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Letter

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Mitigating the Metabolic Liability of Carbonyl Reduction – Novel Calpain Inhibitors with P1´ Extension

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KEYWORDS: Calpain inhibitor, carbonyl reduction, cytosolic stability, cathepsin selectivity.

ABSTRACT: Dysregulation of calpains 1 and 2 has been implicated in a variety of pathological disorders including ischemia/reperfusion injuries, kidney diseases, cataract formation, and neurodegenerative diseases such as Alzheimer´s disease (AD). 2-(3-Phenyl-1*H*)-pyrazol-1-yl)nicotinamides represent a series of novel and potent calpain inhibitors with high selectivity and *in vivo* efficacy. However, carbonyl reduction leading to the formation of the inactive hydroxyamide was identified as major metabolic liability in monkey and human, a pathway not reflected by routine ADME assays. Using cytosolic clearance as a tailored *in vitro* ADME assay coupled with *in vitro* hepatocyte metabolism enabled the identification of analogues with enhanced stability against carbonyl reduction. These efforts led to the identification of P1´ modified calpain inhibitors with significantly improved pharmacokinetic profile including P1´ *N*-methoxyamide **23** as potential candidate compound for non-CNS indications.

The calpains are a family of Ca^{2+}-dependent intracellular cysteine proteases that proteolyze a wide variety of cytoskeletal, membrane-associated and regulatory proteins.[1] Calpains regulate the function and metabolism of proteins which are key to the pathogenesis of several human diseases.[2] In particular, calpain 1 and 2 have been shown to be involved in acute and chronic pathological processes such as ischemia/reperfusion injury, traumatic brain injury (TBI), Alzheimer´s disease (AD), and Multiple Sclerosis (MS). Furthermore, inhibition of calpains is known to exert general cell- and organo-protective effects after a pathological insult. Therefore, calpain has been proposed as an attractive target for diseases such as chronic kidney disease, myocardial infarction, cataract formation, and neurological disorders such as AD or TBI.[3,4]

The majority of calpain inhibitors described so far are active site directed reversible inhibitors with a structure built in a modular concept (**Figure 1**). An electrophilic group, typically a ketoamide or a (masked) aldehyde, is required for covalent interaction with the active cysteine thiol, while the residues on the adjacent sides of this warhead interact with pockets at the subsites of calpain in a manner similar to substrate binding (for a more detailed description of protease substrate designation see lit 5). A variety of potent nonpeptidic calpain inhibitors has been reported, however none of these compounds was advanced into clinical development, either due to lack of selectivity and/or unfavorable PK properties.[6-8]

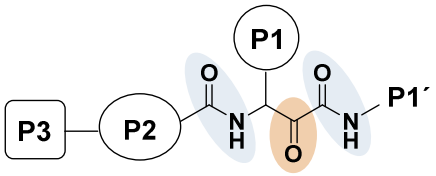


Figure 1: General structure of ketoamide-based calpain inhibitors showing the essential pharmacophores required for activity and selectivity.

Recently we reported on 2-(3-phenyl-1*H*-pyrazol-1-yl)-nicotinamides as highly potent and selective inhibitors of calpain.[9] In particular, *N*-(4-amino-3,4-dioxo-1-phenylbutan-2-yl)-2-(3-phenyl-1*H*-pyrazol-1-yl)nicotinamide **1** (**A-933548**) and its 4-fluoro analogue *N*-(4-amino-3,4-dioxo-1-phenylbutan-2-yl)-2-(3-(4-fluorophenyl)-1*H*-pyrazol-1-yl)nicotinamide **2** (**A-953227**) feature enhanced selectivity versus related cysteine protease cathepsins, favorable microsomal stability, and efficacy in cellular assays (**Figure 2**). In addition, we demonstrated that compound **2** was efficacious in a set of AD related models, and had an overall favorable safety profile.[9]

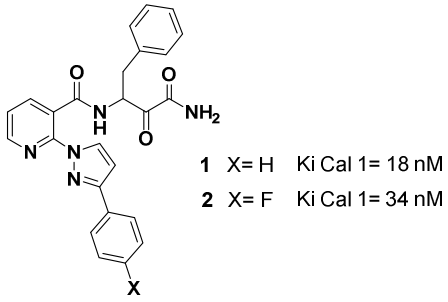


Figure 2: Structure and calpain inhibition of compounds **1** and **2**.

