Supplemental Data

Systemic clearance and brain distribution of carbazole-based cyanine compounds as Alzheimer’s disease drug candidates

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Supplemental Figures and Tables legends

Table S1 Mass spectral characteristics of the in vitro metabolites of SLOH.

Table S2 Mass spectral characteristics of the in vitro metabolites of SLM.

Table S3 Systemic pharmacokinetics parameters of SLOH and SLM in C57BL/6 mice aging 2 months after i.v. administration (n=5, Mean ± SD). Statistical significance in parameters was estimated by Student’s t-test. (SLOH vs SLM group), $^{5}p < 0.05$ and $^{55}p < 0.01$ (n=5, Mean ± SD).

Table S4 Blood cell partitioning, non-specific plasma protein binding, microsomes binding, and brain distribution using brain slices of positive controls (n=3, Mean ± SD).

Table S5 The $P_{app}$ and efflux ratio values of positive controls in Caco-2 cell transport (n=3, Mean ± SD).

Table S6 Liver microsomes stability and hepatocyte stability in mice and human of positive controls (n=3, Mean ± SD).

Fig. S1 Representative MS/MS spectrum of SLOH and SLM.

Fig. S2 Representative SIM chromatograms for $T_{30min}$ incubations of SLOH in liver microsomes supplemented with NADPH.

Fig. S3 Representative MS spectrum for $T_{30min}$ incubations of SLOH in liver microsomes supplemented with NADPH.

Fig. S4 Representative MS/MS spectrum for $T_{30min}$ incubations of SLOH in liver microsomes supplemented with NADPH.

Fig. S5 The fragment ions characteristics of SLOH metabolites for $T_{30min}$ incubations in liver microsomes supplemented with NADPH.

Fig. S6 Representative SIM chromatograms for $T_{30min}$ incubations of SLM in liver microsomes supplemented with NADPH.

Fig. S7 Representative MS spectrum for $T_{30min}$ incubations of SLM in liver microsomes supplemented with NADPH.

Fig. S8 Representative MS/MS spectrum for $T_{30min}$ incubations of SLM in liver microsomes supplemented with NADPH.

Fig. S9 The fragment ions characteristics of SLM metabolites for $T_{30min}$ incubations in liver microsomes supplemented with NADPH.
**Fig. S10** The pharmacokinetics profiles in C57BL/6 mice aging 2 months. A: SLOH, B: SLM. A1 and B1 represent pharmacokinetics profiles; A2 and B2 represent linearity analysis between AUC and dosages ($n=5$, Mean ± SD).

**Fig. S11** The pharmacokinetics and brain exposure in WT and transgenic AD model mice. A: SLOH, B: SLM. A1 and B1 represent pharmacokinetics profiles; A2 and B2 represent brain exposure in ISF. Statistical significance in parameters was estimated by Student’s t-test. (vs WT group), ’*’ $p < 0.05$ and ’**’ $p < 0.01$ ($n=3$, Mean ± SD).

**Fig. S12** The efflux ratio values of SLOH and SLM in the presence or absence of specific OATP2B1 inhibitor (Erlotinib) in Caco-2 cell transport. Statistical significance in parameters obtained from multiple groups was estimated using One-way ANOVA followed by Dunnett’s test. (vs control group), ’*’ $p < 0.05$ and ’**’ $p < 0.01$ ($n=3$, Mean ± SD).

**Fig. S13** The $V_{u,brain}$ values of SLOH and SLM in the presence or absence of P-gp and BCRP inhibitor in brain slice experiment. Statistical significance in parameters obtained from multiple groups was estimated using One-way ANOVA followed by Dunnett’s test. (vs control group), ’*’ $p < 0.05$ and ’**’ $p < 0.01$ ($n=3$, Mean ± SD).
Supplemental Methods

Blood-to-plasma ratio

Aliquots of fresh whole blood and reference plasma (C_P) are spiked with SLOH and SLM (1 μM) and incubated for an hour at 37 °C. At the end of the incubation period, plasma (C_P) is separated from whole blood by centrifugation and all plasma samples are analyzed after protein precipitation using 2 folds acetonitrile (ACN) containing berberine chloride as internal standard (IS) by LC-MS/MS (Analytical method seen below). The blood cell partitioning was calculated as follows.

\[ K_{WB/P} = \frac{C_{WP}}{C_P} \]  \hspace{1cm} (1)

