Comparison of endogenous 4β-hydroxycholesterol with midazolam as markers for CYP3A4 induction by rifampicin

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Abbreviations: CYP3A4; cytochrome P450 3A4, 6β–hydroxycortisol ratio; 6β–hydroxycortisol / cortisol ratio; 4β–hydroxycholesterol ratio, 4β–hydroxycholesterol/cholesterol ratio x 10^4
Abstract

Cytochrome P450 3A4 (CYP3A4) is considered the most important enzyme in drug metabolism and is often involved in drug-drug interactions. When developing new drugs appropriate markers for detecting CYP3A4-induction are needed. The aim of the present study was to compare the endogenously formed 4β–hydroxycholesterol with midazolam clearance in plasma and with 6β–hydroxycortisol / cortisol ratio in urine as markers for CYP3A4 induction. To do this 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), by estimated midazolam clearance and 4β–hydroxycholesterol ratio (both p<0.01) and by 6β–hydroxycortisol ratio (p<0.05). The median fold-induction from baseline was 2.0, 2.6 and 4.0 for estimated midazolam clearance, 1.3, 1.6 and 2.5 for 4β–hydroxycholesterol / cholesterol ratio and 1.7, 2.9 and 3.1 for 6β–hydroxycortisol / cortisol ratio for the three dosing groups (10, 20 and 100 mg). In conclusion, 4β–hydroxycholesterol ratio was 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 e.g. induction or inhibition of cytochrome P450 (CYP) 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 both 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 towards 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, e.g. a multiple ascending dose study. The metabolite 4β-hydroxycholesterol is formed by CYP3A4 and CYP3A5 catalyzed metabolism of cholesterol and has been suggested to be a robust marker for CYP3A-activity (Bodin et al., 2001; Diczfalusy et al., 2011). A previous study have shown that rifampicin, an antituberculosis drug and a well-known inducer of CYP3A-activity, increased 4β-hydroxycholesterol in a dose-dependent way (Kanebratt et al., 2008). In that study rifampicin was given at doses of 20 mg, 100 mg and 500 mg daily for 2 weeks. These doses resulted 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 is dependent 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 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 (Galteau and Shamsa, 2003). The intra- and interindividual variation of this ratio is large and its specificity for CYP3A-activity is debated. The ratio can only be used if the subjects are their own controls (Galteau and Shamsa, 2003).

An alternative probe drug for CYP3A-activity is quinine metabolic ratio, i.e quinine / 3-hydroxy-quinine (Mirghani et al., 2003) in plasma. The advantage with this ratio compared to midazolam AUC is that only one single blood-sample has to be drawn compared to repeated determinations of midazolam.

The primary aim of the present study was to compare the endogenous 4β–hydroxycholesterol ratio and oral midazolam clearance as markers for CYP3A4-induction. The secondary aim was to compare 6β–hydroxycortisol ratio with midazolam clearance and 4β–hydroxycholesterol ratio. An open randomized trial in 24 healthy volunteers was therefore performed. Three different doses of rifampicin, 10 mg, 20 mg and 100 mg rifampicin were given daily to 8 subjects in each group for two weeks to achieve a very low to moderate degree of CYP3A4 induction. The CYP3A4 induction was determined by 4β–hydroxycholesterol ratio in plasma, estimated midazolam clearance and 6β–hydroxycortisol ratio in urine collected during 14 h at baseline and after 2 weeks of rifampicin treatment. A simplified study design is indicated in Figure 1.
Material and Methods

Study design

In an open randomized controlled study, 24 Swedish Caucasian healthy volunteers were randomized to take 10 mg, 20 mg or 100 mg rifampicin per day for 2 weeks to achieve CYP3A induction. There were 8 subjects in each treatment group. Determinations of midazolam AUC, 4β–hydroxycholesterol ratio and 6β–hydroxycortisol ratio were made at baseline, after 2 weeks of medication and 2 weeks after termination of medication. The study was performed at Clinical Pharmacology Trial Unit, Karolinska University Hospital Huddinge during April-June 2011. The study was approved by the local Ethics Committee (Dnr: 2010/1734-31/1) and the Swedish Medical Product Agency and was performed in accordance with the declaration of Helsinki. Written informed consent was obtained from all study participants. The EudraCT number is 2010-023014-31. The full protocol is available from the corresponding author upon request.

