of GSK2251052 and M3 were collected into tubes containing K2 EDTA as the anticoagulant predose and at 0.5, 0.92, 1.08, 1.25, 1.5, 2, 3, 5, 9, 24, 48, 72, and 120 hours. All times listed are from the start of the 1-hour infusion.

**Quantification of GSK2251052 and Metabolite M3 in Monkey and Human Plasma**

The plasma concentrations of GSK2251052 and metabolite M3 were measured using validated methods based on protein precipitation followed by liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. Briefly, a 50-µl (human) or 15-µl (monkey) aliquot of plasma was added to 250 µl (human) or 100 µl (monkey) of methanol containing formic acid (0.1% v/v), [2H2]-GSK2251052, and [2H3]-M3 (100 ng/ml each) in 96-deep-well plates and vortex mixed for 10 minutes. The samples were then centrifuged at 3000g for 5 minutes. For human samples, 225 µl of the supernatants were transferred to clean 96-deep-well plates and dried under warm nitrogen before reconstitution in 60 µl 0.1% formic acid (v/v) in water. Monkey plasma sample supernatants were diluted by adding 25–150 µl of 0.1% formic acid. Between 1- and 5-µl aliquots of the reconstituted or diluted samples were injected into an LC/MS/MS system consisting of a Waters Acquity ultra high-pressure liquid chromatography (Waters Corporation), a Waters HSS T3 column (50 × 2.1 mm, 1.8 µm) at 50°C, and an MDS Sciex 4000 API-4000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Mobile phase A consisted of water containing 0.1% formic acid (v/v), and mobile phase B consisted of methanol:water/formic acid (50:50:0.1%, v/v). The compounds were eluted from the column at a flow rate of 0.7 ml/min and for human samples with a gradient starting at 22% B, which increased linearly to 30% B from 0.5 to 1.51 minutes and returned to 22% B from 1.51 to 1.6 minutes. For monkey samples, the column was eluted isocratically, with 85% mobile phase A and 15% B. Tandem mass spectrometry analyses were performed using a TurboIonSpray interface (Applied Biosystems/MDS Sciex) operated in the positive mode and a probe temperature of 400°C. The analyses were measured by multiple reaction monitoring of the following [M+H]+ transitions: GSK2251052 m/z 238 → 202 and metabolite M3 m/z 252 → 162. The transitions monitored for the internal standards were 3 mass units higher than the corresponding analyte. Data collection and integration were performed using Analyst software (version 1.4.2; Applied Biosystems/MDS Sciex). Quantification was based on analyte/internal standard peak area ratios and calculated using a weighted 1/x2 linear regression model within SMS2000 (version 2.3; GlaxoSmithKline, Research Triangle Park, NC). The operating range of the assays for both analytes, GSK2251052 and M3, was 5–10,000 ng/ml in human plasma and 100–200,000 ng/ml in monkey plasma.

**Pharmacokinetic Analysis**

The plasma pharmacokinetic parameters were estimated by noncompartmental methods with either WinNonlin Professional Edition version 5.2 (Pharsight, Mountain View, CA) using actual pharmacokinetic sampling times.

**Table 1**

Summary of human pharmacokinetic parameters of GSK2251052, M3, and total radioactivity after a single i.v. infusion dose of [13C]-GSK2251052 (1500 mg)

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Analyte</th>
<th>AUC0–24h</th>
<th>AUC0–t</th>
<th>Cmax</th>
<th>CL</th>
<th>Vs</th>
<th>T1/2</th>
<th>Tmax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/l · h/ml</td>
<td>µg/l · h/ml</td>
<td>µg/ml</td>
<td>l/h</td>
<td>l</td>
<td>h</td>
<td>h</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>GSK2251052</td>
<td>72.3 (7.34)</td>
<td>72.5 (7.41)</td>
<td>24.6 (14.5)</td>
<td>20.7 (7.41)</td>
<td>197 (88.7)</td>
<td>11.6 (9.96–12.1)</td>
<td>0.917 (0.50–0.92)</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>101 (12.1)</td>
<td>103 (12.1)</td>
<td>1.77 (9.77)</td>
<td>NA</td>
<td>NA</td>
<td>77.3 (68.1–78.5)</td>
<td>7.00 (5.00–24.0)</td>
</tr>
<tr>
<td>Radioactivity</td>
<td>191 (10.0)</td>
<td>195 (9.90)</td>
<td>24.3 (16.1)</td>
<td>7.70 (9.90)</td>
<td>348 (12.6)</td>
<td>96.0 (86.2–117)</td>
<td>0.917 (0.92–0.92)</td>
<td>1.62</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Radioactivity</td>
<td>141 (20.8)</td>
<td>193 (21.1)</td>
<td>43.7 (10.2)</td>
<td>7.79 (21.1)</td>
<td>116 (17.8)</td>
<td>143 (7.28–16.1)</td>
<td>0.917 (0.92–0.92)</td>
</tr>
</tbody>
</table>