Plasma, liver microsomes and hepatocyte binding by RED device

The stability of the compounds in plasma, liver microsomes and hepatocyte were studied to ensure the reliability of the binding data. Hepatocyte was deactivated by leaving them on a bench for 2 days and going through a freeze thaw cycle thereafter. This rendered the enzymes inactive but not totally denatured. Testosterone was used as a control for CYP3A to ensure no turnover, so that the enzymes were inactivated. All validation compounds were incubated at 37 °C over 4 h, and in triplicate. For plasma stability, 10 mM DMSO solutions of SLM and SLOH were diluted to 40 μM (10% DMSO). Plasma were thawed and 594 μL aliquots dispensed into a 1.5 mL tube, and then pre-warmed at 37 °C in a shaking water bath (60 oscillations/min approx.). The assay was started by addition of 6 μL aliquots to the plasma (final DMSO incubation concentration 0.1%). For liver microsomes without cofactors and deactivated hepatocyte stability, all validation compounds (1 μM) in deactivated liver microsomes (0.5 mg/mL protein concentration, same as the assay condition) or deactivated hepatocytes (0.5 million cells/mL, same as the assay condition) were added to the donor wells. 10 mM DMSO solutions of SLM and SLOH were diluted to 50 μM (10% DMSO). 490 μL aliquots microsomes (0.51 mg/mL) and hepatocytes (0.51 million cells/mL) were dispensed into a 1.5 mL tube, and then pre-warmed at 37 °C in a shaking water bath. The assay was started by addition of 10 μL aliquots of compound solutions to the corresponding biological matrices (final DMSO incubation concentration 0.2%, microsomes concentration 0.50 mg/mL, hepatocytes concentration 0.5 million cells/mL). At 0, 15, 30, 60, 120, 180 and 240 min, 50 μL plasma, microsomes or hepatocytes after protein precipitation were analyzed by LC-
MS/MS. Natural logarithm of peak area ratios (compound peak area normalized by IS peak area) were plotted against time and half-life calculated.

The spiked plasma solutions, microsomes and hepatocytes (400 μL) after observing stability were placed into the sample chamber (indicated by the red ring) with 400 μL of PBS into the adjacent chamber. The plate was sealed with a self-adhesive lid and incubated at 37 °C on an orbital shaker (1.5 g approx.) for 4 h. Aliquots (100 μL) were removed from each side of the insert, and analyzed after protein precipitation using 4 folds acetonitrile (ACN) containing berberine chloride as internal standard (IS) by LC-MS/MS (Analytical method seen below). The experimental procedure outlined in the Thermo Scientific RED device systems brochure was adopted in the present study. The several positive controls were assayed (Table S4).

**In vitro stability in liver microsomes and hepatocytes**

Half-life (t_{1/2}) of SLM or SLOH in liver microsomes was determined in duplicate. In phase I reaction, the compound (1 μM) was incubated with liver microsomes (0.5 mg/mL) in 100 mM sodium phosphate buffer (pH 7.4) including 81 mM Na₂HPO₄ and 19 mM NaH₂PO₄ at 37 °C, the reaction mixture was prewarmed at 37 °C for 2 minutes before adding NADPH (1.0 mM). In phase II reaction, the compound (1 μM) was incubated with liver microsomes (0.5 mg/mL) in 100 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl₂, 10 μg/mL alamethicin, and 2% (w/v) BSA. After pre-incubated on ice for 15 min, the reactions were initiated with the addition of UDPGA (5.0 mM). Aliquots of the reaction mixture at 0, 5, 10, 15, 30 or 45 minutes were added to 4 folds acetonitrile (ACN) containing internal standard (IS), and the samples were centrifuged at 9,659 g for 5 mins before LC-MS/MS analysis of substrate disappearance. For control experiments, NADPH, UDPGA and/or liver microsomes were omitted from these incubations. For the purposes of metabolite identification studies, the concentration of SLOH and SLM in microsomal incubations was raised to 10 μM and the microsomal protein concentration increased to 3 mg/mL. *InVitroGRO™* HT Medium was used for thawing suspension of cryopreserved hepatocytes. Incubations were conducted in a 96-well flat-bottom polystyrene plate in duplicate. Hepatocytes were suspended at 0.5 x 10⁶ viable cells/mL of HT Medium and prewarmed at 37 °C for 10 minutes. Incubations were initiated with the addition of SLM and SLOH (final concentration=1 μM) and were conducted at 37 °C, 75% relative humidity, and 5% CO₂. The reaction was stopped at 0, 5, 15, 30, 60, 90, 120 minutes by the addition of 4 folds ACN containing berberine chloride as internal standard.
(40 nM). The samples were centrifuged at approximately 1,900 g for 10 mins before LC-MS/MS analysis.

