Addressing time-dependent CYP 3A4 inhibition observed in a novel series of substituted amino propanamide renin inhibitors, a case study

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ABSTRACT

Time-dependent inhibitors of CYPs have the potential to perpetrate drug–drug interactions in the clinical setting. After finding that several leading compounds in a novel series of substituted amino propanamide renin inhibitors inactivated CYP3A4 in an NADPH-dependent and time-dependent manner, a search to identify the cause of this liability was initiated. Extensive SAR revealed that the amide bridge present in compound **1** as a possible culprit. Through the installation of a metabolic soft spot distal to this moiety, potent renin inhibitors with improved CYP profile were identified.

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Human cytochrome P450s (CYPs) are membrane-associated heme-containing proteins localized in either the inner membrane of mitochondria or endoplasmic reticulum of cells.[1] Their main function is to catalyze the oxidation of both endogenous (e.g., hormones) as well as xenobiotic substrates. In the latter capacity, CYPs often play a major role in the metabolism and subsequent excretion of pharmaceuticals. Of the 18 families of P450 genes that have been identified and characterized in humans, the CYP3A4 isoform is known to be involved in the clearance of almost half of the drugs currently on the market.[2] The systemic exposure of these drugs can therefore be altered in the presence of agents that can either induce the biosynthesis of CYP3A4 or inhibit its enzyme activity, either reversibly or in a time-dependent manner (TDI). Consequently, it is important to evaluate and minimize the potential of any promising clinical candidate to perpetrate such drug–drug interactions (DDI) throughout the entire drug discovery process.

Hypertension affects more than a billion people worldwide and is one of the major risk factors for strokes, heart attacks, heart failure and arterial aneurysm, as well as the leading cause of chronic renal failure.[3] Despite the availability of numerous treatment options that treat the disease from a variety of unique pathways,[4] most of the patients still can not achieve their targeted blood pressure goals without resorting to combination therapy.[5] Furthermore, since

hypertension is often a consequence of other underlying metabolic disorders such as diabetes or hypercholesterolemia,[6] many of these patients are treated with poly-pharmacy thereby making DDIs a major concern for physicians. Recently, we reported the discovery of a novel and potent series of amino propanamide renin inhibitors for the treatment of essential hypertension (Fig. 1).[7] We were therefore quite alarmed by the observation that several of the most promising compounds were all found to inhibit CYP 3A4 in a time-dependent manner. Consequently, a study aimed at identifying the key culprit(s) responsible for this liability was initiated.

Since the inactivation of CYP3A with compound **1** was found to be an NADPH-dependent and time-dependent process, it was hypothesized that the observed TDI was due to the formation of an active metabolite capable of binding irreversibly to the CYP3A enzyme. However, the search for the exact perpetrator was complicated by two observations. Firstly, the addition of trapping agents such as glutathione (GSH), cyanide and semicarbazide did not prevent the in vitro inactivation of CYP 3A. Secondly, when compound **1** was incubated with either human liver microsomes or human hepatocytes, only a few minor metabolites were observed while the majority (>94%) of compound **1** remained intact (Fig. 2). Consequently a classical SAR approach, where the impact of various structural modifications on CYP3A4 activity was independently evaluated, became necessary. The severity of the CYP3A4 TDI observed for a given modification was gauged by the magnitude of the IC_{50} fold shift observed upon 0 and 30 min pre-incubation.[8]

