EFFECTS OF ROXITHROMYCIN, A NEW SEMISYNTHETIC MACROLIDE, AND TWO ERYTHROMYCINS ON DRUG METABOLIZING ENZYMES IN RAT LIVER

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The effects of a new semisynthetic macrolide, roxithromycin, on drug metabolizing enzymes of rat liver were compared with two erythromycins, the base (EB) and the estolate (EE), after 7 days' treatment with high oral doses (400 and 800 mg/kg daily). Dose-related higher concentrations of roxithromycin were reached in serum and liver than after EB or EE. The two reference erythromycins induced the synthesis of microsomal enzymes and formed inactive cytochrome P-450-metabolite complexes. *N*-Demethylation of erythromycin itself and aminopyrine was increased by the treatment. Liver microsomal enzyme activities were not induced and the inactive cytochrome P-450-metabolite complex was not formed after 400 mg/kg of roxithromycin and only to a very limited extent after 800 mg/kg (10% vs. 50% after EE). At the higher dose microsomal activities were not changed by roxithromycin and only aminopyrine *N*-demethylation was reduced.

The effective antibacterial activity of erythromycin has been recently reviewed[1]. Although this macrolide has been widely used in humans for over three decades, it still poses some problems. Most important are marked variations in the bioavailability of oral preparations because of inactivation by gastric juice, and particular formulations are needed to protect the antibiotic in the stomach. Moreover erythromycin and especially some of the gastro-resistant derivatives, like estolate, may interfere with drug metabolism in man[2~4].

This effect has been related to the ability of these macrolides to influence the hepatic mono-oxygenase system in two different ways. On the one hand, these antibiotics induce the formation of cytochrome P-450 similar to that induced by some steroid hormones like pregnenolone-16-carbonitrile and dexamethasone[5,6]. On the other hand, this leads to increased transformation of the macrolide-$N(CH_3)_2$ group to the NO group and *in vivo* formation of very stable and inactive cytochrome P-450 Fe(II)-metabolite complexes which are characterized by a Soret peak at 456 nm. Once blocked in such a complex, cytochrome P-450 loses all catalytic properties. This dual effect has been shown in rats[7] and man[8].

Thus synthesis of a macrolide which does not influence drug metabolizing enzymes and does not affect detoxification of other compounds may be important for patients concomitantly needing other drugs metabolized by cytochrome P-450. A new semisynthetic macrolide, roxithromycin (formerly RU 28965), recently synthesized by Roussel Uclaf (Paris)[9], was found to have the same antibacterial activity as erythromycin base[10,11] but better stability in acid and improved pharmacokinetic properties in animals and man[12~14].

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This paper compares the effects of roxithromycin, erythromycin base and estolate on drug metabolizing enzymes in rat liver.

Materials and Methods

Chemicals

The three macrolides, roxithromycin, erythromycin base and estolate were kindly supplied by Roussel Uclaf (Paris). All other chemicals were analytical grade.

Animals

Male Crl: CD (SD)BR rats (Charles River, Calco, Italy) weighing 210±15 g were fed a standard diet (Altromin MT) *ad lib* (Rieper, Vandoies, Italy) and housed under controlled conditions (22± 0.5°C, 55% relative humidity, 12: 12-hour light/dark cycle).

Treatments

Rats were given orally (by gastric tube) suspensions of the macrolides in an aqueous solution of 0.25% sodium carboxymethyl-cellulose (CMC) — 400 and 800 mg/kg daily — for 7 consecutive days. Control animals received 10 ml of 0.25% CMC/kg daily. After the last dose the rats were fasted for 18 hours and used for preparation of liver microsomes and other tests.

Assays

1) Drug metabolizing enzymes: Microsomes were prepared as previously described[15] and microsomal protein[16], NADPH-cytochrome c reductase[17], cytochrome b_5[18] and uncomplexed cytochrome P-450[18] were determined. The total amount of cytochrome P-450 was measured by the same procedure[18] after first adding 50 μM potassium ferricyanide to another batch of microsomes.

