Title: Mechanism-based Inactivation of Human Cytochrome P450 3A4 by Two Piperazine-containing Compounds
Authors: Bolles, Fujiwara, Briggs, Nomeir, Furge
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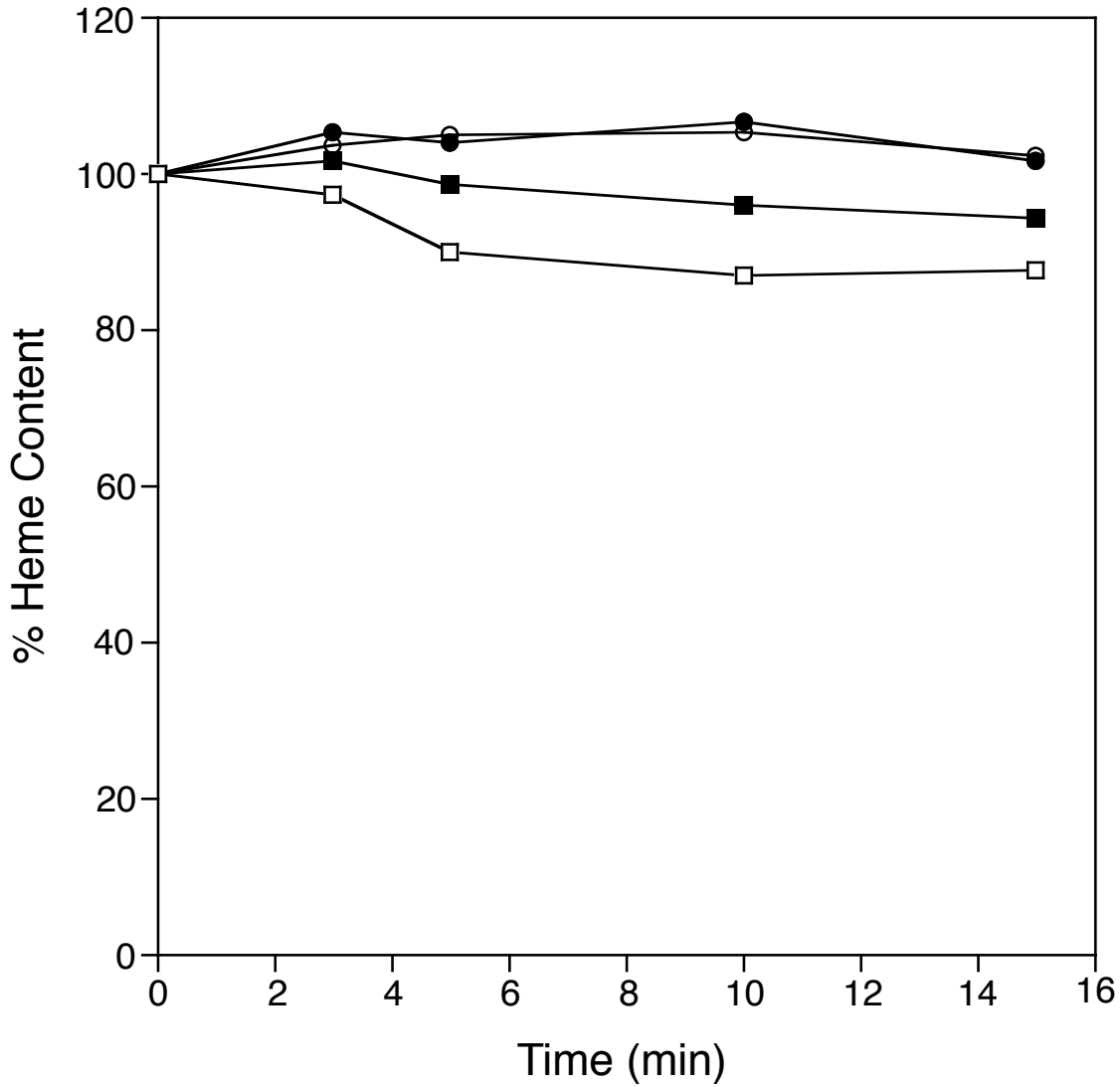

Supplemental Figure 1. Analysis of native heme. CYP3A4 was treated with SCH 66712 (16 μM) (squares) or without SCH 66712 (circles) in the presence (open symbols) or absence (solid symbols) of NADPH for 0, 3, 5, 10, and 15 min. Heme content was analyzed by HPLC using absorbance at 405 nm. Native heme eluted at 22.5 min. After 15 min incubation with SCH 66712 and NADPH (□) there was a ~10% decrease in native heme compared to time zero and control samples.

