Interaction of selected platinum(II) complexes containing *roscovitine*-based CDK inhibitors as ligands with human liver microsomal cytochrome P450

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**Background.** We studied the interaction of oxaliplatin derivatives involving cytotoxic adenine-based cyclin-dependent kinase inhibitors, with human liver microsomal cytochrome P450.

**Methods and Results.** The activities of 9 human liver microsomal CYP forms (CYPs 1A2, 7-ethoxyresorufin O-deethylation; 2A6, coumarin 7-hydroxylation; 2B6, 7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation; 2C8, luciferin-6' methyl ether demethylation; 2C9, diclofenac 4'-hydroxylation, 6'-deoxyluciferin hydroxylation; 2C19, (5)-mephenytoin 4'-hydroxylation; 2D6, bufuralol 1'-hydroxylation, 2E1, chlorozoxazone 6-hydroxylation; 3A4, testosterone 6β-hydroxylation, luciferin-6' benzyl ether debenzylation) were tested using HPLC, fluorescence and luminescence product detection. At 100 μM platinum(II) oxalato complex concentration, CYP inhibition was in general 25%-50%, except for the CYP3A4 form which showed roughly twice the inhibition (72%-95%). At low complex concentration (10 μM), the difference in inhibition of CYP3A4 and other forms was even more pronounced. Dixon and Lineweaver-Burk plots indicated a partially noncompetitive mechanism of CYP3A4 inhibition.

**Conclusions.** The tested complexes significantly inhibit human liver microsomal CYP3A4 activity even at clinically relevant concentrations. This could be a serious drawback for the use of these compounds in clinical practice.

**Key words:** cytochrome P450, platinum(II) complexes, CDK inhibition, *roscovitine* derivatives

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**INTRODUCTION**

Liver microsomal cytochromes P450 (P450s) play a very important role in the process of environmental chemical (xenobiotics) biotransformation as these heme-containing enzymes are responsible for more than one-half of known pathways of drug metabolism in the liver and other organs. P450s form a superfamily of enzymes localized in the liver, lung, gastrointestinal tract, brain, heart, and in other tissues. Within the group of liver microsomal P450s, nine forms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) are active in the metabolism of more than 90% reactions catalyzed by these enzymes. The need for studies on the interaction of P450s with newly designed and synthesized compounds is linked to clinical application and to the fact that many drugs can be transformed by these enzymes to either less active or more active molecules. There is also possible competition of drug for these proteins causing drug interactions leading to increasing the toxic levels of the respective drugs.

The anticancer activity of *cisplatin* (*cis*-diammine-dichloroplatinum(II)) has been known for more than 40 years and during this period a plethora of analogous compounds have been synthesized with the aim of enhancing the cytotoxic effect of the already applied substances, reduce their toxicity, and/or increase their ability to overcome the resistance of various tumors to the action of *cisplatin* or its analogues. The therapeutic activity of these compounds is mainly based on the covalent modification of the DNA molecule. There is much evidence on the formation of various types of covalent DNA adducts and their influence e.g. on DNA replication. The platinum complexes were later followed by the complexes of other transition metals (e.g. ruthenium), however, no such compound has been approved for clinical use to date.

*Carboplatin* (*cis*-diammine-cyclobutane-1,1'-dicarboxylatoplatinum(II)) and *oxaliplatin* (1,2-diaminoclohexane-oxalatoplatinum(II)) are more recent analogs exhibiting generally lower or less severe toxicity. Their use is increasing as in some indications they represent an alternative to *cisplatin* or exhibit effects unattainable for *cisplatin* (e.g. *oxaliplatin* in treatment of colorectal cancer).
from a plant hormone N6-(benzyl)adenine (6-benzylaminopurine) (ref.9). Roscovitine belongs to the group of adenine-based cyclin-dependent kinase (CDK) inhibitors and this compound was successfully tested in vivo on patients with non-small cell lung cancer10,11. CDKs are core components of the cell cycle machinery that govern the transition between phases during cell cycle progression and currently represent one of the alternative enzyme groups with potential to be a target for anticancer therapy12,13.

