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Inhibition of carbonyl reductase activity in pig heart by alkyl phenyl ketones

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Abstract
The inhibitory effects of alkyl phenyl ketones on carbonyl reductase activity were examined in pig heart. In this study, carbonyl reductase activity was estimated as the ability to reduce 4-benzoylpyridine to *S*(−)-α-phenyl-4-pyridylmethanol in the cytosolic fraction from pig heart (pig heart cytosol). The order of their inhibitory potencies was hexanophenone > valerophenone > heptanophenone > butyrophenone > propiophenone. The inhibitory potencies of acetophenone and nonanophenone were much lower. A significant relationship was observed between V_{max}/K_m values for the reduction of alkyl phenyl ketones and their inhibitory potencies for carbonyl reductase activity in pig heart cytosol. Furthermore, hexanophenone was a competitive inhibitor for the enzyme activity. These results indicate that several alkyl phenyl ketones including hexanophenone inhibit carbonyl reductase activity in pig heart cytosol, by acting as substrate inhibitors.

Keywords: *Carbonyl reductase activity, alkyl phenyl ketones, 4-benzoylpyridine, pig heart cytosol, substrate inhibition*

Introduction

A variety of xenobiotic compounds contain a ketone or aldehyde group within their chemical structures. These carbonyl compounds are metabolized to the corresponding alcohols in biological systems. Carbonyl reductase (EC 1.1.1.184) is an enzyme that catalyzes NADPH-dependent reduction of carbonyl compounds [1–5]. Monomeric and tetrameric carbonyl reductases have been purified from the liver, kidney, lung, brain and testis of mammalian species [6–11]. We have recently purified a tetrameric carbonyl reductase from the cytosolic fraction of pig heart [12]. The purified pig heart carbonyl reductase (PHCR) efficiently reduces alkyl phenyl ketones such as hexanophenone and valerophenone, and α-dicarbonyl compounds such as 9,10-phenanthrenequinone and isatin. Interestingly, PHCR also catalyzes the reduction of all-*trans* retinal to all-*trans* retinol, suggesting that it can play a role in retinoid metabolism.

It has been reported that PHCR reduces mainly 4-benzoylpyridine (4-BP) to *S*(−)-α-phenyl-4-pyridylmethanol [*S*(−)-PPOL], as shown in Figure 1 [13]. We have also demonstrated that the enantiomeric excess of *S*(−)-PPOL (89.2% ee) produced in the cytosolic fraction from pig heart (pig heart cytosol) is almost the same as that of *S*(−)-PPOL (87.2% ee) produced in the reaction system of recombinant PHCR [13,14]. Thus, it is reasonable to assume that most of 4-BP reduction in pig heart cytosol is catalyzed by PHCR.

The substrate specificities of carbonyl reductase for various ketone and aldehyde compounds have been demonstrated. However, information on the substrate inhibition of carbonyl reductase by ketone and aldehyde compounds has been very limited. The purpose of the present study was to elucidate a possible mechanism for the substrate inhibition of carbonyl reductase activity in pig heart by a series of alkyl phenyl ketones. The enzyme (carbonyl reductase) activity was

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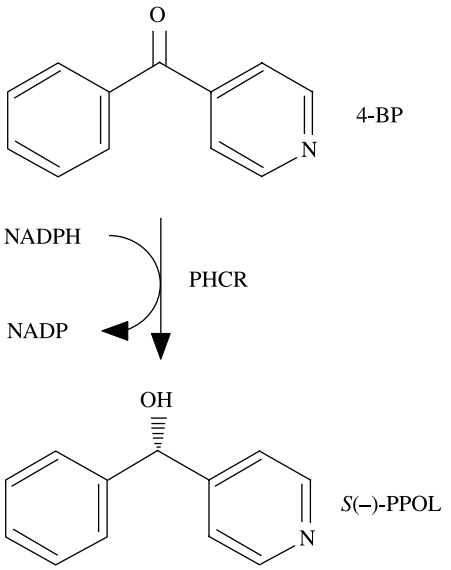

Figure 1. Reduction of 4-BP to *S*(−)-PPOL catalyzed by PHCR.

estimated as the ability to reduce 4-BP to *S*(−)-PPOL, by using pig heart cytosol as the reaction system of PHCR.