The CYP3A4 and CYP1A2 activities in human hepatocytes were assayed by testosterone 6β-hydroxylation (141 ± 23.0 pmol/min/10⁶ cells) and phenacetin O-deethylation (24.3 ± 3.25 pmol/min/10⁶ cells). The other positive controls assays were shown in Table S6. The scaling of in vitro to in vivo clearance based on the well-stirred model [1] (equation 10) was carried out as follows (2-5: microsomes; 6-9: hepatocytes):

$$\text{Cl}_{\text{int, app}} = \frac{0.693 \cdot \text{mL incubation}}{t_{1/2 (\text{min})} \cdot \text{mg microsomes}}$$  \hspace{1cm} (2)\\

$$\text{Cl}_{\text{u, int, app}} = \frac{\text{Cl}_{\text{int, app}}}{f_u, \text{mic}}$$  \hspace{1cm} (3)\\

$$\text{Cl}_{\text{int, scaled}} = \frac{0.693 \cdot \text{mL incubation}}{t_{1/2 (\text{min})} \cdot \text{mg microsomes} \cdot \text{MPPGL} \cdot \text{LWPBW}}$$  \hspace{1cm} (4)\\

$$\text{Cl}_{\text{int, scaled}} = \frac{\text{Cl}_{\text{int, scaled}}}{f_u, \text{mic}}$$  \hspace{1cm} (5)\\

$$\text{Cl}_{\text{int, app}} = \frac{0.693 \cdot \text{mL incubation}}{t_{1/2 (\text{min})} \cdot \text{million cells}}$$  \hspace{1cm} (6)\\

$$\text{Cl}_{\text{u, int, app}} = \frac{\text{Cl}_{\text{int, app}}}{f_u, \text{hep}}$$  \hspace{1cm} (7)\\

$$\text{Cl}_{\text{int, scaled}} = \frac{0.693 \cdot \text{mL incubation}}{t_{1/2 (\text{min})} \cdot \text{million cells} \cdot \text{HPGL} \cdot \text{LWPBW}}$$  \hspace{1cm} (8)\\

$$\text{Cl}_{\text{int, scaled}} = \frac{\text{Cl}_{\text{int, scaled}}}{f_u, \text{hep}}$$  \hspace{1cm} (9)\\

Well-stirred model

$$\text{Cl}_{\text{hep}} = \frac{Q \cdot f_u, \text{plasma} \cdot \text{Cl}_{\text{u, int, scaled}}}{Q + f_u, \text{plasma} \cdot \text{Cl}_{\text{u, int, scaled}}}$$  \hspace{1cm} (10)\\

Where MPPGL is microsomal protein per gram liver (human: 48.8 mg/g Liver; mice: 45 mg/g Liver), HPGL is hepatocellularity (million cells per gram liver) (human: 99; mice: 128) and LWPBW is grams liver per kg body weight (human: 25.7 g/kg; mice: 87.5 g/kg) [2]. Cl\text{int, app} is apparent intrinsic clearance, Cl\text{u, int, app} is unbound apparent intrinsic clearance, Cl\text{int, scaled} is scaled intrinsic clearance, Cl\text{u, int, scaled} is unbound
scaled intrinsic clearance, $C_{hep}$ is plasma hepatic clearance, $Q$ is hepatic blood flow (human: 20.7 mL/min per kg; mice: 90 mL/min per kg) [3].

