Comparison of Endogenous 4β-Hydroxycholesterol with Midazolam as Markers for CYP3A4 Induction by Rifampicin

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ABSTRACT

CYP3A4, considered the most important enzyme in drug metabolism, is often involved in drug-drug interactions. When developing new drugs, appropriate markers for detecting CYP3A4 induction are needed. Our study compared endogenously formed 4β-hydroxycholesterol with the midazolam clearance in plasma and the 6β-hydroxycortisol/cortisol ratio in urine as markers for CYP3A4 induction. To this end, we performed a clinical trial in which 24 healthy subjects were randomized to 10, 20, or 100 mg daily doses of rifampicin for 14 days (n = 8 in each group) to achieve a low and moderate CYP3A4 induction. The CYP3A4 induction could be detected even at the lowest dose of rifampicin (10 mg) via the estimated midazolam clearance, the 4β-hydroxycholesterol ratio (both P < 0.01), and the 6β-hydroxycortisol ratio (P < 0.05). For the three dosing groups (10, 20, and 100 mg), the median fold induction from baseline was 2.0, 2.6, and 4.0 for the estimated midazolam clearance; 1.3, 1.6, and 2.5 for the 4β-hydroxycholesterol/cortisol ratio; and 1.7, 2.9, and 3.1 for the 6β-hydroxycortisol/cortisol ratio. In conclusion, the 4β-hydroxycholesterol ratio is comparable to midazolam clearance as a marker of CYP3A4 induction, and each may be used to evaluate CYP3A4 induction in clinical trials evaluating drug-drug interactions for new drugs.

Introduction

An important aspect of drug development is to predict clinically relevant drug-drug interactions such as induction or inhibition of cytochrome P450 enzymes in the liver or intestine. CYP3A4 is considered to be the most important enzyme catalyzing drug metabolism with broad substrate specificity. CYP3A4 may be involved in drug-drug interactions as a result of drug inhibition or induction (Backman et al., 1996; Gerber et al., 2005; Pal and Mitra, 2006).

For a long time, midazolam has been considered the probe drug of choice for measuring CYP3A4 activity in humans (Fuhr et al., 2007). Pharmaceutical companies use midazolam clearance in healthy volunteers before and after intake of new drug entities to determine the potential to induce or inhibit CYP3A4. However, the pharmaceutical industry is today working toward the use of endogenous substances instead of probe drugs to avoid unnecessary administration of drugs to healthy subjects and patients. The induction properties of a new compound could then be investigated in early pharmacokinetic and tolerability studies with repeated dosing in the development program, such as a multiple ascending dose study.

The metabolite 4β-hydroxycholesterol, which is formed by CYP3A4 and CYP3A5-catalyzed metabolism of cholesterol, has been suggested as a robust marker for CYP3A activity (Bodin et al., 2001; Diczfalusy et al., 2011). A previous study showed that rifampicin, an antituberculosis drug and a well-known inducer of CYP3A activity, increases 4β-hydroxycholesterol in a dose-dependent manner (Kanebratt et al., 2008). In that study, rifampicin was administered at doses of 20, 100, and 500 mg daily for 2 weeks, resulting in 1.5, 2.5, and 4-fold induction of 4β-hydroxycholesterol, respectively. The intraindividual variation in 4β-hydroxycholesterol in untreated subjects is low, with a coefficient of variation between 4.8 and 13.2% during a time period of 3 months (Diczfalusy et al., 2009). The elimination half-life of 4β-hydroxycholesterol is about 17 days, resulting in stable plasma concentrations within subjects (Diczfalusy et al., 2009).

The long half-life excludes 4β-hydroxycholesterol as a marker for rapid changes in CYP3A4 activity. The level of 4β-hydroxycholesterol in plasma depends not only on the CYP3A4/5 activity but also on the concentration of cholesterol. Thus, the 4β-hydroxycholesterol/cholesterol ratio (4β-hydroxycholesterol ratio) is used to adjust for possible changes in the cholesterol level in the subject at different time points.

Another suggested endogenous marker for CYP3A4 activity is the 6β-hydroxycortisol/cortisol ratio (6β-hydroxycortisol ratio) in urine...
Materials and Methods

Study Design. In an open, randomized controlled study, 24 Swedish Caucasian healthy volunteers were randomized to receive 10, 20, or 100 mg of rifampicin per day for 2 weeks to achieve CYP3A4 induction. There were eight subjects in each treatment group. The midazolam AUC, 4β-hydroxycholesterol ratio, and 6β-hydroxycortisol ratio were determined at baseline, after 2 weeks of medication, and at 2 weeks after termination of medication. The CYP3A4 induction was determined by the 4β-hydroxycholesterol ratio in plasma and the estimated midazolam clearance and the 6β-hydroxycortisol ratio in urine collected during 14 hours at baseline and after 2 weeks of rifampicin treatment. A simplified study design is shown in Fig. 1.

