Evaluation of Drug-drug Interaction Potential of Beraprost Sodium Mediated by P450 In Vitro

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Beraprost sodium (BPS), a chemically stable and orally active analogue of prostacyclin, has multiple effects that improve microcirculation, such as antplatelet, vasodilatory, and anti-inflammatory effects similar to those of prostacyclin. BPS has been used for the treatment of chronic occlusive disease from 1992, and for primary pulmonary hypertension (PPH) from 1999. In clinical practice, multiple-drug therapy is a common therapeutic practice, and BPS is also coadministered with many other drugs, e.g., bosentan, cilostazol, and flunidione. Although no severe side effects from drug-drug interactions have been reported, such interactions related to BPS remain unclear.

Drug-drug interactions are mediated by several mechanisms, e.g., metabolic enzyme inhibition, metabolic enzyme induction, and protein binding elimination. The binding rate of beraprost (BP) to human serum protein is about 90% (unpublished observations), and a drug-drug interaction mediated by protein binding was anticipated, but in a coadministration experiment with the high-level protein binding drug flunidione, no changes in the efficacy of BP or effect of BP on the pharmacokinetic parameters of flunidione were observed. Therefore, we focused on the possibility of drug-drug interaction mediated by metabolic enzymes.

BP is metabolized mainly to β-oxidation products, 2,3-dinor-beraprost, and conjugated by gluconic acid, but the major metabolic enzymes of BP remain unknown. Taking account of the β-oxidation products, β-oxidative enzymes may be major metabolic enzymes of BP. However, a drug-drug interaction mediated by β-oxidation was reported at a greater than 3000-fold higher dose (400–500 mg daily) than the clinical dose of BPS (120 μg daily); therefore any drug-drug interaction of BP mediated by β-oxidation appeared negligible.

Except for the β-oxidative enzymes, the cytochrome P450 superfamily comprises major metabolic enzymes for many drugs. In this study, we investigated the possibility of drug-drug interactions mediated by cytochrome P450. To clarify whether P450 plays an important role in the metabolism of BP, we characterized the human P450 isoforms (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9,

INTRODUCTION

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Except for the β-oxidative enzymes, the cytochrome P450 superfamily comprises major metabolic enzymes for many drugs. In this study, we investigated the possibility of drug-drug interactions mediated by cytochrome P450. To clarify whether P450 plays an important role in the metabolism of BP, we characterized the human P450 isoforms (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9,
CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11) involved in BP metabolism using human P450-expressing insect microsomes. Then, to determine whether BP causes a drug-drug interaction via the induction or inhibition of P450, we conducted P450 induction (CYP1A2, CYP2C9, CYP2C19, and CYP3A4) and inhibition (CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) studies using freshly isolated human hepatocytes and human liver microsomes, respectively.

**MATERIALS AND METHODS**

**Chemicals**  BPS was synthesized by Toray Industries, Inc.\(^1\) \(^3\)H–BPS was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Phenacetin, paracetamol, tolbutamide, hydroxytolbutamide, nifedipine, oxidized nifedipine, resorufin, resorufin, \(S(+)-\)-mephenytoin, \(\pm\) -4'-hydroxymephenytoin, \(\pm\) -bufuralol hydrochloride salt, \(\pm\) -bufuralol, maleate salt, 
\(1\) ' -hydroxymidazolam, 6β-hydroxytestosterone, furafylline, tranylcypromine, trimethoprim, and sulfaphenazole were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Coumarin, 7-hydroxycoumarin, paclitaxel, diclofenac sodium, midazolam, testosterone, omeprazole, quinidine, and ketoconazole were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 6α-hydroxyxiclofenac and 4'-hydroxydiclofenac were purchased from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA). \([4-\text{14C}]\) Testosterone was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK).

**Human Cytochrome P450 Isoforms and Microsomes**  Human cytochrome P450 450 isoform (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11)-expressing microsomes prepared from baculovirus-infected insect cells (Supersomes) were purchased from Becton, Dickinson and Co. Human liver microsomes were purchased from Xenotech, LLC (Lenexa, KS, USA).

**Human Hepatocytes**  Cryopreserved and freshly isolated human hepatocytes were purchased from Biopredic International (Rennes, France).

