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Discovery of a novel bicycloproline P2 bearing peptidyl α-ketoamide LY514962 as HCV protease inhibitor

Yvonne Yip, Frantz Victor, Jason Lamar, Robert Johnson, Q. May Wang, Donna Barket, John Glass, Ling Jin, Lifei Liu, Daryl Venable, Mark Wakulchik, Congping Xie, Beverly Heinz, Elcira Villarreal, Joe Colacino, Nathan Yumibe, Mark Tebbe, John Munroe and Shu-Hui Chen*

Lilly Research Laboratory, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA

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Abstract—We describe herein the design, syntheses and evaluation of a number of bicycloproline P2 bearing HCV protease inhibitors endowed with impressive enzyme potency, enzyme selectivity, cellular activity and favorable ADME profiles.

1. Introduction

Hepatitis C virus (HCV) has infected ~170 million people worldwide and has become a global health crisis. Of those infected with HCV, approximately 20 and 4% are likely to develop liver cirrhosis and hepatocellular carcinoma within the next decade. HCV infection is one of the leading causes of liver transplantation.[1] The therapy currently available is interferon (INF), either alone or in combination with ribavirin. Unfortunately, IFN efficacy is poor (at best 50% response rate) and significant side effects are associated with both IFN and IFN combination therapy. This has led to considerable efforts aimed at improving physiochemical property and toxicity profiles of INF-based therapy.[2] These research efforts have resulted in the discovery of several more efficacious and less toxic pegylated INFs[3,4] such as Pegasys and PEG-Intron. Despite the recent progress, most patients infected with HCV genotype 1 of the virus can not be treated successfully with currently available therapy. To address this unmet medical need, scientists from both pharmaceutical industries and academic institutions are aggressively pursuing many new approaches aimed at viral targets. The new viral targets include, but not limited to, NS3 protease, NS3 helicase, NS5B polymerase and full length NS3 protease-helicase.[5] Among those targets, NS3 serine protease has been most extensively characterized as an enzyme. This protease activity resides in the N-terminus of the NS3 domain and requires the HCV protein NS4A as a co-factor for maximal proteolytic activity. NS3 is an essential viral enzyme responsible for the cleavage of NS3–4A, NS4A–4B, NS4B–5A, and NS5A–5B.[6] Thus, it is reasonable to assume that inhibition of NS3 protease activity should inhibit viral maturation and replication. To date, there are various types of substrate based HCV protease inhibitors reported in the literature. These include peptidyl aldehydes,[7] boronic acids,[8] phosphonates,[9] peptidyl carboxylic acids,[10] α-keto-acids,[11] and α-ketoamides.[12] Recent report from Vertex disclosed the design and synthesis of a series of P2 TIQ-Proline bearing tetapeptidyl α-ketoamides such as **2** as shown in Figure 1.[12d] To further develop that series of inhibitors, we focused our effort on bicycloproline P2 containing α-ketoamides[13] such as **3a** and **3b** as shown in Figure 1. Our interest in α-ketoamides stemmed from the fact that these inhibitors can interact with the viral enzyme at the C-terminus binding domain (prime side). This added inhibitor–enzyme interaction at prime-side should further improve binding affinity.[14]

In addition to the keto-bc-proline P2 bearing inhibitors, we have also investigated a number of fluorinated (**4** and **5**), deoxy (**6**), dehydro (**7**), and O-MOM ether (**8**) containing peptidyl ketoamides shown in Figure 2. In conjunction with each P2 subunit, we also incorporated both acidic (**b**) and neutral P1′ residues (**a**, **c** and **d**) as depicted in Figure 2.

