Short Communication

4β-Hydroxycholesterol as an Endogenous Biomarker of CYP3A Activity in Cynomolgus Monkeys

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ABSTRACT

It has been proposed that in humans 4β-hydroxycholesterol is formed mainly by CYP3A-catalyzed metabolism of cholesterol and thus may serve as an endogenous marker for CYP3A activity. The cynomolgus monkey is widely used as one of the nonrodent 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/d in cynomolgus monkeys, the mean serum 4β-hydroxycholesterol levels increased 4-fold from the baseline of 55.3 ± 21.7 to 221 ± 53.4 ng/ml. The mean concentration ratios of 4β-hydroxycholesterol to 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 (area under the curve) of midazolam was markedly decreased by ~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 \( K_{m} \) values of CYP3A8 and CYP3A5 for 4β-hydroxycholesterol formation from cholesterol were 204 and 104 \( \mu \)M, respectively, and \( V_{max} \) 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 occasion of drug combination and potentially impact drug efficacy and safety. It’s known that the human cytochrome P450 (P450) CYP3A subfamily is involved in the metabolism of >50% of marketed drugs. Assessment of drug-drug interactions related to CYP3A is therefore a very important aspect during drug discovery and development. Rifampicin, a well-known strong CYP3A inducer in vivo, increased the plasma concentrations of endogenous 4β-hydroxycholesterol to a similar extent as the CYP3A4 activity index (based on the probe drug quinine) indicated in a trial that enrolled 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 >200 ng/ml in patients treated with antiepileptics (carbamazepine, phenytoin, or phenobarbital) thought to be strong CYP3A inducers similar to 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 (Josephson et al., 2008; Diczfalusy et al., 2009, 2011; Lütjohann et al., 2009), which makes studies simpler and safer by eliminating the use of probe drugs especially in 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) is 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 monkeys and humans in vitro. Furthermore, both cynomolgus monkey and human hepatocytes responded similarly to the 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 humans and cynomolgus monkeys have CYP3A5 (Iwasaki and Uno, 2009). Therefore, cynomolgus monkeys could potentially be a predictive animal model for human CYP3A4/5 induction.

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

ABBREVIATIONS: AUC, area under the serum concentration-time curve; CV%, coefficient of variance; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; P450, cytochrome P450; PXR, pregnane X receptor; QC, quality control; \( t_{1/2} \), terminal half-life.
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/d 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 (predose), 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 dose 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 P450 Enzymes.** To identify P450 enzyme(s) responsible for the biotransformation of cholesterol to 4β-hydroxycholesterol, 100 µM cholesterol was incubated with 200 pmol/ml recombinant cynomolgus CYP2C43, CYP2C75, CYP2C76, CYP2C20, CYP3A8, or CYP3A5 (purchased from Cypex Ltd., Dundee, UK) for 60 minutes at 37°C, and 4β-hydroxycholesterol was determined using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method described below. For $K_m$ and $V_{max}$ estimation, 10, 25, 50, 100, 200, 300, or 500 µM cholesterol was incubated with 100 pmol/ml CYP3A8 or CYP3A5 for 60 minutes at 37°C. 4β-Hydroxycholesterol was determined, and $K_m$ and $V_{max}$ were calculated using the software WinNonlin (version 5.2.1; Pharsight Corporation, Sunnyvale, CA). The incubation system also contained 4 mM MgCl₂ and 1 mM 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-d₄ 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-picoline 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 acetonitrile/water [9:1 (v/v)] and analyzed by LC-MS/MS (Sciex API 4000 mass spectrometer; AB Sciex, Framingham, MA). The mobile phase consisted 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 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 phase 60 minutes at 37°C. 4β-Hydroxycholesterol was determined using the software WinNonlin (version 5.2.1; Pharsight Corporation, Sunnyvale, CA). The incubation system also contained 4 mM MgCl₂ and 1 mM NADPH.

