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## Involvement of Pazopanib and Sunitinib Aldehyde Reactive Metabolites in Toxicity and Drug–Drug Interactions in Vitro and in Patient Samples

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1 Involvement of pazopanib and sunitinib aldehyde reactive  
2 metabolites in toxicity and drug-drug interactions *in vitro*  
3 and in patient samples  
4

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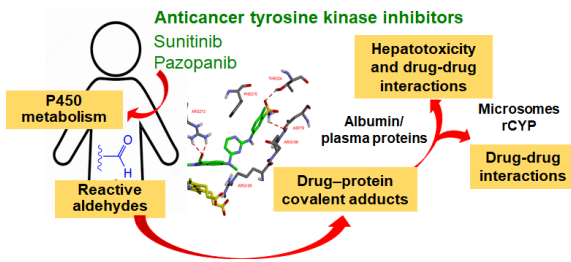
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27 **ABSTRACT:**

28 Tyrosine kinase inhibitors (TKI) are targeted anticancer drugs that have been  
29 successfully developed over the past two decades. To date, many of them  
30 (around 70%) require warnings for liver injury and five of them, including  
31 pazopanib and sunitinib, have Black Box Warning (BBW) labels. Although TKI-  
32 induced hepatotoxicity is the first cause of drug failures in clinical trials, BBW  
33 labels and market withdrawals, the underlying mechanisms remain unclear.  
34 However, the recent discovery of new reactive metabolites (RM) with  
35 aldehyde structures during pazopanib and sunitinib metabolism offers new  
36 perspectives for investigating their involvement in the toxicity of these two  
37 TKI. These hard electrophiles have a high reactivity potential toward proteins  
38 and are thought to be responsible for cytochrome P450 inactivation, drug-  
39 drug interactions (DDI) and liver toxicity. We report here, for the first time,  
40 the presence of these aldehyde RM in human plasma samples obtained during

41 drug monitoring. Docking experiments in the CYP3A4 active site were  
42 performed and showed that pazopanib and sunitinib fitting in the catalytic  
43 site are in accordance with their regioselective oxidation to aldehydes. They  
44 also suggested that aldehyde RM may react with lysine and arginine residues.  
45 Based on these results, we studied the reactivity of the aldehyde RM toward  
46 lysine and arginine residues as potential targets on the protein framework to  
47 better understand how these RM could be involved in liver toxicity and drug-  
48 drug interactions. Adduct formation with different hepatic and plasma  
49 proteins was investigated by LC-MS/MS and adducts between pazopanib or  
50 sunitinib aldehyde derivatives and lysine residues on both CYP3A4 and plasma  
51 proteins were indeed shown for the first time.

52

53 Key words: pazopanib, sunitinib, reactive aldehyde metabolites, lysine adduct,  
54 hepatotoxicity, drug-drug interactions.

55

## 56 **1. INTRODUCTION**

57 Tyrosine kinase inhibitors (TKI) are small molecular weight anticancer drugs  
58 targeting membrane-bound (receptors) and cytoplasmic tyrosine kinases that  
59 have emerged as important targets in oncology, due to their key role in  
60 cellular signaling and their frequent dysregulation in various cancers. Over the  
61 past two decades, many compounds have been successfully developed since  
62 the first-in-class drug, imatinib, was approved in 2001<sup>1</sup>. However, several

63 studies have shown that TKI, including pazopanib and sunitinib, which are oral  
64 drugs prescribed over a long period of time to patients with co-medications  
65 and are also P450 inhibitors, can cause Drug-Drug Interactions (DDI)<sup>2-5</sup>. To  
66 date, among the 33 FDA-approved TKI, 22 (67%) require warnings for liver  
67 injury in drug labelling with Black Box Warning (BBW) labels for five  
68 compounds (lapatinib, pazopanib, ponatinib, sunitinib, and regorafenib)<sup>6,7</sup>.  
69 TKI-induced hepatotoxicity is a major clinical and industrial concern, since  
70 drug-induced liver injury is the first cause of drug failures in clinical trials, BBW  
71 labels and market withdrawals. Shah *et al.* indeed reported that 25–35% of  
72 clinical trial patients treated with TKI showed a low-grade elevation in alanine  
73 and/or aspartate aminotransferases, and approximately 2% of them  
74 developed high-grade increases in serum transaminases, with a wide variability  
75 among TKI<sup>8</sup>.

76 Although little is known about the underlying mechanisms, it is thought that  
77 liver toxicity could be due to reactive metabolites (RM) and as well as being  
78 immune-related. RM reactivity is associated with the presence of electrophilic  
79 moieties or radicals originating from the metabolic transformation, often  
80 called bio-activation, of particular functional groups known as structural alerts  
81 or toxicophores. RMs are able to covalently bind to proteins and DNA, causing  
82 irreversible cellular damage and even cell death as well as DDI through P450  
83 inactivation<sup>9-12</sup>. These so-called P450 time-dependent inhibitions (TDI) or  
84 mechanism-based inhibitions (MBI) are characterized *in vitro* by a

85 concentration-, NADPH-, and time-dependence and involve RM arisen from  
86 parent drug through a previous metabolic enzymatic step<sup>13</sup>.

87 Drug bio-activation to RM that have the propensity to permanently inactivate  
88 the enzyme involved in their generation could lead to enzyme MBI through  
89 various and thoroughly reviewed<sup>14-17</sup> mechanisms, depending on RM structure  
90 and reactivity. In any case, MBI is irreversible *in vivo*, so that the loss of  
91 catalytic activity can only be restored by *de novo* synthesis of the enzyme,  
92 resulting in modifications of drug metabolism (auto-inhibition) and  
93 pharmacokinetic DDI (under or overdosing of co-administered drugs).

94 The formation of different RM has been suggested for several TKI<sup>7</sup>. While RM  
95 formation has been proposed as a causative factor of liver toxicity<sup>9,18</sup>, very  
96 few published data actually support the role of RM in TKI-induced  
97 hepatotoxicity. Until now, studies have mainly focused on the toxic role of *p*-  
98 quinone-imines, for example for lapatinib in HepaRG cells<sup>19-21</sup> and for famitinib  
99 in human primary hepatocytes<sup>22</sup>. Using HepG2 cells, Petrucci *et al.* suggested  
100 that RM rather than the parent drug were involved in pazopanib liver toxicity<sup>23</sup>.

101 Sunitinib oxidative defluorination involving P450 enzymes to generate a  
102 reactive, potentially toxic quinone-imine was also reported more recently<sup>18</sup>.

