Interactions of Olomoucine II with Human Liver Microsomal Cytochromes P450

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ABSTRACT:

Olomoucine II is a cyclin-dependent kinase inhibitor and a potential antineoplastic agent because it can arrest animal cell cycles. This study examines its interactions with human liver microsomal cytochrome P450 (P450) enzymes. Spectroscopic and high-performance liquid chromatography (HPLC) methods were used to estimate the degree of olomoucine II-mediated inhibition of enzymatic activities of eight drug-metabolizing P450s in vitro. In addition, mass spectrometry coupled with HPLC was used to identify an olomoucine II metabolite (2,5-dihydroxyroscovitine) formed in the reaction mixtures, and CYP3A4 was found to be responsible for the hydroxylation of the N9-benzyl ring at position 5, leading to this compound. Olomoucine II significantly inhibited the enzymatic activities of CYP1A2, CYP2C9, and (to a lesser degree) CYP3A4. The results indicate that use of olomoucine II as a drug could affect the activities of CYP3A4, CYP1A2, and CYP2C9 in vivo. Hence, the clinical relevance of these interactions should be carefully evaluated.

Microsomal cytochrome P450 (P450) enzymes in the liver contribute to the first steps of the metabolism of diverse xenobiotics, including various drugs. However, some drugs may have undesirable effects, such as the inhibition or induction of these enzymes. Hence, interactions between P450s and drugs can have profound effects, including (reportedly) drug toxicity (e.g., terfenadine and mibefradil) (Wilkinson, 1996; Welker et al., 1998) and, in several cases, subtherapeutic concentrations of drugs in target tissues (Bachmann et al., 2004). The principal human liver P450s involved in drug biotransformation are CYP3A4, CYP2D6, CYP2C9, CYP2E1, and CYP1A2, although CYP2C19, CYP2A6, and CYP2B6 may also contribute significantly to the metabolism of some drugs or other xenobiotics (Anzenbacher and Anzenbacherová, 2001; Guengerich, 2005). The potential effects of drugs on specific P450 enzyme activities are generally examined using in vitro bacterial systems, each containing single forms of P450, and/or liver microsomal fractions (Phillips and Shephard, 1998).

Olomoucine II is a C2,N9-substituted 6-(2-hydroxybenzyl)amino)purine (ortho-topolin) inhibitor of cyclin-dependent kinases (CDKs). Such compounds have attracted considerable attention because they can inhibit CDK9, thereby blocking the cell division cycle of animal cells. Thus, there is a possibility that they could be used as antineoplastic drugs (Krystof et al., 2002), although little is currently known about the potential interactions between Olomoucine II and human liver microsomal P450s. Hence, in the study presented here the potential for the CDK inhibitor olomoucine II to influence the activities of the principal drug-metabolizing P450s of human liver microsomes (CYP3A4, CYP2C9, CYP2D6, CYP2E1, CYP1A2, CYP2A6, CYP2B6, and CYP2C9) was thoroughly evaluated. In addition, the potential ability of human liver microsomal P450s to catalyze the formation of olomoucine II metabolites was studied, and a metabolite generated in vitro reaction mixtures (2,5-dihydroxyroscovitine) was identified by tandem mass spectrometry.

Materials and Methods

Materials. Olomoucine II (2-[(6-[N-hydroxy-2-benzyl]amino)-9-isopropyl-9H-purine-2-yl]amino)butan-1-ol; CAS 500735-47-7) was obtained from Sigma-Aldrich (Prague, Czech Republic), and two potential metabolites, 2,4-dihydroxyroscovitine and 2,5-dihydroxyroscovitine, were synthesized as detailed in the following section (for structures, see Fig. 1). Diclofenac, 4’-hydroxyclofenac, bufuralol, 1’-hydroxybufuralol, and 6β-hydroxytestosterone were supplied by SAFC Corp. (Safford, UK), 7-Ethoxy-4-(trifluoromethyl)coumarin was purchased from Fluka (Buchs, Switzerland). (+)-N-3-benzylirinanol was supplied by BD Gentest (Woburn, MA). All other chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