**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%), and cholesterol (purity 97%) for bioanalysis were provided by Research Institute for Liver Diseases (Shanghai) Co. Ltd. (Shanghai, China), Toronto Research Chemicals Inc. (Toronto, ON, 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

**TABLE 1**

<table>
<thead>
<tr>
<th>Animals</th>
<th>4β-Hydroxycholesterol (ng/ml)</th>
<th>Cholesterol (ng/ml)</th>
<th>4β-Hydroxycholesterol/Cholesterol Ratio ($\times 10^{-3}$)</th>
</tr>
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<tbody>
<tr>
<td>All (n = 46)</td>
<td>79.5 ± 22.7 (39.6–139; 75.9)</td>
<td>1.05 ± 0.185 (0.685–1.43; 1.04)</td>
<td>0.0755 ± 0.0163 (0.0406–0.108; 0.0752)</td>
</tr>
<tr>
<td>Male (n = 31)</td>
<td>79.7 ± 21.6 (39.6–139; 76.7)</td>
<td>1.03 ± 0.192 (0.685–1.40; 1.02)</td>
<td>0.0769 ± 0.0141 (0.0549–0.107; 0.0754)</td>
</tr>
<tr>
<td>Female (n = 15)</td>
<td>79.0 ± 25.4 (45.4–125; 75.1)</td>
<td>1.09 ± 0.171 (0.766–1.43; 1.05)</td>
<td>0.0727 ± 0.0205 (0.0406–0.108; 0.0754)</td>
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10/90, 0/100, 20/80, and 20/80, respectively. A sample analysis ran 15 minutes. The MRM transition (in the atmospheric pressure chemical ionization positive ionization mode) for each analyte was as follows: 4β-hydroxycholesterol (m/z 385.4 → 109.1) and 4β-hydroxycholesterol-d$_7$ (m/z 392.3 → 109.1). The calibration range was 0.1–25 ng/ml.

For the analysis of midazolam, serum sample was mixed with internal standard 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) for analysis. The mobile phase consisting of 10 mM 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 water (A) and acetonitrile/methanol [1:1 (v/v)] (B) was delivered at a flow rate for analysis. The mobile phase consisting of 10 mM ammonium acetate in organic layer was collected and evaporated to dryness. The residue was standard diazepam, 1% ammonia, followed by methyl oxysterols 24S-, 25-, 27-, 7-,

### 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 the other isobaric oxysterols 24S-, 25-, 27-, 7-, and 7β-hydroxycholesterol (Xu et al., 2013). Especially, an adequate separation from the closest 4α-hydroxycholesterol was demonstrated. Figure 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 biases of −13.3%, −4.0%, and −2.8%, respectively, at the levels of 15, 200, and 400 ng/ml, and the coefficients of variance (CV%) 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 biases of −3.3%, 2.3%, and −8.3%, respectively, for 0.3, 30, and 75 ng/ml, and CV% values of 7.5%, 4.1%, and 4.0%, respectively.

**Basal Levels of 4β-Hydroxycholesterol and Cholesterol in Cynomolgus Monkeys.** The basal levels of 4β-hydroxycholesterol and cholesterol were determined in 31 male and 15 female cynomolgus monkeys (Table 1). The concentrations of 4β-hydroxycholesterol in serum ranged from 39.6 to 139 ng/ml, with a mean of 79.5 ng/ml and a 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 4β-hydroxycholesterol and cholesterol. The concentration ratios of 4β-hydroxycholesterol to cholesterol ranged from 0.0406 × 10$^{-3}$ to 0.108 × 10$^{-3}$, with a mean value of 0.0755 × 10$^{-3}$ for the male and female animals.

**Midazolam Exposure and 4β-Hydroxycholesterol Level after Rifampicin Treatment in Cynomolgus Monkeys.** After multiple oral dosing with 15 mg/kg/d rifampicin (once daily for 14 consecutive days) in 5 male cynomolgus monkeys, midazolam exposure in serum markedly decreased by 94.5%, from 110 ± 34.3 to 5.84 ± 1.79 (mean ± S.D.) ng/ml, for the area under the serum concentration–time curve (AUC$_{0→∞}$) 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4β-Hydroxycholesterol</th>
<th>4β-Hydroxycholesterol/Cholesterol Ratio</th>
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<tbody>
<tr>
<td>$t_{1/2}$ (d)</td>
<td>12.7 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td>221 ± 53.4</td>
<td></td>
</tr>
<tr>
<td>$C_{\text{mean}}$ (ng/ml)</td>
<td>55.3 ± 21.7</td>
<td></td>
</tr>
<tr>
<td>$t_{1/2\text{ratio}}$ (d)</td>
<td>15.3 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Ratio$_{\text{max}}$ (× 10$^{-3}$)</td>
<td>0.385 ± 0.0451</td>
<td></td>
</tr>
<tr>
<td>Ratio$_{\text{mean}}$ (× 10$^{-3}$)</td>
<td>0.0775 ± 0.0130</td>
<td></td>
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</table>