103 We have recently demonstrated the aldehyde RM formation during pazopanib  
104 and sunitinib metabolic activation using P450 biomimetic models and human  
105 liver microsomes (HLM)<sup>24</sup> (Figure 1). Pazopanib aldehyde derivatives have also  
106 been reported in HLM incubations using a metabolomic approach<sup>25</sup>. Aldehyde

107 formation has otherwise been previously reported for several other TKI<sup>26,27</sup>.  
108 Although their involvement in DDI and toxicity was not further investigated,  
109 aldehydes are hard electrophiles with a high reactivity potential toward  
110 proteins and are thought to be responsible for P450 inactivation and  
111 toxicity<sup>28,29</sup>. Wang *et al.* indeed suggested a relationship between aldehyde  
112 formation and elevation of transaminases in pazopanib-treated mice<sup>25</sup>.  
113 Thus, studies are needed to investigate the molecular relationship between  
114 aldehyde RM and P450 inactivation and hepatotoxicity for pazopanib and  
115 sunitinib. Few studies have been carried out for this purpose in patient  
116 samples. We report here, for the first time, evidence of the formation of these  
117 drug-derived aldehydes in treated patients.  
118 Sunitinib and pazopanib are mainly metabolized by CYP3A4 and to a lesser  
119 extent by CYP2C8 leading to metabolites that cause CYP3A4 TDI but do not  
120 affect CYP2C8 activity *in vitro*<sup>3-5,30</sup>. A docking study was performed in CYP3A4  
121 catalytic site to gain more insight on the interactions of aldehyde metabolites  
122 with the protein framework in order to anticipate their putative involvement  
123 in liver toxicity, P450 inhibition and DDI. This study suggested that arginine  
124 and lysine residues, located in the catalytic site and the solvent channel of  
125 CYP3A4, close to the carbonyl aldehyde groups, could be potential targets to  
126 react with aldehyde RM. The reactivity of the aldehyde RM toward arginine  
127 and lysine residues of different hepatic (HLM, recombinant P450) and plasma  
128 (albumin) proteins was thus investigated by LC-MS/MS.

129

130 Figure 1

131

## 132 **2. EXPERIMENTAL SECTION**

133

### 134 **2.1. Patients**

135 Plasma samples were obtained from patients enrolled in the “SUP-R” trial  
136 (Institut Claudius Regaud, Toulouse, France, EUDRACT 2015-001051-68,  
137 NCT02555748). All patients gave written informed consent, and the study  
138 was approved by the Ethics Committee of the Midi-Pyrénées Region (Comité  
139 de Protection des Personnes Sud-Ouest et Outre-Mer III). It was an open-label,  
140 multicenter, non-randomized, phase IV clinical trial to examine the feasibility  
141 of sunitinib and pazopanib dose individualization based on therapeutic drug  
142 monitoring. Patients were treated with 800 mg of pazopanib once daily or  
143 with 50 mg of sunitinib once daily for four weeks, followed by a washout  
144 period of two weeks. Blood samples were obtained at day 1 and day 15 of  
145 cycle 1 and cycle 2. Each blood sample (4 mL in a EDTA tube) was then  
146 processed by centrifugation for 10 minutes at 1500g. Plasma supernatant  
147 was stored in cryotubes at -20°C until subsequent analysis.

148

### 149 **2.2. Docking experiments: computational procedure**



150 The crystallographic structures of CYP3A4 were taken from the Brookhaven  
151 Protein Data Bank. A large number of 3D structures of CYP3A4 are available.  
152 We chose two, making a compromise between the resolution of the structures  
153 and the number of missing residues, preferably the lowest possible. The first  
154 structure (PDB entry 6BCZ) is complexed with a ritonavir-like inhibitor and has  
155 a resolution of 2.23 Å<sup>31</sup>. The second (PDB entry 1TQN) is an apo form and  
156 has a resolution of 2.05 Å<sup>32</sup>.

157 The ligands and water molecules were extracted from the PDB files. MolProbity  
158 software was used to assign the position of hydrogen atoms and the  
159 protonation state of histidines<sup>33</sup>. Hydrogen atoms were independently added  
160 onto the crystal structure of the heme prosthetic group. The ligands of this  
161 study, pazopanib and sunitinib aldehydes, were thoroughly built and fully  
162 optimized before the docking stage with the PM7 Hamiltonian implemented in  
163 the MOPAC2016 quantum chemistry package (MOPAC2016, James J. P.  
164 Stewart, Stewart Computational Chemistry, Colorado Springs, CO, USA,  
165 [HTTP://OpenMOPAC.net](http://OpenMOPAC.net) (2016))<sup>34</sup>. The docking calculations were performed  
166 with the docking program Autodock Vina v1.1<sup>35</sup>. The Autodock graphical  
167 interface AutoDockTools was used to keep polar hydrogens and add partial  
168 charges to the proteins using the Kollman United charges<sup>36</sup>. The search space  
169 was included in a box of 24 × 24 × 24 Å, centered on the binding site of the  
170 ligands and heme. The side chain of the S119, R212, I301, F304 and E374  
171 residues that are exposed on the surface of the catalytic cavity were allowed

Code de champ modifié

172 to rotate during the docking procedures. Flexible torsions of side chains and  
173 ligands were assigned with Autotors, an auxiliary module of AutoDockTools.  
174 For each calculation, 15 poses ranked according to the scoring-function of  
175 Autodock Vina were obtained.

176

### 177 **2.3. Materials and instruments**

178 Sunitinib L-malate and pazopanib hydrochloride were purchased from  
179 Alsachim (Illkirch-Graffenstaden, France). All solvents and commercially  
180 available reagents were used without further purification. Acetonitrile and  
181 methanol were provided by Scharlau (Barcelona, Spain), and formic acid by  
182 Merck (Darmstadt, Germany). Ammonium formate, ammonium  
183 hydrogencarbonate, sodium acetate, sodium cyanoborohydride, sodium  
184 dihydrogenphosphate, disodium hydrogenphosphate, D,L-dithiothreitol,  
185 dimethylsulfoxide (DMSO), human serum albumin, pronase, chymotrypsin,  
186 trypsin and L-arginine were purchased from Sigma-Aldrich (St Quentin-Fallavier,  
187 France). L-Lysine•HCl came from Alfa Aesar (Ward Hill, USA) and calcium  
188 chloride from Acros organics (Illkirch, France). Blank plasma from healthy  
189 donors was provided by « Etablissement Français du Sang » (EFS, Toulouse,  
190 France). Aldehyde metabolites **1a-CHO** and **2-CHO** for control or spiking  
191 experiments were prepared through drug biomimetic oxidation using  
192 manganese porphyrin complexes as catalysts, as previously reported<sup>24</sup>.  
193 Recombinant Human CYP3A4 (Ref. 456202), pooled human liver microsomes

194 (Ref. 452161), NADPH regenerating solutions (Ref. 451220, 451200) were  
195 purchased from Corning (Woburn, USA).