Materials and methods

Materials

4-BP was purchased from Wako Pure Chemicals (Osaka, Japan). *S*(−)- and *R*(+)-PPOL were synthesized from 4-BP as reported previously [13]. All alkyl phenyl ketones (Figure 2) were obtained from Aldrich (Milwaukee, WI) except nonanophenone (Wako Pure Chemicals). All other chemicals were of reagent grade.

Preparation of pig heart cytosol

Pig hearts were supplied from a slaughterhouse and stored at −20°C. The tissues were homogenized in 3 volumes of 10 mM sodium potassium phosphate buffer containing 1.15% KCl (pH 6.0). The homogenates were centrifuged at 105,000 × *g* for 60 min to obtain the cytosolic fraction (pig heart cytosol) which was used for the determination of enzyme activity, as described below.

Assay of carbonyl reductase activity

The enzyme (carbonyl reductase) activity was assayed by determining *S*(−)-PPOL formed from 4-BP in pig heart cytosol. The reaction mixture consisted of substrate (500 μM 4-BP), NADPH-generating system (50 μM $NADP^+$, 1.25 mM glucose-6-phosphate, 50 munits glucose-6-phosphate dehydrogenase and 1.25 mM $MgCl_2$), pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 mL. The reaction mixture was

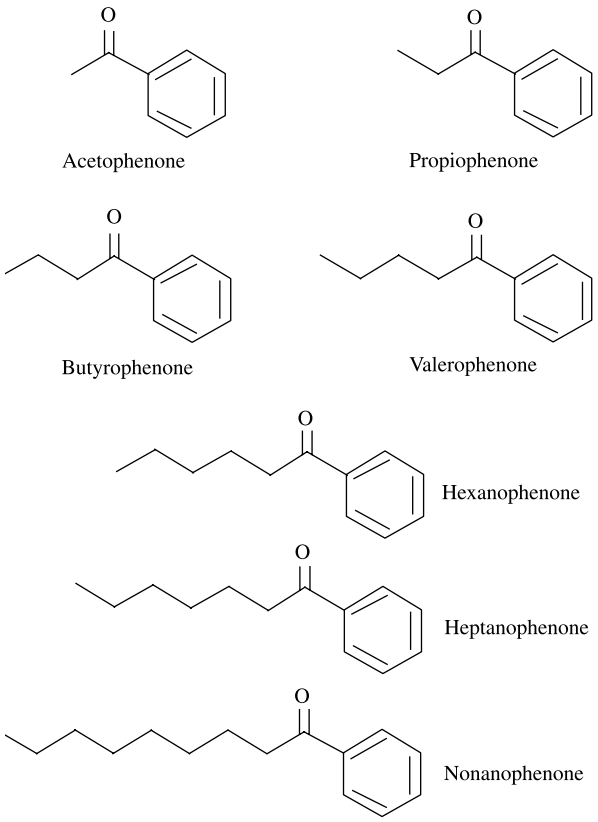

Figure 2. Chemical structures of alkyl phenyl ketones used in this study.