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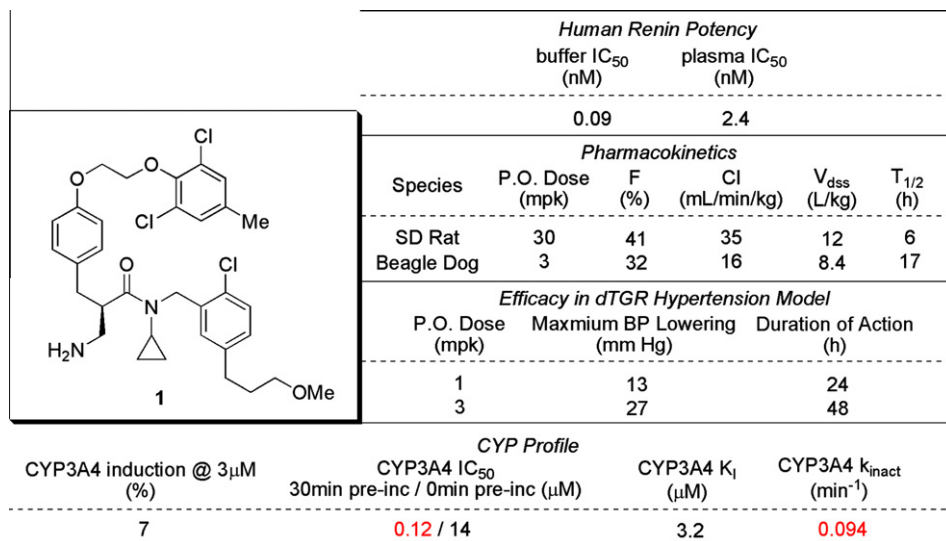*E-mail address:* austin_chen@merck.com (A. Chen).

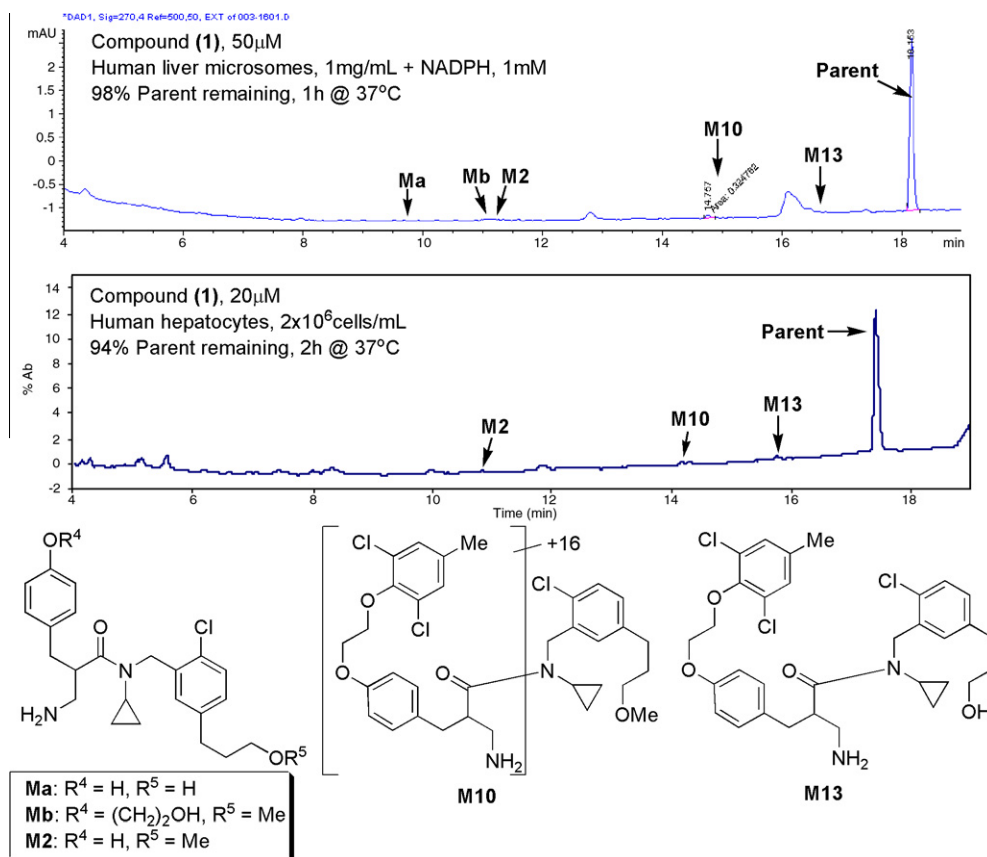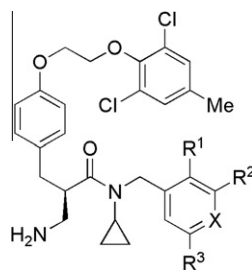**Figure 1.** Profile of a representative amino propanamide renin inhibitor.

Figure 2. In vitro metabolic profile of compound **1**.