Participants. The inclusion criteria were healthy males and females aged 18 years and older, Caucasian, with negative drug screening tests and an agreement to refrain from any other drugs (including herbal supplements) during the study period. All subjects were ascertained to be healthy on the basis of their medical history, physical examination, virology testing (hepatitis B and C, and human immunodeficiency virus), and routine laboratory testing (liver and kidney function and hematology) before they were enrolled in the study. The participants had to completely refrain from alcohol intake on days 0, 1, 14, and 15 of the study when the midazolam doses were administered. During the rest of the study period, only moderate intake of alcohol was allowed. The participants were not allowed to drink grapefruit juice during the entire study period, starting 2 days before the study began. The women of childbearing age had to be using a reliable barrier contraceptive rather than an oral hormone-based contraceptive during the study period, and they also had to have a negative pregnancy test result at the screening visit.

Exclusion criteria were prior drug allergic reactions, signs of infection, use of oral hormone-based contraceptives, intake of any drugs that could influence the enzyme activity of CYP3A4, a positive drug-screening test, pregnancy, breastfeeding, or a history of liver disease.

Interventions. The 24 subjects were randomized to take rifampicin (Rifadin oral suspension; Sanofi S.A., Paris, France) for 2 weeks in one of three different daily doses: 10 mg (n = 8), 20 mg (n = 8), or 100 mg (n = 8). The plasma samples for determining the 4β-hydroxycholesterol ratio and the urine samples for determining the 6β-hydroxycortisol ratio were collected at baseline, after 2 weeks of rifampicin treatment, and at 2 weeks after termination of rifampicin administration. The urine collection started at 6:00 pm the day before the start of the study (day -1) and continued until 8:00 am on day 0 (14 hours). Urine was collected from day 14 at 6:00 pm to day 15 at 8:00 am (14 hours) and from day 27 at 6:00 pm to day 28 at 8:00 am (14 hours).

At the start of the study (day 0), the subjects were given an oral suspension of midazolam in a dose of 4 mg (midazolam APL, 1 mg/ml oral solution; Apoteket APL, Stockholm, Sweden). Blood samples were drawn at nine time points: before the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose for the midazolam determination and the 1′-hydroxymidazolam plasma concentrations. The midazolam AUC was determined at baseline (day 0) and after 2 weeks (day 14) of rifampicin administration. The subjects also took 250 mg of quinine, and a blood sample for quinine analysis was drawn 14 hours after this dose at baseline and after 2 and 4 weeks. Unfortunately, the quinine samples have yet to be analyzed because of methodologic issues.

All subjects were genotyped for CYP3A*1 and CYP3A5*3 by the method described previously elsewhere (Muirhead et al., 2006).

The study design, including the different measurements, is presented in Fig. 1. The subjects were their own controls, and the baseline values were compared with the values after 2 weeks of induction and the values 2 weeks after last dose of rifampicin.

Measurements of 4β-Hydroxycholesterol and Cholesterol. The plasma 4β-hydroxycholesterol determination was performed by isotope dilution gas chromatography-mass spectrometry using [2H6]4β-hydroxycholesterol as the internal standard, as described elsewhere (Bodin et al., 2001; Diczfalussy et al., 2011). The total variation was 8.2% at 23.9 ng/ml. The 4β-hydroxycholesterol/cholesterol ratio was calculated and expressed as mol/mol × 10^4.

Measurements of 6β-Hydroxycortisol and Cortisol. Urinary 6β-hydroxycortisol and cortisol were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using [2H5]6β-hydroxycortisol and [2H5]cortisol as internal standards as described in a previous report (Märde Arrhen et al., 2012).
The total variations were 9.2% (at 255 nM) and 6.9% (at 89 nM) for 6β-hydroxycortisol and cortisol, respectively.

