in vitro and *in silico* Studies of Interaction of Synthetic 2,6,9-Trisubstituted Purine Kinase Inhibitors BPA-302, BP-21 and BP-117 With Liver Drug-Metabolizing Cytochromes P450

Alena ŠPIČÁKOVÁ[1], Petr KRAUS[1,2], Tomáš GUCKÝ[2], Vladimír KRYŠTOF[2], Miroslav STRNAD[2], Václav BAZGIER[3], Michal OTYEPKA[3], Vendula KUBÍČKOVÁ[1], Martin PORUBA[1], Zuzana RÁCOVÁ[1], Iveta ZAPLETALOVÁ[1], Pavel ANZENBACHER[1]

[1]Department of Pharmacology Faculty of Medicine, Palacký University Olomouc, Olomouc, Czech Republic, [2]Laboratory of Growth Regulators, Palacký University Olomouc and Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czech Republic, [3]Regional Centre of Advanced Technologies and Materials, Palacký University Olomouc, Olomouc, Czech Republic

Summary

An evaluation of possible interactions with enzymes of drug metabolism (cytochromes P450, CYP) is an important part of studies on safety and, in general, on the properties of any drug or biologically active compound. The article is focused on the preliminary metabolic study of selected 2,6,9-trisubstituted purine kinase inhibitors with significant anticancer activities which we have developed. The compounds BP-21 and BP-117 represent strong CDK inhibitors and the compound BPA-302 was developed as selective FLT3-ITD kinase inhibitor. Here, emphasis is placed on interactions of these compounds with the nine most important forms of CYP to evaluate the possibility of inhibition of these enzymes. The possibility of their inhibitory effect was studied *in vitro* on selected human liver microsomal CYP enzymes. The most affected enzyme was CYP2C19. Its activity dropped to 22 % of its original value by BPA-302, to 13 % by BP-21 and to 6 % by BP-117 at the highest concentration tested (250 $\mu mol \cdot l^{-1}$). The results suggest that the metabolism of concomitantly administered drugs should not be significantly affected at lower doses. Molecular docking of BPA-302 indicated that it can bind to active site of both CYP2C19 and CYP2D6 enzymes above the heme cofactor corroborating the experimental data.

Key words

Cytochrome P450 inhibition • High performance liquid chromatography • Cyclin-dependent kinase inhibitor • FLT3-ITD inhibitor • Drug metabolism

Corresponding author

P. Anzenbacher, Department of Pharmacology**,** Faculty of Medicine, Palacký University Olomouc, Hněvotínská 3, 77515 Olomouc, Czech Republic. E-mail: anzen@seznam.cz

Introduction

The cyclin-dependent kinases (CDKs) are essential regulators of cell cycle and apoptosis. During the cell cycle, they are activated by cyclins and phosphorylate target molecules, such as transcriptional regulators, which in turn induce the expression of phase-specific genes, stimulate DNA replication, or initiate mitosis. CDK control functions are impaired in most types of cancer cells (Hanahan and Weinberg 2011). Therefore, pharmacological inhibition of CDKs has become a potential therapeutic method for cancer treatment (Otto and Sicinski 2017). (*R*)-roscovitine, also known as seliciclib or CYC202, is pan-selective ATP-competitive inhibitor of CDK1, 2, 5, 7 and 9. It was evaluated in combination with sapacitabine in patients with advanced solid tumors in the first phase of clinical trials and in the second phase of clinical trials in patients with nasopharyngeal tumors (Yeo *et al.* 2009). Clinical trials with CDK inhibitors partially confirmed the anticancer activity of this class of compounds and roscovitine (Cicenas *et al.* 2015); for example, clinical trial with roscovitine indicated a partial response in

a patient with hepatocellular carcinoma and a sustained tumour stabilisation. Effective plasma levels of CDK inhibitors are in the micromolar range (Cicenas *et al.* 2015, van der Biessen *et al.* 2014).