**Characterization of Human Cytochrome P450 Isoforms**  The characterization of human cytochrome P450 isoforms (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11) involved in BP metabolism was performed using Supersomes.\(^16-22\)

\(^3\)H-BPS (4 Gbq/mmol; final concentration, 20 \(\mu\)M) was preincubated at 37°C in Tris-HCl (50 mM, pH 7.4) in the presence of NADPH (1 mM) for 5 min. After preincubation, reactions were initiated by the addition of ice-cold Supersomes (final concentration, 100 nmol/l) and incubated at 37°C for 1 h. The total volume of the mixture was 500 \(\mu\)l. In preliminary experiments, the linearity of the reaction with regard to incubation time and protein concentration was confirmed for each Supersomes. Control reactions were performed in the absence of NADPH. Reactions were terminated by the addition of 500 \(\mu\)l of ice-cold acetonitrile. After centrifugation at 600 \(\times\) g for 10 min, 100 \(\mu\)l of the supernatants were analyzed using HPLC.

**In Vitro Human Cytochrome P450 Induction**  Before the induction experiment, the cytotoxicities of BP and reference inducers (3-methylcholangthrene for CYP1A2, and rifampicin for CYP2C9, CYP2C19 and CYP3A4) were investigated using the neutral red uptake method,\(^23\) with cryopreserved human hepatocytes.

The evaluation of P450 induction was performed using freshly isolated human hepatocytes. Hepatocytes were seeded with the culture medium for cell attachment [CMA: Williams’ E medium containing penicillin (100 IU/ml), streptomycin (100 \(\mu\)g/ml), bovine insulin (4 \(\mu\)g/ml), bovine serum albumin (0.1 %, w/v), and fetal calf serum (10%, v/v)]\(^24-26\) and cultured for 24 h before the initiation of experiments. CMA was replaced with the culture medium for incubation [CMI: Williams’ E medium containing penicillin (100 IU/ml), streptomycin (100 \(\mu\)g/ml), bovine insulin (4 \(\mu\)g/ml), and hydrocortisone hemisuccinate (50 \(\mu\)M)] containing BPS at three noncytotoxic concentrations (1, 10, and 100 \(\mu\)M) or one of the reference inducers (3-methylcholangthrene, 2.5 and 5 \(\mu\)M; and rifampicin, 50 \(\mu\)M), and incubated for 72 h, with daily renewal of CMI containing test compounds. Control cultures with 1% (v/v) DMSO instead of test compounds were run in parallel. Before and 72 h after the addition of BPS or reference inducer, the medium was removed and the probe substrates of the P450 isoforms (phenacetin for CYP1A2, tolbutamide for CYP2C9, \(S(+)-\)-mephenytoin for CYP2C19, and nifedipine for CYP3A4) in minimum essential medium were added at a concentration of 0.2 mM (CYP1A2, CYP2C19, and
CYP3A4) or 1 mM (CYP2C9) and incubated at 37°C for 2 to 6 h. After incubation, cells and supernatants were collected separately, and concentrations of cellular protein and metabolites in supernatants were analyzed.

In Vitro Human Cytochrome P450 Inhibition

The inhibition of human P450 isoforms was investigated by incubating human liver microsomes and the probe substrates corresponding to each P450 isoform (CYP1A2: ethoxyresorufin 0.4 μM; CYP2A6: coumarin 1.5 μM; CYP2C8: paclitaxel 7.5 μM; CYP2C9: diclofenac 10 μM; CYP2C19: S(+)-mephenytoin 30 μM; CYP2D6: (±)-bufuralol 10 μM; and CYP3A4: midazolam and 14C-testosterone 4 μM and 0.2 μM, respectively) with or without test compounds. To the incubation mixture [phosphate buffer (100 mM, 390 μl), the probe substrates (5 μl), and microsomes (0.25–1 mg/ml, 50 μl)], 5 μl of BPS (final concentration, 0.05–1 μM) or one of the inhibitors corresponding to each P450 isoform (CYP1A2: furafylline 1–25 μM; CYP2A6: tranylcypromine 0.02–1 μM; CYP2C8: trimethoprim 10–200 μM; CYP2C9: sulfaphenazole 0.2–10 μM; CYP2C19: omeprazole 2–50 μM; CYP2D6: quinidine 0.01–0.5 μM; and CYP3A4: ketoconazole 0.005–0.1 μM) was added, and the mixture was preincubated at 37°C for 5 min. Control samples with distilled water instead of test compounds were run in parallel. After preincubation, 50 μl of the NADP regeneration system [NADP+, 12.5 mM]; glucose-6-phosphate (33 mM); MgCl₂ (33 mM); glucose-6-phosphate dehydrogenase (4 U/ml); and sodium citrate (0.5 mM)] was added, and the mixture was incubated at 37°C for 5 min (midazolam, 10 min (ethoxyresorufin), or 20 min (coumarin, paclitaxel, diclofenac, S(+)-mephenytoin, bufuralol, and testosterone). Reactions were terminated by the addition of ice-cold acetonitrile (250 μl). After centrifugation at 10000 × g at 4°C for 10 min, 100 μl of the supernatants were analyzed using HPLC or LC/MS/MS.