%CV, % coefficient of variation; NA, not applicable.
Geometric mean (%CV).
Median (range).
(human study), or WinNonlin Enterprise Edition version 4.1 using the nominal sampling times (monkey study). The maximum observed plasma concentration (Cmax), the time at which Cmax was observed, and the time to the last measurable concentration were determined directly from the raw concentration-time data or using WinNonlin. The area under the concentration-time curve from time zero to the last quantifiable time point [AUC(0-t)], and the area from time zero to infinity [AUC(0-∞)] were calculated using the linear up/logarithmic down trapezoidal method. The AUC(0-∞) was estimated as the sum of AUC(0-t) plus Cint/λz, where Cint is the concentration at the last quantifiable time point. The terminal elimination rate constant (λz) was estimated from linear regression analysis of the log-transformed concentration-time profile. The number of points included in the terminal phase was determined by visual inspection of the semilog plots of the concentration-time profiles. The associated apparent terminal elimination half-life (t1/2) was calculated as ln2/λz, where Cint is the concentration at the last quantifiable time point.

Cytosol Incubations. [14C]GSK2251052 (500 mM) was incubated with 4 mg/ml of liver cytosol (monkey and human) containing 7.5 mM NAD+ in 30 mM sodium pyrophosphate buffer at pH 7.4 or 23 mM sodium pyrophosphate buffer at pH 8.4. Incubations without NAD+ or liver cytosol were used as negative controls. Where appropriate, 4-MP was added to incubations to yield a final concentration of 0.6 mM. To verify ADH in the preparations of liver cytosol, human and monkey liver cytosol (4 mg/ml) was incubated with 7.5 mM NAD+, ethanol (0.1%), and 30 mM sodium pyrophosphate buffer at pH 7.4. Incubations in the absence of ethanol or in the presence of 4-MP (0.6 mM) were performed in parallel as controls. To assess the concentration-dependent inhibitory effect of GSK2251052 on its own metabolism, [14C]GSK2251052 (1–50 mM) was incubated with human liver cytosol (1.5 mg/ml) in phosphate buffer, pH 7.4, containing 7.5 mM NAD+ for up to 20 hours.

**In Vitro Sample Preparation and Analysis.** All samples from in vitro incubations, except positive controls with ethanol, were run in triplicate for 1 or 3 hours in a shaking water bath set to 37°C and 100 rpm. Following incubation, individual samples were quenched with acetonitrile such that the ratio of acetonitrile was 45%. Samples were then centrifuged at approximately 21,000g for 5 minutes to remove protein precipitate, and the supernatant was removed to a fresh 1.5 ml vial. Samples were then prepared for radioactivity analysis by combining an aliquot of the incubation mixture (50 μl), with H2O, absolute ethanol, and acetonitrile (200 μl each). The mixture was vortexed, sonicated for 5 minutes to remove protein precipitate, and the supernatant was removed for LSC analysis. Dried samples were analyzed for radioactivity by LSC or AMS at a minimum of six healthy male subjects after a single 1500 mg i.v. infusion dose of [14C]GSK2251052.

