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SHORT COMMUNICATIONS

Effect of rifampicin treatment on *in vitro* drug-metabolizing activities in the pig

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Studies on the inducing effects of rifampicin have revealed that the compound is certainly not a potent inducer of drug metabolism in every species. There are large differences between man and most other species: in man, a strong induction was found [1, 2], in mouse [3–6], rabbit [7] and hamster [3, 4] only a moderate inductive effect, and in rat [3, 4, 6] and guinea-pig [3, 4, 8] the induction was small or even not present at all. In a preceding paper, however, it was demonstrated that rifampicin markedly induces antipyrine and hexobarbital metabolism *in vivo* in the pig [9].

The purpose of the investigation described in this communication was to see whether the increase of biotransformation *in vivo* is associated with an increase in certain hepatic parameters and drug-metabolizing parameters *in vitro* and to investigate selectivity in the process of induction further.

Materials and methods. Rifampicin and rifampicin-quinone were a gift from Lepetit B.V. (Rotterdam, The Netherlands), 6β- and 16α-hydroxytestosterone from Prof. Kirk (Steroid Reference Collection, London, U.K.), 7α-hydroxytestosterone from Dr. Coert (Organon, Oss, The Netherlands), 11β-hydroxytestosterone from Dr. Moolenaar (Dept. of Pathology, Leiden, The Netherlands) and benzphetamine–HCl from Upjohn Co. (Kalamazoo, MI). 1-Chloro-2,4-dinitrobenzene, benzo[*a*]pyrene and *p*-nitroanisole were from Fluka (Buchs, Switzerland), aminopyrine and ethylmorphine from Brocacef (Maarssen, The Netherlands), 7-ethoxycoumarin from Aldrich-Europe (Beerse, Belgium), [4-^{14}C]testosterone (sp. act. 51.9 mCi/mmole) from NEN (Langen, F.R.G.) and biochemicals from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the best quality available and were obtained from Merck (Darmstadt, F.R.G.) or J.T. Baker Chemicals (Deventer, The Netherlands).

Six male Yorkshire pigs weighing *ca* 25 kg were obtained from a specialized farm and kept for acclimatization in straw-bedded cages for at least 1 week before the experiments started. The animals had been free of antibiotics and other drugs for at least 1 month. During treatment the pigs were kept in stainless steel-wired cages and had free access to water. They were fed twice daily. On 7 subsequent days three pigs were treated with rifampicin (capsules in the food; 300 mg p.o. twice daily, generally with an interval of 8–10 hr; it was checked that the capsules were swallowed). Three other pigs were used as controls.

At 9 a.m. on the first day after the last rifampicin administration, the animals were killed by carotidal incision after light anaesthesia with metomidate (20 mg/kg) and azaperone (1.5 mg/kg) i.p. (Janssen Pharmaceutica, Beerse, Belgium). Control pigs were killed at random days, at 9 a.m. Livers were perfused *in situ* with at least 1 l of ice-cold 0.25 M sucrose, and microsomes and 100,000 *g* supernatant were prepared; the supernatant (S100) fraction was stored at −35° until used. The microsomal fraction was suspended in 0.1 M phosphate buffer, pH 7.5, containing 1 mM EDTA and 0.5 mM dithiothreitol, frozen in liquid nitrogen and stored at −85° until used.

The following parameters were investigated according to the literature, with minor modifications: protein concentration in microsomal and cytoplasmic fractions ([10] modified as described in ref. [11]), cytochrome P-450 [12], cytochrome b_5 [13], NADPH-cytochrome *c* reductase [14], NADPH-rifampicin-quinone reduction [15], aniline hydroxylation ([14]; activation studies with acetone, at concentrations up to 450 mM), benzo[*a*]pyrene hydroxylation [16], *p*-nitroanisole *O*-demethylation [14], testosterone hydroxylation [17], 7-ethoxycoumarin *O*-deethylation [18, 19], biphenyl hydroxylation [20], aminopyrine *N*-demethylation [14, 21], ethylmorphine *N*-demethylation and benzphetamine *N*-demethylation (as aminopyrine), UDP-glucuronosyl transferase (with 3-methyl-2-nitrophenol as substrate [22]), glutathione *S*-transferase (with 1-chloro-2,4-dinitrobenzene as substrate [11]), SDS-polyacrylamide gel electrophoresis [23, 24].

