REPEATED ORAL RIFAMPICIN DECREASES THE JEJUNAL PERMEABILITY OF R/S-VERAPAMIL IN RATS

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ABSTRACT:
The main purpose of this rat study was to investigate the effect of rifampicin on the effective permeability (Pe) of R/S-verapamil in the rat jejunum. In addition the effect on metabolism of R/S-verapamil to R/S-norverapamil was examined. In situ single-pass perfusions of the rat jejunum were performed in animals pretreated with oral rifampicin (250 mg/kg/day) or saline (control) over various time periods (1, 4, 7, and 14 days). The jejunal Pe of each of the enantiomers of verapamil and D-glucose was estimated. The appearance ratios of the CYP3A-formed metabolites R- and S-norverapamil were also estimated in the outlet jejunal perfusate. The jejunal Pe of both R- and S-verapamil decreased as an effect of the oral pretreatment with rifampicin. The appearance of R- and S-norverapamil in the jejunum was also affected by the oral pretreatment with rifampicin, with increasing concentrations of R/S-norverapamil being evident after 14 days of rifampicin pretreatment. There was no stereoselectivity in either the Pe of R- and S-verapamil or the metabolic appearance of R- and S-norverapamil. Treatment with oral rifampicin decreased the Pe of R/S-verapamil, which is in accordance with an induction of P-glycoprotein activity in the apical enterocyte membrane. The increase in appearance of R/S-norverapamil in jejunum is in accordance with an induction of CYP3A metabolism in the rat.

Recently, increased attention has been focused on the potential impact that P-glycoprotein (Pgp)\(^1\) may have on intestinal drug absorption and bioavailability of various drugs (Wacher et al., 1995; Benet et al., 1996). In a human cell line (in vitro), some drugs (such as rifampicin, verapamil, or reserpine) induced both cytochrome P-450 enzyme (CYP) 3A and Pgp (Schuetz et al., 1996a,b). In vivo studies in humans have shown that both Pgp and CYP3A in the enterocytes have been induced by oral rifampicin (Fromm et al., 1996; Lown et al., 1996). The metabolic contribution of CYP3A4 in the enterocytes is reported to be significant for drugs such as midazolam, verapamil, cyclosporine, and felodipine, but the importance of Pgp in the enterocyte was not examined in any of those studies (Regardh et al., 1989; Wu et al., 1995; Fromm et al., 1996; Paine et al., 1996). In a human in vivo study, Pgp was reported to partially influence the intestinal absorption/bioavailability of cyclosporine (Lown et al., 1997). Paclitaxel (Taxol) has also been shown to have a higher bioavailability in mice in vivo with a homozygously disrupted mdrla gene [mdrla\(^{-/-}\)] in comparison with wild-type animals (Sparreboom et al., 1997). In an intestinal perfusion study, we have shown that the concentration-dependent jejunal effective permeability (Pe) of R/S-verapamil in humans is probably due to a saturable Pgp-mediated efflux in the enterocyte, but this had no effect on the extent of absorption (Sandstrom et al., 1998a,b). At present, it is uncertain whether the contribution of Pgp to the reduction of intestinal absorption and bioavailability of orally administered drugs in vivo in humans is of any clinical importance.

Rifampicin is a macrocyclic antibiotic that is a strong inducer of some CYPs belonging to the subfamily of CYP3A enzymes (Zilly et al., 1977a,b; Fromm et al., 1996). Recently, it was reported that rifampicin binds to the human glucocorticoid receptor with high affinity, which regulates the expression of many genes, including those encoding interleukins that regulate immune response and CYPs (Calleja et al., 1998). Because rifampicin is also metabolized by CYP3A enzymes, this will cause autoinduction and decrease the oral bioavailability. Rifampicin is also a substrate for the Pgp efflux pump. In mdrla\(^{-/-}\) mice, the dose required to cause induction of CYP3A protein was lower than in mdrla\(^{+/+}\) mice. This is most likely due to enhanced intracellular accumulation of rifampicin in mdrla\(^{-/-}\) mice because they lack the cellular efflux by Pgp (Schuetz et al., 1996a,b).

