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INHIBITION OF THE OXIDATIVE METABOLISM OF THEOPHYLLINE IN ISOLATED RAT HEPATOCYTES BY THE QUINOLONE ANTIBIOTIC ENOXACIN AND ITS METABOLITE OXOENOXACIN, BUT NOT BY OFLOXACIN

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Abstract—Isolated rat hepatocytes obtained from Aroclor-pretreated rats were incubated with theophylline in the presence or absence of the quinolone antibiotics enoxacin, its metabolite oxoenoxacin, or ofloxacin. The hepatocytes converted theophylline by cytochrome P-450 activity mainly to two metabolites: 1,3-dimethyluric acid and 3-methylxanthine.

Enoxacin inhibited the formation of 1,3-dimethyluric acid by 67% at 1.0 mM. Oxoenoxacin or ofloxacin had no inhibitory effect. The oxidation of theophylline to 3-methylxanthine was not inhibited by any of the three compounds. The quinolones had no effect on cell viability.

These results show that the inhibition by enoxacin is not due to the formation of its oxoenoxacin metabolite.

Recently, Wijnands *et al.* [1, 2] reported that, in patients, enoxacin decreased the clearance of theophylline. Similar effects were seen in the rat *in vivo* [3]. Ofloxaxin does not have such an effect [4]. Wijnands *et al.* [5] suggested that the oxo-metabolite of enoxacin, oxoenoxacin, might be responsible for this inhibition; this metabolite is formed from enoxacin by oxidation of the 3′-C atom in the piperazine ring to the keto ("oxo") form [6]. A similar metabolite seems not to be formed from ofloxacin [7].

The aim of the present investigation was to find out if this interaction could be confirmed in rat hepatocytes *in vitro* and whether we could indeed find a more pronounced effect of oxoenoxacin than of its precursor enoxacin. Little work has been done so far on the metabolism of theophylline in isolated hepatocytes. A recent abstract [8] showed that in isolated human and rat hepatocytes *in vitro* the two major metabolites of theophylline were the 1,3-dimethyluric acid (1,3-DMU)‡ and 3-methylxanthine (3-MX) derivatives.

In the present report we show that enoxacin at 0.1 mM inhibits the formation of 1,3-DMU very effectively, while ofloxacin and oxoenoxacin have no effect, even at a 1.0 mM concentration. On the other hand, the formation of 3MX is not inhibited by any of the oxacins at 1.0 mM. These results indicate that enoxacin does not inhibit theophylline metabolism through its own metabolite oxoenoxacin.

MATERIALS AND METHODS

Rats. Male Wistar rats (200 g body wt) which had free access to food and water were used. They were supplied by the breeding unit of the Department of Pharmacology, University of Leiden. Unless specified otherwise, they were pretreated with Aroclor 1254 (Analabs Inc. No Haven, CT. 06473) by a single intraperitoneal injection of 250 mg/kg (Aroclor was dissolved in corn oil at 125 mg/ml). The rats were used for the preparation of hepatocytes or microsomes 3–5 days after this injection.

Hepatocytes. Hepatocytes were prepared routinely according to the method of Seglen, as described by Nagelkerke *et al.* [9], by a perfusion with collagenase in the presence of calcium ions, after a preperfusion with medium that does not contain calcium. The viability of the final preparation was 90–95% as judged by Trypan blue exclusion and lactate dehydrogenase leakage. During a subsequent 2-hr incubation, the viability does not decrease by more than 10–15% (based on lactate dehydrogenase leakage).

Hepatocyte incubations. The incubations were performed in Hanks–Hepes buffer (137 mM NaCl; 5.4 mM KCl; 0.81 mM $MgSO_4$; 0.42 mM Na_2HPO_4; 0.44 mM KH_2PO_4; 20.8 mM HEPES) to which 5.5 mM glucose and 2.5% w/v bovine serum albumin were added. To 5 ml of the above buffer, 3.5 ml of a hepatocyte suspension in Hanks-HEPES buffer was added resulting in a final cell density of approx. 700,000 cells/ml. Incubations were performed under carbogen gas at 37° in a rotatory lab shaker [9] for up to 2 hr. The theophylline concentration was 1 mM, while also (when present) 0.01 to 1 mM of the quinolones was added.

