Journal of Antimicrobial Chemotherapy (1996) **37**, 1111–1119

Inducing properties of rifampicin and rifabutin for selected enzyme activities of the cytochrome P-450 and UDP-glucuronosyltransferase superfamilies in female rat liver

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Important species differences have been reported concerning the induction properties of rifampicin towards enzymes of the P-450 superfamily. Mice, rabbits and humans are far more responsive than rats and guinea pigs. In the present study a strong induction of cytochrome P-450 3A-dependent enzyme activities was observed in female rat liver microsomes after high dose treatment ($\geq$250 mg/kg/day for 9 days) with rifampicin, resulting in an up to 30-fold enhanced hydroxylation rate of testosterone in the 2β-, 6β- and 15β-position *in vitro*. Other cytochrome P-450 isozyme-selective reactions were not, or only marginally, affected. A steep increase in cytochrome P-450 3A activity on a moderate elevation of the dose administered, together with the previously observed lack of efficient induction with doses below 200 mg/kg/day demonstrated that there is a threshold in enzyme induction by rifampicin. For rifabutin such a threshold was not apparent. Induction by rifabutin showed an isoenzyme-selectivity profile similar to that produced by rifampicin, but the maximally achievable induction of cytochrome P-450 3A by rifabutin was about two-fold lower compared with rifampicin. Rifampicin and rifabutin enhanced the glucuronidation of 1-naphthol, 4-hydroxybiphenyl and β-estradiol by a factor of two to three. The potential implications of the enzyme induction by rifampicin derivatives in terms of possible drug–drug interactions are discussed.

Introduction

Rifampicin and rifabutin are two rifamycin derivatives used for the treatment of pulmonary tuberculosis. In addition to being more active than rifampicin against *Mycobacterium tuberculosis in vitro* (Della Bruna *et al.*, 1983; Grosset, 1988), rifabutin proved to be beneficial to patients with chronic drug-resistant pulmonary tuberculosis unresponsive to previous treatments including those containing rifampicin (De Cian, Sassella & Wynne, 1996). Moreover, the results of two large clinical trials in patients with HIV infection have allowed rifabutin to be approved for prophylaxis of *Mycobacterium avium* complex (MAC) infection (Cameron *et al.*, 1992; Sparti *et al.*, 1992), which represents a frequent cause of death in AIDS.

Important species differences have been observed concerning the induction properties

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0305–7453/96/061111 + 09 $12.00/0

of rifampicin towards enzymes of the cytochrome P-450 superfamily, with humans being particularly sensitive. Five to ten mg/kg doses of rifampicin are actually sufficient to induce P-450-dependent enzymes (Acocella *et al.*, 1971; Perucca *et al.*, 1988). This has been the cause of a spectacular drug–drug interaction, the strong interference with concomitantly taken oral contraceptives, which resulted in a significant number of unwanted pregnancies.

Among laboratory animals, mice and rabbits are far more responsive to the inducing properties of rifampicin than rats and guinea-pigs (Strolin Benedetti & Dostert, 1994). The rat hepatic cytochrome P-450 system seems to be resistant to the action of rifampicin, unless very high doses are used. In the early 1970s Barone, Beretta & Tenconi (1972) reported that several parameters related to cytochrome P-450 induction, such as pentobarbital-induced sleeping-time, pentobarbital blood levels, cytochrome P-450 content and hexobarbital hydroxylase, were apparently not affected by the oral administration to rats of a 170 mg/kg/day dose of rifampicin for 10 days. In a more recent study (Adachi *et al.*, 1985), an oral dose of 200 mg/kg/day rifampicin for 7 days to male Wistar rats did not influence the concentrations of hepatic microsomal cytochrome P-450 and cytochrome b_5, and the activities of aniline hydroxylase and aminopyrine demethylase. Results from our own laboratory (data not shown) also indicated that a dose of 150 mg/kg/day of rifampicin for 21 days is not efficient in inducing a variety of drug-metabolizing enzymes in the rat, with the exception of a minor induction of testosterone 2β- and 6β-hydroxylation (3- and 1.4-fold of control values, respectively). Finally, in a recent study Daujat *et al.* (1991) reported an induction of erythromycin demethylase activity and a concomitant increase in cytochrome P-450 3A immunoreactive protein in liver microsomes of female and male Wistar rats after administration of very high doses of rifampicin mixed into the ground chow (1 g/100 g of chow) for 3 weeks. The average daily dose of rifampicin was estimated to be 300 to 600 mg/kg, depending on individual food intake. In the study of Adachi *et al.* (1985), although the administration of a 200 mg/kg/day dose of rifampicin for 7 days did not affect mixed function oxidase activities and p-nitrophenol UDP-glucuronosyltransferase, bilirubin UDP-glucuronosyltransferase was induced.

