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A POSSIBLE MECHANISM OF THE IMPAIRMENT OF HEPATIC MICROSOMAL MONOOXYGENASE ACTIVITIES AFTER MULTIPLE ADMINISTRATION OF PROPRANOLOL IN RATS

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Abstract—The mechanism of selective inhibition of propranolol hydroxylations after multiple administration of the drug was investigated by metabolic inhibition studies in rat liver microsomes. The time course of irreversible binding of a reactive metabolic intermediate(s) of propranolol to liver microsomal protein, which was proposed as the cause of the impairment of enzymatic activities, had a delayed phase followed by a rapid linear rise, while the unmetabolized propranolol remaining in the reaction mixture showed a rapid linear decrease immediately after the onset of incubation. Thus, it was conceivable that the reactive intermediate(s) was not always formed directly from the parent drug, propranolol. Among four primary metabolites of propranolol, 4-hydroxypropranolol was the most potent inhibitor of propranolol hydroxylase activities, and this inhibition was much enhanced by preincubation of 4-hydroxypropranolol with NADPH. The type of inhibition kinetics of propranolol 5- and 7-hydroxylase activities by 4-hydroxypropranolol was changed from a competitive type to a non-competitive type by the preincubation. These results suggest that a reactive metabolite(s) of propranolol which impaired propranolol hydroxylase activities is a further metabolite(s) of 4-hydroxypropranolol.

Propranolol is a non-selective β-adrenergic blocking agent used in the treatment of hypertension, angina pectoris, cardiac arrhythmias and other diseases. The metabolism of propranolol has been studied quantitatively in experimental animals and humans [1, 2]. Propranolol is metabolized to a number of products, some of which are pharmacologically active [3, 4]. As shown in Scheme 1, major products for the primary pathways of propranolol metabolism are ring-hydroxylated products, 4-, 5- and 7-hydroxypropranolol (4-OH-P,‡ 5-OH-P and 7-OH-P, respectively) and a side-chain-oxidized product, NDP in rat liver microsomes [5–7].

Previous reports have shown that propranolol is a potent inhibitor of cytochrome P450-mediated drug metabolism in rats and humans [8–10]. Furthermore, repetitive oral administration of propranolol to rats caused a marked decrease of hepatic microsomal propranolol 4-, 5- and 7-hydroxylase activities [7, 10]. Our previous study suggested that the impairment of the monooxygenase activity by propranolol pretreatment is selective for debrisoquine 4-hydroxylase [7].

Irreversible binding of a metabolic intermediate(s) of propranolol with liver microsomal protein was proposed as the mechanism for the impairment of monooxygenase activities following chronic administration of propranolol [10–12]. Because the irreversible binding required both an NADPH-generating system and oxygen, and was inhibited by SKF-525A, a classical inhibitor of cytochrome P450, it was suggested that propranolol was converted to a chemically reactive metabolic intermediate(s) which bound irreversibly to rat liver microsomal protein by cytochrome P450-dependent monooxygenation [10]. In addition, the correlation between the extent of irreversible binding and that of the impairment of lidocaine 3-hydroxylase activity after the pretreatment of labelled propranolol provided substantial evidence for a role of the irreversibly bound metabolic intermediate(s) of propranolol in the impairment of lidocaine metabolism [12]. But limited information on the mechanism of activation of propranolol has been provided [10–12]. In the present study, we report results on the inhibition of propranolol metabolism by primary propranolol metabolites that binds irreversibly to microsomal protein and thereby impair propranolol hydroxylase activities.

MATERIALS AND METHODS

Chemicals. Propranolol hydrochloride was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]Propranolol hydrochloride labelled at the 4-position (sp. act. 21 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). The radiochemical purity of [^{3}H]propranolol was stated to be at least 97.0% as determined by HPLC on a MCP C18 protein column using a gradient of

