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Metabolism of metronidazole and antipyrine in hepatocytes isolated from mouse and rat

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1. In order to study species-related differences and select a model for the human metabolism of metronidazole and antipyrine, the Michaëlis–Menten kinetics of metabolite formation from the two compounds were investigated in freshly isolated mouse and rat hepatocytes.

2. The average K_m values for the formation of the major metronidazole metabolites ranged from 0·6 to 3 mM. The intrinsic clearance values (V_{max}/K_m) of metronidazole to the acetic acid, hydroxy and glucuronide metabolites were 58 (36–125) and 21 (12–28; $P<0·05$), 156 (63–263) and 36 (19–56; $P<0·05$), and 269 (102–452) and 500 (389–1616; $P<0·05$) nl/min per 10^6 hepatocytes, for mouse and rat, respectively (median with range, $n=6$).

3. The average K_m values for the formation of antipyrine metabolites ranged from 2 to 10 mM. The intrinsic clearance values for production of 3-hydroxymethyl-, nor- and 4-hydroxyantipyrine were 232 (43–519) and 487 (296–793; $P<0·05$), 594 (168–813) and 93 (55–180; $P<0·05$), and 118 (23–505) and 239 (134–501; $P>0·05$) nl/min per 10^6 hepatocytes, for mouse and rat, respectively (median with range, $n=6$).

4. The results demonstrate that metronidazole and antipyrine are metabolized with quantitative, but not qualitative, differences in isolated hepatocytes from mice and rats. Neither species provided an ideal model for the human metabolism of the two compounds.

Introduction

We have proposed the antimicrobial metronidazole as a probe for the study of drug metabolism capacity, optionally in a mixture with antipyrine (Loft *et al.* 1988). Recent investigations on the metabolism of the two drugs in isolated rat hepatocytes and humans indicate that the formation of the two oxidative metabolites from metronidazole and the three from antipyrine are catalysed by different cytochrome P-450 isozymes, respectively (Loft *et al.* 1987, 1988, Loft and Poulsen 1989). Identical metabolites are formed from the two drugs in man and rat; however, their rank orders of quantitative importance differ. In rats, *in vivo* as well as *in vitro*, conjugation is the predominant route of metabolism of metronidazole (LaRusso *et al.* 1978, Allars *et al.* 1985, Loft and Poulsen 1989), whereas oxidative formation of hydroxymetronidazole and metronidazole acetic acid are the major routes of elimination in man (Loft *et al.* 1986, 1987). In rats 3-hydroxymethylantipyrine is the dominant metabolite of antipyrine, as opposed to 4-hydroxyantipyrine formation in humans (Danhof *et al.* 1979, Inaba *et al.* 1980, Kahn *et al.* 1982, Penno and Vesell 1983, Loft *et al.* 1987, Loft and Poulsen 1989). Thus, a species other than the rat may be more relevant to man as a model for the study of metronidazole and antipyrine metabolism. In mice the metronidazole metabolite profile has been reported to

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resemble that in humans (Stambaugh *et al.* 1968), whereas data on antipyrine metabolite formation in mice are not available.

In the present study the kinetics of metabolite formation from metronidazole and antipyrine by freshly isolated hepatocytes from mice and rats were compared.

Materials and methods

Chemicals were obtained as previously described (Loft and Poulsen 1989).

Cell preparation

Laboratory-bred male Theiler mice (25–35 g) and Wistar rats (225–275 g) were housed under constant temperature and humidity in a 12 h light cycle with free access to food (Altromin®) and tap water. Rats and mice were anaesthetized with thiopental 9 and 18 mg per kg body wt i.p., respectively, and the hepatocytes were isolated as previously described with the following exception regarding the mice (Loft and Poulsen 1989). The mouse livers were perfused *in situ* at a flow rate of 5 ml/min by means of a once-through system with a 4·5 m gas-diffusible Silastic® tube in carbogen gas (O_2/CO_2; 95/5; v/v) and hepatocytes from two or three mice were pooled for each experiment. The cell yield as counted in a Burker chamber was $20–50 \times 10^6$ and $200–400 \times 10^6$ per mouse and rat liver, respectively. The viability as assessed by trypan blue exclusion exceeded 90% in all experiments and did not decrease during incubation.