196

197 *LC-MS analyses.* Identification of aldehyde metabolites **1a-CHO** and **2-CHO** in  
198 human plasma were performed by UPLC-MS/MS with an UPLC Waters Acquity  
199 system equipped with a Waters TQ-S micro mass spectrometer (triple  
200 quadrupole detector), an electrospray ionization source (ESI) and MassLynx™  
201 software. UPLC chromatographic separations were performed on an Acquity  
202 UPLC® BEH Shield RP18 (1.7 μm, 2.1 x 50 mm) column (Waters, Milford, MA,  
203 USA) at a flow rate of 0.3 mL/min and a column temperature of 30°C. Eluent  
204 A was ammonium formate 5 mM pH = 3.2 and eluent B was formic acid 0.1%  
205 (v/v) in acetonitrile. Eluting conditions were a linear gradient of A and B as  
206 follows: the percentage of B, initially set at 2%, stayed at 2% until 0.5 min,  
207 increased to reach 60% (t = 3.5 min), 100% (t = 4 min), stayed at 100%  
208 until 4.5 min, then returned to initial conditions over 0.5 min. Afterward, the  
209 system was re-equilibrated for 1 min before the following injection. The mass  
210 spectrometer was operated in positive ionization mode (ESI<sup>+</sup>), using cone  
211 voltages of 60 or 80 V and collision energy of 30 eV. Mass spectra for L-  
212 lysine adduct characterization were acquired in Full Scan mode (*m/z* range  
213 50-650 amu for **1a-CHO** and **2-CHO**). Multiple Reaction Monitoring (MRM)  
214 mode was used for **1a-CHO** and **2-CHO** detection in human plasma. MRM

215 transitions were as follows: for **1a-CHO**:  $m/z$  452>343, 328; for **2-CHO**:  $m/z$   
216 413>340, 268.

217 Identification of lysine adducts of **1a-CHO** and **2-CHO** in protein samples  
218 (human plasma, human serum albumin, HLM, recombinant CYP3A4  
219 (rCYP3A4)) was performed by UPLC-MS/MS with an RS 3000 UHPLC system  
220 (ThermoFisher Scientific) equipped with an AB Sciex QTRAP 4500 mass  
221 spectrometer (triple quadrupole detector), an electrospray ionization source  
222 (ESI) and Analyst™ software. UPLC chromatographic separations were  
223 performed with the same column and under the same eluting conditions,  
224 reported above. The mass spectrometer was operated in positive ionization  
225 mode (ESI<sup>+</sup>), using cone voltages of 40 or 60 V and collision energies of 30  
226 or 40 eV. MRM mode was used for lysine adduct detection in protein samples  
227 with the following MRM transitions: for **1a-CHO**-lysine adduct:  $m/z$   
228 582.2>436.2; for **2-CHO**-lysine adduct:  $m/z$  543.2>326.1.

229

#### 230 **2.4. Preparation of lysine adduct standards**

231 *Reaction of 1a-CHO and 2-CHO with L-lysine:* an aliquot of an aldehyde-  
232 containing medium (ca. 2  $\mu$ mol of **1a-CHO** or **2-CHO** prepared as previously  
233 described<sup>24</sup> was dissolved in H<sub>2</sub>O/CH<sub>3</sub>OH (400  $\mu$ L, 37/63 v/v). Sodium  
234 acetate (160  $\mu$ mol) and L-lysine (80  $\mu$ mol) were then added to afford imine  
235 derivatives.

236 *Preparation of stable adducts.* the one pot reductive amination of the imine  
237 derivatives was performed by NaBH<sub>3</sub>CN (three additions of 20 μL of a 1.3 M  
238 solution in methanol, *i.e.* ca. 40 eq. in regard to **1a-CHO** or **2-CHO**, every 30  
239 minutes) over 120 minutes at 37°C. The crude reaction media were then  
240 analyzed by UPLC-MS.

241

#### 242 **2.5 Spiking experiments of 1a-CHO and 2-CHO in human protein samples.**

243 *Human plasma.* An aliquot of an aldehyde-containing medium (ca. 20 nmol of  
244 **1a-CHO** or **2-CHO**, prepared as previously described<sup>24</sup>, in 0.8 μL of DMSO) was  
245 added in human plasma (250 μL). During incubation at 37°C for 120 minutes,  
246 reductive amination was performed by NaBH<sub>3</sub>CN (four additions of 0.5 μL of  
247 a 1.25 M solution in methanol, *i.e.* ca. 125 eq. in regard to **1a-CHO** or **2-CHO**,  
248 every 30 minutes). The crude reaction media were then digested by  
249 proteases.

250 *Human serum albumin.* An aliquot of an aldehyde-containing medium (ca. 20  
251 nmol of **1a-CHO** or **2-CHO**, prepared as previously described<sup>24</sup>, in 0.8 μL of  
252 DMSO) was added to a mixture of human serum albumin (3 μL of a 40 g/L  
253 solution in 0.1 M phosphate buffer pH = 7.4) and 0.1 M phosphate buffer (pH  
254 = 7.4, final volume = 250 μL). During incubation at 37°C for 120 minutes,  
255 reductive amination was performed by NaBH<sub>3</sub>CN (four additions of 0.5 μL of  
256 a 1.25 M solution in methanol, *i.e.* ca. 125 eq. in regard to **1a-CHO** or **2-CHO**,

257 every 30 minutes). The crude reaction media were then digested by  
258 proteases.

259 *Human liver microsomes/recombinant CYP3A4.* An aliquot of an aldehyde-  
260 containing medium (ca. 20 nmol of **1a-CHO** or **2-CHO**, prepared as previously  
261 described<sup>24</sup>, in 0.8  $\mu$ L of DMSO) was added to a mixture of human liver  
262 microsomes (final protein concentration = 0.5 mg/mL) or recombinant  
263 CYP3A4 (final rCYP3A4 concentration = 20 pmol/mL) and 0.1 M phosphate  
264 buffer (pH = 7.4, final volume = 250  $\mu$ L). During incubation at 37°C for 120  
265 minutes, reductive amination was performed by NaBH<sub>3</sub>CN (four additions of  
266 0.5  $\mu$ L of a 1.25 M solution in methanol, *i.e.* ca. 125 eq. in regard to **1a-CHO**  
267 or **2-CHO**, every 30 minutes). The crude reaction media were then digested  
268 by proteases.

