ABSTRACT:
The disposition and metabolism of LY295501 was studied in mice, rats, and monkeys. This novel diaryl sulfonylurea oncolytic agent is structurally related to sulofenur and shows excellent activity in a broad range of mouse antitumor models. The compound is well absorbed, giving plasma concentrations greater than 200 μg/ml after oral doses of 30–100 mg/kg, where it appears to be completely bound (>99.9%) to plasma proteins. The high degree of protein binding may be a factor in its relatively long half-life, which ranges from about 8 hr in rats and 15 hr in mice to 50 hr in monkeys. While more material was excreted in feces than in urine from mice and rats given single oral doses of [14C]LY295501, urine was the major route of elimination in monkeys. Three major metabolites—all formed via oxidation of the saturated part of the benzodihydrofuran moiety—were characterized in the urine of mice, rats, and monkeys. It is interesting that two of these metabolites are derived from opening of this saturated ring, an unusual metabolic process which represents a significant part of the metabolism of LY295501. As with sulofenur, metabolites of 3,4-dichloroaniline formed after metabolic cleavage of the sulfonylurea linkage were also found in urine. Unlike sulofenur, these do not seem to have major toxicological significance, but their formation does explain the minor methemoglobinemia observed in toxicology studies of LY295501. Even though only trace amounts of LY295501 were found in urine, LY295501 is the predominant drug-related material in plasma, along with small amounts of other, relatively nonpolar, metabolites.

LY295501 is a novel oncolytic agent (1–3) presently in phase I clinical trials. It is a sulfonylurea closely related to the compound sulofenur, the metabolism of which has been previously reported (scheme) (4–7):

Sulofenur

\[
\text{LY295501}
\]

Sulofenur entered clinical trials after it was shown to have a broad spectrum of activity in mouse syngeneic and xenograft tumor models (8). However, it did not show consistent activity, and its dosing was limited by the appearance of anemia with the characteristic of methemoglobin formation (9, 10). Anilines are known to be potent inducers of methemoglobinemia (11), and further work showed that metabolic cleavage liberating the p-chloroaniline moiety of sulofenur and related analogs is associated with the observation of methemoglobinemia in a mouse model (7). LY295501 was developed as a significantly more potent analog of sulofenur which also is highly active in a broad spectrum of mouse antitumor models (1–3). Experiments defining its disposition and metabolism in mice, rats, and monkeys are reported here.

Materials and Methods

Liquid chromatography (LC) was performed using a Waters Instrument Co. (Milford, MA) 600 Pump/990 Photo-Diode Array UV detector and 712 WISP autosampler. Mass spectral data were obtained on a Sciex API III mass spectrometer operating in ionspray (~3600 V) mode with orifice potential of 60 V, m/z 150–800 scanned in 0.5 amu steps over 1 sec. For LC/MS analysis, the LC eluent was split to allow a flow of 20 μl/min into the ionspray source. Collision activated dissociation (CAD) used collisional energy of 30 eV with argon set at 365 × 10^2 atoms/cm^2.

Materials. [14C]LY295501 (N-(2,3-dihydrobenzofuran-5-sulfonyl)-N’-(3,4-dichlorophenyl)-[UL-14C]urea; specific activity 27.8 μCi/mg) was prepared by Donald L. K. Kau (Lilly Research Laboratories, lot 497–3-242) and its radiochemical purity was found to be 98.7% by LC analysis (4.6 × 250 mm DuPont Zorbax ODS column eluted with 1 ml/min of 50% acetonitrile/0.5% sodium phosphate buffer, pH 4.9). Unlabeled LY295501 (N’-(2,3-dihydrobenzofuran-5-sulfonyl)-N’-(3,4-dichlorophenyl)urea; lot 223NK2, purity 98.4%), internal standard LY301058 (N-(N-methylindol-5-sulfonyl)-N’-(3,4-dichlorophenyl)urea) and LY282478 (VI, a synthetic intermediate of LY295501) were prepared at Eli Lilly and Co. 3,4-Dichloroaniline (LY004892; V) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Authentic samples of all other
identified or potential metabolites were prepared and used for qualitative identification of metabolites (12, 13). These are: LY311235 (II), LY317813 (III), LY335937 (IV), LY301226 (VII), LY301202 (VIII), LY009844 (IX), LY307578 (X), LY301222 (XI) and LY349309 (XII). Structures for these compounds are shown in figs. 1 and 2.

Methods: Pharmacokinetic Studies. Oral administration of LY295501 in 10% acacia suspension (3–100 mg/ml) was by gavage to female C3H mice (30 or 300 mg/kg) and male Fischer 344 rats (100 –1000 mg/kg) or by nasogastric intubation to female Rhesus monkeys (10 –100 mg/kg). IV doses of LY295501 were administered by tail vein to mice at 30 mg/kg using a 3.75 mg/ml solution in 1% Tween80/9% absolute ethanol/polyethylene glycol diluted 3:1 with C3H mouse plasma. The limit of solubility for LY295501 (4 mg/ml) in the stock solution precluded comparable iv dosing in rats and monkeys. Blood was collected by cardiac puncture under CO2 anesthesia from three mice at times of 1, 2, 4, 12, 24, 36, 48, and 72 hr, and from three rats at times of 2, 4, 6, 12, 24, 36, 48, and 60 hr. Blood samples (approx. 2 ml aliquots) were collected serially at times of 2, 4, 8, 24, 72, 120, and 168 hr from three monkeys dosed orally with 10 mg/kg LY295501. Three weeks later, these monkeys were given oral doses of 100 mg/kg and blood samples were serially collected at the same times.

