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, (S)-mephenytoin 4'-hydroxylation; 2D6, bufuralol 1'-hydroxylation, 2E1, chlorzoxazone 6-hydroxylation; 3A4, testosterone 6β-hydroxylation, luciferin-6' benzyl ether debenzilation) 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 organs¹. P450s form a superfamily of enzymes localized in the liver, lung, gastrointestinal tract, brain, heart, and in other tissues². 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 drugs³.

The anticancer activity of *cisplatin* (*cis*-diammine-dichloridoplatinum(II)) has been known for more than

40 years⁴ and during this period a plethora of analogous compounds have been synthesized⁵ 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 replication⁶. The platinum complexes were later followed by the complexes of other transition metals (e.g. ruthenium⁷), however, no such compound has been approved for clinical use to date.

Carboplatin (*cis*-diammine-cyclobutane-1,1'-dicarboxylatoplatinum(II)) and *oxaliplatin* (1,2-diaminocyclohexane-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) (ref.⁸).

2-(1-Ethyl-2-hydroxyethylamino)-N6-(benzyl)-9-isopropyladenine (*Roscovitine*; Ros) is a substance derived

from a plant hormone *N*6-(benzyl)adenine (6-benzylaminopurine) (ref.⁹). *Roscovitin* 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 cancer^{10,11}. 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 therapy^{12,13}.

A large group of complexes which combine in their structures diverse transition metals with variously substituted *N*6-(benzyl)adenine derivatives as ligands have been synthesized and tested for anticancer activity¹⁴⁻²⁰. One of the most recently reported²¹ group of complexes involves the platinum(II) oxalato (ox) complexes [Pt(ox)(Ros)₂] $\cdot\frac{3}{4}$ H₂O (1), [Pt(ox)(2OMeRos)₂] $\cdot$ H₂O (2), [Pt(ox)(3OMeRos)₂] $\cdot\frac{1}{2}$ H₂O (3) and [Pt(ox)(4OMeRos)₂] $\cdot\frac{3}{4}$ H₂O (4) containing *Roscovitin* (Ros) and its benzyl-substituted analogues (i.e. 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(2-methoxybenzyl)-9-isopropyladenine (2OMeRos), 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(3-methoxybenzyl)-9-isopropyladenine (3OMeRos) and 2-(1-ethyl-2-hydroxyethylamino)-*N*6-(4-methoxybenzyl)-9-isopropyladenine (4OMeRos)) as N-donor carrier ligands (Fig. 1). These complexes showed moderate (IC₅₀ ranged from 10.8 to 34.4 μ M) 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 μ M, 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, chlorzoxazone, 6-hydroxychlorzoxazone, diclofenac, 4-hydroxydiclofenac, bufuralol, 6-hydroxybufuralol and 6 β -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 Advantcell (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 Advantcell web site (www.advantcell.net, batch reference 102091201). All other chemicals were supplied by Sigma Aldrich (Prague, 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 μ M to 100 μ M. No drug was added to the control sample, only DMF.

Final incubation mixture volumes were: CYP1A2, CYP2A6 and CYP2B6, 100 μ L; CYP2C8, 100 μ L; CYP2C9, 100 and 50 μ L for diclofenac or 6'-deoxyluciferin as substrates; CYP2C19, and CYP2D6, 200 μ L; CYP2E1, 500 μ L; CYP3A4 activities, 500 μ L for testosterone and 50 μ L for luciferin derivative as substrate. The final concentrations of microsomal P450 were as follows: CYP1A2, CYP2A6 and CYP2B6, 0.35 μ M; CYP2C8, 1.20 μ M; CYP2C9, 0.18 μ M and 0.68 μ M for diclofenac or 6'-deoxyluciferin as substrates; CYP2C19, 0.25 μ M; CYP2D6, 0.35 μ M; CYP2E1, 0.16 μ M; CYP3A4 activities, 0.14 μ M for testosterone and 1.20 μ M 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), diethyldithiocarbamate (CYP2E1) (ref.^{2,24}), 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 $0.5K_M$, K_M and

$2K_M$). 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)₂]. $\frac{3}{4}$ H₂O (1), [Pt(ox)(2OMeRos)₂].H₂O (2), [Pt(ox)(3OMeRos)₂]. $\frac{1}{2}$ H₂O (3) and [Pt(ox)(4OMeRos)₂]. $\frac{3}{4}$ H₂O (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 μ M, 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 μ M. Here all for CYP forms are affected markedly, (inhibition typically