N-Demethylation of erythromycin[19] and aminopyrine[20], *O*-demethylation of 4-nitroanisol and hydroxylation of aniline[21] were assayed using both untreated and treated (50 μM ferricyanide) microsomes.

2) The *in vivo* formation of complexes absorbing at 456 nm was measured with a microsomal suspension (2 mg protein/ml) after addition of 50 μM potassium ferricyanide to the reference cuvette. The difference spectrum was recorded from 400 to 500 nm[7]. The amount of complexed cytochrome P-450 was calculated[8] from its Soret peak at 456 nm with a molar extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$.

3) The binding spectra of erythromycins with cytochrome P-450 Fe(III) were recorded from 360 to 500 nm after addition to microsomes (2 mg protein/ml)[7] of increasing amounts of the drugs dissolved in ethanol.

4) Macrolide concentrations were measured in serum and liver by a microbiological assay using *Bacillus subtilis* ATCC 6633 as test organism[22].

5) Statistical significance of the results was analyzed by DUNNETT's test[23].

Results

After 7 days' treatment with high oral doses of roxithromycin, erythromycin base and estolate (400 and 800 mg/kg daily), drug levels of all macrolides in serum and liver were dose-related (Table 1). However, roxithromycin reached levels 5 to 70 times higher than the two erythromycins (Table 1).

The dose-related increases in liver weight, in the concentration of microsomal protein, NADPH-cytochrome c reductase and cytochrome b_5 showed that the estolate was a strong inducer of hepatic drug metabolizing enzymes (Table 2). The concentration of CO-binding free cytochrome P-450 was also significantly raised (Table 3). However, part of the total cytochrome P-450 (30% and 48% respectively after 400 and 800 mg/kg) was dose-dependently bound in complexes with erythromycin metabolites and did not bind CO (Table 3). After repeated administration of estolate, the reverse type I binding spectrum of estolate was also dose-dependently increased (Table 3). Correspondingly,

Table 1. Erythromycins (E) and roxithromycin concentrations in serum and liver of pretreated rats[a].

Group	Treatment (mg/kg daily for 7 days)	Serum (μg/ml)	Liver (μg/g)
Control	—	ND	ND
E base	400	0.06±0.01	0.64±0.09
	800	0.07±0.01	1.52±0.48
E estolate	400	0.08±0.01	1.60±0.36
	800	1.09±0.19	14.28±2.54
Roxithromycin	400	3.12±0.53	35.02±9.75
	800	5.38±1.29	67.47±10.93

Results are mean±SEM of 5~6 rats.
[a] Rats were killed 18 hours after the last erythromycin dose.
ND: Not detectable.

Table 2. Effects of erythromycins (E) and roxithromycin on hepatic drug-metabolizing enzymes.

Group	Treatment (mg/kg daily for 7 days)	Liver weight/ body weight (%)	Microsomal protein (mg/g liver)	NADPH-cytochrome c reductase (nmol/ minute/ mg protein)	Cytochrome b_5 (nmol/mg protein)
Control	—	2.98±0.06	28.64±0.60	90±5	0.409±0.015
E base	400	3.05±0.09	27.39±1.27	95±12	0.356±0.015
	800	3.18±0.05	31.41±1.42	134±7[a]	0.515±0.028[b]
E estolate	400	3.34±0.06[b]	28.32±1.06	157±14[b]	0.488±0.015[b]
	800	3.81±0.10[b]	32.69±0.88[b]	182±21[b]	0.590±0.024[b]
Roxithromycin	400	3.10±0.04	27.19±1.35	96±12	0.462±0.022
	800	4.08±0.15[b]	26.23±0.91	123±6	0.355±0.017

Results are mean±SEM of 5~6 rats.
[a] $P<0.05$ against control. [b] $P<0.01$ against control.

Table 3. Effects of erythromycins (E) and roxithromycin on the hepatic mono-oxygenase system.

