Bioorganic & Medicinal Chemistry 24 (2016) 686–692

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Design and synthesis of an activity-based protein profiling probe derived from cinnamic hydroxamic acid

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ARTICLE INFO

Article history:
Received 8 October 2015
Revised 14 December 2015
Accepted 22 December 2015
Available online 23 December 2015

Keywords:
Hepatitis C virus (HCV)
Cinnamic hydroxamic acid
Benzothiophene
Hydroxamic acid
Histone deacetylase
Matrix metalloproteinases
Activity-based protein profiling

ABSTRACT

In our continued effort to discover new anti-hepatitis C virus (HCV) agents, we validated the anti-replicon activity of compound **1**, a potent and selective anti-HCV hydroxamic acid recently reported by us. Generally favorable physicochemical and in vitro absorption, distribution, metabolism, and excretion (ADME) properties exhibited by **1** made it an ideal parent compound from which activity-based protein profiling (ABPP) probe **3** was designed and synthesized. Evaluation of probe **3** revealed that it possessed necessary anti-HCV activity and selectivity. Therefore, we have successfully obtained compound **3** as a suitable ABPP probe to identify potential molecular targets of compound **1**. Probe **3** and its improved analogs are expected to join a growing list of ABPP probes that have made important contributions to not only the studies of biochemical and cellular functions but also discovery of selective inhibitors of protein targets.

1. Introduction

HCV infection affects 3.2 million people in the United States.[1] If untreated, HCV is a major cause of liver diseases including chronic hepatitis, cirrhosis and hepatocellular carcinoma. By 2007, HCV had caused more deaths than HIV,[2] incurring hefty direct medical costs. Worldwide, HCV poses a major health risk and burden.[3] HCV is estimated to infect 160–180 million people in the world and the vast majority remain untreated.[4] More alarmingly, among the 20 countries that have the highest prevalence of HCV infection, 12 have low or lower-middle incomes,[5] severely restricting their ability to access new and effective treatments.

For many years, the standard of care (SOC) was pegylated interferon in combination with ribavirin,[6] a regimen undermined by a lack of efficacy[6,7] and the occurrence of severe side-effects.[8] In the past decade, novel therapies have been vigorously pursued to improve and eventually replace SOC.[9] Efforts on direct-acting antivirals (DAAs) have led to the marketing of inhibitors that target viral non-structural (NS) proteins NS3/4A, NS5A or NS5B. When combined with SOC, NS3/4A protease inhibitors markedly improved outcomes and shortened treatment duration.[10,11] However, protease inhibitors suffer from rapid emergence of drug resistance caused by viral mutations.[12] Very recently, NS5A and NS5B inhibitors were used as backbones in all-oral, interferon-free and ribavirin-free regimens, with a cure rate of >90% within 12 weeks.[13] Nevertheless, drug resistance still remains a concern. In addition, varied responses to new treatments by different genotypes have been observed.[14] Moreover, the prohibitively high costs of newly approved regimens have severely restricted their global access.[15]

Taken together, there is still an urgent need to explore therapeutics with distinct mechanisms of action that can be combined with approved DAAs. To this end, host-targeting antivirals (HTAs) hold promise because HTAs are generally less prone to induce resistance and more likely to be active across different HCV genotypes.[16,17] HTAs, including those targeting cyclophilin A and miR-122, have been investigated in clinical trials,[17] supporting the notion that HTAs are promising anti-HCV agents even though their long-term side effects need to be assessed.

Recently, we discovered novel hydroxamic acids that showed excellent anti-HCV activity and a high therapeutic index (TI = CC_{50}/EC_{50}) (Fig. 1).[18] Our structure–activity relationship (SAR) studies revealed trends that are summarized in Figure 1. Most importantly,

Abbreviations: ABPP, activity-based protein profiling; ADME, absorption, distribution, metabolism, and excretion; DAAs, direct-acting antivirals; DPBS, Dulbecco's phosphate-buffered saline; HCV, hepatitis C virus; HDACs, histone deacetylases; HTAs, host-targeting antivirals; 2mA, 2′-*C*-methyl adenosine; MMPs, matrix metalloproteinases; NADPH, nicotinamide adenine dinucleotide phosphate; RT-qPCR, reverse transcription and quantitative PCR; SAR, structure–activity relationship; SOC, standard of care; TACE, tumor necrosis factor-α-converting enzyme; TI, therapeutic index; UDPGA, uridine 5′-diphosphoglucuronic acid.