However, further ADME and pharmacokinetic profiling (PK) revealed significant species differences in metabolism. Whereas in rodents ketoamides **1** and **2** are mainly hydrolyzed to the corresponding ketoacid and then oxidized to give the homologous acids, non-CYP mediated carbonyl reduction to the hydroxyamide was identified as the major metabolic pathway in cynomolgus monkey and human (**Figure 3**).

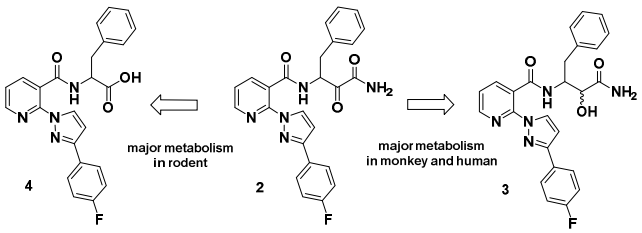


Figure 3: Species specific metabolism of compound **2**. Hydroxyamide **3** and acid **4** are inactive (Ki values > 10.000 nM).

In general, carbonyl reduction is mediated by multiple cytosolic reductases, most of them belonging to the superfamilies of NADPH-dependent short-chain dehydrogenases/reductases and aldo-keto reductases. These enzymes are ubiquitous, found in many tissues including liver, lung, brain, kidney and blood, and show broad and overlapping substrate specificities. Differences in multiplicity and tissue distribution can cause specific cellular metabolism for carbonyl-containing compounds and hence different metabolic pathways across species.[10, 11] Further investigation of compound **2** suggested that the carbonyl reductase activities impacting the ketoamide moiety were cytosolic and NADPH-dependent. Preclinical PK studies with **2** were consistent with data from *in vitro* metabolism, demonstrating good to moderate bioavailability (F) in rat and dog (F = 68% and 30%, respectively), but low bioavailability in monkey (F = 4.5%).[9] Significant formation of the hydroxyamide metabolite was observed in monkey, with the hydroxyamide present in 82 fold excess compared to parent compound, while less was formed in rat and dog (2.3 and 0.5 fold, respectively – based on AUC). Consistently, data from a first-in-human Phase 1 study testing single doses up to 800 mg showed that compound **2** had low bioavailability (F_e ~ 10%), short effective half-life ($t_{1/2}$), and significant formation of the hydroxyamide metabolite (95 fold excess of hydroxyamide metabolite to parent).

On the basis of these results our goal was to identify calpain inhibitors with enhanced stability against carbonyl reduction, which should translate into an improved PK profile in humans. Carbonyl reduction as metabolic liability is not reflected by standard *in vitro* stability assays, such as intrinsic liver microsomal clearance (mCl_{int}), which is routinely employed during lead optimization. Hence we have developed a new screening assay based on liver cytosolic stability as a tailored assay, coupled with other tier 1 *in vitro* ADME assays. Compounds with improved cytosolic stability were evaluated for hydroxyamide metabolite formation in monkey and human hepatocytes, followed by monkey PK to assess *in vivo* carbonyl reduction with PK parameters (CL, $t_{1/2}$ and oral F). In addition, *in vivo* metabolite formation was determined. Herein we describe the synthesis and characterization of novel calpain inhibitors with P1′ extension as means to enhance stability versus carbonyl reduction. From a set of different analogues prepared in this series, cyclopropyl and O-methyl were identified as most favorable P1′ moieties balancing calpain inhibition, selectivity and overall metabolic stability, also showing an improved PK profile across preclinical species.

Cytosolic Stability: Intrinsic cytosolic clearance ($cytCl_{int}$) was implemented as tailored *in vitro* ADME assay to screen for compounds with enhanced stability against reductases. In this assay we measured the percentage of parent compound lost over time in cytosolic incubations, and used this data to calculate intrinsic clearance (*see Supporting Information*). Clearance ranges were adjusted to hepatic plasma flow of human and monkey in analogy to mCl_{int}, with the following qualifiers assigned to evaluate cytosolic stability of a compound (Table **1**).