Group	Treatment (mg/kg daily for 7 days)	Uncomplexed cytochrome P-450 (nmol/mg protein)	Total cytochrome P-450 (nmol/mg protein)	Complexed cytochrome P-450 (nmol/mg protein)	Induced P-450/ control P-450[a]	Reverse type I binding spectrum ($OD_{422\sim390}\times10^{-3}$)
Control	—	0.609±0.033	0.596±0.032	ND	1.00	ND
E base	400	0.654±0.018	0.734±0.040	0.060±0.004	1.23	ND
	800	0.700±0.052	0.859±0.056[b]	0.207±0.036	1.44	ND
E estolate	400	0.774±0.052[b]	1.154±0.113[c]	0.348±0.068	1.94	104±23
	800	0.776±0.052[b]	1.519±0.220[c]	0.733±0.129	2.55	280±60
Roxithromycin	400	0.562±0.091	0.520±0.085	ND	0.87	ND
	800	0.527±0.029	0.593±0.018	0.059±0.010	0.99	ND

Results are mean±SEM of 5~6 rats.
[a] Ratio of cytochrome P-450 in treated and control rats.
[b] $P<0.05$ against control. [c] $P<0.01$ against control.
ND: Not detectable.

demethylation of erythromycin itself and of aminopyrine rose significantly, particularly after breaking the inactive complex with potassium ferricyanide (Table 4).

Aniline hydroxylation and 4-nitroanisole demethylation were not significantly affected by the treatment (data not shown). Erythromycin base influenced most of the parameters studied (Tables 2~4) but to a lesser extent than estolate and only at the 800 mg/kg dose.

Table 4. Effects of erythromycins (E) and roxithromycin on macrolide and aminopyrine demethylation.

Group	Treatment (mg/kg daily for 7 days)	Substrate of macrolide demethylation	Macrolide demethylase (nmol HCHO/10 minutes/mg protein)		Aminopyrine *N*-demethylase (nmol product/30 minutes/mg protein)	
			Without potassium ferricyanide (%)[a]	With potassium ferricyanide (%)[a]	Without potassium ferricyanide (%)[a]	With potassium ferricyanide (%)[a]
Control	—	Roxithromycin	4.3±0.3[b]	5.5±0.4[b]	3.6±0.3 (100)	4.2±0.4 (100)
		E base	8.6±0.4	9.5±0.6		
		E estolate	8.8±1.0	10.8±1.0		
E base	400	E base	12.7±1.2 (149)	19.2±1.1[c](201)	3.3±0.3 (92)	4.2±0.4 (100)
	800	E base	25.5±3.0[d](298)	36.7±4.9[d](384)	3.9±0.4 (108)	6.0±0.3 (143)
E estolate	400	E estolate	18.6±3.1[d](211)	28.6±2.7[d](265)	5.7±0.6[d](158)	6.6±0.5[c](157)
	800	E estolate	19.9±1.4[d](225)	33.1±1.5[d](307)	6.9±1.0[d](192)	10.8±1.1[d](257)
Roxithromycin	400	Roxithromycin	8.2±0.6[d](188)	10.3±0.9[d](188)	3.7±0.7 (102)	5.0±1.1 (119)
	800	Roxithromycin	5.3±0.2 (122)	7.6±0.4 (139)	1.8±0.2[c](50)	2.3±0.2[c](55)
	800	E base	8.0±0.6 (93)	12.3±0.8 (129)		
	800	E estolate	6.3±0.4 (71)	9.2±0.4 (86)		

Results are mean±SEM of 5~6 rats.

[a] Activity is expressed as a percentage of the corresponding control.

[b] $P<0.01$ against E base and estolate. [c] $P<0.05$ against control. [d] $P<0.01$ against control.

