**Title**

# Investigation of Metabolism and Disposition of GSK1322322, a PDF Inhibitor, in Healthy Humans Using Entero-Test® For Biliary Sampling

Authors:

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## Supplemental Data

## Introduction

The major metabolites of GSK1322322 were structurally characterized using samples from rat isolated perfused rat liver and rat balance excretion studies using radiolabelled in vitro and in vivo studies. These studies are briefly described as follows.

## Materials and Methods

### Isolated Perfused Rat Livers (IPRL)

To characterize the major metabolites of GSK1322322 in rat bile, an isolated perfused rat liver experiment was conducted. Three male rats (Sprague-Dawley strain supplied by Charles River Laboratories, Inc., Raleigh, NC) were used in this study. Animals were surgically cannulated and connected to the perfusion apparatus using an established procedure (Curtis et al., 1971). The dose was delivered at a target dose of 30 mg/kg [$^{14}$C]GSK1322322 into the constantly mixing perfusate reservoir approximately 15-30 minutes following the initiation of perfusion. Liver perfusion continued for four hours after dosing.

Post-dose bile was collected, and stored at -70°C if not in use. The biliary recovery of the total administered radioactivity was 61%, 31% and 62% from rat livers 1, 2 and 3, respectively. The weight of bile produced during the perfusion experiment by rats 1, 2 and 3 were *ca* 2.9, 1.7 and 2.3 g, respectively. All the bile samples were pooled to give a single representative sample of bile, vortex mixed and then centrifuged at *ca* 12,000 $g_{av}$ (Eppendorf 5415C, Brinkman Instruments, Westbury, NY) at ambient temperature for 5 minutes. The resulting supernatant was injected onto a semi-preparative column for isolation of M6 and M9. NMR analysis was used to determine their definite structures. The semi-preparative HPLC and NMR conditions for the separation and analysis are detailed in Table 1 and Table 2.

**In Vivo Rat Bile Collection and Metabolite Characterization**

Three bile duct-cannulated (BDC) male adult Sprague Dawley rats (Hilltop Lab Animals, Inc,; Scottdale, PA, USA) were administered orally with [$^{14}$C]GSK1322322 at a nominal dose level of 100 mg/kg (dose vehicle was 1% methylcellulose) as part of a rat mass balance/excretion study (conducted at Covance Laboratories Inc. Madison, WI). The 0-24 hour rat bile was collected, pooled and mixed; the resulting sample was stored at -70° C when not in use. The pooled bile samples were thawed at room temperature and centrifuged at *ca* 3,000 $g_{av}$ (Eppendorf 5415C, Brinkman Instruments, Westbury, NY) at room temperature for 5 minutes to remove any particulates prior to HPLC-MS$^n$ analysis. During these analyses, radio detection was used in parallel with the mass spectrometer to assist the identification of radio-labeled drug related materials and to confirm peak assignments. The HPLC and the mass spectrometric conditions used for analyzing the samples are detailed in Table 3.

## Results

As summarized in Table 4 (of Supplemental Data), M6 and M9 were structurally characterized by NMR and LC-MS$^n$ analysis in these two studies, with the accurate mass data obtained during the in vivo rat study. Metabolites M1 to M4 were also characterized using accurate mass HPLC-MS$^n$.

## References

Curtis CG, Powell GM, Stone SL (1971). Perfusion of the Isolated Rat Liver. *J Physiol.* **213**:14-15.

**Table 1 HPLC Conditions for the Isolation of [$^{14}$C]GSK1322322 and Related Metabolites from IPRL Bile**

| | | | |
|---|---|---|---|
| HPLC System: | Agilent 1100 series | | |
| Software Version: | Chemstation REV B.02.01-SR1 | | |
| Column: | Phenomenex Synergi Polar RP-80 250x10 mm 4µ | | |
| Guard Column: | Polar 10x10mm | | |
| Temperature: | Ambient | | |
| Solvent A: | 25 mM Ammonium Formate pH 3.78 | | |
| Solvent B: | Methanol | | |
| Flow rate: | 4.73 mL/min | | |
| Solvent gradient: | Time (min) | % A | % B |
| | 0 | 70 | 30 |
| | 10 | 70 | 30 |
| | 65 | 15 | 85 |
| | 66 | 0 | 100 |
| | 75 | 0 | 100 |
| Sample Preparation: | IPRL: Sample was vortexed, centrifuged and the resulting supernatant was diluted 1:1 with buffer and subsequently utilized for the HPLC separation<br>Parent: 100µL of 5mM solution were diluted with 200µL of buffer and subsequently utilized for the HPLC separation | | |
| Injection Volume: | IPRL: 400µL Manual Injection<br>Parent: 300µL Manual Injection | | |
| UV Detector [wavelength]: | Agilent 1100 series Model G1315A DAD [220, 230, 254, 280, 310 nm] | | |
| Fraction Collector: | Agilent 1100 series Model G1364C Analyt-FC | | |
| Online Radiodetector: | LabLogic β–RAM Model 3B | | |
| Mass Spectrometer | ThermoQuest Finnigan LCQDuo | | |
| Ionization mode: | Electrospray – positive ion | | |
| Software version: | Xcalibur 2.0 | | |
| Split ratio to MS: | Approximately 1% | | |
| Scanning mass ranges: | 100 – 1200 (full scan MS) | | |