**TABLE 2**

Mean percentage of cumulative radioactivity recovered from six healthy male subjects after a single 1500 mg i.v. infusion dose of [14C]GSK2251052

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Urine</th>
<th>Feces</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>% dose</td>
<td>% dose</td>
<td>% dose</td>
</tr>
<tr>
<td>0–24</td>
<td>51.2</td>
<td>0.1</td>
<td>51.3</td>
</tr>
<tr>
<td>0–48</td>
<td>69.2</td>
<td>1.3</td>
<td>70.5</td>
</tr>
<tr>
<td>0–72</td>
<td>76.9</td>
<td>4.3</td>
<td>81.2</td>
</tr>
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<td>0–96</td>
<td>81.1</td>
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<td>86.4</td>
<td>7.6</td>
<td>94.0</td>
</tr>
<tr>
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<td>7.6</td>
<td>95.0</td>
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<td>7.6</td>
<td>95.8</td>
</tr>
<tr>
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<td>88.8</td>
<td>7.6</td>
<td>96.4</td>
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</tr>
<tr>
<td>0–336</td>
<td>90.5</td>
<td>7.6</td>
<td>98.2</td>
</tr>
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a fresh vial. An aliquot (10 μl) of each supernatant, except 7-ethoxycoumarin, was mixed with 5.0 ml Ultima Gold scintillation cocktail and analyzed using liquid scintillation counting. Data obtained from liquid scintillation counting were used to assess the recovery of radiocarbon in sample extracts.

Portions of the replicates were then pooled using equal volumes to produce a single representative sample for each incubation condition. The pooled portions were diluted with water 10-fold prior to analysis using HPLC as described previously in the Metabolite Profiling and Identification section, except that radiochemical detection was performed by a PerkinElmer Radiomatic 625TR series flow scintillation analyzer with Laura version 3.4.11 software and Ultima Flo M scintillation fluid (3 ml/min).

Experiments to Assess the Formation of NADH. GSK2251052 (final concentrations of 0.001, 0.01, 0.1, 1.0, and 10 mM) was incubated with human liver cytosol (1.5 mg/ml), NAD⁺ (0.22 mM), and sodium pyrophosphate buffer, pH 8.8 (22 mM), or potassium phosphate buffer, pH 7.4 (50 mM), at room temperature. The incubations, in triplicate, were conducted in a 96-well plate with the reactions started by addition of GSK2251052. The NADH produced during GSK2251052 oxidation in the incubations was measured at an absorbance of 340 nm using a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). Incubations with the ADH inhibitor 4-MP (0.6 mM) were included as positive inhibitor controls. Incubations without enzyme, NAD⁺, or GSK2251052 were included as negative controls. Incubations containing ethanol in the absence of GSK2251052 were conducted to confirm metabolic activity of the enzyme preparations. Cytosolic and purified ADH incubation samples were analyzed by UV absorbance detection at 340 nm as a function of time (every 60 seconds for 1 hour, or every 2 minutes for 120 minutes). Rates of NADH formation were calculated by monitoring the decrease in NADH formation, and were expressed as the rate (slope).

Fig. 4. Representative HPLC radiochromatogram of pooled urine [0–120 hours (top) and 120–336 hours (bottom)] following a single 1500-mg i.v. infusion of GSK2251052 in humans. CPM, counts per minute.
GSK2251052 (final concentrations of 1 and 10 μM) was incubated with purified ADH (0.025 units/ml), NAD⁺ (7.5 mM), and potassium phosphate buffer, pH 7.4 (50 mM), at room temperature, and samples were analyzed by UV detection as described previously.

Results

Clinical Safety and Tolerability Data. All subjects completed the study as planned and received the correct treatment in the fasting state. The treatments were well tolerated with no deaths, serious adverse events, or withdrawals due to adverse events (AEs) reported. Three subjects (50%) reported AEs, all of which were mild in intensity (headache, diarrhea, infrequent bowel movements, and insomnia) and resolved during the study without treatment or intervention. Two subjects experienced multiple AEs (infrequent bowel movements and headache; insomnia and headache). None of the AEs were considered to be related to the study drug.