Metabolites (i.e., **Ma** and **M13**) derived from the de-methylation of the methoxypropyl group found in compound **1** were observed in vitro (Fig. 2) and this led us to focus the initial SAR study on the P₃ arene plate (Table 1). Although the extent of TDI observed was slightly tempered by modifying the nature of the *meta*-substituent, TDI was not restricted to derivatives bearing the methoxypropyl tail. Indeed, analogues possessing either a cyano residue (i.e., **3**) or more polar capping groups such as amide **4** or sulfone **5** all suffered from TDI. Furthermore, replacement of the 2-chloro-

benzene plate found in compounds **1** with pyridine (i.e., **6**), pyridine N-oxide (i.e., **7**) or quinoline (i.e., **8** and **9**) also failed to decrease the severity of time-dependent CYP3A4 inactivation.

Given the number of bio-transformations that can be envisioned for primary amines,[11] we then turned our attention to the aminomethyl warhead. Unfortunately, its replacement by an ethyl amine (i.e., **10**), an amide (i.e., **11**) or even simple hydrogen (i.e., **12**) still afforded compounds with significant potential to perpetrate DDI (Table 2). Furthermore, the small improvement in TDI

Table 1
SAR of select renin compounds: P₃ arene modifications

Compd	R^1	R^2	R^3	X	Renin IC_{50}[a,b] (nM)		CYP3A4 IC_{50}[c] (μM)		
					Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
1	Cl	H	$(CH_2)_3OMe$	CH	0.09	2.4	0.12	14	110
2	Cl	H	$(CH_2)_2OMe$	CH	0.04	2.9	0.12	14	110
3	Cl	H	$(CH_2)_3CN$	CH	0.10	4.6	0.10	12	120
4	Cl	H	$CH_2N(Me)Ac$	CH	2.9	250	0.21	7	35
5	Cl	H	$(CH_2)_2SO_2Me$	CH	3.1	720	0.10	5	50
6	Cl	H	$(CH_2)_3OMe$	N	0.19	2.2	0.14	12	90
7	Cl	H	$(CH_2)_3OMe$	N^+-O^-	0.3	1.8	0.17	12	70
8	–CH=CH–CH=CH–		$(CH_2)_3OMe$	N	0.17	4.4	0.10	12	120
9	–N=CH–CH=CH–		$(CH_2)_3CN$	CH	0.08	1.5	0.08	10	140

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.

Table 2
SAR of select renin compounds: warhead modifications
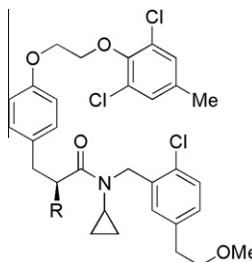
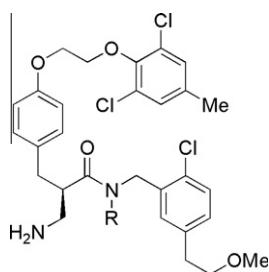| Compd	R	Renin IC_{50}[a,b] (nM)		CYP3A4 IC_{50}[c] (μM)		
		Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
2	CH_2NH_2	0.04	2.9	0.12	14	110
10	$C(Me)NH_2$[d]	0.43	59	0.20	12	60
11	$C(=O)NH_2$[d]	17	7000	0.41	22	55
12	H	470	—	0.30	12	40

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.
[d] Compound was tested as a racemate.

realized with these analogues came at the expense of their renin potency.

Although metabolites derived from the hydrolysis of compound **1**'s amide bond were not detected in vitro, if formed, the observed TDI may be ascribed to reactive intermediates generated from further oxidation of the resulting cyclopropylamine metabolite.[12,13] To test this hypothesis, several analogues lacking the cyclopropyl-amine moiety were synthesized and tested. Replacement of the cyclopropyl group by methyl (i.e., **13**), cyclobutyl (i.e., **14**), cyclo-propylmethyl (i.e., **15**) or cyclobutylmethyl (i.e., **16**) again afforded similar time-dependent inhibitors of CYP3A4 (Table 3). These ana-logues also proved to be inferior inhibitors of renin.

