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INACTIVATION OF HUMAN LIVER CYTOCHROME P-450 BY THE DRUG METHOXSALEN AND OTHER PSORALEN DERIVATIVES

MARINA TINEL,* JACQUES BELGHITI,† VERONIQUE DESCATOIRE,* GILLES AMOUYAL,* PHILIPPE LETTERON,* JEAN GENEVE,* DOMINIQUE LARREY* and DOMINIQUE PESSAYRE*‡

*Unité de Recherches de Physiopathologie Hépatique (INSERM U 24), and †Clinique Chirurgicale, Hôpital Beaujon, 92118 Clichy, France

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Abstract—The effects of psoralen derivatives on cytochrome P-450 have been studied in human liver microsomes. CO-binding cytochrome P-450 was decreased by 33% after 10 min of incubation with 1.5 mM EDTA, an NADPH-regenerating system and 20 μM methoxsalen (8-methoxypsoralen). No destruction of cytochrome P-450 was observed when either NADPH or methoxsalen was omitted. A similar (27%) decrease in CO-binding required a 100-times higher concentration of allylisopropylacetamide (2 mM). The activities of 7-ethoxycoumarin deethylase and benzo(*a*)pyrene hydroxylase were decreased by about 50% in the presence of 12.5 μM methoxsalen. At this low concentration, neither cimetidine nor SKF 525-A or piperonyl butoxide had any significant inhibitory effect. Monooxygenase activities were also decreased in the presence of 12.5 μM bergapten (5-methoxypsoralen) or 12.5 μM psoralen, but not with 12.5 μM trioxsalen (trimethylpsoralen). CO-binding cytochrome P-450 was not decreased after 10 min of incubation with 1.5 mM EDTA, an NADPH-regenerating system and 20 μM trioxsalen. We conclude that methoxsalen is an extremely potent suicide inhibitor of cytochrome P-450 in human liver microsomes. Bergapten and psoralen are also inhibitory whereas trioxsalen has little effects. In the latter derivative, a methyl group is attached on the furan ring and may hinder its metabolic activation and the inactivation of cytochrome P-450.

Psoralens are a family of furocoumarin derivatives present in several plants, including some edible ones such as figs, celery, parsley or parsnip [1–4]. Several psoralen derivatives, including methoxsalen (8-methoxypsoralen), psoralen, bergapten (5-methoxypsoralen) and trioxsalen (trimethylpsoralen) (Fig. 1), have potent photosensitizing properties [2], which are used in the treatment of vitiligo and psoriasis, two frequent skin diseases which may affect 1 and 2%, respectively of the world's population [2, 5]. In the so-called "PUVA-therapy" of psoriasis, the psoralen derivative is given orally, followed by exposure to long wavelength ultraviolet light [6]; this PUVA-therapy is highly effective [6].

We recently reported that methoxsalen is activated by rat liver cytochrome P-450 into reactive metabolites which covalently bind to microsomal proteins and inactivate cytochrome P-450 [7]. As a consequence of both competitive inhibition by methoxsalen itself, and suicide inactivation by methoxsalen metabolites, the drug is a potent inhibitor of rat liver cytochrome P-450 [7]. Its potency was usually greater than that of the potent experimental inhibitors SKF 525-A or piperonyl butoxide, and was much greater than that of the drug cimetidine [7].

Cytochrome P-450 isozymes present in humans are not exactly the same as those present in rats [8]. It was therefore of interest to see whether similar effects also occur in human liver microsomes. We report herein the effects of methoxsalen and other psoralen derivatives on human liver cytochrome P-450.

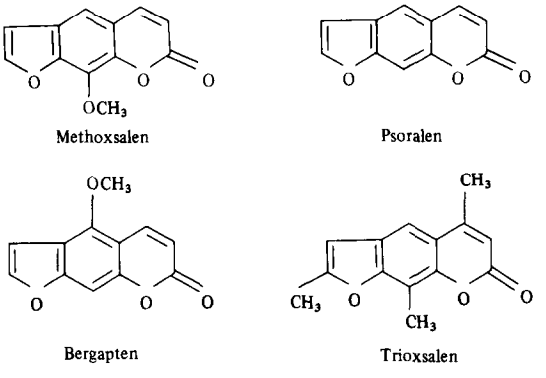

Fig. 1. Chemical structure of methoxsalen (8-methoxypsoralen), psoralen, bergapten (5-methoxypsoralen) and trioxsalen (trimethylpsoralen).