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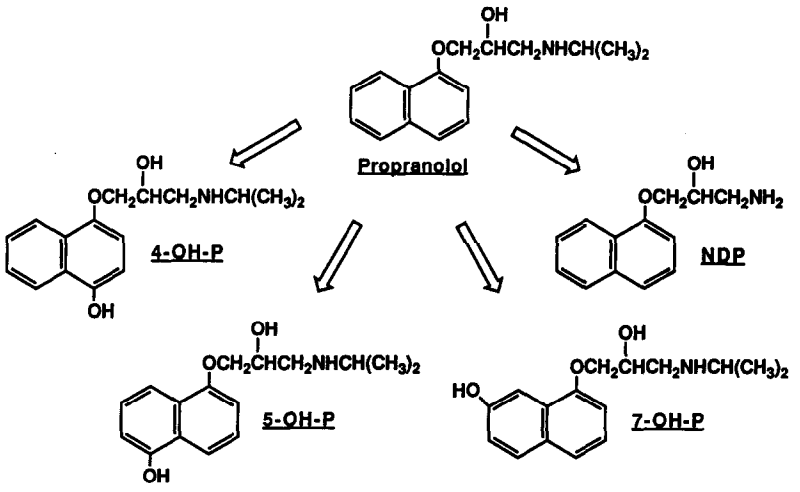
‡ Abbreviations: 4-OH-P, 5-OH-P, 7-OH-P, 4-, 5- and 7-hydroxypropranolol; NDP, *N*-desisopropylpropranolol; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase.



Scheme 1. Major products for the primary pathways of propranolol metabolism in rat liver microsomes.

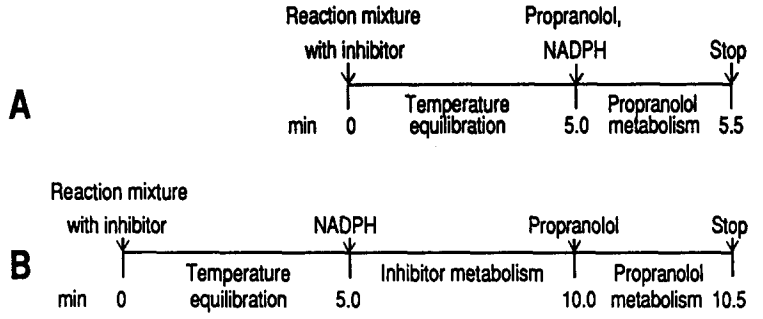
0.01 M trifluoroacetic acid to 0.01 M trifluoroacetic acid-acetonitrile (25:5). 4-OH-P and NDP were provided by ICI Pharmaceuticals Co. (Macclesfield, U.K.). 5-OH-P and 7-OH-P were synthesized according to the method of Oatis *et al.* [3]. 4-Hydroxybunitrolol was supplied by Nippon C.H. Boehringer Sohn Co., Ltd (Osaka, Japan). G-6-P, G-6-PDH and NADPH were purchased from the Oriental Yeast Co., Ltd (Tokyo, Japan). All other chemicals and solvents were of analytical grade.

Preparation of hepatic microsomes. Male Wistar rats weighing 250–300 g were obtained from Takasugi Experimental Animals (Kasukabe, Japan). The rats were killed by decapitation, and hepatic microsomal fractions were prepared according to the method of Omura and Sato [13]. Protein concentrations were determined by the method of Lowry *et al.* [14].

Incubation of labelled or non-labelled propranolol with hepatic microsomes. To examine time courses of propranolol metabolism and the irreversible binding of a reactive propranolol metabolite(s) to hepatic microsomal protein, a 1-mL incubation mixture containing 10 mM G-6-P, 2 units G-6-PDH, 0.5 mM NADPH, 8 mM $MgCl_2$, 1 mg microsomal protein and 2 μM unlabelled propranolol or 2 μM (0.2 μCi) [^{3}H]propranolol in 0.15 M potassium phosphate buffer (pH 7.4) was used. After 5 min preincubation under air at 37°, reaction was started by the addition of propranolol and NADPH. The incubation was performed for several time periods from 0 to 10 min. The reaction was stopped by the addition of 1 mL of 10% trichloroacetic acid, when the irreversible binding between a labelled reactive metabolic intermediate(s) and rat liver microsomal protein was measured. On the other hand, it was stopped by the addition of 1 mL of 1 N NaOH including sodium bisulfite (25 mg/mL) as an antioxidant to the reaction mixture for avoiding degradation of 4-OH-P, when the concentration of propranolol and 4-OH-P was measured.