incubated at 37°C for 10 min and boiled for 2 min to stop the reaction. After centrifugation at 5,000 rpm, the supernatant (20 μL) was subjected to HPLC for the determination of the reduction products [*S*(−)- and *R*(+)-PPOL] of 4-BP. HPLC was carried out using a Tosoh DP-8020 HPLC apparatus (Tosoh, Tokyo, Japan) equipped with a Daicel Chiralpak AD-RH column (Daicel, Tokyo, Japan) and a Tosoh UV-8020 monitor (254 nm). A mixture of 20 mM borate buffer (pH 9.0)-acetonitrile (6:4, v/v) was used as a mobile phase at a flow rate of 0.5 mL/min. In the case of inhibition experiments, alkyl phenyl ketones were dissolved in methanol, and added to the reaction mixture at a concentration of 500 μM. The final concentration of methanol did not exceed 2% (v/v), and this concentration did not affect the enzyme reaction. Furthermore, the inhibition of 4-BP reduction (carbonyl reductase activity) by hexanophenone was kinetically analyzed using Lineweaver-Burk plots. Velocity (v) was expressed as nmol/min/mg protein. Protein concentration was determined by the method of Lowry et al. [15] with bovine serum albumin as the standard.

Reduction of alkyl phenyl ketones

The reduction of alkyl phenyl ketones was measured spectrophotometrically by monitoring the decrease in the

absorbance of NADPH at 340 nm ($\varepsilon = 6220\ M^{-1}cm^{-1}$). The reaction mixture consisted of substrate (alkyl phenyl ketone), 0.3 mM NADPH, pig heart cytosol and 100 mM sodium potassium phosphate buffer (pH 6.0) in a final volume of 0.5 mL. The enzyme reaction was initiated by the addition of alkyl phenyl ketones at various concentrations to the reaction mixture. The parameters (K_m and V_{max}/K_m) for the reduction of alkyl phenyl ketones were kinetically determined. One unit of enzyme activity was defined as the amount catalyzing the oxidation of 1 μmol of NADPH/min at 37°C.

Results

Inhibitory effects of alkyl phenyl ketones on carbonyl reductase activity

The inhibitory effects of various alkyl phenyl ketones on carbonyl reductase activity were examined in pig heart cytosol, using 4-BP as substrate. As shown in Figure 3, hexanophenone was the most potent inhibitor for the enzyme activity. Several alkyl phenyl ketones also exhibited significant inhibitions against the enzyme activity. The order of their inhibitory potencies for carbonyl reductase activity was hexanophenone > valerophenone > heptanophenone > butyrophenone > propiophenone. The inhibitory potencies of acetophenone and nonanophenone were much lower.

Kinetic parameters for the reduction of alkyl phenyl ketones

The kinetic parameters (K_m and V_{max}/K_m) for the reduction of alkyl phenyl ketones in pig heart cytosol are summarized in Table I. The kinetic parameters for the reduction of acetophenone and nonanophenone were not determined because of the lower catalytic activities. Of the alkyl phenyl ketones tested, hexanophenone exhibited the highest V_{max}/K_m value. Furthermore, V_{max}/K_m values of valerophenone, heptanophenone, butyrophenone and propiophenone decreased with the order of their inhibitory potencies for carbonyl reductase activity.

Table I. Kinetic parameters for the reduction of alkyl phenyl ketones in pig heart cytosol.

Ketones	K_m (mM)	V_{max}/K_m (munit/mg/mM)
Propiophenone	0.249 ± 0.046	28 ± 5
Butyrophenone	0.084 ± 0.014	109 ± 25
Valerophenone	0.048 ± 0.009	270 ± 47
Hexanophenone	0.037 ± 0.011	410 ± 13
Heptanophenone	0.078 ± 0.013	244 ± 13

The values are the mean ± S.D. of three or four experiments

Relationship between V_{max}/K_m *values and inhibitory potencies*

Figure 4 shows the relationship between V_{max}/K_m values for the reduction of alkyl phenyl ketones and their inhibitory potencies for carbonyl reductase activity in pig heart cytosol. As expected, a significant regression line ($r = 0.937$, $P < 0.05$) was obtained from the plots for these alkyl phenyl ketones.

Kinetic mechanism for the inhibition of carbonyl reductase activity by hexanophenone

The inhibition of carbonyl reductase activity (the reduction of 4-BP to *S*(−)-PPOL) by hexanophenone was kinetically examined in pig heart cytosol. As shown in Figure 5, hexanophenone was found to competitively inhibit the enzyme activity.