MATERIALS AND METHODS

Materials. Methoxsalen and psoralen were kindly given by Promedica Laboratories (Levallois-Perret, France). Bergapten was a gift from Goupil Laboratories (Cachan, France). Trioxsalen was purchased from Sigma Chemical Co. (St Louis, MO). Allylisopropylacetamide was a gift from Hoffman-LaRoche (Nutley, NJ).

Human liver microsomes. The overall approach was similar to that used in two previous studies on the effects of troleandomycin [9] and erythromycin [10] on human liver cytochrome P-450. A liver specimen removed by surgical biopsy or during an hepatic

‡ To whom correspondence should be addressed at: INSERM U 24, Hôpital Beaujon, 92118 Clichy, France.

tumor resection was obtained in 15 patients undergoing elective abdominal surgery for various reasons (digestive carcinomas, gall-bladder stones, liver abscess, hepatic tumors) and in whom a histologic examination of the liver was medically required. We excluded alcoholic patients or those who had taken drugs known to induce microsomal enzymes [11] during the two weeks preceding surgery; patients taking other drugs were not excluded, inasmuch as drugs had to be taken for premedication and anesthesia anyway. Patients were premedicated with alimemazine, atropine and hydroxyzine and were anesthetized with droperidol, enflurane, fentanyl, thiopental sodium, and pancuronium bromide.

Part of the liver specimen was placed in Bouin's fluid, and sent to the pathologist. Another fragment of the surgical liver biopsy, or parts of the removed normal liver during tumor resection, were stored at −20° until the conclusion of the pathologist was available. Only those liver fragments with a normal liver histology were used in the present study.

The liver fragment was thawed, blotted dry, weighed and homogenized in 3 vol. of ice-cold 0.154 M KCl, 0.01 M sodium–potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged at 100,000 *g* for 60 min. Microsomal pellets were stored at −20° until analyzed, 1–60 days later (usually, 1 day; in a few cases, 2–6 days; once, 40 days and once, 60 days).

Rat liver microsomes. For comparison, a few studies were also made with rat liver microsomes. Male Sprague–Dawley rats [Crl:CD (SD)BR] were purchased from Charles River France (Saint-Aubin-lès-Elbeuf, France). Animals were fed *ad libitum* with a normal standard diet (Autoclavé 113, UAR, France). Rats were killed when they weighed 180 to 200 g, and hepatic microsomes were prepared as described above.

In vitro *destruction of cytochrome P-450.* The *in vitro* destruction of cytochrome P-450 was determined as previously reported [7, 12]. Hepatic microsomes from 125 mg of liver were incubated in 1 ml of 0.6 mM KCl, 0.025 mM sodium–potassium phosphate buffer, pH 7.4 containing EDTA (1.5 mM), methoxsalen or trioxsalen (20 or 200 μM), and the following NADPH-regenerating system: NADPH (0.4 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (3 enzyme units·ml^{-1}) and $MgCl_2$ (6 mM). (Microsomal protein in the assay was 5.4 ± 1.8 mg·ml^{-1} (mean ± SD) with human liver microsomes and 4.4 ± 0.7 with rat liver microsomes.) In some flasks, the psoralen derivative was omitted. In other flasks, the NADPH-regenerating system was omitted. Half of the flasks were kept in ice throughout and served as zero-time samples. The other flasks were incubated at 37° for 10 min, and then placed in ice again. After adding 1 ml of buffer, the microsomal suspension was divided into two cuvettes, and cytochrome P-450 was then determined as described by Omura and Sato [13]. Similar incubations were made with allylisopropylacetamide (20, 200, or 2000 μM). Microsomal protein was determined by the technique of Lowry *et al.* [14].

Monooxygenase activities. Monooxygenase activities were assayed by incubating the substrates (0.25 mM) with hepatic microsomes and the NADPH-regenerating system described above, at 37° for 10 min. Benzo(*a*)pyrene hydroxylase activity was determined by the technique of Kuntzman *et al.* [15]. 7-Ethoxycoumarin deethylase activity was assayed according to Greenlee and Poland [16].