Table 1. Ranges for Intrinsic Cytosolic Clearance ($cytCl_{int}$) and Qualifiers Assigned

qualifier	cyno [µL/min/mg]	hu [µL/min/mg]
stable	0 - 18	0 - 14
moderate	18 – 90	14 – 70
instable	> 90	> 70

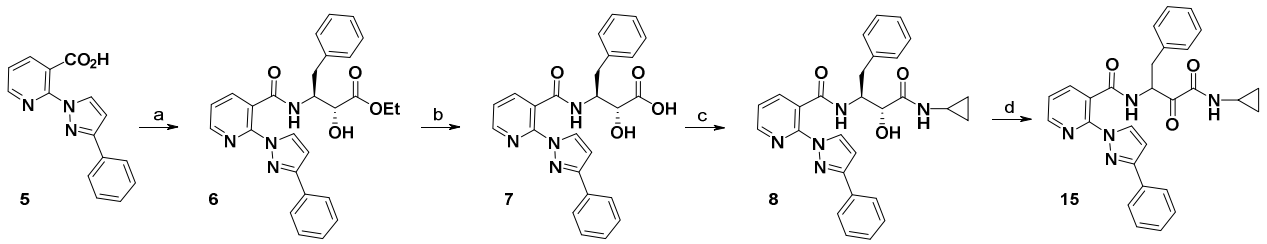
In Vitro Metabolite/Parent Ratio in Hepatocytes: As stated above, enhanced cytosolic stability is supposed to translate into decreased formation of hydroxyamide metabolites in human and monkey hepatocytes, which should be reflected by a change in the ratio of hydroxyamide metabolite to the corresponding parent compound. Thus, the metabolite to parent ratio (M/P ratio) was determined as an additional parameter for a set of advanced compounds (*see Supporting Information*).

Table 2. Intrinsic Cytosolic Stability ($cytCl_{int}$) and Metabolite to Parent Ratio (M/P) in Hepatocytes for Compound 2

	cytCl*		M/P ratio	
compd	cyno	hu	cyno	hu
2	39	> 70	3.4	8.0

* [µL/min/mg]

Scheme 1 Synthetic Route to *N*-substituted (4-Amino-3,4-dioxo-1-phenylbutan-2-yl)-2-((3-phenyl-1H-pyrazol-1-yl)nicotinamides 9 and analogues [a]



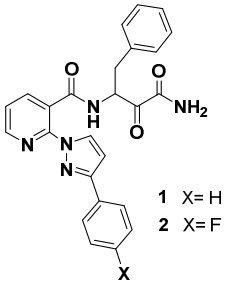
[a]Reagents and conditions**:** a) ethyl (*2S*,3R**)-3-amino-2-hydroxy-4-phenylbutanoate hydrochloride, EDCI, HOBt, triethlyamine, CH_2Cl_2, 5°C – rt.; b) LiOH, THF/H_2O, 10°C - rt.; c) cyclopropylamine, EDCI, HOBt, triethlyamine, CH_2Cl_2, 5°C – rt.; d) EDCI, dichloroacetic acid, DMSO, rt.

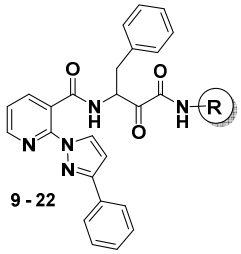
Table **2** depicts $cytCl_{int}$ and M/P ratios obtained for compound **2**. CytCl is moderate in cynomolgus monkey and high in human, which correlates well with the M/P ratios observed. The data also agree with the PK in these species (see Table **5**), clearly reflecting the liability for carbonyl reduction.

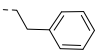
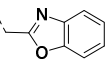
Synthesis and Screening: Scheme **1** outlines the synthesis of cyclopropyl 2-hydroxy-4-phenyl-3-(2-(3-phenyl-1*H*-pyrazol-1-yl)nicotinamido)butanoate **15** as a general example for the synthesis of compounds **9 – 32** comprising different P1´ moieties. Starting from 2-(3-phenyl-1H-pyrazol-1-yl)nicotinic acid **5**, coupling with ethyl (*2S*,3R**)-3-amino-2-hydroxy-4-phenylbutanoate gave (*2S*,3R**)-2-hydroxy-4-phenyl-3-((2-(3-phenyl-1H-pyrazol-1-yl)pyridin-3-yl)amino)butanoic acid **6**. Ester cleavage and coupling of acid **7** with cyclopropylamine yielded hydroxyamide **8**. Final oxidation using either Pfitzner-Moffat conditions[12] or, in the case of alkoxy-substituted amides, 2-iodobenzoic acid[13] gave ketoamides **9 – 32** as racemic mixtures.[14,15] Detailed experimental procedures for the preparation of key compounds **15** and **23** are given in the Supporting Information.