In the case of the determination of inhibition of propranolol hydroxylase activities by primary metabolites of propranolol, the incubation conditions at a propranolol concentration of 2 μM described above were used, except for the use of 0.5 mg microsomal protein and 30 sec incubation of propranolol to determine the initial velocities of propranolol 4-, 5- and 7-hydroxylation. Five minutes preincubation prior to 30 sec incubation of propranolol was employed in the absence and the presence of NADPH with an inhibitor of 2 μM shown in A and B of Scheme 2, respectively. The reaction was stopped by the addition of 1 mL of 1 N NaOH including sodium bisulfite (25 mg/mL). The inhibition kinetics of propranolol 5- and 7-hydroxylase activities by 4-OH-P was determined using propranolol concentrations of 0.5 ~ 2 μM and 4-OH-P concentrations of 0 ~ 20 μM.

Determination of irreversible binding of a labelled material(s) to hepatic microsomes. Irreversible binding of a labelled material(s) after incubation of [^{3}H]propranolol with hepatic microsomes was measured as described by Nakagawa *et al.* [15]. After the termination of the incubation by the addition of 1 mL of 10% trichloroacetic acid, 4 mL of the same solvent was added to the reaction mixture. The resultant precipitate was collected by centrifugation (2000 *g*, 10 min) and was resuspended in 4 mL of 7.5% trichloroacetic acid. After centrifugation, the washed pellet was extracted with 4 mL of 80% methanol (twice), 80% hot methanol (twice), methanol–ether (1:1, v/v, twice) and 80% methanol (twice). After the last extraction, no further radioactivity could be removed from the pellet. The thoroughly extracted precipitate thus obtained was dissolved in 1 N NaOH, and this was mixed with 10 mL of scintillation medium for the determination of radioactivity of a material(s) which bound irreversibly to microsomal protein. The radioactivity in these samples was measured by a liquid scintillation counter (Beckman LS-1800). The scintillation medium used consisted of one volume of Triton X-100 and two volumes of toluene phosphor including 4 g of 2,5-diphenyloxazole and 100 mg of



Scheme 2. Protocols of the incubation of propranolol and an inhibitor after preincubation in the absence (A) and presence (B) of NADPH with an inhibitor.

1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per 1000 mL of toluene.

Measurement of propranolol and its metabolites. Propranolol, 4-OH-P, 5-OH-P and 7-OH-P and NDP in the incubation mixture were assayed by the HPLC method described previously [7]. After the termination of the reaction by 1 N NaOH, 4-hydroxybunitrolol was added as internal standard. Propranolol and its metabolites were extracted with ethyl acetate, and then the organic phase was evaporated to dryness and the residue was dissolved in HPLC mobile phase (CH_3CN:CH_3OH:H_2O:CH_3COOH = 22:22:56:1). The sample was applied to a reversed-phase column (Inertsil ODS, Gasukuro Kogyo Ltd, Tokyo). The fluorescent intensity of propranolol and its metabolites was continuously monitored with an excitation wavelength at 310 nm and an emission wavelength at 380 nm.

RESULTS

Time courses of in vitro *metabolism of propranolol and the irreversible binding of a reactive propranolol metabolite(s) to hepatic microsomal protein*

Incubation of [^{3}H]propranolol with rat liver microsomes and an NADPH-generating system resulted in irreversible binding of a ^{3}H-labelled material(s) to microsomal protein as reported previously [10]. Interestingly, in the time course of this binding, a lag phase before a linear increase of the formation of the irreversible binding material(s) was observed (Fig. 1). The binding reached a plateau 7 min after the onset of incubation. But unmetabolized propranolol remaining in the reaction mixture showed a rapid linear decrease up to 3 min after the onset of incubation as shown in Fig. 1. The unmetabolized propranolol concentration was close to the detection limit of 3 pmol/mL 5 min after the onset of incubation (Fig. 1). The amount of a major primary metabolite of propranolol, 4-OH-P, increased with time and exhibited a maximum concentration 2 min after the onset of incubation, and then decreased to negligible levels at 7 min (Fig. 1). The binding of the labelled material(s) to microsomal protein was less than the half maximum even when approximately 90% of propranolol disappeared from the reaction mixture at a 3-min time point (Fig. 1). The binding kept a rapid linear rise even after this point.