**LC-HR MS (LTQ-Orbitrap XL hybrid MS) conditions for metabolites identification of SLOH and SLM**

LC-MS experiments were carried out on a Thermo Electron LTQ-Orbitrap XL hybrid MS (ThermoFinnigan, Bremen, Germany) equipped with an ESI interface. An Accela HPLC system (ThermoElectron) was equipped with an autosampler, a vacuum degasser unit and a quaternary pump. The MS employing positive ionization was calibrated using calibration standards mixture allowing for mass accuracies less than 5 ppm in external calibration mode. The ionization voltage was 4.2 KV and the capillary temperature was set at 300 °C. Nitrogen was used as both the sheath gas (40 units) and auxiliary gas (10 units). The resolving power was 15000 for full-scan and 7500 for the MS$^2$ scans. Chromatographic separation was achieved on a Luna ODS C18 column (150 × 2.1 mm, 5 μM; Phenomenex, Torrance, CA, USA). The mobile phase was composed of A (ACN containing 0.1% formic acid), B (Water containing 0.1% formic acid) using a gradient elution of 10%-10% A, 90%-90% B at 0-5 min, 10%-30% A, 90%-70% B at 5-8 min, 30%-90% A, 70%-10% B at 8-20 min, 90%-10% A, 10%-90% B at 20-21 min and hold for 4 min. The flow rate was set at 0.3 mL-min$^{-1}$. The auto-sampler was conditioned at 4 °C and the injection volume was 5 μL.

A Thermo Xcaliber 2.1 workstation was used for the data acquisition and processing. For the computer-based MDF approach, representative structures with predicted mass defect windows were set as filtering templates for screening homologous compounds. Thermo Scientific™ MetWorks™ and Mass Frontier software automated structural elucidation.

**Bio-sample preparations and LC-MS/MS (TQD) analysis for quantitation of SLOH and SLM**

The LC-MS/MS condition was reported previously [4]. Briefly, under positive mode of Waters Xevo TQD, SLM (m/z 437 $>$ 347 as quantifier ion and m/z 437 $>$ 168 as qualifier ion), SLOH (m/z 467 $>$ 333 as quantifier ion and m/z 467 $>$ 154 as qualifier ion) and berberine (as internal standard, m/z 336 $>$ 320 as quantifier ion and m/z 336 $>$ 278 as qualifier ion) were simultaneously monitored. Plasma, urine and bile samples preparation method was completed as previously described [4]. For brain sample, brain was weighted and PBS buffer (pH=7.4) was added based on 10-fold volume of each brain weight. Brain samples were cut into smaller pieces before homogenization. Then 20 μL of homogenate were vortex
mixed with nitrogen-dried IS for 1 min. 40 µL of ACN was then added to precipitate the protein. After vortexed for 1.5 mins, the samples were centrifuged at 9,659 g for 5 mins. The supernatant was transferred to an autosampler vial and an aliquot of 1 µL was injected onto the LC-MS/MS system for analysis.

References

[1] Obach, R.S. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. Drug Metab. Dispos. 27, 1350-1359 (1999).

[2] Barter, Z.E. et al. Scaling factors for the extrapolation of in vivo metabolic drug clearance from in vitro data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. Curr. Drug Metab. 8, 33-45 (2007).

[3] Davies, B. & Morris, T. Physiological parameters in laboratory animals and humans. Pharm. Res. 10, 1093-1095 (1993).