A large group of complexes which combine in their structures diverse transition metals with variously substituted N6-(benzyl)adenine derivatives as ligands have been synthesized and tested for anticancer activity14-20. One of the most recently reported group of complexes involves the platinum(II) oxalato (ox) complexes [Pt(ox)(R)-(Ros)]·¾H2O (1), [Pt(ox)(2OMeRos)]·H2O (2), [Pt(ox)(3OMeRos)]·½H2O (3) and [Pt(ox)(4OMeRos)]·¾H2O (4) containing Roscovitine (Ros) and its benzyl-substituted analogues (i.e. 2-(1-ethyl-2-hydroxyethylamino)adenine (2OMeRos), 2-(1-ethyl-2-hydroxyethylamino)-(3-methoxybenzyl)-9-isopropyladenine (3OMeRos) and 2-(1-ethyl-2-hydroxyethylamino)-N6-(4-methoxybenzyl)-9-isopropyladenine (4OMeRos)) as N-donor carrier ligands (Fig. 1). These complexes showed moderate (IC50 ranged from 10.8 to 34.4 μM) and in most cases higher, as compared with cisplatin and oxaliplatin, in vitro cytotoxic activity against cisplatin sensitive (A2780) and resistant (A2780cis) ovarian carcinoma, malignant melanoma (G-361), lung carcinoma (A549), cervix epitheloid carcinoma (HeLa), breast adenocarcinoma (MCF7) and osteosarcoma (HOS) human cancer cell lines. Moreover, the representative complex 1 was not hepatotoxic up to 100 μM and in most cases higher, as compared with cisplatin and oxaliplatin, in vitro cytotoxic activity against cisplatin sensitive (A2780) and resistant (A2780cis) ovarian carcinoma, malignant melanoma (G-361), lung carcinoma (A549), cervix epitheloid carcinoma (HeLa), breast adenocarcinoma (MCF7) and osteosarcoma (HOS) human cancer cell lines. Moreover, the representative complex 1 was not hepatotoxic up to a concentration of 50.0 μM, as observed on the primary cultures of human hepatocytes. These published21 results favored the complexes 1-4 for further biological studies, such as in vivo experiments on mouse L1210 leukemia model (unpublished results) or the present reported interaction with human liver microsomal P450s.

**Fig. 1.** General structural formula of studied complexes, where R = H for complex (1); R = 2-methoxy for (2); R = 3-methoxy for (3); R = 4-methoxy for (4).

**MATERIALS AND METHODS**

**Chemicals**

Complexes 1-4 were prepared as described in detail previously21. For determination of CYP activities, chloroxazone, 6-hydroxychloroxazone, diclofenac, 4-hydroxydiclofenac, bufuralol, 6-hydroxybufuralol and 6β-hydroxytestosterone were supplied by Cerilliant Corporation (Round Rock, Texas, USA). P450-Glo® substrates luciferin ME (luciferin-6’-methyl ether), luciferin H (6’-deoxyluciferin) and luciferin BE (luciferin-6’ benzyl ether) for evaluation of CYP2C8, CYP2C9 and CYP3A4 activities by luminescence spectrometry were products of Promega (Madison, Wisconsin, USA) obtained through East Port (Prague, Czech Republic). 7-ethoxy-4-(trifluoromethyl)coumarin was supplied by Fluka (Buchs, Switzerland). Cryopreserved human liver microsomes (pooled) were purchased from Advancell (Barcelona, Spain). Microsomes were obtained by approval of the local Ethics Committee and in accordance with the ethic regulations of the country of origin (Spain). They were from 5 males and 5 females with protein content 38.4 mg/mL; the CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1 and CYP3A4 enzyme activities are accessible at the Advancell web site (www.advancell.net, batch reference 102091201). All other chemicals were supplied by Sigma Aldrich (Czech Republic).

**Determination of CYP activities**

Activities of individual CYP forms were measured according to published protocols. All the tested microsomal CYP activities and corresponding methods of specific product detection are listed in the Table 1.

The stock solutions of complexes 1-4 in N,N’-dimethylformamide (DMF) were prepared before each CYP activity assay. The concentration of Pt in stock solutions was 20 mM and was verified using ICP–MS (7000x Series, Agilent Technologies, USA). The stock solutions of the tested drugs were added to reaction mixtures to obtain the desired final concentration which covered two orders of magnitude, from 1 μM to 100 μM. No drug was added to the control sample, only DMF.