Incubations

For each of six groups of two or three mice and six rats, ten round-bottom flasks were incubated with 6×10^6 viable hepatocytes as previously described (Loft and Poulsen 1989). Metronidazole 0·2, 0·5, 1·0, 5·0 and 10·0 mM, and antipyrine 0·2, 0·5, 1·0, 5·0 and 20·0 mM were added to a final volume of 3 ml in each flask. Samples (750 µl) were collected after 30, 60 and 120 min of incubation at 37°C in a carbogen atmosphere and immediately centrifuged at 4000 *g*. The supernatant was kept at −20°C until analysis.

Analytical procedures

The content of cytochrome P-450 after ultrasonication of the hepatocytes was measured according to the method of Omura and Sato (1964). The protein content was measured by the method of Lowry *et al.* (1951). The concentrations of metronidazole and antipyrine and their metabolites in the incubation medium were determined as previously described (Loft *et al.* 1986, 1987, Loft and Poulsen 1989). Analysis of ultrasonicated cell pellet after centrifugation of samples showed similar intra- and extracellular concentrations of substrates and metabolites. The medium from incubations with antipyrine was analysed before and after hydrolysis with glucuronidase/arylsulphatase at pH 4·5 and 37°C for 3 h.

Calculations

The rate of increase in the concentration of each metabolite was compared between the three sampling time points by three-factorial analysis of variance with substrate concentration and animal as the two other factors. Scheffe's test was used for *post hoc* comparison of means.

The V_{max} and K_m were determined for the formation of each metabolite in the hepatocytes from each rat, or group of two or three mice, by weighted nonlinear least-squares regression as previously described (Loft and Poulsen 1989). The rate of metabolite formation was calculated from the concentrations measured after incubation for 30 min, except in rat hepatocyte incubations with metronidazole, in which the 120 min value was used. The intrinsic clearance (CL_i) for production of metabolites was calculated as V_{max}/K_{max}.

The kinetic constants were compared between species by the Mann–Whitney *U*-test and the cytochrome P-450 and protein content by the unpaired *t*-test. *P*-values less than 0·05 were considered statistically significant.

Results

Metronidazole metabolites accumulated at constant rate during the 120 min of incubation with suspensions of freshly isolated hepatocytes from rats as previously demonstrated (figure 1; Loft and Poulsen 1989). In the mouse experiments the appearance rate of metronidazole acetic acid, hydroxymetronidazole and metronidazole glucuronide was linear after 30 min of incubation but had decreased after 120 min. Thus, for the three metronidazole metabolites the average formation

