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THE INHIBITION OF DRUG OXIDATION BY ANHYDROERYTHROMYCIN, AN ACID DEGRADATION PRODUCT OF ERYTHROMYCIN

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Abstract—The inhibition of steroid 6 β-hydroxylase activity by anhydroerythromycin, an acid breakdown product of erythromycin, has been studied and compared to the effects of erythromycin using liver microsomes from control and dexamethasone pretreated rats and human liver microsomes. Both anhydroerythromycin and erythromycin were found to be demethylated, thus both fulfil the prerequisites for possible metabolite–cytochrome P450 complex formation. The formation of a metabolite–cytochrome P450 complex was demonstrated for anhydroerythromycin by preincubating NADPH fortified microsomes with anhydroerythromycin. This complex formation could be reversed by incubating the microsomes in 50 μM potassium ferricyanide. Anhydroerythromycin was a more potent inhibitor of androst-4-ene-3,17-dione (androstenedione) 6 β-hydroxylation than erythromycin. Kinetic analysis shows that there are probably two cytochromes P450 involved in androstenedione 6 β-hydroxylation in control rat microsomes both of which are inhibited by anhydroerythromycin. There are at least two forms of cytochrome P450 responsible for androstenedione 6 β-hydroxylation in microsomes from dexamethasone pretreated rats but only the high affinity form is inhibited by anhydroerythromycin. "Atypical" kinetics were observed in human microsomes but inhibition of androstenedione 6 β-hydroxylation was observed with 5 μM anhydroerythromycin at all androstenedione concentrations used. Inconsistencies have been observed in the literature with respect to clinical interactions observed with erythromycin. Since anhydroerythromycin appears to be a more potent inhibitor of androstenedione 6 β-hydroxylation than erythromycin, we speculate that the variable blood levels of anhydroerythromycin found after dosing with erythromycin may explain these discrepancies.

Erythromycin is a commonly prescribed macrolide antibiotic which has been implicated in clinically significant drug interactions. While interactions have been reported with theophylline [1–3] carbamazepine [4] and warfarin [5] these results remain controversial [6] and there is little agreement between studies. With respect to theophylline, a controlled study by Pfeifer *et al.* [7] showed a non-statistically significant decrease in theophylline clearance when theophylline was administered after chronic erythromycin dosing, although three out of nine subjects showed a 20–25% decrease in theophylline clearance. Some other studies examining the interaction between theophylline and erythromycin have shown no effect [8–10] while others have shown a significant decrease [1–3] in theophylline clearance.

The bioavailability of erythromycin and its salts, and esters is variable among different formulations [11]. Erythromycin and its sulfate salt are acid labile and are easily degraded to anhydroerythromycin in acidic solutions corresponding to the conditions which occur in the stomach [12, 13]. The amount of anhydroerythromycin formed is likely to be a function of gastric residence time and the nature of the formulation used. It has been proposed [14] that the decomposition of erythromycin involves a consecutive two step mechanism from erythromycin to erythromycin enol ether to anhydroerythromycin but a more recent study [15] suggests an equilibrium between erythromycin and erythromycin enol ether, coupled to a direct conversion reaction from erythromycin to anhydroerythromycin. In biopharmaceutical studies examining various oral formulations of erythromycin, plasma concentrations of anhydroerythromycin similar to those for erythromycin have been observed.† Anhydroerythromycin has also been detected in the urine of patients receiving intravenous erythromycin.†

Many studies have examined the effects of erythromycin and triacetyloleandomycin, another macrolide antibiotic, on drug metabolism [16–23]. These macrolide antibiotics are both inducers and inhibitors of cytochrome P450 [24]. For example, induction of rabbit cytochrome P450 3c by triacetyloleandomycin occurs by the stabilization of mRNA and protein [25] whereas inhibition is believed to be due to the formation of a stable cytochrome–metabolite complex [26]. It has been postulated that the tertiary amine function, R-N $(CH_3)_2$ of the macrolides, is demethylated and oxidised by cytochrome P450 to a nitroso metabolite which forms a stable inactive complex with the iron (II) of cytochrome P450. The complex is unstable in the ferric state and treatment of the microsomes with ferricyanide regenerates the activity of the cytochrome P450 [27].