[4] Zhou, W., Wu, X., Li, J., Hu, X. & Tam, K. Quantification of permanent positively charged compounds in plasma using one-step dilution to reduce matrix effect in MS. Bioanalysis 8, 497-509 (2016).
| Metabolite | M*   | Formula      | Metabolic pathway            | Retention time (tR) | Fragment ions                                                                 |
|-----------|------|--------------|------------------------------|---------------------|-----------------------------------------------------------------------------|
| Parent    | 467.2329 | C_{30}H_{31}N_{2}O_{3} | N.A.                       | 16.8                | 423.2074, 377.1659, 347.1551, 333.1396, 320.1310, 319.1226, 154.0654        |
| M1        | 467.2329 | C_{30}H_{31}N_{2}O_{3} | Cis-trans isomer            | 15.4                | 423.2074, 377.1645, 347.1536, 333.1380, 320.1310, 319.1226, 154.0647        |
| M2        | 453.2173 | C_{29}H_{29}N_{2}O_{3} | O-demethylation              | 16.0                | 409.1898, 377.1645, 347.1530, 333.1378, 320.1290, 319.1226, 154.0645        |
| M3        | 483.2278 | C_{30}H_{31}N_{2}O_{4} | Hydroxylation               | 15.8                | 439.2010, 393.1593, 363.1488, 349.1331, 336.1250, 335.1172, 154.0648        |
| M4        | 483.2278 | C_{29}H_{29}N_{2}O_{4} | Hydroxylation               | 16.1                | 365.1641, 347.1530, 333.1378, 321.1379, 319.1226, 154.0647                 |
| M5        | 469.2122 | C_{29}H_{29}N_{2}O_{4} | O-demethylation+ hydroxylation | 15.6                | 425.1865, 393.1604, 363.1495, 349.1341, 336.1264, 335.1186, 154.0652        |
| M6        | 469.2122 | C_{29}H_{29}N_{2}O_{4} | O-demethylation+ hydroxylation | 15.6                | 407.1760, 377.1645, 333.1391, 320.1309, 319.1226, 154.0652                 |
| M7        | 409.1911 | C_{27}H_{25}N_{2}O_{2} | O-dealkylation              | 16.0                | 377.1648, 365.1648, 347.1530, 333.1387, 320.1309, 319.1233, 154.0650        |
| M8        | 425.1860 | C_{27}H_{25}N_{2}O_{3} | O-dealkylation+ hydroxylation | 15.5                | 407.1760, 377.1658, 363.1498, 333.1396, 320.1310, 319.1233, 154.0654        |
| M9        | 365.1648 | C_{27}H_{25}N_{2}O | N-dealkylation              | 16.4                | 321.1380, 319.1233, 154.0648                                               |
| M10       | 381.1598 | C_{27}H_{25}N_{2}O | N-dealkylation+ hydroxylation | 15.3                | 337.1334, 335.1186, 154.0650                                               |
| M11       | 381.1598 | C_{27}H_{25}N_{2}O | N-dealkylation+ hydroxylation | 15.6                | 337.1335, 335.1186, 154.0650                                               |
| M12       | 381.1598 | C_{27}H_{25}N_{2}O | N-dealkylation+ hydroxylation | 16.2               | 363.1487, 334.1462, 320.1209, 319.1230, 167.0728                           |
| M13       | 407.1754 | C_{27}H_{25}N_{2}O | N-dealkylation+ hydroxylation+dehydration | 15.8               | N.A.                                                                                     |
| M14       | 423.1703 | C_{27}H_{25}N_{2}O | O-dealkylation+carbonylation        | 16.0                | 379.1445, 333.1389, 320.1209, 319.1235, 154.0651                           |
| M15       | 451.2016 | C_{29}H_{27}N_{2}O_{3} | O-demethylation+hydroxylation+dehydration | 15.8               | N.A.                                                                                     |
| M16       | 441.2067 | C_{27}H_{25}N_{2}O | unknown                      | 15.8                | N.A.                                                                                     |

All metabolites were observed in liver microsomes from preclinical mice and humans. (N.A.: unidentified).
| Metabolite | M⁺     | Formula       | Metabolic pathway                  | Retention time (t<sub>R</sub>) | Fragment ions                                      |
|-----------|--------|---------------|-----------------------------------|-------------------------------|---------------------------------------------------|
| Parent    | 437.2224 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>2</sub> | N.A.                             | 17.1                          | 361.1696, 347.1541, 334.1454, 333.1376, 319.1228, 168.0807 |
| M1        | 437.2224 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>2</sub> | Cis-trans isomer                 | 15.7                          | 361.1696, 347.1541, 334.1454, 333.1376, 319.1228, 168.0807 |
| M2        | 423.2067 | C<sub>28</sub>H<sub>27</sub>N<sub>2</sub>O<sub>2</sub> | O-demethylation                  | 16.3                          | 361.1693, 347.1538, 334.1452, 333.1374, 168.0806    |
| M3        | 453.2173 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>3</sub> | Hydroxylation                    | 16.3                          | 363.1497, 335.1534, 319.1226, 168.0804            |
| M4        | 439.2016 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>3</sub> | O-demethylation+ Hydroxylation   | 15.8                          | 421.1917, 361.1703, 347.1548, 334.1466, 333.1388, 168.0810 |
| M5        | 379.1805 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>3</sub> | O-dealkylation                   | 16.3                          | 361.1703, 347.1545, 334.1457, 333.1379, 168.0809    |
| M6        | 395.1754 | C<sub>29</sub>H<sub>29</sub>N<sub>2</sub>O<sub>3</sub> | O-dealkylation+ Hydroxylation    | 15.8                          | 377.1646, 347.1540, 334.1460, 333.1382, 168.0807    |
| M7        | 335.1543 | C<sub>24</sub>H<sub>19</sub>N<sub>2</sub> | N-dealkylation                   | 16.7                          | 319.1232, 168.0808                                  |
| M8        | 351.1492 | C<sub>24</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub> | N-dealkylation+ Hydroxylation    | 15.9                          | N.A.                                              |
| M9        | 421.1911 | C<sub>28</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub> | O-demethylation+hydroxylation+dehydration | 16.6                          | 361.1703, 347.1544, 334.1460, 333.1382, 168.0811    |
| M10       | 437.1860 | C<sub>28</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub> | O-dealkylation+carbonylation     | 16.4                          | 379.1810, 361.1703, 347.1545, 334.1459, 333.1381, 168.0809 |
| M11       | 393.1598 | C<sub>28</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub> | O-dealkylation+carbonylation     | 16.3                          | 347.1534, 334.1451, 333.1373, 168.0804            |