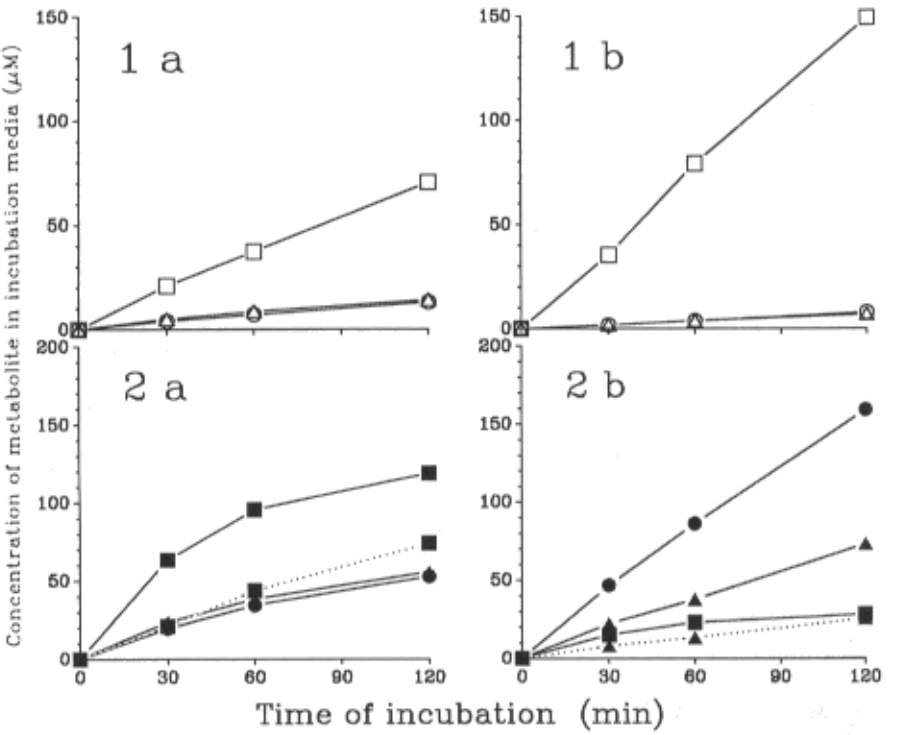

Figure 1. Appearance of metabolites of (**1**) metronidazole and (**2**) antipyrine in (*a*) mouse and (*b*) rat hepatocytes.

Suspensions of mouse (**a**) and rat (**b**) hepatocytes were incubated with metronidazole (**1**) or antipyrine (**2**) 5 mM for 120 min. The time course of metabolite appearance was similar for the other substrate concentrations. Metronidazole acetic acid (○——○); hydroxymetronidazole (△——△); metronidazole glucuronide (□——□); 3-hydroxymethylantipyrine (●——●); norantipyrine (■——■); conjugates of norantipyrine (■·····■); 4-hydroxyantipyrine (▲——▲); conjugates of 4-hydroxyantipyrine (▲·····▲). Values are means of six experiments.

rates estimated from the 120 min sampling time point were 12–25% lower than the corresponding values estimated from the 30 min time point ($P<0{\cdot}05$; figure 1).

In the rat experiments the accumulation rates of the three primary antipyrine metabolites were constant during the first 60 min of incubation, but decreased significantly during the last 60 min as previously demonstrated (figure 1; Loft and Poulsen 1989). In the mouse hepatocytes the appearance rates of the three antipyrine metabolites decreased by 20–25% from the 30 min to the 60 min sampling time point and were further reduced after 120 min of incubation ($P<0{\cdot}05$; figure 1). In incubations with rat as well as mouse hepatocytes the accumulation rate of norantipyrine decreased by approximately 75% from the first 30 min to the last 60 min of incubation. In the mouse experiments about half of the measured amounts of norantipyrine appeared as conjugates, whereas minimal amounts of conjugates of 4-hydroxyantipyrine were detected (figure 1). By contrast, in the rat experiments half of the measured amounts of 4-hydroxyantipyrine appeared as conjugates, whereas minimal amounts of conjugates of norantipyrine were detected (figure 1; Loft and Poulsen 1989).

Table 1. Michaëlis–Menten constants for the production of metabolites from metronidazole Suspensions of freshly isolated mouse and rat hepatocytes were incubated with metronidazole 0·2 to 10 mM.

	Metronidazole acetic acid			Hydroxymetronidazole			Metronidazole glucuronide		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
Mice	98* (52–125)	2·0 (0·7–5·1)	58·3* (36–125)	105* (41–114)	0·57 (0·4–1·4)	156* (63–263)	435* (270–690)	1·7 (1·0–4·0)	269* (102–452)
Rats	69 (28–104)	2·6 (0·9–6·0)	21·0 (12–28)	41·9 (23–52)	1·1 (0·4–1·5)	35·7 (19–56)	848 (515–1141)	1·4 (0·5–2·6)	500 (389–1616)

V_{max} values are expressed as pmol product per min per 10^6 hepatocytes; K_m values as mM; $CL_i = V_{max}/K_m$ as nl/min per 10^6 hepatocytes.
* $P < 0·05$ compared with the corresponding value from the rat experiments.
Values are median with range, $n = 6$.