Plasma Levels of LY295501. Plasma was isolated by centrifugation of blood and stored at −70°C until analysis. Because of the low solubility of LY295501 and the internal standard in aqueous media, stock solutions of 0.1 mg/ml LY301058 (internal standard) and 0.002–0.4 mg/ml LY295501 were prepared in acetonitrile, a solvent in which these sulfonylureas decompose slowly to give the corresponding sulfonamide and aniline fragments. Solutions thus had to be used on the day prepared and kept in a refrigerator when not in use. To each 100 µl aliquot of plasma was added 150 µl internal standard solution, varying amounts of LY295501 stock solutions (standard curve only), and acetonitrile to give a total of 300 µl acetonitrile added. After centrifugation at approximately 1000g for 15 min, 20 µl of supernatant was injected onto a 5×250mm Zorbax RX-C8 column eluted at 2 ml/min with 26% acetonitrile/25mM sodium phosphate buffer, pH7. Retention times for LY295501 and LY301058 were approximately 14 and 19 min, respectively. Peak areas were calculated based on UV absorbance at 254 nm. Using a quadratic fit of the standard curve, concentrations of 2–600 µg/ml (1–600 µg/ml in rat plasma) were measured within ±4% (both intra- and inter-day precision, 3-day validation).

Plasma Protein Binding of LY295501. Plasma was prepared by centrifugation of blood (200g for 10–15 min; stored at −70°C until analysis) which was collected in heparinized tubes from female C3H mice, male Fischer 344 rats, female Rhesus monkeys, and a male human subject. Stock solutions of LY295501 in dimethyl sulfoxide at 100-fold final concentration were prepared immediately before use to give 100 or 500 µg LY295501 in 1 ml plasma or Krebs-Ringer solution, pH7.4 (used as recovery control). Samples were incubated at 37°C for 20 min and transferred to Amicon #4104 Centrifree micropartition units (Beverly, MA), and the units were centrifuged for 20 min at 1900g at 37°C. Filtrates were analyzed over a standard curve range of 0.1–10 µg/ml (prepared in filtrate from control monkey plasma) using the chromatography conditions described above. Recovery of LY295501 in dimethyl sulfoxide at 100-fold final concentration was prepared immediately before use to give 100 or 500 µg LY295501 in 1 ml plasma or Krebs-Ringer solution, pH7.4 (used as recovery control). Samples were incubated at 37°C for 20 min and transferred to Amicon #4104 Centrifree micropartition units (Beverly, MA), and the units were centrifuged for 20 min at 1900g at 37°C. Filtrates were analyzed over a standard curve range of 0.1–10 µg/ml (prepared in filtrate from control monkey plasma) using the chromatography conditions described above. Recovery of LY295501 in Krebs-Ringer buffer at pH7.4 was >90% at the concentrations used, indicating there was little nonspecific binding to the filter units.

Balance/Metabolism Studies. Female C3H mice (22 ± 2 g, Charles River, Wilmington, MA), male Fischer 344 rats (220 ± 20 g, Charles River), and female Rhesus monkeys (4–5 kg, Charles River) were placed in appropriate steel metabolism cages and allowed food and water ad libitum (Purina chow
5002, 5001 and 5048 (St. Louis, MO, respectively) post dose. All animal species were fasted 16 hr prior to oral dosing. Oral doses of 100 mg/kg [14C]-LY295501 were administered as described above to three groups of two mice each (50 μCi/kg), three rats (20 μCi/kg), or three monkeys (50 μCi/kg). Urine and feces were collected at 24, 48, 72, 96, and 120 hr time intervals in mice and rats, and at 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 hr in monkeys. Urine was collected over dry ice for the first 24 hr. Radiocarbon in aliquots of the dose solutions (before and after dosing) and urine samples was determined by scintillation counting (Aquasure Scintillation Cocktail, DuPont, Wilmington, DE) using a TriCarb system (Packard Instrument Co., Meriden, CT). Efficiencies were 90% or greater for [14C] using an external standard. Radiocarbon in feces was determined by combustion analysis (306 Packard Oxidizer, Packard Instrument Co.) using approximately 0.5 g dried aliquots of a dried fecal suspension prepared by addition of 3 volumes of a 5% lauryl sulfate aqueous solution to feces. Residual radiocarbon in mice and rats was determined by dissolution of carcasses in 2.5 volumes (to weight) of boiling 10% KOH in absolute ethanol. Aliquots of 0.5 g were then treated with 200 μl hydrogen peroxide (30% w/v), glacial acetic acid, and water in that order, and counted using 12 ml of scintillation cocktail.