RESULTS

Effects of methoxsalen on CO-*binding cytochrome P-450*

CO-binding cytochrome P-450 was not modified when human liver microsomes were incubated at 37° for 10 min with 1.5 mM EDTA and either methoxsalen (200 μM) alone or an NADPH-regenerating system alone (Fig. 2). However, when human liver microsomes were incubated for 10 min with 1.5 mM EDTA and both an NADPH-regenerating system and methoxsalen (either 20 or 200 μM), there was a marked decrease (either 33 or 50%, respectively) in CO-binding cytochrome P-450 (Fig. 2). The effects of methoxsalen were much less with rat liver microsomes: no decrease was observed with 20 μM methoxsalen and the decrease seen with 200 μM methoxsalen (9%) was not statistically significant (Fig. 2).

For comparison, we studied the effects of allylisopropylacetamide, a prototype of a drug leading to the suicide inactivation of cytochrome P-450. No destruction of cytochrome P-450 was observed after incubation of microsomes from one human liver with 1.5 mM EDTA, an NADPH-regenerating system and either 20 or 200 μM allylisopropylacetamide for 10 min (not shown). Even with a 10-times higher concentration of this suicide substrate (2 mM), there was only a 27%-decrease in CO-binding cytochrome P-450 after 10 min of incubation with human liver microsomes, 1.5 mM EDTA and an NADPH-regenerating system (Fig. 3).

Effects of methoxsalen on monooxygenase activities

The 7-ethoxycoumarin deethylase activity and the benzo(*a*)pyrene hydroxylase activity of human liver microsomes were determined in the presence of various concentrations of methoxsalen (6.25–50 μM); half-inhibition of these activities was observed at 12.5 μM methoxsalen (Fig. 4).

In other experiments, we compared the effect of methoxsalen (12.5 μM) with those of other cytochrome P-450 inhibitors (12.5 μM); at this low concentration, neither cimetidine, nor SKF 525-A or piperonyl butoxide had any significant effect on the 7-ethoxycoumarin deethylase and the benzo(*a*)-pyrene hydroxylase activity of human liver microsomes (Fig. 5).

Effects of other psoralen derivatives

The 7-ethoxycoumarin deethylase activity or the benzo(*a*)pyrene hydroxylase activity of human liver microsomes were also inhibited in the presence of 12.5 μM psoralen or 12.5 μM bergapten (5-methoxypsoralen), but not in the presence of trioxsalen (Fig. 6).

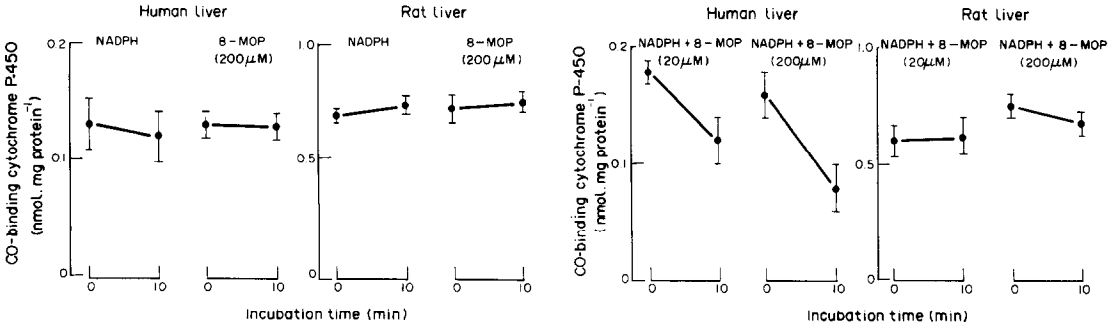

Fig. 2. Destruction of cytochrome P-450 by methoxsalen metabolites. Human liver or rat liver microsomes were incubated at 37° for 10 min with 1.5 mM EDTA, an NADPH-regenerating system and methoxsalen ("8-MOP"). In some flasks either methoxsalen or the NADPH-regenerating system were omitted. Other flasks were kept in ice throughout and served as zero-time samples. CO-binding cytochrome P-450 in the incubated and zero-time samples was determined as the CO-difference spectrum of dithionite-reduced microsomes [13]. Results are means ± SEM for 3–5 human livers. The asterisks indicate significant differences from values in the zero-time sample (Student's *t*-test for dependent data), $P < 0.05$.