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

CYP450	CYP activity	Detection	Reference
1A2	7-ethoxyresorufin O-deethylation	fluorescence	27
	7-ethoxyresorufin O-deethylation	HPLC	27
2A6	coumarin 7-hydroxylation	fluorescence	27
2B6	7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation	fluorescence	28
	7-ethoxy-4-(trifluoromethyl) coumarin O-deethylation	HPLC	29
2C8	luciferin-6' methyl ether demethylation (P450-Glo substrate)	luminescence	Tech. Bulletin No 325 www.promega.com
2C9	diclofenac 4'-hydroxylation	HPLC	30
	6'-deoxyluciferin hydroxylation (P450-Glo substrate)	luminescence	Tech. Bulletin No 325 www.promega.com
2C19	(S)-mephenytoin 4'-hydroxylation	HPLC	www.cypex.co.uk Cypex 2C19 QC assays
2D6	bufuralol 1'-hydroxylation	HPLC	30
2E1	chlorzoxazone 6-hydroxylation	HPLC	31
3A4	testosterone 6 β -hydroxylation	HPLC	32
	luciferin-6' benzyl ether debenzilation (P450-Glo substrate)	luminescence	Tech. Bulletin No 325 www.promega.com

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 6 β -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 $[\text{Pt}(\text{ox})(\text{Ros})_2] \cdot \frac{3}{4}\text{H}_2\text{O}$ (1) concentrations for inhibition of CYP3A4, testosterone 6 β -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.³⁴⁻³⁷).

We reported earlier³⁸ 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 Dixon^{26,39} 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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REFERENCES

1. Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences* 2001;58(5-6):737-47.
2. Guengerich FP. Human cytochrome P450 Enzymes. In: Ortiz de Montellano PR, editor. *Cytochrome P450. Structure, mechanism and biochemistry*. New York: Kluwer Academic/Plenum; 2006. p. 377-530.
3. Bachmann KA, Lewis JD, Fuller MA, Bonfiglio MF. *Drug Interactions Handbook*. Hudson, OH: 2nd Edition. Lexi-Comp; 2004.
4. Rosenberg B, Vancamp L, Trosko JE, Mansour VH. Platinum Compounds - a New Class of Potent Antitumour Agents. *Nature* 1969;222(5191):385-6.
5. Gielen M, Tiekink ERT. *Metallotherapeutic Drugs and Metal-Based Diagnostic Agents: The Use of Metals in Medicine*. London: 1st. John Wiley & Sons, Ltd; 2005.
6. Brabec V. DNA modifications by antitumor platinum and ruthenium compounds: Their recognition and repair. *Progress in Nucleic Acid Research and Molecular Biology* 2002;71:1-68.
7. Kostova I. Ruthenium complexes as anticancer agents. *Current Medicinal Chemistry* 2006;13(9):1085-107.
8. Chabner B, Amrein P, Druker B, Michaelson M, Mitsiades C, Goss P, et al. Antineoplastic agents. In: Brunton L, Lazo J, Parker K, editors. *Goodman and Gilman's The pharmacological basis of therapeutics*. New York: McGraw-Hill; 2006. p. 1315-1403.
9. Davies PJ. *Plant hormones*. Dordrecht: third. Springer; 1997.
10. Meijer L, Borgne A, Mulner O, Chong JPJ, Blow JJ, Inagaki N, Inagaki M, Delcros JG, Moulinoux JP. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *European Journal of Biochemistry* 1997;243(1-2):527-36.
11. Benson C, Kaye S, Workman P, Garrett M, Walton M, de Bono J. Clinical anticancer drug development: targeting the cyclin-dependent kinases. *British Journal of Cancer* 2005;92(1):7-12.
12. Diaz-Padilla I, Siu LL, Duran I. Cyclin-dependent kinase inhibitors as potential targeted anticancer agents. *Investigational New Drugs* 2009;27(6):586-94.
13. Ljungman M, Paulsen MT. The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. *Molecular Pharmacology* 2001;60(4):785-9.
14. Dvorak L, Popa I, Starha P, Travnicek Z. In Vitro Cytotoxic-Active Platinum(II) Complexes Derived from Carboplatin and Involving Purine Derivatives. *European Journal of Inorganic Chemistry* 2010;22:3441-8.