All metabolites were observed in liver microsomes from preclinical mice and humans. (N.A.: unidentified)
| Compound | Dose (mg/kg) | $AUC_{0-\infty}$ (ng/mL*H) | $Cl_{f}$ (L/h per Kg) | $Vd_{ss}$ (L/Kg) | $t_{1/2}$ (h) | $MRT_{0-\infty}$ (H) |
|----------|-------------|----------------------------|----------------------|-----------------|--------------|-------------------|
| SLM      | 0.2         | 240 ± 74.0                 | 0.890 ± 0.240        | 1.50 ± 0.630    | 6.40 ± 4.0   | 3.50 ± 0.950      |
|          | 0.6         | 580 ± 170                  | 0.930 ± 0.140        | 2.10 ± 0.540<sup>6</sup> | 6.00 ± 1.10 | 3.70 ± 0.860<sup>6</sup> |
|          | 1.0         | 140 × 10 ± 240             | 0.710 ± 0.130        | 2.20 ± 0.610<sup>6</sup> | 9.30 ± 3.30 | 3.20 ± 1.00<sup>6</sup> |
|          | 2.0         | 320 × 10 ± 1000 × 10       | 0.750 ± 0.180        | 1.70 ± 0.550<sup>6</sup> | 7.10 ± 0.550 | 2.50 ± 0.180<sup>6</sup> |
|          | 4.0         | 500 × 10 ± 880             | 0.830 ± 0.160        | 2.10 ± 0.200    | 6.50 ± 2.00 | 3.00 ± 0.520<sup>6</sup> |
| SLOH     | 0.2         | 220 ± 61.0                 | 0.930 ± 0.240        | 1.00 ± 0.330    | 6.90 ± 3.70 | 2.70 ± 1.50       |
|          | 0.6         | 860 ± 310                  | 0.770 ± 0.0800       | 1.20 ± 0.580<sup>6</sup> | 5.50 ± 1.10 | 2.30 ± 0.780      |
|          | 1.0         | 230 × 10 ± 130 × 10        | 0.610 ± 0.140        | 0.830 ± 0.430<sup>6</sup> | 5.80 ± 0.160 | 1.80 ± 0.100      |
|          | 2.0         | 320 × 10 ± 86.0            | 0.640 ± 0.0200       | 1.00 ± 0.360<sup>6</sup> | 4.60 ± 0.760 | 1.80 ± 0.170<sup>6</sup> |
|          | 4.0         | 620 × 10 ± 580             | 0.710 ± 0.130        | 1.00 ± 0.350    | 5.80 ± 1.70 | 1.70 ± 0.0800<sup>6</sup> |
| Parameters                                      | Compounds     | Species  | Species  |
|------------------------------------------------|---------------|----------|----------|
|                                                |               | Mouse    | Human    |
| Blood to plasma ratio                          | Chlorthalidone| 6.20 ± 0.200 | 11.0 ± 3.20 |
|                                                | Atenolol      | 98.0 ± 0.210 | 96.0 ± 1.20 |
|                                                | Propranolol   | 0.220 ± 0.0200 | 6.50 ± 0.480 |
| Unbound plasma (fu, plasma %)                  | Atenolol      | 100      | 100      |
| Unbound Liver microsomes (fu, mic %)           | Propranolol   | 76.0 ± 0.700 | 61.0 ± 1.60 |
| Unbound brain distribution (Vu,brain mL/g brain)| Verapamil     | 36.0 ± 9.70  | —        |
|                                                | Gabapentin    | 2.20 ± 1.00  | —        |
| Compounds                  | Parameters                  | $P_{app}$ (A-B) (cm/s) $\times 10^{-6}$ | Efflux ratio |
|---------------------------|-----------------------------|----------------------------------------|--------------|
| Sodium fluorescein (1 μM input) |                             | 1.40 ± 0.260                           | —            |
| Rhodamine 123 (1 μM input)  |                             | 0.730 ± 0.0200                         | —            |
| Atenolol (1 μM input)      |                             | 0.480 ± 0.0900                         | —            |
| Propranolol (1 μM input)   |                             | 17.0 ± 1.30                            | —            |
| Verapamil (2 μM input)     |                             | 23.0 ± 1.20                            | —            |
| Digoxin (2 μM input)       |                             | 3.50 ± 0.340                           | 5.00 ± 1.50  |
| Topotecan (2 μM input)     |                             | 1.20 ± 0.0900                          | 4.60 ± 1.70  |
| Compounds                          | Species                        | $\text{Cl}_{\text{int, app}}$ (μL/min/mg) | Mouse   | Human       |
|-----------------------------------|--------------------------------|----------------------------------------|---------|-------------|
| Propranolol (1 μM input) (Microsomes) |                | 0.0740 ± 0.00200                       | 0.0220 ± 0.00100 |
| Verapamil (1 μM input) (Microsomes) |                | 0.290 ± 0.00500                       | 0.0690 ± 0.00300 |
| Phenacetin (1 μM input) (Hepatocytes) (mL/min/10^6 cells) |            | 0.0330 ± 0.00800                       | —       | —           |
| Verapamil (1 μM input) (Hepatocytes) (mL/min/10^6 cells) |            | 0.0310 ± 0.00100                       | —       | —           |
Fig. S1
Fig. S5

