4β-Hydroxycholesterol as an endogenous biomarker of CYP3A activity in cynomolgus monkeys

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3 tables
2 figures
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247 words in Abstract
436 words in Introduction
1200 words in Result and Discussion

Abbreviations:
PXR, pregnane X receptor
LC-MS/MS, liquid chromatography-tandem mass spectrometry
APCI, atmospheric pressure chemical ionization
ESI, electrospray ionization
AUC, area under the serum concentration-time curve
C_{max}, maximum serum concentration
t_{1/2}, terminal half-life
QC, quality control
CV\%, coefficient of variance
Abstract

It has been proposed that in humans 4β-hydroxycholesterol is formed mainly by cytochrome P450 3A (CYP3A) catalyzed metabolism of cholesterol and thus may serve as an endogenous marker for CYP3A activity. Cynomolgus monkey is widely used as one of the non-rodent preclinical safety species in pharmaceutical research. In the current study, the potential application of 4β-hydroxycholesterol as an endogenous biomarker of CYP3A in response to drug treatment was evaluated in cynomolgus monkeys. Following multiple oral administration of rifampicin (a known CYP3A inducer) at 15 mg/kg/day in cynomolgus monkeys, the mean serum 4β-hydroxycholesterol levels increased 4-fold from the base line of 55.3±21.7 to 221±53.4 ng/mL. The mean concentration ratios of 4β-hydroxycholesterol/cholesterol increased 5-fold. The data suggest that 4β-hydroxycholesterol formation from cholesterol metabolism was induced by rifampicin treatment in monkeys. This observation correlated with the metabolism of midazolam (a probe substrate of CYP3A activity) monitored in the same study. The serum exposure (AUC) of midazolam was markedly decreased by approximately 95%, confirming the induction of CYP3A catalytic activity by rifampicin treatment in monkeys. The formation of 4β-hydroxycholesterol from cholesterol was specifically mediated by recombinant cynomolgus CYP3A8 and CYP3A5. The Km values of CYP3A8 and CYP3A5 for 4β-hydroxycholesterol formation from cholesterol were 204 and 104 μmol/L, respectively, and Vmax values were 0.600 and 0.310 pg/pmol/min, respectively. The results suggest that 4β-hydroxycholesterol can be used as an endogenous biomarker to
identify strong CYP3A inducers in cynomolgus monkeys, which may help to evaluate drug-drug interaction potential of drug candidates in preclinical settings.
Introduction

Drug-drug interactions derived from metabolizing enzymes potentially occur on the occasions of drug combinations, and potentially impact drug efficacy and safety. It’s known that the human cytochrome P450 3A (CYP3A) subfamily is involved in the metabolism of more than 50% of marketed drugs. Assessment of drug-drug interaction related to CYP3A is therefore a very important aspect during the drug discovery and development.

Rifampicin, a well-known strong CYP3A inducer in vivo, increased the plasma concentrations of endogenous 4β-hydroxycholesterol to the similar extents as the CYP3A4 activity index (based on the probe drug quinine) indicated in a trial enrolling 24 healthy volunteers (Kanebratt et al., 2008). The average plasma concentration of 4β-hydroxycholesterol in healthy volunteers was approximately 30 ng/mL, while the average level increased to over 200 ng/mL in the patients treated with the antiepileptics (carbamazepine, phenytoin or phenobarbital) that were thought to be strong CYP3A inducers similar as rifampicin (Bodin et al., 2001). Research in vitro showed that biotransformation of cholesterol to 4β-hydroxycholesterol is specifically catalyzed by human CYP3A4/5 (Bodin et al., 2001). 4β-Hydroxycholesterol is replacing probe drugs to be an indicator of CYP3A activity in humans (Diczfalusy et al., 2009; Diczfalussy et al., 2011; Josephson et al., 2008; Lütjohann et al., 2009), which makes studies simpler and safer by eliminating the use of probe drugs especially in the patients who are at high risk of CYP3A involved drug-drug interactions during their therapies.
Nonhuman primates, including cynomolgus (Macaca fascicularis) and rhesus (Macaca mulatta) monkeys, have been used in pharmaceutical development as preclinical models because they are physiologically and anatomically similar to humans. Cynomolgus monkey CYP3A8 is 93% identical at the amino acid level to human CYP3A4 (Carr et al., 2006), and cynomolgus monkey pregnane X receptor (PXR) was highly homologous to human PXR (96%) (Kim et al., 2010). A high degree of correlation in PXR transactivation by 30 compounds was observed between cynomolgus monkey and human in vitro. Furthermore, both cynomolgus monkey and human hepatocytes responded similarly to CYP3A inducers rifampicin and hyperforin, showing comparable increases of RNA expression and enzyme activity between cynomolgus monkey CYP3A8 and human CYP3A4 (Kim et al., 2010). In addition, both human and cynomolgus monkey have CYP3A5 (Iwasaki and Uno, 2009). Therefore, cynomolgus monkey could potentially be a predictive animal model for human CYP3A4/5 induction.