Analytical Methods For the characterization of human P450 isoforms, BP and its metabolites were analyzed using HPLC. The HPLC system consisted of a pump (Kontron 420), gradient former (Kontron 425), autosampler (Kontron 460), and UV detector (Kontron 335). The mobile phases were as follows: for paracetamol (CYP1A2), A: sodium acetate (2 mM)/acetonitrile (98/2, pH 2.6, adjusted with H₂SO₄), B: acetonitrile; for hydroxytolbutamide (CYP2C9), A: sodium acetate (10 mM pH 4.3, adjusted with H₂SO₄), B: acetonitrile; for (±)-4'-hydroxymercaptoin (CYP2C19), A: sodium acetate (5 mM)/acetonitrile (85/15, pH 4.0, adjusted with H₂SO₄), B: acetonitrile; and for oxidized nifedipine (CYP3A4), A: Tris (5 mM pH 9.5, adjusted with H₂SO₄), B: acetonitrile/ethanol (50/50). Linear gradient systems were used for the analyses of paracetamol, hydroxytolbutamide, and (±)-4'-hydroxymercaptoin in the following manners: for paracetamol, 13 to 21 min (100% A–50% B); for hydroxytolbutamide, 7 to 15 min (41% B–56% B); and for (±)-4'-hydroxymercaptoin, 5 to 12 min (100% A–25% B). An isocratic system was employed for the analysis of oxidized nifedipine at the concentration of 38% B. The flow rate for each analysis was 1 ml/min. The analytical columns were as follows: for paracetamol, Nucleosil C₁₈, 10 cm × 4.6 mm (Agilent Technologies); for hydroxytolbutamide, Nucleosil C₁₈, 15 cm × 4.6 mm (Agilent Technologies); and for (±)-4'-hydroxymercaptoin and nifedipine, LiChrospher RP-18 12.5 cm × 4.6 mm (Agilent Technologies). The detection of metabolites was performed based on UV absorption (280 nm for paracetamol, 230 nm for hydroxytolbutamide, 220 nm for (±)-4'-hydroxymercaptoin, and 240 nm for nifedipine). Protein concentrations of hepatocytes were measured using the Bradford method. Each value
was calculated using a bovine serum albumin standard curve.

For P450 inhibition, metabolites of the probe substrates were analyzed using HPLC or LC/MS/MS. The HPLC and LC/MS/MS systems consisted of two pumps (Shimadzu LC-10A), an autosampler (Shimadzu SIL-10A), column oven (Shimadzu CTO-10A), UV detector (Shimadzu SPD-10A), and fluorescence (Shimadzu RF-10A), RI (PerkinElmer 515TR) or MS/MS (Applied Biosystems API4000) detector. The mobile phases were as follows: for resorufin (CYP1A2), phosphate buffer (20 mM pH 7.0)/acetonitrile (87/13); for 14C-6β-hydroxytestosterone (CYP3A4), A: methanol/acetonitrile/water (43/14/1/55.9), B: methanol/acetonitrile/water (75/1.9/23.1); and for 7-hydroxycoumarin (CYP2A6), 6α-hydroxyxypaclitaxel (CYP2C8), 4′-hydroxydiclofenac (CYP2C9), (±)-4′-hydroxymephénytoïne (CYP2C19), 1′-hydroxybufuralol (CYP2D6), and 1′-hydroxymidazolam CYP3A4), A: formic acid/water (0.1/99.9), B: formic acid/acetonitrile (0.1/99.9). Except for the analysis of resorufin, linear gradient systems were used: for 14C-6β-hydroxytestosterone, 0 to 25 min (100% A–100% B), and for the other metabolites, 0 to 10 min (20% B–80% B). The analytical columns were as follows: for resorufin, Inertisil ODS-3, 15 cm × 4.6 mm (GL Science); for 14C-6β-hydroxytestosterone, Hypersil ODS-5 15 cm × 4.6 mm (Chemco Scientific); and for 7-hydroxycoumarin to 1′-hydroxymidazolam, Hypersil C18, 15 cm × 2.0 mm (YMC). The column temperature was 40°C for resorufin, 45°C for 6β-hydroxytestosterone, and ambient for 7-hydroxycoumarin to 1′-hydroxymidazolam. The detection of metabolites was performed using fluorescence (excitation: 575 nm/emission: 595 nm for resorufin), radioactivity (14C-6β-hydroxytestosterone), and mass spectrometry. The flow rate of the liquid scintillator was 3 ml/min. Q1/Q3 (m/z) of the metabolites were 163/107 for 7-hydroxycoumarin, 871/286 for 6α-hydroxyxypaclitaxel, 312/231 for 4′-hydroxydiclofenac, 235/150 for (±)-4′-hydroxymephénytoïne, 278/141 for 1′-hydroxybufuralol, and 249/192 for 1′-hydroxymidazolam.34

