Evaluation of the inhibition performance of pyrrole derivatives against CYP450 isoforms

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Abstract
Cytochrome P450 is a heme-containing superfamily accountable for the oxidation of various pharmacologically active drugs. The aim of this study was to conduct in silico and in vitro activity assessments of recently synthesized pyrrole-based compounds against three major CYP450 isoforms – CYP1A2, CYP2D6 and CYP3A4. The in vitro study contained specific cytochrome P450 isoform inhibitors and substrates (for CYP1A2, CYP2D6, and CYP3A4) to determine the inhibition performance of the tile compounds at 1 µM concentration. The in vitro data showed that none of the implemented molecules (MI_a-e and MII_a-e) are capable of inhibiting neither of the CYP isoforms. In addition, the potential interactions of the title compounds and the evaluated CYP isoforms were displayed after molecular docking with Glide (Schrödinger). Induced-fit simulations and binding free energy (MM/GBSA) calculations were applied to elucidate the accessibility in each CYP isoform. None of the pyrrole-based compounds exerted significant inhibition towards the applied isoenzymes. Two of the best scored compounds were visualized in the active site of CYP3A4 (PDB: 2V0M). Overall, good correlation between the in vitro results and the free binding MM/GBSA recalculations was observed.

Keywords
CYP450, molecular docking, choline, MM/GBSA, induced-fit docking

Introduction
Cytochrome P450 is a heme-containing superfamily of enzymes which are accountable for the oxidation of the vast majority of the pharmacologically active drugs (Manikandan et al. 2018). The cytochrome P450 system includes 57 isoforms of which five (CYP1A2, CYP2D6 and CYP3A4) are accountable for more than 90% of the metabolic reactions. The most common isoform (CYP3A4) is located in the liver bile duct and jejunum columnar epithelial cells. In the former it catalyzes the metabolism of approximately 50% of the registered therapeutic agents (McDonnell et al. 2013). The issues associated with the use of CYP450 inhibitors are of great importance. The inhibition of CYPs increases the plasma levels of pharmacologically active compounds, such as the influence of the CYP450 inhibitors on the response of antithrombotic therapy in some patients (Deodhar et al. 2020). Such unwanted interactions could be genetically determined or in the majority of the cases could occur during the concurrent use of CYP inhibitors. Thus, the initial assessments of the CYP450 inhibition capacities avoid the potential adverse reactions (Ogu et al. 2000).

Pyrrole is a five membered N-containing heterocyclic compound with a huge range of pharmacological effects. In the last several decades, the interest in the pyrrole de-
with resorufin applied as a substrate and ketoconazole as an inhibitor. The fluorescence for the different isoforms was measured at Ex/Em = 406/468 nm for CYP1A2; Ex/Em = 535/587 nm for CYP3A4 and Ex/Em = 390/468 nm for CYP2D6, respectively.

**Statistical analysis**

The different CYP450 isoform activity was normalized as percentage of the untreated control set as 100% and the results were expressed as mean values and standard deviation (±SD) (Graph Pad Prizm). Statistical analysis was performed by one-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Dunnet’s test) to assess the statistical differences in case of normal distribution. Values of p < 0.05 and p < 0.001 were considered statistically significant.

**Molecular docking**

**Selection and preparation of proteins**

The crystallographic structures of the employed in this study CYP isoforms – CYP1A2 (PDB ID: 2HI4), CYP2D6 (PDB ID: 4WNU) and CYP3A4 (PDB ID: 2V0M), resolved with the co-crystallized ligands alpha-naphthoflavone, quinidine and ketoconazole, respectively, were downloaded from the Protein Data Bank (PDB). The Protein Preparation module in Maestro (Schrödinger Release 2023-1: Protein Preparation Wizard; Epik, Schrödinger, LLC, New York, NY, 2023.) was used for the protein refinements. Hydrogen bonds and ion states at physiological pH were applied followed by the removal of non-active water molecules. Subsequently, the energies of the crystallographic structures were minimized with the OPLS4 force field. The grid box was generated around the co-crystallized ligands by using the Receptor Grid Generation module in Maestro.

**Ligands preparation**

The chemical structures of compounds MI_a, MI_b, MI_c, MI_d, MI_e, MI_l, MI_b, MI_l_c, MI_l_d and MI_l_e were drawn in the 2D sketcher module of Maestro, and converted to the corresponding 3D structures with Ligprep (Schrödinger Release 2023-1: LigPrep, Schrödinger, LLC, New York, NY, 2023). Utilizing the module, hydrogen bonds and ionization states at pH 7.0 ± 2.0 were obtained. The charged groups were neutralized. Furthermore, the ligands’ energies were minimized with the OPLS4 force field.