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http://dx.doi.org/10.1016/j.bmc.2015.12.035
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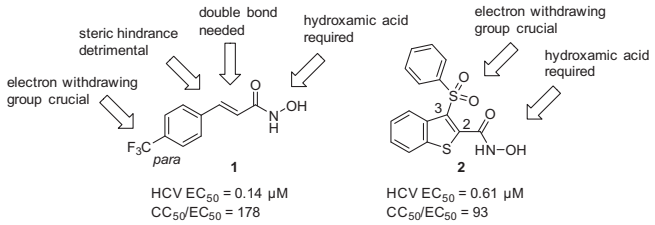


Figure 1. Lead hydroxamic acids and SAR trends.

a hydroxamic acid functionality was required for the anti-HCV activity while electron-withdrawing groups such as CF_3 and sulfone in compounds **1** and **2**, respectively, enhanced both activity and selectivity. Our preliminary studies suggest that these hydroxamic acids act on previously unappreciated targets most likely host cell factors. To unambiguously identify the mechanism of action and molecular target(s) of these new compounds, we decided to use ABPP.[19] Herein we report the design, synthesis, and biological evaluation of an ABPP probe based on the structural features of compound **1**.

2. Results and discussion

2.1. Biochemical assays

We reported structurally simple hydroxamic acids **1** and **2** based on the cinnamic hydroxamic acid and benzo[*b*]thiophen-2-hydroxamic acid core structures, respectively (Fig. 1).[18] Both compounds showed potent and selective anti-HCV activity in a subgenomic replicon. To confirm that both compounds truly inhibited HCV replication, we performed a secondary assay based on reverse transcription and quantitative PCR (RT-qPCR) with 2′-*C*-methyl adenosine (2 mA) as a positive control. As shown in Figure 2, both compounds reduced the replicon RNA in a dose-dependent manner, supporting our conclusion that they are *bona fide* inhibitors of HCV replication.

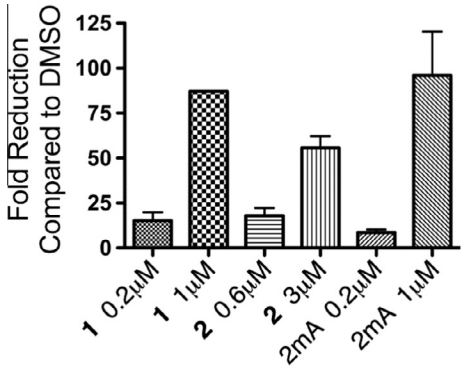


Figure 2. Fold reduction of HCV replicon RNA levels determined using RT-qPCR for cells treated with compounds **1**, **2** and 2 mA.

We attempted to identify potential molecular target(s) of compounds **1** and **2** by screening them against a number of purified enzymes that have been commonly targeted by hydroxamic acids. Among the targets were histone deacetylases (HDACs), matrix metalloproteinases (MMPs) and tumor necrosis factor-α-converting enzyme (TACE) (Table 1). Two major HCV viral targets NS3/4A and NS5B were also included. As we have reported previously,[18] compound **2** possessed weak inhibition of a full panel of 11 human HDACs, suggesting that blocking human HDACs was unlikely to be solely responsible for compound **2**'s anti-HCV activity. However, its inhibition of selected members of human MMPs (MMP9, 12, and 14) and HDAC8 was significant and might contribute to the anti-HCV activity.

We also performed an identical biochemical screening of compound **1** which revealed minimal inhibition of HCV NS3/4A and NS5B (Table 1). Similarly, it did not possess substantial inhibition of MMPs and TACE. However, compound **1** did show good activity against HDAC6 and 8, suggesting that at least for compound **1** inhibition of HDAC6 and/or 8 cannot be fully discounted. However, we previously demonstrated that known selective HDAC6 and HDAC8 inhibitors were not effective as anti-HCV agents.[18] The differential inhibition patterns exhibited by compounds **1** and **2** suggest that they may block different molecular target(s). Alternatively, it is also possible that compounds **1** and **2** share the same primary target(s); therefore their inhibition of HDACs and MMPs, respectively, may be considered as undesired off-targets. These possible scenarios highlight challenges in target identification and prompt us to adopt an unbiased approach like ABPP.