* Corresponding author. Tel.: +1-317-276-2076; fax: +1-317-276-1177; e-mail: chen_shu-hui@lilly.com

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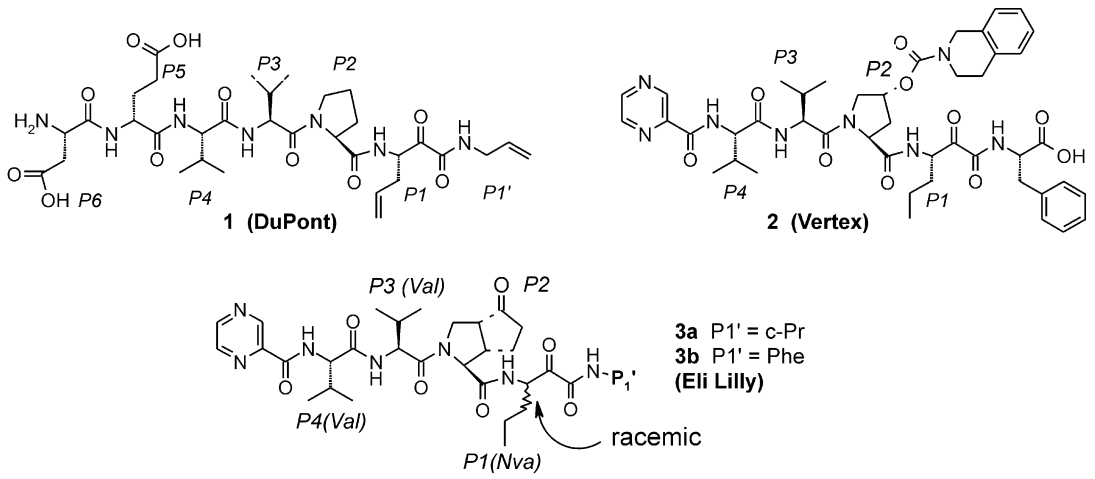


Figure 1. Representative ketoamide containing HCV protease inhibitors.

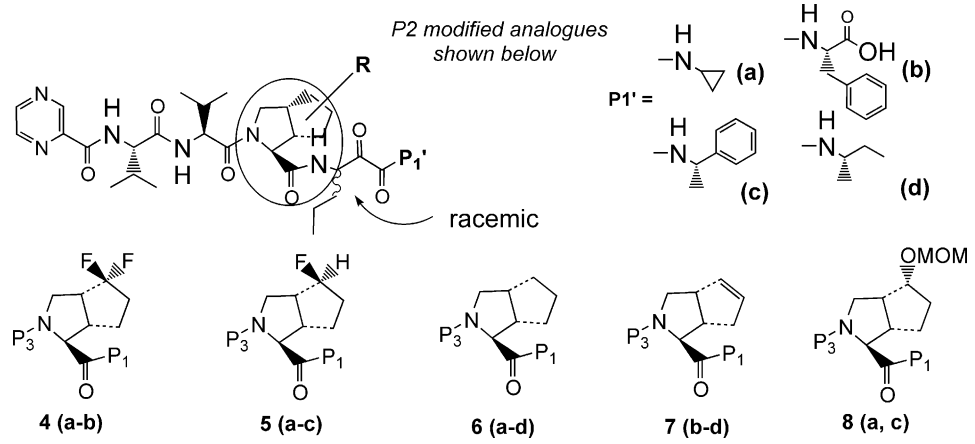


Figure 2. Bicycloproline P2 bearing inhibitors.

In this communication, we report the synthesis, biological evaluation (including enzyme binding affinity, enzyme selectivity against a panel of related protease enzymes, cellular activity obtained from replicon assay, and XTT cytotoxicity data determined in Huh-7 liver cell lines), and in vivo exposure data on a few selected inhibitors.