Preparation of 2,4-dihydroxyroscovitine and 2,5-dihydroxyroscovitine derivatives. The precursors (2,6-dichloro-9-isopropylpurine, 2,4-dihydroxy-benzylamine hydroiodide, and 2,5-dihydroxybenzylamine hydrochloride) re-
required for synthesizing the possible olomoucine II metabolites were prepared as described previously (Otyepka et al., 2000; Dolezal et al., 2006, 2007). The appropriate dihydroxybenzylamine isomers (1 mmol) for the metabolites were then added individually, together with triethylamine (6 mmol), to a suspension of 2,6-dichloro-9-isopropylpurine (1 mmol) in ethyl acetate. Each reaction mixture was stirred at 78°C for 5 h and then cooled to room temperature. Next, the precipitate was filtered off, washed with cold water (2 × 10 ml) and diethyl ether (3 × 10 ml), and then dried at 60°C to constant weight. The resultant 2-chloro-6(dihydroxybenzyl)amino-9-isopropylpurines were further substituted with (R/S)-2-aminobutan-1-ol (160°C, 3 h). The products, 2,4-dihydroxyroscovitine and 2,5-dihydroxyroscovitine, were purified by extraction in ethyl acetate and crystallization from ethanol. Product identities were confirmed by melting point determination on a Boetius stage, thin-layer chromatography using WF254 silica gel 60 plates (Merck, Darmstadt, Germany), elemental analysis (C, H, and N) using a EA1108 CHN analyzer (Fisons Instruments, San Carlos, CA), and NMR spectroscopy using a Bruker Avance AV 300 spectrometer (Bruker, Vienna, Austria).

Microsomes and recombinant enzymes. Pooled human liver microsomes (cryopreserved), prepared and sourced in accordance with the regulations of the local ethics committee, were supplied by Advancell (Barcelona, Spain; http://www.advancell.net). The final microsomal mixture reportedly consisted of microsomes obtained from five males and five females, with a protein content of 38.4 mg/ml. Details of the CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, and CYP3A4 enzymatic activities of the mixture can be accessed from the Advancell Web site (http://www.advancell.net, hatch reference 102091201). *Escherichia coli* bacterial membrane structures (Bactosomes) containing recombinant human P450 enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, and CYP3A4) enzymatic activities of the mixture can be accessed from the Advancell Web site (http://www.advancell.net, hatch reference 102091201). *Escherichia coli* bacterial membrane structures (Bactosomes) containing recombinant human P450 enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, and CYP3A4) coexpressed with human NADPH-cytochrome P450 reductase were purchased from Cypex (Dundee, UK).

Methods. Determination of P450 activities. The following activities of the selected human liver microsomal P450s were assayed according to well-established protocols: CYP3A, testosterone 6β-hydroxylation (Guengerich et al., 1986); CYP2E1, p-nitrophenol hydroxylation (Tassaneeyakul et al., 1993); CYP2C9, diclofenac 4-hydroxylation (Crespi et al., 1998a); CYP1A2, 7-ethoxyresorufin O-deethyl (Chang and Waxman, 1998); CYP2D6, bufuralol 1′-hydroxylation (Crespi et al., 1998b); CYP2C19, S-nephentoin 4′-hydroxylation (http://www.cypex.co.uk/2c19info.htm); CYP2A6, coumarin 7-hydroxylation (Waxman and Chang, 1998); and CYP2B6, 7-ethoxy-4-(trifuoro)methyl)coumarin O-deethyl (Donato et al., 2004). The CYP1A2, CYP2A6, and CYP2B6 activities were calculated from spectral data acquired with a GENios absorbance-fluorescence-luminescence reader (Tecan Austria, Vienna, Austria), whereas the activities of CYP2C9, CYP2D6, CYP2C19, CYP2E1, and CYP3A4 were determined by monitoring amounts (in nanomoles) of metabolites formed (4′-hydroxyclofenac for CYP2C9, 1′-hydroxybufuralol for CYP2D6, 4′-hydroxyphenytoin for CYP2C19, p-nitrochlor for CYP2E1, and 6β-hydroxysterosterone for CYP3A4) by reverse-phase HPLC using a Shimadzu class VP system (Shimadzu, Kyoto, Japan) equipped with a LiChroCART 250-4 LiChrophor 100 RP-18 column (Merck) and UV or fluorescence detection, according to the cited literature.