Compounds **9 - 32** were evaluated for enzyme inhibition using kinetic fluorescence assays as described in lit. 9 (*selectivity data only shown for selected compounds*). In addition, all compounds were routinely submitted to tier 1 mCl in rat and human hepatocytes and cytosolic stability screening (Tables **3** and **4**).

SAR of P1´ Modification: Ethylamide **9** had been prepared during our efforts to investigate the SAR around 2-(3-phenyl-1*H*)-pyrazol-1-yl)nicotinamides such as **1** and **2**. Interestingly, although showing significantly reduction in calpain inhibition, **9** displayed good cytosolic stability and low M/P ratios (0.5 for cyno and 0.2 for human, respectively), which prompted us to examine the SAR for the P1´ position more thoroughly. Previous efforts had shown that the presence of the P1´ amide NH is essential for calpain inhibition, therefore tertiary amides were not included. Due to the better calpain inhibition, compound **1** was used as starting point for optimization. Altogether, about 80 analogues with different P1´ extensions were prepared, including a set of different alkyl-, O-alkyl-, aryl-, and hetaryl residues. Selected examples are depicted in Tables **3** and **4** (*selectivity versus related cysteine protease cathepsins shown in the Supporting Information*).





compd	R	Cal 1 Ki [nM]	mCl*		cytCl*	
			rat	hu	cyno	hu
1	-	18	22	6	74	40
2	-	34	15	23	39	>70
9	Et	6908	45	>70	16	28
10	CH_3	380	24	18	12	21
11		480	>100	64	4	12
12		2600	81	49	5	4
13		470	>100	>70	8	5
14		950	53	37	6	8
15		205	64	27	12	4
16		490	>100	>70	8	8
17		1350	nd	nd	7	9
18		550	>100	52	7	9
19		>5000	81	24	10	4
20		110	>100	>70	17	19
21		330	>100	>70	38	11
22		210	>100	>70	11	16

* [µL/min/mg]

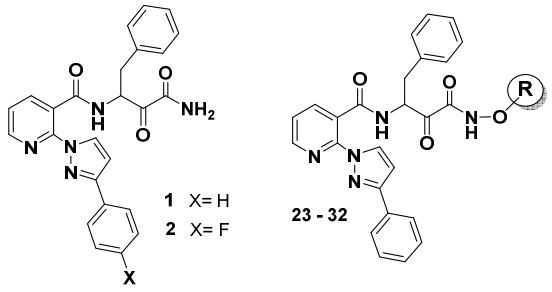
Table 3. Inhibition of Calpain 1 and Microsomal and Cytosolic Stability of Compounds 1, 2 and 9 – 22

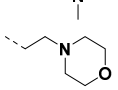
Table 5. Pharmacokinetics of Compounds 1, 2, 15 and 23 in Cynomolgus Monkeys Following a Single IV or PO Dose

	IV					PO						
compd	Dose	$t_{1/2}$°	$AUC_{0\text{-}inf}$	CL_p	V_{ss}	Dose	$t_{1/2}$°	C_{max}	t_{max}	$AUC_{0\text{-}inf}$	F	M/P_{AUC}
1	1	1.1	660	1.5	1.1	1	-	-	-	-	-0	*nd*
2	1	1.1	520	2.0	1.8	10	6.3	0.02	13.3	230	4.5	82.4
15	1	1.4	370	3.0	6.8	3	7.5	9.3	1.3	75	6.8	0.9
23	1	7.8	740	1.4	9.6	3	7.4	276	2.5	1310	58.9	0.04

Data provided as mean; ° harmonic mean; Units: Dose (mg/kg); t1/2 (hr); Vss (L/kg); $AUC_{0\text{-}inf}$ (ng•h/mL); CL_p (L/h•kg); C_{max} (ng/mL); t_{max} (h); F (%); IV: intravenous; PO: oral. M/P_{AUC}: AUC ratio of hydroxyamide metabolite to parent.

