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Article

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Overcoming Time Dependent Inhibition (TDI) of Cytochrome P450 3A4 (CYP3A4) Resulting from Bioactivation of a Fluoropyrimidine Moiety

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ABSTRACT

Herein we describe structure activity relationship (SAR) and metabolite identification (Met-ID) studies that provided insight into the origin of time dependent inhibition (TDI) of cytochrome P450 3A4 (CYP3A4) by compound **1**. Collectively, these efforts revealed that bioactivation of the fluoropyrimidine moiety of **1** led to reactive metabolite formation via oxidative defluorination and was responsible for the observed TDI. We discovered that substitution at both the 4- and 6-positions of the 5-fluoropyrimidine of **1** was necessary to ameliorate this TDI as exemplified by compound **19**.

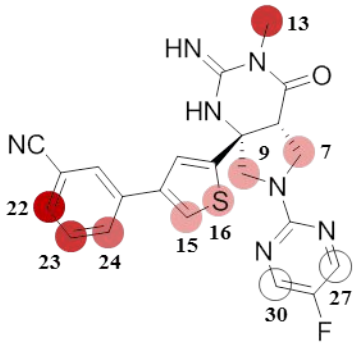
INTRODUCTION

More than half of all drugs are metabolized by cytochrome P450 3A4 (CYP3A4), and thus inhibition of this enzyme by any new therapeutic is highly undesirable due to the potential for drug-drug interactions (DDI) in patients.[1] Time dependent inhibition (TDI) of CYP3A4 is of particular concern because of its potential etiology in reactive metabolite formation and resulting potential for hepatotoxicity.[2] As a consequence, drug discovery efforts generally include *in vitro* assays early in the screening funnel for measurement of both direct and time dependent CYP3A4 inhibition. Many functional groups capable of causing TDI upon bioactivation and reactive metabolite formation have been identified, and related mitigation strategies have been devised.[2] Among those reports, CYP3A4 TDI due to bioactivation of a pyrimidine moiety is less prevalent.[3]

From our efforts on design and development of BACE1 inhibitors for the potential treatment of Alzheimer's disease,[4] we identified compound **1** as a lead in our fused pyrrolidine series that demonstrated robust lowering of amyloid beta ($A\beta_{40}$) in the cerebral spinal fluid (CSF) of rats upon oral administration.[5] Unfortunately, among other off-target liabilities, compound **1** demonstrated CYP3A4 TDI. As a result, we initiated extensive SAR studies to understand and subsequently overcome this effect while preserving BACE1 potency and pre-clinical *in vivo* activity. In this manuscript, we present evidence that bioactivation of the pyrimidine moiety and subsequent reactive metabolite formation is responsible for the observed TDI with compound **1**. We also detail the accompanying SAR and ultimate remediation of the TDI while identifying analog **19** that maintained the overall *in vitro* and *in vivo* profile of the original lead compound in that chemical series.

RESULTS AND DISCUSSION

Discovery of compound **1** was an important milestone for our BACE1 inhibitor program as it represented a distinctly different arrangement of substituents in the enzyme active site than had been previously reported in the literature.[5] The fused pyrrolidine ring enabled facile exploration of the S2" subsite through derivatization of the nitrogen of said ring. After early SAR exploration, we identified *N*-pyrimidine-substituted derivatives as particularly interesting given their overall balance of *in vitro* and cellular potency and *in vivo* activity, albeit with a significant TDI liability in the series. The profile of compound **1** – no direct inhibition of CYP3A4 (co-incubation IC_{50} > 30 μM) with significant TDI (pre-incubation IC_{50} = 1.2 μM) – suggested that one or more metabolites derived upon pre-incubation likely inhibited CYP3A4. Given the interest in this general structure, we began a concerted effort to engineer this liability out of the series.



1

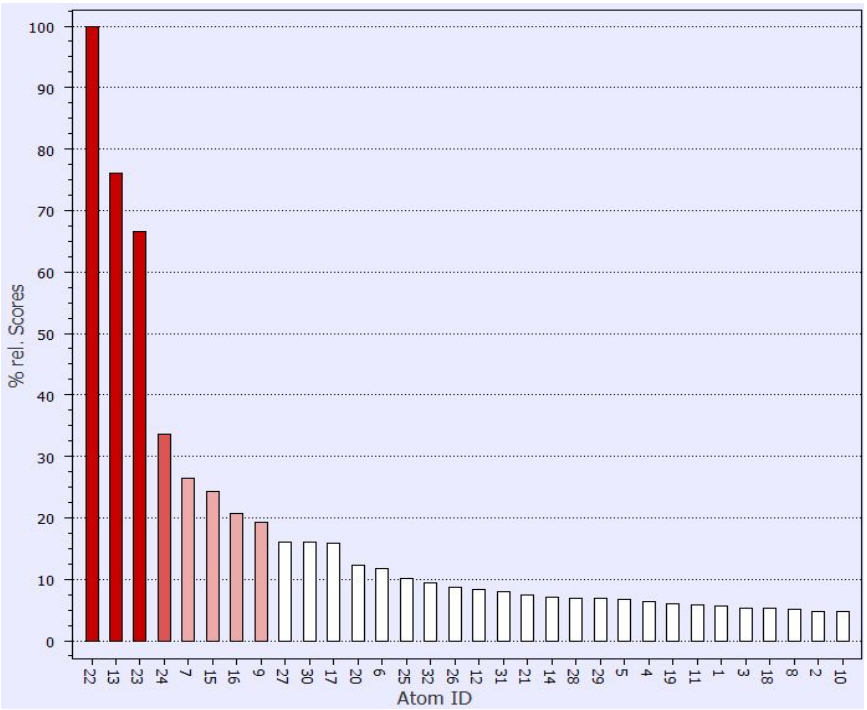
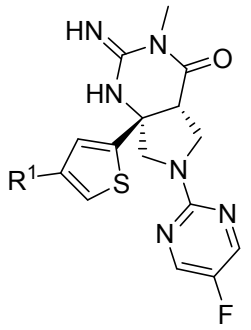
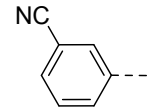
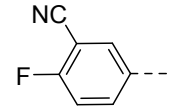
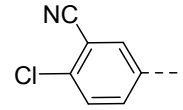
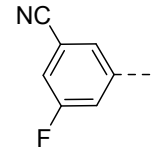
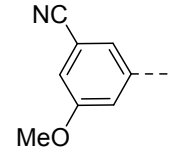
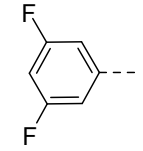
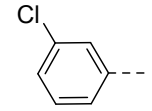


Figure 1. Most likely sites of potential metabolism of compound **1** by CYP 3A4 as predicted by MetaSite. The sites are represented by the red circles on the structure, and the likelihood of metabolism at those sites is expressed by the percentage of relative score.