Since the rat is widely used in toxicological investigation, and was repeatedly used in the induction studies mentioned above, the present study aimed at examining further whether induction of the rat hepatic cytochrome P-450 system occurs when very high doses of rifampicin are used, and comparing the effects of the two rifampicin derivatives in this respect. Furthermore, it was intended to investigate whether rifampicin induction is limited to the cytochrome P-450 3A subfamily or involves other P-450-dependent monooxygenase and UDP-glucuronosyltransferase enzymes.

Methods

Chemicals

Rifampicin, batch no. 80H3294, was obtained from Sigma (St. Louis, MO, USA). Rifabutin, batch no. 1023L155, was provided by Farmitalia Carlo Erba. [^{3}H]Estrone and [^{3}H]β-estradiol were from NEN Du Pont (Bad Homburg, Germany) and from Amersham Buchler (Braunschweig, Germany), respectively. Dithiothreitol, UDP-glucuronic acid, β-estradiol, bovine serum albumin, Tween 20, Brij 58, Lubrol PX and steroid standards were purchased from Sigma (Deisenhofen, Germany). 1-Naphthol,

estrone and 4-hydroxybiphenyl were from Fluka (Buchs, CH). NADH, NAD (free acid, grade II), NADPH, 7-ethoxyresorufin and 7-pentoxyresorufin were obtained from Boehringer Mannheim (Mannheim, Germany). Resorufin was a product of Pierce (Rockford, Illinois, USA). Triton X-100 was from Zinsser Analytic (Frankfurt a.M., Germany). All other chemicals were of analytical grade or of the purest grade commercially available.

Animal treatment

Female Wistar rats (average initial weight 200 g) were obtained from IFFA CREDO (Italy). The animals were housed individually in standard cages on Vermiculite bedding at 21°–22°C with a light/dark cycle of 12 h. All animals had free access to standard lab chow (4RF21 diet type, Mucedola, Italy) and water *ad libitum*. The animals were allowed to adjust to the environmental conditions 5 days prior to treatment. Initially, 24 rats were assigned to four treatment groups of five animals each, and a control group of four animals. A few days later ten additional animals (two groups of five) were introduced in the study for reasons detailed later in the text. Rifampicin and rifabutin were administered orally (gavage) as an aqueous suspension containing 0.5% methyl cellulose and 0.4% Tween 80. Doses are expressed as mg/kg, as the two compounds have very similar molecular weights (822.96 and 847.12 for rifampicin and rifabutin, respectively). Animals of the first four groups received 300 or 450 mg/kg/day of rifampicin or rifabutin for nine days, in a volume of 1 mL/100 g body weight. The 300 and 450 mg/kg daily doses were administered once a day until day six, then as 150 or 225 mg/kg doses twice daily at intervals of 8 h, because significant signs of toxicity had been observed after 5 days of treatment. For the same reason, two additional treatment groups of rats were introduced in the study and were treated for 9 days with a daily dose of 250 mg/kg of either rifampicin or rifabutin, given in portions of 125 mg/kg twice daily at an interval of 8 h. Animals of the control group were treated for the same period with the vehicle only.