Participants

The inclusion criteria were healthy males and females with the age of 18 years and above, Caucasian, with negative drug screening test and acceptance to refrain from drugs including herbal drugs during the study period. All subjects were ascertained to be healthy on the basis of medical history, physical examination, virology testing (hepatitis B and C, HIV) and routine laboratory testing (liver and kidney function and hematology) before enrolled in the study. The participants had to completely refrain from alcohol during day 0, 1, 14 and 15 of the study when midazolam doses were taken. During the rest of the study period only moderate intake of alcohol was allowed. The participants were not allowed to drink grape-fruit juice during the whole study period with start 2 days before the study start. Women of childbearing age should accept using reliable barrier contraceptive during the study time, but not oral hormone-based contraceptives and should have a negative pregnancy test at the screening visit.
Exclusion criteria were prior experiences of allergic drug reactions, signs of infection, use of oral hormone-based contraceptives, intake of any drugs that could influence enzyme-activity of CYP3A4, positive drug screening test, pregnancy, breast-feeding or history of liver disease.

Staff at the Clinical Pharmacology Trial Unit was responsible for randomization procedures, drug administration and blood and urine sampling.

A total of 28 healthy volunteers were screened and 24 subjects fulfilled the inclusion criteria and completed the study.

**Interventions**

The 24 subjects were randomized to take rifampicin (Rifadin®, Sanofi, oral suspension) in one of three different daily doses; 10 mg (n=8), 20 mg (n=8) or 100 mg (n=8) for 2 weeks. Plasma samples for determinations of 4β–hydroxycholesterol ratio and urine for determination of 6β–hydroxycortisol ratio were collected at baseline, after 2 weeks of rifampicin treatment and 2 weeks after termination of rifampicin administration. The urine collection started at 6 p.m. the day before the start of the study (day-1) and continued until 8 a.m. on day 0 (14 hours). Urine was also collected day 14 at 6 p.m. to day 15 at 8 a.m. (14 hours) and at day 27 at 6 p.m. to day 28 at 8 a.m. (14 hours). At the start of the study (day 0) subjects were given an oral suspension of midazolam in a dose of 4 mg (Midazolam APL, oral solution 1 mg/ml, Apoteket APL). Blood samples were drawn at 9 time-points: before dose (0), after 30 minutes, 1h, 2h, 3h, 4h, 6h, 8h and 10h after the dose for the determination of midazolam and 1'-hydroxymidazolam plasma concentrations. Midazolam AUC was determined at baseline (day 0) and after 2 weeks (day 14) of rifampicin medication. The subjects also took 250 mg quinine and a blood sample for quinine analysis was drawn 14 hours after this dose at baseline, after 2 and 4 weeks. Unfortunately, the quinine samples have not been analyzed yet due to methodological issues.

All subjects were genotyped for CYP3A*1 and CYP3A5*3 by the method described previously (Mirghani et al., 2006).
The study design including the different measurements is presented in Figure 1. The subjects were their own controls and the baseline values were compared with the values after 2 weeks of induction and also 2 weeks after last dose of rifampicin.

**Measurements of 4β-hydroxycholesterol and cholesterol**

Plasma 4β-hydroxycholesterol was determined by isotope dilution gas chromatography-mass spectrometry using [2H6] 4β-hydroxycholesterol as internal standard as described earlier (Bodin et al., 2001; Diczfalussy et al., 2011). The total variation was 8.2% at 23.9 ng/mL.

Cholesterol was determined by a commercial enzymatic method (Cholesterol CHOD-PAPP, Roche Diagnostics GmbH, Mannheim, Germany) run on a Roche/Hitachi Modular instrument. The Cv was 1.3% (at 5 mmol/L).

The 4β-hydroxycholesterol / cholesterol ratio was calculated and expressed as mol/mol x 10⁴.

**Measurements of 6β-hydroxycortisol and cortisol**

Urinary 6β-hydroxycortisol and cortisol were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using [2H4]6β-hydroxycortisol and [2H4]cortisol as internal standards as described in a previous report (Mårde Arrhen et al., 2012). The total variations were 9.2% (at 255 nmol/L) and 6.9% (at 89 nmol/L) for 6β-hydroxycortisol and cortisol, respectively.

**Measurements of midazolam**

The concentration of midazolam and its metabolite 1’-hydroxymidazolam were determined by liquid chromatography tandem mass spectrometry (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 [2H4]midazolam and [2H4]1’-hydroxymidazolam. 200 µL of the internal standard solution was added 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 x 50 mm 1.7 μm), 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 Micromass Quattro Premier XE mass spectrometer operating in positive electrospray ionization (ESI) mode utilizing selected reaction monitoring (SRM) for the transitions m/z 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 9 time-points: before dose (0), after 30 minutes, 1h, 2h, 3h, 4h, 6h, 8h and 10h after the dose. The kinetics of midazolam did not allow us to accurately extrapolate midazolam kinetics to infinity; instead the AUC_{0-10h} was used. As an estimate of “midazolam clearance” the dose / AUC_{0-10h} was calculated for each midazolam dose given (n=48), referred to as “midazolam” in the figures.