**Data Analysis** All data are expressed as the mean of two to four experiments, and the results of P450 induction and P450 inhibition were statistically analyzed using Dunnett’s multiple-comparison with SAS software (SAS Institute Inc., Cary, NC, USA).

In P450 induction, the metabolite formation rate was calculated from the metabolite concentration, incubation time, and protein concentration, and it was used to calculate the relative activity ($R_m$) as follows:

$$R_m = M/M_0 \times 100,$$

where $M$ is the metabolite formation rate in the sample with test compound, and $M_0$ is the metabolite formation rate in the sample without test compound.

In P450 inhibition, the remaining activity ($R_c$) was calculated according to the following equation:

$$R_c = (1 - C/IC_{50} + C),$$

where $C$ is the concentration of metabolite in the sample with test compound, and $C_0$ is the concentration of metabolite in the sample without test compound.

**RESULTS**

BP was slightly metabolized in the presence of CYP2C8 (Table 1). Most (97.1%) of the total integrated radioactivity was recovered as unchanged BP at the end of incubation. The appearance of two peaks more polar than BP, called M1 (RT, 27.5 min) and M2 (RT, 30.5 min), was noted. They accounted for 1.35% and 1.55% of total integrated radioactivity respectively. BP was not metabolized by the other P450 isoforms (CYP1A1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11).

In the preliminary cytotoxicity study, BP provoked weak alterations only at the highest concentration (1000 μM). Neutral red uptake was inhibited by 63.7% in the presence of BP at this concentration. Neither cell morphology nor neutral red uptake was modified in the presence of BP at a concentration of 300 μM or lower. Based on these results, the three noncytotoxic concentrations of BP chosen for the induction study were 1, 10, and 100 μM.

In the presence of BP, the enzymatic activities of all P450 isoforms examined in the P450 induction study (CYP1A2, CYP2C9, CYP2C19, and CYP3A4) were not increased, but rather appeared to be reduced (Table 2). Concerning tolbutamide hydroxylase (CYP2C9), BP altered its activity significantly by 48.9%, 67.6%, and 65.5% at 1, 10, and 100 μM,
Table 1. BPS Biotransformation by Human Recombinant Cytochrome P450 Isoforms

<table>
<thead>
<tr>
<th>P450 isoforms</th>
<th>BPS (%)</th>
<th>M1 (%)</th>
<th>M2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.3</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>51.2</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>50.5</td>
<td>BLQ</td>
<td>BLQ</td>
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<tr>
<td>CYP2B6</td>
<td>51.0</td>
<td>BLQ</td>
<td>BLQ</td>
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<td>BLQ</td>
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<td>CYP2C19</td>
<td>50.2</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>51.0</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>50.3</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>50.6</td>
<td>BLQ</td>
<td>BLQ</td>
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<tr>
<td>CYP4A11</td>
<td>51.0</td>
<td>BLQ</td>
<td>BLQ</td>
</tr>
</tbody>
</table>

Results are expressed as the percentage of the sum of all integrated peaks. Data are presented as mean of two experiments. BLQ, Below the limit of quantitation = 600 counts per minute.