**Measurements of Midazolam.** The concentration of midazolam and its metabolite 1'-hydroxymidazolam were determined by LC-MS/MS (Dostalek et al., 2010; Zhang et al., 2010; Kaartama et al., 2011). Sample preparation was performed by protein precipitation with acetonitrile containing internal standards \[^{[H]}\text{midazolam}\] and \[^{[H]}\text{1'-hydroxymidazolam}\]. We added 200 µl of the internal standard solution to 100 µl of sample. After vortexing and centrifugation, 7 µl of the supernatant was injected onto the LC-MS/MS system. Separation of the analytes was achieved on an Acquity UPLC BEH C18-column (2.1 × 50 mm 1.7 µm) (Waters, Milford, MA), using a gradient run with mobile phase A (11 mM ammonium formate) and mobile phase B (0.1% formic acid in acetonitrile). The analytes were detected using a Micro- mass Quattro Premier XE mass spectrometer (Waters) operating in positive electrospray ionization mode using selected reaction monitoring for the transitions m/z (mass-to-charge ratio) 326 → 291 for midazolam and m/z 342 → 324 for 1'-hydroxymidazolam, respectively. The transitions m/z 330 → 295 and m/z 346 → 328 were used for the internal standards for midazolam and 1'-hydroxymidazolam, respectively. The concentrations of both internal standards were 2.0 ng/ml.

The midazolam concentrations could be determined at all nine time points: before the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose. The kinetics of midazolam did not allow us to accurately extrapolate the midazolam kinetics to infinity; instead, the AUC0-the dose. The kinetics of midazolam did not allow us to accurately extrapolate before the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose (0), after 30 minutes, and at 1, 2, 3, 4, 6, 8, and 10 hours after the dose

The baseline values of estimated midazolam clearance, calculated as midazolam dose/AUC0-10 h (midazolam) and were correlated with the 6β-hydroxycholesterol ratio (P < 0.01) but not with the 6β-hydroxycholesterol ratio (P = 0.30) (Fig. 2).

There was no statistically significant difference between the men and women in the baseline values of the three markers.

**Change of Midazolam Clearance, 4β-Hydroxycholesterol Ratio, and 6β-Hydroxy cortisol Ratio during Rifampicin Treatment.** The CYP3A induction after 2 weeks of daily 10, 20, or 100 mg of rifampicin treatment could be detected by all three markers, also at the lowest dose of rifampicin (Figs. 3 and 4). Statistical analysis comparing the 2-week values with the baseline values in the different dosing groups showed that midazolam clearance and the 4β-hydroxycholesterol/cholesterol ratio were comparable as markers of induction (P > 0.01) (Fig. 3). The 6β-hydroxycholesterol ratio in urine showed more divergent results between the subjects, and the statistical analysis indicated that this marker was somewhat inferior to the 4β-hydroxycholesterol ratio (P < 0.05) (Fig. 3). In the fold-induction analysis, 4β-hydroxycholesterol was superior in detecting a statistically significant difference between the three dosing groups (P < 0.001) compared with P < 0.01 for midazolam clearance and P = 0.03 for the 6β-hydroxycholesterol ratio (Kruskal Wallis test) (Fig. 4). However the fold induction of midazolam clearance was larger than the fold induction of 4β-hydroxycholesterol/cholesterol ratio in all dose groups (Fig. 4).

There was no statistical significant difference in fold induction between the men and women in any of the three CYP3A markers.

**4β-Hydroxycholesterol Ratio and 6β-Hydroxy cortisol Ratio 2 Weeks after Termination of Rifampicin Administration.** In Fig. 3 the values of the 6β-hydroxy cortisol ratio and 4β-hydroxy cholesterol ratio can be followed through the study period from baseline, after 2 weeks of rifampicin treatment, and at 2 weeks after termination of rifampicin treatment (4 weeks after the study’s start). Midazolam clearance was not determined after 4 weeks. Both the 6β-hydroxy cortisol ratio and the 4β-hydroxy cholesterol ratio could detect the decrease in CYP3A activity after termination of rifampicin treatment, week 4 compared with week 2. Four weeks after the study’s start, the 6β-hydroxy cortisol ratio had returned to base-line values (Fig. 3). In contrast, 4β-hydroxy cholesterol ratio still showed significantly higher values 4 weeks after the study’s start compared with baseline in all three dosing groups due to the long half-life of this compound (Fig. 3).

**Discussion**

We have shown that the endogenous 4β-hydroxy cholesterol could be used as a biomarker to evaluate CYP3A induction and that it gives comparable results to the commonly used probe drug midazolam. We used 4β-hydroxy cholesterol in our previous studies on CYP3A...
induction (Kanebratt et al., 2008; Wide et al., 2008; Habtewold et al., 2012), showing concordance with the response to other markers of CYP3A activity such as quinine (Kanebratt et al., 2008) or the cortisol ratio in urine (Mårde Arrhen et al., 2012). This is to our knowledge the first study in which the $4\beta$-hydroxycholesterol ratio is compared head to head with midazolam as a marker of CYP3A induction.