Final incubation mixture volumes were: CYP1A2, CYP2A6 and CYP2B6, 100 μL; CYP2C8, 100 μL; CYP2C9, 100 and 50 μL for diclofenac or 6’-deoxyluciferin as substrates; CYP2C19, and CYP2D6, 200 μL; CYP2E1, 500 μL; CYP3A4 activities, 500 μL for testosterone and 50 μL for luciferin derivative as substrate. The final concentrations of microsomal P450 were as follows: CYP1A2, CYP2A6 and CYP2B6, 0.35 μM; CYP2C8, 1.20 μM; CYP2C9, 0.18 μM and 0.68 μM for diclofenac or 6’-deoxyluciferin as substrates; CYP2C19, 0.25 μM; CYP2D6, 0.35 μM; CYP2E1, 0.16 μM; CYP3A4 activities, 0.14 μM for testosterone and 1.20 μL for luciferin derivative as substrate. The reaction mixtures of all CYP activities tested were buffered by 75 mM K-phosphate buffer, pH 7.4.

For each enzyme assay, a preliminary experiment was done to determine the K_m and V_max for a given enzyme...
reaction and to obtain the values of substrate concentrations suitable for the inhibition experiments (as a rule, substrate concentration was chosen in the range corresponding to the value of the $K_M$). Inhibition experiments were routinely performed with up to seven concentrations of the tested drug. As a rule, inhibition experiments with known reference inhibitors were implemented in cases where a significant degree of inhibition was presumed, namely, with sulfaphenazole (CYP2C9) (ref.22), methoxsalen (CYP2A6) (ref.23), diethylidithiocarbamate (CYP2E1) (ref.24), 3-isopropenyl-3-methyldiamantane (CYP2B6) (ref.25), and ketoconazole (CYP3A4) (ref.2).

Selection of established inhibitors was based on the data from the literature.

A TECAN Infinite M200 microplate reader (Tecan Austria, Vienna, Austria) was used for detection of the fluorescence- and luminescence-based assays. HPLC analyses were performed using the Dionex UltiMate 3000 system (Thermo Scientific brand).

Inhibition of individual CYP activities by complexes 1-4 was in all cases evaluated by plotting the respective remaining activity against the inhibitor concentration; as a rule, the results were expressed as the means of two to five independent determinations with the difference between duplicates being lower than 15%.

When an inhibition was pronounced, the $K_i$ values were determined from Dixon plots with three substrate concentrations used (corresponding to 0.5$K_M$, $K_M$ and 2$K_M$). To acquire information on the mechanism of inhibition, both Dixon and Lineweaver-Burk plots were used26.

To analyze the course of the enzyme kinetics, parameters of enzyme kinetics ($K_M$, $V_{max}$) as well as the intercepts of the respective plots were obtained using the Sigma Plot 8.0.2 (SPSS, Chicago, Illinois, USA) and GraphPad Prism 5 (GraphPad Software, San Diego, California, USA) scientific graphing software.

RESULTS AND DISCUSSION

Four oxaliplatin derivatives were studied: ([Pt(ox)(Ros)]·¾H2O (1), [Pt(ox)(2OMeRos)]·H2O (2), [Pt(ox)(3OMeRos)]·½H2O (3) and [Pt(ox)(4OMeRos)]·¾H2O (4)) and the activities of nine forms of CYP enzymes present in human liver microsomes (CYPs 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4). The results show that within complex concentration ranges up to 10 μM, corresponding approximately to levels attainable in human plasma33, all CYP activities were affected only slightly (with effect of inhibition less than 25%) except CYP3A4 activity, which shows inhibition 33-47% (Fig. 2A).

To obtain information on whether complexes at higher concentrations are able to inhibit CYP enzymes in a more pronounced extent, compounds 1-4 were also tested for ten times higher concentration equal to 100 μM. Here all for CYP forms are affected markedly, (inhibition typically

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**Table 1.** The list of CYP activities tested and references to corresponding methods of individual CYP activity product detection.