Clinical Pharmacokinetic Results. Six healthy fasted males received a single i.v. dose of 1500 mg of [14C]GSK2251052 (15 μCi) infused over 1 hour. The pharmacokinetic parameters for whole blood and plasma radioactivity, GSK2251052, and M3 are presented in Table 1, and the concentration-time profiles for plasma radioactivity are shown in Fig. 3. The bioanalytical method was robust and performed well, with quality control samples for both GSK2251052 and M3 demonstrating accuracy and precision in the range of 0.0–6.2% and 1.5–6.6%, respectively, during analysis of the study samples. The half-life of radioactivity (96 hours) was notably longer than that observed for the parent compound (11.6 hours). The plasma concentration for M3 peaked at 7 hours, and the elimination half-life was 77.3 hours, approaching that observed for total plasma radioactivity. The plasma AUC(0–∞) values for GSK2251052 and M3 were 37 and 53% of the radioactivity AUC(0–∞) value, respectively. Relative to total body water [42 l (Davies and Morris, 1993)], total radioactivity and GSK2251052 were both highly distributed in tissues with volume of distribution at steady state (Vss) values of 348 and 197 l, respectively, and plasma clearance (CLp) for GSK2251052 was 20.7 l/h. Blood-to-plasma-concentration ratios ranged from 1.03 to 2.03 through 24 hours postdose, and the calculated percentage of radioactivity associated with red blood cells ranged from 44 to 71.9% through 24 hours postdose. The AUC(0–∞) of radioactivity in plasma and whole blood were generally similar (2% difference).

Mass Balance and Excretion in Urine and Feces. The mean total recovery of radioactivity was 98.2% (range of 92.7–99.9%) (Table 2), with the majority of the dose (90.5%) excreted in the urine and fecal elimination representing a minor route of elimination (7.6% of the dose). Approximately 80% of the dose was recovered during the first 72 hours, and by 192 hours, 95% of the administered dose had been recovered. Excretion of residual radioactivity into the urine continued throughout the 336-hour collection period, which is consistent with the long plasma half-life of M3.
Metabolite Profiling and Characterization. Low levels of radioactivity present in plasma precluded metabolite profiling analysis of this particular matrix using conventional approaches. Representative HPLC radiochromatograms of pooled urine (0–120 and 120–336 hours) and fecal extracts (0–120 hours) are shown in Figs. 4 and 5. Individual and mean quantitative data for metabolites from the six subjects are presented in Table 3. GSK2251052 and metabolite M3 represented a mean of 28.9 and 49.7% of the administered dose, respectively, through 336 hours postdose in urine. Three minor metabolites were also detected in urine and identified by mass spectrometry (Supplemental Table 1), but were below the LLQ: M1 (deboronation, oxidation), M2 (deboronation), and M7 (N-acetylated M3). The proposed metabolic scheme for GSK22520152 in humans is shown in Fig. 6.

Mean combined extraction and reconstitution efficiencies of radioactivity from the fecal homogenates were 94.8%. The principal components in feces were metabolites M3 and M1, which represented a mean of 3.3 and 1.4% of the dose through 120 hours postdose, respectively. Unchanged GSK2251052 was not detected in feces. Overall, at least 83% of the administered radioactivity was identified in urine and feces.

In Vitro Investigations of the Enzyme Responsible for the Formation of M3. The in vitro metabolism of [14C]GSK2251052 and the formation of the oxidative metabolite M3 were investigated in selected tissue subcellular fractions from cynomolgus monkey (liver S9, cytosol, microsomes, as well as, lung and kidney S9) and human (liver S9, cytosol, and microsomes). No metabolism of [14C]GSK2251052 or formation of metabolite M3 was observed in monkey liver, lung, or kidney S9, or human liver microsomes in the presence of an NADPH regeneration system (unpublished data). In contrast, in the presence of NAD+, low (≤10%) but detectable metabolism of [14C]GSK2251052 and the formation of M3 was observed in monkey liver S9, cytosol, and microsomes (Table 4), but not in monkey lung and kidney S9, or in human liver S9, cytosol, or microsomes (unpublished data). Additionally, M3 was not observed in monkey liver S9 or cytosol containing NAD+ cofactor following incubation in the presence of the ADH inhibitor 4-MP.