The fold induction of CYP3A activity measured by the $4\beta$-hydroxycholesterol ratio in our study at the doses of 20 and 100 mg were in good concordance with the results from our previously performed study with the same doses: 1.6 and 2.5 (Fig. 4) compared with 1.5 and 2.5 (Kanebratt et al., 2008). In our previously study, a higher rifampicin dose of 500 mg daily was also investigated, resulting in a 4-fold increase in $4\beta$-hydroxycholesterol levels (Kanebratt et al., 2008).

As seen in Fig. 4, the magnitude of the induction determined by oral midazolam clearance was larger than the induction determined by $4\beta$-hydroxycholesterol ratio. The reason for this is probably the influence of the induction on both systemic clearance as well as bioavailability of the orally administered midazolam. Another reason can be that the $4\beta$-hydroxycholesterol ratio is not at steady state because of the slow

### Table 1

Baseline demographic data: number of males and females and median values for age and body mass index (BMI) for all participants in the different rifampicin dosing groups.

<table>
<thead>
<tr>
<th>Rifampicin daily dose</th>
<th>ALL (n = 24)</th>
<th>10 mg (n = 8)</th>
<th>20 mg (n = 8)</th>
<th>100 mg (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>12/12</td>
<td>6/2</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>Age, yr (range)</td>
<td>25 (20–37)</td>
<td>24 (21–27)</td>
<td>28 (21–33)</td>
<td>24 (20–37)</td>
</tr>
<tr>
<td>BMI, kg/m$^2$ (range)</td>
<td>22.8 (16.7–32.9)</td>
<td>22.1 (18.1–32.9)</td>
<td>20.7 (16.7–26.4)</td>
<td>24.0 (18.4–27.1)</td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of three different markers for CYP3A-activity: estimated midazolam clearance (ml/h) calculated as the midazolam dose/AUC$_{0-10\ h}$, $4\beta$-hydroxycholesterol ratio ($4\beta$-OHchol R), and $6\beta$-hydroxycortisol ratio ($6\beta$-OHcortisol R) at baseline, after CYP3A induction had been achieved by 2 weeks of rifampicin treatment (10, 20, or 100 mg daily), and at 2 weeks after rifampicin administration was terminated (4 weeks). The midazolam clearance was not determined after 4 weeks. Statistically significant differences were calculated using Wilcoxon matched-pairs single rank test.
turnover of 4β-hydroxycholesterol (half-life of 17 days) and is not yet reflecting the induced CYP3A levels.

Midazolam clearance and 4β-hydroxycholesterol ratio seem to be somewhat superior to 6β-hydroxycortisol ratio as markers for detecting CYP3A induction (Fig. 4). This might be explained by the extensive variation in cortisol secretion into the circulation that is influenced by several factors such as stress, infections and a circadian rhythm, making 6β-hydroxycortisol ratio less stable as a marker.

Midazolam is extensively metabolized by the intestinal CYP3A4 and hence midazolam clearance also measures intestinal CYP3A4 activity. Whether 4β-hydroxycholesterol is synthesized in the intestine is not known.

Including 4β-hydroxycholesterol ratio very early in drug development programs such as in the multiple ascending dose study, often the second clinical study performed, is advantageous because that will give an early indication whether CYP3A induction is an issue and at what doses that would start to occur. If there are no signs of induction, it is unlikely this will be a problem. However, if there is a signal of induction on the 4β-hydroxycholesterol ratio, to fully understand the impact of concomitant drugs, a study with either midazolam or the more relevant drugs later in the development program should be performed to assess the magnitude of the effect.

The long elimination half-life of 4β-hydroxycholesterol (17 days) results in stable circulating concentrations (Diczfalusy et al., 2009). This is an advantage during determinations under steady-state conditions, but it makes this marker less appropriate for investigations of rapid changes in CYP3A activity (i.e., the decrease of activity due to potent inhibitors of the enzyme). However, we have previously shown that in 22 HIV patients undergoing ritonavir-boosted treatment with atazanavir, known to inhibit CYP3A activity, the concentration of plasma 4β-hydroxycholesterol decreased significantly (Josephson et al., 2008). Similarly, patients treated with the CYP3A inhibitor itraconazole daily for 1 week on two occasions exhibited a significant decrease in serum 4β-hydroxycholesterol during both treatment periods (Lütjohann et al., 2009). These two studies show that 4β-hydroxycholesterol might be used to demonstrate inhibition of CYP3A activity as well. Interestingly, in the present study we show that despite the long half-life of 4β-hydroxycholesterol this marker seemed to be comparable with the 6β-hydroxycortisol ratio in detecting the decrease of CYP3A4 activity after termination of rifampicin administration (Fig. 3). However, although the 6β-hydroxycortisol ratio had returned to the baseline levels 2 weeks after the last rifampicin dose, the 4β-hydroxycholesterol ratio had not entirely returned to baseline (Fig. 3).