The administered doses proved to have a clear toxic effect, and are actually higher than the highest doses used in chronic toxicity studies (Scampini *et al.*, 1993; Brughera *et al.*, 1995). The 250 mg/kg/day dose was chosen as a compromise between the toxic dose and the doses previously reported to be ineffective in inducing xenobiotic-metabolizing enzymes in rat (see Introduction). All surviving animals were killed by cervical dislocation after a 24-h fasting period (16 h after the last drug administration). Livers were removed within three minutes of death, washed with saline solution (0.9% NaCl), dried briefly on blotting-paper, weighed, then immediately frozen in liquid nitrogen and subsequently stored at −80°C until preparation of subcellular fractions.

Subcellular fractionation

The livers were homogenized separately in sucrose solution (250 mM, containing 10 mM Tris/HCl, pH 7.4) with an Ultra-Turrax homogenizer to give 25% (w/v) homogenates, which were subsequently centrifuged for 15 min at 600*g* and 4°C. The pellet was discarded and the remainder was subjected to a second centrifugation for 20 min at 10,000*g* and 4°C. The resulting supernatant was then spun for 60 min at 100,000*g* and 4°C. The obtained microsomal pellet was resuspended in a volume of homogenization

buffer equivalent to the initial liver weight, portions were snap-frozen in liquid nitrogen and stored at −80°C until assayed.

Assay procedures

Protein concentrations were determined according to the Bradford method (1976) using bovine serum albumin as a standard. O-dealkylating activities for 7-ethoxyresorufin (EROD) and 7-pentoxyresorufin (PROD) were recorded in liver microsomes by monitoring fluorimetrically the generation of resorufin ($\lambda_{excitation}$ = 522 nm, $\lambda_{emission}$ = 586 nm) (Burke & Mayer, 1974, 1976; Lubet *et al.*, 1990). The assay for the regio- and stereoselective testosterone hydroxylations indicative for the activities of individual cytochrome P-450 isozymes was performed as described by Oesch *et al.* (1992). The different metabolites were identified by their respective retention times, compared with external standards. UDP-Glucuronosyltransferase activities (UDP-GT) were determined in liver microsomes with 1-naphthol, 4-hydroxybiphenyl, estradiol and estrone as substrates as described by Bock & White (1974), Rao *et al.* (1976), and Bock *et al.* (1979).

Statistics

Data are presented as the average of the respective groups ± S.D. or, in the case of the high dosage group (450 mg/kg/day), as separate data from two respective animals. Significance of the influence of treatment on the recorded parameters was analyzed for all but the high dosage groups using a MANOVA procedure for multiple comparisons including cases with unequal sample sizes, as introduced by Spjotvoll & Stoline (1973).

Results

General effects of rifamycin treatment

Following rifampicin treatment, significant signs of toxicity were observed in rats of the medium and high dose groups (300 and 450 mg/kg/day) after 5 days: hypothermia, dyspnoea, reduction of spontaneous activity, loss of body weight, reduction of food consumption, tremor, ruffled fur and staggering. As rifampicin has an intense orange colour, orange staining of fur, mucosae and urine was observed. After 10 days, two animals of the medium dosage group (300 mg/kg/day) and three animals of the high dosage group (450 mg rifampicin/kg/day) had died. This result is in apparent contrast to the results of Daujat *et al.* (1991), who did not report any mortality following rifampicin treatment during a 21 day feeding period with an estimated daily intake of 300 to 600 mg rifampicin per kg body weight. In Daujat's study, where rifampicin was mixed into the ground chow, the actual daily doses may have been overestimated.