**Docking protocol**

The molecular docking was carried out with the licensed software Glide (Schrödinger Release 2021-3: Glide, Schrödinger, LLC, New York, NY, 2021.). The program implements an empirically based scoring algorithm, which includes three options: High-throughput screening (HTS), Standard-Precision (SP) and Extra-Precision (XP) modes. For the current study the most precise docking mode – XP, was used. To further explore the calcu-
lated active conformations, the Induced-fit docking (IFD) mode in Maestro was implemented. The IFD observes the protein's active amino acids as fully flexible, which leads to exhaustive sampling. Finally, MM/GBSA (Molecular Mechanics-Generalized Born Surface Area) recalculation were used to determine the binding free energies of the acquired complexes.

Results and discussion

In vitro assays

The CYP450 superfamily of enzymes are involved in the metabolic pathways of various pharmacologically active compounds. The assessment of CYP450-mediated interactions is important because of the involvement in undesirable drug-drug interactions. Therefore, an initial evaluation toward the CYP450 activity for each active molecule is required in the process of drug development (Manikandan et al. 2018). The examined structures were initially synthesized by Tzankova et al. 2023. The series MI_a-e and MII_a-e were studied for possible inhibitory effects on some isoforms of Cytochrome P450 – CYP1A2; CYP2D6, which revealed genetic polymorphism; and CYP3A4, which metabolized over 60% of the drugs on the market.

The examined compounds (at concentration 1 µM) did not reveal any statistically significant inhibitory effects on the different isoforms (Figs 2–4). The risk for possible drug interactions between these compounds and drugs, substrates of the exam isoforms, does not exist. Inhibitory effects exerted only the classical inhibitors: α-Naphthoflavone, a classical CYP1A2 inhibitor, at concentration 1 µM, inhibited CYP1A2 enzyme activity with 50%, compared to the control (pure CYP1A2) (Fig. 2).

![Figure 2](image-url) **Figure 2.** Effects of two series MI_a-e and MII_a-e, as well as α-Naphthoflavone (at concentration 1 µM), on CYP1A2 enzyme activity. ***P < 0.001 vs control (pure CYP1A2).

![Figure 3](image-url) **Figure 3.** Effects of two series MI_a-e and MII_a-e, as well as Quinidine (at concentration 1 µM), on CYP2D6 enzyme activity. ***P < 0.001 vs control (pure CYP2D6).
Quinidine, a classical CYP2D6 inhibitor, at concentration 1 µM, inhibited CYP2D6 enzyme activity with 55%, compared to the control (pure CYP2D6) (Fig. 3).

Ketoconazole, a classical CYP3A4 inhibitor, at concentration 1 µM, inhibited CYP3A4 enzyme activity with 45%, compared to the control (pure CYP3A4) (Fig. 4).

The in vitro results demonstrated that none of the applied pyrrole-based compounds (MI_a-e and MII_a-e) are capable of inhibiting the CYP1A2, CYP2D6 and CYP3A4 isoforms. The results could be related to the unavailability of the MAO-B inhibitors to be placed in the active site of the CYP450 isoforms. It could be hypothesized that the presented compounds are metabolized by non-specific arylamidase. The latter theory is in relation with the similarity of the title compounds with the antidepressant Isocarboxazid. Isocarboxazid (Marplan) is a non-selective, irreversible monoamine oxidase (MAO) inhibitor classified as a hydrazide antidepressant. An arylamidase purified from guinea pig liver microsomes is known to be responsible for the hydrolysis of Isocarboxazid (Mori and Sato 1975), therefore, the hydrolysis of the hydrazide group to a hydrazine and an acid could be plausible for the title structures. Similar metabolism after hydrolysis was discussed for another hydrazide-containing compound – Ipronizide (Nelson et al. 1978). The study was followed by the development of an in silico approach to examine the active conformations and the scores of the complexes ligand-CYP450.

**Molecular docking**

The in silico calculations could provide data about the additional optimization of the novel compounds (Iliev et al. 2023), thus molecular docking in the active sites of the CYP450 isoforms was conducted. The conducted dockings simulations evaluated the correlation between the experimental and the theoretical results.