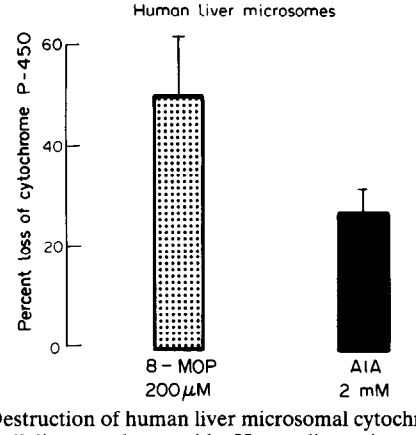

Fig. 3. Destruction of human liver microsomal cytochrome P-450 by allylisopropylacetamide. Human liver microsomes were incubated at 37° for 10 min with 1.5 mM EDTA, an NADPH-regencrating system and 2 mM allylisopropylacetamide ("AIA"); some flasks were kept in ice throughout and served as zero-time samples. Cytochrome P-450 in the incubated and zero-time samples was then measured as the CO-difference spectrum of dithionite-reduced microsomes. Results are means ± SEM for 7 determinations. For comparison, the percent decrease observed after 10 min of incubation with 200 μM methoxsalen ("8-MOP") is also reported (calculated from data shown in Fig. 2).

CO-binding cytochrome P-450 was not decreased when human liver microsomes were incubated for 10 min with 1.5 mM EDTA, an NADPH-regenerating system and 20 μM trioxsalen (not shown). Even with 200 μM trioxsalen, the decrease in CO-binding cytochrome P-450 (21%) was not statistically significant (not shown). No decrease in CO-binding cytochrome P-450 was observed when rat liver microsomes were incubated for 10 min with 1.5 mM EDTA, trioxsalen (20 or 200 μM) and an NADPH-regenerating system (not shown).

DISCUSSION

Methoxsalen

We have reported recently that methoxsalen is activated by rat liver cytochrome P-450 into chemically reactive metabolites that covalently bind to microsomal proteins and inactivate cytochrome P-450 [7]. The results of the present investigation show that methoxsalen produces similar effects in human liver microsomes (Figs 2 and 4). Indeed, methoxsalen decreased CO-binding cytochrome P-450, in the presence of an NADPH-regenerating system (Fig. 2), and was a potent inhibitor of monooxygenase activities, producing half-inhibition at concentrations of about 12.5 μM only (Fig. 4). Methoxsalen undergoes very little *O*-demethylation in rats, dogs and humans [7, 17, 18]. In contrast, it is extensively oxidized on its furan ring, with the presumed formation of a reactive epoxide [17, 18]. Indeed, we have found in rats that 0.1 mM 1,1,1-trichloropropene 2,3-oxide, a potent inhibitor of microsomal epoxide hydrolase [19], doubled the *in vitro* covalent binding of a reactive methoxsalen metabolite to microsomal proteins [7]. By analogy with what is currently proposed in the case of other olefinic or arene compounds [20–22], we have suggested that methoxsalen may be activated, on the outer double bond of its furan ring, to form first an extremely unstable radicaloid species [7]. This radicaloid species may covalently bind to the active site of cytochrome P-450 or may degrade to the epoxide and other reactive metabolites that may be conjugated with glutathione or may covalently bind to other protein sites in microsomes and elswhere [7].

Interestingly, the per cent loss of CO-binding observed after incubation with methoxsalen (20 or 200 μM) and an NADPH-regenerating system was much greater in human liver microsomes than in rat liver microsomes (Fig. 2), suggesting that some human cytochrome P-450 isozymes may be uniquely sensitive to the destructive effects of methoxsalen. Indeed, still unpublished experiments in rats suggest that although many different isozymes are destroyed to some extent, nevertheless some of them (including those induced by glucocorticoids) may be more effectively affected.