Further optimization of roscovitine led to development of significantly more potent CDK inhibitors BA-12 and BP-14 (Zatloukal *et al.* 2013, Gucký *et al.* 2013). Both these compounds have shown anti-tumor activity in hepatocellular carcinoma (Haider *et al.* 2013). Interestingly, parallel modification of inhibitor BP-14, in which the 6-benzylaminopurine core was replaced with a 6-anilinopurine, led to compound BPA-302 with significantly different kinase selectivity and anticancer activity. BPA-302 displays nanomolar potency on the oncogenic receptor tyrosine kinase FLT3-ITD and selective cytotoxic activity in acute myeloid leukemia cell lines expressing FLT3-ITD (Gucký *et al.* 2018). There is hope that novel variants of CDK inhibitors will be even more successful. Results (Wang *et al.* 2020) are very promising, however, clinical trials will bring hopefully more significant outcomes (e.g. with novel CDK inhibitors). Details of *in vitro* studies as well as clinical trials are presented in (Cicenas *et al.* 2015). These findings stimulated our interest in further characterization of these compounds as drug candidates in terms of metabolic stability, drug interactions and pharmacokinetics.

CYPs are the most important enzymes of the first steps of drug metabolism (Anzenbacher and Anzenbacherová 2001, Anzenbacher and Zanger 2012, Ortiz de Montellano 2005, Zanger and Schwab 2013). These hemoproteins are localized in many tissues of the human organism (e.g. liver, lungs, brain, heart, kidney, and intestines). Subcellular localization of CYP enzymes is typically membrane of endoplasmatic reticulum and mitochondria (Šrejber *et al.* 2018). The main function of CYPs is formation of more polar metabolites of drugs either by inserting of polar group into parent molecule (e.g. hydroxylation) or by liberation of a present functional group (e.g. demethylation of a methoxy group). Fifty-seven human CYP forms are known; in pharmacology, focus is given to CYPs present in human liver, important in biotransformation of drugs (Anzenbacher and Anzenbacherová 2001, Anzenbacher and Zanger 2012, Guengerich 2003). Monitoring of drug-drug interactions is very important in the case of new potential drugs (Špičáková *et al.* 2016, Špičáková *et al.* 2019, Tománková *et al.* 2016).

We investigated the interactions between liver microsomal cytochromes P450 (CYP), which are the main enzymes of the first phase of metabolism of xenobiotics, and three kinase inhibitors: BP-21, BP-117 (both CDK inhibitors) and BPA-302 (FLT3-ITD inhibitor). Studies of interactions of these three potential drugs with CYPs and detection of possible metabolites formed by CYP enzymes are important for evaluation of drug-drug interactions, which may cause serious adverse effects (Anzenbacher and Anzenbacherová 2001). Molecular docking of BPA-302 was then performed to find how it interacts not with most sensitive CYPs.

For molecular docking, compound BPA-302 has been chosen as the most perspective kinase inhibitor among the three CDK inhibitors studied here. This compound (according to the results obtained) also interacts not only with the CYP2C19 form (a representative of structurally related CYP2C forms), but also with the CYP2D6 - known to metabolize about 20 % of clinically used drugs metabolized by CYPs (Zanger and Schwab 2013).

Methods

Chemicals and reagents

BPA-302 (N^2-(4-amino-cyclohexyl)-9-cyclopentyl-N^6-(4-morpholin-4-ylmethyl-phenyl)-*9H*-purine-2,6-diamine), BP-21 (4-{9-cyclopentyl-6-((6-furan-2-yl-pyridin-3-ylmethyl)-amino)-*9H*-purine-2-ylamino}-cyclohexanol) and BP-117 (1-{9-cyclopentyl-6-((6-furan-2-yl-pyridin-3-ylmethyl)-amino)-*9H*-purine-2-ylamino}-2-methyl-propan-2-ol) were synthesized and characterized according to described procedures (Gucký *et al.* 2013, Gucký *et al.* 2018). Structures of tested compounds are shown in Fig. 1.