Based on the evidence of cynomolgus monkey as an animal model for human prediction and 4β-hydroxycholesterol as the biomarker of human CYP3A4/5 activity, the present study is demonstrating that 4β-hydroxycholesterol is also an endogenous indicator of the cynomolgus monkey CYP3A8/5 activity, which may assist to assess the CYP3A induction potential of drug candidates at the preclinical stage.
Materials and Methods

Chemicals and reagents

Rifampicin (purity 97%) and midazolam injection (5 mg/mL) were purchased from Aladdin (Shanghai, China) and Nhwa Pharmaceutical Corporation (Shanghai, China), respectively. Reference standards of midazolam (purity 99.05%), 4β-hydroxycholesterol (purity 98%), cholesterol (purity 97%) for bioanalysis were provided by Research Institute for Liver Diseases (Shanghai) Co. Ltd. (Shanghai, China), Toronto Research Chemicals Inc. (Ontario, Canada) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively.

Animals

Male and female cynomolgus (Macaca fascicularis) monkeys (2.7 – 5.5 kg; 3.3 – 5.3 years old) were purposely bred in Hainan, China. Thirty-one male and 15 female cynomolgus monkeys were investigated for the basal concentrations of cholesterol and 4β-hydroxycholesterol in serum. The animals were fed standard monkey feed commercially provided by Beijing Keao Xieli Co., Ltd. (Beijing, China).

CYP3A induction by rifampicin treatment in monkeys

Five male animals were orally administered 2 mg/kg midazolam (1 mg/mL in 0.9% NaCl solution) on Day 1 and Day 16. During the period from Day 2 to Day 15, the animals were orally given 15 mg/kg/day rifampicin (7.5 mg/mL suspension formulated in 0.5% (w/v) aqueous Methocel E5) once daily. On Day 1 and Day 16, blood samples were collected from the animals at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours following each midazolam administration. In addition, blood collections
were conducted prior to each rifampicin dosing on Days 2, 3, 5, 8, 11, 14 and 15, and on Days 17, 19, 22, 25, 29, 32 and 36.

**In vitro incubation in recombinant cynomolgus monkey CYPs**

To identify CYP(s) responsible for the biotransformation of cholesterol to 4β-hydroxycholesterol, 100 μmol/L of cholesterol was incubated with 200 pmol/mL of recombinant cynomolgus CYP2C43, CYP2C75, CYP2C76, CYP2C20, CYP3A8 or CYP3A5 (purchased from Cypex Ltd., Scotland, UK) for 60 minutes at 37°C, and 4β-hydroxycholesterol was determined using an LC-MS/MS method described below. For Km and Vmax estimation, 10, 25, 50, 100, 200, 300 or 500 μmol/L of cholesterol was incubated with 100 pmol/mL of CYP3A8 or CYP3A5 for 60 minutes at 37°C. 4β-Hydroxycholesterol was determined, and Km and Vmax were calculated using the software WinNonlin (version 5.2.1, Pharsight Corporation, California, USA). The incubation system also contained 4 mmol/L MgCl$_2$ and 1 mmol/L NADPH.