The striking potency of methoxsalen in human liver microsomes is also apparent by comparison with other drugs (Figs 3 and 5). Allylisopropylacetamide is a prototype of a drug leading to the formation of N-alkylated porphyrins, and to the suicide inac-

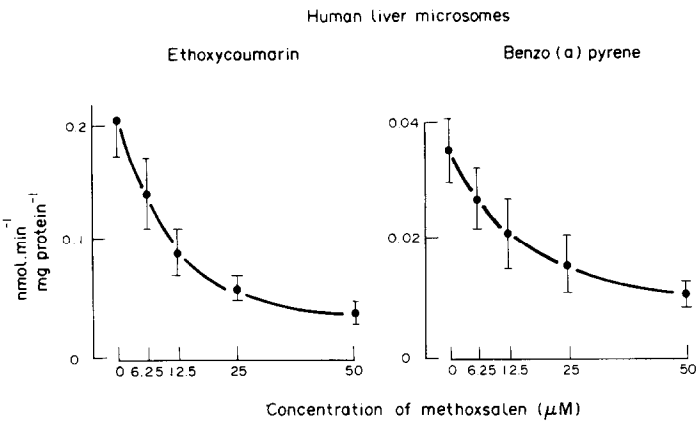

Fig. 4. Inhibitory effects of methoxsalen on the 7-ethoxycoumarin deethylase and benzo(*a*)pyrene hydroxylase activity of human liver microsomes. 7-Ethoxycoumarin (0.25 mM) or benzo(*a*)pyrene (0.25 mM) were incubated at 37° for 10 min with an NADPH-regenerating system and human liver microsomes in the presence of various concentrations of methoxsalen. Results are means ± SEM for 3 human livers.

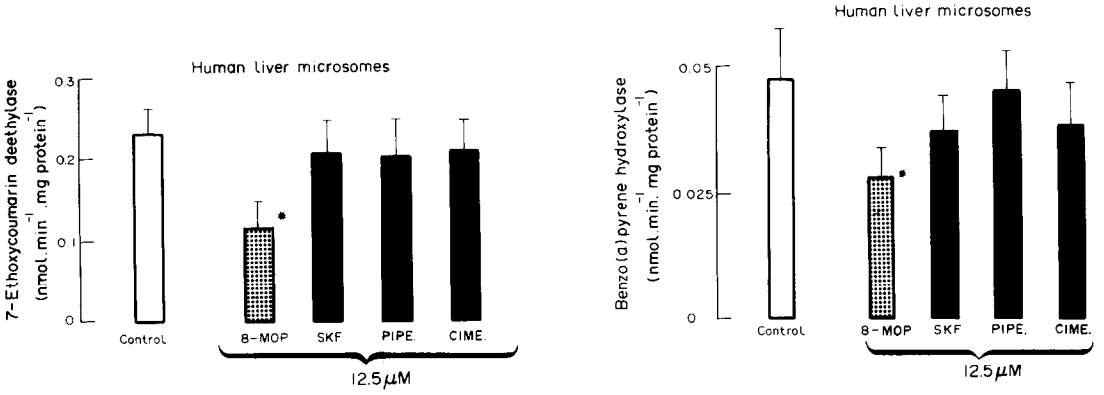

Fig. 5. Comparison of the effects of methoxsalen with those of other, unrelated, cytochrome P-450 inhibitors. 7-Ethoxycoumarin (0.25 mM) or benzo(*a*)pyrene (0.25 mM) were incubated at 37° for 10 min with an NADPH-regenerating system and human liver microsomes, in the presence or absence ("Control") of various inhibitors of cytochrome P-450 (12.5 μM). The inhibitors were: methoxsalen ("8-MOP"), SKF 525-A ("SKF"), piperonyl butoxide ("PIPE") or cimetidine ("CIME"). Results are means ± SEM for 5 human livers. The asterisks indicate significant differences from control values (Student's *t*-test for dependent data), $P < 0.05$.