269

## 270 **2.6. Human liver microsomes and recombinant CYP3A4 incubations.**

271 Microsomal incubations were performed according to supplier protocol. An  
272 aliquot of human liver microsomes (final protein concentration = 2 mg/mL)  
273 was added to a mixture of NADPH regenerating solutions A (50  $\mu$ L), B (10  
274  $\mu$ L) and 0.1 M phosphate buffer (pH = 7.4, final volume = 250  $\mu$ L). After pre-  
275 incubation at 37°C for 5 minutes, microsomal incubations were started by the  
276 addition of pazopanib **1** or sunitinib **2** in DMSO solutions (final substrate  
277 concentration = 100  $\mu$ M, final DMSO concentration = 0.2%). The mixture was  
278 then kept at 37°C for 30 minutes to ensure aldehyde formation. Reductive

279 amination was then performed by NaBH<sub>3</sub>CN (four additions of 0.5 μL of a 1.25  
280 M solution in methanol, *i.e.* 100 eq. in regard to **1** or **2**, every 30 minutes,  
281 final methanol concentration = 0.8%) over 120 minutes at 37°C. The  
282 incubation media were then subjected to proteolytic digestion. The same  
283 protocol was followed with recombinant CYP3A4 (final rCYP3A4  
284 concentration = 80 pmol/mL).

285

## 286 **2.7. Protein digestion**

287 Protein denaturation was achieved by heating the mixture to 60°C over 30  
288 minutes. Disulfide bonds were reduced by addition of D, L-dithiothreitol (DTT,  
289 50 μL of a 50 mM solution in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH = 8). The mixture was then  
290 incubated at 60°C for one hour. Protein digestion was performed by the  
291 addition of trypsin, chymotrypsin, pronase E (100 μL of a 12.5 mg/mL  
292 solution of each protease in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH = 8), acetonitrile (50 μL)  
293 and calcium chloride (100 μL of a 25 mM solution in water). The mixture was  
294 kept overnight at 37°C. Proteolytic digestion was stopped by the addition of  
295 5 μL of formic acid 1% in water. The mixture was then centrifuged for 20  
296 minutes at 4000 g at room temperature. The supernatants were then  
297 analyzed by UPLC-MS to characterize lysine or arginine adducts of sunitinib  
298 and pazopanib aldehyde metabolites. The following modifications to the  
299 above-described proteolytic digestion protocol were made for:

300 *i) HLM incubations in the presence of pazopanib 1:* after protein denaturation,  
301 incubation media were centrifuged for 20 minutes at 4000 g at 40°C. The  
302 protein pellets were then subjected to proteolytic digestion [DTT (20 µL of a  
303 125 mM solution in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH = 8), proteases (20 µL of a 62.5  
304 mg/mL solution of each protease in 50 mM NH<sub>4</sub>HCO<sub>3</sub> pH = 8) and calcium  
305 chloride (10 µL of a 250 mM solution in water)];

306 *ii) HLM incubations in the presence of sunitinib 2:* after protein denaturation,  
307 incubation media were centrifuged for 20 minutes at 4000 g at 40°C. The  
308 protein pellets were then treated by DTT and incubated at 60°C for 1 hour  
309 before proteolytic digestion;

310 *iii) rCYP3A4 incubations in the presence of pazopanib 1:* after proteolytic  
311 digestion, supernatants of 10 incubations were pooled before UPLC-MS  
312 analyses.

313

### 314 **3. RESULTS AND DISCUSSION**

#### 315 **3.1. Aldehyde identification in patient plasma samples**

316 Aldehyde metabolites **1a-CHO** and **2-CHO** have been recently prepared *in vitro*  
317 using chemical models<sup>24</sup> or HLM<sup>24,25</sup> (Figure 1). These aldehydes resulted from  
318 the oxidation of the alcohol derivatives **1-CH<sub>2</sub>OH** and **2-CH<sub>2</sub>OH** previously  
319 reported, respectively in human plasma for pazopanib **1**<sup>37,38</sup> and in rats,  
320 monkeys and dogs for sunitinib **2**<sup>39</sup>. Wang *et al.* have also detected **1b-CHO**  
321 in mice feces and urine<sup>25</sup>, that we did not observe using biomimetic P450



322 models or HLM. Speed *et al*<sup>0</sup> reported the formation of a series of mono-  
323 oxygenated metabolites giving the same product ions (*m/z*: 415, fragments  
324 at *m/z*: 342, 299) and consistent with oxidation on sunitinib  
325 indolylidene/dimethylpyrrole moiety. Although the exact position of the  
326 oxidation could not be elucidated by LC-MS alone, such metabolites, detected  
327 in rat, monkey and human feces, are consistent with **2-CH<sub>2</sub>OH**. In addition,  
328 Speed *et al* have reported unidentified metabolites, some of them cannot be  
329 ruled out as aldehydes.

330 The formation of aldehyde RM **1a-CHO** and **2-CHO** *in vivo* was investigated in  
331 plasma samples from patients treated, respectively, with pazopanib or  
332 sunitinib. Screening for **1a-CHO** and **2-CHO** was performed by UPLC-MS/MS at  
333 two characteristic MRM transitions for each compound to ensure specificity,  
334 in comparison with aldehyde standards prepared beforehand by biomimetic  
335 oxidations<sup>24</sup>. Despite reactivity of the aldehyde function, **1a-CHO** and **2-CHO**  
336 have been unambiguously detected in patient plasma samples during drug  
337 monitoring at 3.96 min (*m/z*: 452>343; 452>328) for pazopanib (Figure S-  
338 1B, C) and at 4.22 min (*m/z*: 413>268; 413>340) for sunitinib (Figure S-2B,  
339 C), consistently with the retention times of the corresponding standards  
340 (Figures S-1A and S-2A). Since no other peak was shown in the  
341 chromatograms (Figures S-1B, C and S-2B, C), **1b-CHO** was not detected in  
342 patient plasma samples. The exact quantification of these metabolites could  
343 not be carried out since the response factor of metabolites could not be

344 determined; but the time course relative evolution of the aldehyde contents  
345 based on the related peak areas was established during drug monitoring at  
346 different days of treatment cycles. Figure 2 illustrates the aldehyde profiles  
347 over time (**1a-CHO**,  $m/z = 452>343$  and **2-CHO**,  $m/z = 413>340$ ) that  
348 paralleled those of the parent drugs, both for pazopanib and sunitinib,  
349 suggesting that the rate of aldehyde formation was lower than their rate of  
350 elimination through bio-transformation, which is consistent with their  
351 reactivity.

352 Moreover, monitoring at another MRM characteristic transition for each  
353 aldehyde RM (**1a-CHO**,  $m/z = 452>328$  and **2-CHO**,  $m/z = 413>340$ ) gave  
354 the same profiles, thus supporting the specificity of aldehyde detection.

355

## 356 **Figure 2**

357

358 The detection of drug-derived aldehydes in plasma of patients treated with  
359 pazopanib or sunitinib is very important and provides evidence that the  
360 reactive, potentially toxic metabolites are in fact generated *in vivo*. It also  
361 confirms that metalloporphyrin-catalyzed biomimetic oxidations or HLM are  
362 useful drug oxidation models<sup>24</sup> to provide relevant metabolites that can be  
363 used as authentic standards and advance their study.