Incubation experiments with human hepatocytes and human li-ver microsomes revealed the formation of phenol-bearing metabo-lites (i.e., **Ma** and **M2**, Fig. 2) via the oxidative cleavage of the 2-(2,6-dichloro-4-methyl-phenoxy)ethoxy northern terminus (vide supra). Further oxidative processing of these phenol metabolites could in theory deliver reactive intermediates capable of binding irreversibly to CYPs.[14] In this regard, compounds **17** and **18** were prepared and tested (Table 4). Again, the TDI liability was not suc-cessfully addressed with these modifications.
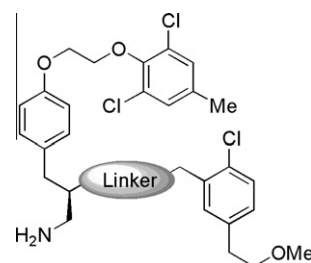
Having sequentially ruled out some of the most likely suspects, we then proceeded to evaluate the impact the amide functionality has on TDI. Gratifyingly, removal of the carbonyl oxygen (i.e., **19**)

Table 3
SAR of select renin compounds: P_1 modifications

Compd	R	Renin IC$_{50}$[a,b] (nM)		CYP3A4 IC$_{50}$[c] (µM)		
		Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
2		0.04	2.9	0.12	14	110
13	Me	0.47	26	0.30	12	40
14		13	3100	0.10	18	180
15		9.7	3700	0.09	9	100
16		210	26,000	0.10	13	130

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.

Table 5
SAR of select renin compounds: P_1 modifications

Compd	Linker	Renin IC$_{50}$[a,b] (nM)		CYP3A4 IC$_{50}$[c] (µM)		
		Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
2		0.04	2.9	0.12	14	110
19		190	50,000	1.7	9.8	5.8
29		3.5	1900	4	14	3.5
40		16	1300	11	19	1.7

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.

led to a significant improvement with respect to the extent of TDI observed (Table 5). The renin enzyme unfortunately, did not tolerate this functional group deletion. In order to hopefully regain some of the loss in renin potency, analogues where the nitrogen in **19** was replaced by a carbon (i.e., **29** and **40**) were synthesized (Scheme 1). Briefly, palladium-catalyzed formylation of the known aryl bromide **20** with sodium formate as the reducing agent[15] afforded aldehyde **21** and subsequent olefination with ylide **22** was carried out quantitatively. α,β-Unsaturated ester **23** thus obtained could then be hydrogenated in EtOAc with PtO$_2$ as the catalyst.[16] Hydrolysis of the resulting ester **24** to carboxylic acid **25** with aq lithium hydroxide followed by coupling with (−)-pseudo-ephedrine[17] both proceeded without incidents. Alkylation of the

lithium enolate generated from amide **26** with chiral iodide **39** occurred with >95:5 diastereo-selectivity.[17] Subsequent reductive removal of the Myer's auxiliary was best accomplished with lithiated borane ammonia complex which delivered alcohol **27** with no loss in stereochemical integrity.[18] Finally, diphenyl phosphorazidate-mediated conversion[19] of **27** to azide **28** followed by Staudinger reduction[20] afforded compound **29**. Synthesis of iodide **39** used in the alkylation step described earlier began with the Suzuki-coupling of aryl bromide **30** with vinylboronic acid pinacol ester.[21] Iridium-catalyzed hydroboration of the resulting styrene **31** delivered, after oxidative workup with NaBO$_3$, alcohol **32**.[22] Iodometh-

Table 4
SAR of select renin compounds: northern terminus
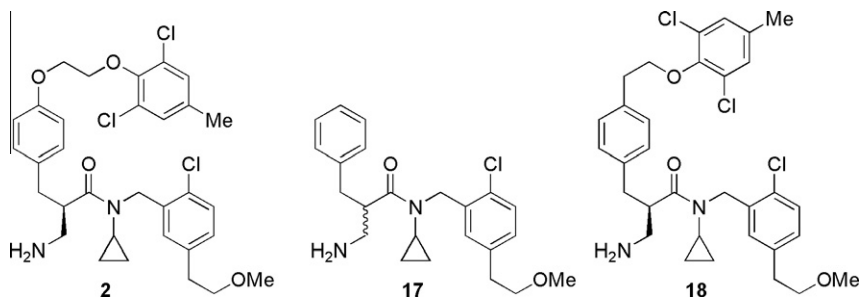
Compd	Renin IC$_{50}$[a,b] (nM)		CYP3A4 IC$_{50}$[c] (µM)		
	Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
2	0.04	2.9	0.1	14	110
17	2900	—	0.1	12	120
18	0.29	21	0.25	14	60