7-Ethoxyresorufin and ethoxy-4-(trifluoro-methyl)coumarin were supplied by Fluka (Buchs, Switzerland). All other chemicals were purchased from Sigma Aldrich CZ (Prague, Czech Republic). All other common laboratory chemicals with the HPLC or analytical grade were obtained from the same company. Pooled human liver microsomes (HLM) were obtained from Biopredic (Rennes, France). Microsomes were obtained in accordance with ethical regulations of the country of origin (France). They were from seventeen donors (ten males and seven females) with a protein content of 25 mg/ml and total CYP content of 14.55 μmol·l^{-1}; CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5 enzyme activities were verified before the experiment with minimal consumption of the sample.
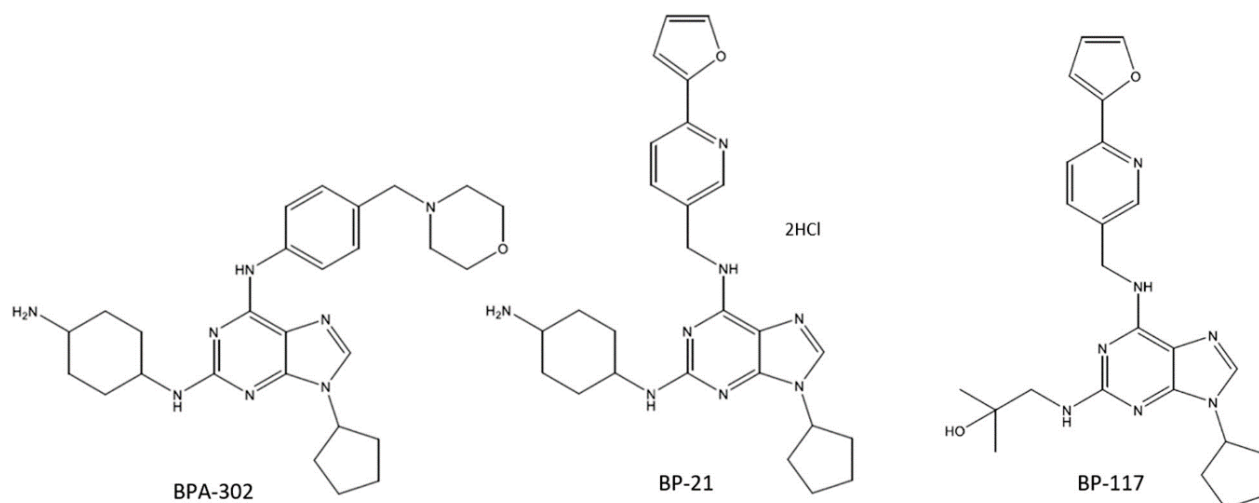
Fig. 1. Structures of tested compounds: BPA-302, BP-21 and BP-117.

Table 1. Incubation conditions for individual CYP assays used in the inhibition study.

CYP	Substrate concentration ($\mu mol \cdot l^{-1}$)	Substrate	Reaction catalyzed by CYP	Content CYP (nmol)	Reaction volume (μl)	Quench reagent	Method of detection
1A2	5.2	7-ethoxyresorufin	*O*-deethylation	35	100	200 µl methanol	fluorescence ex. 535 nm; em. 585 nm
2A6	15	coumarin	7-hydroxylation	35	100	200 µl methanol	fluorescence ex. 325 nm; em. 450 nm
2B6	15	EFC	*O*-deethylation	35	100	200 µl methanol	fluorescence ex.410 nm; em. 510 nm
2C8	150	luciferin-ME	demethylation	1000	50	luciferin detection reagent	luminescence
2C9	20	diclofenac	4´-hydroxylation	35	200	50 µl ACN/CH$_3$COOH	UV, 280 nm
2C19	10	luciferin-H EGE	6´-hydroxylation	250	50	luciferin detection reagent	luminescence
2D6	14.3	bufuralol	1´-hydroxylation	67	200	20 µl 70 % HClO$_4$	fluorescence ex. 252 nm; em. 302 nm
2E1	26	chlorzoxazon	6β-hydroxylation	160	1000	50 µl 42.5 % H$_3$PO$_4$	UV, 287 nm
3A4	100	testosterone	6β-hydroxylation	100	500	100 l Na$_2$CO$_3$/NaCl	UV, 245 nm

Enzyme assays

All microsomal CYP activities were assayed according to well established protocols.

Following enzyme assays were performed to determine activities of specific CYP enzymes: CYP1A2, 7-ethoxyresorufin *O*-deethylation (Chang and Waxman 1998); CYP2A6, coumarin 7-hydroxylation (Soucek 1999); CYP2B6, 7-ethoxy-4-(trifluoromethyl) coumarin (EFC) *O*-deethylation (Donato *et al.* 2004); CYP2C8 luciferin-6´methyl ether (luciferin-ME) demethylation; CYP2C19, deoxyluciferin ethylene glycol ester (luciferin-H EGE) 6´-hydroxylation (Promega Technical

Bulletin No.325, http://www.promega.com); CYP2C9, diclofenac 4′-hydroxylation (Crespi *et al.* 1998a); CYP2D6, bufuralol 1′-hydroxylation (Crespi *et al.* 1998b); CYP2E1, chlorzoxazone 6β-hydroxylation (Lucas *et al.* 1996); CYP3A4/5, testosterone 6β-hydroxylation (Guengerich *et al.* 1986).