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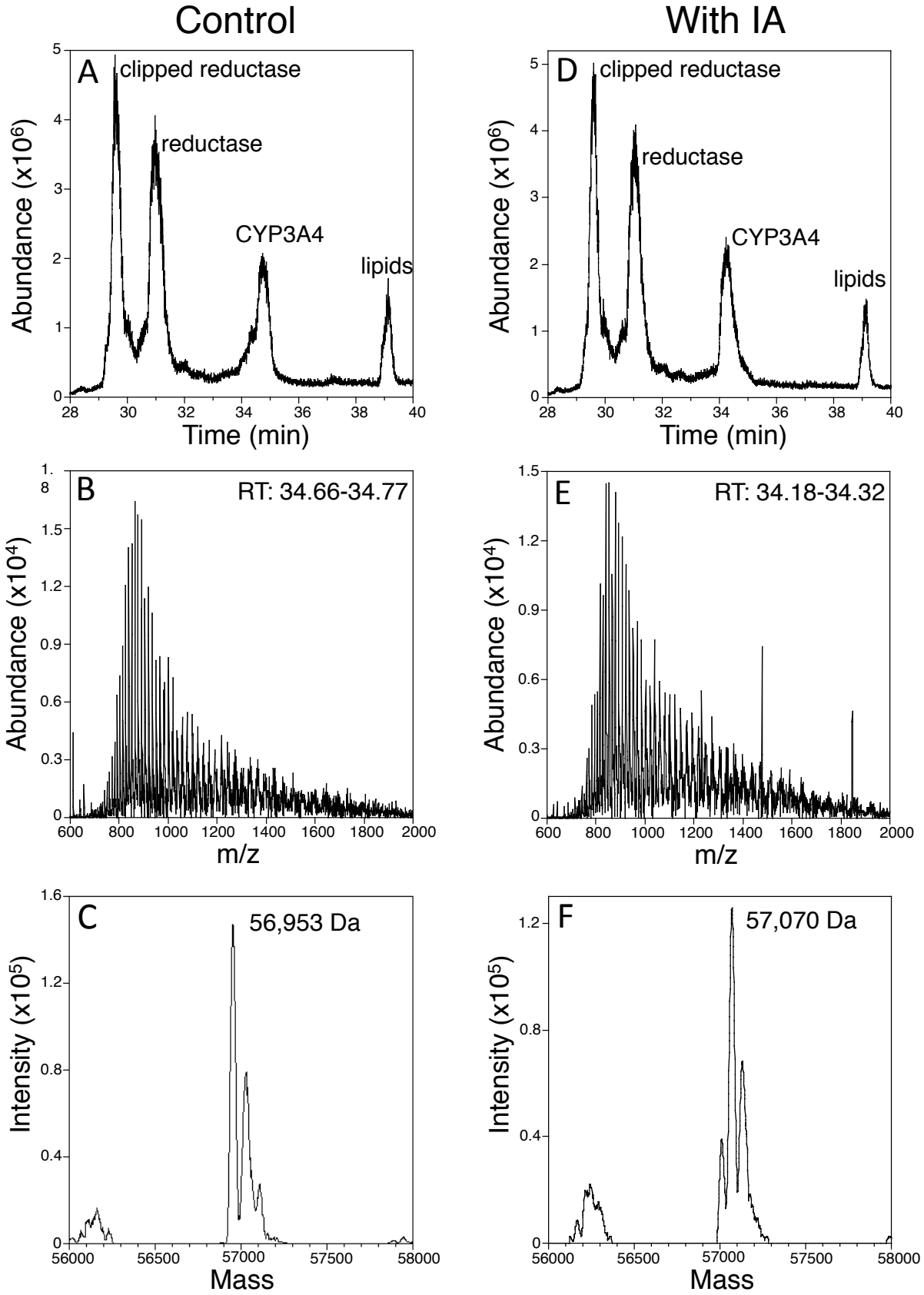

Supplemental Figure 2. LC-ESI-MS analysis of CYP3A4 incubated with iodoacetamide. (A) Chromatogram of reaction with no iodoacetamide. (B) ESI-MS of recombinant CYP3A4 from panel A. (C) Deconvolution of MS in panel B yielded mass of 56,953 Da for CYP3A4. (D) Chromatogram of reaction with iodoacetamide (500 μM) for 1 h. (E) ESI-MS of recombinant CYP3A4 from panel D. (F) Deconvolution of MS in panel E yielded mass of 57,070 Da for CYP3A4. The mass increase is consistent with formation of iodoacetamide alkylation reactions at two cysteines. Chromatograms (A) and (D) show separation of reconstituted system by HPLC and elution of clipped reductase, reductase, CYP3A4, and lipids.