With rifabutin, signs of toxicity similar to those seen with rifampicin were observed in rats of the medium and high dose groups (300 and 450 mg/kg/day) after 5 days of treatment. Rifabutin has a red-violet colour, thus, red-violet staining of fur, mucosae and urine was observed. After 10 days, three animals of the high dosage group (450 mg rifabutin/kg/day) had died. At the end of the treatment period significant, an apparently dose-dependent decrease in body weight and increase in liver-to-body-weight ratio was

observed on treatment with rifabutin (Table I). A similar tendency which, however, did not reach statistical significance, was obtained on rifampicin treatment, maybe as a consequence of the death of two out of five animals in the group treated with 300 mg/kg.

Induction of enzyme activities

The most pronounced effect on enzymatic activities observed with the two rifamycin derivatives was the very strong induction of testosterone hydroxylation in the 2β-, 6β- and 15β-position (results reported in detail in Table II). This change in the metabolic pattern is specific for the induction of (an) isoenzyme(s) of the cytochrome P-450 3A subfamily, as testosterone 15β-hydroxylation is, as far as is known, exclusively carried out by this subfamily and 2β- and 6β-hydroxytestosterone are the two other major testosterone metabolites formed by cytochromes P-450 3A. At the daily dosage of 250 mg/kg there was no significant quantitative difference between the two compounds. By contrast, the effect of rifampicin on the cytochrome P-450 3A activities was more than doubled on increasing the dose to 300 mg/kg while the same increase in dosage did not enhance the effect of rifabutin treatment. The difference between rifampicin and rifabutin induction of cytochrome P-450 3A was statistically significant at this dosage level ($P < 0.01$). This difference was similarly apparent with the groups treated with the highest doses (450 mg/kg/day), although statistical evaluation of the effects was made impossible by the death of animals.

An increase in testosterone 16β-hydroxylation, a reaction earlier ascribed exclusively to the cytochromes P-450 2B1 and 2B2, paralleled the induction of the cytochrome P-450 3A-dependent testosterone hydroxylations, especially after rifampicin. However, no increase in 7-pentoxyresorufin O-depentylation, a cytochrome P-450 2B1/2B2-specific enzymatic reaction, was discernible. A concomitant increase in testosterone 16β-hydroxylation and in testosterone hydroxylations representative of cytochrome P-450 3A activity has already been observed (M. Arand, unpublished observation). Regression analysis of the 16β-hydroxylation activity versus 2β- 6β- and 15β-hydroxylation activities on the basis of the data from individual animals ($n = 26$) resulted in correlation coefficients ranging between 0.81 and 0.85. The parallelism of the changes in 16β-hydroxylation of testosterone with several cytochrome P-450 3A but not with cytochrome P-450 2B-mediated activities indicates that the observed

Table I. Effects of rifampicin (RIM) and rifabutin (RIB) treatment on body weight and liver weight of female rats

	Dose (mg/kg/day)	n	Final body weight mean ± S.D. (g)	Liver weight mean ± S.D. (% of body weight)
Control	0	4	196 ± 5	2.90 ± 0.30
RIM	250	5	164 ± 21	3.22 ± 0.30
	300	3	179 ± 6	3.96 ± 0.44
	450	2	140, 178	380, 5.03
RIB	250	5	171 ± 21	3.60 ± 0.44
	300	5	150 ± 17*	4.91 ± 0.48**
	450	2	134, 179	4.60, 5.66

* = $P < 0.05$; ** = $P < 0.01$ (compared with control).