The validation of the proteins were done through re-docking procedures provided in our recent study (Mateev et al. 2022). After the validation of the crystallographic structures, all 10 ligands were docked into the three CYP isoforms to observe the potential correlation between the in vitro and in silico results. The data from the docking simulations is provided in Table 1. The co-crystallized ligands were docked into their native enzymes for reference.

From the docking calculations, it was found that only MI_b could be situated in the active site of CYP1A2. However, the compounds showed docking score of -2.66 kcal/mol and returned no MM/GBSA data, not IFD. Moreover, quinidine showed score of -9.89 kca/mol which is not comparable to the pyrrole-based compound.

Interestingly, the results from the docking simulations in CYP3A4 demonstrated that all ligands can be situated in the active site of CYP3A4 and good XP scores were recorded. Subsequently, IFD docking led to good results as well. However, after rescoring the enzyme-ligand complexes, it was found that only MI_c, MII_e, MI_a and MII_b returned scores of -13.82, -6.35, -5.39 and -3.37 kcal/mol, respectively. The scores were not comparable to the native CYP3A4 inhibitor ketoconazole which demonstrated MM/GBSA score of -117.64. Therefore, it could be concluded that none of the ligands poses significant CYP3A4 inhibition capacity. However, MI_c and MII_e returned low binding scores, which do not correlated with the in

![Figure 4. Effects of two series MI_a-e and MII_a-e, as well as Ketoconazole (at concentration 1 µM), on CYP3A4 enzyme activity. *** P < 0.001 vs control (pure CYP3A4).](image-url)
vitro experiments where all compounds showed zero inhibition capacity. Therefore, the next step was to analyse and visualize the active conformation of both pyrrole-based compounds in 2D and 3D panels (Fig. 5).

Based on the visualized stabilization forces, the hem group and Phe304 were the major drug-binding residues in CYP3A4. The Hem group formed a stable hydrogen bond with the p-bromophenyl moiety of MI_c. However, in the case of MII_e, only hydrophobic interactions were found. Therefore, MI_c formed a more stable complex with the active site of CYP3A4 and the lower binding energy was observed. Several weak hydrophobic interactions were formed between the two ligands and the active amino residues Ile120, Leu210, Leu211, Phe241, Ala305, Ile369, Ile370, Gln371 and Leu372 of CYP3A4.

Table 1. Binding energies of the title ligands with human CYP1A2, CYP2D6 and CYP3A4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CYP1A2</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>XP Glide</td>
<td>IFD</td>
<td>MM/GBSA</td>
</tr>
<tr>
<td>MI_a</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MI_b</td>
<td>-2.66</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MI_c</td>
<td>n.r.</td>
<td>n.r.</td>
<td>-3.13</td>
</tr>
<tr>
<td>MI_d</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MI_e</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MII_a</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MII_b</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MII_c</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MII_d</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
</tr>
<tr>
<td>MII_e</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
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*n.d.: not determined; n.r.: no result.

Figure 5. Visualized major intermolecular interactions of the pyrrole-based compounds MI_c (A, B) and MII_e (C, D) in the active site CYP3A4 (PDB: 2V0M) after employing IFD and MM/GBSA recalculations. The interactions are provided in both 3D and 2D forms. The enzyme is depicted in grey, the active amino acids were shown and ligands are presented as green sticks.
Leu482 and Leu483. After the XP docking, the active pose of MI_c demonstrated that the carbonyl group from the ester group was in close proximity to the hem structure. In contrast, the more hardware demanding, and more precise IFD displayed different orientation in the active site of CYP3A4. During the latter simulations, the p-bromophenyl moiety in the pyrrole ring was facing the Hem moiety and a stable hydrogen bond was formed. Thus, the implementation of IFD for virtual simulations in various CYPs isoforms is essential considering the enhanced reliability of the acquired results, which was also underlined in recent studies (Angelov et al. 2022; Mateev et al. 2022).

Conclusion

In conclusion, none of the evaluated compounds exerted any statistically significant inhibitory effects on CYP1A2, CYP2D6 and CYP3A4 compared to the applied standards. Subsequently, all 10 ligands were docked into the former isoforms to observe the potential correlation between the in vitro and in silico results. Two compounds (MI_c and MII_e) showed moderate docking scores in CYP3A4 (PDB: 2V0M) and were visualized in the active site of the enzyme. The in silico simulations confirmed the advantage of the IFD and MM/GBSA recalculations compared to the default Schrodinger’s searching and scoring algorithms.

Acknowledgments

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References