Table 2. Michaëlis–Menten constants for the production of metabolites from antipyrine. Suspensions of freshly isolated mouse and rat hepatocytes were incubated with antipyrine 0·2 to 20 mM.

	3-Hydroxymethylantipyrine			Norantipyrine			4-Hydroxyantipyrine		
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m
Mice	433* (205–582)	1·7 (1·0–4·7)	232* (43–519)	1260* (708–3488)	2·9* (0·4–3·4)	594* (168–813)	708 (179–3489)	7·2 (2·0–18)	118 (23–505)
Rats	1110 (587–1655)	2·1 (2·0–2·6)	487 (296–793)	656 (4·8–792)	7·6 (4·0–10·6)	93 (55–180)	712 (362–917)	2·8 (1·4–5·8)	239 (134–501)

V_{max} values are expressed as pmol product per min per 10^6 hepatocytes; K_m values as mM; $CL_i = V_{max}/K_m$ as nl/min per 10^6 hepatocytes.
* $P < 0·05$ compared with the corresponding value from the rat experiments.
Values are median with range, $n = 6$.

In all experiments the data fitted a single Michaëlis–Menten equation. In the typical experiment the coefficients of variation of the K_m and V_{max} estimates were 25% and 10%, respectively, i.e. much less than the variation between experiments.

The Michaëlis–Menten kinetics of formation of metabolites from metronidazole in isolated rat and mouse hepatocytes are given in table 1. The K_m values did not differ significantly between the species, whereas the V_{max} and CL_i values for production of hydroxymetronidazole and metronidazole acetic acid from metronidazole were higher in mouse than in rat hepatocytes ($P<0.05$), with reversed relationship for formation of the glucuronide conjugate ($P<0.05$; table 1).

The Michaëlis–Menten kinetics of antipyrine metabolite formation are given in table 2. The V_{max} and CL_i for production of 3-hydroxymethylantipyrine from antipyrine was higher in the rat than in the mouse hepatocytes ($P<0.05$), but the relationship were reversed for production of norantipyrine and included a lower K_m value ($P<0.05$), whereas there were no significant species-related differences concerning 4-hydroxyantipyrine and the K_m for 3-hydroxymethylantipyrine formation.

The cytochrome P-450 content was 577 ± 230 and 560 ± 60 pmol per 10^6 hepatocytes or 208 ± 83 and 276 ± 69 pmol per mg protein in the rat and mouse experiments, respectively (mean $\pm$ SD, $n=4$, $P>0.5$).

Discussion

In mouse hepatocytes the formation of metronidazole glucuronide accounted for 56% of the sum of the CL_i values of metronidazole, whereas oxidative formation of hydroxymetronidazole and metronidazole acetic acid accounted for 32% and 12%, repectively. In rat hepatocytes the formation of metronidazole glucuronide, hydroxymetronidazole and metronidazole acetic acid accounted for 92%, 5% and 3%, in agreement with values obtained in rats *in vivo* and in perfused liver (LaRusso *et al.* 1978, Allars *et al.* 1985). In CD1 mice, as well in humans, less than 15% of the total amount recovered in urine has been reported to be excreted as the glucuronide, whereas hydroxymetronidazole and metronidazole acetic acid accounted for 35–45% and 15–20%, respectively (Stambaugh *et al.* 1968, Loft *et al.* 1986, 1987). This apparent difference between the Theiler mice used in this study and the reported results in CD1 mice is unexplained, but could be related to strain or *in vivo–in vitro* differences. Thus, regarding the quantitative metabolism of metronidazole, isolated hepatocytes from Theiler mice resemble rats more than humans.