The concentrations of 1’-hydroxymidazolam were lower than those of the parent drug and could only be determined at certain time-points. We therefore decided not to use those for the calculations of midazolam disposition.

Statistical methods

Statistical analyses were performed using GraphPad Prism software version 5.03 (San Diego, CA, USA) and R 2.11.1. In the statistical analysis of comparisons of the different markers for CYP3A4 Wilcoxon matched-pairs signed rank test was used (fig 3). In the statistical analysis of baseline demography and in fold-induction between the three dosing-groups one-way Kruskal-Wallis test was used (Fig 4). In the correlation analysis linear regression was carried out (Fig 2).
Results

Baseline data

A total of 24 healthy volunteers were included, 12 males and 12 females, with a median age of 25 years and a median BMI of 22.8. The baseline demography of all participants is presented in Table 1. There was no statistically significant difference at baseline between the 3 groups regarding estimated midazolam clearance and 4β–hydroxycholesterol ratio. The median 6β–hydroxycortisol ratio at baseline was somewhat higher at baseline in the subjects randomized to the 100 mg rifampicin dose compared to those given lower doses (p<0.01). There were two subjects carrying one CYP3A5*1 allele. Both subjects were in the 100 mg rifampicin group. These two subjects with one active allele behaved as the other 6 subjects in this dose group expressing CYP3A5*3/*3 i.e. with no CYP3A5-activity.

Baseline values of estimated midazolam clearance calculated as midazolam dose/AUC_{0-10h} (midazolam) were correlated to 4β–hydroxycholesterol ratio (p<0.01) but not with 6β–hydroxycortisol ratio (p=0.30) (Fig 2).

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

Change of midazolam clearance, 4β–hydroxycholesterol ratio and 6β–hydroxycortisol ratio during rifampicin treatment

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

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

**4\( \beta \)-hydroxycholesterol ratio and 6\( \beta \)-hydroxycortisol ratio 2 weeks after termination of rifampicin administration**

In Figure 3 the values of 6\( \beta \)-hydroxycortisol ratio and 4\( \beta \)-hydroxycholesterol ratio can be followed through the study period from baseline, after 2 weeks of rifampicin treatment and 2 weeks after termination of rifampicin treatment (4 weeks after study start). Midazolam clearance was not determined after 4 weeks. Both 6\( \beta \)-hydroxycortisol ratio and 4\( \beta \)-hydroxycholesterol ratio could detect the decrease in CYP3A-activity after termination of rifampicin treatment, week 4 compared with week 2. Four weeks after study start the 6\( \beta \)-hydroxycortisol ratio had returned to base-line values (Fig 3). In contrast, 4\( \beta \)-hydroxycholesterol ratio still showed significantly higher values 4 weeks after study start compared to 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–hydroxycholesterol could be used as a biomarker to evaluate CYP3A-induction and that it gives comparable results to the commonly used probe drug midazolam. 4β-hydroxycholesterol has been used 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 4β-hydroxycholesterol ratio is compared head to head with midazolam as a marker of CYP3A induction.

The fold-induction of CYP3A-activity measured by 4β-hydroxycholesterol ratio in the present study at the doses of 20 mg 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 to 1.5 and 2.5 (Kanebratt et al., 2008). In the previously performed study a higher rifampicin dose of 500 mg daily was also investigated resulting in a 4-fold increase in 4β-hydroxycholesterol levels (Kanebratt et al., 2008).

As seen in Figure 4, the magnitude of the induction determined by oral midazolam clearance was larger than the induction determined by 4β–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 4β–hydroxycholesterol ratio is not at steady state because of the slow 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 a marker 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. If 4β-hydroxycholesterol is synthesised 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 since 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, in order 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 in order 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 makes this marker less appropriate for investigations of rapid changes in CYP3A activity, i.e. 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 caused 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 6β–hydroxycortisol ratio in detecting the decrease of CYP3A4-activity after termination of rifampicin administration (Fig 5). However, while 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 (Fig 5).
The advantages and disadvantages of the three markers for CYP3A-activity studied here are summarized in Table 2. In addition to measuring the CYP3A-activity, 4β-hydroxycholesterol ratio and 6β-hydroxycortisol ratio are also affected by CYP3A5 genotype (Diczfalusy et al., 2008; Hassan et al., 2013). In contrast, midazolam clearance is not affected by 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 2 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, at any time, is required. For midazolam a probe drug has to be given, followed by 8-10 blood samples to be drawn during 8-10 hours. A single blood sample after midazolam could not predict the clearance for the drug (Rogers et al., 2002). On the other hand, as described above, midazolam is superior to 4β-hydroxycholesterol in detecting inhibition of CYP3A4 (Table 2). 6β-hydroxycortisol ratio can also measure CYP3A-inhibition (Li et al., 2010).