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.
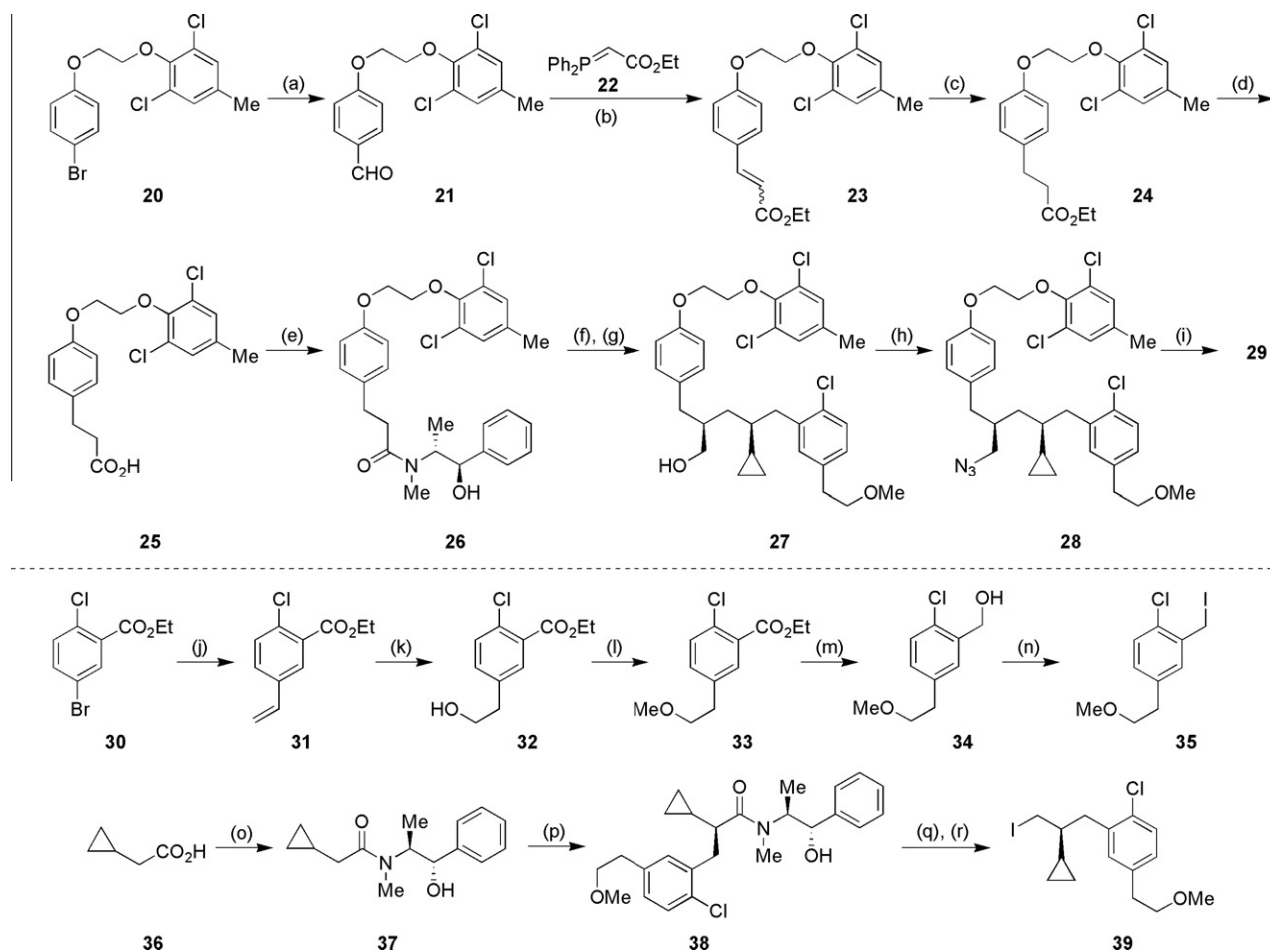
Scheme 1. Synthesis of **29**: (a) Pd(PPh₃)₂Cl₂, CO, NaCO₂H, DMF, 110 °C, 8 h, 77%; (b) **22**, THF, 24 h, >99%; (c) PtO₂, H₂, EtOAc, 14 h, 92%; (d) 2 N aq LiOH, MeOH, THF, 16 h, >99%; (e) (ClCO)₂, CH₂Cl₂, cat. DMF, 1 h, then (−)-pseudoephedrine, NEt₃, THF, 0 °C, 91%; (f) 2.5 equiv LDA, 8 equiv LiCl, THF, −78 °C, 1 h, then 0 °C, 30 min, then **39**, 0 °C to rt, 20 h; (g) 4 equiv LDA, 4.2 equiv BH₃·NH₃, THF, 0 °C to rt, 20 h, 36% yield over two steps; (h) (PhO)₂P=O(N₃), DBU, DMSO, rt to 80 °C, 4 h, 82%; (i) PPh₃, H₂O, THF, 50 °C, 2 h, 94%; (j) Pd(OAc)₂, PPh₃, vinylboronic acid pinacol ester, 2 N aq Na₂CO₃, EtOH, DMF, 90 °C, 8 h, 