Statistical analysis was done by Student's *t*-test for unpaired data; if standard deviations of the two compared groups were not of the same order, Student's *t*-test was done with the natural logarithm of the data. Data were assumed to be significant when $P < 0.05$.

Apparent enzyme parameters were obtained by using a Hanes plot [25].

Results and discussion. The results are summarized in Table 1. Rifampicin treatment of pigs during 7 days (300 mg p.o., twice daily) increased the relative liver weight by 33% and the microsomal protein content by 110% (mg/g body wt). Cytochrome P-450 (nmole/mg microsomal protein) increased 4-fold, which was more than in all other species studied, including man [3]. The content of cytochrome b_5 increased to 220% (nmole/mg protein). The activity of NADPH-cytochrome P-450 reductase, a third enzyme involved in the mixed-function oxidation of xenobiotics, was also induced when expressed in the reduction of cytochrome *c* (+70%). This reductase is also able to reduce rifampicin-quinone to rifampicin [26]; this activity was induced to about the same extent (+50%).

The activity of a number of phase-I metabolic reactions was investigated after rifampicin induction. The *N*-demethylation of ethylmorphine, benzphetamine and aminopyrine all increased considerably (+190%, +260% and +380%). Benzo[*a*]pyrene hydroxylation increased *ca* 9-fold. The activity of biphenyl 4-hydroxylation almost doubled, but no biphenyl 2-hydroxylation could be measured, either in control microsomes, or in those of rifampicin-treated pigs. 7-Ethoxycoumarin *O*-deethylation increased *ca* 5-fold and *p*-nitroanisole *O*-demethylation 4-fold. Like in the *in vivo* experiment [9], the only one of the known main metabolites of antipyrine that could be recovered was 4-hydroxyantipyrine (to be published elsewhere). For testosterone, the main metabolites were 2β- and 6α-hydroxytestosterone in both types of microsomal preparations. Minor metabolites were 7α-, 11β- and 16α-hydroxytestosterone. The biotransformation to the 2β-hydroxymetabolite increased *ca* 4-fold, while the pathway to the 6α-metabolite increased 5-fold. The oxidation to the three minor metabolites increased too.

The only phase-I activity that apparently could not be induced was aniline hydroxylation. 'Activation' with acetone, however, did increase the activity in microsomes of rifampicin-treated pigs *ca* 3-fold. In control microsomes, the activity declined after addition of 45 mM acetone. With concentrations higher than 150 mM, the activity tended to increase to *ca* 1.5 times the initial value. For microsomes of rifampicin-treated animals, the apparent K_m increased also.