Analysis of compound **1** by MetaSite[6] suggested that the most likely routes of liver-based metabolism were oxidation of several sites on the cyanophenyl ring and *N*-demethylation. Additional, potential reactive sites were flagged on the pyrrolidine, thiophene, and, to a lesser extent, pyrimidine rings (Figure 1). From this analysis, of particular interest to us was the potential for oxidation on the cyanophenyl ring to generate reactive intermediates. Given that we had previously demonstrated tolerance for modification of this ring,[5,7] in contrast to known limited tolerance for modification of the *N*-methyl moiety,[8] we investigated the impact of substitution and ultimately removal of the cyanophenyl ring. As shown in Table 1, blocking of the potential metabolic sites of the cyanophenyl ring with halogens, which should have also served to deactivate other sites on the ring, or with a methoxy group failed to significantly alter the TDI profile (modest attenuation with α-halo analogues **2** and **3**, no change with β-substituted analogues **4** and **5**). Removal of the cyano moiety entirely from the phenyl ring also did not change the TDI liability (analogues **6** and **7**). Interestingly, even the complete absence of the cyanophenyl ring did not alleviate the TDI issue in analog **8**, suggesting that moiety was not responsible for the observed TDI in this series.

Table 1. CYP 3A4 TDI SAR around the cyanophenyl ring



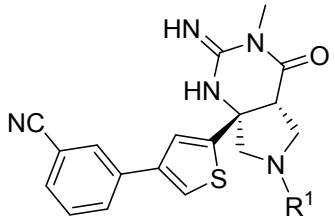
Cpd	R^1	BACE1 K_i (nM)[a]	CYP 3A4 pre-incubation IC_{50} (μM)[b]
1		4	1.2
2		4	5.7
3		10	5.5
4		2	1.6
5		1	1.6
6		10	1.0
7		1	1.2
8	Br	100	0.9

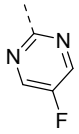
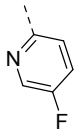
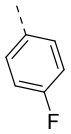
[a]Protocols for determination of K_i values against purified human BACE1 and of BACE1 IC_{50} values in HEK293 cells have been previously described.[4d] [b]Pre-incubation CYP3A4 IC_{50} was obtained following 30 minute pre-incubation in human liver microsomes supplemented with NADPH.

Concurrent with the above efforts, we examined SAR around the changes to the *N*-heteroaryl substituent on the pyrrolidine ring (Table 2). Replacement of the fluoropyrimidine moiety with a fluoropyridine in analog **9**[5] led to a modest attenuation of the TDI, and most strikingly, both fluorophenyl analog **10**[5] and unsubstituted pyrimidine analog **11**[5] did not inhibit CYP3A4 after pre-incubation (IC_{50} > 30 μM). While replacement of a phenyl with a pyrimidine

and fluorination of aryl or heteroaryl rings are techniques often used to increase metabolic stability,[9] these data suggested that combination of these modifications in the 5-fluoropyrimidine moiety of compound **1** was responsible for the observed TDI.

Table 2. CYP3A4 TDI SAR around the pyrrolidine *N*-heteroaryl substituent

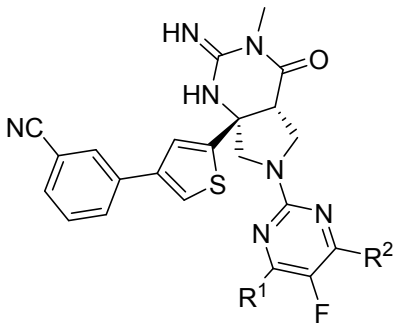


Cpd[a]	R^1	BACE1 K_i (nM)[b]	CYP 3A4 pre-incubation IC_{50} (μM)[c]
1		4	1.2
9		6	6.4
10		26	>30
11		11	>30

[a]Compounds were reported previously and used here to build hypothesis.[5] [b]Protocols for determination of K_i values against purified human BACE1, and BACE1 IC_{50} values, have been previously described.[4d] [c]Pre-incubation CYP3A4 IC_{50} was obtained following 30 minute pre-incubation in human liver microsomes supplemented with NADPH.

The above observation presented a significant potential challenge for this series in our BACE inhibitor program since, as previously disclosed,[5] 5-fluoropyrimidine analog **1** provided the best overall balance of potency, selectivity over cathepsin-D, and good pharmacokinetics in rat. We therefore examined SAR at the 4- and 6-positions of the 5-fluoropyrimidine moiety with the intent to identify modifications that attenuated the TDI liability while maintaining the overall profile of compound **1** (Table 3). To that end, mono substitution on the fluoropyrimidine with either alkyl (analogues **12** and **13**) or alkoxy moieties (analogues **14**,[10] **15,** and **16**) did not mitigate the CYP3A4 TDI issues. In contrast, mono substitution with amino moieties such as pyrrolidine or morpholine (analogues **17** and **18**) or disubstitution on the fluoropyrimidine ring (**19** and **20**) led to significant improvement in the TDI liability. While all of the above modifications were generally well-tolerated in terms of BACE1 affinity, among the selected analogs with an improved TDI profile that were evaluated *in vivo* in rat, only compound **19** exhibited a similar reduction in levels of CSF Aβ_{40} to that of compound **1** (54% vs. 52% for compounds **19** and **1**, respectively) following a 10 mg/kg oral dose. In addition, compound **19** displayed an acceptable PK profile when dosed orally (10 mg/kg) in Wistar Han rats, having bioavailability and a C_{max} of 54% and 5.8 μM respectively (Table 4).

Table 3. CYP3A4 TDI SAR around substitution on the *N*-fluoropyrimidine moiety



Cpd	R^1	R^2	K_i (nM)	CYP 3A4	Rat CSF Aβ_{40}

			BACE1[a]	pre-incubation IC_{50} (μM)[b]	% Reduction (dose, mpk)
1	H	H	4	1.2	52 (10)
12	Me	H	0.7	0.6	38 (10)
13	Et	H	1	0.9	49 (10)
14	OMe	H	1	1.3	45 (30)
15	OEt	H	1	0.7	20 (30)
16	O-*n*Pr	H	1	1.2	12 (30)
17		H	3	>30	2 (10)
18		H	1	>30	17 (10)
19	Me	Me	1	13	54 (10)
20	Me	OMe	0.7	11	ND

[a]Protocols for determination of K_i values against purified human BACE1, and BACE1 IC_{50} values, have been previously described.[4d] [b]Pre-incubation CYP3A4 IC_{50} was obtained following 30 minute pre-incubation in human liver microsomes supplemented with NADPH.

Table 4. Rat pharmacokinetic parameters of compound 19

$AUC_{(0-\infty)}$ (μM*hr)[a]	C_{max} (μM)[a]	F%	PPB (% bound)	Cl (mL/min/kg)[b]	Vd,ss (L/kg)[b]	$t_{1/2}$ (h)[b]
34	5.8	54	99.7	5.6	1.0	2.1

[a] data from 10 mg/kg oral dose. [b] data from 2 mg/kg intravenous dose.