Species differences have been reported for the induction properties of rifampicin by the P-450 enzymes. Mice, rabbits, and humans are more sensitive than rats and guinea pigs (Zilly et al., 1975; Oesch et al., 1996). In 1996 Oesch et al. suggested that there is a threshold dose for rifampicin-mediated P-450 induction in rat liver (in vivo) at approximately 250 mg/kg/day (Oesch et al., 1996). It is possible that the metabolism and/or biliary secretion of rifampicin is saturable at higher doses because there seems to be a large response in induction after an increase in the dose above 250 mg/kg/day (Oesch et al., 1996). This is supported by the fact that it is rifampicin itself, and not its metabolites, that is the active inducing agent (Calleja et al., 1998). Such a dose-dependent induction in rats is analogous to the higher sensitivity for induction seen for mdrla\(^{-/-}\) mice (Schuetz et al., 1996a,b). The purpose of this rat study was to investigate whether pretreatment with rifampicin for various time

\(^1\)Abbreviations used are: Pgp, P-glycoprotein; Ar, appearance ratio; CYP, cytochrome P-450 enzyme; Pe, effective permeability; PEG, polyethylene glycol.
periods would change the jejunal transport and metabolism of R-and S-verapamil.

Materials and Methods

Drugs. The racemic verapamil and norverapamil were gifts from Knoll AG (Darmstadt, Germany). Rifampicin mixture was purchased from the hospital pharmacy (Rimactan, 20 mg/ml mixture, Ciba-Geigy AG, Schweiz) in Uppsala. All other chemicals were of analytical grade.

Animals. Sixty male Sprague-Dawley rats [(Crl:CD(SD)BR) from Charles River AB, Uppsala, Sweden] were housed under controlled conditions (22.4°C, 50% air humidity, 12-h light/dark cycle) at the animal house at the Biomedical Center at Uppsala University. The rats arrived at the animal house at least 1 week before the start of the experiments and had free access to tap water and pellet food (R36; Lactamin AB, Vadstena, Sweden). The study was approved by the Animal Ethics Committee in Uppsala (C11/97).

Design. The animals were divided into 10 groups, with six rats in each group. Five groups were given rifampicin (Rimactan, 20 mg/ml mixture, Ciba-Geigy AG, Schweiz) by soft oral gavage (250 mg/kg/day) for 1 to 14 days. The other five groups received saline by soft gavage and served as a control for the rifampicin-treated groups. Single-pass perfusion experiments were performed in the jejunum on one treated and one control group on days 1, 4, 7, 14, and 21 after the start of the treatment.

Surgery. The anesthesia and surgery were performed in accordance with a previously validated in situ intestinal perfusion method in rats (Fagerholm et al., 1996). The rats were fasted overnight before the perfusion experiment. Breathing was facilitated by inserting a plastic tube into the trachea. By making a midline longitudinal incision of approximately 1 cm, the abdominal cavity was opened and a 10-cm segment of the proximal jejunum (beginning 5 cm from the stomach) was isolated and cannulated with plastic tubing (4 mm o.d.). The perfusion rate was 0.2 ml/min, maintained by a syringe pump (model 22; Harvard Apparatus Company, Holliston, MA). The outlet perfusate was quantitatively collected at intervals on ice in fractions after 45, 60, 75, 90, and 105 min.

Perfusion Solution. The perfusion solution consisted of a 70 mM phosphate buffer (pH 6.5) with 5.4 mM KCl, 48 mM NaCl, 10 mM d-glucose, 35 mM mannitol, 1 g/liter polyethylene glycol (PEG; PEG 4000, molecular weight ~ 4000), and 100 mg/liter of racemic verapamil (Knoll AG). Trace amounts of d-[3H]glucose and 14C-PEG (Amersham Laboratories, Buckinghamshire, England) were added to the solution as a volume and viability marker.

Analytical Methods. An enantioselective HPLC method for R/S-verapamil and R/S-norverapamil was performed on a Chiral AGP column (4 × 150 mm; Chrometech, Stockholm, Sweden), with a Chiral ACG precolumn (3 × 10 mm). The pump was a Shimatzu LC-9A (Kyoto, Japan) and the flow rate applied was 1 ml/min. Temperatures above room temperature (22°C) were achieved by heating the column and the mobile phase in a heat bath. The detection was made with a Jasco FP-920 fluorescence detector (Tokyo, Japan) (ex = 232, em = 310 nm). The mobile phase consisted of a phosphate buffer with an ionic strength of 0.01 and a pH of 7.6 with 22% (v/v) acetonitrile at 30°C (Sandstrom et al., 1999). The perfusate samples were diluted in mobile phase and 50.1 μl was injected on the column. The limit of quantification (±S.D.) for R- and S-verapamil was 5.5 ± 0.3 ng/ml for each of the enantiomers. The limit of quantification (±S.D.) for R- and S-norverapamil was 2.9 ± 0.2 ng/ml. The concentrations of d-[3H]glucose and 14C-PEG 4000 in the intestinal perfusate were determined by liquid as described previously (Fagerholm et al., 1996).