Table 1. Effect of rifampicin treatment on *in vitro* hepatic parameters in pigs

	Controls $\bar{x} \pm$ S.D. ($n = 3$)	Rifampicin-treated $\bar{x} \pm$ S.D. ($n = 3$)
Liver (% of body wt)	1.8 ± 0.3	2.4 ± 0.2*
Microsomal protein (mg/g liver)	12.6 ± 3.4	20.4 ± 3.8 n.s.
Cytoplasmic protein (mg/g liver)	58.8 ± 2.3	77.9 ± 6.6**
Cytochrome P-450 (nmole/mg microsomal protein)	0.59 ± 0.05	2.32 ± 0.76**
Cytochrome b_5 (nmole/mg microsomal protein)	0.38 ± 0.02	0.80 ± 0.24*
NADPH-cytochrome *c* reductase V_{max}†	55 ± 5	94 ± 13**
NADPH-rifampicin-quinone reductase V_{max}	1000 ± 130	1500 ± 260*
Ethylmorphine *N*-demethylase V_{max}	8300 ± 2500	24300 ± 1900***
Benzphetamine *N*-demethylase V_{max}	1600 ± 600	5800 ± 1600*
Aniline hydroxylase (−acetone) V_{max}	0.49 ± 0.11	0.52 ± 0.03 n.s.
(+450 mM acetone; % of initial value)	144 ± 40	279 ± 18**
Benzo[*a*]pyrene hydroxylase V_{max}‡	0.54 ± 0.16	4.56 ± 0.65***
Biphenyl 4-hydroxylase V_{max}	1.49 ± 0.36	2.62 ± 0.25**
Aminopyrine *N*-demethylase V_{max}	2800 ± 1200	13500 ± 4100*
7-Ethoxycoumarin *O*-deethylase V_{max}	1.03 ± 0.26	5.2 ± 0.6**
p-Nitroanisole *O*-demethylase V_{max}	1260 ± 270	5100 ± 800**
UDP-Glucuronosyl transferase V_{max}	130 ± 20	140 ± 40 n.s.
Glutathione *S*-transferase V_{max}	8700 ± 1000	7100 ± 1400 n.s.
K_m (mM)	0.13 ± 0.01	0.25 ± 0.09*
Testosterone 2β-hydroxylase *V*	0.97 ± 0.15	4.0 ± 0.2***
Testosterone 6β-hydroxylase *V*	1.9 ± 0.3	9.7 ± 1.2***
Testosterone 7α-hydroxylase *V*	0.08 ± 0.01	0.82 ± 0.05***
Testosterone 11β-hydroxylase *V*	0.20 ± 0.05	1.09 ± 0.11***
Testosterone 16α-hydroxylase *V*	0.06 ± 0.00	0.34 ± 0.04***
Soret peak CO–cytochrome P-450 complex (nm)	449.3 ± 0.1	448.4 ± 0.1***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s. not significant.
† *V* and V_{max} in nmole/mg protein per min.
‡ Units/mg protein per min.

There seem to be at least two forms of aniline-hydroxylating cytochrome: one with low apparent K_m and V_{max} ('control' cytochrome P-450; not induced by rifampicin; inhibited by acetone *in vitro*), the other with high apparent V_{max}, but also high apparent K_m (induced by rifampicin; not or only slightly inhibited by acetone). Similar situations were found in mice [3] and rats [27, 28].

The two phase-II activities studied were not inducible by the antibiotic. Of the microsomal UDP-glucuronosyl transferase, both apparent V_{max} and K_m remained the same. Although the cytoplasmic protein increased after rifampicin treatment (+32% in nmole/g liver), the apparent V_{max} of cytoplasmic glutathione *S*-transferase was not induced. The apparent K_m increased *ca* 2-fold, which indicates competitive inhibition of the activity by rifampicin or (one of) its metabolites. Indeed, *in vitro* addition of rifampicin inhibited glutathione *S*-transferase, and with rifampicin-quinone inhibition was even greater.

SDS-Polyacrylamide gel electrophoresis clarified that rifampicin has a marked effect on the different forms of cytochrome P-450 (Fig. 1). Of interest is the induction of a protein with mol. wt of *ca* 52,000 and the relative decrease of proteins of 60,000 and 55,500.

The Soret peak of the reduced CO–cytochrome P-450 complex shifted to the blue region from 449.3 to 448.4 nm.

It is clear that rifampicin is a potent inducer of drug metabolism in the pig and that there is good agreement between the profound induction observed *in vivo* [9] and *in vitro*. Pigs seem to be very sensitive to induction by the antibiotic, which is in contrast with findings for other laboratory animals like the rat, mouse, hamster, rabbit and guinea-pig.