For quantitation of metabolites, each 0–24 hr urine sample was filtered (0.2μ, Gelman, Ann Arbor, MI), and 150 μl injections were analyzed by LC/UV using a 5×250 mm Zorbax RX-C8 column at 30°C eluted with 17% acetonitrile/25mM sodium phosphate, pH 7, buffer at 1.5 ml/min for 5 min followed by linear change to 26 min to 50% acetonitrile. Column effluent was collected in 0.5 min fractions, and 12 ml Aquasence scintillation cocktail (DuPont) was added to each fraction for subsequent scintillation counting. The detection limit was based on 3X background and corresponded to 5002, 5001 and 5048 (St. Louis, MO, respectively) post dose. All animal species were fasted 16 hr prior to oral dosing. Oral doses of 100 mg/kg [14C]-LY295501 were administered as described above to three groups of two mice each (50 μCi/kg), three rats (20 μCi/kg), or three monkeys (50 μCi/kg). Urine and feces were collected at 24, 48, 72, 96, and 120 hr time intervals in mice and rats, and at 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 hr in monkeys. Urine was collected over dry ice for the first 24 hr. Radiocarbon in aliquots of the dose solutions (before and after dosing) and urine samples was determined by scintillation counting (Aquasure Scintillation Cocktail, DuPont, Wilmington, DE) using a TriCarb system (Packard Instrument Co., Meriden, CT). Efficiencies were 90% or greater for [14C] using an external standard. Radiocarbon in feces was determined by combustion analysis (306 Packard Oxidizer, Packard Instrument Co.) using approximately 0.5 g dried aliquots of a dried fecal suspension prepared by addition of 3 volumes of a 5% lauryl sulfate aqueous solution to feces. Residual radiocarbon in mice and rats was determined by dissolution of carcasses in 2.5 volumes (to weight) of boiling 10% KOH in absolute ethanol. Aliquots of 0.5 g were then treated with 200 μl hydrogen peroxide (30% w/v), glacial acetic acid, and water in that order, and counted using 12 ml of scintillation cocktail.

For quantitation of metabolites, each 0–24 hr urine sample was filtered (0.2μ, Gelman, Ann Arbor, MI), and 150 μl injections were analyzed by LC/UV using a 5×250 mm Zorbax RX-C8 column at 30°C eluted with 17% acetonitrile/25mM sodium phosphate, pH 7, buffer at 1.5 ml/min for 5 min followed by linear change to 26 min to 50% acetonitrile. Column effluent was collected in 0.5 min fractions, and 12 ml Aquasence scintillation cocktail (DuPont) was added to each fraction for subsequent scintillation counting. The detection limit was based on 3X background and corresponded to <0.05% of dose for each metabolite. For quantitation of plasma metabolites, a similar LC/UV/radioactivity analysis was carried out, except an isocratic system (25% acetonitrile/25mM sodium phosphate buffer, pH 7) was used. To each 100 μl plasma sample was added 300 μl acetonitrile, and the mixture was vortexed thoroughly and centrifuged. The supernatant was filtered (0.45μ Ultrafree-MC, Millipore) and 25–50 μl aliquots analyzed by LC.

In a comparative study, 20 mg/kg (50–53 μCi/kg) doses of [14C]-3,4-dichloroaniline (V) in 10% uacca suspension were given by oral gavage either to 2 male Fischer 344 rats or to 2 female C3H mice. The radiolabeled 3,4-dichloroaniline (specific activity 8.29 μCi/mg) was prepared by hydrolysis of [14C]LY295501 and found to have a radiochemical purity of 99.2% by TLC analysis. The animals were placed in appropriate metabolism cages (the two mice in one cage; the two rats in separate cages) and urine was collected for 48 hr. Other experimental details were analogous to the balance studies described above.

**Results**

**Pharmacokinetic Studies.** Oral doses of LY295501 ranging from 10 to 1000 mg/kg were given to female C3H mice, male Fischer 344 rats, and female Rhesus monkeys. A representative comparison of plasma levels in mice, rats, and monkeys is shown in fig. 3, and a summary of pharmacokinetic parameters is given in table 1. High plasma levels were observed in all three species, with Cmax between 200 and 500 μg/ml after 30–100 mg/kg doses. Fig. 3 illustrates the long plasma half-life of LY295501, which varies from 7–8 hr in rats to 14–17 hr in mice and around 50 hr in monkeys. Data obtained after a 10 mg/kg oral dose in rats are not shown. In this case, plasma concentrations declined from 21 ± 5 μg/ml observed at 2 hr to below the quantitation limit (1 μg/ml) by 6 hr, and no pharmacokinetic parameters could be calculated. Notable in the plasma level data from the 1000 mg/kg oral dose in rats is that Cmax (430 ± 10 μg/ml) was reached after 4 hr and the levels remained essentially constant through 24 hr (350 ± 90 μg/ml) before declining at a rate (t1/2 = 8 hr) similar to the half-life observed after the 100 mg/kg dose (7 hr). An iv bolus dose of 30 mg/kg LY295501 was also given to mice and these plasma concentrations are also shown in fig. 3. The parameters Cmax, t1/2, and AUC were comparable to those from oral doses (table 1), and an oral bioavailability of 78% for the 30 mg/kg oral dose was calculated for mice. The calculated volume of distribution (Vd) is low (close to the estimated volume of plasma in these mice), which is consistent with the essentially complete plasma protein binding of LY295501 described below. Note that the Tmax observed in that case was at 2 hr (earliest timepoint measured was 1 hr), which is not logical after iv dosing. However, this could simply be a result of the variability seen in the mouse data (fig. 3), especially considering the low Vd, which may imply a relatively insignificant distribution phase with this compound. More detailed data would be required to resolve this point.