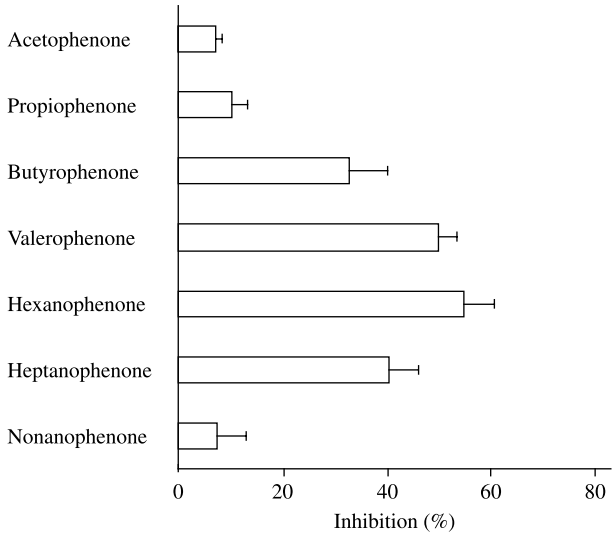

Figure 3. Inhibitory effects of alkyl phenyl ketones on carbonyl reductase activity in pig heart cytosol. 4-BP at a concentration of 500 μM was used as the substrate. The concentration of alkyl phenyl ketones added as inhibitor was 500 μM. Each bar represents the mean ± S.D. of three to six experiments.

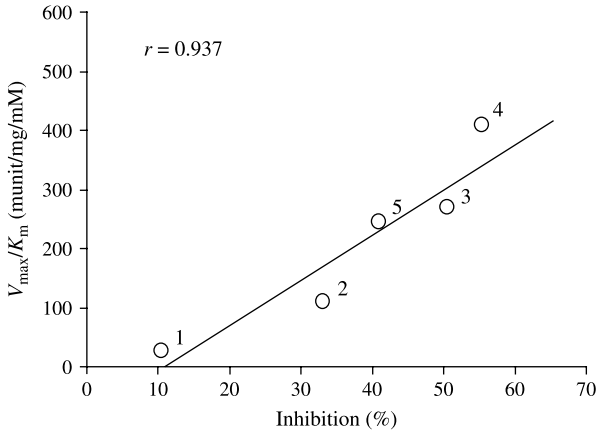

Figure 4. Relationship between V_{max}/K_m values for the reduction of alkyl phenyl ketones and their inhibitory potencies for carbonyl reductase activity in pig heart cytosol. Plot: 1, propiophenone; 2, butyrophenone; 3, valerophenone; 4, hexanophenone; 5, heptanophenone.

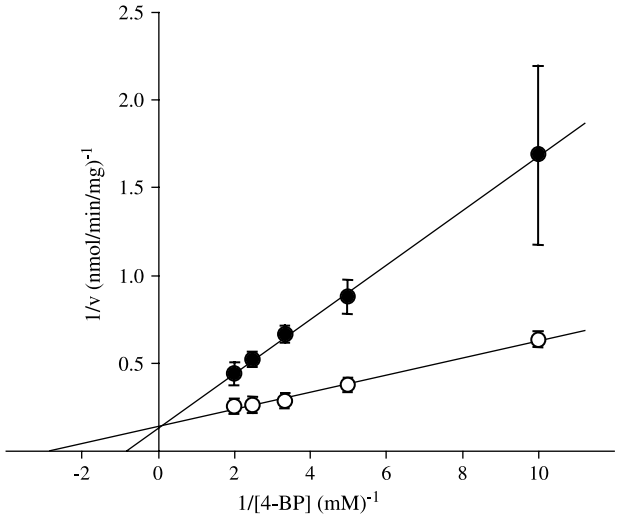

Figure 5. Lineweaver-Burk plots for the reduction of 4-BP to *S*(−)-PPOL in the absence and in the presence of hexanophenone. ○, in the absence of hexanophenone; ●, in the presence of hexanophenone (500 μM). Each point represents the mean ± S.D. of three or four experiments.