2.2. Physicochemical and in vitro ADME properties

To estimate whether our lead compounds have drug-like properties required for therapeutic application in HCV, compounds **1** and **2** were assessed for their physicochemical and in vitro ADME properties (Table 2). First, we tested the aqueous solubility in Dulbecco's phosphate-buffered saline (DPBS, pH 7.4). Compounds **1** and **2** showed good and excellent solubility, respectively. Second, both compounds were stable in mouse and human plasma. Third, while compound **1** exhibited excellent phase I stability in human liver microsomes, it was less stable in mouse liver microsomes. Similarly, compound **2** showed great phase I stability in human

Table 1
Evaluation of compound **1** against HDACs, MMPs, TACE, HCV NS3/4A, and HCV NS5B

Enzyme	IC_{50} (μM)	Enzyme	IC_{50} (μM)	Enzyme	IC_{50} (μM)
HDAC1	4.11	MMP1	>100	TACE	21.5
HDAC2	6.36	MMP2	>100	HCV NS3/4A	>200
HDAC3	5.37	MMP3	>100	HCV NS5B	<5% inhibition at 10 μM
HDAC4	>100	MMP7	>100		
HDAC5	34.7	MMP8	>100		
HDAC6	0.183	MMP9	>100		
HDAC7	>100	MMP10	>100		
HDAC8	0.954	MMP12	>100		
HDAC9	>100	MMP13	>100		
HDAC10	6.32	MMP14	>100		
HDAC11	5.53				

Table 2
Physicochemical and in vitro ADME parameters of compounds **1** and **2**

Compd	Aqueous solubility (μM)	Plasma stability $t_{1/2}$ (h)		Microsomal phase I stability $t_{1/2}$ (min)		Microsomal phase II stability $t_{1/2}$ (min)	
		Mouse	Human	Mouse	Human	Mouse	Human
1	648	23.2	>24	46.2	693	14.8	533
2	3346	>24	>24	78.8	770	1.7	1.4

Plasma and microsomal stabilities were determined in triplicate as described in Sections 4.3.4 and 4.3.5, respectively.

liver microsomes but reduced, albeit still good, stability in mouse liver microsomes. When the phase II (glucuronidation) stability was measured, compound **1** showed superb stability in human liver microsomes; however its stability in mouse liver microsomes was markedly lower. Compound **2** exhibited minimal phase II (glucuronidation) stability in microsomes from both species. Taken together, although compound **1**'s microsomal stability in mouse still needs improvement, it possesses good physicochemical and in vitro ADME properties Therefore, compound **1** represents a favorable chemotype from which an ABPP probe can be designed.

2.3. Probe design

Even though we have uncovered clues about the molecular target(s) of our lead compounds through biochemical screening, it remains a challenge to identify the targets of our leads in an unbiased fashion. We decided to use ABPP,[19] which offers sensitivity and specificity advantages over traditional affinity chromatography approaches. A strength of ABPP is that it can be used in intact HCV replicon cells to allow interrogation of the native cellular proteome. ABPP has been increasingly used to identify the protein targets of small molecules in complex proteomes including those targeted by hydroxamic acid-based HDAC inhibitors[20] and those involved in host–virus interaction.[21]

Successful application of ABPP requires a probe consisting of three key moieties. First, a binding group is needed to interact with the target; second, a reactive group is incorporated to covalently cross-link the ABPP probe to the target upon UV irradiation; third, a handle is appended to facilitate the enrichment of bound targets. Elaborating on our lead compound **1**, we designed probe **3** (Fig. 3), in which a cinnamic hydroxamic acid moiety was retained. To preserve high anti-HCV activity and selectivity, we also strived to incorporate an electron-withdrawing group based on our previous SAR studies. To that end, we introduced a benzophenone functionality[22] (highlighted in red) has been commonly used as an UV-activated photoaffinity label. The ketone functionality in benzophenone also serves as an electron-withdrawing group. A terminal alkyne (highlighted in green) functions as a bio-orthogonal chemical handle, upon which a biotin-azide can then be appended via a copper(I)-catalyzed click reaction,[23,24] allowing for subsequent enrichment of the target proteins using streptavidin-coated beads. Proteins bound to beads can then be purified

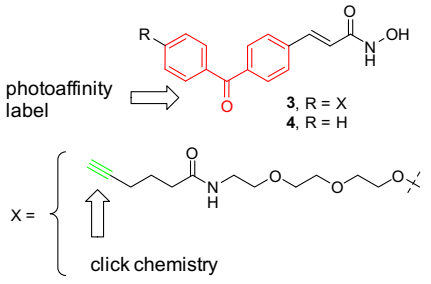
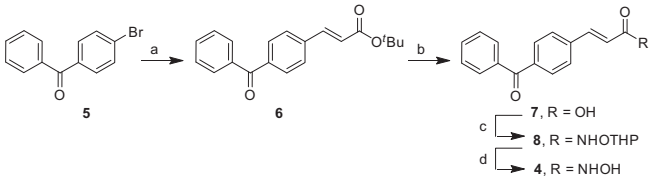


Figure 3. Design of an ABPP probe based on hydroxamic acid **1**.