364

## 365 **3.2. Molecular docking of aldehyde metabolites**

366 Since molecular docking is a useful approach to predict molecular interactions,  
367 docking experiments were performed to elucidate whether these electrophilic  
368 aldehyde RM could be involved in CYP3A4 TDI and also in liver toxicity, through  
369 covalent coupling with nucleophilic amino acid residues on P450 protein  
370 framework. Aiming to identify putative amino acid targets that could interact  
371 with the carbonyl aldehyde groups, **1a-CHO** and **2-CHO** were docked in  
372 CYP3A4 catalytic site (PDB entries: 1TQN and 6BCZ). For both aldehyde RM,  
373 binding modes were very close to those of parent molecules (see  
374 supplementary data for docking of parent molecules Figures S-3, S-4). These  
375 observations supports the regioselective oxidation of pazopanib (-C<sub>7</sub>-CH<sub>3</sub>)  
376 and sunitinib (-C<sub>11</sub>-CH<sub>3</sub>) respectively to **1a-CHO** and **2-CHO** by CYP3A4.

377

378 ***Pazopanib aldehyde*** - Binding modes found with 1TQN and 6BCZ crystals were  
379 quite similar for **1a-CHO**. In both structures, the first pose was oriented in the  
380 same way so that the indazole ring was nearly coplanar with the heme center,  
381 and the sulfonamide group made hydrogen bonds with Asp76 and Thr224  
382 (1TQN, Figure 3A) and with Asp76 (6BCZ, Figure 3B). The predicted binding  
383 free energies were -12.3 and -11.6 kcal/mol for 1TQN and 6BCZ, respectively.  
384 It is noteworthy that the carbonyl aldehyde group of pazopanib RM was close  
385 to the basic amino acid Arg212 (3.4 or 2 Å between the guanidino and the  
386 carbonyl groups, Figure 3A, 3B respectively), thus suggesting a possible  
387 reaction between **1a-CHO** and Arg212.

388

389 **Sunitinib aldehyde** - With 1TQN, **2-CHO** indolinone ring was inserted between  
390 Phe215 and Arg106, and the terminal trialkylammonium group was located in  
391 front of the heme. Furthermore, the interaction could be stabilized by  
392 hydrogen bonds between the aldehyde function and Glu374 and Arg105  
393 (Figure 3C). The predicted binding free energy was -10.1 kcal/mol. With the  
394 6BCZ crystal, Autodock Vina provided two alternative poses with similar  
395 energy, which were arranged head to tail with each other. In the first pose  
396 ( $\Delta G = -9.7$  kcal/mol), the indolinone ring was stacked with the heme  
397 macrocycle, and the trialkylammonium group was placed between the Arg106  
398 and Phe215 residues (Figure 3D). The carboxaldehyde group formed  
399 hydrogen bonds with the NH groups of Glu374 and Arg105 residues (Figure  
400 3E). The second pose ( $\Delta G = -9.6$  kcal/mol) was, for its part, similar to the  
401 conformation found with 1TQN and exhibited the same non-bonding  
402 interactions, thus suggesting a possible reaction between **2-CHO** and these  
403 amino acid residues.

404

405 **Figure 3**

406

407 Docking experiments showed that several arginine residues are located in  
408 CYP3A4 active site, in close vicinity of **1a-CHO** and **2-CHO** carboxaldehyde  
409 groups (Figure 3). They also revealed the presence of a large number of

410 accessible lysine residues (37) on the protein surface, and more specifically  
411 in the solvent channel of CYP3A4 (Figure S-5). These results suggest that  
412 interactions between aldehyde RM and arginine residues (in the active site) or  
413 lysine residues (when metabolites leave the active site) could be expected  
414 and potentially be responsible for the formation of covalent protein-drug  
415 adducts and thereby for CYP3A4 TDI. In addition, covalent coupling of **1a-**  
416 **CHO** and **2-CHO** on CYP3A4 could also be involved in immune-related liver  
417 injury. RM coupling to P450 enzymes could indeed trigger an auto-immune  
418 response leading to hepatotoxicity as it has been previously reported for  
419 other drugs<sup>41,42</sup>. CYP3A4 therefore appears to be a potential target for  
420 aldehydes, this does not exclude the reaction of aldehydes with other proteins  
421 that can contribute to toxicity.

422

### 423 **3.3. Proof of concept of protein-drug coupling**

424 To support the hypothesis that reactions between pazopanib or sunitinib  
425 aldehydes and arginine or lysine residues may occur *in vivo*, we investigated  
426 the formation of arginine or lysine adducts in different hepatic (recombinant  
427 CYP3A4, HLM) and plasma (albumin) protein samples. Because of the  
428 presence of a free amino group on its side chain, lysine could be a good target  
429 for reactive electrophiles like aldehyde RM. Lysine adducts with different  
430 drugs have indeed been previously reported *in vitro*<sup>43,44</sup> and *in vivo*<sup>45</sup>.  
431 Reactions between aldehydes and amines affording imine derivatives are

432 known to be reversible in aqueous media. Thus, adduct formation was shown  
433 after reductive amination of imine derivatives by NaBH<sub>3</sub>CN *in vitro*.  
434 To facilitate the identification of lysine and arginine adducts in protein  
435 samples, adduct formation was first investigated with lysine and arginine  
436 amino acids in preliminary experiments. The reaction between **1a-CHO** or **2-**  
437 **CHO** generated through biomimetic oxidations catalyzed by  
438 metalloporphyrins<sup>24</sup> and free lysine or arginine (40 mole eq.) yielded **1a-CHO**  
439 or **2-CHO** lysine adducts. As shown in Figure 4A, in the full scan  
440 chromatogram, lysine adduct of **1a-CHO** was detected at 3.20 min and was  
441 characterized by the presence of the [M+H]<sup>+</sup> ion (*m/z*: 582) and the MS/MS  
442 fragmentation pattern corresponding to the loss of lysine (*m/z*: 436) and  
443 lysine fragmentation (*m/z*: 130, 218). Fragmentation of the bond between  
444 N8 and the indazole ring provided ions at *m/z*: 291 and 190 with subsequent  
445 lysine fragmentation. These results showing the formation of covalent  
446 adducts between free lysine and **1a-CHO** confirmed the reactivity of this  
447 aldehyde RM toward the lysine ε-amino group. Similarly, lysine adduct of **2-**  
448 **CHO** was detected at 3.43 min and was characterized by the [M+H]<sup>+</sup> ion (*m/z*:  
449 543) in the mass spectrum and by the MS/MS fragmentation pattern  
450 corresponding to the loss of lysine (*m/z*: 397), the loss of lysine and the  
451 diethylamine side chain (*m/z*: 326) or the diethylamine-ethylamine side chain  
452 (*m/z*: 283) (Figure 4B). MS/MS fragmentation pattern did not allow to  
453 exclude the coupling between **2-CHO** and the lysine α-amino group. However,

454 a small peak at  $m/z$  469 (magnified in the frame) corresponding to the loss  
455 of the  $\alpha$ -amino acid moiety could be in favor of the coupling at the  $\epsilon$ -amino  
456 group. Thus, by analogy with **1a-CHO**, the structure of the adduct resulting  
457 from the coupling of **2-CHO** at the lysine  $\epsilon$ -amino group was shown in Figure  
458 4B.