**LC-MS/MS analysis of 4β-hydroxycholesterol, cholesterol and midazolam**

The assay for 4β-hydroxycholesterol and cholesterol in monkey serum was adapted from a previously reported method for human plasma (Xu et al., 2013). Briefly, serum sample was mixed with internal standard (4β-hydroxycholesterol-d7 and cholesterol-d7) and 1 mol/L potassium hydroxide, and incubated at 37°C for 1 hour. The incubation was stopped by adding hexane and diluted with water. After centrifugation, the supernatant was collected and evaporated to dryness. The residue was reconstituted with derivatization reagent containing 1.5 mg 4-dimethylaminopyridine, 5.0 mg 2-methyl-6-nitrobenzonic anhydride, 4.0 mg...
2-picolinic acid, 10 µL triethylamine and 75 µL pyridine. The reconstituted solution was further mixed with hexane. After centrifugation, the supernatant was collected and evaporated to dryness, and the residue was reconstituted with 200 µL of acetonitrile/water (9:1, v/v), and analyzed by LC-MS/MS (Sciex API 4000 mass spectrometer). The mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was delivered at a flow rate of 0.5 mL/min with the gradient condition as follows: the proportion of mobile phases A and B at 0.3, 11, 11.1, 13 and 13.1 min was 30/70, 2/98, 2/98, 2/98 and 30/70, respectively. A sample analysis ran 15 minutes. The multiple reaction monitoring transition (in the ESI positive ionization mode) for the dipicolinate derivative of each analyte was as follows: 4β-hydroxycholesterol (m/z 613.5→490.5), 4β-hydroxycholesterol-d7 (m/z 620.5→497.5), cholesterol (m/z 492.5→369.5) and cholesterol-d7 (m/z 499.5→376.5).

For in vitro incubations, the incubation mixture was quenched by adding ice-cold acetonitrile containing internal standard 4β-hydroxycholesterol-d7. The supernatant after centrifugation was evaporated to dryness and then reconstituted with 200 µL of methanol/0.1% formic acid in water (8:2, v/v) for LC-MS/MS analysis (Sciex API 5000 mass spectrometer). The mobile phase consisting of 0.1% formic acid in water (A) and methanol (B) was delivered at a flow rate of 0.5 mL/min with the gradient condition as follows: the proportion of mobile phases A and B at 0, 2, 5, 6, 9, 10 and 15 min was 20/80, 10/90, 10/90, 0/100, 0/100, 20/80 and 20/80 respectively. A sample analysis ran 15 minutes. The multiple reaction monitoring transition (in the APCI
positive ionization mode) for each analyte was as follows: 4β-hydroxycholesterol (m/z 385.4→109.1), 4β-hydroxycholesterol-d7 (m/z 392.3→109.1). The calibration range was from 0.1 to 25 ng/mL.

For the analysis of midazolam, serum sample was mixed with internal stand diazepam, 1% ammonia followed by methyl t-butyl ether. The upper organic layer was collected and evaporated to dryness. The residue was reconstituted and injected to LC-MS/MS (Sciex API 4000 mass spectrometer; Applied Biosystems) for analysis. The mobile phase consisting of 10 mmol/L ammonium acetate in water (A) and acetonitrile/methanol (1:1, v/v) (B) was delivered at a flow rate of 0.5 mL/min with the gradient condition as follows: the proportion of mobile phases A and B at 0.2, 2, 2.05, 3 and 3.05 min was 48/52, 30/70, 5/95, 5/95 and 48/52, respectively. LC-MS/MS analysis was carried out using the multiple reaction monitoring transition (in the ESI positive ionization mode) for each analyte: midazolam (m/z 326.1→291.1) and diazepam (m/z 285.1→193.1).

Pharmacokinetic analysis

The pharmacokinetic (PK) parameters were derived from the serum concentration versus time profile in each animal, using the noncompartmental analysis of WinNonlin (Version 5.2.1, Pharsight). The concentration ratios of 4β-hydroxycholesterol to cholesterol were also used for a limited PK analysis to obtain the maximum ratio (Ratio_{max}), the basal ratio prior to rifampicin dosing (Ratio_{base}) and the terminal half-life (t_{1/2,\, ratio}).
Results and Discussion

*LC-MS/MS analysis of 4β-hydroxycholesterol, cholesterol and midazolam*

The assay for 4β-hydroxycholesterol and cholesterol in monkey serum was adapted from a validated LC-MS/MS method for human plasma that exhibited good chromatographic separations between 4β-hydroxycholesterol and other isobaric oxysterols 24S-, 25-, 27-, 7α-, 7β-hydroxycholesterol (Xu et al., 2013). Especially, an adequate separation from the closest 4α-hydroxycholesterol was demonstrated. Fig. 1 shows the chromatograms of 4α-hydroxycholesterol, 4β-hydroxycholesterol and cholesterol in a monkey serum sample prior to rifampicin treatment. For the determination of 4β-hydroxycholesterol and cholesterol concentrations in serum, the standard curves were fit with a linear regression, and the calibration range was from 5 to 500 ng/mL for 4β-hydroxycholesterol and from 20 to 2000 µg/mL for cholesterol.