Because of the inconsistencies in the literature concerning clinical interactions with erythromycin and the variability in blood levels of anhydroerythromycin found after oral dosing†, we

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speculated that anhydroerythromycin may be a potent inhibitor of drug metabolism and that the variability in the extent of drug interaction with erythromycin may be due to the presence of varying amounts of anhydroerythromycin. In this communication we present *in vitro* evidence that anhydroerythromycin is a more potent inhibitor than erythromycin of cytochrome P450 3A associated metabolic activity, as measured by steroid 6 β-hydroxylation capacity in both rat and human liver microsomes.

MATERIALS AND METHODS

Materials. NADP, NADPH, bovine serum albumin, isocitrate dehydrogenase, dexamethasone, unlabelled androst-4-ene-3,17-dione (androstenedione*) and 3β-hydroxy steroid dehydrogenase were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [4-^{14}C]Androstenedione (sp. act. 59 mCi/mmol) was purchased from Amersham Australia (Sydney). 16β-Hydroxyandrostenedione was prepared enzymatically by the action of 3β-hydroxy steroid dehydrogenase on 3β, 16β-dihydroxyandrost-5-ene-17-one (Prof. D.N. Kirk and the MRC Steroid Reference Collection, Queen Mary's College, London, U.K.) as described by Sheets and Estabrook [28].

Other hydroxylated testosterone and androstenedione standards were obtained from the MRC Steroid Collection or Steraloids, Inc. (Wilton, NH, U.S.A.).

Erythromycin and anhydroerythromycin were obtained from Faulding Pharmaceuticals (Salisbury, Australia). All solvents and other miscellaneous chemicals were at least analytical reagent grade.

Animals. Male Hooded Wistar rats (200 g) were obtained from the Animal Resource Centre (Gilles Plains, Australia). Dexamethasone treated rats received dexamethasone, 100 mg/kg in arachis oil, intraperitoneally for 4 consecutive days. Rats were killed 24 hr after the last injection. The liver homogenates from rats were pooled and microsomes were prepared by differential centrifugation, and stored at −70° prior to use.

Assays. Protein concentrations were determined by the method of Lowry *et al.* [29] and cytochrome P450 was quantitated by the method of Omura and Sato [30].

Erythromycin and anhydroerythromycin demethylase activities were measured in a 1 mL reaction mixture containing microsomal samples diluted to 0.25 mg/mL protein, isocitrate (4 mM), isocitrate dehydrogenase (0.4 I.U.), $MgCl_2$ (8 mM), 1 mM NADPH and erythromycin or anhydroerythromycin in 0.1 M potassium phosphate buffer (pH 7.4). After a 15 min incubation at 37°, the formaldehyde formed was assayed by the method of Nash [31]. Blank samples were prepared without erythromycin or anhydroerythromycin and were assayed in an identical manner. For each experiment, data are expressed as the mean of triplicate observations.

Microsomal androstenedione hydroxylase activity was assayed essentially by the procedure of Gustafsson and Ingelman-Sundberg [32] and Waxman *et al.* [33]. Microsomal fractions were incubated in a 2-mL reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.8 I.U.). $MgCl_2$ (8 mM), 1 mM NADP and androstenedione (3.5–175 μM, 130 μCi/mmol) for 15 min at 37°, after which the incubation mixtures were extracted twice with ethyl acetate. In inhibition studies, erythromycin and anhydroerythromycin were added in 20 μL DMSO and control samples with and without DMSO were always included in the experiments. DMSO had no significant effect on the reaction. The organic phase was evaporated to dryness under N_2, reconstituted in a small volume of ethylacetate and applied to TLC plates (silica gel 60, F_{254} type, 20 × 20 cm × 0.25 mm thickness; E. Merck, Darmstadt, F.R.G.). Plates were developed twice in the solvent system ($CHCl_3$: ethyl acetate 1:2, v/v) as described by Waxman *et al.* [32]. Zones corresponding to hydroxylated androstenedione standards were visualized under UV light and scraped into vials for scintillation spectrometry (ACS Amersham Australia, Sydney). Data are expressed as the mean of three replicates.

Dissociation of the anhydroerythromycin metabolite complex. Dissociation of the metabolite complex formed between anhydroerythromycin and the microsomal P450 was carried out by incubation of the preformed metabolite complex at 4° with potassium ferricyanide (50 μM final concentration). Thirty minutes later the microsomes were reisolated by centrifugation and rewashed to remove any residual ferricyanide. The microsomes were then assayed as described above for androstenedione hydroxylase activity.