Parent

(M1)

(M2)

(M3)

(M4)

(M5)

(M6)

(M7)

(M8)

(M9)

(M10, M11)

(M12)

(M14)
Fig. S6

HLM +066+F1-130min_MDF

Multiple Mass Defect Filtered file

RT: 0.00 - 24.98 SM: 7G

N-dealkylation + O (M6)

P (M0); M1

N-side chain cutting off (M7)

P-CH₃ (M2)

N-side chain cutting off + O (M8)

P+O (M3)

O-demethylation + hydroxylation + dehydration (M9)

P-CH₂ + O (M4)

O-demethylation + carboxylation (M10)

O-dealkylation (M5)

O-dealkylation + carboxylation (M11)
Fig. S7

HLM+066+F1-1-30min

| Run | RT (min) | AV (A) | FTMS + c ESI Full ms1 (100.00-800.00) |
|-----|---------|-------|---------------------------------|
| #819 | 15.68 | 1 | F: FTMS + c ESI Full ms1 |
| #851 | 16.27 | 1 | F: FTMS + c ESI Full ms1 |
| #855 | 16.35 | 1 | F: FTMS + c ESI Full ms1 |
| #827 | 15.83 | 1 | F: FTMS + c ESI Full ms1 |

| m/z | Relative Abundance |
|-----|--------------------|
| 439.2013 | 
| 437.2220 | 
| 379.1807 | 
| 379.1839 | 
| 372.5400 | 
| 372.5400 | 
| 370.1839 | 
| 340.5296 | 
| 421.9006 | 
| 437.1556 | 
| 329.2321 | 
| 336.2216 | 
| 317.1520 | 
| 317.1520 | 
| 340.8085 | 
| 362.3254 | 
| 372.3769 | 
| 423.2067 | 
| 423.2067 | 
| 423.2067 | 
| 437.1556 | 
| 392.2877 | 
| 392.2877 | 
| 392.2877 | 
| 393.1596 | 

M1 M2 M3 M4 M5 M6 M7 M8

- Relative Abundance
- m/z Values

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Fig. S10

- A1: Concentration (μg/mL) vs. Time (min) for different dosages (0.2 mg/kg, 0.6 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 4.0 mg/kg).

- A2: Concentration (μg/mL) vs. Dosage (μg/kg) with a linear relationship, $R^2 = 0.9780$.

- B1: Concentration (μg/mL) vs. Time (min) for different dosages (0.2 mg/kg, 0.6 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 4.0 mg/kg).

- B2: Concentration (μg/mL) vs. Dosage (μg/kg) with a linear relationship, $R^2 = 0.9701$. 


Fig. S12
Fig. S13