Table II. Effects of rifampicin (RIM) and rifabutin (RIB) treatment on cytochrome P-450-dependent monooxygenase activities in female rat liver

	Dose (mg/kg/day)	n	Cytochrome P-450-dependent monooxygenase activity (mean ± S.D.) (pmol/mg/min) EROD	PROD	2α-OH,	2β-OH,	6α-OH,	6β-OH,	7α-OH,	15β-OH,	16α-OH,	16β-OH,	Androstenedione formation
Control	0	4	55.5 ± 17.1	11.9 ± 0.3	20 ± 8	35 ± 10	108 ± 6	163 ± 22	864 ± 122	21 ± 6	104 ± 10	30 ± 19	289 ± 59
RIM	250	5	91.3 ± 47.5	14.6 ± 3.9	20 ± 7	466 ± 219*	104 ± 9	859 ± 291**	763 ± 155	232 ± 106*	255 ± 77	75 ± 29	436 ± 97
	300	3	126.0 ± 47.9	16.1 ± 1.3	18 ± 5	1085 ± 281**	137 ± 13	1934 ± 295**	1099 ± 175	574 ± 133**	162 ± 23	159 ± 54**	573 ± 103**
	450	2	65.8, 137.3	16.5, 18.6	22, 28	1349, 1449	125, 151	2219, 2336	1211, 1253	720, 740	197, 221	157, 163	385, 442
RIB	250	5	126.9 ± 26.6	17.1 ± 4.0	16 ± 5	577 ± 126**	98 ± 10	942 ± 169**	1145 ± 212	304 ± 54**	257 ± 89	88 ± 17	373 ± 49
	300	5	139.2 ± 21.0*	13.7 ± 3.7	24 ± 4	552 ± 92**	123 ± 14	946 ± 113**	1365 ± 416	283 ± 39**	301 ± 43**	89 ± 16	415 ± 56
	450	2	78.8, 198.4	11.5, 19.2	14, 17	478, 1005	102, 109	1010, 1556	998, 1161	264, 505	233, 254	83, 100	422, 462

EROD = 7-ethoxyresorufin O-deethylase; PROD = 7-pentoxyreforufin O-dependthylase; OH, = site of testosterone hydroxylation. * = $P < 0.05$, ** = $P < 0.01$ (compared with control)
Underlining denotes significant difference between RIM and RIB groups at the same dosages. Singly underlined = $P < 0.05$, doubly underlined = $P < 0.01$

Table III. Effects of rifampicin (RIM) and rifabutin (RIB) treatment on UDP-glucuronosyltransferase activities in female rat liver

	Dose (mg/kg/day)	n	UDP-glucuronosyltransferase activity (nmol/mg/min) 1-naphthol	4-hydroxybiphenyl	estradiol	estrone
Control	0	4	10.2 ± 3.3	10.3 ± 0.5	1.13 ± 0.21	0.27 ± 0.02
RIM	250	5	22.2 ± 3.4*	16.7 ± 1.3*	1.91 ± 0.52	0.24 ± 0.02
	300	3	20.7 ± 2.5	18.0 ± 1.7*	2.57 ± 0.25	0.29 ± 0.00
	450	2	15.9, 30.1	15.3, 18.6	2.30, 2.74	0.20, 0.24
RIB	250	5	23.0 ± 5.5*	21.9 ± 2.2**	3.07 ± 0.67**	0.31 ± 0.03
	300	5	27.1 ± 5.1**	24.1 ± 3.6**	3.38 ± 0.69**	0.25 ± 0.05
	450	2	29.8, 35.9	30.9, 33.9	3.43, 4.80	0.29, 0.34

* = $P < 0.05$; ** = $P < 0.01$ (compared with control).
Single underlining denotes significant difference ($P < 0.05$) between RIM and RIB groups.

increase in testosterone 16β-hydroxylation can actually be ascribed to a 16β-hydroxylation activity of the cytochrome P-450 3A isoenzyme(s) induced by the rifamycins. Other P-450-mediated reactions were only marginally enhanced (testosterone 16α-hydroxylation, androstenedione formation, 7-ethoxyresorufin deethylation) or not at all affected by rifampicin and rifabutin (Table II).