459 **Figure 4**

460

461 Based on these results (Figure 4), the characteristic MRM transitions at  $m/z$ :  
462 582>436 for **1a-CHO** lysine adduct and at  $m/z$ : 543>326 for **2-CHO** lysine  
463 adduct were selected in order to detect covalent adducts between these drug  
464 aromatic aldehydes and several proteins (Figures 5 and 6, traces A).

465 Hepatic proteins are expected to be the primary targets of reactive aldehydes  
466 since they are involved in forming the latter, thus making HLM and  
467 recombinant P450 (rCYP) relevant models *in vitro*. Plasma proteins, including  
468 albumin, have also been used as surrogate proteins since plasma samples are  
469 easier to collect than liver tissues from patients in a clinical perspective<sup>45-47</sup>.

470 In protein samples, proteolytic digestion was performed with a protease  
471 cocktail (trypsin, chymotrypsin and pronase) prior to LC-MS analyses to ensure  
472 lysine adduct detection. These analyses were performed with a QTRAP LC-MS  
473 apparatus for high sensitivity (this is why the retention times were slightly  
474 different in comparison with Figure 4). The assignment of the chromatogram  
475 peaks was done by spiking blank biological matrices (human plasma, human

476 albumin, HLM and rCYP3A4) with **1a-CHO** or **2-CHO** generated by biomimetic  
477 oxidations catalyzed by metalloporphyrins as previously reported<sup>24</sup>. The  
478 formation of lysine adducts of **1a-CHO** or **2-CHO** in plasma and albumin (3.03  
479 min, *m/z*: 582>436, Figures 5B or 6B, respectively) and with HLM and  
480 rCYP3A4 proteins (3.34 min, *m/z*: 543>326, Figures 5C or 6C, respectively)  
481 was demonstrated by the presence of MRM peaks at the same retention time  
482 as with free lysine (Figures 4 A and B). Lysine adducts were then detected in  
483 HLM incubations and in incubation with rCYP3A4, supporting that **1a-CHO**  
484 generated in HLM and rCYP3A4 incubations was able to readily react with  
485 lysine residues on the P450 protein framework (Figure 5D). Similar results  
486 were obtained for sunitinib biotransformation in HLM and rCYP3A4, since  
487 lysine adducts of **2-CHO** were also detected in these conditions (Figure 6D).

488 **Figure 5**

489 **Figure 6**

490

491 Since docking experiments revealed that **1a-CHO** and **2-CHO** carbonyl  
492 aldehyde groups are in close vicinity of arginine residues in CYP3A4 active  
493 site, we also investigated the putative formation of arginine-aldehyde  
494 adducts. Arginine guanidinium group can react with aldehydes to afford Schiff  
495 bases, as has been shown with unsaturated aldehydes and formaldehyde<sup>48-50</sup>.  
496 Thus, the approach described above for lysine was also applied to examine if  
497 arginine adducts in different protein samples could be formed. Although we



498 found low amounts of arginine adducts of **1a-CHO** and **2-CHO** after reaction  
499 with free arginine (data not shown), we did not detect any arginine adduct in  
500 protein samples after proteolytic digestion (data not shown). This could be  
501 explained by the lower nucleophilicity of the guanidinium group when  
502 compared to lysine primary amine in these biological conditions.

503 Until now, **1a-CHO** and **2-CHO** lysine adducts were not detected in patient  
504 plasma samples, probably due to their very low concentrations. However, this  
505 work advances the field by showing that aldehyde metabolites of pazopanib  
506 and sunitinib (**1a-CHO** and **2-CHO**) are found in patient samples and that these  
507 aldehyde RMs are able to react with proteins on lysine residues (shown *in*  
508 *vitro*).

509 Among other potential causative factors (mitochondrial toxicity, bile salt  
510 export pump and UDP-glucuronosyltransferase inhibition, genetic and  
511 immunologic polymorphisms<sup>6,20,51-56</sup>, it could be hypothesized that the  
512 coupling of aldehydes with lysine residues of proteins (CYP3A4 and hepatic  
513 proteins) may be involved in hepatotoxicity. Lysine adduct formation could  
514 also explain CYP3A4 TDI and hence may contribute to pazopanib and sunitinib  
515 DDI.

516 **Figures legend**

517 **Figure 1:** Structures of pazopanib (1), sunitinib (2) and their related alcohol (1-CH<sub>2</sub>OH and 2-  
518 CH<sub>2</sub>OH) and aldehyde metabolites (1-CHO and 2-CHO).

519

520 **Figure 2:** Analyses of patient plasma samples during pazopanib or sunitinib treatment. 1)  
521 patients treated with pazopanib : detection of aldehyde **1a-CHO** (▲:  $m/z = 452 > 343$ ) along  
522 with pazopanib (□) in the plasma samples of three patients (panels 1A, 1B, and 1C,  
523 respectively). 2) patients treated with sunitinib : detection of aldehyde **2-CHO** (■:  $m/z =$   
524  $413 > 268$ ) along with sunitinib (○) in the plasma samples of three patients (panels 2A, 2B,  
525 and 2C, respectively). D1C1: day 1, cycle 1; D15C1: day 15, cycle 1; D1C2: day 1, cycle 2;  
526 D15C2: day 15, cycle 2.

527

528 **Figure 3:** Molecular docking into the catalytic cavity of crystal structure of CYP3A4: A) **1a-**  
529 **CHO** / 1TQN; B) **1a-CHO** / 6BCZ; C) **2-CHO** / 1TQN, D) **2-CHO** / 6BCZ. The red dotted lines  
530 represent the non-bonding interactions (*i.e.* Hydrogen bonds,  $\pi$ -stacking or  $\pi$ -cation).

531

**Figure 4:** Lysine adducts A) with pazopanib aldehyde **1a-CHO**. LC-MS chromatograms in Full  
Scan mode, B), mass spectra. C) with sunitinib aldehyde **2-CHO**. LC-MS chromatograms in Full  
Scan mode, D) mass spectra, insert frame: magnified MS ( $m/z$  from 450 to 500). Adduct  
structures in inserts.