To understand the molecular basis for CYP3A4 TDI in this series, with a particular interest in identification of reactive metabolites derived from fluoropyrimidine moiety, we conducted metabolite identification studies on compound **1** in two different systems – human liver microsomes and recombinant human CYP3A4. Both systems produced *N*-demethylation[11] and a variety of oxidized products. In addition, in the recombinant CYP3A4 system, a minor

metabolite (0.26%) with a molecular weight corresponding to a net oxidation and defluorination with general structure **A** was observed by LCMS (Figure 2). Analogous *N*-demethylated oxidative defluorination metabolism was also observed at much lower levels (0.07%). To trap the reactive intermediate formed in this oxidative defluorination process, the incubation with recombinant CYP3A4 was repeated in presence glutathione (GSH), and an LCMS signal was observed corresponding to a GSH adduct of general formula **B** (Figure 2). Prompted by these results, reinvestigation of the incubation of compound **1** in human liver microsomes showed that an oxidative defluorination metabolite was indeed formed in that system as well at very low levels (0.01%).

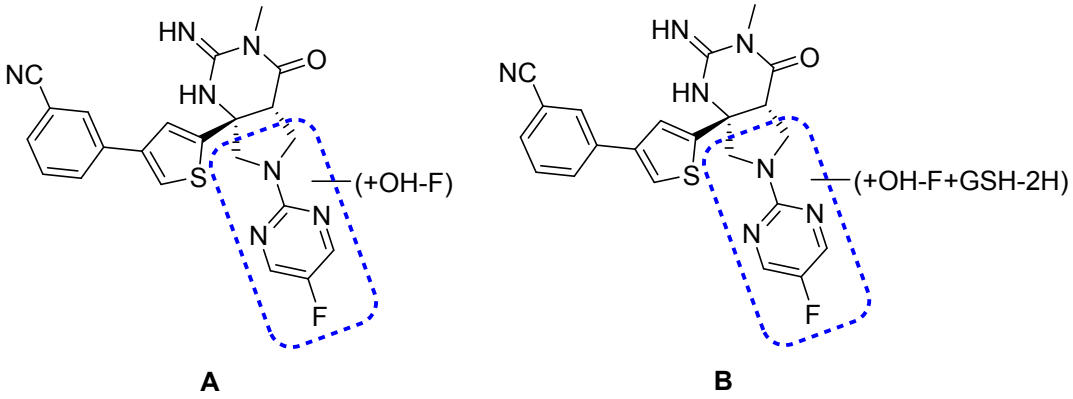


Figure 2. Key metabolites observed following incubation with rCYP3A4 in absence or presence of glutathione (metabolite **A** or adduct **B**, respectively).

In thinking about the mechanism and products for the observed oxidative defluorination, we considered that a net ipso process was most likely, and thus the incubation products in the absence or presence of glutathione would be compounds **21** and **22**, respectively (Figure 3). En route to those observed species, we proposed that either epoxide **23**, likely formed by direct oxidation of the pyrimidine ring,[12] or the resulting quinone iminium intermediate **24** were the possible reactive intermediates that underwent GSH addition to form adduct **22**. Oxidative defluorination product **21** was likely formed by two electron reduction of quinone iminium

intermediate **24** by NADPH mediated by P450 reductase.[13] While the specific reasons for the TDI SAR for pyrimidine substitution in Table 3 are unclear, we speculate that substitution that decreases the ability to form epoxide **23** and quinone iminium intermediate **24**, either through steric or electronic changes, would resolve the TDI.

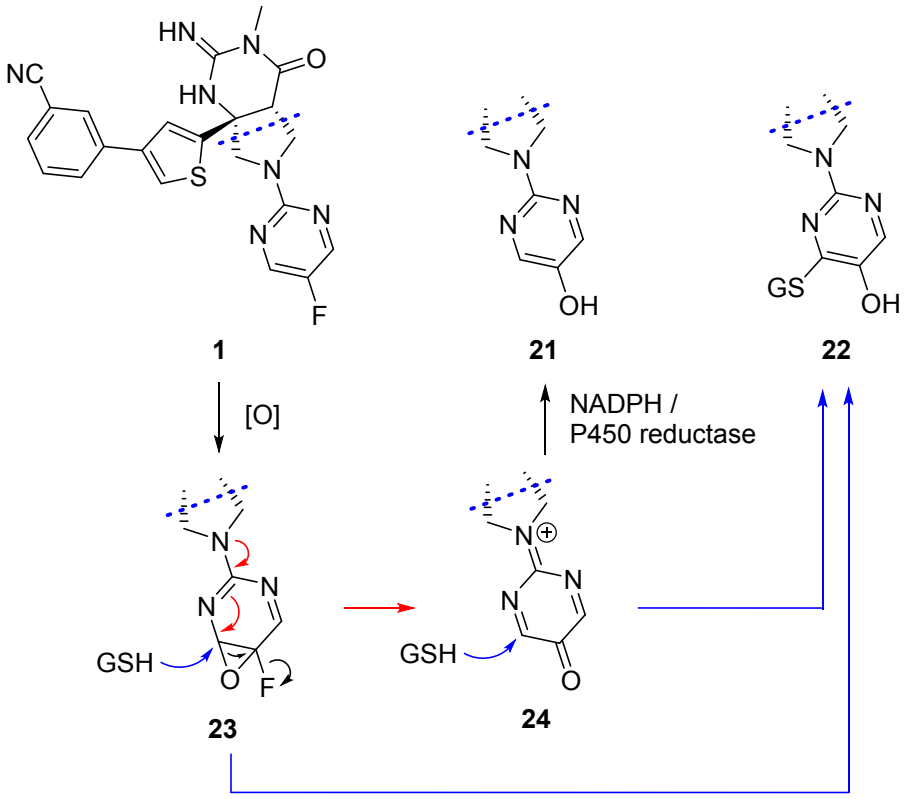


Figure 3. Possible mechanisms for formation of reactive intermediates and resulting oxidative defluorination metabolite **21** and glutathione adduct **22**.

To provide additional evidence that the CYP3A4 TDI for compound **1** was due to formation of reactive intermediates and that compound **19** addressed that issue, we determined the rate constant (k_{obs}) for 6β-hydroxylation of testosterone in human liver microsomes in the presence of each compound at two different concentrations (Figure 4). For compound **1**, inhibition of the CYP3A4-mediated hydroxylation was observed at both 2 μM and 10 μM, and the magnitude was dependent on both the concentration of **1** and time (Figure 4A), key indicators

of the involvement of metabolically derived reactive intermediates. In fact, the k_{obs} for compound **1** at 10 μM was nearly identical to that of mifepristone (0.69 min^{-1} and 0.66 min^{-1}, respectively), a known time- and metabolism-based inhibitor of CYP3A4.[14] Gratifying and in concert with its more moderate TDI, the profile of compound **19** represented a significant improvement over that of compound **1** (Figure 4B), specifically, there was no inhibition of testosterone 6β-hydroxylation observed at 2 μM, and the k_{obs} at 10 μM was reduced relative to **1** (of 0.022 min^{-1} versus 0.069 min^{-1}, respectively).