Most of the glucuronidation reactions monitored in this study appeared to be enhanced to some extent by both treatments (Table III). Rifabutin significantly induced the glucuronidation of the model substrates 1-naphthol, 4-hydroxybiphenyl and estradiol by a factor of 2.4 to 3, while the estrone glucuronidation remained essentially unaffected. Rifampicin treatment displayed a qualitatively similar yet less pronounced, pattern of effects on the glucuronidation activities. In this context, it must be pointed out that the most prominent inductions reported for the respective glucuronidation reactions do not exceed a factor of five to seven-fold the basal activity, while many cytochrome P-450-dependent enzyme activities can be induced 50-fold or more.

Discussion

The results of the present study clearly show that, despite the resistance of the rat to the inducing properties of rifampicin, induction of the hepatic cytochrome P-450 system, mainly the P-450 3A subfamily, occurs in the rat after high doses of rifampicin. The fact that doses of 150–200 mg/kg/day have not been effective in earlier studies (see Introduction) together with our observation of a steep increase in induction in consequence of a relatively modest increase of dose from 250 to 300 mg/kg/day indicates that there is a threshold dose for rifampicin-mediated P-450 induction in the rat liver. Below this dose some apparently saturable process may inactivate/sequester rifampicin, thereby rendering it ineffective in terms of enzyme induction. Above this threshold rifampicin behaves as a strong inducer of cytochrome P-450 3A in rat liver. A concomitant induction of a variety of glucuronidation reactions also occurred, although to a minor extent compared to cytochrome 3A activity (1.8–2.5-fold).

Rifabutin proved to be an enzyme inducer similar to rifampicin, with quantitative differences only. While the maximally achievable induction of P-450 3A by rifabutin was about two-fold lower as compared to rifampicin, the influence on the glucuronidation reactions appeared to be similar or slightly higher. No indication of a threshold-dose for efficient enzyme induction by rifabutin was obtained at the doses used in the present study.

Induction of P-450 3A, as observed with the rifamycins, has potential implications in terms of possible drug–drug interactions since the members of the P-450 3A subfamily have a broad substrate specificity including a significant number of therapeutic drugs, such as dihydropyridine-derived calcium antagonists (Gonzalez, 1992; Murray, 1992), oral contraceptives (Murray, 1992), anticoagulants (Namkung *et al.*, 1988), dapsone (Fleming *et al.*, 1992) and macrolide antibiotics (Murray, 1992). Moreover, the induction of some UDP-glucuronosyltransferases, observed in female rat liver in the present study, may act synergistically to enhance the clearance of drugs administered simultaneously with these rifamycin derivatives. However, in the light of the present knowledge, any extrapolation of the rat data to the human situation cannot be made. On the one hand, the effects of rifampicin and rifabutin on the urinary excretion of 6β-hydroxycortisol in healthy volunteers (Perucca *et al.*, 1988) and on erythromycin *N*-demethylase and 7-ethoxyresorufin O-deethylase in patients (Ged *et al.*, 1989) are in

favour of similarities between rats and humans as regards the induction of the cytochrome P-450 3A subfamily and the absence of induction of the cytochrome P-450 1A subfamily. On the other hand, cytochrome P-450-dependent pathways induced in humans by rifampicin and, to a lesser degree, by rifabutin, such as antipyrine oxidative pathways (Perucca *et al.*, 1988), deserve further investigation so that a satisfactory comparison between rat and man as regards the selectivity of the induction by these agents can be made. The same applies to glucuronosyltransferases. Preliminary data suggest that azidothymidine glucuronidation is not induced in humans (Strolin Benedetti, Duchene & Olliaro, 1991; Strolin Benedetti & Dostert, 1994) after administration of rifabutin or rifampicin for 10 days at daily doses, which have been shown to induce P-450-dependent enzymes (Perucca *et al.*, 1988).

Acknowledgements

The authors wish to thank Mrs H. Wagner for excellent technical assistance and Miss L. Magné for skillful secretarial assistance.

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(Received 2 August 1995; returned 19 October 1995; revised 2 January 1996; accepted 7 February 1996)