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Probing Mechanisms of CYP3A Time-Dependent Inhibition Using a Truncated Model System

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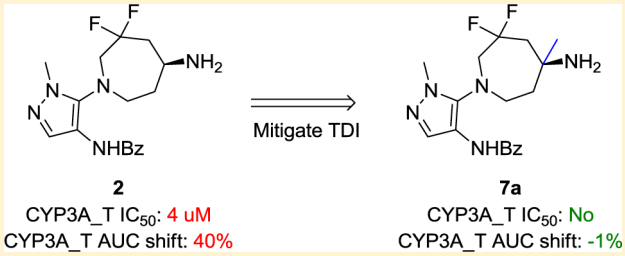
Supporting Information

ABSTRACT: Time-dependent inhibition (TDI) of cytochrome P450 (CYP) enzymes may incur serious undesirable drug−drug interactions and in rare cases drug-induced idiosyncratic toxicity. The reactive metabolites are often generated through multiple sequential biotransformations and form adducts with CYP enzymes to inactivate their function. The complexity of these processes makes addressing TDI liability very challenging. Strategies to mitigate TDI are therefore highly valuable in discovering safe therapies to benefit patients. In this Letter, we disclose our simplified approach toward addressing CYP3A TDI liabilities, guided by metabolic mechanism hypotheses. By adding a methyl group onto the α carbon of a basic amine, TDI activities of both the truncated and full molecules (**7a** and **11**) were completely eliminated. We propose that truncated molecules, albeit with caveats, may be used as surrogates for full molecules to investigate TDI.



KEYWORDS: *TDI, metabolic mechanism, CYP3A, Pim kinases*

Members of the cytochrome P450 (CYP) 3A subfamily are responsible for the metabolism of roughly half of all marketed pharmaceutical drugs.[1−3] Altered CYP activity resulting from time-dependent inhibition (TDI) can lead to clearance changes of drugs, thus contributing to drug−drug interactions (DDI)[4] that can cause efficacy and safety problems.[1,5,6] These concerns are becoming increasingly relevant in the face of growing polypharmacy.[4,5,7] CYP inhibition is a collective term for a decrease in CYP activity and can be classified into three groups: reversible, quasi-irreversible, and irreversible.[8,9] More specifically, the latter two modes often fall under the heading of TDI because in these cases CYP inactivation is caused by the formation of either an adduct or a metabolic-inhibitory (or -intermediate) complex (MIC) between the enzyme and reactive metabolites of the drug.[6,8] The inactivating species can result from sequential biotransformations through multiple routes and the complexity of these possibilities makes the accurate analysis of TDI challenging.[7,8,10,11] Previously, researchers have demonstrated the possibility of dialing out TDI by (i) blocking metabolic sites that lead to the generation of reactive metabolites, (ii) diverting the site of bioactivation to other metabolic hotspots, and (iii) reducing the binding affinity of the substrates to the CYP enzymes through the modulation of their physicochemical properties.[12−15] Despite some successes, dialing out TDI may still be complicated by multiple species responsible for mechanism-based inhibition (MBI).[16,17] Our present work takes a simplified approach toward studying the TDI mechanisms by removing potential sites of metabolism.

Aside from having potent PIM kinase inhibition,[18] compound **1** (Figure 1) was found to have potent CYP3A TDI activity. Two methods were used to assess the TDI potency as described by Kenny et al.[19] In an area under the curve (AUC) shift dilution assay, **1** inhibited CYP3A with a TDI IC_{50} of below 0.1 μM and an AUC shift of 57.3% when

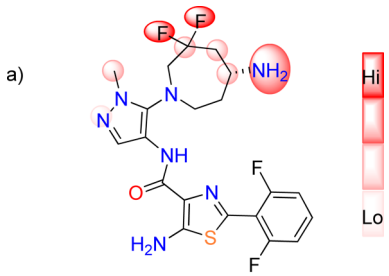


Figure 1. (a) Compound **1** shown with likely sites of metabolism by the CYP3A enzyme, as predicted by MetaSite without reactivity correction. Predicted metabolic hotspots are represented by red circles. (b) CYP3A TDI parameters for **1**, using testosterone (or midazolam) as the probe substrate.