Human microsomes. Human microsomes were obtained from Clinical Pharmacology, Flinders University of South Australia. The clinical details of the patients have been published previously [34].

Statistical analysis. Statistical analysis was carried out using two factor ANOVA.

RESULTS

Demethylation of erythromycin and anhydroerythromycin in rat liver microsomes

Anhydroerythromycin and erythromycin were both demethylated by microsomes from both control and dexamethasone treated rats. Anhydroerythromycin demethylase activity was 0.64 and 2.82 nmol/mg/min in liver microsomes from control and dexamethasone pretreated rats, respectively; erythromycin demethylase activity was 0.28 and 7.42 nmol/mg/min in liver microsomes from control and dexamethasone pretreated rats respectively. Eadie–Hofstee analyses of demethylation reactions in liver microsomes from dexamethasone pretreated rats were linear. This was indicative of a single enzyme reaction with a similar K_m value for both erythromycin and anhydroerythromycin demethylation (Table 1).

In vitro *inhibition and inactivation of androstenedione 6 β-hydroxylases in rat liver microsomes*

When erythromycin and anhydroerythromycin

* Abbreviations: androstenedione, androst-4-ene-3,17-dione; DMSO, dimethyl sulfoxide.

Table 1. Demethylation of erythromycin and anhydroerythromycin in pooled microsomes from dexamethasone treated rats

	Erythromycin demethylase	Anhydroerythromycin demethylase
K_m (μM)	113.8	101.2
V_{max} (nmol/mg/min)	8.9	3.4

Liver microsomes from rats pretreated with dexamethasone were incubated under air with a NADPH generating system for 30 min with erythromycin or anhydroerythromycin. Substrate concentrations ranged from 25 to 500 μM. The data were transformed to an Eadie–Hofstee plot from which K_m and V_{max} values were calculated.

Table 2. Effect of potassium ferricyanide treatment of 6 β-hydroxylation of androstenedione by microsomes from dexamethasone treated rats preincubated with anhydroerythromycin

Preincubation conditions	Potassium ferricyanide treatment	6 β-Hydroxylase activity (nmol/mg/min)
Complete	−	5.03 ± 0.17*
Complete	+	7.58 ± 0.18
−NADP	−	7.84 ± 0.33
−NADP	+	7.97 ± 0.38

Liver microsomes (2.5 mg/mL) from dexamethasone treated rats were incubated under air with 1 mM anhydroerythromycin for 45 min, with or without NADP in a NADPH generating system. Some microsomes were also incubated with potassium ferricyanide and then reisolated as described in Materials and Methods. Androstenedione hydroxylase activity was assayed as described with aliquots of microsomal protein (0.125 mg/mL) and 175 μM androstenedione. Results are the means ± SD of six observations from two separate experiments.

* Statistically different $P < 0.01$.

were incubated with microsomes (0.05 mg/mL) from control or from dexamethasone treated rats, varying degrees of inhibition of the 6 β-hydroxylation of androstenedione were observed. At a concentration of 0.5 mM, anhydroerythromycin was a more potent inhibitor than erythromycin. The percentage activity in the presence of erythromycin was 102% in control and 90% in dexamethasone treated microsomes, respectively, whereas the percentage activity in the presence of anhydroerythromycin was 42% in control and 57% in dexamethasone treated microsomes, respectively. Thus, inhibition is apparent in microsomes from both control and dexamethasone treated animals with anhydroerythromycin whereas no inhibition is observed with erythromycin in microsomes from control rats.

The formation of a complex between an anhydroerythromycin metabolite and the Fe(II) cytochrome P450 was demonstrated (Table 2). There was a decrease in the androstenedione 6 β-hydroxylase capacity of microsomes from dexamethasone treated rats which had been preincubated with NADP in a NADPH generating system and anhydroerythromycin when compared to microsomes which had been similarly preincubated, but without NADP. Microsomes which had been preincubated with anhydroerythromycin and a NADPH generating system were also treated with ferricyanide prior to determining their androstenedione 6 β-hydroxylase capacity. As shown in Table 2, ferricyanide effectively dissociated the anhydroerythromycin metabolite–Fe(II) cytochrome P450 complex restoring 6 β-hydroxylase capacity.