tivation of cytochrome P-450 [23]. However, reproduction of the 30% decrease in CO-binding observed with 20 μM methoxsalen (Fig. 2) required a 100-times higher concentration (2 mM) of allylisopropylacetamide (Fig. 3). SKF 525-A [24] and piperonyl butoxide [25] are transformed into metabolites forming inactive complexes with the iron of cytochrome P-450. Both are considered as being potent inhibitors of cytochrome P-450. Nevertheless, SKF 525-A and piperonyl butoxide had no inhibitory effect when used at a concentration of 12.5 μM (Fig. 5), although methoxsalen at the same concentration produced half-inhibition of monooxygenase activities (Figs 4, 5). The doses of methoxsalen used in the treatment of psoriasis are rather low ($0.6\ mg \cdot kg^{-1}$), leading to serum concentrations of about 1 μM [26]. Judging from the high volume of distribution ($3\ l \cdot kg^{-1}$), concentrations in several tissues must be higher, however [26]. Furthermore, because of its irreversible nature, a slow inactivation of cytochrome P-450 may, with time, become quite noticeable. Indeed, administration of methoxsalen has been shown to decrease the clearance of antipyrine [27] and the metabolic activation of acetaminophen [28] in humans. Ingestion of psoralen derivatives from food has not been accurately estimated, although it is suggested that a maximal level of 1 mg per 24 hr may be ingested in the summer [2]. Whether this low intake has any consequence on drug metabolism remains unknown at that time.

Other psoralen derivatives

The present results also extend to humans (Fig. 6) previous findings in rats [12] showing that bergapten (5-methoxypsoralen) and psoralen are likewise potent suicide inhibitors of cytochrome P-450. In the chemical structure of 5-methoxypsoralen and psoralen, the furan ring is identical to that of methoxsalen (Fig. 1), and may be similarly activated [12].

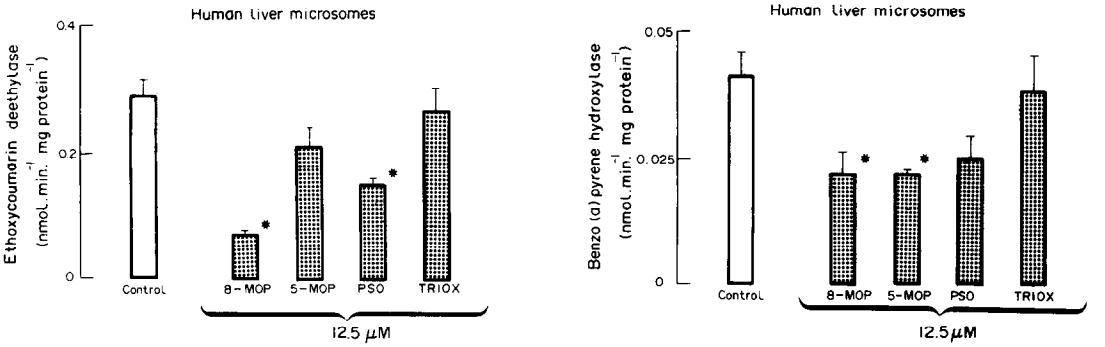

Fig. 6. Comparison of the inhibitory effects of methoxsalen with those of other psoralen derivatives. 7-Ethoxycoumarin (0.25 mM) or benzo(*a*)pyrene (0.25 mM) were incubated at 37° for 10 min with an NADPH-regenerating system and human liver microsomes, in the presence or absence ("Control") of various psoralen derivatives (12.5 μM). The derivatives were: methoxsalen ("8-MOP"), bergapten ("5-MOP"), psoralen ("PSO"), and trioxsalen ("TRIOX"). Results are means ± SEM for 3 human livers. The asterisks indicate significant differences from control values (Student's *t*-test for dependent data), $P < 0.05$.

Contrasting with the marked effects of methoxsalen, bergapten and psoralen, those of trioxsalen appeared very trivial, indeed (Fig. 6). Similar observations have been made in rats [12]. In the chemical structure of trioxsalen, a methyl group is attached on the furan ring (Fig. 1). The metabolism of trioxsalen has been studied in mouse liver microsomes [29]. There was no evidence for ring hydroxylation [29]. In contrast, trioxsalen was extensively oxidized on the methyl groups, particularly that attached on the furan ring [29]. Conceivably, this methyl group may sterically hinder the oxidation of the furan ring [12] and is, indeed, oxidized in its stead [29].

We conclude that methoxsalen destroys CO-binding cytochrome P-450, and markedly decreases monooxygenase activities in human liver microsomes. Bergapten and psoralen also decrease monooxygenase activities in human liver microsomes, whereas trioxsalen does not. In the chemical structure of this derivative, a methyl group is attached on the furan ring and may hinder its metabolic activation and the inactivation of cytochrome P-450.

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