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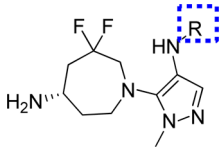
testosterone[20] was used as a probe substrate for one binding site of CYP3A. A similar result (IC_{50} of <0.1 μM and AUC shift of 55.6%) was obtained with midazolam as the probe substrate for the second binding site of CYP3A. Furthermore, a manual kinetic assay allowed for measurement of both the maximal rate of enzyme inactivation (k_{inact}) and the inhibitor concentration that supports half the maximal rate of inactivation (K_I), thereby confirming TDI activity. Compound **1** has a k_{inact} of 0.06 min^{-1} and K_I of 1.2 μM, resulting in a high ratio of k_{inact}/K_I of 52 mL/min/μmol. The high degree of TDI exhibited by **1** is comparable to potent known clinical time-dependent CYP3A inhibitors such as mifepristone[16] (k_{inact} = 0.08 min^{-1}, K_I = 1.3 μM, and k_{inact}/K_I = 62 mL/min/μmol). Major metabolites of compound **1** were identified through compound incubation with human hepatocytes. Not surprisingly, the azepane ring was the hotspot for phase I and II metabolism (Supporting Information, Figure s1). Phase I metabolism such as oxidation was predicted by MetaSite[21] (Figure 1). In order to mitigate TDI activity, truncated tool molecules were designed to probe the molecular mechanism of metabolism by focusing on the major metabolic hotspot.

Given the preponderance of metabolism on the azepane ring, fragments **2**–**4** (Table 1) were designed with a range of polarity

Table 1. CYP3A TDI of Substituted Pyrazoles vs Calculated Physicochemical Properties



		CYP3A_T (M)[a]		calculated[b]	
ID	R	IC_{50} (μM)	%AUC shift	tPSA	cLogP
1	*c*	<0.1 (<0.1)	57.3 (55.6)	115	3.5
2	Bz	4.3 (5.0)	40.1 (28.7)	76	2.3
3	Cbz	2.5 (7.7)	22.5 (−21.1)	85	2.7
4	pic[d]	no (no)	11.8 (7.2)	89	1.7

[a]CYP3A AUC shift dilution assay with testosterone (or midazolam) as the probe substrate. IC_{50} values were calculated from the AUC shift assay. "No" in IC_{50} column indicates there is no evidence of TDI. [b]tPSA, topological polar surface area; cLogP, calculated partition coefficient. [c]See Figure 1. [d]Picolinoyl.

(tPSA: 76–89) and lipophilicity (cLogP: 1.7–2.7). Indeed, the more lipophilic compounds (i.e., **2** and **3** compared to **4**) were found to have moderate TDI activity in AUC shift dilution assays with either testosterone or midazolam as the probe substrate. The overall reduced magnitude of TDI activity relative to **1** (cLogP: 3.5) is probably related to the decreased lipophilicity of **2** and **3**. Fragment **4** represents a set of picolinoyl analogues with various azepane substitutions (Supporting Information, Table s1) that all had no CYP3A TDI activity. Redirection of metabolism to the picolinoyl group, reduction of metabolism due to lower lipophilicity, or both factors together may be responsible for this observation.

Guided by the hypothetical metabolic pathways delineated in Figure 2, we investigated TDI structure–activity relationships (SARs) of benzoyl derivatives with various azepane substitutions (Table 2). To gauge the impact of stereochemistry on TDI activity, two pairs of enantiomers (e.g., **5a**/**5b** and **7a**/**7b**) were compared. There was no difference in TDI potency of the enantiomeric pairs, which agreed well with the fact that both