Kinetics of mono-oxygenase inhibition by anhydroerythromycin in rat liver microsomes

Eadie–Hofstee analysis of the inhibition of androstenedione 6 β-hydroxylase in microsomes from control animals revealed at least two forms of cytochrome P450 involved in the 6 β-hydroxylation both of which were inhibited by anhydroerythromycin (Fig. 1). In microsomes from dexamethasone pretreated animals, at least two forms of cytochrome P450 were also revealed by Eadie–Hofstee analysis, however, essentially only the high affinity form(s) was inhibited by anhydroerythromycin. The inhibition of 6 β-hydroxylation by 50 μM anhydroerythromycin throughout both profiles (Fig. 1.) is obviously greater than that observed with 50 μM erythromycin.

In vitro *inhibition of androstenedione 6 β-hydroxylases in human liver microsomes*

Anhydroerythromycin was found to be a more

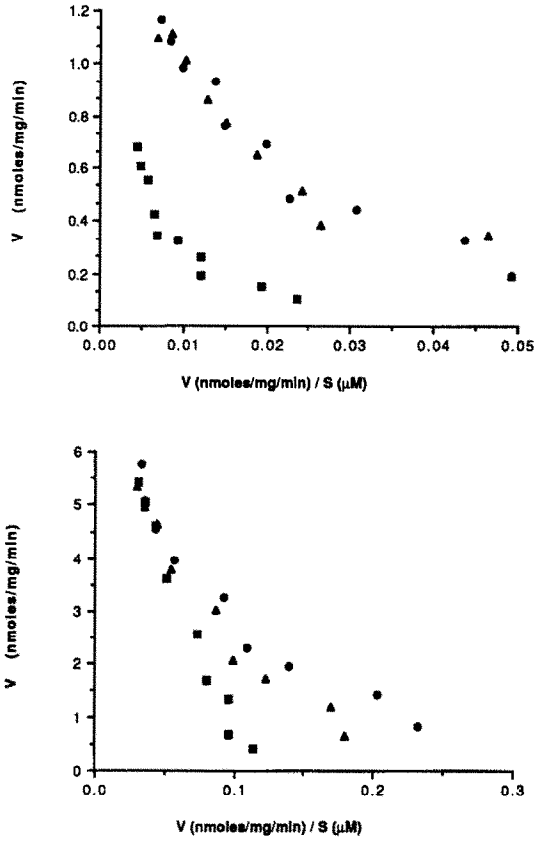

Fig. 1. Representative Eadie–Hofstee plots for androstenedione 6 β-hydroxylation by liver microsomes from control rats (upper panel) and dexamethasone treated rats (lower panel); without inhibitor (●), in the presence of 50 μM erythromycin (▲) and in the presence of 50 μM anhydroerythromycin (■). The incubations contained 0.125 mg/mL microsomal protein (upper panel), 0.05 mg/mL microsomal protein (lower panel), and androstenedione concentrations were 3.5–175 μM.

Table 3. Inhibition of the 6 β-hydroxylation of androstenedione by erythromycin and anhydroerythromycin in human liver microsomes

	6 β-Hydroxylase activity*	
	H_5	H_6
Erythromycin	59	94
Anhydroerythromycin	43	23

Human liver microsomes (0.125 mg/mL) were incubated under air with a NADPH generating system with 87.5 μM androstenedione. Inhibitor concentration was 0.5 mM.
* % Activity in the absence of inbibitor.

potent inhibitor of androstenedione 6 β-hydroxylation than erythromycin in liver microsomes from two human subjects (Table 3). The degree of inhibition varied between the two subjects. Interestingly, patient H_6 had no recorded drug history, whereas patient H_5 had received both

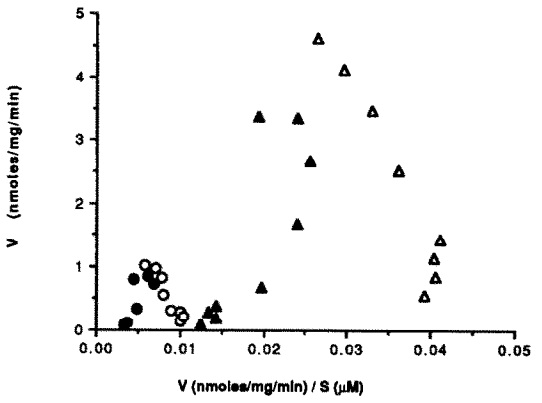

Fig. 2. Representative Eadie–Hofstee plots for androstenedione 6 β-hydroxylation by human liver microsomes: H_5 without inhibitor (△) and in the presence of 5 μM anhydroerythromycin (▲); H_6 without inhibitor (○) and in the presence of 5 μM anhydroerythromycin (●). The incubations contained 0.125 mg/mL microsomal protein and androstenedione concentrations were 3.5–175 μM.

phenytoin and dexamethasone. Induction of cytochrome P450 3A proteins is reflected by the 6 β-hydroxylase activities measured in the H_5 liver (Fig. 2).