86%; (k) [IrCl(COD)]₂, DPPP, pinacolborane, THF, 24 h, then NaBO₃, H₂O, 2 h, 69%; (l) NaH, MeI, THF, 30 min; (m) 1.5 M DIBAl-H in toluene, CH₂Cl₂, −78 °C to rt, 16 h, 98% over two steps; (n) I₂, imidazole, PPh₃, CH₂Cl₂, 0 °C to rt, 1.5 h, 77%; (o) (ClCO)₂, CH₂Cl₂, cat. DMF, 1 h, then (+)-pseudoephedrine, NEt₃, THF, 0 °C, 60%; (p) 2.5 equiv LDA, 10 equiv LiCl, THF, −78 °C, 1 h, then 0 °C, 30 min, then **35**, 0 °C to rt, 20 h, 88%; (q) 4 equiv LDA, 4.2 equiv BH₃·NH₃, THF, 0 °C to rt, 20 h, 85% yield; (r) I₂, imidazole, PPh₃, CH₂Cl₂, 0 °C to rt, 1.5 h, 90%. Abbreviations: COD = 1,5-cyclooctadiene; DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene; DIBAl-H = diisobutylaluminum hydride; DPPP = 1,3-bis(diphenylphosphino)propane; LDA = lithium diisopropyl-amide.