Rifampicin treatment induced a large variety of phase-I enzyme activities, and the large variety of induced parameters may indicate a rather aspecific induction, in accordance with Heubel and Netter's results for mice [5]. However, the strong induction of benzo[*a*]pyrene metabolism, the increase of the formation of the 4-hydroxymetabolite of antipyrine and the clear blue-shift of the Soret peak of the CO–cytochrome P-450 complex indicate that in the pig

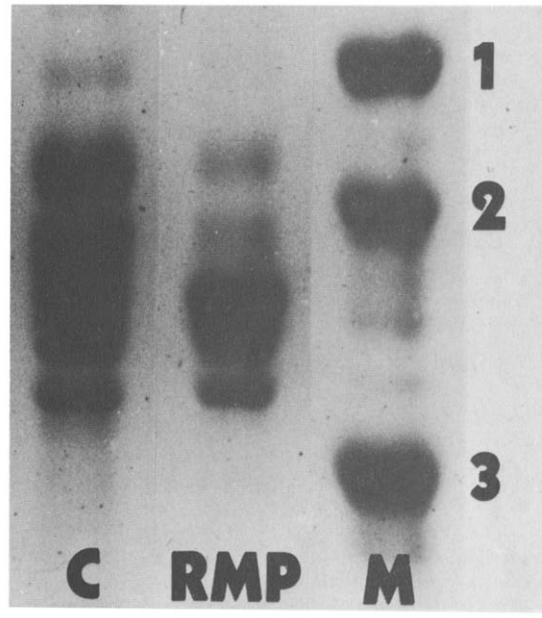

Fig. 1. SDS-Polyacrylamide gel electrophoresis of microsomes of control and rifampicin-treated pigs. Electrophoresis occurred from top to bottom; staining was done with Coomassie Brilliant Blue. C, Control microsomes; RMP, microsomes of rifampicin-treated pigs; M, marker proteins (from top to bottom: 1, bovine serum albumin; 2, catalase; 3, glutamate dehydrogenase).

a form of P-450 whose activities are coupled with aryl hydrocarbon hydroxylation is preferently induced.

It is interesting to note that the induction of drug metabolism by rifampicin in man shows a resemblance, with respect to changes in *in vivo* parameters [1] and in *in vitro* parameters [29]. The pig therefore seems to be a good animal model for studying the mechanism of induction by rifampicin. Especially this species is useful for further *in vivo* and *in vitro* studies in which the induction by the antibiotic and other inducers (phenobarbital, 3-methylcholanthrene) is compared, for instance with respect to the induction of metabolic pathways of hexobarbital, antipyrine and other test substances. The purification of pig microsomal cytochrome P-450 will be a useful tool [18].

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Inhibition of aromatic amino acid decarboxylase and depletion of biogenic amines in brain of rats treated with α-monofluoromethyl *p*-tyrosine: similitudes and differences with the effects of α-monofluoromethyldopa

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Monofluoromethyldopa (MFMD) is a selective, irreversible inhibitor of aromatic amino acid decarboxylase (AADC) [1, 2], so potent that the decarboxylation of Dopa and 5-hydroxytryptophane (5-HTp) become rate-limiting in the biosynthesis of catechol- and indoleamines, respectively [3, 4]. In our attempts to restrict AADC inhibition and amine depletion to catecholaminergic neurons, we decided to take advantage of the substrate specificity and the neuronal localization of tyrosine hydroxylase (TH), the first and normally rate-limiting enzyme of catecholamine biosynthesis. For this purpose, we needed a compound which did not inhibit AADC directly but was transformed by the catalytic action of TH into an AADC inhibitor of the potency of MFMD. We wish to report that α-monofluoromethyl *p*-tyrosine (MFMT) has the required properties.

Materials and methods. MFMD, MFMT and its methyl ester (MFMT-Me) were synthesized in our laboratory. α-Methyl *p*-tyrosine, methyl ester (MT-Me) and Dopa were obtained from Sigma Chemical Co.; haloperidol used was