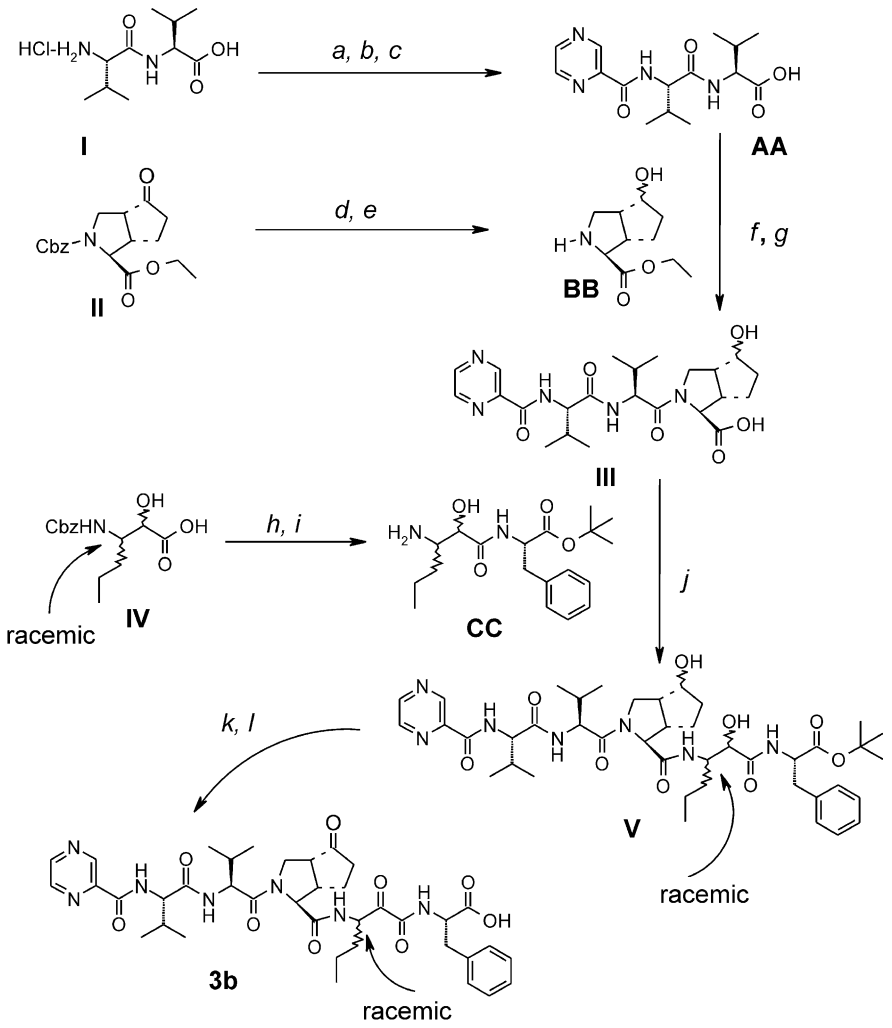
2. Synthesis of keto-bc-proline P2 bearing inhibitor 3b

As shown in Scheme 1, the P4–P3 dipeptidyl acid **AA** was prepared from the commercial available dipeptide **I** in three steps. HOAt and DCC mediated coupling[15] of **AA** and the hydroxyl bc-proline ester **BB** (prepared in two steps from the known bicycloproline derivative **II**)[16] afforded, after the ethyl ester hydrolysis, the hydroxyl acid **III** (64%). As also depicted in Scheme 1, the P1–P1′ building block **CC** was synthesized from the known norvaline (Nva) based hydroxyl acid **IV**[17] via a two-step route as shown in Scheme 1. Finally, the PyBOP mediated coupling of **III** and **CC** yielded the desired adduct **V** (68%), which was further converted to the final product **3b** (76%) upon Dess–Martin oxidation and acid deprotection.