**Table 2 NMR Conditions for the Analysis of GSK1322322 and its Metabolites**

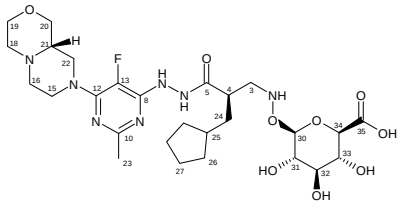
| | |
|---|---|
| NMR Spectrometer: | Bruker AVANCE II 700MHz |
| Software Version: | TopSpin 2.0.4 |
| Probe: | 5 mm TCI cryoprobe |
| Nucleus Detected: | Proton |
| Solvent: | 1:1 Acetonitrile-d3:$D_2O$ |
| Experiments: | 1D $^{1}H$<br>2D $^{1}H$-$^{1}H$ gCOSY<br>2D $^{1}H$-$^{13}C$ gHSQC<br>2D $^{1}H$-$^{13}C$ gHMBC |

| | |
|---|---|
| NMR Spectrometer: | Bruker AVANCE II 600MHz |
| Software Version: | TopSpin 2.0.4 |
| Probe: | 5mm TFI cryoprobe |
| Nuclei Detected: | Proton<br>Fluorine |
| Solvent: | 1:1 Acetonitrile-d3:$D_2O$ |
| Experiments: | 1D $^{1}H$<br>1D $\{^{1}H\}$ $^{19}F$ |

**Table 3 HPLC-MS Conditions for the Analysis of [$^{14}$C]GSK1322322 and its Metabolites**

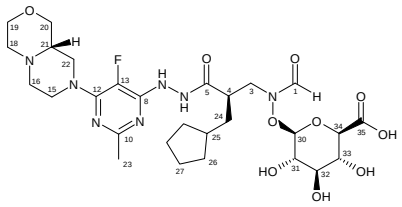
| | | | |
|---|---|---|---|
| HPLC Pump: | Agilent 1100 | | |
| Autosampler: | CTC LC-PAL | | |
| Column: | Synergi Polar-RP 4u, 250 x 4.6mm, 4u | | |
| Solvent A: | 25mM ammonium formate, pH 3.78 | | |
| Solvent B: | Methanol | | |
| Flow rate: | 1.0 mL/min | | |
| Gradient: | Time (min) | %A | %B |
| | 0 | 95 | 5 |
| | 5 | 95 | 5 |
| | 80 | 15 | 85 |
| | 80.2 | 0 | 100 |
| | 85 | 0 | 100 |
| | 85.2 | 95 | 5 |
| | 90 | 95 | 5 |
| On-line Radiodetector | IN/US Beta Ram model 4 | | |
| Split ratio: | ~10% to MS | | |
| Mass Spectrometer: | ThermoElectron LTQ XL with Xcalibur 2.0.7<br>ThermoElectron LTQ-Orbitrap with Xcalibur 2.0.7 | | |
| Ionisation mode: | Electrospray, positive ion | | |