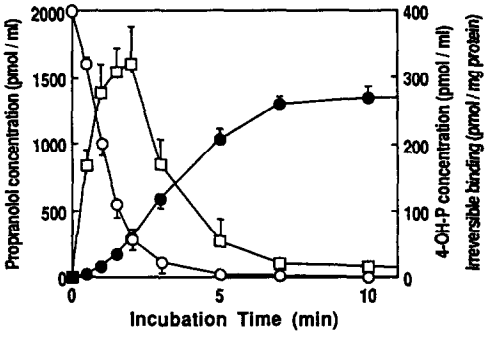


Fig. 1. Time courses for the disappearance of propranolol (○), the formation and disappearance of 4-OH-P (□) and the irreversible binding of [^{3}H]propranolol metabolites (●) to rat liver microsomal protein. Unlabelled propranolol (2 μM) or [^{3}H]propranolol (2 μM, 0.2 μCi/mL) was incubated with rat liver microsomes (1 mg protein/mL) and an NADPH-generating system as described in Materials and Methods. Data are means ± SE for three determinations using different microsomes obtained from three rats.

Inhibition of propranolol hydroxylase activities by primary metabolites of propranolol and the effect of preincubation of microsomes with inhibitors and NADPH

Table 1 shows inhibitory effects (% of control activity) of 4-OH-P, 5-OH-P, 7-OH-P and NDP on propranolol hydroxylase activities in rat liver microsomes. All the metabolites tested had some inhibition potency on propranolol hydroxylase activity, and inhibitory effects of 4-OH-P were more potent than those of the other inhibitors. In addition, as shown in Table 1, the inhibition of propranolol 5- and 7-hydroxylation by 4-OH-P was enhanced by 5-min preincubation of microsomes with 4-OH-P and NADPH, but the inhibition by the other metabolites was not affected by preincubation.

Kinetics of the inhibition of propranolol hydroxylase activities by 4-OH-P and the effect of preincubation on the inhibition

Figure 2A shows the Lineweaver–Burk plots of

Table 1. Inhibition of propranolol 4-, 5- and 7-hydroxylase activities in rat liver microsomes by primary metabolites of propranolol

Inhibitor		Activity (% of control) 4-Hydroxylation	5-Hydroxylation	7-Hydroxylation
4-OH-P	(A)*	ND	74.5 ± 2.0†	75.4 ± 2.5†
	(B)	ND	34.9 ± 4.0‡	37.2 ±7.4‡
5-OH-P	(A)	81.8 ± 1.1	ND	85.5 ± 1.9
	(B)	88.9 ± 4.6	ND	87.3 ± 2.7
7-OH-P	(A)	95.5 ± 1.9	92.5 ± 2.3	ND
	(B)	96.4 ± 1.1	95.1 ± 1.6	ND
NDP	(A)	93.3 ± 2.2	95.3 ± 3.5	93.2 ± 2.1
	(B)	91.8 ± 3.1	96.2 ± 0.6	95.2 ± 1.6

Results are expressed as per cent of the activity in the case of no inhibitor. Values are means ± SE of three determinations using different microsomes obtained from three rats.

* Incubations were carried out with 0.5 mg microsomal protein/mL for 30 sec at propranolol and inhibitor concentrations of 2 μM. (A) and (B) show that inhibition experiments were performed by the procedures described in (A) and (B) of Scheme 2, respectively. The other incubation conditions were described in Materials and Methods.

† Significant difference between addition of inhibitor and control values ($P < 0.05$) by Student's *t*-test.

‡ Significant difference between addition of inhibitor and control values ($P < 0.01$) by Student's *t*-test.

ND, not determined.