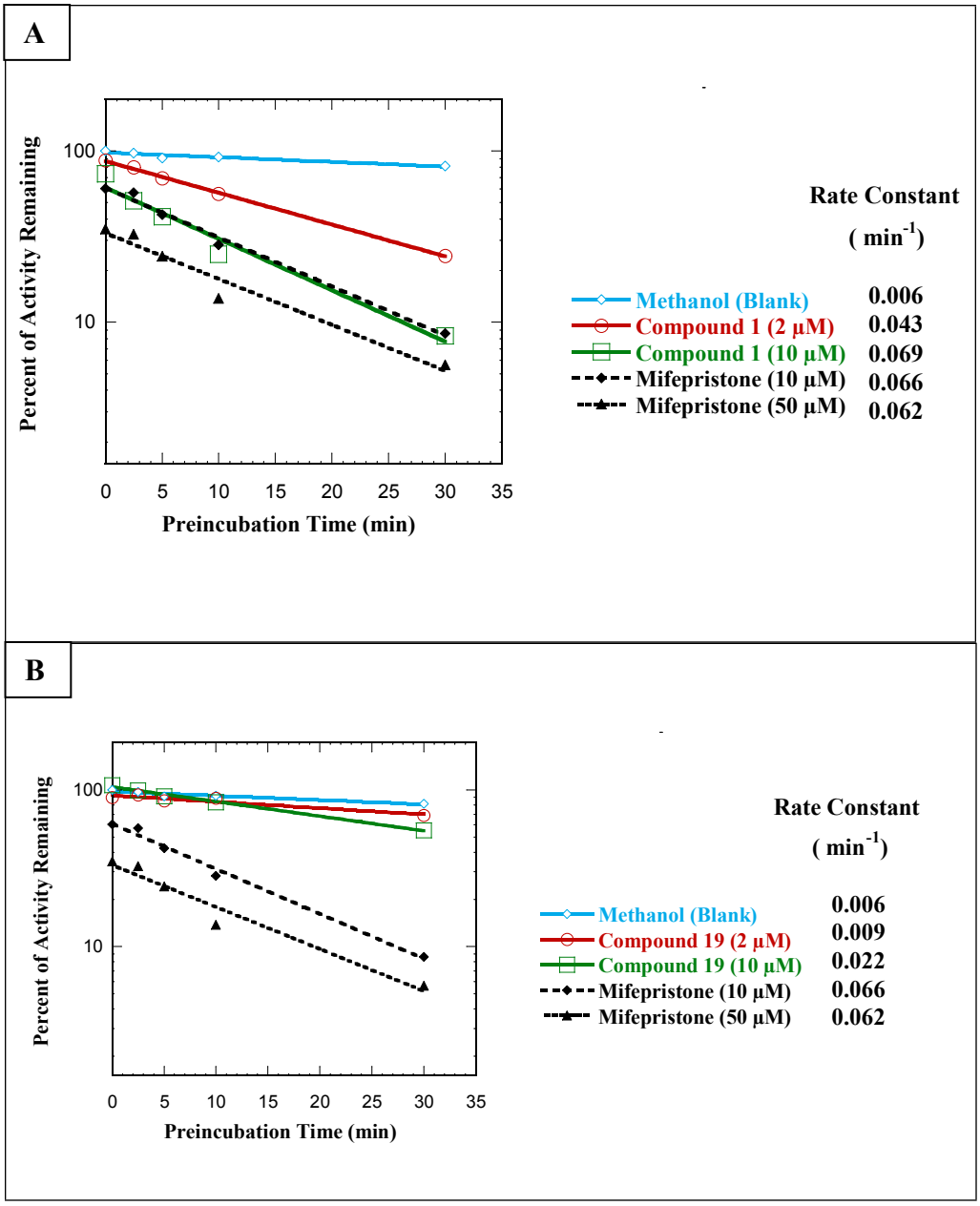


Figure 4. Time-dependent inhibition of testosterone 6β-hydroxylation by compound **1** (Panel A) and **19** (Panel B) in human liver microsomes. The k_{obs} for each compound was measured at concentrations of 2 µM (red circle) and 10 µM (green square) of compound. Mifepristone at 10 µM and 50 µM (black diamond and triangle, respectively) is included as a positive control. Methanol (blue diamond) is included as a blank negative control. The number of replicates from each experiment is given as "n". Panel A (Compound **1**): n=3 for each data point except at 30 min at 10 µM concentration of **1** (n=2). CV < 20 % for all data points. Panel B (Compound **19**): n=3 for all data points. CV < 20 % for all data points.