Incubation mixtures contained 100 mmol·l⁻¹ potassium phosphate buffer (pH 7.4), NADPH-generating system (0.8 mmol·l⁻¹ NADP⁺, 5.8 mmol·l⁻¹ isocitrate, 0.3 unit/ml of isocitrate dehydrogenase and 8 mmol·l⁻¹ MgCl₂), HLM and individual probe substrate. Assay conditions are listed in Table 1.

For determination of metabolites formed from specific substrates, Prominence LC-20A HPLC system (Shimadzu, Kyoto, Japan) with UV (6β-hydroxy-chlorzoxazone, 4′-hydroxydiclofenac, 6β-hydroxytesto-sterone) or with fluorescence detection (resorufin, 7-hydroxycoumarin, 7-hydroxy-4-(trifluoro-methyl)cou-marin, 1′-hydroxybufuralol) was used. A TECAN Infinite M200 absorbance/fluorescence/ luminescence reader (Tecan, Vienna, Austria) was used for the detection of luciferin luminescence.

Enzyme inhibition studies

K_M and V_{max} values were determined for each enzyme assay to find the optimal concentration of the specific substrates for the inhibition experiments. Substrate concentration was chosen close to K_M. Data were analyzed using Sigma Plot v. 12.0 graphing software (Jandel Scientific, Chicago, IL, USA). Inhibition experiments were performed as three duplicate experiments at 0, 3.9, 7.8, 15.63, 31.25, 62.5, 125, and 250 µmol·l⁻¹ concentration of each compound; individual values differed less than 10 %. Experimental conditions with tested compounds were the same as for determination of individual CYP activities (Table 1). Reaction mixtures were preincubated with potential inhibitors (BPA-302, BP-21 and BP-117) at 37 °C for 30 min. Inhibition of individual CYP activities was in all cases evaluated by plotting respective remaining activity versus the inhibitor concentration.

Statistical analysis

Microsomal CYP activities obtained from enzyme inhibition studies were evaluated using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). All experimental data obtained from these studies were compared with data of controls (without the presence of tested inhibitor) using one-way ANOVA

combined with Duncan multiple range test on the significance level of 0.01. Statistical analyses were performed with the Statistica software package (ver. 12; TIBCO Software, Inc., Palo Alto, CA, USA).

Molecular docking

The compound BPA-302 was prepared using MarvinSketch 15.1.5.0 software package (http://www.chemaxon.com) for molecular docking. Polar hydrogens were explicitly considered and Kollman charges assigned using AutoDock Tools program suite (Morris *et al.* 2009). Autodock Vina program (Trott *et al.* 2010) was used for docking of BPA-302 on a grid box containing the complete cytochrome P450 enzyme structure, which was centered close to the heme cofactor. The crystal structures of CYP2C19 (i.e., CYP2C19 with bound 4-hydroxy-3,5-dimethylphenyl)(2-methyl-1-benzofuran-3-yl)methanone henceforth 0XV, Protein Data Bank (PDB)ID: 4GQS (Reynald *et al.*, 2012)) and CYP2D6 (with bound (4aR,6R,8aS)-8a-(2,4-difluorophenyl)-6-(1H-pyrazol-4-yl)-4,4a,5,6,8,8a-hexa-hydropyrano(3,4-d)(1,3)thiazin-2-amine henceforth SI6, PDBID: 4XRZ (Brodney *et al.* 2015)) were used to construct the enzyme docking templates after removal of the bound ligand (0XV or SI6).