Data Analysis. Calculations of the absorption parameters were made from the outlet concentrations of the perfusate leaving the jejunal segment during steady state. Steady-state conditions were considered to have been achieved when the concentration of 14C-PEG 4000 leaving the segment was stable (within 45 min in all rats) (Fagerholm et al., 1996; Sandstrom et al., 1998a,b).

The Peff was calculated according to the parallel tube model which has been used in other animal studies (Levitt et al., 1988; Fagerholm et al., 1996):

\[
\text{P}_{\text{eff}} = \frac{-\ln(C_{\text{in}}(\text{Cin})/(2 \times \text{ml})} {\text{Q}}
\]

where Q is the flow rate, and C_{in} and C_{out} are the inlet and outlet perfusate concentrations. The C_{out} was corrected for the net absorption/secretion of water and 2 ml is the cylindrical surface area of the jejunal segment with a length (L) of 10 cm and the intestinal radius (r) of 0.23 cm.

The fraction absorbed (f_{a}) was calculated according to eq. 2, assuming that the fraction of drug that disappeared during the passage through the segment was absorbed (Lennernas et al., 1992):

\[
f_{\text{a}} = 1 - \frac{(C_{\text{out}}(\text{PEGin})/(2 \times \text{ml})} {C_{\text{in}}(\text{PEGout})}
\]

The appearance ratio (A_{r}) for each enantiomer of norverapamil, the CYP3A-formed metabolites, was calculated as shown in eq. 3:

\[
A_{r} = \frac{C_{\text{out}}(\text{norverapamil})}{C_{\text{in}}(\text{verapamil})}
\]

Results and Discussion

Jejunal Transport of R- and S-Verapamil. The Peff of each enantiomer of R/S-verapamil in the rifampicin-treated rats compared to control rats was different (p < .05) on days 1, 4, and 14, but not on days 7 and 21 (Table 1 and Fig. 1, A and B). One rat was excluded in each rifampicin-treated group on days 1 and 7 because of leakage from the perfused jejunal segment. In three study groups (days 1, 4, and 14) the jejunal Peff decreased by approximately 25% (Table 1 and Fig. 1, A and B). This is consistent with an increased
efflux of each enantiomer of verapamil as a consequence of an induction of Pgp in the apical membrane of the enterocyte. However, on day 7 no statistical difference could be detected. Furthermore, there was no difference in the intestinal $P_{\text{eff}}$ between the $R$- and $S$-enantiomers of verapamil in any of the groups. The group of rats perfused on day 21 did not demonstrate any decreased jejunal $P_{\text{eff}}$ (Table 1 and Fig. 1, A and B).

It has been reported previously that verapamil is a Pgp substrate in the rat enterocyte (Saitoh and Aungst, 1995; Sandstrom et al., 1998a, b). We have previously found that there was no detectable difference between the jejunal $P_{\text{eff}}$ values for $R$- and $S$-verapamil in humans (in vivo) or rats (in situ), which is consistent with data in the present study (Sandstrom et al., 1998a, b, 1999). This has also been shown to be valid for the transport of $R/S$-verapamil in cancer cells in vitro (Haussermann et al., 1991). The lack of stereoselectivity of Pgp-mediated intestinal efflux transport, clearly demonstrated by us in humans and rats, is of utmost pharmacological importance to consider for the otherwise well known stereoselective pharmacokinetics and pharmacodynamics for $R/S$-verapamil in humans (Kroemer et al., 1992; Fromm et al., 1996; Sandstrom et al., 1998a, b, 1999). The recovery of $^{14}$C-PEG 4000 (>90%) and the $P_{\text{eff}}$ of d-glucose were high in all animals, which suggests that the tissue integrity and viability were maintained throughout the study (Table 1).