CONCLUSIONS

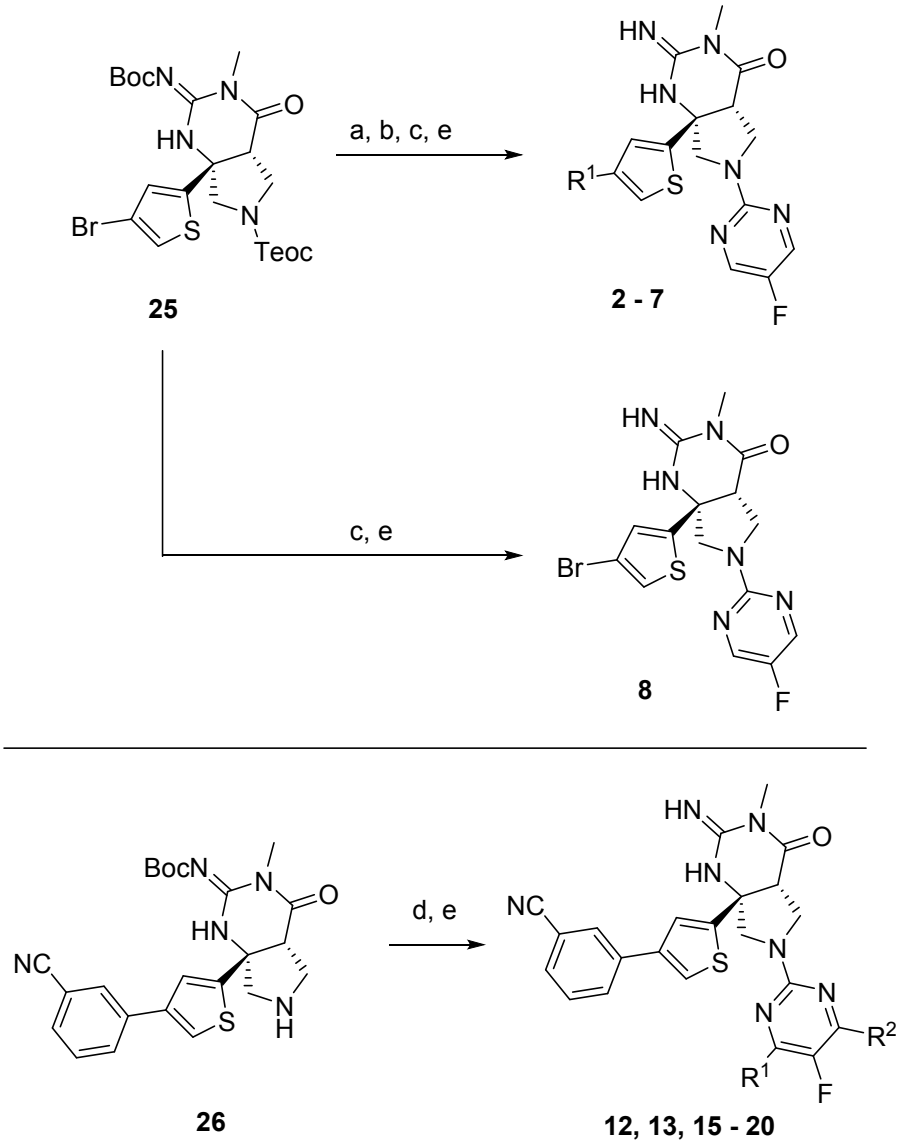
Manifestation of CYP TDI as a result of bioactivation of drug molecules is a complex process, and identification of structural elements in the molecules responsible for the TDI remains a very difficult task. Once identified, SAR studies to mitigate this off-target liability without major deterioration of potency, pharmacokinetics, and related *in vivo* activity is a major challenge in drug discovery. Over the years, many functional groups prone to manifest TDI upon bioactivation have been identified and mitigation strategies developed. However, TDI originating from pyrimidine moieties are rare, and thus related mitigation strategies are less well characterized. Our SAR analysis and investigations coupled with Met-ID studies supported that reactive metabolite generation through bioactivation of the fluoropyrimidine moiety of compound **1** was responsible for its observed CYP3A4 TDI[15] and ultimately that substitution of the fluoropyrimidine could mitigate this TDI liability while maintaining a desirable overall molecular profile in analog **19**.

CHEMISTRY

Intermediates **25** and **26**, which we previously reported,[5] served as key intermediates for synthesis of all of the novel analogues described in this article (Scheme 2). Synthesis of analogues **2 – 7** began with Suzuki cross-coupling of compound **25** with the analogous aryl boronic acids followed by fluoride-mediated deprotection of the Teoc group, *N*-arylation of the corresponding free pyrrolidine with 2-chloro-5-fluoropyrimidine, and TFA-mediated deprotection of the Boc group to afford the desired final compounds. Analog **8** was made via fluoride-mediated deprotection of the Teoc group from intermediate **25** directly followed by *N*-arylation of the corresponding free pyrrolidine with 2-chloro-5-fluoropyrimidine and TFA-mediated deprotection of the Boc group. Analogues **12, 13**, and **15 - 20** were prepared from

intermediate **26** via *N*-arylation with the corresponding substituted 2-chloro-5-fluoro pyrimidines followed by TFA-mediated deprotection of the Boc group to afford the desired final compounds. The protocols for the synthesis of substituted 2-chloro pyrimidines used for the preparation of analogues in Table 3 are described in the supporting information. Syntheses of compounds **1**, **9**, **10**, **11** and **14** were reported previously.[5]

Scheme 2. Synthesis of compounds 2 - 8 and 12, 13, and 15 - 20.



Reagents and conditions: (a) R^1-Boronic acid, dichloro[1,1'-bis(diphenylphosphino)ferrocene]-palladium(II) dichloromethane, *tert*-butanol, K_2CO_3, 60 °C; (b) TBAF, THF, 0 °C; (c) 2-chloro-5-fluoropyrimidine, $Pd_2(dba)_3$, (2-biphenylyl)di-*tert*-butylphosphine (JohnPhos) and Na-O*t*Bu, 50 °C; (d) 2-Chloro-4-R^1-5-fluoro-6-R^2-pyrimidine, $Pd_2(dba)_3$, (2-biphenylyl)di-*tert*-butyl-phosphine (JohnPhos), and Na-O*t*Bu, 60 °C; (e) TFA in dichloromethane, rt.

EXPERIMENTAL SECTION

General. CYP3A4 TDI was assessed by measuring inhibition of 6β-hydroxylation of testosterone by rCYP3A4 after 30 minutes of pre-incubation of the analogues in the presence of NADPH to allow for metabolite formation and is reported here as the pre-incubation IC_{50}. Direct inhibition of CYP3A4 was measured without NADPH pre-incubation and is reported here as the co-incubation IC_{50}. Protocols for more detailed measurement of k_{obs} for compounds **1** and **19** are in the supplemental material. Measurement of BACE1 K_i and IC_{50} values and determination of rat CSF $A\beta_{40}$ lowering *in vivo* were carried out following our previously described protocols.[4d]

Synthesis. All the reagents and solvents obtained from commercial sources were used without further purification. Air sensitive chemistries were performed under an atmosphere of nitrogen or argon. Purification of the final compounds to >95% purity were carried out either using a prepacked silica gel cartridge (Analogix, Biotage, or ISCO) or a reverse phase C18 column (mobile phase, A = 0.05% TFA in water and B = 0.05% TFA in acetonitrile, gradient = 10% to 95% B over 10 min). All NMR data unless otherwise specified were collected using either at 400 or 600 MHz on a Varian or Bruker instrument. Chemical shifts are reported in ppm relative to the residual solvent peak in the indicated solvent, and for ^1H NMR, multiplicities, coupling constants in Hertz, and numbers of protons are indicated parenthetically. Microwave assisted reactions were performed in a Smith synthesizer from Personal Chemistry. Purity and MS information was obtained via LC-electrospray-mass spectroscopy with a C18 column using a gradient of 5% to 95% MeCN in water with 0.05% TFA as the mobile phase. The purity of the

samples was assessed using a UV detector at 254 nm. An additional analytical reverse phase HPLC system was used to assess the purity of final compounds using an ELSD detector and a UV detector monitoring both 220 nm and 254 nm.