**Plasma Protein Binding.** Using human as well as mouse, rat, and monkey plasma, protein binding of LY295501 at 100 and 500 μg/ml was measured using an ultrafiltration technique described in Methods. Binding was 99.9% or greater in all measurements. Levels of free LY295501 in most cases were below the quantitation limit of 0.1 μg/ml even with the 500 μg/ml samples. While radiolabeled LY295501 might theoretically be used to obtain a more sensitive measurement, in this unusual case the radiochemical purity of the [14C]LY295501 material (98.7%) indicated that impurities (1.3%) would be substantially greater than the level of free drug (<0.1%).

**Balance Studies.** The excretion of radiocarbon after a 100 mg/kg oral dose of [14C]LY295501 in all three species is shown in table 2. In mice and rats, the majority of radiocarbon was eliminated within 24 hr, but in monkeys only about 21% of the dose was eliminated in that time. Such a slow rate of elimination in monkeys is consistent with the long (50 hr) plasma half-life. In both rodent species, about twice as much radiocarbon was eliminated in feces as in urine compared with the larger nonrodent species (monkeys), where more was recovered in urine. Recovery of radiocarbon was low in the monkeys (69% total in urine and feces). Two factors accounting for this low recovery are that residual radiocarbon was not measured (monkeys not sacrificed) and...
the very slow elimination of [14C]LY295501 (1.7% of the radioactivity was recovered in urine and feces on the last day of this 10-day study).

Identification of Metabolites in Urine. Urine collected 0–24 hr after an oral dose of 100 mg/kg [14C]LY295501, and corresponding to the balance studies described above, was used for the initial identification of metabolites. From preliminary work, there was tentative evidence for a number of metabolites, and all compounds shown in figs. 1 and 2 (I-XII) were available, either for initial identification or for subsequent structural confirmation. As outlined in Methods, a reversed phase LC system was developed which separated all known metabolites and any observed unknowns. The amounts of LY295501 and identified metabolites in urine samples were quantified by radioscintillation counting of collected fractions. The results are shown in table 3.

Trace amounts of LY295501 (I) itself were found in LC/MS analysis of rat and mouse urine. Using LC/UV analysis, the peak corresponding to this material in one mouse urine sample matched the retention time (40.5 min) and UV spectrum ($\lambda_{max}$ 205 and 258 nm) of authentic material. However, quantifiable amounts of LY295501 were found in only one of the three mouse urine samples (0.43% of actual administered [14C]LY295501 dose) and not in any rat or monkey samples (< 0.05% of dose). Analysis by negative ion LC/MS revealed the parent ion m/z 385 for [M-H]$^-$ for a standard of LY295501. In

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**TABLE 1**

<table>
<thead>
<tr>
<th>Dose (mg/kg):</th>
<th>Mouse</th>
<th>Rat</th>
<th>Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg/m²):</td>
<td>30</td>
<td>30</td>
<td>300</td>
</tr>
<tr>
<td>Route:</td>
<td>IV$^b$</td>
<td>PO</td>
<td>PO</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>280 ± 20</td>
<td>220 ± 80</td>
<td>540 ± 30</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>2</td>
<td>4</td>
<td>1$^d$</td>
</tr>
<tr>
<td>$t_{1/2}$ (hr)$^e$</td>
<td>14</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-Inf&lt;/sub&gt; (µg-hr/ml)</td>
<td>5500</td>
<td>4300</td>
<td>11000</td>
</tr>
<tr>
<td>Clearance (ml/hr/kg)</td>
<td>5.5</td>
<td>4300</td>
<td>11000</td>
</tr>
<tr>
<td>Vol. distribution (ml/kg)</td>
<td>76</td>
<td>4300</td>
<td>11000</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>78</td>
<td>76</td>
<td>78</td>
</tr>
</tbody>
</table>

$^a$ In mice and rats, 3 animals were sacrificed at each timepoint, whereas a series of blood samples was taken over time from 3 monkeys. Numbers are reported with ± SD.

$^b$ Intravenous administration.

$^c$ Oral administration.

$^d$ Earliest timepoint measured.

$^e$ Calculated over the following intervals: mouse, 4–72 hr; rat 100 mg/kg, 6–36 hr; rat 1000 mg/kg, 36–60 hr; monkey, 8–168 hr.

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**FIG. 3.** Comparative plasma levels of LY295501 in female rhesus monkeys (○) or male Fischer 344 rats (□) given an oral dose of 100 mg/kg, compared with those in female C3H mice given an oral (□) or iv (■) dose of 30 mg/kg.

Error bars represent the SD (not shown when smaller than symbol size).
TABLE 2
Excretion of \(^{14}\)C\尽早195501 in mice, rats, and monkeys

An oral dose of 100 mg/kg \(^{14}\)C\尽早195501 was given to three groups of two female C3H mice (50 \(\mu\)Ci/kg), three male Fischer 344 rats (20 \(\mu\)Ci/kg), or three female rhesus monkeys (10 \(\mu\)Ci/kg). Shown is the per cent of dose excreted in the fractions listed \(\pm\) SD.