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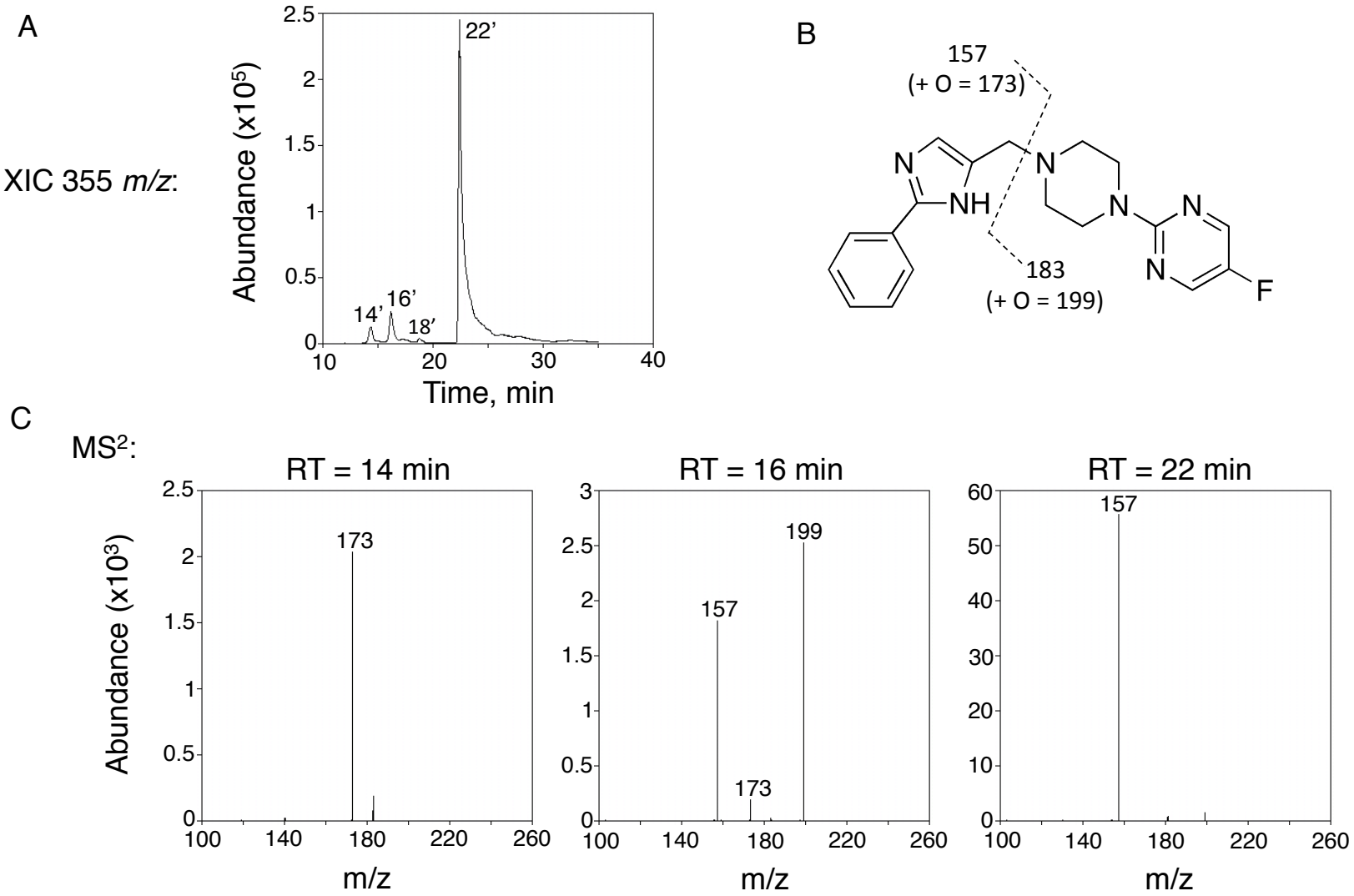

Supplemental Figure 3. Analysis of SCH 66712 metabolites formed by CYP3A4. CYP3A4 was incubated with SCH 66712 and NADPH, and the resulting mixtures were prepared for MS as described previously {Nagy, 2011 #636}. (A) Extracted ion chromatogram of the molecular ions $[M + H]^{+1}$ with *m/z* of 355 representing mono-oxygenation of SCH 66712. (B) MS^2 fragmentation patterns for SCH 66712. (C) MS^2 of the metabolites eluting at 14, 16, and 22 min from panel A. The mass pairs of 173/183 are consistent with mono-oxygenation on the phenyl end of SCH 66712 while the mass pairs of 157/199 are consistent with mono-oxygenation on the piperazine/heteroaromatic ring end of SCH 66712. MS/MS