For 4β-hydroxycholesterol, the quality control (QC) samples in the analysis run exhibited the bias of -13.3%, -4.0% and -2.8%, respectively, at the levels of 15, 200 and 400 ng/mL, and the CV% (coefficient of variance) of 0.7%, 6.8% and 4.8%, respectively. For cholesterol at the QC levels of 60, 800 and 1600 µg/mL, the bias values were -5.0%, -1.0% and -5.0%, respectively, and the CV% values were 11.9%, 6.6% and 5.7%, respectively.

For the determination of midazolam concentrations in serum, the standard curves were fit with a linear regression, and the calibration range was from 0.1 to 100 ng/mL. The QC samples in the analysis run exhibited the bias of -3.3%, 2.3% and -8.3%, respectively, for 0.3, 30 and 75 ng/mL, and the CV% of 7.5%, 4.1% and 4.0%, respectively.
respectively.

Basal levels of $4\beta$-hydroxycholesterol and cholesterol in cynomolgus monkeys

The basal levels of $4\beta$-hydroxycholesterol and cholesterol were determined in 31 male and 15 female cynomolgus monkeys (Table 1). The concentrations of $4\beta$-hydroxycholesterol in serum ranged from 39.6 to 139 ng/mL with the mean of 79.5 ng/mL and the median of 75.9 ng/mL. The basal levels of cholesterol in serum ranged from 0.685 to 1.43 mg/mL. The mean and median concentrations were 1.05 and 1.04 mg/mL, respectively. The male and female animals had similar basal levels of $4\beta$-hydroxycholesterol and cholesterol. The concentration ratios of $4\beta$-hydroxycholesterol/cholesterol ranged from 0.0406 to 0.108 ($\times 10^{-3}$) with the mean value of 0.0755 ($\times 10^{-3}$) for the male and female animals.

Midazolam exposure and $4\beta$-hydroxycholesterol level after rifampicin treatment in cynomolgus monkeys

After multiple oral dosing of 15 mg/kg/day rifampicin (once daily for 14 consecutive days) in 5 male cynomolgus monkeys, the midazolam exposure in serum markedly decreased by 94.5% from 110±34.3 to 5.84±1.79 (mean±SD) hr×ng/mL for AUC$_{0-\infty}$ and by 94.1% from 58.4±24.3 to 3.42±1.18 ng/mL for C$_{\text{max}}$ at the single oral dose level of 2 mg/kg (Table 2), confirming the activity of CYP3A was elevated. In line with the elevation of CYP3A activity, the mean concentration of endogenous $4\beta$-hydroxycholesterol in serum increased 4-fold from the base line of 55.3±21.7 to 221±53.4 ng/mL, and the concentration ratio of $4\beta$-hydroxycholesterol/cholesterol increased 5-fold from 0.077±0.013 to 0.385±0.045 ($\times 10^{-3}$). The base line of
cholesterol in serum was 0.695±0.158 mg/mL, and during the 14-day rifampicin treatment the average concentrations ranged from 0.516 to 0.628 mg/mL. The elevated 4β-hydroxycholesterol/cholesterol level returned to the base line with the half-life of 15.3±4.3 day after the rifampicin treatment termination. In terms of 4β-hydroxycholesterol, the t1/2 was 12.7±5.0 day. The data are listed in Table 3. As shown by Fig. 2, the normalization of 4β-hydroxycholesterol concentration by the respective cholesterol level minimized the variability of the elevated 4β-hydroxycholesterol levels in monkeys.