General Procedure A: Synthesis of analogues 2 – 7 from intermediate 25

To a solution of bromide **25** (1 equiv.) and appropriately substituted aryl boronic acid (1.5 equiv.) in *t*-BuOH (4 mL) was added dichloro[1,1'-bis(diphenylphosphino)-ferrocene]palladium(II) dichloromethane (0.15 equiv.) followed by aqueous 1N K_2CO_3 (1.5 equiv.). The resulting mixture was heated at 60 °C for 1 h, or until the reaction was determined to be complete, before it was cooled, diluted with ethyl acetate and washed with water. The organic layer was dried, concentrated and the residue was purified by silica gel chromatography using 0 to 100% ethyl acetate in hexanes to provide the product. To this product was added 1M TBAF (2.8 equiv.) in THF at 0 °C and the solution was stirred at room temperature for 4 h or until the reaction was determined to be complete. The reaction mixture was poured into a saturated aqueous solution of $NaHCO_3$ and extracted with ethyl acetate. The organic layer was concentrated and residue was purified by silica gel chromatography using 100% ethyl acetate to provide a free pyrrolidine intermediate.

To a solution of the above pyrrolidine intermediate (1 equiv.) and 2-chloro-5-fluoropyrimidine (1.2 equiv.) in toluene (5 mL/mmol of pyrrolidine) were added tris(dibenzylidene-acetone)dipalladium(0) (0.05 equiv.), (2-biphenylyl)di-*tert*-butylphosphine (JohnPhos, 0.13 equiv.) and Na-O*t*Bu (2.7 equiv.). The resulting solution was degassed and heated at 50 °C for 16 h or until the reaction was determined to be complete, then cooled to room temperature, filtered through a pad of Celite, and the crude material was purified via silica gel

chromatography using ethyl acetate in hexanes as the eluent to give an *N*-arylated product. The Boc protecting group was removed through treatment of this material with 20% TFA/CH_2Cl_2 (2.5 mL/mmol). The deprotected compounds were purified by reverse phase HPLC (C18) using 0 to 90% water / acetonitrile with 0.05% TFA.

2-Fluoro-5-(5-((4aR,7aR)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (2)

Analog **2** was prepared from intermediate **25** and (3-cyano-4-fluorophenyl)boronic acid according to General Procedure A. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.25 (s, 2H), 7.77 - 7.70 (m, 2H), 7.43 - 7.41 (m, 1H), 7.33 (s, 1H), 7.28 - 7.23 (m, 1H), 4.57 (d, *J* = 12.2 Hz, 1H), 4.36 (s, 1H), 3.98 (d, *J* = 12.5 Hz, 1H), 3.89 - 3.82 (m, 2H), 3.37 (s, 3H). *m/z*: 466.3.

2-Chloro-5-(5-((4aR,7aR)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (3)

Analog **3** was prepared from intermediate **25** and (4-chloro-3-cyanophenyl)boronic acid according to General Procedure A. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.26 (s, 2H), 7.80 (d, *J* = 1.8 Hz, 1H), 7.67 (dd, *J* = 2.1, 7.9 Hz, 1H), 7.54 (d, *J* = 8.2 Hz, 1H), 7.47 (s, 1H), 7.35 (s, 1H), 4.59 (d, *J* = 12.5 Hz, 1H), 4.36 (s, 1H), 3.99 (d, *J* = 12.2 Hz, 1H), 3.90 - 3.83 (m, 2H), 3.38 (s, 3H). *m/z*: 482.2.

3-Fluoro-5-(5-((4aR,7aR)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (4)

Analog **4** was prepared from intermediate **25** and (3-cyano-5-fluorophenyl)boronic acid according to General Procedure A (12% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.55 (br, 1H), 8.25 (s, 2H), 7.59 (s, 1H), 7.50 (d, *J* = 1.5 Hz, 1H), 7.44 (ddd, *J* = 1.3, 2.5, 9.4 Hz, 1H), 7.30 (ddd, *J* = 1.2, 2.5, 7.7 Hz, 1H), 7.24 (d, *J* = 1.5 Hz, 1H), 4.31 - 4.21 (m, 2H), 4.13 (d, *J* = 12.4 Hz, 1H), 3.94 (dd, *J* = 8.6, 11.2 Hz, 1H), 3.70 (t, *J* = 8.8 Hz, 1H), 3.36 (s, 3H). *m/z*: 466.3.

3-(5-((4aR,7aR)-6-(5-Fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)-5-methoxybenzonitrile (5)

Analog **5** was prepared from intermediate **25** and (3-cyano-5-methoxyphenyl)boronic acid according to General procedure A (19% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.43 (s, 2H), 7.89 (d, *J* = 0.7 Hz, 1H), 7.68 (s, 1H), 7.60 (s, 1H), 7.52 - 7.45 (m, 1H), 7.21 (d, *J* = 0.7 Hz, 1H), 4.47 (d, *J* = 12.1 Hz, 1H), 4.26 - 4.12 (m, 3H), 4.08 (dd, *J* = 7.3, 9.5 Hz, 1H), 3.89 (s, 3H), 3.35 (s, 3H). *m/z*: 478.3.

(4aR,7aR)-7a-(4-(3,5-Difluorophenyl)thiophen-2-yl)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyloctahydro-4H-pyrrolo[3,4-d]pyrimidin-4-one (6)

Analog **6** was prepared from intermediate **25** and (3,5-difluorophenyl)boronic acid according to General Procedure A. ^{1}H NMR (600 MHz ,$CDCl_3$) δ 8.27 (s, 2 H), 7.45 (s, 1H), 7.32 (s, 1H), 7.02 (d, *J* = 6.4 Hz, 2H), 6.76 (t, *J* = 8.7 Hz, 1H), 4.58 (d, *J* = 12.2 Hz, 1H), 4.35 (s, 1H), 3.99 (d, *J* = 12.5 Hz, 1H), 3.91 - 3.82 (m, 2H), 3.37 (s, 3H). *m/z*: 459.3.

(4aR,7aR)-7a-(4-(3-Chlorophenyl)thiophen-2-yl)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyloctahydro-4H-pyrrolo[3,4-d]pyrimidin-4-one (7)

Analog **7** was prepared from intermediate **25** and (3-chlorophenyl)boronic acid according to General Procedure A. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.22 (s, 2H), 7.50 (s, 1H), 7.40 (d, *J* = 7.6 Hz, 1H), 7.36 (s, 1H), 7.30 (t, *J* = 7.9 Hz, 1H), 7.27 - 7.24 (m, 1H), 4.31 (d, *J* = 11.3 Hz, 1H), 4.22 (t, *J* = 10.4 Hz, 1H), 3.96 (dd, *J* = 2.0, 12.2 Hz, 1H), 3.80 (t, *J* = 10.1 Hz, 1H), 3.65 (t, *J* = 9.2 Hz, 1H), 3.30 (s, 3H). *m/z*: 457.3.