Although M3 was not observed in human liver cytosolic and purified ADH enzyme incubations, ADH involvement in GSK2251052 metabolism was further assessed by spectrophotometric measurement of NADH produced in the incubations as a result of oxidation to an aldehyde intermediate of M3. In human liver cytosol and purified ADH enzyme incubations with GSK2251052, a time-dependent
increase in absorbance at 340 nm was observed. This increase in absorbance was dependent on the ADH cofactor, NAD+, and was not observed in the presence of the ADH inhibitor, 4-MP (Fig. 7).
represented the significant majority of the plasma radioactivity AUC(0-\(t\)). Additionally, the elimination half-life of M3 (77.3 hours) approached that of plasma radioactivity, 96 hours. Although an acyl glucuronide conjugate of M3 had not been previously observed in other metabolism studies (unpublished data), analysis of acidified plasma from this clinical study provided confirmation that such a conjugate, if

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Treatment</th>
<th>(\text{AUC}_{(0-\infty)}^{b,c})</th>
<th>(C_{\text{max}}^{b})</th>
<th>CL (\text{h/l/kg})</th>
<th>(Vss^{b})</th>
<th>MRT (h)</th>
<th>(T1/2^{b})</th>
<th>(T_{\text{max}}^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSK2251052</td>
<td>Alone</td>
<td>60.7 (51.3–76.7)</td>
<td>46.0 (17.3–99.1)</td>
<td>0.60 (0.46–0.68)</td>
<td>3.04 (1.66–3.97)</td>
<td>4.9 (3.6–5.8)</td>
<td>5.4 (4.8–5.9)</td>
<td>0.92 (0.92–0.92)</td>
</tr>
<tr>
<td></td>
<td>Plus 4-MP</td>
<td>161 (148–18)</td>
<td>68.6 (21.9–461)</td>
<td>0.22 (0.19–0.24)</td>
<td>2.53 (1.99–2.88)</td>
<td>11 (10–12)</td>
<td>9.2 (8.6–9.7)</td>
<td>0.92 (0.92–0.92)</td>
</tr>
<tr>
<td>M3</td>
<td>Alone</td>
<td>52.3 (42.8–67.0)</td>
<td>4.15 (3.49–4.54)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3.0 (3.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Plus 4-MP</td>
<td>11.0 (9.4–13.0)</td>
<td>0.36 (0.32–0.42)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>9.0 (9.0–48)</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable.

* \(N = 3\) treatment, and data from males shown only for brevity.

**Values are the mean and (range), except for \(T_{\text{max}}\), which is shown as the median (range).

For M3, \(\text{AUC}_{(0-\infty)}\).

Fig. 8. Individual plasma concentration-time profiles of GSK2251052 and M3 from LC/MS/MS analyses following a single i.v. infusion administration of GSK2251052 at 35 mg/kg alone or in combination with 4-MP at 35 mg/kg to male monkeys.
present in plasma, would not hydrolyze and interfere with the quantitative assessment of M3.

Consistent with the plasma pharmacokinetic data, analysis of urine demonstrated that metabolite M3 was a significant pathway of clearance for GSK2251052, consisting of approximately 50% of the dose recovered in that matrix. M3 was considered major as it represented greater than 10% of drug-related exposure, and therefore, according to the International Conference on Harmonization (Non-Clinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals, International Conference on Harmonization Guidance M3(R2), 2009; http://www.emea.europa.eu/pdfs/human/ich/028695en.pdf), was investigated further in the relevant toxicity species (unpublished data). In addition to M3, three other minor metabolites of GSK2251052 were also identified in this study. Metabolite M1, which results from deboronation and oxidation of the propanol side chain, represented 1.4% of the dose recovered in the feces. M1 was also detected in urine but below quantifiable levels, similar to the two other minor metabolites, M2 and M7. Metabolite M3 results from a simple oxidation of the propanol side chain to the corresponding carboxylic acid derivative; however, it has been determined previously that the metabolite does not appear to have significant antibacterial activity (Peter DeMarsh, internal communication).