Inhibition of cytochrome P450 enzymes in microsomal fractions by olomoucine II. Preliminary experiments were performed using microsomal preparations in the absence of olomoucine II to determine the Michaelis constant (K_m) and limiting velocity (V_max) of each P450 investigated and to determine suitable substrate concentrations for the inhibition experiments. In general, inhibition assays were then routinely performed with substrate concentrations corresponding to the K_m values of the respective enzymes and six concentrations of olomoucine II (10, 50, 100, 150, 200, and 400 μM) obtained by diluting a 25 mM stock solution of olomoucine II in 100% dimethyl sulfoxide as appropriate) plus olomoucine II-free controls. In addition, because some organic solvents can inhibit P450 activities, even in relatively low concentrations (Chaur et al., 1998; Bushy et al., 1999), the organic solvent concentrations in the final reaction mixtures were generally less than 1% (v/v), and organic solvent controls without the tested compounds were included in all cases, under otherwise identical experimental conditions. Experimental conditions in inhibition experiments were the same as those for determination of individual P450 activities: preincubation of reaction mixture with olomoucine II as a potential P450 inhibitor for 30 min at 37°C was performed for all samples. The amount of human liver microsomes (expressed as the amount of P450 in picomoles and concentration of human liver microsomal protein in milligrams per milliliter in the reaction vessel) in the reaction mixture was determined by dilutions in accordance with protocols for determination of P450 activities as follows: 35 pmol of P450 and 0.500 mg of protein/ml for CYP1A2, 34 pmol of P450 and 0.500 mg of protein/ml for CYP2A6, 34 pmol of P450 and 0.400 mg of protein/ml for CYP2B6, 35 pmol of P450 and 0.250 mg of protein/ml for CYP2C9, 50 pmol of P450 and 0.375 mg of protein/ml for CYP2C19, 67.3 pmol of P450 and 0.500 mg of protein/ml for CYP2D6, 160 pmol of P450 and 0.250 mg of protein/ml for CYP2E1, and 100 pmol of P450 with 0.297 mg of protein/ml for CYP3A4.

The inhibitory effect of olomoucine II on each of the P450 activities examined was then evaluated by plotting its concentration against the activities, expressed as means (in nanomoles of product per minute per nanomole of P450), obtained from two parallel assays at each substrate concentration for which there was a between-duplicate difference lower than 15%. In each case when significant inhibition of a P450 was detected, the inhibition constant (K_i) was determined by constructing Dixon plots, using the scientific graphing software Sigma Plot 8.0.2 (SPSS, Chicago, IL) and data obtained from assays with three substrate concentrations (corresponding to 1/2K_m, K_m, and 2K_m).

The degree of P450 inhibition was then evaluated using Microsoft Excel.

Metabolite formation and its inhibition by carbon monoxide. The effects of carbon monoxide on rates of formation of the olomoucine II metabolite (2,5-dihydroxyroscovitine) identified in the analyses described below catalyzed by the P450s in the microsomal preparations were also assessed, as follows. Portions of the preparations containing 250 pmol of P450s were incubated in 1 ml of a 50 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP, 3.7 mM isocitric acid, and 0.5 unit/ml isocitric acid dehydrogenase) and 5 mM MgCl_2. Carbon monoxide (Linde Technoplyn, Prague, CZ) was applied by gentle bubbling through the reaction mixture for 1 min after a 10-min preincubation period at 37°C. Diluted olomoucine II solutions were then added to the reaction mixtures to a final concentration of 100 μM, and the samples were incubated in a shaking water bath for 30 min at 37°C in sealed air-tight test tubes. Reactions were stopped by adding 20 μl of 70% HClO_4 (to precipitate proteins), the samples were vigorously stirred and centrifuged (5000 rpm for 6 min), and then 200 μl of each supernatant was transferred into an autosampler vial. The amounts of the metabolite formed were then determined by HPLC using a Shimadzu class VP system, equipped with a LiChroCART 250-4 LiChrophor 100 RP-18 column.
and 29% acetonitrile (v/v) in 7.35 mM potassium phosphate buffer (pH 2.2) as the mobile phase.