The formation of norantipyrine was the quantitatively dominant metabolic pathway of antipyrine in the mouse hepatocytes. In support of this, the *N*-demethylation pathway represented at least 40% of a dose of radiolabelled antipyrine in mice *in vivo* as estimated by their exhalation of $^{14}CO_2$ (La Delfa *et al.* 1989). In contrast, 3-hydroxymethylantipyrine formation dominated in rat hepatocytes in agreement with *in vitro* and *in vivo* findings (Danhof *et al.* 1979, Inaba *et al.* 1980, Kahn *et al.* 1982). Accordingly, metabolism of antipyrine by neither rat nor mouse hepatocytes completely resembles its metabolism in humans where 4-hydroxyantipyrine is the dominant pathway (Penno and Vesell 1983, Loft *et al.* 1987). Whether the appearance of all three antipyrine metabolites in suspensions of mouse hepatocytes corresponds to the *in vivo* situation remains to be demonstrated.

We have suggested that each of the oxidative metabolites of metronidazole and antipyrine are formed by one or more distinct cytochrome P-450 isozyme(s) (Loft *et al.* 1987, 1988, Loft and Poulsen 1989). Regarding the antipyrine metabolites the formation of 3-hydroxymethylantipyrine and norantipyrine was quantitatively dominant in rats and mice, respectively, whereas the CL_i to 4-hydroxyantipyrine was similar in the two species. This apparent difference in regulation between the species of the formation of the three antipyrine metabolites lends further support to the view that they are formed by different P-450 isozyme(s) (Danhof *et al.* 1979, Inaba *et al.* 1980, Kahn *et al.* 1982, Penno and Vesell 1983, Loft *et al.* 1987, 1988, Loft and Poulsen 1989). By the same token, the higher CL_i of metronidazole to metronidazole acetic acid and hydroxymetronidazole in mice than in rat hepatocytes supports the suggestion that the involved isozymes are different from those involved in 3-hydroxymethylantipyrine and 4-hydroxyantipyrine formation from antipyrine. The parallel differences in the formation rates of norantipyrine from antipyrine and metronidazole acetic acid and hydroxymetronidazole from metronidazole in rat and mouse hepatocytes could support the proposal that identical or co-regulated isozymes are involved. This would, however, be inconsistent with the main bulk of evidence from studies in humans and isolated rat hepatocytes of the covariation of rates of metabolite formation from the two drugs under various conditions, including the effects of specific enzyme induction and inhibition, sex and co-interactions (Loft *et al.* 1987, Loft and Poulsen 1989). Moreover, the K_m for norantipyrine formation was significantly lower in mouse than in rat hepatocytes, indicating qualitative differences between the species in the P-450 isozyme(s) involved in this reaction.

The lack of linearity in metabolite appearance with mouse hepatocytes could be related to further metabolism, or to a decrease in the metabolic performance due to loss of viability during incubation. The latter possibility was not supported by changes in the ability to exclude trypan blue during incubation. The particular nonlinearity in accumulation of norantipyrine in hepatocytes from both species is in agreement with another study of antipyrine metabolism in isolated hepatocytes (Chenery *et al.* 1987) and was probably caused by the instability of this metabolite in its free form (Eichelbaum *et al.* 1981). Thus, the appearance rate of conjugates of norantipyrine departed only slightly from linearity in the mouse experiments (figure 1). In mouse hepatocytes, approximation to linear metabolite appearance could be assumed from 30 to 60 min of incubations and by using the 30 min values only minor and unimportant underestimations of the initial metabolite production rates could result.

Mouse and rat hepatocytes contained almost identical amounts of cytochrome P-450 and protein, giving scope to the inter-species comparison of the metabolite formation rates expressed per 10^6 hepatocytes. Moreover, the metabolic capacity was similar in hepatocytes from the two species as judged by the sum of the CL_i values for production of the metronidazole and antipyrine metabolites, although the ranking order of magnitude differed. In fact, the inter-species differences in the median values of the kinetic constants were less than the variation within one of the two species.

In conclusion we have demonstrated that metabolites relevant for humans are formed from metronidazole and antipyrine in suspensions of freshly isolated hepatocytes from mouse and rat according to Michaëlis–Menten kinetics with significant species-related differences in ranking order and magnitude of kinetic

constants and intrinsic clearance. From a quantitative point of view neither mouse nor rat hepatocytes are ideal *in vitro* models for the human metabolism of metronidazole and antipyrine.

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