*Basal concentration of 4β-hydroxycholesterol in serum prior to the first rifampicin dose.

*Calculation from the basal concentrations of 4β-hydroxycholesterol and cholesterol in serum prior to the first rifampicin dose.
that the activity of CYP3A was elevated. In line with the elevation of CYP3A activity, the mean concentration of endogenous 4β-hydroxycholesterol in serum increased 4-fold from the baseline of 55.3 ± 21.7 to 221 ± 53.4 ng/ml, and the concentration ratio of 4β-hydroxycholesterol to cholesterol increased 5-fold from 0.077 ± 0.013 × 10⁻³ to 0.385 ± 0.045 × 10⁻³. The baseline 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 baseline with a half-life of 15.3 ± 4.3 days after termination of the rifampicin treatment. In terms of 4β-hydroxycholesterol, the t½ was 12.7 ± 5.0 days. 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 been reported that rifampicin at 15 mg/kg/d in cynomolgus monkeys provided a Cmax value comparable to that from the treatment with 600 mg/d in humans (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 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/d rifampicin once daily for 7 days) and 94% in human subjects (600 mg/d rifampicin once daily for 5 days) (Kim et al., 2010). In line with the decrease of midazolam exposure, the 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/d 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 change in 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 observations were reported for efavirenz in human immunodeficiency virus type 1-positive patients. Following oral repeated administrations of efavirenz (600 mg/d once daily for at least 21 days) to patients, the median midazolam index (plasma concentration ratio of 1-hydroxymidazolam to midazolam) increased 5-fold compared with the placebo group (Fellay et al., 2005), whereas the median 4β-hydroxycholesterol level (after treatment with 600 mg/d for 28 days) in plasma increased only 2-fold (Josephson et al., 2008).

The present study also indicated that endogenous 4β-hydroxycholesterol had a long elimination half-life of >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 that even if the exposure to CYP3A inhibitors was maintained for a few days, the endogenous 4β-hydroxycholesterol level did not decrease as markedly as it increased in induction studies. Repeated oral administrations of ketoconazole at 400 mg/d (once daily for 4 days) resulted in a decrease of only 17% in the 4β-hydroxycholesterol level in healthy human subjects (Goodenough et al., 2011), and oral dosing of itraconazole (once daily for 1 week at 400 mg/d) led to a decrease of 25% in patients with onychomycosis (Lüttjohann et al., 2009).

**In Vitro P450 Identification and Kinetics Estimation in Recombinant P450 Enzymes.** No 4β-hydroxycholesterol was detected after cholesterol was incubated with recombinant cynomolgus CYP2C20, CYP2C43, CYP2C75, or CYP2C76, but it was detected with CYP3A8 and CYP3A5. The K_m and V_max values were estimated to be 204 μM and 0.600 pmol/min, respectively, for cynomolgus CYP3A8 and 104 μM and 0.310 pmol/min for cynomolgus CYP3A5. The K_m values for cynomolgus monkey CYP3A8 and CYP3A5 estimated in this study were higher than reported for human CYP3A4, although similar V_max was observed (Shinkyo and Guengerich, 2011).
In summary, this study showed that cynomolgus monkeys and humans 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 classic 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. Han Hsu.

Conducted experiments: Zhang, Wu, Shu.

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

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

References


Address correspondence to: Ke Li, Drug Safety Sciences Asia Pacific, Janssen Research & Development, 25F Shinmay Union Square, 999 South Pudong Rd., Shanghai 200120, China. E-mail: kli30@its.jnj.com