**Table 4 Summary of Structural Information for Major Metabolites of GSK1322322**

<table>
<tr><th>ID</th><th>Mass Spectral data (m/z)[1]</th><th>NMR data in<br>1:1 acetonitrile-$d_3$/$D_2O$</th></tr>
<tr><td>M1</td><td>Full mass: 496.2666<br>MS$^2$: 223.1229, 267.1490, 283.1678, 435.2519, 478.2572</td><td>NA</td></tr>
<tr><td>M2</td><td>Full mass: 496.2669<br>MS$^2$: 223.1229, 267.1490, 283.1678, 435.2517, 478.2572</td><td>NA</td></tr>
<tr><td>M3</td><td>Full mass: 496.2668<br>MS$^2$: 223.1229, 267.1490, 311.1628, 435.2517, 478.2572</td><td>NA</td></tr>
<tr><td>M4</td><td>Full mass: 494.2513<br>MS$^2$: 223.1227, 267.1487, 283.1676, 311.1623, 433.2355, 466.2574, 476.2412.</td><td>NA</td></tr>
<tr><td>M6</td><td>  <br>Full mass: 628.3088<br>MS2: 283.1679, 419.2566, 434.2680, 452.2784, 610.3007.<br>.</td><td>
<table>
<tr><th>Atom #</th><th>$^1$H δ (ppm)</th><th>$^{13}$C δ (ppm)</th></tr>
<tr><td>3</td><td>nd</td><td>nd</td></tr>
<tr><td>4</td><td>nd</td><td>nd</td></tr>
<tr><td>5</td><td>•</td><td>nd</td></tr>
<tr><td>8</td><td>•</td><td>nd</td></tr>
<tr><td>10</td><td>•</td><td>nd</td></tr>
<tr><td>12</td><td>•</td><td>nd</td></tr>
<tr><td>13</td><td>•</td><td>nd</td></tr>
<tr><td>15</td><td>nd & 4.18</td><td>44.9</td></tr>
<tr><td>16</td><td>nd</td><td>nd</td></tr>
<tr><td>18</td><td>nd</td><td>nd</td></tr>
<tr><td>19</td><td>3.63 & 3.82</td><td>65.4</td></tr>
<tr><td>20</td><td>3.27 & 3.77</td><td>67.0</td></tr>
<tr><td>21</td><td>nd</td><td>nd</td></tr>
<tr><td>22</td><td>nd & 4.06</td><td>45.6</td></tr>
<tr><td>23</td><td>2.20</td><td>24.0</td></tr>
<tr><td>24</td><td>1.24 & 1.57</td><td>36.0</td></tr>
<tr><td>25</td><td>1.83</td><td>37.0</td></tr>
<tr><td>26</td><td>1.03 & 1.86</td><td>31.5</td></tr>
<tr><td>27</td><td>1.49 (br)</td><td>24.5</td></tr>
<tr><td>30</td><td>4.49</td><td>103.7</td></tr>
<tr><td>31</td><td>3.15</td><td>71.5</td></tr>
<tr><td>32</td><td>3.36</td><td>ambiguous</td></tr>
<tr><td>33</td><td>3.36</td><td>ambiguous</td></tr>
<tr><td>34</td><td>3.53</td><td>74.1</td></tr>
<tr><td>35</td><td>•</td><td>nd</td></tr>
</table>
F13= -169.1 ppm<br>Many signals broadened and/or doubled due to dynamic processes whose rate occurs at or near that of the NMR timescale</td></tr>
</table>

| ID | Mass Spectral data (m/z)[1] | NMR data in 1:1 acetonitrile-$d_3$/$D_2O$ |
|---|---|---|

M9[2]



Full mass: 656.3041
$MS^2$: 223, 267, 293, 446, 462, 480, 638

| Atom # | $^1H$ δ (ppm) | $^{13}C$ δ (ppm) |
|---|---|---|
| 1 | 7.80 | 161.1 |
| 3 | 3.37 & 3.77 | 51.9 |
| 4 | 3.20 | 41.2 |
| 5 | • | nd |
| 8 | • | nd |
| 10 | • | nd |
| 12 | • | nd |
| 13 | • | nd |
| 15 | nd & 4.20 | 44.6 |
| 16 | nd | nd |
| 18 | nd | nd |
| 19 | 3.65 & 3.85 | 65.1 |
| 20 | 3.32 & 3.80 | 66.7 |
| 21 | nd | nd |
| 22 | nd & 4.09 | 45.3 |
| 23 | 2.19 | 24.1 |
| 24 | 1.24 & 1.56 | 35.6 |
| 25 | 1.85 | 36.9 |
| 26 | 1.02 & 1.89 | 31.6 |
| 27 | 1.50 (br) | 24.5 |
| 30 | 4.99 | 105.2 |
| 31 | 3.31 | ambiguous |
| 32 | ambiguous | ambiguous |
| 33 | ambiguous | ambiguous |
| 34 | ambiguous | ambiguous |
| 35 | • | nd |

F13= -169.3 ppm

Many signals broadened and/or doubled due to dynamic processes whose rate occurs at or near that of the NMR timescale

1. Positive ion, if accurate mass, the experimental masses are within 5ppm accuracy from theoretical masses, unless noted specifically.
2. $MS^2$ of M9 is nominal mass.
3. NA- no analyzed.