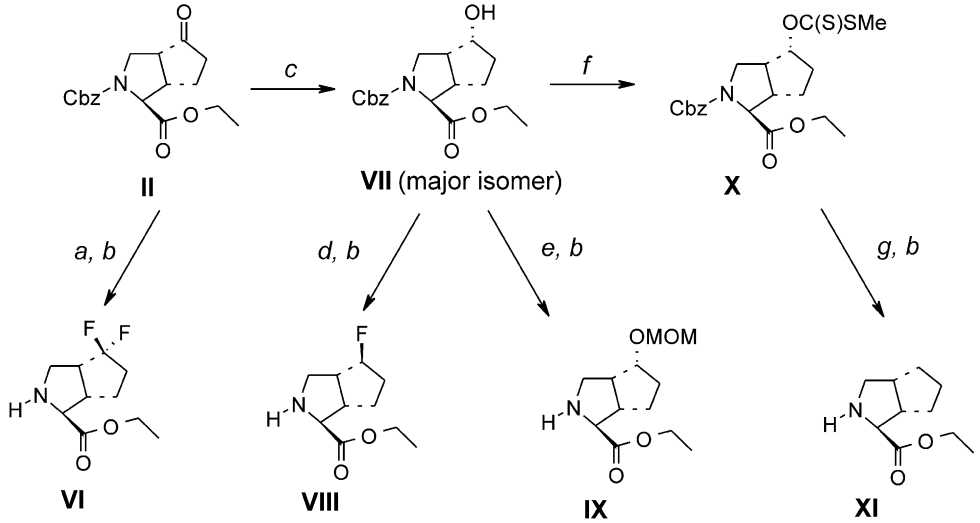
3. Modifications on P2 bicycloproline

After completing the synthesis of **3a** and **3b**, we shifted our attention to the preparation of various P2 modified inhibitors as exemplified in Figure 2. As can be seen from Scheme 1, the *gem*-difluoro bc-proline derivative **VI** was prepared from **II** via DAST mediated fluorination followed by hydrogenation. Reduction of the keto moiety in **II** with $NaBH_4$ in ethanol afforded, after silica gel chromatographic separation, the α-hydroxyl intermediate **VII** as the major product, which was further converted to the β-fluoro derivative **VIII** via monofluorination. The OMOM ether bearing bc-proline **IX** was obtained from **VII** via *O*-alkylation followed by *N*-deprotection. Further removal of the hydroxy functionality from **VII** was achieved using Barton deoxygenation sequence,[18] affording the corresponding deoxy bc-proline derivative **XI** shown in Scheme 2.

The preparation of the dehydro bc-proline intermediate **XIV** began with palladium catalyzed Cbz–Boc exchange reaction. The resulting product **XII** was treated with $NaBH_4$ to afford the hydroxylated intermediate **XIII** (as a ~4:1 mixture of isomers favoring the α-hydroxyl bearing isomer), which was then converted to the



Scheme 1. Synthesis of HCV protease inhibitor **3b**. Reagents and conditions: (a) $SOCl_2$, MeOH; (b) pyrazine carboxylic acid, PyBOP; (c) 2 N NaOH, MeOH, then 1 N HCl; (d) $NaBH_4$, EtOH; (e) H_2, $Pd(OH)_2$, C, EtOH; (f) **AA**, HOAt, DCC, then **BB**, *i*-Pr_2EtN; (g) 2 N NaOH, EtOH, then 1 N HCl; (h) **IV**, PyBOP, then HCl–H_2N–PheObu-t, *i*-Pr_2EtN; (i) H_2, Pd/C, EtOAc; (j) **III**, PyBOP, then **CC**, *i*-Pr_2EtN; (k) Dess–Martin, THF; (l) TFA, CH_2Cl_2.

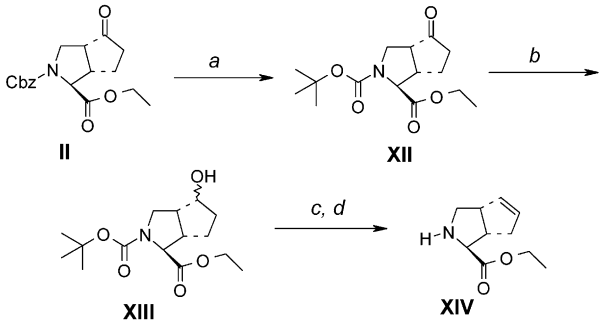


Scheme 2. Preparation of P2 modified building blocks. Reagents and conditions: (a) 2.5 equiv DAST, CH_2Cl_2; (b) H_2, $Pd(OH)_2$, C, EtOH; (c) $NaBH_4$, EtOH; (d) 1.2 equiv DAST, CH_2Cl_2; (e) *i*-Pr_2EtN, MOMCl; (f) NaH, CS_2, MeI, THF; (g) *n*-Bu_3SnH, AIBN/Tol.

