METABOLISM OF 4-AMINOPIPERIDINE DRUGS BY CYTOCHROME P450S: MOLECULAR AND QUANTUM MECHANICAL INSIGHTS INTO DRUG DESIGN

- Supporting Information -

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Experimental Procedures

Microsomal Clearance Assay. Human liver microsomal clearance assay is a standard high-throughput ADME screening protocol at Pfizer, a half-life-based approach as previously reported [1]. Pooled human liver microsomes were obtained from Pfizer Global Supply. Each incubation contained Pfizer internally synthesized or centrally stocked 4-aminopiperidine compounds (1µM), pooled human liver microsomes (0.25µM protein), NADPH regenerating system (1mM $NADP^+$, 5mM isocitric acid and 1unit/mL isocitric dehydrogenase), $MgCl_2$ (1 mM) and potassium phosphate buffer (100mM at pH 7.4). This mixture was incubated at 37 ºC for 0, 5, 10, 20, 30 and 60 min before quenching with acetonitrile. Control incubations were prepared using the same procedure without adding the cofactor NADPH. Individual 4-aminopiperidines were then analyzed using HPLC/MS/MS standard protocols on an API 3000 or 4000 mass spectrometry (Applied Biosystems) in Multiple Reaction Monitoring (MRM) mode. The ratios of the peak area responses of 4-aminopiperidines to that of the internal standard were used to calculate the half-life of parent compounds, as determined by the slope of the corresponding time-course curve of individual 4-aminopiperidines.

P450 Isoform Phenotyping Assay. P450 reaction phenotyping study is a standard ADME screening protocol at Pfizer, which has been applied to assess the *in vitro* metabolism of studied 4-aminopiperidines by recombinant CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 enzymes. 4-Aminopiperidines (1µM) were incubated with individual CYPs at a final P450 concentration of 150pM in the 0.1M potassium phosphate buffer (pH=7.4) with 3mM $MgCl_2$. The reaction mixture was pre-incubated at 37°C before adding NADPH regenerating solution (1mM

NADPH), which contained 10mM $NADP^+$, 55mM Isocitric acid and 55unit/mL of isocitrate dehydrogenase. A 50μL aliquot of incubate was sequentially removed at 0, 5, 10, 20, 30 and 60 min and quenched with 100μL acetonitrile, which were then centrifuged at 2000 rpm for 10 min. Control incubations were prepared using the same procedure but lacking the NADPH cofactor. Individual samples (10uL) were then analyzed using HPLC/MS/MS standard protocols on an API 4000 mass spectrometry (Applied Biosystems) in MRM mode using a monolithic C18 column (Onyx, 4.6mm x 50mm). Chromatographic separation was achieved using the mobile phases consisted of solvent A (9.9% acetonitrile, 90% water and 0.1% formic acid) and B (89.9% acetonitrile, 10% water and 0.1% formic acid) at a flow rate of 4.5mL/min. The gradient system was set as: 18% B, 0-0.5 min; 18%-82% B, 0.5-1.5 min; 88% B, 1.5-1.6min; and 18% B, 1.6-1.7 min. The ratios of the peak area responses of 4-aminopiperidines to that of the internal standard were used to calculate the rate of parent compound disappearance. Positive controls were phenacetin, bupropion, paclitaxel, diclofenac, S-mephenytoin, dextromethorphan and testosterone, for CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 assays, respectively.

Molecular Docking and Quantum Chemical Calculations. A well-established and evaluated docking method was applied in current study [2]. The chemical structure of 4-aminopiperidines was constructed using Chem3D (CambridgeSoft Corporation, Cambridge, MA) with geometries optimized by molecular mechanics method MM2. The derived molecular structure was further optimized by DFT/B3LYP (Becke three-parameter Lee-Yang-Parr) method using a 6-31G** basis set in Gaussian 03 (Gaussian, Inc., Wallingford, CT). To prepare for docking input files, the energetically minimized structure of 4-aminopiperidines was modified by AutoDockTools (The Scripps Research Institute, La Jolla, CA) with Gasteiger atomic charges assigned and

flexible torsions defined. For protein templates, three-dimensional coordinates of CYP3A4 were collected from both Protein Data Bank [PDB code: 1TQN, 1W0E, 1W0F, 1W0G, 2J0D, 2V0M and 3NXU] and Pfizer protein structure database (13 structures with various substrate/inhibitor bound during co-crystallization). Based on the dynamic analysis of all templates over 30 well-characterized CYP3A4 substrates (in-house Pfizer compounds), a slightly modified CYP3A4 structure including the alteration of several key active site residues was chosen for the molecular docking study of 4-aminopiperidines. The structure was further modified with polar hydrogens, Kollman partial charges and solvation parameters added using AutoDockTools. AutoGrid 4.0 (The Scripps Research Institute) was applied to define the active site space of CYP3A4, which pre-calculates the grids of *van der* Waals, hydrogen bonding, electrostatics, torsional, and solvation interactions. Docking process was accomplished on Pfizer LSF (load sharing facility) Linux GRID using AutoDock 4.0 (The Scripps Research Institute), which searched globally optimized conformation and orientation of 4-aminopiperidines using Lamarckian genetic algorithm, a hybrid of genetic algorithms and an adaptive local search method, with 50 million evaluations performed for each output binding pose that was visualized using PyMOL (Schrödinger, LLC). In addition, the same DFT/B3LYP method in a 6-31G** basis set was applied to structurally optimize designed 4-aminopiperidine fragments and their α-carbon radical species. The activation energy was calculated accordingly.

Supporting Data

Table. Measured distance between the 4-amino nitrogen of 4-aminopiperidines and the oxygen of CYP3A4 Serine119's hydroxyl group, and the distance between the α-carbon and the heme iron of CYP3A4, for the reported binding poses in Figure 2

4-Aminopiperidines	Distance between 4-amino nitrogen and the oxygen of S119 hydroxyl (Å)	Distance between α-carbon and heme iron of CYP3A4 (Å)
Astemizole	**3.5**	**3.3**
Bamipine	**3.7**	**3.0**
Benperidol	**3.5**	**3.3**
Bezitramide	**3.7**	**3.4**
Cisapride	**3.4**	**3.4**
Clebopride	**3.7**	**3.4**
Domperidone	**3.5**	**3.1**
Enzastaurin	**3.9**	**3.1**
Fentanyl	**4.0**	**3.3**
Indoramin	**3.7**	**3.1**
Lorcainide	**4.0**	**3.5**
α-Methylfentanyl	**3.6**	**3.2**
Pimozide	**3.8**	**3.3**
Sabeluzole	**3.7**	**3.6**
Timiperone	**3.6**	**3.3**

Reference

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