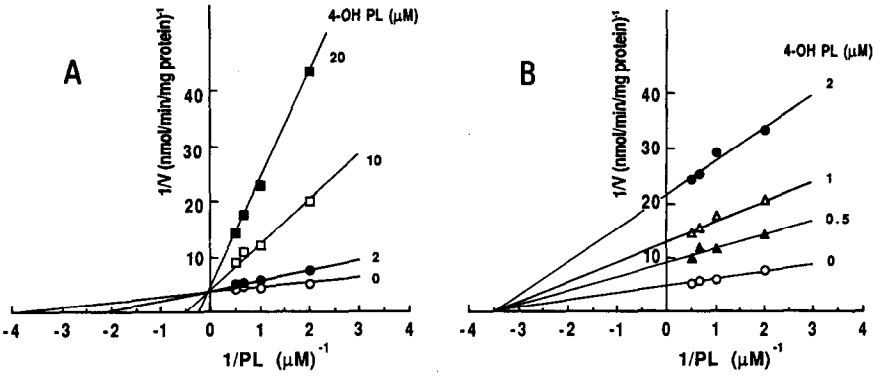


Fig. 2. Lineweaver–Burk plot of propranolol 7-hydroxylase activity in the presence of the inhibitor, 4-OH-P, in rat liver microsomes. Incubations were performed at propranolol concentrations of 0.5 ~ 2 μM, and at 4-OH-P concentrations of 0 ~ 20 μM (A) and 0 ~ 2 μM (B). Other preincubation and incubation conditions in (A) and (B) are shown in the footnote of Table 1. The inhibitor constant (K_i) for competitive inhibition is 0.677 μM in (A). Illustrations in (A) and (B) show typical results for two and three rats, respectively.

the rates of 7-OH-P formation from propranolol in the presence of various concentrations of 4-OH-P. Clear competitive inhibition was observed. On the other hand, in the experiments (B in Scheme 2) shown in Fig. 2B, 4-OH-P was preincubated with microsomes in the presence of NADPH for 5 min to allow metabolism of 4-OH-P to take place. The inhibition of propranolol 7-hydroxylation thus obtained was observed even if concentrations of 4-OH-P lower than those used in the experiments (A in Scheme 2) shown in Fig. 2A were added. The type of inhibition kinetics was apparently non-competitive, and a marked decrease in V_{max} was observed (Fig. 2B). A similar competitive inhibition by 4-OH-P (Fig. 3A) and a marked decrease in V_{max} at a constant K_m by preincubation of microsomes with 4-OH-P and NADPH were obtained in the inhibition of propranolol 5-hydroxylation (Fig. 3B).

DISCUSSION

Irreversible binding of radioactivity to hepatic microsomal protein after incubation of labelled propranolol was observed in rat [10, 12] and human [11] liver microsomes, and the binding in hepatic microsomes after chronic oral administration of labelled propranolol was also observed in rats [10, 12]. It was proposed that the irreversible binding

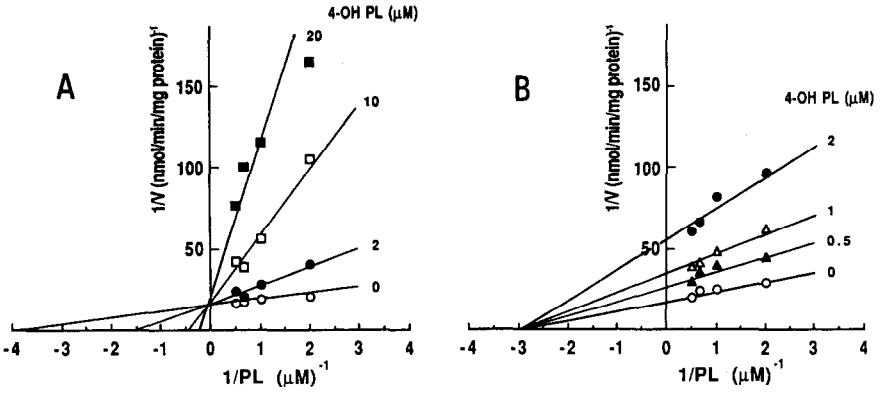


Fig. 3. Lineweaver–Burk plot of propranolol 5-hydroxylase activity in the presence of the inhibitor, 4-OH-P, in rat liver microsomes. The preincubation and incubation conditions are shown in the footnote of Fig. 2. The inhibitor constant (K_i) for competitive inhibition is 0.657 μM in (A). Illustrations in (A) and (B) show typical results for two and three rats respectively.

of a reactive metabolic intermediate(s) formed from propranolol metabolism played a major role in the selective impairment of hydroxylase activities in propranolol metabolism [10, 12]. In the present study, the extent of *in vitro* irreversible binding of propranolol to rat liver microsomal protein was almost equal to that shown by Schneck and Pritchard [10].