However, even if $R$- and $S$-verapamil are substrates for Pgp in the small intestine in both rats and humans, the measured $P_{\text{eff}}$ values were sufficiently high to predict a complete intestinal absorption (Fagerholm et al., 1996; Sandstrom et al., 1998a, b). Our earlier studies in humans and rats were performed as single-dose studies (without pretreatment of any Pgp inducer). Therefore, it is of great interest to specifically examine the effect on intestinal $P_{\text{eff}}$ in rats pretreated with...
a well known Pgp inducer. Rifampicin has previously been shown to induce Pgp in human enterocytes in vivo and in cell cultures in vitro (Lown et al., 1996; Schuetz et al., 1996a,b). In humans, the multidrug resistance (MDR1) mRNA expression in the enterocyte increased by an average of 4.3-fold after rifampicin treatment for 7 days (Lown et al., 1996). In the present intestinal perfusion study in rats, the jejunal P<sub>er</sub> tended to decrease after oral treatment with rifampicin (250 mg/kg/day). This is most probably attributable to increased expression of an efflux protein which, based on previous experience, should be the Pgp. In one other rat study it was shown that oral treatment with rifampicin at 100 mg/kg/day for 3 to 4 days gave a high increase in Pgp content, but only slightly increased the CYPA3A expression in the liver (Salphati and Benet, 1998). However, even if the P<sub>er</sub> for R- and S-verapamil was affected by rifampicin treatment in the present study, the reduction was too small to influence the extent of intestinal absorption of R- and S-verapamil (Fagerholm et al., 1996).

**Metabolism of R/S-verapamil.** The A<sub>H</sub> of each enantiomer of R/S-norverapamil was reduced in the rifampicin-treated rats compared with the control rats on days 1, 4, and 7, which suggests that the CYPA3A metabolism of R- and S-verapamil was inhibited (Fig. 1, C and D). However, on days 14 and 21, the A<sub>H</sub> for R/S-norverapamil showed a tendency to higher in the rifampicin-treated animals than in the control rats (Fig. 1, C and D). An analysis of variance test between the rifampicin-treated groups revealed a difference between days (p < .001) for both R- and S-norverapamil. An additional analysis revealed that there were differences (p < .05) between day 21 and all the other days and between days 7 and 14 for both R- and S-norverapamil in the rifampicin-treated animals. This observation suggests that it takes longer than 7 days for CYPA3A to be induced to increase the appearance of R- and S-norverapamil in the outlet perfusate. It is also of interest to note that there was no difference in the A<sub>H</sub> between R- and S-norverapamil (Fig. 1, C and D).

Although induction of CYPA3A liver enzymes is difficult to achieve in rats, it has been reported that treatment of rifampicin at a level of at least 250 mg/kg/day for 9 days should be adequate (Oesch et al., 1996). This dose regimen gave an induction of CYPA3A enzymes in the rat liver. Therefore, it seems logical to assume that the same oral treatment should also induce CYPA3A enzymes in the gut wall because the enterocyte is exposed to a higher rifampicin concentration. In addition, glucocorticoids have been reported to induce the metabolic capacity for the degradation of erythromycin in the rat enterocyte (Watkins et al., 1987). An induction of Pgp seems possible because they may also respond to the same local enterocyte concentrations. The proximal region of the rat small intestine will be exposed to the highest concentrations of rifampicin. This is the region where we performed our perfusion experiments and where the CYPA3A enzymes have the highest expression (Kolars et al., 1992). It seems reasonable to assume that the proximal small intestine in rats is a region with high expression of efflux proteins (such as Pgp; Lown et al., 1996).

Previously, we presented evidence that R- and S-verapamil are metabolized to their corresponding enantiomers of norverapamil in the rat enterocyte, most likely by the CYPA3A enzymes (Sandstrom et al., 1998a,b). The A<sub>H</sub> values for R- and S-norverapamil found in the present study were significantly higher on days 14 and 21 than on the earlier days of the experiment for the rifampicin-treated group. In addition, they were statistically lower on days 4 and 7 than on the control rats. Altogether, this suggests that the induction of CYPA3A enzymes in the rat takes more than 7 days before it has any influence on the A<sub>H</sub> for R- and S-norverapamil at day 21 was most likely due to a combination of both induction of metabolism and less inhibition of the metabolism because the treatment with rifampicin had ended.

**Conclusion**

In the study the effective jejunal permeability of verapamil was decreased after pretreatment with rifampicin (Table 1, Fig. 1, A and B). This was probably due to induction of Pgp in the enterocytes of the small intestine, demonstrating a higher efflux activity. The effect on the metabolism, measured as the A<sub>H</sub> for the CYPA3A metabolite norverapamil, was not significantly different until 14 days of rifampicin pretreatment had occurred, which suggests that the induction of CYPA3A activity takes a longer time to achieve than the induction of Pgp in the rat. Finally, because the interplay between the expression and the regulation of CYPA3A and Pgp and their effect on absorption and metabolism of drugs in the intestine are not well understood, extensive evaluation is needed in the future.

**References**


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