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‡ Abbreviations used: 1,3-DMU, 1,3-dimethyluric acid; HEPES, N-2-hydroxyethylpiperazone-N′-2-ethane sulfonic acid; 3-MX, 3-methylxanthine; 1-MU, 1-methyluric acid; LDH, lactate dehydrogenase.

Table 1. Retention times of the various compounds

Theophylline	960
Theobromine	890
1,3-Dimethyluric acid	660
3-Methylxanthine	720
3-Methyluric acid	295
1-Methylxanthine	655
1-Methyluric acid	260

Analysis of incubation. After 60 or 120 min a 300 μl sample was taken, and 600 μl ice-cold methanol was added on ice. After centrifugation, a sample of 10–20 μl was injected on HPLC. As internal standard, a constant concentration of theobromine was added in the methanol used to precipitate the cell protein at the end of the incubation.

HPLC separation of theophylline and its metabolites. The equipment used was a Waters gradient HPLC system consisting of two pumps (510 and M-6000A) in combination with an Apple IIe computer and chromatochart Y software (Interactive Microware, Inc., State College, PA 16804, U.S.A.). Detection was at 254 nm (Waters, model 440). The column was a Spherisorb ODS II, 5 μM (15 × 0.3 cm). The eluents were: 0.05 M sodium phosphate, pH 8.0 in water, and methanol. Eluent flow was 0.45 ml/min. The gradient was as follows: 0–66 sec from 0% to 10% methanol; 66–990 sec from 10% to 40 methanol; 990–1390 sec from 40 to 70% methanol; 1390–1590 sec remain at 70% methanol; 1590–2475 sec from 70 to 0% methanol.

Table 1 gives the retention time of various theophylline metabolites; due to variations between separate runs, in individual experiments the retention times may vary ±30 sec. Therefore, theobromine was added as an internal standard.

RESULTS

Metabolites formed by hepatocytes in vitro

Virtually exclusively two metabolites were formed during incubation of theophylline with isolated rat liver hepatocytes: 1,3-DMU, the main metabolite, and 3MX. None of the other potential metabolites was formed in measurable quantities under our conditions (Figs 1–3). The extent of 1,3-DMU and 3-MX production still increased after 1 hr of incubation: after 2 hr an additional approx. 60% rise in metabolite concentration was observed.

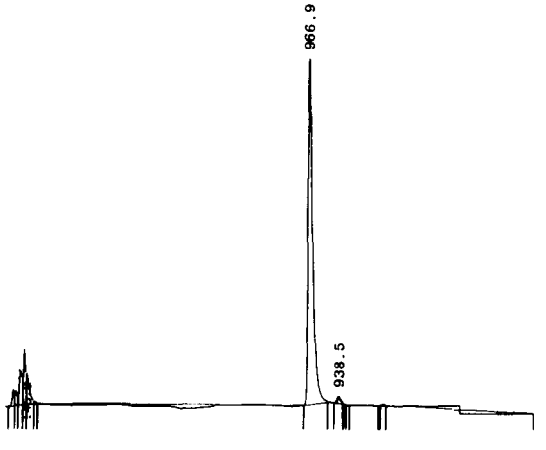

Fig. 1. HPLC analysis of cell incubation without theophylline added. The only (major) peak is the theobromine internal standard (120 min incubation).