<table>
<thead>
<tr>
<th>Fraction (hr)</th>
<th>Mice</th>
<th>% Dose/Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–24</td>
<td>18.56 ± 1.55</td>
<td>27.23 ± 2.11</td>
</tr>
<tr>
<td>24–48</td>
<td>6.81 ± 3.03</td>
<td>5.56 ± 0.19</td>
</tr>
<tr>
<td>48–72</td>
<td>3.27 ± 0.38</td>
<td>0.74 ± 0.03</td>
</tr>
<tr>
<td>Total(^a)</td>
<td>31.05 ± 6.93</td>
<td>34.49 ± 2.17</td>
</tr>
<tr>
<td>Feces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–24</td>
<td>35.48 ± 4.83</td>
<td>43.59 ± 2.06</td>
</tr>
<tr>
<td>24–48</td>
<td>16.24 ± 1.98</td>
<td>8.67 ± 1.21</td>
</tr>
<tr>
<td>48–72</td>
<td>6.03 ± 0.74</td>
<td>2.64 ± 0.87</td>
</tr>
<tr>
<td>Total(^a)</td>
<td>61.27 ± 3.37</td>
<td>56.24 ± 0.71</td>
</tr>
<tr>
<td>Cage wash</td>
<td>0.63 ± 0.15</td>
<td>0.38 ± 0.05</td>
</tr>
<tr>
<td>Residual (carcass):</td>
<td>1.73 ± 0.09</td>
<td>1.89 ± 0.04</td>
</tr>
<tr>
<td>Total:</td>
<td>94.07 ± 2.70</td>
<td>92.61 ± 1.61</td>
</tr>
</tbody>
</table>

\(^a\) Collections made up to 120 hr in mice and rats and up to 240 hr in monkeys.

\(^b\) Monkeys were not euthanized at the end of this experiment, so residual radiocarbon was not measured.

TABLE 3
Urinary metabolites of \(^{14}\)C\尽早195501 in mice, rats, and monkeys

An oral dose of 100 mg/kg \(^{14}\)C\尽早195501 was given to three groups of two female C3H mice (50 \(\mu\)Ci/kg), three male Fischer 344 rats (20 \(\mu\)Ci/kg), or three female rhesus monkeys (10 \(\mu\)Ci/kg), and urine samples were collected (over dry ice) over 24 hr. Metabolites were profiled by reverse-phase LC with quantitation by radioactivity. Shown is the per cent of dose represented by each metabolite in the 0–24 hr urine fractions \(\pm\) the standard deviation.

<table>
<thead>
<tr>
<th>Compound (LY#)</th>
<th>Percentage of (^{14})C\尽早195501 Dose in 0–24 hr Urine:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse</td>
</tr>
<tr>
<td>295501 (I)</td>
<td>NC</td>
</tr>
<tr>
<td>311235 (II)</td>
<td>8.37 ± 1.19</td>
</tr>
<tr>
<td>317813 (III)</td>
<td>ND</td>
</tr>
<tr>
<td>335937 (IV)</td>
<td>4.08 ± 0.37</td>
</tr>
<tr>
<td>004892 (V)</td>
<td>NC</td>
</tr>
<tr>
<td>301226 (VII)</td>
<td>1.59 ± 0.20</td>
</tr>
<tr>
<td>301202 (VIII)</td>
<td>1.30 ± 0.41</td>
</tr>
<tr>
<td>Unknowns</td>
<td>8.29 ± 1.62</td>
</tr>
<tr>
<td>Total % dose in fraction:</td>
<td>23.86 ± 2.91</td>
</tr>
</tbody>
</table>

\(^\) Not calculated (none detected in two of the three samples in this group); ND, None detected (<0.05% of dose).

LC/MS/MS analysis, formation of the benzodihydrofuran sulfonamide fragment was observed at m/z 198 for LY295501. The formation of analogous sulfonamide fragments was found in LC/MS/MS analysis of all the sulfonlyurea metabolites as described below.

The major metabolite found in urine of mice, rats, and monkeys was the benzyl oxidation product II. A consistent 35–41% of radioactivity in the urine in all three species was accounted for by II (of course, the overall percentage of the dose, 4.6–17%, accounted for by II varies more widely because of the different amounts of total radioactivity found in urine samples from the different species; see table 3). Its LC retention time (34.5 min) and UV spectrum (\(\lambda_{max}\) 203 and 258 nm) matched that of standard in all three species. Analysis of this metabolite in rat urine by [-] ion LC/MS/MS further confirmed this assignment since the observed m/z 401 and 214 are equivalent to one extra oxygen atom (+16) compared with m/z 385 and 198 for LY295501.

A major metabolite found in rat and monkey urine—but not mouse—was III. Constituting about 10% of total radioactivity in the 0–24 hr urine fractions in the rat and 23% in the monkey, III elutes at approximately 19 min in the LC system and possesses the same basic UV spectral characteristics (\(\lambda_{max}\) at 203 and 256 nm, consistent with authentic standard) as LY295501 and II. Mass spectral evidence also confirmed the presence of III (m/z 403 and 216 by [-] ion LC/MS analysis of rat urine). This ring-opened compound is formally equivalent to a hydrolysis product of LY295501.