Results

*Inhibition of specific CYP activities in HLM*

In the present study, the potential inhibition of nine microsomal CYPs by kinase inhibitors BPA-302, BP-21 and BP-117 was studied. At first, enzyme kinetics of individual CYP forms was determined using standard substrates. Experiments assessing the inhibition of enzyme activities of nine CYP forms by BPA-302, BP-21 or BP-117 were then performed. Fig. 2 shows summarized results of the inhibition experiments (Fig. 2A, for BPA-302; Fig. 2B, for BP-21; Fig. 2C for BP-117). Compound BPA-302 inhibits significantly the CYP2C19 activity to 22 % of the corresponding control; this is however apparent only at higher concentrations (62.5 - 250 µmol·l⁻¹, Fig. 2A). It also inhibits the CYP2D6 activity in a concentration-dependent manner; however, the extent of inhibition is relatively small (decrease of activity to 50 - 35 % at two highest levels of the inhibitor). Both the BP-21 and BP-117, are able to inhibit the CYP2C9 (to 19 % of corresponding control, and to 17 % of corresponding control, respectively), and CYP2C19 (to 13 %

of corresponding control, and to 6 %, respectively) activities at the highest concentration of inhibitor (250 $\mu mol \cdot l^{-1}$, Fig. 2B, 2C). In all cases of tested compounds (BPA-302, BP-21 and BP-117) the CYP2C19 was the most affected and its IC_{50} value was determined as follows: $IC_{50} = 28.86 \pm 12.69 \ \mu mol \cdot l^{-1}$; $34.07 \pm 4.91 \ \mu mol \cdot l^{-1}$; $23.88 \pm 9.02 \ \mu mol \cdot l^{-1}$, respectively.