It has reported that rifampicin at 15 mg/kg/day in the cynomolgus monkey provided the Cmax value comparable to that from the treatment of 600 mg/day in the human (Kim, et al., 2010). The same dosing regimen of rifampicin and midazolam was applied to the present study, except that the dosing duration was extended from 7 days to 14 days. The change of midazolam exposure observed in this study was similar to the reported results of 92% in cynomolgus monkeys (15 mg/kg/day rifampicin; once daily for 7 days) and 94% in human subjects (600 mg/day rifampicin; once daily for 5 days) (Kim, et al., 2010). In line with the decrease of midazolam exposure, 4β-hydroxycholesterol concentration in serum increased 4-fold in the monkey, which was very similar to the observation following the induction by rifampicin in healthy volunteers (500 mg/day; once daily for 14 days) (Diczfalusy, et al., 2009). Moreover, the terminal half-life of 4β-hydroxycholesterol was 13 days (for 4β-hydroxycholesterol/cholesterol, 15 days) in cynomolgus monkeys, which was close to the 4β-hydroxycholesterol half-life of 17 days observed in humans.
It was observed that the fold of the change of 4β-hydroxycholesterol level was not as drastic as that of midazolam exposure in this study. In response to the rifampicin treatment in the monkey, there was a 10-fold change for midazolam exposure but only 4-fold for 4β-hydroxycholesterol level. Similar observation was reported for efavirenz in HIV-1 positive patients. Following oral repeated administrations of efavirenz (600 mg/day, once daily for at least 21 days) to patients, the median midazolam index (plasma concentration ratio of 1-hydroxymidazolam/midazolam) increased 5-fold compared with the placebo group (Fellay J, et al., 2005), whereas the median 4β-hydroxycholesterol level (after the treatment of 600 mg/day for 28 days) in plasma increased only 2-fold (Josephson F, et al., 2008).

The present study also indicated that the endogenous 4β-hydroxycholesterol had a long elimination half-life of more than 10 days after its level was elevated. Due to the long half-life, the 4β-hydroxycholesterol level may appear unchangeable for a rapid decline of CYP3A activity such as an inhibition effect caused by a short exposure to a reversible inhibitor. It was reported even if the exposure to CYP3A inhibitors was maintained for a few days, the endogenous 4β-hydroxycholesterol levels did not decrease as markedly as it increased in induction studies. Repeated oral administrations of ketoconazole at 400 mg/day (once daily for 4 days) resulted in a decrease of only 17% in the 4β-hydroxycholesterol level in healthy human subjects (Goodenough AK, et al., 2011), and the oral dosing of itraconazole (once daily for 1 week at 400 mg/day) led to a decrease of 25% in patients with onychomycosis (Lütjohann D, et al., 2009).
In vitro CYP identification and kinetics estimation in recombinant CYPs

No 4β-hydroxycholesterol was detected after cholesterol was incubated with recombinant cynomolgus CYP2C20, CYP2C43, CYP2C75 or CYP2C76, but with CYP3A8 and CYP3A5. The values of Km and Vmax were estimated to be 204 μmol/L and 0.600 pg/pmol/min for cynomolgus CYP3A8, and 104 μmol/L and 0.310 pg/pmol/min for cynomolgus CYP3A5. The Km values for cynomolgus monkey CYP3A8 and CYP3A5 estimated in this study were higher than reported for human CYP3A4, although similar Vmax was observed (Shinkyo and Guengerich, 2011).

In summary, this study showed that cynomolgus monkey and the human exhibited similar changes in midazolam exposure and in 4β-hydroxycholesterol concentration after rifampicin treatment at an exposure comparable dose level, which echoed the reported high homology between cynomolgus monkey CYP3A8 and human CYP3A4.

The formation of 4β-hydroxycholesterol from cholesterol was specifically catalyzed by CYP3A8/5 in cynomolgus monkeys. 4β-Hydroxycholesterol is not as sensitive as the classical CYP3A probe midazolam, and may be more beneficial for induction studies than for inhibition investigations. As an endogenous biomarker, 4β-hydroxycholesterol can be used to identify potential strong CYP3A inducers in cynomolgus monkeys, which may help to evaluate drug-drug interaction potential of drug candidates in preclinical settings.

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Authorship Contributions

Participated in research design: Li, Zhao, Wang, Gu, and Han Hsu

Conducted experiments: Zhang, Wu, and Shu

Performed data analysis: Li, Zhang, Feng, and Gu

Wrote or contributed to the writing of the manuscript: Li, Zhao, and Wang
References:


Footnotes:

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FIG. 1. LC-MS/MS chromatograms of 4β-hydroxycholesterol (A) and cholesterol (B) in a monkey serum sample prior to rifampicin treatment.