Table 4. Inhibition of Calpain 1 and Microsomal and Cytosolic Stability of Compounds 1, 2 and 23 – 32



compd	R	Cal 1 Ki [nM]	mCl*		cytCl*	
			rat	hu	cyno	hu
1	H	18	22	6	74	40
2	-	34	15	23	39	>70
23	CH3	120	21	11	1	2
24	Et	110	32	10	<1	<1
25		75	96	20	<1	2
26		220	>100	>70	29	33
27		185	44	11	1	<1
28		90	78	17	2	8
29		150	59	15	3	3
30		120	>100	21	1	4
31		1060	12	9	3	2
32		265	17	6	2	<1

* [µL/min/mg]

In general, in comparison to compound **1**, analogues with P1´ *N*-alkyl extension (Table **3**) showed a robust enhancement in cytosolic stability, which might be attributed to the inability of carbonyl reductases to adapt to P1´ extended ketoamides as substrates. However, in parallel we also observed a significant reduction in calpain inhibition.

The most potent compound in this subseries was benzylamide **20** with a K_i of 108 nM showing"only" 6 fold reduction in calpain inhibition. Results for compounds **20 - 22** suggest that, in terms of calpain inhibition, an aromatic moiety in P1´ is better tolerated. However, these analogues were not suitable for further advancement due to insufficient stability in liver microsomes (mCl > 70 and 100 µL/min/mg, respectively), probably due to the enhanced lipophilicity in P1´. In the case of P1´ *N*-alkoxy analogues **23 – 32**, we again observed a significant improvement in stability versus carbonyl reduction (Table **4**). In this subseries the negative impact of P1´ extension on calpain inhibiton was less pronounced: in general the P1´ *N*-alkoxy amides were more potent than the corresponding *N*-alkyl analogues. Compounds **23 – 26** and **28 – 30** showed Ki values for calpain in the range of 75 to 150 nM, which is 4 to 7 fold reduction in calpain inhibition compared to **1**. The P1´ comprising amine analogues **31** and **32** did not show any remarkable properties. Although displaying the highest calpain potency in this series, compounds **24**, **25**, and **28** did not fulfill our criteria for cathepsin selectivity (*Supporting Information*), and thus were not advanced.

Altogether, from the different amides prepared in this effort, cycloalkyl amide **15** and *N*-methoxy amide **23** displayed the best balance between calpain inhibition, microsomal and cytosolic stability, and selectivity versus cysteine protease cathepsins. To determine cellular efficacy, cpds. **15** and **23** were examined in a functional tissue assay using the prevention of NMDA-induced intracellular spectrin cleavage in rat hippocampal slice cultures as read-out.[16, 17] Although featuring diminished calpain inhibition *in vitro,* cellular efficacy was in a range comparable to primary amide **1** with compounds **15** and **23** showing IC_{50} values of 750 nM and 2150 nM, respectively. For both compounds enhanced cytosolic stability was reflected by reduced hydroxyamide metabolite formation in hepatocytes with M/P ratios of < 0.1 (cyno) and 0.2 (hu) for **15**, and < 0.1 (cyno, hu) for **23**, respectively.

ADME and Monkey PK: To determine whether the improvement in *in vitro* metabolic stability is translated to the *in vivo* situation, we advanced both compounds to further profiling in monkey PK, using primary ketoamide **1** as reference. *In vivo* monkey pharmacokinetic behavior of compounds **1**, **2**, **15** and **23** are summarized in Table **5.** Consistent with *in vitro* metabolism, **23** demonstrated a significant reduction in the formation of the hydroxyamide metabolite and improved monkey PK profile: low clearance, long half-life, and high oral bioavailability. Despite good cytosolic stability and low M/P ratio in cynomolgus monkey hepatocytes, compound **15** showed suboptimal *in vivo* monkey PK, which could be explained by increased Cyp-mediated clearance observed in liver microsomes for **15** compared to **23** (39 versus < 23 µL/min/mg).