Inhibition of microsomal olomoucine II metabolism by specific inhibitors of individual P450 enzymes. In another series of assays, we examined the effects on olomoucine II metabolite formation of the following specific inhibitors of CYP1A2, CYP2C9, CYP3A4, CYP2E1, CYP2D6, CYP2A6, CYP2B6, and CYP2C19 activity: 22.5 μM furafylline, 3 μM sulfaphenazole, 2 μM ketoconazole, 75 μM diethyldithiocarbamate, 0.3 μM quinidine, 1 μM 8-methoxypsoralen, 2 μM 7-pentoxysresorufin, and 1 μM S-benzylisovanilin, respectively (Goldstein et al., 1994; Baldwin et al., 1995; Newton et al., 1995; Dierks et al., 2001; Suzuki et al., 2002). In these experiments, each of the inhibitors was preincubated with the P450-containing reaction mixture (see Metabolite formation and its inhibition by carbon monoxide) before addition of olomoucine II to a final concentration of 25 μM.

Identification of P450s involved in metabolite formation in Bactosomes. Bactosomes, each containing a single recombinant P450, were used in an attempt to identify P450s that participate in the metabolism of olomoucine II, following protocols recommended by the supplier (Cypex). Incubations were performed in 0.1 M Tris-HCl buffer (pH 7.4) containing 5 pmol of the relevant P450 (together with P450 reductase), 5 mM MgCl₂, an NADPH-generating system (0.5 mM NADP, 3.7 mM isocitric acid, and 0.5 unit/ml isocitric acid dehydrogenase), and 50 μM olomoucine II. The reactions were terminated by adding 20 μl of 70% HClO₄. Samples were then centrifuged at 5000 rpm for 6 min before transferring 200 μl of the resulting supernatants to autosampler vials and analyzing them as described above (see Metabolite formation and its inhibition by carbon monoxide).

Identification of the olomoucine II metabolite by mass spectrometry. To assess the possibility that interactions between olomoucine II and enzymes in the human liver microsomal preparations may result in the production of metabolites, olomoucine II was incubated with microsomal preparations (in triplicate) in 1 ml of Tris-HCl buffer (pH 7.4) containing an NADPH-generating system (0.5 mM NADP, 3.7 mM isocitric acid, and 0.5 unit/ml isocitric acid dehydrogenase), 5 mM MgCl₂, and 250 pmol of microsomal P450s. After a 10-min preincubation at 37°C, olomoucine II was added to the reaction mixtures to a final concentration of 50 μM. The samples were then incubated for 30 min at 37°C before the reactions were stopped through addition of 20 μl of 70% HClO₄. After centrifugation at 5000 rpm for 6 min, 200 μl of each supernatant was used to isolate putative olomoucine II metabolites by HPLC, using methods described above (see Metabolite formation and its inhibition by carbon monoxide).

The fractions containing putative olomoucine II metabolites were evaporated to half their initial volumes under a nitrogen stream, combined, and injected into an ion trap LCQ Fleet (Thermo Fisher Scientific, Waltham, MA) mass spectrometer equipped with an electrospray interface, operated in (+)ESI-MS/MS mode with previously optimized analytical parameters (5 kV spray voltage, 300°C capillary temperature, sheath gas flow rate 60 arbitrary units, auxiliary gas flow rate 15 arbitrary units, and sweep gas flow rate 8 arbitrary units). A putative olomoucine II metabolite was detected and identified as 2,5-dihydroxypsorococitine. Ion fragments of the compound formed in the (+)ESI-MS/MS experiments correlated well with a molecular structure and theoretical fragmentation mechanisms obtained using Mass Frontier 5.0 software (HighChem, Ltd., Bratislava, Slovakia).