Molecular modelling

The BPA-302 was docked into crystal structures of CYP2C19 and CYP2D6 in order to assess its binding to active sites of the respective enzymes. Both enzymes structures contained bound ligand (0XV see Methods), which was removed before the docking experiment. An applicability of the used docking method was evaluated by re-docking of the ligand, which was present in the used crystal structures. In both cases the docking identified the native ligand position as the most favorable (Figs. 3A and 3C).
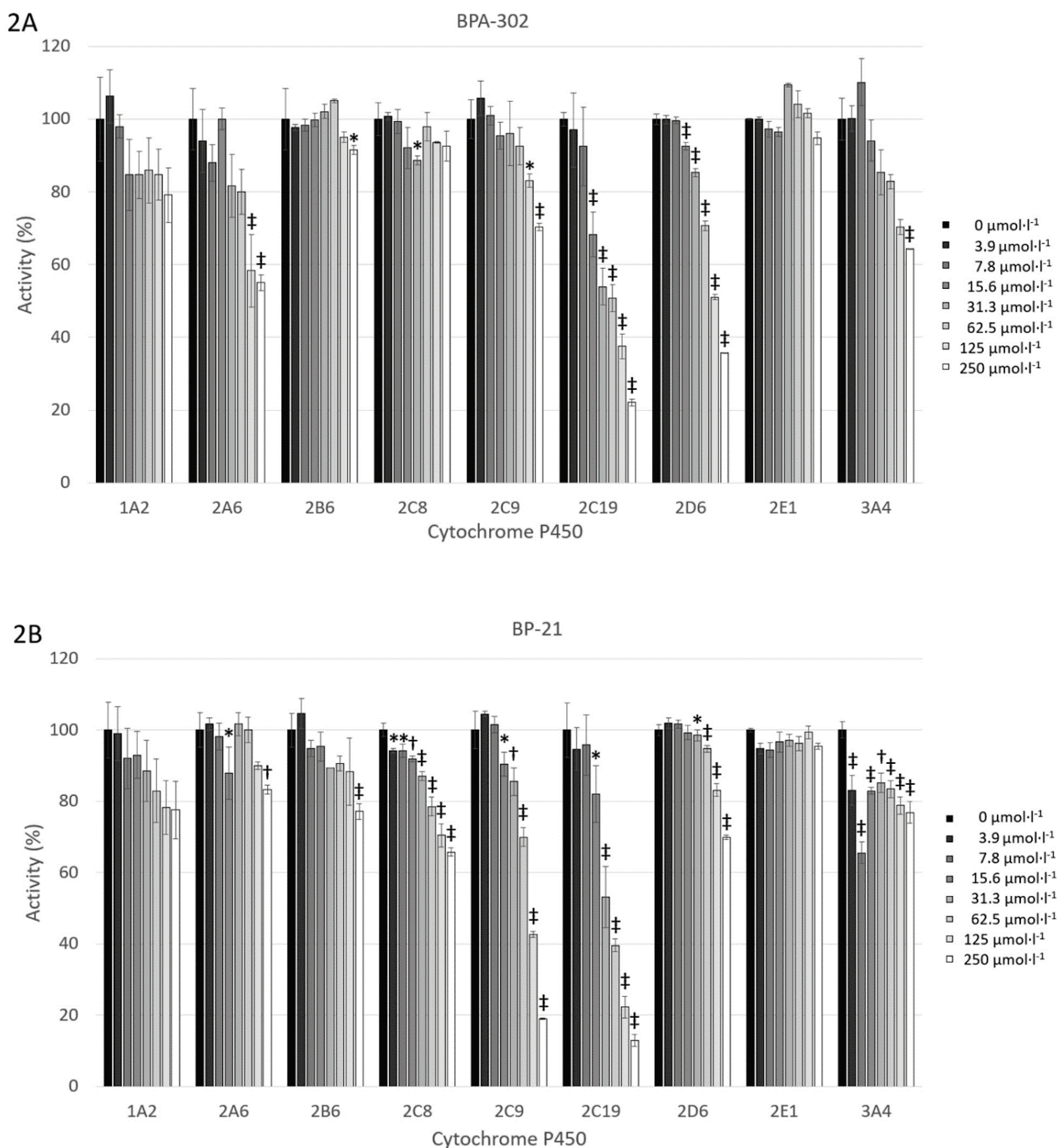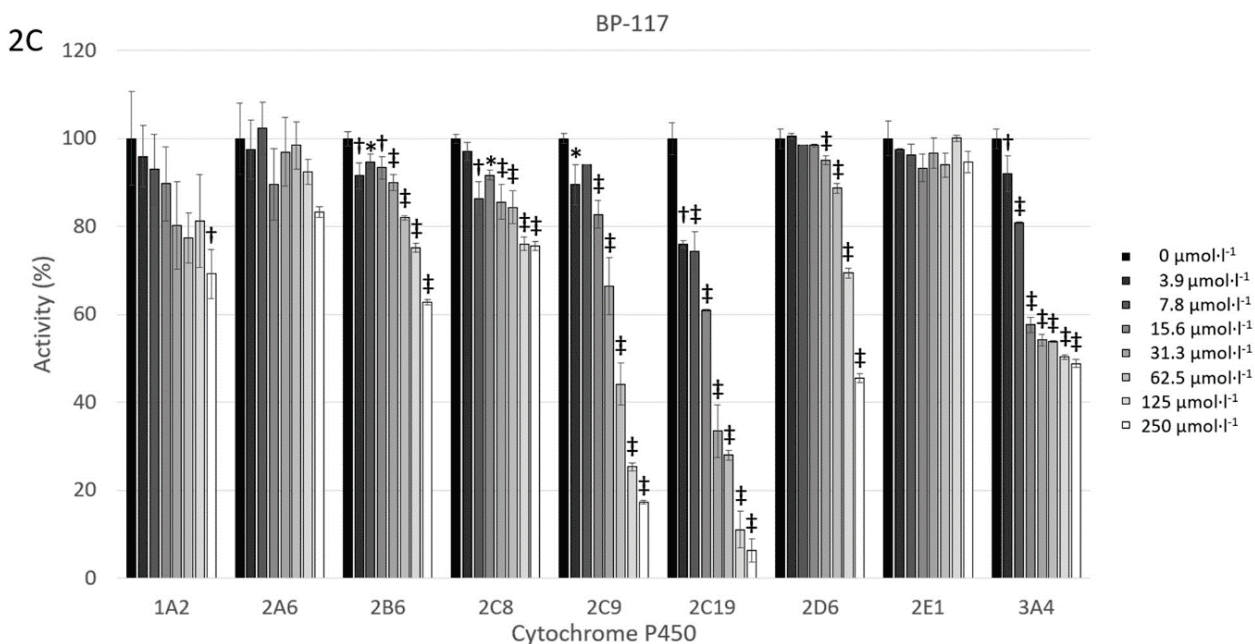