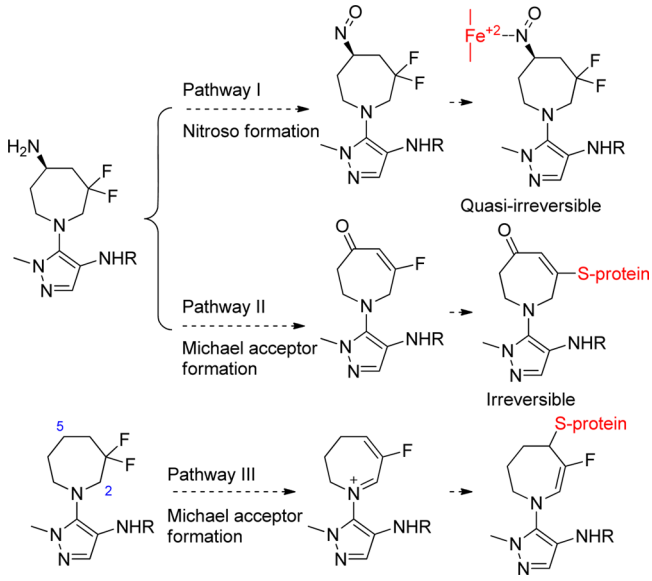


Figure 2. Proposed mechanisms of action.

compound **1** and its enantiomer (CYP3A IC_{50} of <0.1 μM and AUC shift of 54%) demonstrated potent TDI activity. Therefore, racemic compounds **6** and **9** were used in further studies. In striking contrast with **2**, compounds **5a,b** and **6** lacked difluoro substitution and showed no TDI liability. This implies that fluorine groups played a critical role in the formation of reactive intermediates. In this regard, the lack of TDI activity for **5a,b** seemed to negate the quasi-irreversible pathway hypothesis (Figure 2). In addition, a dialysis experiment (data not shown) confirmed **1** as an irreversible CYP inhibitor and therefore invalidated the quasi-irreversible pathway I.

As in pathway II, we hypothesized that a Michael acceptor[22−24] generated through an oxidative cleavage of the amine and HF elimination could react with a CYP enzyme covalently. In fact, glutathione trapping experiment of compound **1** supported this hypothesis (Supporting Information, Figure s2). Logically, **7a,b** were designed to block the α position of the basic amine from being oxidized and thus prevent the formation of a Michael acceptor. Satisfyingly, **7a,b** were both devoid of TDI activity despite having significantly higher lipophilicity (cLogP: 3.3) than compound **2** (cLogP: 2.3).

Compound **8**, which has a tertiary amine in place of the primary amine on **2**, was designed for two purposes; first to block the nitroso group formation and second to slow down the α carbon oxidation by steric hindrance. Encouraged by the lack of TDI activity of **8**, we made the fully decorated analogue **S4** (Supporting Information, Table s2). Despite with slightly decreased activity compared to **1**, **S4** still maintained strong TDI activity surprisingly. Compound **9**, which features a secondary alcohol and gem-difluoro group, had weak TDI activity with midazolam as the probe substrate. This is consistent with the hypothesis that secondary alcohol being oxidized to the ketone ultimately gives rise to the same Michael acceptor intermediate. Further confirming this hypothesis, the fully assembled analogue **S5** (Supporting Information, Table s2) had strong TDI potency. However, one cannot exclude the possibility that the TDI activities of **S4** and **S5** are net results of metabolic modifications of multiple sites of the molecules.

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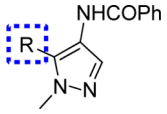
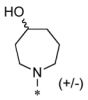
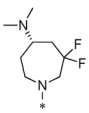
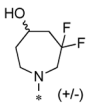
Table 2. CYP3A TDI of Substituted Pyrazoles: Probing the Main Site of Metabolism

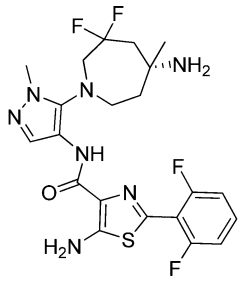


ID[d]	R	CYP3A_T[a]		CYP3A_M[a]		Calculated[b]		
		IC_{50} (μM)	%AUC shift	IC_{50} (μM)	%AUC shift	tPSA	cLogP	cpKa[c]
2		4.3	40.1	5.0	28.7	76	2.3	9.2
5a		no	10.3	no	11.4	76	2.2	10.1
5b		no	-22	no	8.9	76	2.2	10.1
6		no	-8.5	no	6.2	70	2.3	4.8
7a		no	-0.99	no	13.4	76	3.3	9.1
7b		no	7	no	3.8	76	3.3	9.1
8		no	-0.45	no	1.2	53	3.1	6.8
9		no	11.8	>10	28	70	2.4	4.8
10		1.7	37.9	0.84	38.3	50	3.4	4.8

[a]Same as that in Table 1. [b]Same as that in Table 1. [c]Calculated logarithm of acid dissociation constant for the most basic group. [d]Compounds **6** and **9** are racemic, **10** is achiral, and the rest are all single enantiomers.