for the peak from panel A at 18 min is not shown but is also consistent with mono-oxygenation on the piperazine/heteroaromatic ring end of SCH 66712.

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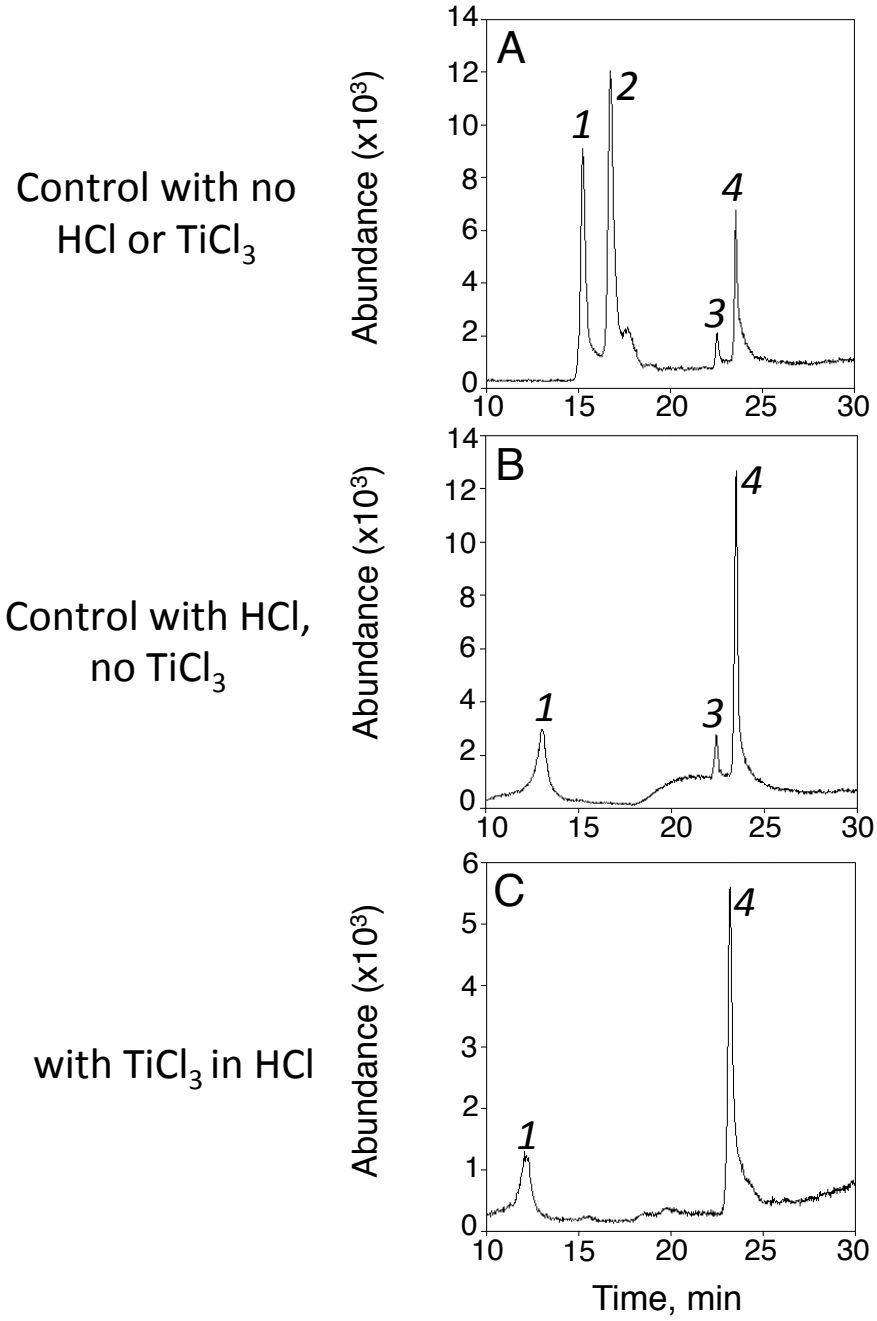

Supplemental Figure 4. Identification of *m/z* 355 isomeric products. Extracted ion chromatograms (*m/z* 355) of CYP2D6 metabolite samples after (A) no treatment, (B) HCl treatment only, or (C) $TiCl_3$ in HCl treatment (Note, CYP3A4 and CYP2D6 make the same metabolites, as previously reported by our group, but in different proportions; compare Supplemental Figure 3A with Supplemental Figure 4A; also see {Nagy, 2011 #636}). The addition of HCl lead to loss of product peak 2. Addition of $TiCl_3$ in HCl resulted only in the loss of