In contrast, roxithromycin did not induce biosynthesis of microsomal enzymes. After 400 mg/kg daily, liver weight, microsomal protein concentration, NADPH-cytochrome c reductase activity, cytochrome b_5 (Table 2), CO-binding free cytochrome P-450 content were not changed and no cytochrome P-450-metabolite complexes were formed (Table 3). Moreover, roxithromycin showed no reverse type I binding spectrum with microsomes from treated rats (Table 3). The new macrolide was demethylated by microsomes from untreated animals but significantly less than the two erythromycins (about 50% of the base and estolate) (Table 4). Microsomes from roxithromycin-treated rats demethylated the macrolide itself to a greater extent but this reaction was still very low and similar to demethylation of the base and estolate in untreated control rats (Table 4). Aminopyrine *N*-demethylation was not affected by roxithromycin (Table 4).

One week's treatment with the higher dose of roxithromycin caused a significant increase in liver weight (Table 2). However, microsomal protein concentration, NADPH-cytochrome c reductase activity and cytochrome b_5 content did not significantly increase (Table 2). In contrast, the concentration of uncomplexed cytochrome P-450 was slightly reduced even though the total amount, free and complexed, was not different from controls (Table 3). Only 10% of the total cytochrome P-450 content was complexed (Table 3).

Roxithromycin demethylation was not induced by the treatment with roxithromycin itself; when the base and estolate were used as substrates, demethylation by microsomes of roxithromycin treated rats was not different from that of untreated animals (Table 4). Only aminopyrine demethylation was significantly reduced by treatment with the higher dose of roxithromycin (Table 4); the other activities (4-nitroanisole *O*-demethylation and aniline hydroxylation) were not affected (data not shown).

Discussion

Repeated administration of erythromycin and some of its derivatives (*e.g.* propionate, estolate) to humans and rats induces biosynthesis of microsomal enzymes and promotes formation of metabolites which bind and inactivate cytochrome P-450. The oxidative metabolism of other drugs may also be affected. The hydrophobic character of the erythromycin molecule and the presence of an accessible *N*-dimethylamino group seem mainly responsible for these effects[24]. In fact erythromycin estolate, being more hydrophobic than the base, is also more active in this respect.

The characteristics of the new semisynthetic macrolide roxithromycin differ from those of erythromycins. Roxithromycin is more stable in acid solution and reaches much higher blood levels in man and animals[12~14]. It does not induce biosynthesis of microsomal enzymes and does not form cytochrome P-450-metabolite complexes despite the fact that it reaches high levels in the liver. Only a very small part of cytochrome P-450 is complexed after the higher roxithromycin dose tested, 800 mg/kg daily, but it must be underlined that this dose is 160-fold higher than the usual dosage schedule of roxithromycin in man (5 mg/kg daily).

Therefore roxithromycin does not appear to interact significantly with oxidized microsomal cytochrome P-450; no binding spectrum is detectable even at the highest dose tested. The demethylation of the new macrolide is low in untreated animals in comparison with erythromycin base and estolate and it is induced only after treatment with 400 mg/kg. In fact the higher dose is not able to induce roxithromycin demethylation probably because of the smaller amount of free cytochrome P-450 which also affects aminopyrine metabolism. Demethylation is the main metabolic pathway of macrolides, giving metabolites, probably nitrosoalkanes, which bind and inactivate cytochrome P-450-Fe(II)[7,24]. Consequently, the lower the demethylation rate, the smaller the amount of inactivating metabolites. Other macrolides like josamycin and midecamycin, which are demethylated slowly, do not form cytochrome P-450-metabolite complexes[25]. Preliminary *in vitro* results have shown that roxithromycin is less concentrated than the base (about half) in the hepatocyte microsomal fraction and this could

partly explain its lower metabolism (unpublished data).

In conclusion the evidence that roxithromycin does not induce the hepatic mono-oxygenase system and does not inactivate cytochrome P-450, in contrast to erythromycin base and estolate, suggests that it should not interfere with other drugs which are *N*- or *O*-demethylated or hydroxylated by the liver. The slight demethylation of roxithromycin is in agreement with its reported longer half-life[12~14]. All these properties of roxithromycin suggest that this new macrolide may offer some advantages for therapeutic purposes.

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