desired dehydro bc-proline building block **XIV** via a base mediated beta-elimination reaction followed by TFA promoted *N*-deprotection as shown in Scheme 3.

4. Synthesis of non-keto bicycloproline P2 bearing inhibitors

With various modified bicycloproline building blocks in hand, we proceeded with the synthesis of five additional bc-proline bearing HCV protease inhibitors outlined in Figure 2. Both acidic and neutral P1′ moieties were incorporated into the target molecules for P1′ SAR study. Generally speaking, these tetrapeptidyl ketoamides (**4**–**8**) were prepared via a four-step sequence as exemplified for the synthesis of deoxy-bc-proline ketoamide **6a**. As shown in Scheme 4, the HOAt/DCC mediated coupling[15] between the dipeptidyl acid **AA** (already described in Scheme 1) with the requisite bc-proline P2 unit **XI** provided the tripeptidyl ester **XV**, which was then converted to its corresponding acid **XVI** upon base hydrolysis. The PyBOP mediated coupling of the acid **XVI** and the P1–P1′ unit **CC**′ (prepared in a similar manner to that described for **CC** in Scheme 1) gave rise to the adduct **XVII**, which was next oxidized with Dess–Martin periodinane to provide the desired ketoamide inhibitor **6a**. The same reaction sequence was used to prepare the rest of the inhibitors shown in Table 1. The overall yields for all final products are listed in ref 23. The structures of these inhibitors were secured on the basis of their NMR and mass spectroscopic analyses.

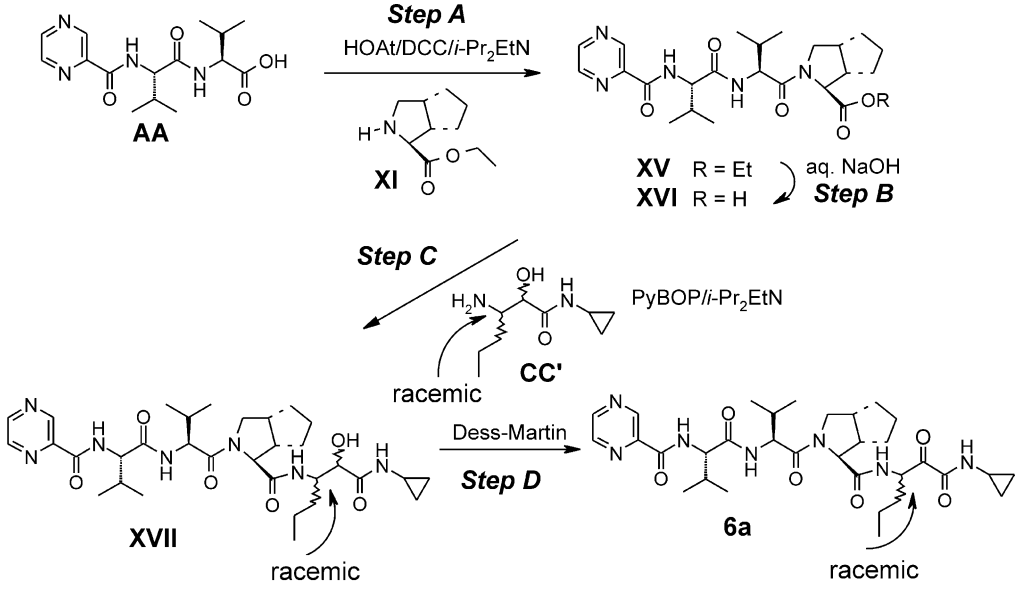


Scheme 3. Preparation of dehydro bicycloproline XIV. Reagents and conditions: (a) H_2, Pd/C, Boc_2O, EtOAc; (b) $NaBH_4$, EtOH; (c) Tf_2O, *i*-Pr_2EtN or DBU, CH_2Cl_2; (d) TFA, CH_2Cl_2.