To assess the enzyme responsible for the formation of M3, a series of in vitro studies were conducted using both human and monkey hepatic and nonhepatic tissue subcellular fractions. The limited formation of M3 in these experiments in the presence of NADPH suggested that the metabolism of \(^{14}\text{C}\)GSK2251052 was likely not mediated by cytochrome P450 enzymes. Additionally, the low (≤10%), but detectable, formation of M3 observed in monkey liver S9 in the presence of NAD\(^+\) was eliminated in the presence of 4-MP, a known inhibitor of monkey liver ADH (Makar and Tephly, 1975). Although M3 was not detected in human liver cytosolic or purified ADH incubations fortified with NAD\(^+\), the NADH produced during GSK2251052 oxidation could be assessed by the UV spectrophotometric measurement of the conversion of NAD\(^+\) to NADH, which results in a time-dependent increase in absorbance at 340 nm. The NADH formation in these enzyme preparations was dependent on the ADH cofactor, NAD\(^+\), and was not observed in the presence of the ADH inhibitor 4-MP. Class I ADH enzymes (ADH1A, 1B, and 1C) are highly sensitive to 4-MP, and demonstrate substrate inhibition at high concentrations (Riveros-Rosas et al., 1997). Indeed, a concentration-dependent inhibitory effect of GSK2251052 on its own metabolism was observed as noted by the overlapping 340-nm absorbance spectra at the highest concentration of GSK2251052 (100 μM) in the presence of NAD\(^+\) compared with that observed in the presence of the ADH inhibitor, 4-MP. In contrast, at a substrate concentration of 1 μM, time-dependent absorbance changes were only observed with incubations containing NAD\(^+\), and the absorbance spectrum was identical in the absence or presence of cofactor and the ADH inhibitor 4-MP. Taken together with consideration that M3 resulted from oxidation of the propanol side chain to the corresponding carboxylic acid, the in vitro data indicated that ADH may be involved in metabolism of \(^{14}\text{C}\)GSK2251052. To test this hypothesis, a pharmacokinetic interaction study was designed and conducted in monkeys with GSK2251052 and 4-MP. An i.v. administration of GSK2251052 to monkeys, in combination with an oral dose of 4-MP, markedly decreased the plasma clearance of GSK2251052. The resultant increase in GSK2251052 plasma exposure was observed with a concordant reduction in M3 exposure (Fig. 8). Additionally, the terminal half-life and MRT of GSK2251052 were markedly increased in the presence of 4-MP, with no impact on Vss. Collectively, these experiments provide strong evidence that ADH, potentially in association with aldehyde dehydrogenase (ALDH), is the enzyme(s) involved in the metabolism of GSK2251052 and formation of M3. Both ADH and ALDH are polymorphic enzymes and critical players in ethanol metabolism; ADH1B catalyzes the oxidation of ethanol to acetaldehyde, and ALDH2 catalyzes the oxidation of acetaldehyde to acetate. Functional polymorphisms in the ADH1B and ALDH2 genes (Agarwal, 2001) have a significant influence on the activities of both enzymes, and are stratified by race, with the ADH1B*2 (increased catalytic activity) and ALDH2*2 (reduced catalytic activity) alleles being common in East Asians and virtually absent in Caucasians (Eriksson et al., 2001).
Theoretically, subjects with increased or rapid ADH activity may quickly metabolize GSK2251052, leading to lower parent concentrations, which in turn may impact efficacy. Subjects with reduced or inactive ALDH activity may slowly or ineffectively metabolize the aldehyde intermediate to the acid (M3).

In conclusion, following i.v. administration, the recovery of [14C]GSK2251052 and related components from urine and feces was essentially complete, with urine being the principal route of excretion (Fig. 9). Metabolite M3, a product of oxidation of the propanol side chain, was the primary metabolite of GSK2251052 and the predominant circulating component in plasma. Our data suggest that M3 is formed by ADH, potentially in association with ALDH. These are polymorphic enzymes, and therefore, the clinical exposure of GSK2251052 and M3 may vary between certain ethnic populations (Enomoto et al., 1991; Chen et al., 2009).

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Contributed new reagents or analytic tools: Conde.
Performed data analysis: Bowers, Chism, O’Mara, Yueh, Huynh, Reese, Tenero, Kurtinecz.

Wrote or contributed to the writing of the manuscript: Bowers, Young, Chism, O’Mara, Sigafoos, Yueh, Huynh, Reese, Cunningham, Tomayko, Stump, Tenero, Kurtinecz, Patel, Dumont.

References

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