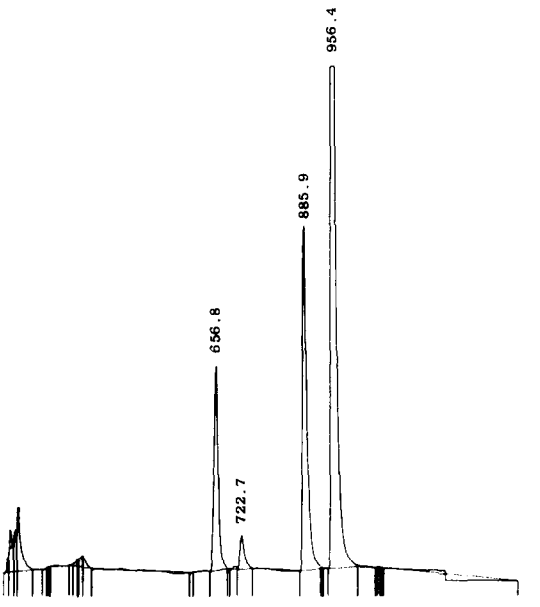

Fig. 2. HPLC analysis of cell incubation with theophylline after a 120 min incubation. The peaks shown are 1,3-DMU (657 sec) 3MX (723 sec), theobromine (886 sec) and theophylline (956 sec).

In non-induced rats, the rate of metabolism was much slower than in the induced rats: the difference was some tenfold (not shown). Because in both uninduced and induced rats 1,3-DMU and 3MX were the only metabolites (and the ratio between their formation was very similar), we have used for inhibition studies exclusively the induced rats.

Effect of enoxacin, oxoenoxacin and ofloxacin on theophylline metabolism in the isolated rat hepatocytes

In order to test whether the quinolones would inhibit theophylline oxidation, we incubated hepatocytes with three concentrations of the quinolones: 0.01, 0.1 and 1.0 mM. At the lowest concentration none of the quinolones showed an appreciable effect; a 10% inhibition of 1,3-DMU formation by enoxacin seems of border-line significance. However, at the 0.1 mM concentration there clearly was inhibition of 1,3-DMU formation by enoxacin: approximately 35% inhibition (Table 2). The others, ofloxacin and oxoenoxacin had no effect. At a 1.0 mM concentration the same applied: now enoxacin inhibited theophylline oxidation to 1,3-DMU by 67%. The formation of this metabolite was inhibited only marginally by ofloxacin and oxoenoxacin: 9 and 15% respectively. The extent of inhibition was the same after 1 hr and 2 hr incubations as shown in Table 2.

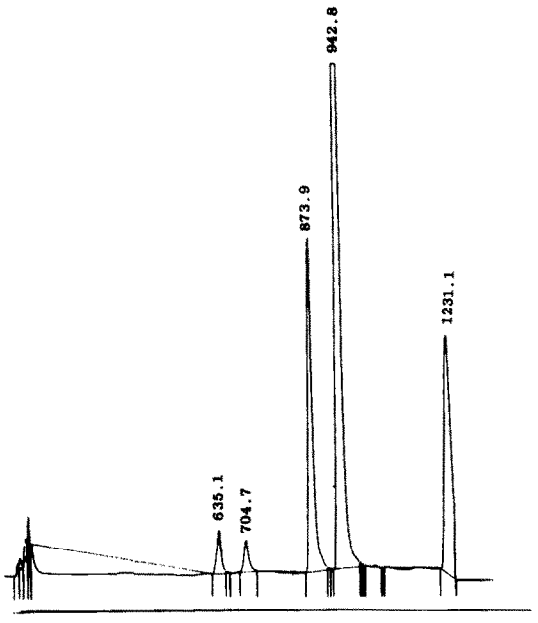

Fig. 3. HPLC analysis of cell incubation with theophylline and enoxacin (1 mM) after 120 min. The peaks shown are 1,3-DMU (635), 3MX (705), theobromine (874), theophylline (943) and enoxacin (1231).

Interestingly, the formation of 3MX was not at all affected by any of the quinolones: even 1.0 mM enoxacin did not at all decrease formation of 3-MX, while it inhibited the formation of 1,3-DMU by almost 70%.