532 **Figure 5:** LC-MS MRM chromatograms of lysine adducts of pazopanib aldehyde **1a-CHO** (RT =  
533 3.0 min,  $m/z$ : 582 > 436). A) standard lysine adduct obtained through reaction of **1a-CHO**  
534 with free lysine; B) in plasma and albumin spiked with **1a-CHO** generated during biomimetic  
535 oxidations with metalloporphyrins; C) in HLM and rCYP3A4 spiked with **1a-CHO** generated

536 during biomimetic oxidations with metalloporphyrins; D) in HLM and rCYP3A4 incubations. B,  
537 C, D: adducts were detected after proteolytic digestion.

538

539 **Figure 6:** LC-MS MRM chromatograms of lysine adducts of sunitinib aldehyde **2-CHO** (RT = 3.3  
540 min,  $m/z$ : 543.2>326.1). A) Standard lysine adduct obtained through reaction of **2-CHO** with  
541 free lysine; B) in plasma and albumin spiked with **2-CHO** generated during biomimetic oxidations  
542 with metalloporphyrins; C) in HLM and rCYP3A4 spiked with **2-CHO** generated during  
543 biomimetic oxidations with metalloporphyrins; D) in HLM incubations and rCYP3A4 incubations.  
544 B, C, D: adducts were detected after proteolytic digestion.

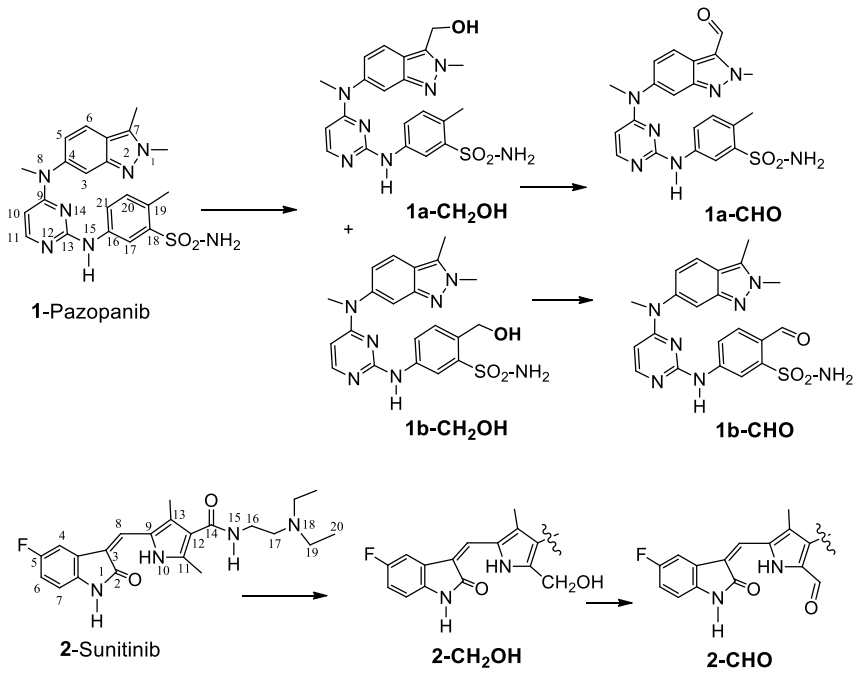
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551 **Figure 1**

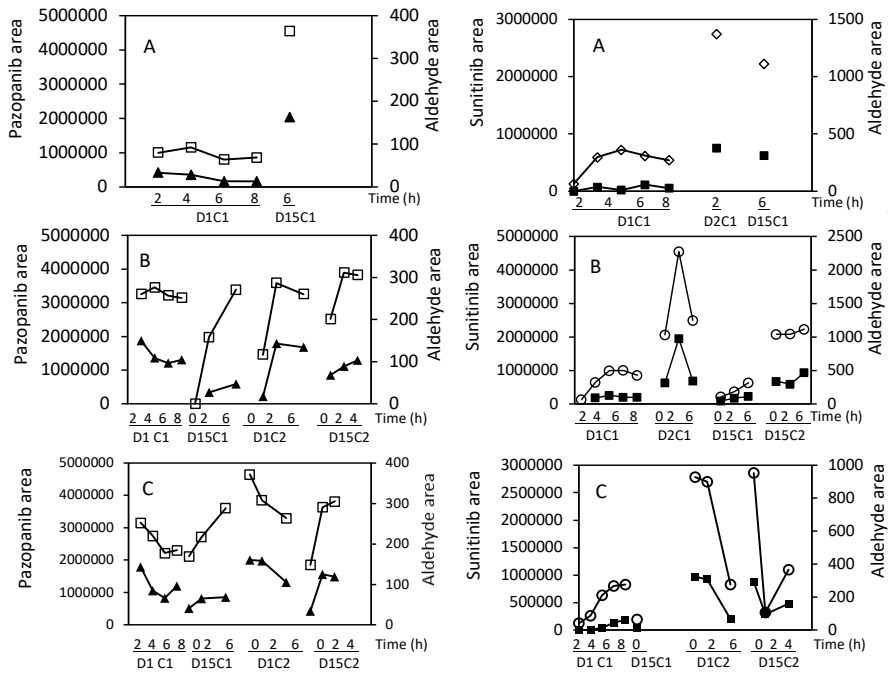
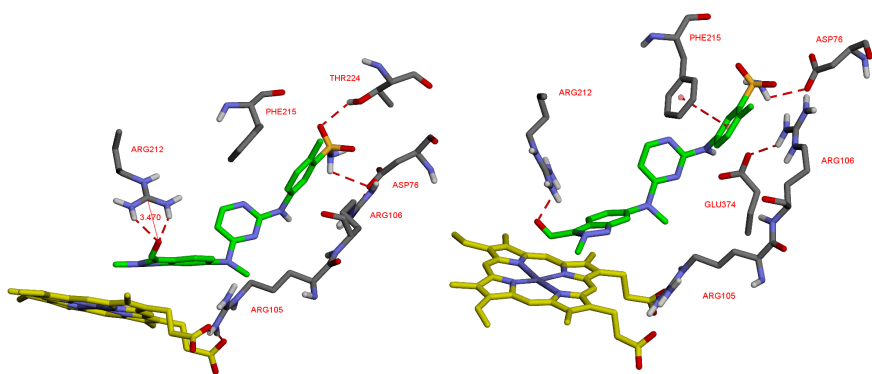