The advantages and disadvantages of the three markers for CYP3A activity studied here are summarized in Table 2. In addition to measuring the CYP3A3-activity, the 4β-hydroxycholesterol ratio and 6β-hydroxycortisol ratio are also affected by the CYP3A5 genotype (Diczfalusy et al., 2008; Hassan et al., 2013). In contrast, midazolam clearance is not affected by the CYP3A5 genotype in vivo (Fromm et al., 2007; Kharasch et al., 2007; Tomalik-Scharte et al., 2008; Miao et al., 2009). In Caucasians, few individuals express CYP3A5, as demonstrated here with only two subjects of 24 having one active CYP3A5 allele, similar to earlier reports (Diczfalusy et al., 2008). In black Tanzanians, a major part of the population expresses CYP3A5 (Diczfalusy et al., 2008).

A major advantage with 4β-hydroxycholesterol is that no probe drug needs to be administrated and only one blood sample is required at any time. For midazolam, when a probe drug is given, it must be followed by 8 to 10 blood samples drawn during 8 to 10 hours. A single blood sample after midazolam cannot predict the clearance for the drug (Rogers et al., 2002). On the other hand, as described earlier, midazolam is superior to 4β-hydroxycholesterol in detecting inhibition of CYP3A4 (Table 2). Also, 6β-hydroxycortisol ratio can measure CYP3A inhibition (Li et al., 2010).

Because of the long half-life of 4β-hydroxycholesterol, the study period in which CYP3A4 induction is determined must be at least 2 months.

### TABLE 2

| Advantages and disadvantages of different markers of CYP3A4/5 activity: comparison of midazolam clearance (midazolam), 4β-hydroxycholesterol ratio (4β-OHchol R), and 6β-hydroxycortisol ratio (6β-OHcortisol R) |
|---------------------------------|----------------|----------------|
| Probe drug administration required | Yes | No | No |
| Number of blood samples to be drawn | 8–10 during 8–10 h | 1 urine sample | |
| Detection of CYP3A4 induction by low dose of rifampicin | Yes | Yes | Yes |
| Detection of CYP3A4 inhibition | Yes | Yes, but limited | Yes |
| CYP3A5 measured in addition to CYP3A4 in vivo | No | Yes | Yes |
| Measurement of intestinal CYP3A4 | Yes | Not known | No |
| Duration of study | Short term | Long term (>2 weeks) | Short term |
weeks. In contrast, a much shorter study is possible for detecting induction via midazolam clearance and the 4\(\beta\)-hydroxycortisol ratio.

The two markers of CYP3A activity, 4\(\beta\)-hydroxycholesterol and midazolam, provide similar information and have their own advantages and disadvantages. Major advantages of the former include that it is endogenous and no drug must be given; with the latter, it can be used to record rapid changes in CYP3A activity such as inhibition.

The 4\(\beta\)-hydroxycortisol ratio was not only comparable with midazolam clearance in demonstrating CYP3A induction, but there was also a significant correlation between the baseline values of midazolam clearance and the 4\(\beta\)-hydroxycholesterol ratio. This indicates that the 4\(\beta\)-hydroxycortisol ratio might also be used as a marker to evaluate CYP3A activity at baseline and not only during induction (Fig. 2). It should, however, be remembered that only 29% \((r^2 = 0.29)\) of the variation in the 4\(\beta\)-hydroxycholesterol ratio is related to the variation in midazolam clearance.

In conclusion, to use an endogenous biomarker such as 4\(\beta\)-hydroxycholesterol in clinical trials evaluating drug-drug interactions for new drugs is safer, easier, and cheaper than using probe drugs. In addition, CYP3A4 induction could be monitored in the patients in whom it is not suitable to administer probe drugs, such as children, pregnant women, and elderly.

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Contributed new reagents or analytic tools: Nylén, Rönnquist-Niïi, Diczafalussy.

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Wrote or contributed to the writing of the manuscript: Björkhem-Bergman, Bäckström, Nylén, Rönnquist-Niïi, Bredberg, Andersson, Bertilsson, Diczafalussy.

References