Due to the long half-life of 4β-hydroxycholesterol a study in which CYP3A4- induction is to be detected the study period should be at least 2 weeks. In contrast, for detecting an induction with midazolam clearance and 6β-hydroxycortisol ratio the study can be much shorter.

The two markers of CYP3A-activity 4β-hydroxycholesterol and midazolam give similar information with their own pros and cons. Major advantages of the former are that it is endogenous and no drug has to be given and with the latter that it can be used to record rapid changes of CYP3A-activity e.g. inhibition.

4β-Hydroxycholesterol 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 4β-hydroxycholesterol ratio. This indicates that 4β-hydroxycholesterol ratio might be used also 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 4β-hydroxycholesterol ratio is related to the variation in midazolam clearance.

In conclusion, to use an endogenous biomarker, such as 4β-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 patient categories in which it is not suitable to give probe drugs, such as children, pregnant women and elderly.

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**Author contributions:**

Participated in research design: LBB, TB, EB, TBA, LB, UD.

Conducted experiments: LBB, TB, HN, YRN, UD.

Contributed new reagents or analytic tools: HN, YRN, UD.

Performed data analysis: LBB, TB, HN, YRN, EB, TBA, LB, UD.

Wrote or contributed to the writing of the manuscript: LBB, TB, HN, YRN, EB, TBA, LB, UD.
References


Footnotes:
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Figure Legends:

Figure 1. Simplified design of the study and the different measurements carried out at baseline (day 0), after 2 weeks of rifampicin medication (day 14) and 2 weeks after rifampicin medication was terminated (day 28).

Figure 2. Correlation between baseline values of estimated midazolam clearance calculated as midazolam dose/AUC$_{0-10h}$ (midazolam) and 4β–hydroxycholesterol ratio ($4\beta$-OHchol R) or 6β–hydroxycortisol ratio ($6\beta$–OHcortisol R). Linear regression analysis shows a significant correlation between midazolam and $4\beta$-OHchol R but not between midazolam and $6\beta$–OHcortisol R.

Figure 3. Comparison of three different markers for CYP3A-activity: estimated midazolam clearance (ml/h) calculated as midazolam dose/AUC$_{0-10h}$, $4\beta$–hydroxycholesterol ratio ($4\beta$-OHchol R) and $6\beta$–hydroxycortisol ratio ($6\beta$–OHcortisol R) at baseline and after CYP3A induction achieved by rifampicin treatment for 2 weeks (10 mg, 20 mg or 100 mg daily) and 2 weeks after rifampicin administration was terminated (4 weeks). Midazolam clearance was not determined after 4 weeks. Statistically significant differences were calculated using Wilcoxon matched pairs single rank test.

Figure 4. Fold-induction of CYP3A4 after 2 weeks of rifampicin treatment (10, 20 and 100 mg) compared to baseline measured by three different markers; estimated midazolam clearance calculated as midazolam dose/AUC$_{0-10h}$ (midazolam), $4\beta$–hydroxycholesterol ratio ($4\beta$–OHchol R) and $6\beta$–hydroxycortisol ratio ($6\beta$–OHcortisol R). Statistically significant differences between the three dosing groups using Kruskal-Wallis test are shown. The numbers at the top designate the median-fold induction in each dosing-group.
**Table 1. Baseline demographic data.** Number of males and females and median values for age and BMI (range within parentheses) for all participants in the different rifampicin dosing groups.

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<th>ALL (n=24)</th>
<th>10 mg Rifampicin (n=8)</th>
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<th>100 mg Rifampicin (n=8)</th>
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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).

<table>
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<th>Midazolam</th>
<th>4β–OHchol R</th>
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<td>1 urine sample</td>
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Baseline:
4β-hydroxycholesterol ratio
6β-hydroxycortisol ratio
Midazolam AUC
Genotyping CYP3A5

2 weeks:
4β-hydroxycholesterol ratio
6β-hydroxycortisol ratio
Midazolam AUC

4 weeks:
4β-hydroxycholesterol ratio
6β-hydroxycortisol ratio

Fig 1

Day 0

Rifampicin
10 mg, 20 mg or 100 mg / day

Day 14

No medication

Day 28
Fig 2

$r^2 = 0.29$
$p < 0.01$

$r^2 = 0.05$
$p = 0.30$
Fig 4

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