Conclusion: Our goal was to identify calpain inhibitors with enhanced stability versus carbonyl reduction. Based on the results obtained for compound **2**, we developed cytosolic clearance as *in vitro* ADME assay reflecting carbonyl reduction as metabolic liability. In addition, we confirmed that compounds with good cytosolic stability showed significant reduction in formation of hydroxyamide metabolites *in vitro* and *in vivo*. CytCl and hepatocyte M/P ratio were then used routinely as tailored *in vitro* ADME assays in our screening funnel. Starting from ethyl amide **9** as lead, we identified P1´ extension as reliable approach to address carbonyl reduction as metabolic liability. Incorporation of different P1´ moieties led to a robust enhancement of cytosolic stability in our lead series, although this modification also resulted in diminished calpain inhibition. Systematic investigation of the SAR in P1´ revealed that the corresponding cyclopropyl and *N*-methoxy amides **15** and **23** showed the best balance between calpain potency, cathepsin selectivity and overall ADME properties. Further profiling in preclinical PK studies showed improved PK profiles, in particular for compound **23**.

In conclusion, *N*-(4-(methoxyamino)-3,4-dioxo-1-phenylbutan-2-yl)-2-(3-phenyl-1*H*-pyrazol-1-yl)nicotinamide **23** represents the most favorable analogue from this series combining enhanced metabolic stability, good potency against calpain, selectivity versus related cysteine protease cathepsins, in particular cathepsins L and S, and favorable PK with good oral bioavailabilities across pre-clinical species. The brain to plasma drug concentration of < 0.05 in rat suggests that compound **23** does not penetrate into the brain and is peripherally restricted. In addition, *N*-methoxy amide **23** did not show cross-reactivity in the standard receptor and enzyme panels (CEREP and MDS Enzyme Profiling Screen, up to 10 µM concentration), or any liability in hERG, and rat cardiovascular safety (*data not shown*). Based on these results, compound **23** might have potential as candidate calpain inhibitor for non-CNS indications like kidney diseases, ischemia/reperfusion injury, and cataract.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures for the preparation of compounds **15** and **23**; selectivity of compounds **1**, **2** and **9** - **32** versus related cysteine protease cathepsins B, K, L, and S; determination of intrinsic cytosolic stability in cynomolgus monkey and human liver cytosol; determination of hydroxyamide metabolite parent ratio in cynomolgus monkey and human hepatocytes; pharmacokinetic behaviour and determination of hydroxyamide metabolite parent ratio in cynomolgus monkey; ^{1}H NMR and ^{13}C spectra for compounds **15** and **23**.

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The authors declare the following competing financial interest(s): The authors are current or former employees of AbbVie (or Abbott Laboratories prior to separation), and may own company stock.

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ABBREVIATIONS

ABBREVIATIONS

ADME, absorption, distribution, metabolism, excretion; Cal, calpain; Cat, cathepsin; cyno, cynomolgus monkey; cytCl, cytosolic clearance; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; F_e, estimated bioavailability; HATU, [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorphosphat]; hERG, human *Ether-à-go-go*-Related Gene; HOBt, hydroxybenzotriazole; mCl, microsomal clearance; PK, pharmacokinetics.

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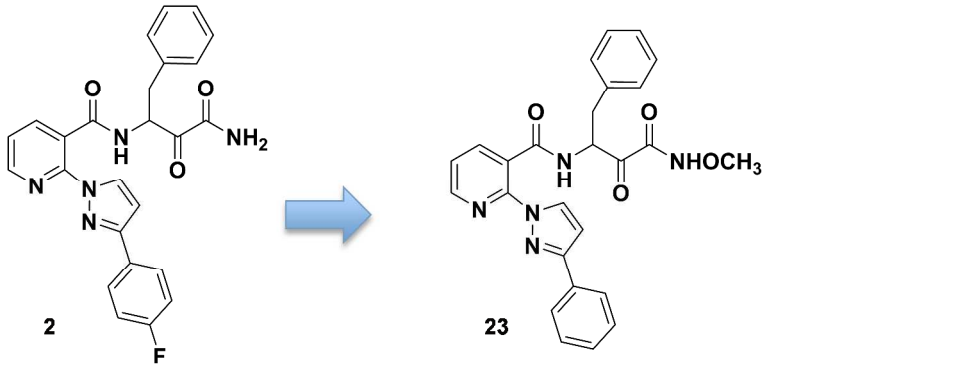

cytCl (cyno/hu) = 74 / 40 µL/min/mg
F (cyno) = 4.5 %
Low stability vs. carbonyl reduction

cytCl (cyno/hu) = 1 / 2 µL/min/mg
F (cyno) = 58.9 %
Highly stable against carbonyl reduction