15. Starha P, Popa I, Travnicek Z. Palladium(II) oxalato complexes involving N6-(benzyl)-9-isopropyladenine-based N-donor carrier ligands: Synthesis, general properties, H-1, C-13 and N-15{H-1} NMR characterization and in vitro cytotoxicity. *Inorganica Chimica Acta* 2010;363(7):1469-78.
16. Starha P, Popa I, Travnicek Z, Vanco J. N6-Benzyladenosine Derivatives as Novel N-Donor Ligands of Platinum(II) Dichlorido Complexes. *Molecules* 2013;18(6):6990-7003.
17. Travnicek Z, Starha P, Vanco J, Silha T, Hosek J, Suchy P, Prazanova G. Anti-inflammatory Active Gold(I) Complexes Involving 6-Substituted-Purine Derivatives. *Journal of Medicinal Chemistry* 2012;55(10):4568-79.
18. Travnicek Z, Szucova L, Popa I. Synthesis, characterization and assessment of the cytotoxic properties of cis and trans-[Pd(L)(2)Cl-2] complexes involving 6-benzylamino-9-isopropylpurine derivatives. *Journal of Inorganic Biochemistry* 2007;101(3):477-92.
19. Vrzal R, Starha P, Dvorak Z, Travnicek Z. Evaluation of in vitro cytotoxicity and hepatotoxicity of platinum(II) and palladium(II) oxalato complexes with adenine derivatives as carrier ligands. *Journal of Inorganic Biochemistry* 2010;104(10):1130-2.
20. Liskova B, Zerkankova L, Novakova O, Kostrhunova H, Travnicek Z, Brabec V. Cellular Response to Antitumor cis-Dichlorido Platinum(II) Complexes of CDK Inhibitor Bohemine and Its Analogues. *Chemical Research in Toxicology* 2012;25(2):500-9.
21. Travnicek Z, Starha P, Popa I, Vrzal R, Dvorak Z. Roscovitine-based CDK inhibitors acting as N-donor ligands in the platinum(II) oxalato complexes: Preparation, characterization and in vitro cytotoxicity. *European Journal of Medicinal Chemistry* 2010;45(10):4609-14.
22. Brown HS, Galetin A, Hallifax D, Houston JB. Prediction of in vivo drug-drug interactions from in vitro data - Factors affecting prototypic drug-drug interactions involving CYP2C9, CYP2D6 and CYP3A4. *Clinical Pharmacokinetics* 2006;45(10):1035-50.
23. Kharasch ED, Hankins DC, Taraday JK. Single-dose methoxsalen effects on human cytochrome P-450 2A6 activity. *Drug Metabolism and Disposition* 2000;28(1):28-33.
24. Bourrie M, Meunier V, Berger Y, Fabre G. Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. *Journal of Pharmacology and Experimental Therapeutics* 1996;277(1):321-32.
25. Stiborova M, Borek-Dohalska L, Hodek P, Mraz J, Frei E. New selective inhibitors of cytochromes P4502B and their application to antimutagenesis of tamoxifen. *Archives of Biochemistry and Biophysics* 2002;403(1):41-9.
26. Segel IH. *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems.* New York: Wiley-Interscience; 1993.
27. Waxman DJ, Chang TKH. Spectrofluorometric analysis of CYP2A6-catalyzed coumarin 7 hydroxylation. In: Phillips IR; Shephard EA, editors. *Cytochrome P450 protocols.* Totowa (NJ): Humana Press; 1998. p. 111-116.
28. Donato MT, Jimenez N, Castell JV, Gomez-Lechon MJ. Fluorescence-based assays for screening nine cytochrome P450 (P450) activities in intact cells expressing individual human P450 enzymes. *Drug Metabolism and Disposition* 2004;32(7):699-706.
29. Morse MA, Lu J. High-performance liquid chromatographic method for measurement of cytochrome P450-mediated metabolism of 7-ethoxy-4-trifluoromethylcoumarin. *Journal of Chromatography B* 1998;708(1-2):290-3.
30. Crespi CL, Chang TKH, Waxman DJ. Determination of CYP2C9-catalyzed diclofenac 4'-hydroxylation by high-performance liquid chromatography. In: Phillips IR; Shephard EA, editors. *Cytochrome P450 protocols.* Totowa (NJ): Humana Press; 1998. p. 129-40.
31. Lucas D, Menez JF, Berthou F. Chlorzoxazone: An in vitro and in vivo substrate probe for liver CYP2E1. *Cytochrome P450, Pt B* 1996;272:115-23.
32. Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ. Characterization of Rat and Human-Liver Microsomal Cytochrome-P-450 Forms Involved in Nifedipine Oxidation, a Prototype for Genetic-Polymorphism in Oxidative Drug-Metabolism. *Journal of Biological Chemistry* 1986;261(11):5051-60.
33. Brunton L, Lazo J, Parker K, Goodman and Gilman's *The pharmacological basis of therapeutics.* New York: McGraw-Hill; 2006.
34. Hendrychova T, Berka K, Navratilova V, Anzenbacher P, Otyepka M. Dynamics and Hydration of the Active Sites of Mammalian Cytochromes P450 Probed by Molecular Dynamics Simulations. *Current Drug Metabolism* 2012;13(2):177-89.
35. Williams PA, Cosme J, Vinkovic DM, Ward A, Angove HC, Day PJ, Vonrhein C, Tickle IJ, Jhoti H. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* 2004;305(5684):683-6.
36. Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, Johnson EF. The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-angstrom resolution. *Journal of Biological Chemistry* 2004;279(37):38091-4.
37. Johnson EF, Sansen S, Reynald RL, Yano JK, Schoch GA, Stout CD. Update on CYP crystal structures and applications. *Drug Metabolism Reviews* 2006;38:25.
38. Masek V, Anzenbacherova E, Machova M, Brabec V, Anzenbacher P. Interaction of antitumor platinum complexes with human liver microsomal cytochromes P450. *Anti-Cancer Drugs* 2009;20(5):305-11.
39. Burlingham BT, Widlanski TS. An intuitive look at the relationship of K-i and IC50: A more general use for the Dixon plot. *Journal of Chemical Education* 2003;80(2):214-8.