Discussion

Carbonyl reductases have been shown to be inhibited by various drugs and flavonoids [16–18]. For example, nonsteroidal anti-inflammatory drugs such as diclofenac sodium, flufenamic acid and indomethacin effectively inhibit carbonyl reductase purified from rabbit liver, using befunolol as a substrate [17]. Quercetin and quercitrin belonging to flavonoids are well-known inhibitors of carbonyl reductases purified from several tissues [7,8,19,20]. In the present study, we found that alkyl phenyl ketones have the ability to inhibit carbonyl reductase activity in pig heart cytosol. Furthermore, the order of their inhibitory potencies for carbonyl reductase activity was hexanophenone > valerophenone > heptanophenone > butyrophenone > propiophenone > acetophenone = nonanophenone.

To elucidate a possible mechanism for the substrate inhibition of carbonyl reductase activity by alkyl phenyl ketones, V_{max}/K_m values for the reduction of alkyl phenyl ketones were further determined. Hexanophenone, valerophenone, heptanophenone, butyrophenone and propiophenone have a straight-chain alkyl group of five, four, six, three and two carbon atoms, respectively, within their chemical structures (see Figure 2). Of the alkyl phenyl ketones tested, hexanophenone exhibited the highest V_{max}/K_m values. In addition, a significant relationship was observed between V_{max}/K_m values for the reduction of alkyl phenyl ketones and their inhibitory potencies for carbonyl reductase activity in pig heart cytosol. Based on these V_{max}/K_m values and inhibitory potencies, we propose the possibility that a hydrophobic cleft, which fits most efficiently to a straight-chain alkyl group of five carbon atoms, is located in the substrate-binding domain of PHCR present in pig heart cytosol (Figure 6).

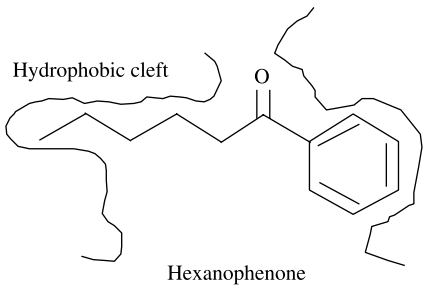

Figure 6. Proposed substrate-binding domain of PHCR.

Furthermore, the inhibition of carbonyl reductase activity by hexanophenone was kinetically analyzed in pig heart cytosol. As expected, hexanophenone was confirmed to be a competitive inhibitor for the enzyme. This finding indicates that hexanophenone inhibits enzyme activity by acting as a substrate inhibitor, and also supports the idea that a hydrophobic cleft corresponding to a straight-chain alkyl group of five carbon atoms in length is located in the substrate-binding domain of PHCR present in pig heart cytosol.

The stereochemistry of ketone reduction has been reported for a number of xenobiotics [13,21–26]. In this study, carbonyl reductase activity was estimated as the ability to reduce 4-BP to *S*(−)-PPOL in pig heart cytosol. Hexanophenone at a concentration of 500 μM had the ability (55.3 ± 5.9%) to inhibit the reduction of 4-BP to *S*(−)-PPOL (carbonyl reductase activity) by acting as a substrate inhibitor, as shown in Figure 3. The reduction of 4-BP in pig heart cytosol involves, in part, the formation of *R*(+)-PPOL that is the enantiomer of *S*(−)-PPOL. Interestingly, hexanophenone at a concentration of 500 μM also exhibited a similar inhibition (51.0 ± 5.9%) against the reduction of 4-BP to *R*(+)-PPOL.

In conclusion, the present study provides evidence that several alkyl phenyl ketones including hexanophenone inhibit carbonyl reductase activity in pig heart cytosol, by acting as substrate inhibitors. Further studies are in progress to elucidate the detailed mechanism for the substrate inhibition of recombinant PHCR by alkyl phenyl ketones and α-dicarbonyl compounds.

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