product peak 3 indicating *N*-hydroxylation for that product peak. Both product peaks 1 and 4 were resistant to change by $TiCl_3$ in HCl and are therefore carbon hydroxylations. Note that after treatment with HCl and $TiCl_3$, product peak 1 retention time was slightly shifted to ~13 min; however, all fragmentation patterns matched those for peak 1 eluting at ~15 min in the control sample of panel A. Previously we also observed a change in retention time in the first product peak of $TiCl_3$ in HCl treated metabolites (Livezey, 2014).

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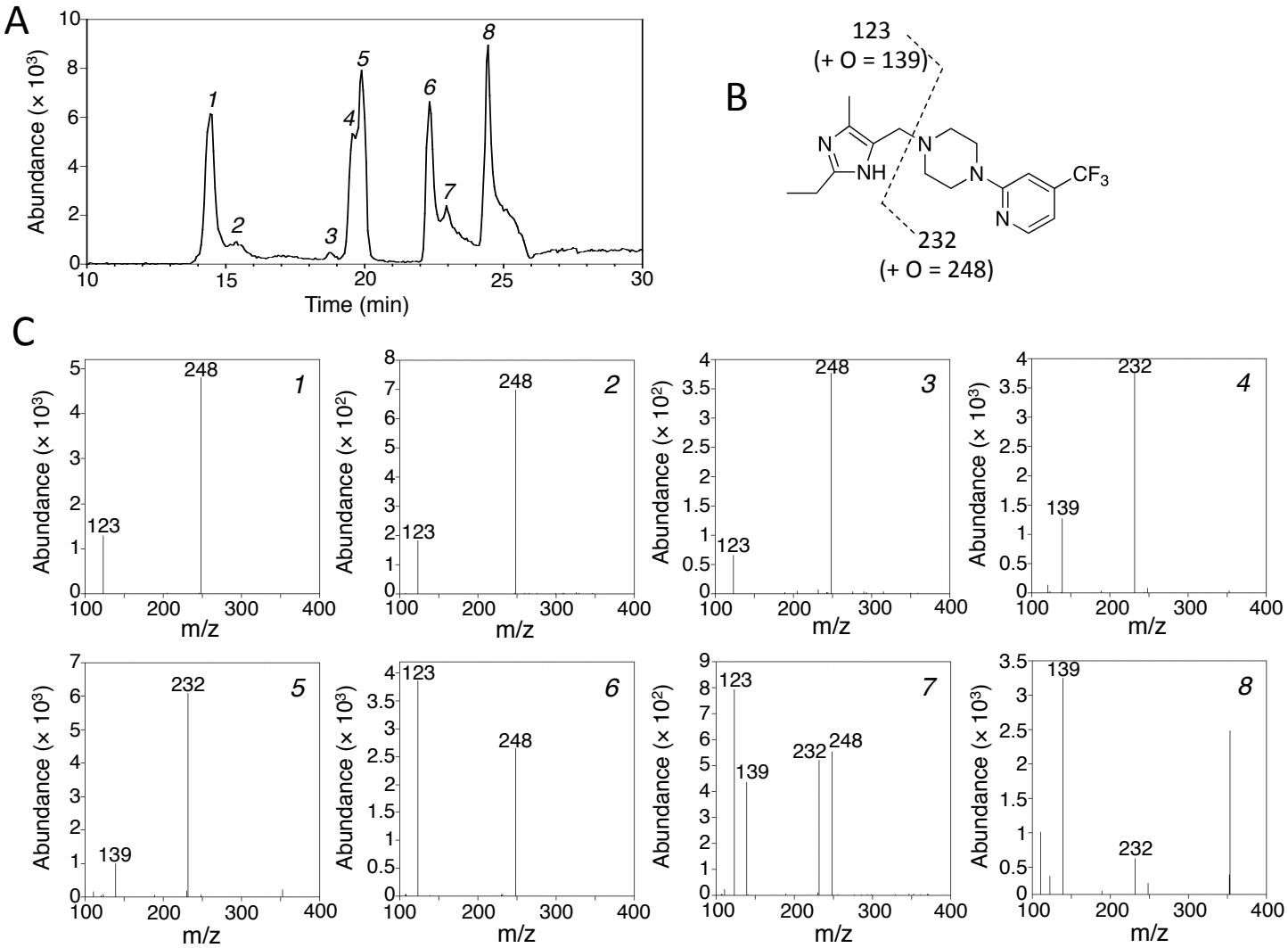

Supplemental Figure 5. Analysis of EMTPP metabolites formed by CYP3A4. CYP3A4 was incubated with EMTPP and NADPH, and the resulting mixtures were analyzed by LC-ESI-MS. (A) Extracted ion chromatogram of the molecular ions $[M + H]^{+1}$ with *m/z* of 370 representing mono-oxygenation of EMTPP. (B) MS^2 fragmentation patterns for EMTPP. (C) MS^2 of the metabolites at peaks *1-8* from panel A. EMTPP breaks in MS^2 fragmentation at the bond between the methylene carbon and the piperazine ring. MS^2 with fragments of 123/248 *m/z* are consistent with mono-oxygenation on the piperazine/heteroaromatic ring ends of EMTPP (product peaks *1*, *2*, *3,* and *6*) (note peak 5 tailed into peak 6 resulting in both appearance of 123/248 *m/z* pair as well as residual 139/232 *m/z* ions). MS^2 with fragments of 139/232 *m/z* are consistent with mono-oxygenation on the imidazole ring ends of EMTPP (product peaks *4, 5, 7,* and *8*).