One other major metabolite in mouse and rat urine (also a minor metabolite in monkey urine) was found to have a UV absorption spectrum (\(\lambda_{max}\) 205, 258 nm) similar to that of LY295501, but with a much shorter retention time in the reversed phase LC system used (6–7 min). The short retention time was consistent with a much more polar compound. Analysis of the corresponding peak in rat urine by LC/MS indicated the presence of a carboxy group (in addition to m/z 417 for the apparent [M-H]\(^-\) ion, a fragment was noted at m/z 373, equivalent to loss of 44, or CO\(_2\)). Like the sulfonlyureas described above, LC/MS/MS analysis gave an ion corresponding to the sulfonamide moiety (here m/z 230, compared with m/z 198 for LY295501), which in this case also showed loss of 44 (to m/z 186). This indicated that the carbonyl group is present on the terminal carbon of a ring-opened material, identifying the material as IV, as shown in fig. 1. The lactone corresponding to IV was subsequently prepared synthetically (12), which, under the conditions of this analysis (reversed phase LC of aqueous solutions), was identical to the ring-opened carbonylate IV.

In previous studies, metabolic cleavage of the related compound sulforufin led to observation of metabolites derived from its sulfonamide and aniline moieties (4, 7). In the case of LY295501, formation of 3,4-dichloroaniline (V) and the sulfonamide VI was also found to occur. In fact, VI could be detected qualitatively in rat urine by LC/UV analysis (retention time 8.5 min and \(\lambda_{max}\) 206, 244, and 278 nm), but not in mouse or monkey urine. However, since \(^{14}\)C\尽早195501 labeled in the 3,4-dichloroaniline ring was used in these studies, amounts of this sulfonamide could not be quantitated. In contrast, V could be quantitated and was also found only in rat urine (representing <0.5% of dose). Its retention time (47.5 min) and characteristic UV spectrum (\(\lambda_{max}\) 206, 244, and a lower, broader, characteristic absorption at 304 nm) matched authentic material.

Again, consonant with the metabolism of sulforufin, the major urinary excretion products expected from V would be the \(\alpha\)-sulfate-conjugated oxidation products VII and VIII (4, 7, 13, 14) Both VII (retention time 12 min) and VIII (retention time 9 min) are ionic compounds and have short retention times in the reversed phase LC system used. The two metabolites exhibit characteristic UV spectra (\(\lambda_{max}\) 207, 244, and 302 nm) very similar to that of LY295501, but with a much longer retention time in the reversed phase LC system used. The two metabolites exhibit characteristic UV spectra (\(\lambda_{max}\) 205, 258 nm) similar to that of LY295501, and possess the same basic UV spectral characteristics (\(\lambda_{max}\) at 203 and 256 nm, consistent with authentic standard) as LY295501 and II. Mass spectral evidence also confirmed the presence of III (m/z 403 and 216 by [-] ion LC/MS analysis of rat urine). This ring-opened compound is formally equivalent to a hydrolysis product of LY295501.

Compounds IX, X, XI (fig. 1), and XII (fig. 2) are other possible metabolites of LY295501. All four compounds were synthesized, but these compounds could not be found in the urine of any of the three species.
animal species. Collectively, the identified metabolites accounted for greater than 65% of the urinary radiocarbon in mice and monkeys and greater than 80% in rats. The remainder of the radioactivity was accounted for by a number of small peaks.

**Metabolism of 3,4-Dichloroaniline.** Oral doses of 20 mg/kg [\(^{14}\)C]3,4-dichloroaniline (V) were also given to either 2 male Fischer 344 rats or 2 female C3H mice to compare its metabolism with that of LY295501. Urine was collected for 48 hr, over which time 96% of the dose was recovered in mouse (92% in the first 24 hr) and 72–76% in the rat urine (70–75% in the first 24 hr). Using an LC/radiocarbon analysis similar to that described above, it was found in both species that 3,4-dichloroaniline (V) accounted for approximately 1–2% of the dose, but VII accounted for 30–35% and VIII accounted for 7–8% of the dose. The formation of the o-sulfates VII and VIII as major metabolites is consistent with the metabolism of p-chloroaniline and similar anilines (14). 3,4-Dichloroacetanilide (IX), for which a standard was also available, was not observed. One other polar metabolite (eluting before VIII in the reversed phase system) was notable in mouse (around 25% of dose), but not in rat (<5% of dose). This metabolite was not identified, nor were 3–5 other minor (representing around 10% of dose or less) observed metabolites.

**Metabolites of LY295501 in Plasma.** Since pharmacokinetic studies of LY295501 had been carried out before radiolabeled compound was available, separate plasma metabolism studies were carried out using [\(^{14}\)C]LY295501. Plasma was collected after single oral doses of [\(^{14}\)C]LY295501 at times of 1, 2, 4, 6, and 24 hr in mice (30 mg/kg dose); 3, 5, 7 and 24 hr in rats (100 mg/kg dose); and 2, 8, 24, 48, and 72 hr in monkeys (100 mg/kg dose). After precipitation of plasma proteins with acetonitrile, the supernatant was profiled directly by LC, and LY295501 and the observed metabolites were quantitated by radioactivity (see *Methods*). Isocratic LC conditions were used to better separate the relatively nonpolar metabolites found in plasma (13.3 min retention time for LY295501 in this system). Initial work using the gradient LC conditions used for urine profiling indicated that the more polar urinary metabolites were not found in plasma.