Kinetics of mono-oxygenase inhibition by anhydroerythromycin in human liver microsomes

Eadie–Hofstee plots of androstenedione 6 β-hydroxylation were "atypical" (Fig. 2). Lineweaver–Burk plots gave calculated K_m values approaching infinity (data not shown). However inhibition of the 6 β-hydroxylation of androstenedione by 5 μM anhydroerythromycin was observed at all concentrations of androstenedione used.

DISCUSSION

The metabolic oxidation of the $N(CH_3)_2$ function of macrolide antibiotics, such as erythromycin, has been implicated in the formation of the cytochrome P450–macrolide complex. The complexed P450 is in the ferrous state and inactive. The results of the present investigation show that anhydroerythromycin was also similarly metabolized to complex with the Fe(II) of cytochrome P450. The formation of an inhibitor complex was demonstrated when microsomes were preincubated with a NADPH generating system and anhydroerythromycin (Table 2). The complex could be reversed by incubating with 50 μM ferricyanide, as shown by the restoration of 6 β-hydroxylation capacity.

In studies using high inhibitor concentrations (0.5 mM), and non-saturating androstenedione concentrations, anhydroerythromycin was apparently a more potent inhibitor than was erythromycin with respect to androstenedione 6 β-hydroxylation.

Steroid 6 β-hydroxylase activity has been found to be associated with cytochromes P450 3A [35, 36]. A multiplicity of proteins have been established in the cytochrome P450 3A family with purification

[37–40] cloning studies [41–45]. In the rat, cytochrome P450 3A1 is inducible whilst cytochrome P450 3A2 is constitutive and male-specific. The human P450 3A family has been shown to be composed of at least four genes [42–46]. Cytochromes P450 3A3 and 3A4 are apparently inducible by both dexamethasone and the macrolide antibiotics [42]. Therefore, in Eadie–Hofstee plots for dexamethasone induced liver microsomes, it is not unexpected to find that androstenedione 6 β-hydroxylation is biphasic (Fig. 1), indicating that at least two enzymes are involved in the reaction. Only the high affinity enzyme(s) is inhibited by 50 μM anhydroerythromycin, i.e. the cytochrome P450 3A induced by dexamethasone is not inhibited. In contrast, previous studies using macrolides as inducers have suggested that macrolide induced cytochrome P450 3A is inhibited by the formation of a nitrosoalkane complex [16, 18].

In microsomes from control rats only one cytochrome P450 3A protein, cytochrome P450 3A2, has been identified. The Eadie–Hofstee data (Fig. 1) suggest either two constitutive rat 3A proteins or the involvement of another cytochrome P450 in androstenedione 6 β-hydroxylation. Both of these cytochromes P450 were inhibited by 50 μM anhydroerythromycin.

The data shown in both Eadie–Hofstee plots, from control and from dexamethasone treated microsomes, show little inhibition with 50 μM erythromycin as compared to 50 μM anhydroerythromycin.

The Eadie–Hofstee plots for human liver microsomes are "atypical". Substrate activation has been suggested previously for the cytochrome P450 3A family in a study [47] which measured progesterone 6 β-hydroxylation in human microsomes and in which a curvilinear double-reciprocal plot was obtained. Our data also suggest substrate activation, however, the anhydroerythromycin is also able to bind to and inhibit the cytochrome suggesting a distinct effector site for the activator steroid.

In conclusion, these data show that anhydroerythromycin is a more potent inhibitor of cytochrome P450 3A associated metabolic activity than is erythromycin in both rat and human liver microsomes. Potentially, this is of clinical significance, as variable blood levels of anhydroerythromycin may explain inconsistencies in the literature on the interactions of erythromycin with other drugs.

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