All peptidyl ketoamides synthesized were evaluated in the following bioassays: (1) enzyme binding assay against truncated NS3 enzyme;[19] (2) a few selected compounds were evaluated against a panel of relevant cellular proteases;[20] (3) HCV replication surrogate assay (replicon) for cellular activity;[21] (4) cytotoxicity assay in a liver cell line (Huh-7 cells).[22] Promising inhibitors emerging from these testing were further evaluated in rats for drug exposure determination in liver and systemic circulation.

5. Enzyme inhibition assay[19]

All α-ketoamides were found to be tight-binding inhibitors for HCV NS3 protease. Therefore, the inhibition constant, K_i, was determined using Morrison equation.[19] Careful inspection of the data listed in Table 1 revealed the following trends: (1) all P1′ acid bearing inhibitors exhibited greater enzyme binding affinity relative to their respective neutral P1′ bearing counterparts, with K_i value ranging from 17 nM (**3b**) to 176 nM (**4b**). (2) Within cyclopropyl P1′ bearing analogues, the enzyme inhibition efficiency decreases according to following order: **6a** (deoxy) > **5a** (mono-F) > **4a** (*gem*-di-F) > **8a** (O-MOM ether) > **3a** (keto). (3) When compared with other neutral P1′ bearing inhibitors, c-Pr terminus bearing ones demonstrated similar or better enzyme inhibition activity.



Scheme 4. Synthesis of deoxy-bicycloproline P2 inhibitor **6a**.

6. Enzyme selectivity[20]

To identify potent and selective HCV NS3 protease inhibitor, compound **6a** was selected, on the basis of its activity demonstrated in enzyme binding assay and replicon assay (Table 2), for further evaluation against a panel of related cellular proteases. When tested in a single-dose experiment at 100 μM, **6a** exhibited only minimal inhibitory effect against chymotrypsin (63%), kallikrein (0%), thrombin (12%), plasmin (5%), and trypsin (0%). Inhibition of cellular elastase, cathepsin B, cathepsin G, and cathepsin L by compound **6a** was seen, however, at least 10× selectivity window with the exception of cathepsin B (an enzyme involved in cancer metastases).

7. Replicon assay[21]

All newly synthesized inhibitors were tested in the replicon assay at 25 or 50 μM. After careful reviewing the data shown in Table 1, several trends can be gleaned from this data set: (1) all P1′ phenyl alanine (Phe) bearing inhibitors (**3b**–**7b**) were inactive in this assay. In sharp contrast to this finding, with the exception of **3a**, all of the neutral P1′ bearing compounds demonstrated inhibitory effect in this assay. (2) Based on the percentage inhibition obtained @ 25 μM with various c-Pr P1′ bearing inhibitors (**3a**, **4a**, **5a**, **6a**, and **8a**), it is evident that compound **6a** (deoxy-bc-proline as P2) was the most potent inhibitor. (3) When compared with c-Pr P1′ bearing inhibitors (**5a**, **6a**, and **8a**), their corresponding (s)-MeBn P1′ containing counterparts (**5c**, **6c**, and **8c**) exhibited superior activity. Judging from the IC_{50} data listed in Table 1, it is clear that compound **6c** ($IC_{50} = 3.4$ μM) was about 2-fold more potent than **6a** ($IC_{50} = 7.0$ μM). (4) Based on the inhibitory data obtained with **6a** and **6d** in the replicon assay, it appears that (s)-butyl P1′ bearing inhibitor **6d**, despite relatively reduced enzyme binding affinity, displayed similar replicon activity to that obtained with c-Pr P1′ bearing analogue **6a**.