Fig. 3 illustrates levels of LY295501 and the observed metabolites relative to total radioactivity for the monkey. The great majority of total circulating radioactivity was accounted for by LY295501: 85% at the C\(_{\text{max}}\) (470 µg/ml for LY295501 versus 560 µg-equiv/ml for radiocarbon, at 8 hr) and 72% for the AUC\(_{0-72\text{hr}}\) (23000 versus 32000 µg-equiv-hr/ml, respectively). The most prominent metabolite found in plasma was XI (retention time 18.6 min in the isocratic system). This metabolite exhibits a characteristic UV spectrum (\(\lambda_{\text{max}}\) 256 nm) with a band shape distinct from LY295501 and other metabolites. However, it is minor compared with LY295501, with a C\(_{\text{max}}\) at 48 hr of 23 µg-equiv/ml and AUC\(_{0-72\text{hr}}\) of 1300 µg-equiv-hr/ml. Both values are just 4% of those for total radiocarbon. (However, the AUC for XI is underestimated relative to LY295501 and total radiocarbon since its plasma levels were highest from 24–72 hr.) An even smaller amount of the major urinary metabolite II (retention time 6.7 min in isocratic system) was found, with a C\(_{\text{max}}\) of 8 µg-equiv/ml at 24 hr and an overall AUC\(_{0-72\text{hr}}\) of just 1% that of the total circulating material. Another metabolite, X (retention time 10.7 min in isocratic system), was also found in plasma, but not in urine. A standard for X was again available, and average levels of X in monkey plasma are shown in fig. 3. However, it was not found at all time points in any monkey nor was it present at all in one of the three monkeys. No other significant metabolites could be discerned in plasma using either the gradient or the isocratic LC analysis.

Similar results were obtained with plasma from rats and mice dosed with [\(^{14}\)C]LY295501 (results not shown). In both species, parent accounted for the vast majority of circulating material (C\(_{\text{max}}\) and AUC > 80% of the total circulating material). The most prominent metabolite was XI, but it constituted less than 10% of the total circulating material in both rats and mice. The major urinary metabolite II was again observed, but at even lower levels.

**Discussion**

Pharmacokinetic studies were carried out in species used for antitumor models (female C3H mice) (1, 2) and toxicology evaluation (Fischer 344 rats and Rhesus monkeys) (2, 3) of LY295501, and these results are summarized in table 1. As fig. 2 also illustrates, plasma levels of LY295501 were high (C\(_{\text{max}}\) up to 500 µg/ml) and persisted for a long period (half-lives from 7 up to 50 hr). One factor which helps explain both of these observations is the extensive binding of LY295501 in plasma, which was found to be >99.9% using a protein filtration technique. While the nature of this binding has not been characterized for LY295501, it is known that sulofenur and other sulfonylureas bind tightly to serum albumin (1, 6, 15).

Consistent with the above data, the oral bioavailability of LY295501 was high in mice (78%). However, iv dosing of LY295501 was possible only in mice because of its limited solubility in the formulation used. Note that in rats and monkeys the C\(_{\text{max}}\) for LY295501 was in the range 400–500 µg/ml after a 100 mg/ml oral dose, compared with 220 µg/ml in mouse after a 30 mg/ml dose. This suggests the oral bioavailability is also high in rats and monkeys. In all three species, AUC values were substantially greater after the higher oral dose (3–6 times), though not in proportion to the 10-fold increase in dose. This may mean that a limit to oral absorption is reached in the higher dose range of 100–1000 mg/kg.

Excretion studies with [\(^{14}\)C]LY295501 resulted in greater elimination of radiocarbon into feces than into the urine of rats and mice (table 2). In contrast, more radiocarbon was found in the urine than in feces from monkeys given [\(^{14}\)C]LY295501. The same trend was observed with sulofenur, and in humans given oral doses of [\(^{14}\)C]-sulofenur, the urine contained >90% of radiocarbon eliminated (6).

Urine was used for the initial identification of potential metabolites of LY295501, since the major urinary metabolites of another analog in this oncolytic series, sulofenur (8), were already known (4, 5). Sulofenur is very similar in structure to LY295501, having indane and p-chloroaniline moieties in place of the dihydrobenzofuran and 3,4-dichloroaniline groups of LY295501. The two major routes of sulofenur metabolism involve both the oxidation of its indane moiety to give hydroxindane, ketoindane, or indene metabolites, as well as cleavage of the sulfonylurea linkage to give indanesulfonamide and p-chloroaniline-derived metabolites (4,5). While not as much is known about transformation of the analogous dihydrobenzofuran group in LY295501, work with other compounds suggests that metabolites such as II and III might be also formed (16). Also, if the sulfonylurea linkage of LY295501 is cleaved, VII and VIII would be expected metabolites of 3,4-dichloroaniline (7,13). Therefore, compounds II, III, and V–XI were prepared as possible metabolites. As discussed below, all of these with the exception of IX were found to be either urinary or plasma metabolites of LY295501. In addition, IV was identified as a metabolite in rat, mouse, and monkey urine.

As with sulofenur, essentially no parent LY295501 was found in the mouse, rat, or monkey urine (fig. 1, table 3). The hydroxy metabolite II was the major urinary metabolite observed in mice, rats, and monkeys. This metabolite, along with III and IV, accounted for over half of the radiocarbon excreted in urine within 24 hr from these species. While no attempt was made in this study to characterize the mechanism leading to formation of these metabolites, II was formed.
after incubation of LY295501 with rat liver microsomes in the presence of NADPH (data not shown). Thus, a direct cytochrome P450 oxidation similar to that observed for sulofenur and for hypoglycemic sulfonylureas such as tolbutamide is implicated (4, 5).