<table>
<thead>
<tr>
<th>CYP450</th>
<th>CYP activity</th>
<th>Detection</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>1A2</td>
<td>7-ethoxyresorufin O-deethylation</td>
<td>fluorescence</td>
<td>27</td>
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<tr>
<td></td>
<td>7-ethoxyresorufin O-deethylation</td>
<td>HPLC</td>
<td>27</td>
</tr>
<tr>
<td>2A6</td>
<td>coumarin 7-hydroxylation</td>
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<td>27</td>
</tr>
<tr>
<td>2B6</td>
<td>7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation</td>
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<td>2C8</td>
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<td>luminescence</td>
<td>Tech. Bulletin No 325 <a href="http://www.promega.com">www.promega.com</a></td>
</tr>
<tr>
<td>2C9</td>
<td>diclofenac 4´-hydroxylation 6´-deoxyluciferin hydroxylation (P450-Glo substrate)</td>
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<td>30 <a href="http://www.promega.com">www.promega.com</a></td>
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<tr>
<td>2C19</td>
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<td>HPLC</td>
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<td>31</td>
</tr>
<tr>
<td>3A4</td>
<td>testosterone 6β-hydroxylation luciferin-6´ benzyl ether debenzylation (P450-Glo substrate)</td>
<td>HPLC, luminescence</td>
<td>32 <a href="http://www.promega.com">www.promega.com</a></td>
</tr>
</tbody>
</table>
Fig. 2. Overview of inhibition rates of CYP activities by complexes 1-4 at 10 μM (A) and 100 μM (B) concentration. Results expressed as extent of inhibition that is 100%, full inhibition. Individual activities determined as described in Materials and methods. Mean and standard deviation values are shown.

Fig. 3. Effect of individual complexes (circles (1), squares (2), triangles (3) and diamonds (4)) on specific activity of CYP3A4, testosterone 6β-hydroxylation, in a dose-dependent manner. Concentrations of 1-4 in the reaction mixture were 0, 3, 6, 12, 25, 50 and 100 μM.

Fig. 4. Dixon plot at lower range of complex[Pt(ox)(Ros)₂]·½H₂O (1) concentrations for inhibition of CYP3A4, testosterone 6β-hydroxylation, at three substrate concentrations (50 μM, squares; 100 μM, triangles; 200 μM, circles). 1/v, reciprocal velocity.
between 20-50%). At 100 μM concentration, similar to the effects observed at 10 μM level, the most prominent effect was observed at the CYP3A4 form which was nearly completely inhibited with complexes 3 and 4 (inhibition 95% and 93% respectively), inhibition of CYP3A4 with complexes 1 and 2 was 72% and 77% (Fig. 2B).

In most cases, the inhibition even at high drug concentration (100 μM) hardly reaches 50%. The most prominent inhibition even at 10 μM concentration was observed for CYP3A4. This is apparently due to the fact that the active site of this enzyme is able to accommodate bulky substrates (as e.g. macrolide antibiotics and steroids) (ref.26-28).

We reported earlier28 that oxaliplatin exhibits only negligible effect on P450s even at very high concentration. With this compound, we observed the inhibition of CYP2C9 enzyme however only by about 20% and at extremely high concentration of the drug (400 μM). Hence, this study should also show whether the introduction of roscovitine-based N-donor ligand to the parent drug (oxaliplatin) will affect the complex-P450s interaction. Radical changes in the manner of interaction with P450s, i.e. that the complexes studied here inhibit the CYP enzymes, are probably related to the fact that relatively polar oxaliplatin does not enter the active site of CYP enzymes, with the exception of CYP2C9. On the other hand roscovitine and its derivatives are less polar and possibly interact with CYP enzymes to a greater extent.

Apparently the inhibition patterns are determined mainly by the properties of the active site of the respective CYP enzyme. The position of the methoxy group (none (1); 2-methoxybenzyl (2); 3-methoxybenzyl (3); 4-methoxybenzyl (4)) does not seem to affect the inhibition of CYP3A4 activity in a systematic manner.

Experimental data obtained for CYP3A4 inhibition (Fig. 3) were analyzed by Dixon24,25 and Lineweaver-Burk26 plots to evaluate the possible mechanisms of enzyme inhibition.

However the results did not yield unambiguous answer as the courses of Dixon and Lineweaver-Burk plots did not correlate well with standard diagnostic courses of these plots described for individual inhibition mechanisms as at higher concentrations the results are apparently influenced by the strong binding of compounds tested, to enzyme proteins. Analysis of data reduced to lower range of complex concentrations (as represented in Fig. 4 for complex 1) corresponds to partially noncompetitive mechanism of inhibition.

CONCLUSION

Complexes 1-4 inhibit CYP3A4 activity even at clinically relevant concentration. This could be a serious drawback for the use of these compounds in clinical practice as the majority of drugs are metabolized by this enzyme. This facilitates their elimination or, in some cases of pro-drugs, to formation of pharmacologically active species.

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Author contributions: VM, PS, ZT, EA: study design; VM, PS: manuscript writing; VM, MH, MM, DM: data collection and analysis; VM, ZT, EA: data interpretation; PS: compounds synthesis; all authors: manuscript revision.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

REFERENCES