For a short time after the onset of incubation, the formation of irreversible binding showed a delayed phase followed by a rapid linear rise, while the disappearance of propranolol from the incubation mixture was rapid and linear with respect to incubation time. Furthermore, the irreversible binding continued to increase linearly with respect to time, even after the propranolol concentration reached a very low level in the reaction mixture (Fig. 1). It seems likely that the linear increase in the binding relates to the disappearance of 4-OH-P rather than propranolol. These phenomena suggest that a reactive metabolic intermediate(s) which binds to microsomal protein is not an intermediate(s) produced directly from propranolol, but is formed during further metabolism of a primary metabolite(s) of propranolol such as 4-OH-P.

The extent of inhibition of propranolol hydroxylase activities by primary propranolol metabolites was determined (Table 1). The effects of 4-OH-P, 5-OH-P and 7-OH-P on propranolol 4-, 5- and 7-hydroxylase activities, respectively, were not determined, because each inhibitor, being a metabolite of propranolol, interfered with the measurement of each metabolite. However, their effects may be estimated from the data of Table 1, because propranolol 4-, 5- and 7-hydroxylation are probably catalysed by a common cytochrome P450 isozyme(s). This reason can be drawn from the fact that propranolol 5- and 7-hydroxylase activities, as well as propranolol 4-hydroxylase activity, were demonstrated to decrease markedly in rat liver microsomes after propranolol pretreatment, and thus the cytochrome P450 isozyme impaired by propranolol pretreatment was suggested to be debrisoquine 4-hydroxylase [7]. The inhibition of propranolol hydroxylase activities by 4-OH-P was more potent than that by any other primary metabolite of propranolol (Table 1). In addition, a marked inhibition by 4-OH-P was observed after preincubation in the presence of NADPH.

The type of inhibition kinetics of propranolol 5- and 7-hydroxylase activities by 4-OH-P was competitive after preincubation in the absence of NADPH (Figs 3A and 2A, respectively). It was observed that 4-OH-P was rapidly eliminated in rat liver microsomes only in the presence of an NADPH-generating system (i.e. 90% elimination of an initial concentration of 2 μM in 5 min). 4-OH-P was reported to be metabolized sequentially to di-hydroxylated metabolites [16, 17]. These findings suggest that oxidation of 4-OH-P is catalysed by the same cytochrome P450 isozyme(s) as propranolol 5- and 7-hydroxylase, at least partially.

On the other hand, the inhibitory effect was much enhanced after 4-OH-P was incubated for 5 min with microsomes in the presence of NADPH to allow further metabolism of 4-OH-P (Table 1), despite the inhibitor (4-OH-P) concentration being very low at the end of the 5-min preincubation, i.e. at the start of the incubation of propranolol in the reaction mixture. The inhibition kinetics of propranolol 5-hydroxylation (Fig. 3B) and 7-hydroxylation (Fig. 2B) were switched from competitive to non-competitive type inhibition and marked decreases in V_{max} were observed. These observations suggest that a metabolic intermediate(s) formed by metabolism of 4-OH-P during preincubation with NADPH probably bound irreversibly to the cytochrome P450 isozyme(s) catalysing propranolol 5- and 7-hydroxylation and impaired them.

No information on the chemical structure of the reactive intermediate(s) formed from propranolol metabolism has been provided. The irreversible binding was reported to be caused by the cytochrome P450-catalysed metabolism of propranolol to a reactive intermediate(s) [10, 11]. Reduction of the binding by reduced gluthathione in rat and

human liver microsomes indicates that the reactive intermediate is electrophilic [10, 11]. Nelson and Powell [18] suggested that the 4-hydroxylation of propranolol proceeded partially via a 3,4-arene oxide intermediate. Since aromatic epoxides are electrophilic, 3,4-arene oxide was suspected to be one of reactive intermediates of propranolol, but the evidence for this postulate was not obtained [11]. Our present observations suggest that the binding species is not a metabolic intermediate of propranolol preceding 4-hydroxylation, but a further metabolite(s) of 4-OH-P.

In summary, kinetic studies on the inhibition of propranolol metabolism by its metabolites in rat liver microsomes have shown that a metabolite(s) of 4-OH-P impaired hepatic microsomal monooxygenase activities by reducing the V_{max} values of propranolol 5- and 7-hydroxylation. This finding suggests that 4-OH-P is a proximate metabolite for a reactive intermediate(s) that inactivates propranolol hydroxylases.

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