Results

Olomoucine II Inhibition of Specific P450 Enzyme Activities in Human Liver Microsomes. Olomoucine II had no apparent effects on the measured CYP2A6 and CYP2E1 activities and only weakly interacted with CYP2B6, CYP2C8, CYP2C9, and CYP2D6. These interactions are unlikely to be clinically relevant, because the enzymatic activities were not significantly influenced at physiologically relevant concentrations of the potential inhibitor (≤50 μM) (Fig. 2). However, olomoucine II clearly inhibited the enzymatic activities of CYP1A2, CYP2C9, and CYP3A4 at such concentrations (Fig. 2). The inhibition of these three drug-metabolizing P450s by olomoucine II is shown in more detail in Fig. 3.

In the presence of the highest concentration of olomoucine II (400 μM), the enzymatic activities of CYP1A2, CYP2C9, and CYP3A4 decreased to 24, 26, and 35% of their respective control values, as shown by the plots in Fig. 3. The IC₅₀ values for these P450s were calculated and were as follows: IC₅₀ of 17.0 ± 1.9 μM for CYP1A2, IC₅₀ of 39.1 ± 3.4 μM for CYP2C9, and IC₅₀ of 18.0 ± 3.7 μM for CYP3A4. The degrees to which the enzymatic activities of CYP1A2 and CYP2C9 (and possibly CYP3A4) were inhibited indicated that olomoucine II and these P450s interacted quite specifically. Hence, Dixon plots were used to reveal the mechanisms behind these enzyme inhibitions and to determine the respective Kᵢ values.

The Dixon plot for CYP1A2 indicates that it is subject to inhibition by olomoucine II via a mixed mechanism, with a Kᵢ of 50 μM (Fig. 4). In contrast, the Dixon plot for CYP2C9 (Fig. 5), together with the Lineweaver-Burk plot (data not shown), indicates that olomoucine II noncompetitively inhibits it, with an estimated Kᵢ of 75 μM. The results also indicate that olomoucine II noncompetitively inhibits CYP3A4 (data not shown).

Metabolic Identification by MS Analysis. To assess the possibility that interactions between olomoucine II and P450 enzymes resulted in the formation of metabolites, human liver microsomal preparations were incubated with the compound. A putative metabolite in the resulting mixtures was then detected by HPLC with UV detection (λ = 289 nm). The presence of an olomoucine II-like substance in the samples was further verified by drying and combining the supernatants, fractionating them by HPLC, collecting and semidrying the corresponding UV fractions, and finally injecting them
into an ion trap mass spectrometer with an electrospray interface. (+)ESI-MS-chromatograms of the potential metabolite revealed a single peak with a retention time of 6.3 min in channel m/z 387, corresponding to a quasimolecular ion of a dihydroxyroscovitine molecule. The putative olomoucine II metabolite was unequivocally identified as 2,5-dihydroxyroscovitine by comparing its chromatographic retention and mass spectra with those obtained using synthetic standards (2,4-dihydroxyroscovitine and 2,5-dihydroxyroscovitine). The mass spectrum of authentic 2,5-dihydroxyroscovitine, obtained from (+)ESI-MS chromatograms at m/z 387, and the fragmentation patterns of the quasimolecular ion of the metabolite are shown in Fig. 6.

Influence of CO and Specific P450 Inhibitors on the Formation of the Olomoucine II Metabolite. A carbon monoxide inhibition experiment corroborated the role of P450s in the metabolism of olomoucine II, because the amount of 2,5-dihydroxyroscovitine formed was reduced by 85% after addition of carbon monoxide (results not shown). In addition, the effects of specific P450 inhibitors on formation of the metabolite were examined to identify the particular P450s involved in olomoucine II metabolism. The only potent inhibitor of olomoucine II 5-hydroxylation seemed to be ketoconazole, a specific inhibitor of CYP3A4, which resulted in reductions in the amount of 2,5-dihydroxyroscovitine formed to approximately 1% of control levels (results not shown).