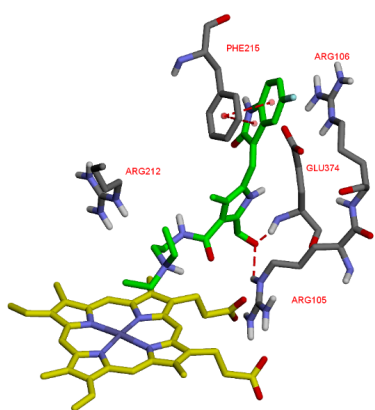
Figure 2

552

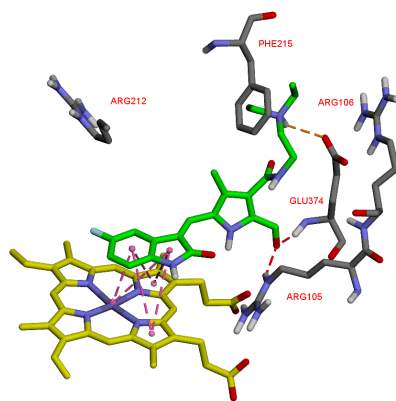


A) 1a-CHO / 1TQN

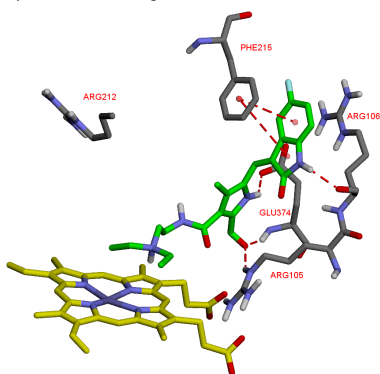
B) ) 1a-CHO / 6BCZ



C) 2-CHO / 1TQN



D) 2-CHO / 6BCZ (Pose 1)



E) 2-CHO / 6BCZ (Pose 2)

Figure 3

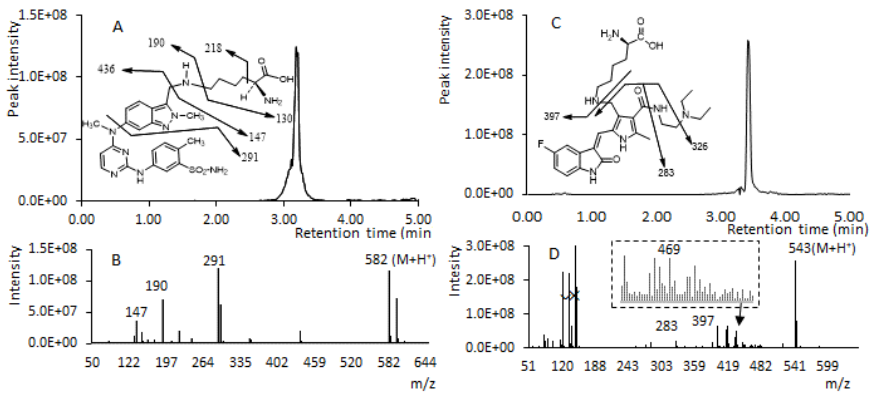


Figure 4

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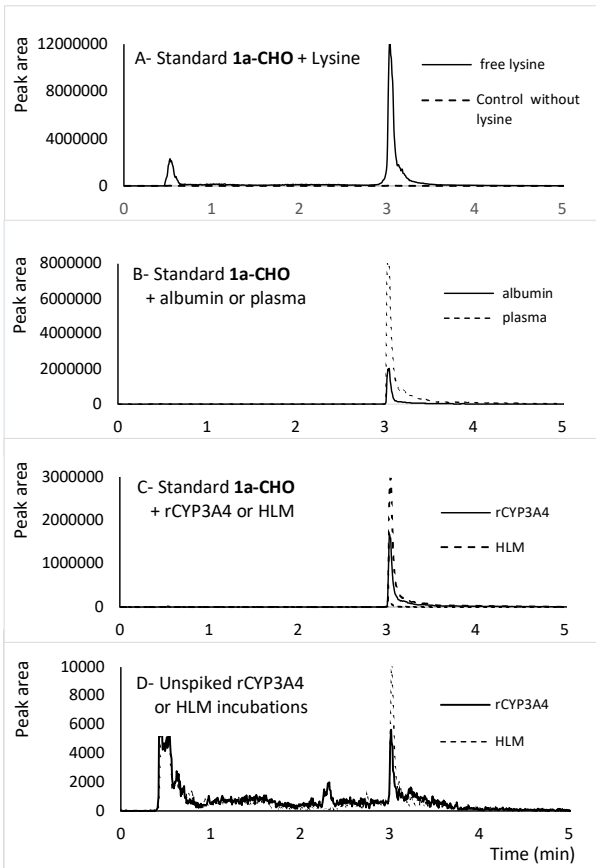
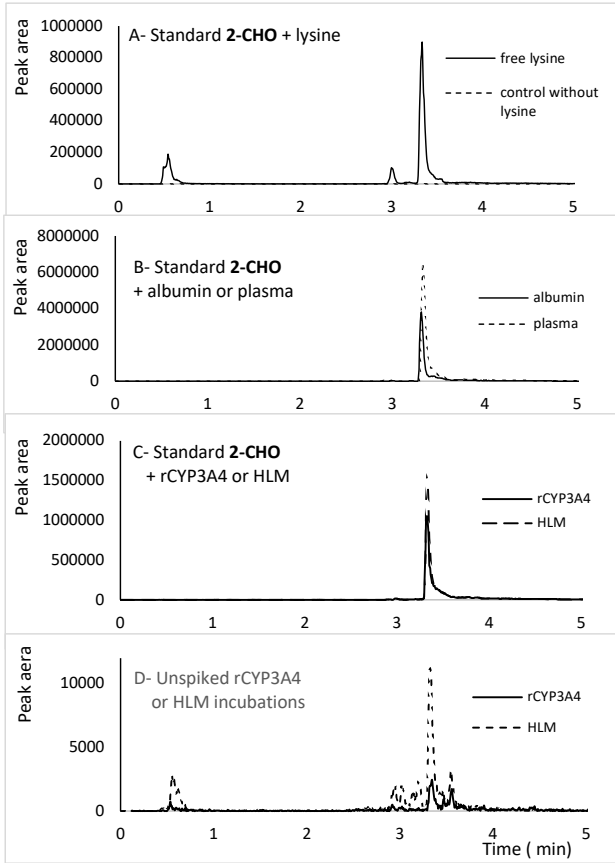


Figure 5

555



556



557 **Figure 6**

558

559 **Supporting information:**

560 **Figure S-1:** Typical chromatograms of pazopanib aldehyde **1a-CHO** in patient plasma.

561 **Figure S-2:** Typical chromatograms of sunitinib aldehyde **2-CHO** in patient plasma.

562 **Figure S-3:** Molecular docking of pazopanib and sunitinib into CYP3A4 catalytic cavity.

563

564 **Figure S-4:** Molecular docking of pazopanib showing the orientation of C19 near the heme.

565 **Figure S-5:** Lysine residues in CYP3A4 protein framework.

566

567

568

569

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#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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