Fig. 2. Effects of studied kinase inhibitor (**A**) BPA-302, (**B**) BP-21 and (**C**) BP-117 on enzyme activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 in HLM. Inhibition of enzyme activity is expressed as the activity remaining relative to control (100 %, without kinase inhibitor) in percent. Concentration of an individual kinase inhibitor in the reaction mixture was 0, 3.9, 7.8, 15.6, 31.3., 62.5, 125 and 250 $\mu mol \cdot l^{-1}$. Activities represent the mean ± standard deviation; N = 3. A significant decrease of metabolite concentration measured in experimental sample vs. control (sample with 0 $\mu mol \cdot l^{-1}$ of kinase inhibitor) is labeled (*$p < 0.01$, †$p < 0.001$, ‡$p < 0.0001$).
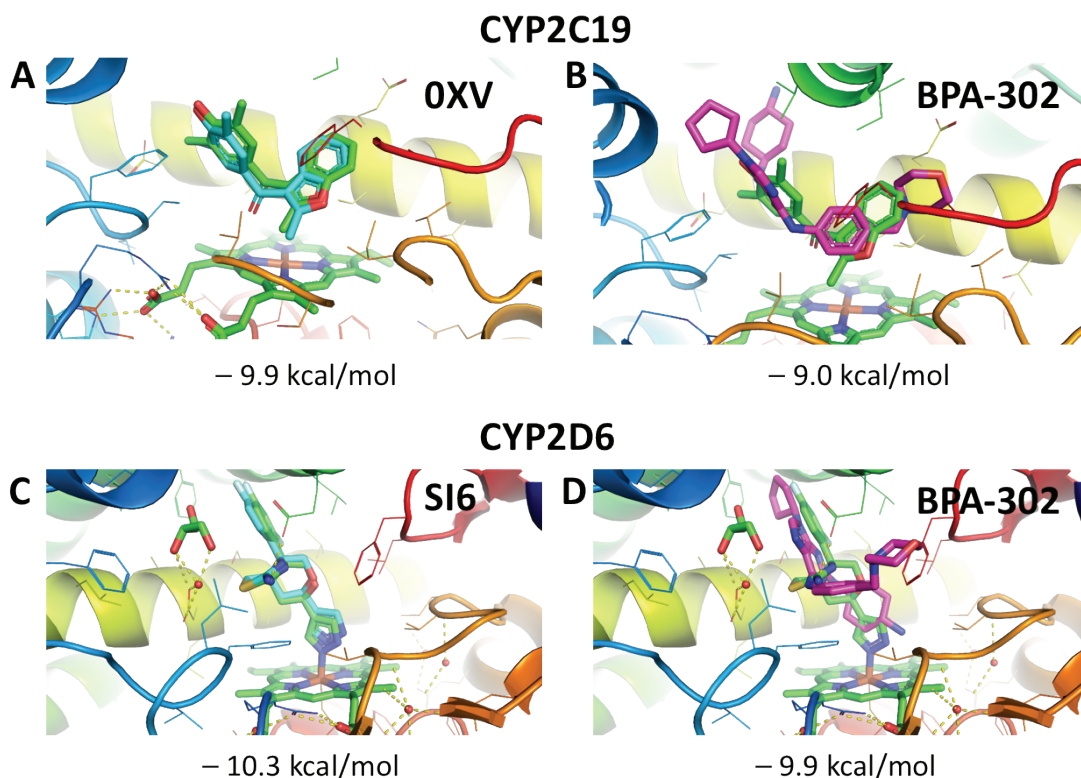