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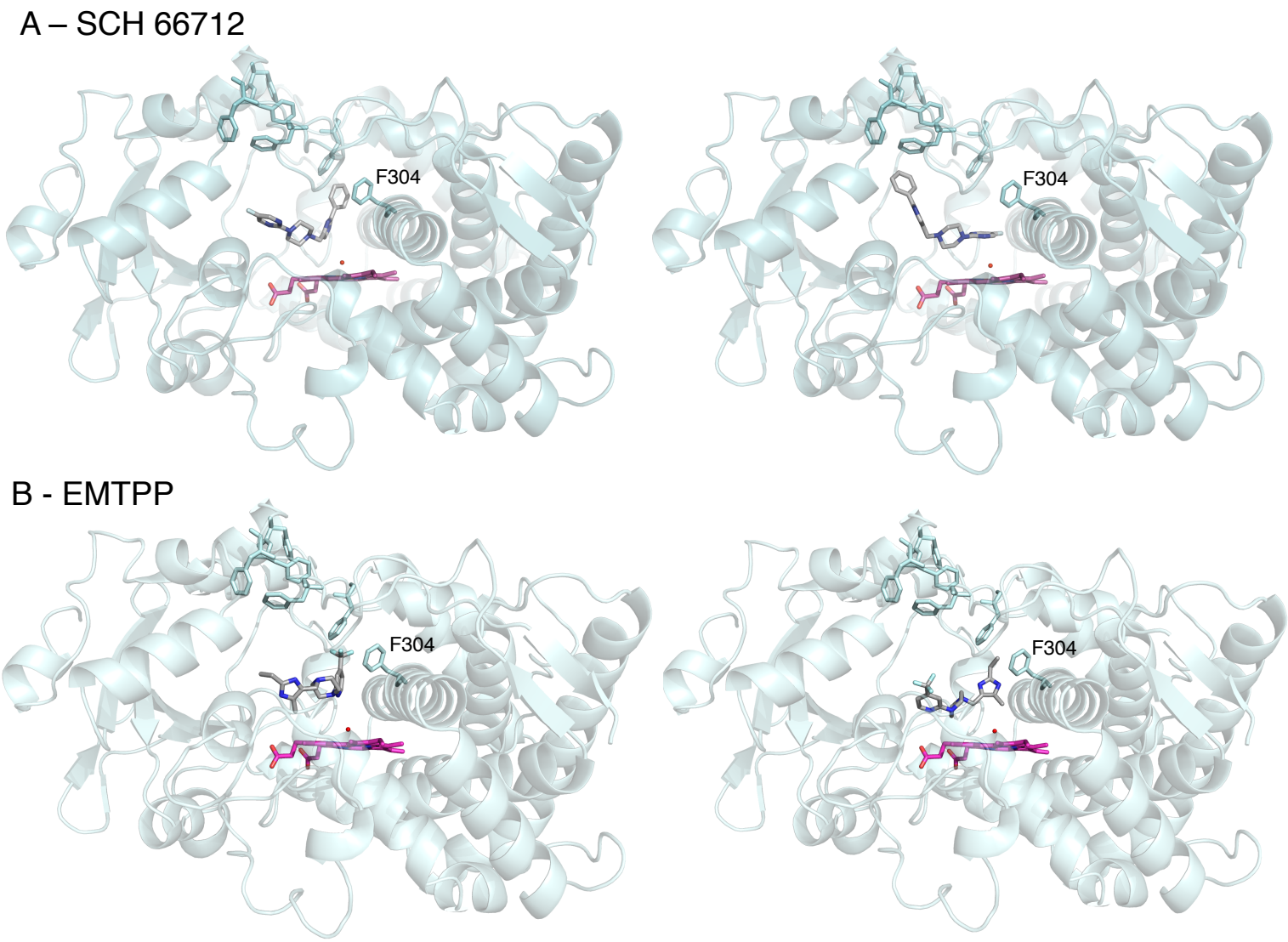

Supplemental Figure 6. Molecular docking of inactivators in the active site of CYP3A4 (3NXU). An oxygen, shown in red, was inserted 1.7 Å above the heme iron as described in the Methods. The heme is shown in magenta. The residues composing the phenylalanine cluster of CYP3A4 are shown as sticks and include: 213, 215, 219, 220, 241, and 304. (A) The lowest free energy binding pose for SCH 66712 (on the left) showed π–π stacking geometry between Phe304 (labeled F304) and the phenyl group of SCH 66712. The majority of docking poses showed this orientation. Docking with the phenyl ring oriented in the direction of the phenylalanine cluster in the roof of CYP3A4 was also observed (on the right), though with lower binding free energy and frequency of observation. (B) The lowest free energy binding pose for EMTPP (on the left) was oriented with the -CF_3 group pointing toward Phe304 while other orientations included the imidazole ring pointing toward Phe304 (on the right). No π–π stacking geometries were observed with EMTPP and residues in the CYP3A4 active site.