FIG. 2. Mean serum concentrations of 4β-hydroxycholesterol (A), mean concentration ratios of 4β-hydroxycholesterol to cholesterol (B) and individual concentration ratios of 4β-hydroxycholesterol to cholesterol (C) in male cynomolgus monkeys (animal No. 1001 – 1005) following repeated oral dosing of 15 mg/kg/day rifampicin from Day 2 to Day 15 (once daily).
TABLE 1

*Basal concentrations of 4β-hydroxycholesterol and cholesterol in cynomolgus monkeys*

<table>
<thead>
<tr>
<th>Animal</th>
<th>4β-Hydroxycholesterol</th>
<th>Cholesterol</th>
<th>4β-Hydroxycholesterol/cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ng/mL)(^a)</td>
<td>(mg/mL)(^b)</td>
<td>(×10(^{-3}))(^a)</td>
</tr>
<tr>
<td>All</td>
<td>79.5 ± 22.7</td>
<td>1.05 ± 0.185</td>
<td>0.0755 ± 0.0163</td>
</tr>
<tr>
<td>(n = 46)</td>
<td>(39.6 - 139; 75.9)</td>
<td>(0.685 - 1.43; 1.04)</td>
<td>(0.0406 - 0.108; 0.0752)</td>
</tr>
<tr>
<td>Male</td>
<td>79.7 ± 21.6</td>
<td>1.03 ± 0.192</td>
<td>0.0769 ± 0.0141</td>
</tr>
<tr>
<td>(n = 31)</td>
<td>(39.6 - 139; 76.7)</td>
<td>(0.685 - 1.40; 1.02)</td>
<td>(0.0549 - 0.107; 0.0754)</td>
</tr>
<tr>
<td>Female</td>
<td>79.0 ± 25.4</td>
<td>1.09 ± 0.171</td>
<td>0.0727 ± 0.0205</td>
</tr>
<tr>
<td>(n = 15)</td>
<td>(45.4 - 125; 75.1)</td>
<td>(0.766 - 1.43; 1.05)</td>
<td>(0.0406 - 0.108; 0.0754)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD (range; median)
TABLE 2

*Pharmacokinetic parameters of midazolam in male cynomolgus monkeys (n = 5) following a single oral dose of 2 mg/kg midazolam before and after repeated dosing of 15 mg/kg/day rifampicin*<sup>a</sup>

<table>
<thead>
<tr>
<th>Treatment day</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt; (hr)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (hr)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (hr×ng/mL)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (hr×ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.9 ± 0.1</td>
<td>1.5 ± 0.7</td>
<td>58.4 ± 24.3</td>
<td>110 ± 34.1</td>
<td>110 ± 34.3</td>
</tr>
<tr>
<td>Day 16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.9 ± 0.2</td>
<td>1.1 ± 0.5</td>
<td>3.42 ± 1.18</td>
<td>6.09 ± 2.05</td>
<td>5.84 ± 1.79</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SD

<sup>b</sup>AUC<sub>0-8</sub> for Day 1 and AUC<sub>0-4</sub> for Day 16

<sup>c</sup>Following 14 daily oral administrations of rifampicin from Day 2 to Day 15
TABLE 3

Pharmacokinetic analysis based on 4β-hydroxycholesterol concentrations and concentration ratios of 4β-hydroxycholesterol to cholesterol in male cynomolgus monkeys (n = 5) following rifampicin treatment

<table>
<thead>
<tr>
<th>4β-Hydroxycholesterol</th>
<th>t_{1/2} (day)</th>
<th>C_{max} (ng/mL)</th>
<th>C_{base} (ng/mL)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.7 ± 5.0</td>
<td>221 ± 53.4</td>
<td>55.3 ± 21.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4β-Hydroxycholesterol/cholesterol</th>
<th>t_{1/2, ratio} (day)</th>
<th>Ratio_{max} (×10^{-3})</th>
<th>Ratio_{base} (×10^{-3})^b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15.3 ± 4.3</td>
<td>0.385 ± 0.0451</td>
<td>0.0775 ± 0.0130</td>
</tr>
</tbody>
</table>

^a Basal concentration of 4β-hydroxycholesterol in serum prior to the first rifampicin dosing

^b Calculation from the basal concentrations of 4β-hydroxycholesterol and cholesterol in serum prior to the first rifampicin dosing