Preparation of (4aR,7aR)-7a-(4-bromothiophen-2-yl)-6-(5-fluoropyrimidin-2-yl)-2-imino-3-methyloctahydro-4H-pyrrolo[3,4-d]pyrimidin-4-one (8)

Following removal of the Teoc group from intermediate **25** using a procedure analogous to that described in the relevant section of General Procedure A, the resulting free pyrrolidine (0.027 g, 0.063 mmol) was mixed with toluene (0.5 mL), sodium *tert*-butoxide (0.021 g, 0.219 mmol), $Pd_2(dba)_3$ (0.007 g, 7.64 μmol) and BINAP (0.01 g, 0.016 mmol). The resulting mixture was degassed and back filled with nitrogen (x3). To this solution was added a solution of 2-chloro-5-fluoropyrimidine (0.016 g, 0.121 mmol) in toluene (0.1 mL), and the mixture was heated at 60 °C for 12 h. The reaction mixture was filtered through a pad of Celite, and the crude material was purified via silica gel chromatography using 0 to 100% ethyl acetate in hexanes as the eluent to give an *N*-arylated product. The Boc protecting group from the *N*-arylated product was removed through treatment with 20% TFA/CH_2Cl_2 (1 mL). The deprotected compound was purified by reverse phase HPLC (C18) using water/acetonitrile with 0.1% formic acid, and was converted to its HCl salt to provide compound **8** (0.0013 g, yield 5%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.37 (d, *J* = 0.9 Hz, 2H), 7.54 (d, *J* = 1.4 Hz, 1H), 7.22 (d, *J* = 1.5 Hz, 1H), 4.38 (d, *J* = 12.2 Hz, 1H), 4.21 – 3.99 (m, 4H), 3.33 (s, 3H). *m/z*: 427.2.

General Procedure B: Synthesis of analogues 12, 13, 15 – 20 from intermediate 26.

To a solution of the pyrrolidine **26** (1 equiv.) and an appropriately substituted 2-chloro-5-fluoro pyrimidine (1.2 equiv.) in toluene (5 mL/mmol of pyrrolidine) were added tris(dibenzylidene-acetone)dipalladium(0) (0.1 equiv.), (2-biphenylyl)di-*tert*-butylphosphine (JohnPhos, 0.15 equiv.), and Na-O*t*Bu (2.2 equiv.). The resulting solution was degassed, and heated at 60 °C for 2 h, or until the reaction was determined to be complete, and then cooled to rt. The reaction mixture was filtered through a pad of Celite, and the crude material was purified via silica gel chromatography using ethyl acetate in hexanes as the eluent to give an *N*-arylated product. The Boc protecting group of the *N*-arylated product was removed through treatment with 20% TFA/CH_2Cl_2 (2.5 mL/mmol). The deprotected compounds were purified by reverse phase HPLC (C18) using 0−90% water/acetonitrile with 0.05% TFA.

3-(5-((4aR,7aR)-6-(5-Fluoro-4-methylpyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (12)

Analog **12** was prepared from intermediate **26** and 2-chloro-5-fluoro-4-methylpyrimidine following General Procedure B (0.035 g, 35% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.27 (d, *J* = 2.2 Hz, 1H), 8.05 (t, *J* = 1.5 Hz, 1H), 7.98 (td, *J* = 1.5, 8.1 Hz, 1H), 7.90 (d, *J* = 1.5 Hz, 1H), 7.70 (d, *J* = 1.1 Hz, 1H), 7.66 (d, *J* = 7.7 Hz, 1H), 7.59 (t, *J* = 8.0 Hz, 1H), 4.49 (d, *J* = 12.4 Hz, 1H), 4.27 - 4.06 (m, 4H), 3.35 (s, 3H), 2.44 (d, *J* = 2.2 Hz, 3H). *m/z*: 462.3.

3-(5-((4aR,7aR)-6-(4-Ethyl-5-fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (13)

Analog **13** was prepared from intermediate **26** and 2-chloro-4-ethyl-5-fluoropyrimidine following General Procedure B (0.063 g, 60% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.24 (d, *J* = 2.2 Hz, 1H), 8.04 (s, 1H), 7.97 (d, *J* = 7.7 Hz, 1H), 7.90 (d, *J* = 1.5 Hz, 1H), 7.69 (d, *J* = 1.1 Hz, 1H), 7.66 (d, *J* = 7.7 Hz, 1H), 7.59 (t, *J* = 7.9 Hz, 1H), 4.49 (d, *J* = 12.1 Hz, 1H), 4.29 - 4.05 (m, 4H), 3.35 (s, 3H), 2.84 - 2.74 (m, 2H), 1.28 (t, *J* = 7.5 Hz, 3H). *m/z*: 476.3.

3-(5-((4aR,7aR)-6-(4-Ethoxy-5-fluoropyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (15)

Analog **15** was prepared from intermediate **26** and 2-chloro-4-ethoxy-5-fluoropyrimidine following General Procedure B. ^{1}H NMR (400 MHz, CD_3OD) δ 8.23 (d, *J* = 4.0 Hz, 1H), 8.06 (s, 1H), 7.99 (d, *J* = 7.7 Hz, 1H), 7.93 (d, *J* = 1.5 Hz, 1H), 7.73 (s, 1H), 7.67 (d, *J* = 7.7 Hz, 1H), 7.60 (t, *J* = 8.0 Hz, 1H), 4.58 - 4.51 (m, 3H), 4.24 (s, 4H), 3.35 (s, 3H), 1.46 (t, *J* = 7.1 Hz, 3H). *m/z*: 492.3.

3-(5-((4aR,7aR)-6-(5-Fluoro-4-propoxypyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (16)

Analog **16** was prepared from intermediate **26** and 2-chloro-5-fluoro-4-propoxypyrimidine following General Procedure B. ^{1}H NMR (400 MHz, CD_3OD) δ 8.13 (d, *J* = 3.7 Hz, 1H), 8.05 (t, *J* = 1.5 Hz, 1H), 7.98 (td, *J* = 1.5, 8.1 Hz, 1H), 7.91 (d, *J* = 1.5 Hz, 1H), 7.71 (d, *J* = 1.1 Hz, 1H), 7.67 (td, *J* = 1.5, 7.7 Hz, 1H), 7.59 (t, *J* = 7.8 Hz, 1H), 4.54 - 4.49 (m, 1H), 4.45 (t, *J* = 6.6 Hz, 2H), 4.28 - 4.06 (m, 4H), 3.35 (s, 3H), 1.91 - 1.80 (m, 2H), 1.03 (t, *J* = 7.3 Hz, 3H). *m/z*: 506.3.

3-(5-((4aR,7aR)-6-(5-Fluoro-4-(pyrrolidin-1-yl)pyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (17)

Analog **17** was prepared from intermediate **26** and 2-chloro-5-fluoro-4-(pyrrolidin-1-yl)pyrimidine following General Procedure B (0.059 g, 51% yield). ^{1}H NMR (400 MHz ,CD_3OD) δ 8.06 (s, 1H), 8.00 (d, *J* = 7.7 Hz, 1H), 7.94 (d, *J* = 1.1 Hz, 1H), 7.91 (d, *J* = 7.0 Hz, 1H), 7.76 (d, *J* = 1.1 Hz, 1H), 7.67 (d, *J* = 7.7 Hz, 1H), 7.60 (t, *J* = 7.9 Hz, 1H), 4.36 - 4.12 (m, 5H), 3.98 - 3.87 (m, 2H), 3.85 - 3.72 (m, 2H), 3.35 (s, 3H), 2.18 - 2.05 (m, 2H), 2.04 - 1.92 (m, 2H). *m/z*: 517.3.