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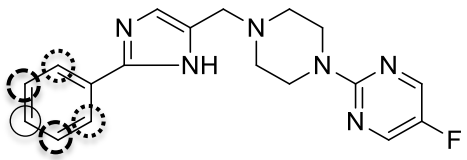
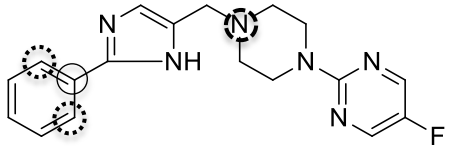
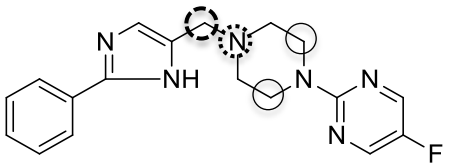
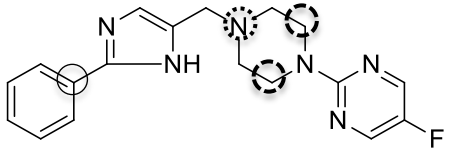
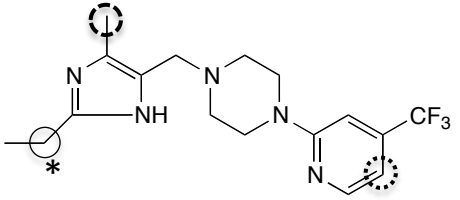
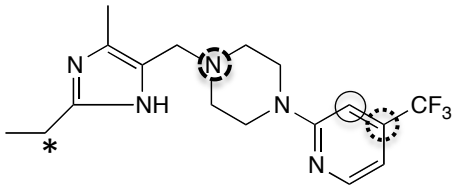
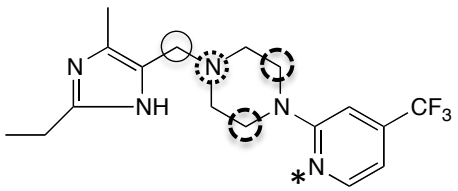
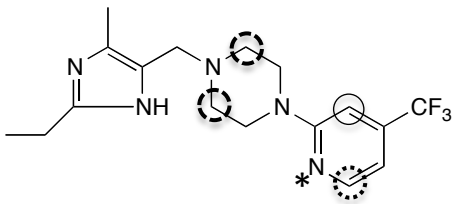
Supplemental Table I – Predicted sites of metabolism on SCH 66712 and EMTPP by CYP2D6 and CYP3A4 and correlation with observed metabolites in mass spectrometry.

	CYP	SMARTCyp	Consistent with observed products	RS Predictor	Consistent with observed products
SCH 66712	2D6		◯ -Yes ◌ -Yes ◌ -Yes		◯ -Yes ◌ -No ◌ -Yes
	3A4		◯ -Yes ◌ -No ◌ -No		◯ -Yes ◌ -Yes ◌ -No
EMTPP	2D6		◯ -Yes ◌ -No ◌ -Yes		◯ -Yes ◌ -Yes ◌ -Yes
	3A4		◯ -Yes ◌ -Yes ◌ -No		◯ -Yes ◌ -Yes ◌ -Yes

*Ranked atom sites of probable reactivity are indicated by circles. Atoms circled with a solid circle (◯) are predicted to be the most probable site of metabolism followed by atoms circled with dashed circle (◌) and finally atoms circled with dotted circle (◌). Observed metabolism of any type at the predicted atoms is indicated by "yes" in the "Metabolite Observed" column. Asterisks (*) indicate the sites of metabolism confirmed experimentally.