Table 6
SAR of select renin compounds: second appendage

Compd	R	Renin IC₅₀[a,b] (nM)		CYP3A4 IC₅₀[c] (µM)		
		Buffer	Plasma	30 min Pre-Inc	0 min Pre-Inc	Shift
1	−	0.09	2.4	0.12	14	110
41	OMe	0.02	3.5	1.3	2	1.5
42	§-N⟳O	0.03	2.3	3.2	3.0	<1

[a] See Ref. 9 for assay protocols.
[b] Average of at least two replicates.
[c] See Ref. 10 for assay protocols.

ane methylation of alcohol **32**, DIBAl-H reduction of ester **33** and Arbusov iodination of alcohol **34** then furnished iodide **35**. Treatment of lithiated amide **37**, itself synthesized from cyclopropyl-acetic acid **36** and (+)-pseudoephedrine, with iodide **35** also proceeded with complete stereochemical control. Again, removal of the Myer's auxiliary from amide **38** was best accomplished with lithiated borane ammonia complex. Finally, Arbusov iodination cleanly delivered iodide **39**. Unfortunately, although compound **29** and its diastereomer **40** were both much cleaner than compound **2** in terms of TDI, neither compound was sufficiently potent against renin to warrant further profiling.

The exact mechanism by which the amide moiety promotes the observed time-dependent inactivation of CYP3A remains unclear. If the amide itself was indeed the culprit, one could, as was observed with compounds **29** and **40**, dial out the TDI liability through the installation of an appropriate amide isostere. Furthermore, since independent docking studies have revealed that the amide itself does not participate in any key stabilizing interaction with the re-nin enzyme, it should be possible to design an amide-free, potent renin inhibitor as long as the proper orientation of the flanking substituents are preserved.[23] Indeed, our successful realization of this strategy will be disseminated in a separate communication. An alternative explanation can also be envisioned, wherein the re-

moval of the amide moiety instead served to reduce the formation of non-amide derived reactive intermediate(s).[24] Consequently, it should be possible to keep the amide but to also introduce a distal metabolic soft spot to divert the metabolism to a more innocuous area in the molecule. In this regard, analogues possessing a second appendage on the P_3 aromatic plate were prepared and tested (Table 6).

We were gratified to observe that these modifications not only led to a significant improvement in TDI but did so without jeopardizing their respective potency against renin. Furthermore when these compounds were incubated with human liver microsomes so that their respective in vitro clearance can be determined,[25] compound **1** (Cl_{int} = 15 mL/min/kg) was indeed found to be more metabolically stable than both compounds **41** (Cl_{int} = 32 mL/min/kg) and **42** (Cl_{int} = 45 mL/min/kg).

In conclusion, we have described our effort to identify the key functionality responsible for the time-dependent CYP3A4 inhibition observed with the leading compounds in our recently disclosed novel series of renin inhibitors. Two unique strategies to overcome this liability were also successfully implemented.

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9. *Buffer assay*: recombinant human renin (3 pg/µL) in assay buffer (PBS1X, 1 mM EDTA, 0.1% BSA, pH 7.4), human tetradecapeptide (**1**–**14**) substrate (5 µM in 10 mM HCl), hydroxyquinoline sulfate (30 mM in H$_2$O) and assay buffer were pre-mixed at 4 °C at a ratio of 100:30:10:145. 47.5 µL per well of this pre-mix was transferred into polypropylene plates. Test compounds were dissolved and diluted in DMSO and 25 µL added to the pre-mix, then incubated at 37 °C for 3 h. At the end of the incubation period, 5 µL of the renin reaction (or standards in the assay buffer) were measured for AngI accumulation. The percentage of renin inhibition (AngI decrease) was calculated for each concentration of compound and an IC$_{50}$ was determined by curve fitting software. *Plasma assay*: citrate-plasma from human volunteers was pooled, aliquoted and stored frozen at −20 °C. Renin activity in pooled plasma was supplemented with recombinant human renin (150 pg/mL final concentration) in order to increase the readout of the assay. 5 µL of renin inhibitors, at various concentrations in DMSO, was added to 80 µL of a mixture (7:1) of human plasma and a fast trapping primary AngI antibody (anti-AngI: AS1, bleed 6, prediluted 1:10 in horse serum) diluted initially 1:26.5 in assay buffer (PBS1X, 1 mM EDTA, 0.1% BSA, pH 7.4) and then diluted 1:3.3 in 3 M Tris, 200 mM EDTA, pH 7.2 (final anti-serum dilution 1:50,000) and was incubated at 37 °C for 2 h. As was done in the buffer assay, 12 µL of the renin reaction (or standards in the assay buffer) were measured for AngI accumulation by immunoassay.
10. CYP3A4 IC$_{50}$ shift assay: 0.25 mg/mL of human liver microsomes, 0.03–200 µM of test compound, and 1 mM NADPH in 125 mM phosphate buffer pre-incubated for 0 or 30 min at 37 °C. Then, 500uM of testosterone (CYP3A substrate) added and incubation carried-out for additional 15 min at 37 °C. Samples are then quenched with acetonitrile, centrifuged and formation of 6β-hydroxy-testosterone monitored by HPLC-MS/MS.
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24. The authors would like to thank the reviewer for proposing this alternative scenario.
25. *Metabolic stability assay*: Incubations performed at 37 °C in 125 mM phosphate buffer in presence of 1 mg/mL human liver microsomes, NADPH regenerating system and 1uM of test compound. Incubates quenched at *t* = 2, 5, 10, 20, 45 and 75 min with acetonitrile, centrifuged, and supernatant analyzed by HPLC-MS/MS to monitor disappearance of test compound over-time. Cl$_{int}$ calculated based on initial K_e.