The formation of metabolites III and IV from LY295501 is a more interesting observation. Formation of III corresponds to a direct hydrolysis of the saturated ring in the benzodihydrofuran moiety of LY295501. However, we found only one other reference to the metabolic opening of a benzodihydrofuran ring system (16), and the evidence presented by those metabolism chemists for the analogous transformation of their structurally unrelated series of compounds indicated P450 oxidation was involved. Similarly, formation of III was observed only after incubation of LY295501 in the presence of NADPH, which suggested it might be the product of initial oxidation to give XII, followed by reduction of the ring-opened tautomeric aldehyde XIII to give III, as postulated in fig. 2. Oxidation of XIII would also explain the formation of compound IV. Compound XII was then prepared as a possible metabolite of LY295501, but XII was not observed in urine or plasma. Thus, while XII is a logical potential intermediate in the formation of both III and IV, as shown in fig. 2, there is no direct evidence for this route in these studies.

Cleavage of the sulfonylurea linkage leads to compounds V-IX, which were found to be minor metabolites (note that, potentially, metabolites such as II, III, and IV could also be further metabolized to give V). Of these, VI contains no radiolabel and was not quantitated. Compounds VII and VIII are themselves major urinary metabolites of 3,4-dichloroaniline (V), as shown by metabolism of [14C]3,4-dichloroaniline itself in rats and mice (see Results). Like p-chloroaniline, 3,4-dichloroaniline (V) is a potent inducer of methemoglobin formation and associated hemolytic anemia caused by the reaction of oxyhemoglobin with its hydroxylamine metabolite (17). These were the major toxicities found for sulofenur in animal studies (3, 7) and were found to be dose limiting in clinical trials of sulofenur (9, 10). A series of metabolism studies eventually led to the conclusion that metabolic release of the aniline was responsible for the observed methemoglobinemia and hemolytic anemia (4–7). In contrast, myelosuppression and intestinal effects were the major toxicities observed in animal studies of LY295501 (2, 3). Since LY295501 was found to be around 5-fold more potent in vitro than sulofenur in cytotoxicity assays (1, 2), this relative potency may explain why those toxicities, typical for cytotoxic oncolytic compounds, were mainly observed for LY295501 (2, 3). However, formation of these aniline metabolites does explain the (relatively minor) hemolytic anemia and methemoglobinemia observed in rat and monkey toxicity studies of LY295501 (2, 3, 7).

Collectively, the identified metabolites accounted for greater than 65% of the urinary radiocarbon in mice and monkeys and greater than 80% in rats; a number of small peaks accounted for the remainder. Only trace amounts of LY295501 were found in urine. The potential metabolites IX, X, XI, and XII were not found as urinary metabolites in this study (authentic samples of all four compounds were available).

In plasma, by contrast, LY295501 itself accounted for the vast majority of circulating material in monkey plasma (70–85%; fig. 4). No one metabolite accounted for the remainder, but relatively small amounts (1% of total AUC) of the major urinary metabolite II were observed. Somewhat greater amounts (4% of total AUC) of a nonpolar oxidation product, XI, were also found. Direct oxidation by cytochrome P450 of saturated groups to a dehydrogenated product is known to occur in a number of cases, and similar oxidation of sulofenur (which contains an indane moiety) to an indene metabolite is also known (5). Presumably, the benzofuran metabolite XI is found in plasma but not in urine because it is relatively nonpolar (quite similar to LY295501 itself) and not readily excreted into the urine without further metabolism. Another relatively nonpolar metabolite not found in urine, but found in small amounts in some plasma
samples (<1% of total AUC), is the keto metabolite, **X**, which might be formed via oxidation of **II**. In plasma from rats and mice dosed with [14C]LY295501, very similar results were obtained (data not shown), where >80% of total circulating material was accounted for by parent LY295501 and small amounts of the nonpolar metabolite **XI** were observed.

The chromatography and excretion methods described above for the urine and plasma matrices were not useful for work with feces. Identification of potential fecal metabolites was not pursued further in these studies. With sulofenur there was much more excretion of radioactivity into the urine of monkeys than for mice or rats (4), which matches the trend observed with LY295501. It is anticipated that, like sulofenur (4), urinary excretion of radioactivity associated with LY295501 will predominate even more in humans. Because of the extensive metabolism of LY295501 in mice, rats, and monkeys, it will be necessary to carry out a human metabolism study with [14C]LY295501 in its further clinical development, which will provide direct evidence for this point. If a substantial amount of fecal excretion were found in that study, that would provide an impetus for identification of fecal (and biliary) metabolites.

In summary, high levels of LY295501 are found in plasma (Cmax up to 500 μg/ml) after oral doses of 100–300 mg/kg) and the compound exhibits a long half-life (7–50 hr). Both of these characteristics may be influenced by the high degree of plasma protein binding found for LY295501 (>99.9%). Urine was the major route of excretion in monkey, and, by analogy with sulofenur, might be the major route of excretion in human as well. A number of oxidative metabolites were identified in urine, along with two additional less polar metabolites in plasma, which can all be accounted for by initial oxidation of the saturated portion of the benzodihydropyran moiety of LY295501. Despite the observation that essentially no LY295501 was found in urine, the vast majority of drug-related material circulating in plasma was accounted for by LY295501 itself. Finally, cleavage of this sulfonylurea was found to occur, leading to sulfonamide and aniline fragments as well as metabolites thereof.

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**References**