Table 1. Biological evaluation of bc-proline bearing HCV protease inhibitors

Compd	HCV K_i (μM)	% Inhibition @25 μM	Cytotoxicity (μM)
	PNA assay	Replicon assay	
3a	0.51	−9	>100
3b	0.017	−20	>100
4a	0.37	64	>100
4b	0.176	−49 @50 μM	>100
5a	0.30	84	>100
5b	0.082	−8	>100
5c	0.315	97	33% inhib. @100 μM
6a	0.123	93 ($IC_{50} = 7.0$ μM)	>100
6b	0.068	−88	>100
6c	2.60	97 ($IC_{50} = 3.4$ μM)	>100
6d	1.80	90	>100
7b	0.090	−57	>100
7c	0.88	97	33% inhib. @100 μM
7d	0.625	91	Not tested
8a	0.415	80	>100
8c	0.332	98	33% inhib. @100 μM

Table 2. In vivo exposure of **6a** after oral dosing @ 50 mg/kg

Liver exposure		Plasma exposure		
$C_{ave\ (0–8\,h)}$	$C_{8\,h}$	$AUC_{(0–8\,h)}$	C_{max}	$C_{8\,h}$
6.29 μM	0.50 μM	2.86 μg×h/mL	2.25 μg/mL	0.01 μg/mL

8. Cytotoxicity assay[22]

In view of the data listed in Table 1, it is evident all of inhibitors tested exhibited were considered to be non-cytotoxic with IC_{50} values >100 μM. It is also clear that (s)-MeBn P1′ bearing inhibitors (**5c**, **7c**, and **8c**) seemed to be more cytotoxic than other P1′ carrying counterparts.

9. In vivo target tissue drug exposure

With the aim of identifying orally active HCV protease inhibitors with significant drug exposures in the target tissue (liver), compounds **3a**, **3b**, and **6a** were dosed orally to male Sprague–Dawley rats at a dose of 50 mg/kg. The compounds were dosed as suspensions in a wet granulation vehicle (povidone/lactose/polysorbate). Blood was collected by orbitakl sinus and cardiac puncture (terminal) bleeding procedures at selected time points following dosing. Liver samples were obtained after intracardiac perfusion of the livers with saline to remove residual blood in the liver. Plasma and liver drug concentrations were determined by LC/MS/MS.

When inhibitors **3a** and **3b** were dosed to rats orally, only minimal amounts of the respective inhibitors were found in the liver samples of the treated animals. Despite these disappointing results, we next studied the deoxy-bicyclo-proline P2 bearing inhibitor **6a**. As can be seen in Table 2, upon oral administration of **6a** to rats, rather high concentrations of **6a** were found in the liver samples with C_{ave} (0–8 h) of 6.29 μM and $C_{8\,h}$ of 0.50 μM. In addition, impressive drug exposures were also detected in the plasma samples, albeit less than that found in the liver samples. In light of the replicon IC_{50} value and the liver exposure achieved with **6a**, it is conceivable that this compound may be able to inhibit the NS3 protease function in vivo.

10. Conclusion

We synthesized and evaluated six types of bicycloproline P2 bearing α-ketoamides as HCV protease inhibitors. Generally speaking, the P1′ phenyl alanine containing inhibitors (e.g., **3b** $K_i = 17$ nM) possessed greater enzyme inhibitory activity in comparison to their neutral P1′ bearing counterparts. However, none of the acids (**3b**–**7b**) demonstrated activity in the replicon assay. This is likely due to the poor lipophilic nature of these acids. In contrast, essentially all of the neutral P1′ bearing inhibitors exhibited activity in the

replicon assay without inherent cytotoxicity. One of such promising compounds, **6a**, also demonstrated favorable drug exposure in liver and plasma upon oral dosing. In view of these data, we believe that further optimization of **6a** could lead to a novel class of peptidyl α-ketoamides with therapeutic potential.

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