During all incubations we recorded the leakage of lactate dehydrogenase (LDH) as an indicator of cell damage, because it was possible that one of the quinolones might cause cell death, and thereby inhibit metabolism of theophylline non-specifically. However, none of the oxacin's resulted in damage to the hepatocytes after the 2 hr incubation (initial LDH leakage between 10 and 15%; at the end of the 2 hr incubation this was 15–20%).

Oxidative metabolism of theophylline by rat liver microsomes

Liver microsomes from rats pretreated with Aroclor were used in an attempt to detect metabolism of theophylline *in vitro*. Although there was some formation of 1,3-DMU, This was so little that it could not reliably be quantitated. Therefore, we have not tried to inhibit this formation with any of the quinolones. The low rate of conversion by rat liver microsomal cytochrome P-450 is in agreement with other reports ([10]; Dr. Brian Houston, Univ. of Manchester, Dept. of Pharmacy, personal communication). In the rabbit liver, as well as in human liver microsomes higher levels are found [11, 12].

DISCUSSION

In man the major metabolite of theophylline is 1,3-DMU, while appreciable 3-MX and 1-methyluric acid (1-MU) is excreted in urine as well; upon chronic administration also 3-MX becomes a major metabolite [13]. It seems, therefore, that the isolated rat hepatocyte system offers a good model for two of the three major metabolites. In the rat, 1,3-DMU is the major metabolite (certainly in the 3-methyl-

Table 2. Effect of quinolones on theophylline oxidation to 1,3-dimethyluric acid in incubations with isolated hepatocytes

Conc. (mM)	Oxacin	Incubation (hr)	Activity (% of control) Exp. I	Exp. II	Exp. III	mean	Inhibition (%)	
0.01	Enoxacin	1	95					
		2	84					
0.1	Enoxacin	1	77	63	73	70	30	34
		2	79	50	57	62	38	
1.0	Enoxacin	1	43	29	35	36	64	67
		2	39	22	32	31	69	
0.01	Ofloxacin	1	102					
		2	112					
0.1	Ofloxacin	1	118	103	102	108	—	0
		2	129	97	96	107	—	
1.0	Ofloxacin	1	112	93	82	96	4	9
		2	99	81	80	87	13	
0.01	Oxoenoxacin	1	109					
		2	111					
0.1	Oxoenoxacin	1	105	104	104	104	—	0
		2	100	86	93	93	—	
1.0	Oxoenoxacin	1	81	98	90	90	10	14
		2	83	89	78	83	17	

Hepatocytes were incubated at 1 mM theophylline in the presence of 0.01–1.0 mM of the quinolones for either 1 or 2 hr. The rate at which 1,3-DMU was formed was approx. 90 nmol/g protein/hr over the first hour: for 3-MX this was approx. 20 nmol/mg protein/hr. The activity in the presence of the quinolones is given as percentage of control for 3 separate experiments.

cholanthrene-induced rat), while 1-MU is excreted at approx. 1/3 the level of 1,3-DMU [14]. Therefore, the finding that in our isolated hepatocytes no 1-MU (or 1MX) but rather 3-MX was formed was somewhat surprising. The rats we used do form 1MU *in vivo* as has been shown before [15]. Recently, Riche *et al.* [8] also reported the formation of 1,3-DMU and 3-MX by rat and human isolated hepatocytes *in vitro*. Gorodischer *et al.* [16] using rat liver slices also found no formation of 1-MU, but did not separate 1,3-DMU and 3-MX in their thin layer chromatography system, so that it is not clear whether they also had 3-MX production. The fact that only the formation of 1,3-DMU (oxidation at C8) is inhibited by (only) enoxacin, while the formation of 3-MX (N-demethylation) is unaffected suggests that these are formed by different cytochrome P-450 species, one of which only is able to bind enoxacin. This is in agreement with recent findings in human microsomes [12].

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