Incubation of Olomoucine II with Recombinant P450 Enzymes. The experiments described in the previous section indicated that CYP3A4 plays a major role in olomoucine II metabolism. Incubation of olomoucine II with E. coli bacterial membrane fractions containing recombinant P450s (1A2, 2A6, 2B6, 2D6, 2E1, 2C9, 2C19, and 3A4) confirmed the participation of CYP3A4 in olomoucine II 5-hydroxylation (Fig. 7).

Discussion

The inhibition experiments showed that olomoucine II influenced the enzymatic activities of at least three P450s: CYP1A2, CYP2C9, and CYP3A4 (Figs. 2 and 3). Standard Dixon plots, used to examine the character of enzyme inhibition, indicated that CYP1A2 enzymatic activity was subjected to a mixed inhibition mechanism, whereas olomoucine II noncompetitively inhibited CYP2C9 and CYP3A4. In each case, the IC50 values were estimated to be in the tens of micromolar concentration range.

The results also revealed the formation of an olomoucine II-like metabolite in the liver microsomal fraction. Analysis of this metabolite by HPLC-(+)ESI-MS/MS showed that it possessed an extra hydroxyl group on the N6-benzyl substituent of the parent compound. Comparative analysis of the potential olomoucine II metabolite with synthetic 2,5-dihydroxy- and 2,4-dihydroxyroscovitine derivative standards confirmed the 5-hydroxylation of the aromatic ring of the putative olomoucine II metabolite. Next, we were interested in identifying the particular P450(s) that participated in the formation of the metabolite. Initially, the role of P450 enzymes in the reaction was confirmed by using carbon monoxide to inhibit metabolite formation. Carbon monoxide is known to be a universal P450 inhibitor because it binds strongly to the heme iron, forming a complex that cannot mediate the catalytic reaction because of its inability to bind molec-
higher polarity. However, a higher IC50 value could be advantageous on the benzene ring of olomoucine II, apparently resulting in its magnitude higher. This difference could be due to the presence of a hydroxy structural similarities to olomoucine II (Fig. 1), were published during the microsomal fraction.

We then used inhibitors of specific P450s to identify the P450s responsible for the formation of the olomoucine II metabolism. Of these, ketoconazole, a specific CYP3A4 inhibitor, was found to inhibit the hydroxylation of olomoucine II most strongly. In addition, the lack of, or little, metabolite formation when Bactosomes expressing with Bactosomes expressing a single P450. In contrast, there was a of the olomoucine II aromatic ring when olomoucine II was incubated with Bactosomes expressing 1,2-di-hydroxyroscovitine from olomoucine II.

Nevertheless, the results concerning the metabolism of olomoucine II and its inhibition of P450 activities do not exclude the possibility that olomoucine could significantly interact with CYP3A4 (which was largely responsible for the formation of the metabolite) or CYP1A2 and/or CYP2C9, which were also inhibited by olomoucine II in the microsomal fraction.

The results of a similar study with roscovitine (seliciclib), with structural similarities to olomoucine II (Fig. 1), were published during the preparation of this article (McCue and Stuart, 2008). It is interesting to note that this compound was shown to inhibit the activity of CYP3A4 and CYP2C9, albeit with IC50 values in the micromolar concentration range, whereas the results presented in this article show IC50 values in the tens of micromolar range, i.e., an order of magnitude higher. This difference could be due to the presence of a hydroxy group on the benzene ring of olomoucine II, apparently resulting in its higher polarity. However, a higher IC50 value could be advantageous for olomoucine II, because plasma levels of these compounds are likely to be in the lower (micromolar) concentration ranges (McCue and Stuart, 2008). On the other hand, the results presented here and those published by McCue and Stuart (2008) indicate that the potential for drug interactions involving these prospective antineoplastic compounds cannot be excluded and should therefore be thoroughly tested.

References


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