To probe the mechanism in a different way, we designed **10** by removing the basic amine or alcohol. In view of the significantly increased lipophilicity, it was not surprising that **10** had nearly the same magnitude of TDI activity as **2**. However, from a mechanistic point of view, it prompted us to add metabolic pathway III, involving oxidation at the α carbon (C2) to the endocyclic nitrogen of the azepane ring. The fully decorated analogue **S6** (Supporting Information, Table s2) exhibited even stronger TDI activity than **10**. Collectively, these data imply that fully decorated molecules with higher lipophilicity are more prone than fragments to undergo metabolic modifications leading to TDI activities. In addition, TDI investigation is complicated by sequential metabolic modifications and multiple-site metabolisms at play. Truncated molecules with fewer sites for biotransformation may provide a straightforward way of understanding the mechanism of action of the main metabolic site.

Coupled with maintaining PIM target potency (reports to be published separately), the potential strategies to mitigate TDI were applied to the full molecules. In fact, inclusion of a methyl group alpha to the primary amine in **11** (Figure 3) not only maintained potent PIM inhibition but also obviated the TDI activity similar to analogous fragments **7a,b**. With this data, the pathway II hypothesis of generating a Michael acceptor was further supported. In summary, the TDI potency of appropriate



11
CYP3A_T(M) IC_{50}: 8.4(>10) μM
CYP3A_T(M) AUC shift: 26(21)%
k_{inact}/K_I: no TDI

Figure 3. CYP3A TDI of compound **11** with testosterone (or midazolam) as the substrate probe.

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fragments trends well with the full molecules. The absolute TDI potencies are reduced for fragments that are in all cases less lipophilic than full molecules. We concluded that as a general strategy in mitigating TDI, carefully selected fragments may be used to investigate the underlying metabolic mechanisms. The benefits of using fragments include simpler syntheses, shorter turnaround time, better material economy, and more focused study of the major metabolic soft spots than full molecules.

The syntheses of **1**−**11** are outlined in Schemes s1−s3 (Supporting Information). Gem-difluoro azepanes were not readily available, for which novel routes were developed starting from α,β-unsaturated ketones (Scheme s1). Michael addition of trimethylsilyl azide to *t*-butylcarboxyl (Boc) protected azepine (**S16a,b**) provided the desired adducts (**S17a,b**). Subsequent ketone reduction, Boc removal, and SnAr reactions afforded azepane substituted nitro pyrazoles (**S20a,b**). Staudinger reduction of the azide followed by Boc protection transformed azides **S20a,b** to Boc-protected amines **S20d,e**, respectively. Hydroxyl azepane **S19c** was readily available commercially and was converted to **S20c** by SnAr reaction with **S13**. Desired products **1**−**4**, **7**, **10**, and **11** were then obtained in a sequence of hydroxyl oxidation to ketone using Dess−Martin periodinane, difluorination using Deoxo-Fluor, catalytic hydrogenation of the nitro group, amide coupling, and acidic removal of the Boc group. Similarly, compound **8** was prepared by Boc removal of **S22a**, methylation through reductive amination, catalytic hydrogenation of the nitro group, and amide coupling in sequence.

In summary, molecular fragments were used as surrogates to probe the TDI SAR and mechanism. A hypothesis-driven approach was instrumental in designing key molecules devoid of TDI liabilities, e.g., **7a, 7b**, and **11**. In understanding TDI SAR, it is important to closely track physiochemical properties, particularly lipophilicity. Metabolic hotspot migration should be anticipated when designing molecules to mitigate metabolism. Our work adds to the SAR database of culprit pharmacophores incurring TDI beyond the traditional motifs.[6,17] In addition, robust syntheses of decorated azepanes, broadly useful for other chemical series, are described.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, compound characterization, assay protocols, additional TDI data, and metabolite identification data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00191.

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Notes

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■ ABBREVIATIONS

TDI, time-dependent inhibition; CYP, Cytochrome P450; DDI, drug−drug interactions; SAR, structure−activity relationship; SnAr, nucleophilic aromatic substitution

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