3-(5-((4aR,7aR)-6-(5-Fluoro-4-morpholinopyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (18)

Analog **18** was prepared from intermediate **26** and 4-(2-chloro-5-fluoropyrimidin-4-yl)morpholine following General Procedure B (0.021 g, 59% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (s, 1H), 7.99 (d, *J* = 8.1 Hz, 1H), 7.96 (d, *J* = 7.7 Hz, 1H), 7.91 (d, *J* = 1.1 Hz, 1H), 7.70 - 7.64 (m, 2H), 7.59 (t, *J* = 7.9 Hz, 1H), 4.29 - 4.06 (m, 5H), 3.99 - 3.91 (m, 4H), 3.83 - 3.75 (m, 4H), 3.33 (s, 3H). *m/z*: 533.3.

3-(5-((4aR,7aR)-6-(5-Fluoro-4,6-dimethylpyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (19)

Analog **19** was prepared from intermediate **26** and 2-chloro-5-fluoro-4,6-dimethylpyrimidine following General Procedure B (0.040 g, 38% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.04 (t, *J* = 1.5 Hz, 1H), 7.96 (td, *J* = 1.5, 7.9 Hz, 1H), 7.89 (d, *J* = 1.5 Hz, 1H), 7.67

(td, *J* = 1.5, 7.9 Hz, 1H), 7.65 (d, *J* = 1.5 Hz, 1H), 7.59 (t, *J* = 7.9 Hz, 1H), 4.43 (d, *J* = 12.1 Hz, 1H), 4.21 - 4.06 (m, 3H), 4.01 (dd, *J* = 8.1, 10.3 Hz, 1H), 3.34 (s, 3H), 2.34 (d, *J* = 2.6 Hz, 6H). *m/z*: 476.3.

3-(5-((4aR,7aR)-6-(5-Fluoro-4-methoxy-6-methylpyrimidin-2-yl)-2-imino-3-methyl-4-oxooctahydro-7aH-pyrrolo[3,4-d]pyrimidin-7a-yl)thiophen-3-yl)benzonitrile (20)

Analog **20** was prepared from intermediate **26** and 2-chloro-5-fluoro-4-methoxy-6-methylpyrimidine following General Procedure B (0.10 g, 47% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.03 (t, *J* = 1.8 Hz, 1H), 7.96 (ddd, *J* = 7.9, 1.9, 1.2 Hz, 1H), 7.88 (d, *J* = 1.5 Hz, 1H), 7.66 (dt, *J* = 8.0, 1.4 Hz, 2H), 7.58 (t, *J* = 7.8 Hz, 1H), 4.45 (d, *J* = 12.3 Hz, 1H), 4.20 (dd, *J* = 10.3, 8.5 Hz, 1H), 4.15 – 4.01 (m, 3H), 3.99 (s, 3H), 3.34 (s, 3H), 2.29 (d, *J* = 2.9 Hz, 3H). *m/z*: 492.3.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Synthetic procedures for preparation of 2-chloro-5-fluoro-4-methylpyrimidine (**27**), 2-chloro-4-ethyl-5-fluoropyrimidine (**28**), 2-chloro-5-fluoro-4-methoxypyrimidine (**29**), 2-chloro-4-ethoxy-5-fluoropyrimidine (**30**), 2-chloro-5-fluoro-4-propoxypyrimidine (**31**), 2-chloro-5-fluoro-4-(pyrrolidin-1-yl)pyrimidine (**32**), 4-(2-chloro-5-fluoropyrimidin-4-yl)morpholine (**33**), and 2-chloro-5-fluoro-4,6-dimethylpyrimidine (**34**)

Incubation protocol for compound **1** with CYP3A4 in the presence and absence of glutathione

Materials and methods for measurement of k_{obs} for **1** and **19**, HPLC traces for compounds **2 – 8**. **12**, **13**, **15 – 20**

Molecular Formula Strings

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ABBREVIATIONS USED

Cat-D, cathepsin D; TDI, time dependent inhibition; CYP, cytochrome P450; NADPH, reduced form of Nicotinamide adenine dinucleotide phosphate; rCYP3A4, recombinant cytochrome P450 3A4.

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11. An *N*-demethylated metabolite of compound **1**, predicted by MetaSite, was observed in our initial cold Met-ID study.

12. Direct epoxidation of pyrimidine ring and subsequent addition of GSH was proposed based on metabolism that causes TDI of aromatics and heteroaromatics. Please see reference 2b.

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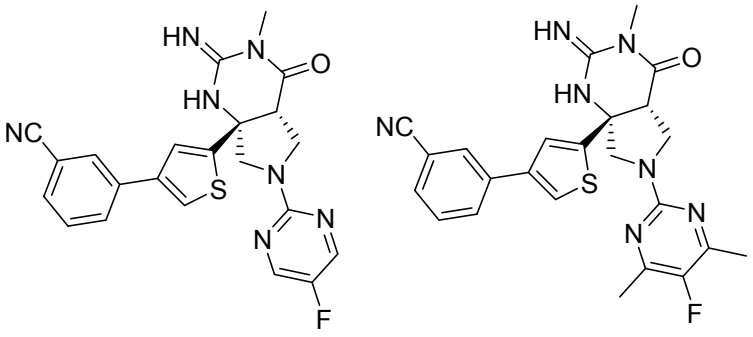
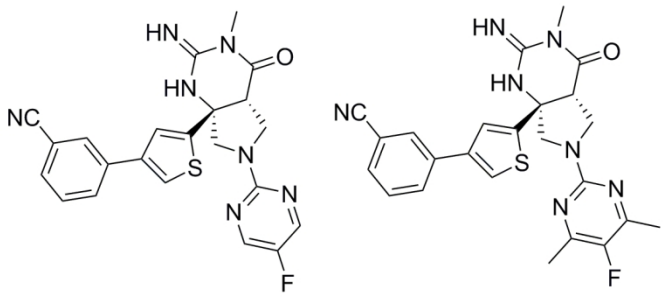
15. Given the observation of CYP3A4 TDI with compound **1,** despite the lack of direct inhibition, measurement of TDI should be included early in the screening funnel along with direct inhibition studies.

Table of content graphics



	1	**19**
CYP3A4 co-incubation; pre-incubation IC_{50} (μM)	>30; 1.2	>30; 13

Table of Contents graphic



	1	**19**
CYP3A4 co-incubation; pre-incubation IC_{50} (μM)	>30; 1.2	>30; 13

150x83mm (300 x 300 DPI)