Fig. 3. Structures of CYP2C19 (panels **A** and **B**) and CYP2D6 (**C** and **D**) active sites with heme cofactor and ligands shown in stick models. Carbon atoms of crystal structures–of hemes and docked structures 0XV (CYP2C19, Figs. A and B) and SI6 (CYP2D6, Figs. C and D) are shown in green, oxygen atoms in red. The CYP I-helix, a typical structural motif of both CYPs, is shown by yellow curl. Reliability of docking procedure used documented by a re-docking of 0XV and SI6 (in panels A and C in turquoise) close to the original positions of 0XV and SI6 observed in (in panels A and C in green). BPA-302 docked to CYP2C19 and CYP2D6, in panels B and D, in magenta (crystal positions of PDB ligands 0XV and SI6 in both cases again in green for comparison). The energies in kcal/mol are estimated binding affinities by Autodock Vina. Hydrogens are not shown for clarity.

The docking experiment of BPA-302 into CYP2C19 active site (Fig. 3B) showed that BPA-302 binds into the CYP active site with a phenyl ring positioned above the heme cofactor. The bonding afinity - 9.0 kcal/mol was only slightly weaker than the binding activity of 0XV -9.9 kcal/mol, which may indicate rather comparative binding affinities of both ligands to CYP2C19 active site. In the case of CYP2D6 docking experiment, BPA-302 bounded to the active site with affinity of -9.9 kcal/mol, which was only slightly weaker that the predicted affinity of SI6 (-10.3 kcal/mol). These results clearly indicate that BPA-302 can bind to active sites of both CYP2C19 and CYP2D6 enzymes above the heme cofactor corroborating the experimental data.

Discussion

2,6,9-trisubstituted purines have been developed as CDK inhibitors and (*R*)-roscovitine entered clinical trials as candidate anticancer drug. Its pharmacokinetics and metabolism was already described (McClue and Stuart 2008, Nutley *et al.* 2005). Few its analogues were also studied in terms of metabolic stability and interactions with CYPs (Chmela *et al.* 2001, Rypka *et al.* 2002, Šiller *et al.* 2009).

Results of the *in vitro* experiments with related compounds show that neither of the three kinase inhibitors show reasonable inhibitory effect on enzyme activities of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, and 3A4. In contrast, compound BPA-302 inhibits significantly the CYP2C19 activity to 22 % of the corresponding control; this is however apparent only at the higher concentration (62.5 - 250 µmol·l⁻¹, Fig. 2A). This compound is also able to inhibit also the CYP2D6 activity in a concentration-dependent manner. Given the fact that BPA-302 is acting at low nanomolar concentrations specifically in acute myeloid leukemia models (Gucky *et al.* 2018), inhibition of these two CYPs seems not to be clinically relevant issue. The docking experiment showed that BPA-302 can bind into active site of both CYP2C19 and CYP2D6 with estimated binding affinity of -9.0 and -9.9 kcal/mol, respectively, close to the values obtained with bioactive ligands 0XV and SI6 known to strongly bind to these two CYP proteins (Reynald *et al.* 2012, Brodney *et al.* 2015). Both BP-21 and BP-117 are able to inhibit the CYP2C9 (to 19 % of corresponding control, and to 17 % of corresponding control, respectively) and CYP2C19 (to 13 % of corresponding control, and to 6 %, respectively)

activities at the highest concentration of inhibitor (250 µmol·l⁻¹, Fig. 2B, 2C). Hence, the docking experiment confirmed the possibility of a relatively strong interaction of the kinase inhibitor BPA-302 with these two CYP enzymes.

The determined IC_{50} values of BPA-302, BP-21 and BP-117 ($IC_{50} = 28.86 \pm 12.69$ µmol·l⁻¹; 34.07 ± 4.91 µmol·l⁻¹; 23.88 ± 9.02 µmol·l⁻¹, respectively) indicate a strong interaction of tested compounds with CYP2C19. Drugs metabolized by this enzyme are often proton pump inhibitors (PPIs), such as omeprazole and pantoprazole, as well as the prodrug clopidogrel, which is activated to effective anticoagulant (McTavish *et al.* 1991). Co-administration of BP-117 with drugs metabolised by CYP2C19 could lead (because of its interaction with PPI) to increased gastric acid production, gastric ulcers, duodenal ulcers, etc.; in the case of clopidogrel it could lead to bleeding (McTavish *et al.* 1991, Zanger and Schwab 2013). In other words, a possibility of this effect should be considered when higher doses and concentrations are used and clinical investigation of this interaction should be considered and further examined. On the other hand, the real possibility of drug interaction in clinical praxis is less probable as the plasma levels of CDK inhibitors (as reported from clinical data) are in the micromolar range (Cicenas *et al.* 2015, van der Biessen *et al.* 2014).

Taken together, the results obtained show that the kinase inhibitors studied are promising drug candidates, at least because of their relatively low potential to inhibit the liver CYP enzymes. However, the possibility of this interaction should be considered, namely, because of known inter-individual variations in drug metabolism (Anzenbacher and Zanger 2012, Zanger and Schwab 2013). Apparently, the studies of this kind deserve a continuation aimed e.g. at CYP3A4 and other CYP enzymes. Possibly, studies on the interaction with CYP enzymes should be oriented not only to these involved in drug metabolism, but also with other CYP forms, activity of which may be influenced by interaction with biologically active substances and xenobiotics (as e.g. CYPs involved in steroidogenesis).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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