Table 2. Relative Activities of P450 Isoforms in Human Hepatocytes after 72-h Incubation with Test Compounds

<table>
<thead>
<tr>
<th>P450 isoforms</th>
<th>Enzymatic activity</th>
<th>Test compound</th>
<th>0</th>
<th>1</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin deethylase</td>
<td>BPS</td>
<td>100±5</td>
<td>83±4</td>
<td>4746±1237*</td>
<td>3602±1045*</td>
<td>85±6</td>
<td>78±7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-Methylcholanthrene</td>
<td>100±5</td>
<td>100±5</td>
<td>85±6</td>
<td>78±7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide hydroxylase</td>
<td>BPS</td>
<td>100±12</td>
<td>52±10*</td>
<td>214±17*</td>
<td>214±17*</td>
<td>34±11*</td>
<td>214±17*</td>
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</tr>
<tr>
<td></td>
<td>Rifampicin</td>
<td>100±12</td>
<td>100±12</td>
<td>32±7*</td>
<td>34±11*</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4′-hydroxylase</td>
<td>BPS</td>
<td>100±16</td>
<td>84±6</td>
<td>70±4</td>
<td>70±4</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Rifampicin</td>
<td>100±16</td>
<td>100±16</td>
<td>84±9</td>
<td>70±4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Nifedipine oxidase</td>
<td>BPS</td>
<td>100±6</td>
<td>70±4</td>
<td>198±30*</td>
<td>198±30*</td>
<td>42±5</td>
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<td></td>
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<tr>
<td></td>
<td>Rifampicin</td>
<td>100±6</td>
<td>100±6</td>
<td>58±6</td>
<td>42±5</td>
<td></td>
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</table>

Data are presented as mean±S.D. of four experiments. *Relative activity was obtained by dividing the metabolite formation rate of the sample with a test compound by that of the control sample. *p<0.05

respectively.

BP showed no inhibitory effects on any P450 isoform (CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) examined in the P450 inhibition study (Table 3). The remaining activities of all P450 isoforms were not altered in the presence of BP at the concentration of 0.05 to 1 μM.

**DISCUSSION**

A previous study using rats (in vivo) demonstrated that BP induced no metabolic enzymes, including cytochromes (unpublished observations). In addition, metabolite analysis of human samples demonstrated that the major metabolites of BP were β-oxidation products and glucuronic acid conjugates of BP and β-oxidation products. However, despite the extensive use of BPS, no studies evaluating drug-drug interactions using human enzymes or cells have been carried out.

To initiate the present study, the characterization of human P450 isoforms (CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11) involved in BP metabolism was performed. BP was slightly metabolized by CYP2C8, and the metabolite formation rate was calculated as 3 pmol/h/μmol P450. The metabolite formation rate of paclitaxel, used as a probe substrate in the assay system, was reportedly 216 pmol/h/μmol P450 in the product document of Supersomes; therefore BP appeared to be a minor substrate of...
CYP2C8. On the other hand, BP was readily metabolized in human cells, and the major metabolites of BP were β-oxidation products, as described above. Based on these results, it appears that CYP2C8 is not the major metabolic enzyme of BP. Other prostaglandins (E1, E2, A1, A2, B1, and B2) were also not metabolized by P450 isoforms; in the metabolism of prostaglandins, P450 isoforms would play a minor role. Hence, even if the coadministration of drugs inhibits P450 isoforms, this would not affect BP metabolism.

To determine the possibility of P450 induction by BP, substrate-specific assays were conducted using freshly isolated human hepatocytes treated with various noncytotoxic concentrations of BP. BP did not induce any P450 isoforms (CYP1A2, CYP2C9, CYP2C19, and CYP3A4); on the contrary, it reduced the activity of CYP2C9. BP exhibited neither cytotoxicity in the induction study nor direct inhibition in the inhibition study; therefore the reduction might be caused by decreased P450 production or time-dependent P450 inhibition by BP. A decrease in P450 content was reported when PG-I\textsubscript{2} was continuously administered to rats, but the reason was not clear. For prostaglandin E\textsubscript{2}, down-regulation of CYP2B1 through EP2 receptors was reported, but whether prostaglandin I\textsubscript{2} or its analogues have the ability to down-regulate P450 isoforms has not yet been clarified. In addition to the possibility of P450 down-regulation, the possibility of time-dependent P450 inhibition by BP cannot be ruled out, but considering the high concentrations used in this induction study (at least 500-fold higher than clinical C\textsubscript{max}) and short plasma half-life of BP (0.89–1.11 h), BP would not provoke P450 induction or reduction in clinical use.

In the P450 inhibition study, BP did not inhibit any P450 isoform at a concentration of 1 μM or less. As noted above, 1 μM was about 500-fold higher than clinical C\textsubscript{max}; therefore we think that BP would not exhibit any inhibitory effects on P450 in clinical use.

As mentioned above, BP was only slightly metabolized by P450 isoforms and did not induce or inhibit these isoforms. Therefore it is concluded that BP is not involved in drug-drug interactions mediated by P450 isoforms in clinical use.

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