Scheme 1. Reagents and conditions: (a) *t*-butyl acrylate, $Pd(OAc)_2$, $P(o\text{-tolyl})_3$, NEt_3, DMF, 120 °C, 33%; (b) TFA, DCM, 90%; (c) NH_2OTHP, EDC, DCM, 82%; (d) HCl/Et_2O, DCM, 77%.

and identified by mass spectrometry.[25] To alleviate the steric hindrance around the terminal alkyne and to ensure an efficient click reaction, a polyethylene glycol-based spacer is inserted between the benzophenone functionality and the alkyne group.

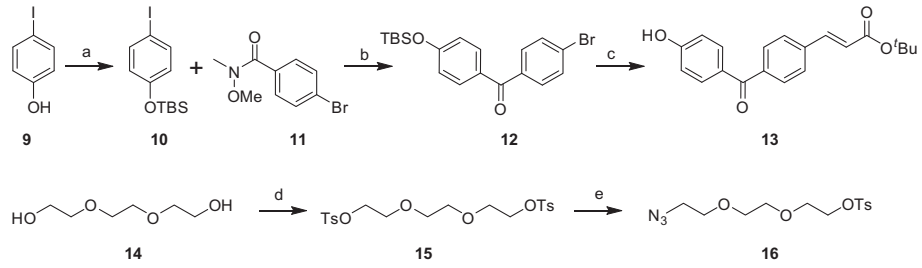
2.4. Chemical synthesis

Before we committed to the synthesis of probe **3**, we prepared and evaluated compound **4** (Fig. 3), a truncated analog of probe **3**. The synthesis of compound **4** was straightforward (Scheme 1). Bromide **5** underwent a Heck reaction to give *tert*-butyl ester **6**, which was then converted into the corresponding hydroxamic acid **4** through a series of standard chemical transformations, i.e. hydrolysis, introduction of a THP-protected hydroxylamine, and finally removal of the THP protective group. With compound **4** in hand, we tested its anti-HCV activity (EC_{50}) in the HCV subgenomic replicon model and cellular toxicity (CC_{50}). Compound **4** showed an EC_{50} value of 480 nM, which was about 3-fold less active than the parent compound **1** (Table 3). Reduced anti-HCV activity might be attributed to the steric hindrance imposed by the benzophenone functionality in comparison with the less bulky trifluoromethyl group as seen in compound **1**. Alternatively, the ketone functionality, with its relatively lesser electron-withdrawing capacity, might account for compound **4**'s reduced anti-HCV activity. Nonetheless, very modest loss of activity suggested that probe **3**, which contained key structural features of compound **4**, warranted further exploration.

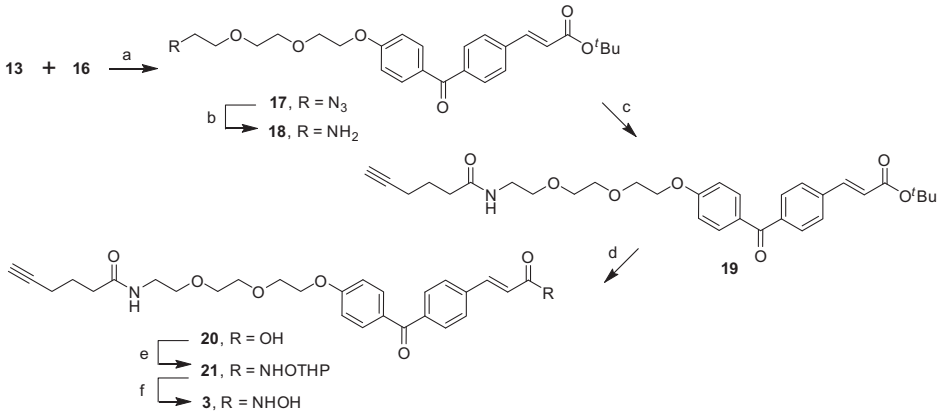
Table 3
Anti-HCV activity and toxicity of probe **3** and its analogs

Compd	% inhibition at 10 μM		EC_{50} (μM)	CC_{50} (μM)	TI
	HCV	Viability			
1			0.14[a]	25[a]	178[a]
4			0.48	16	33
3			1.2	25	21
20	<5	<5			
Biotin-azide	<10	<5			
2 mA			0.20[a]	54[a]	270[a]

[a] Reported previously.[18] TI, therapeutic index, $TI = CC_{50}/EC_{50}$. Percentages of inhibition, EC_{50} and CC_{50} values were determined in at least duplicate, and the averages were reported.



Scheme 2. Reagents and conditions: (a) TBSCl, imidazole, DMF, 90%; (b) ^{i}PrMgBr, LiCl, THF, 86%; (c) *t*-butyl acrylate, $Pd(OAc)_2$, P(*o*-tolyl)$_3$, NEt_3, DMF, 87%, 120 °C; (d) TsCl, NEt_3, DCM, 87%; (e) NaN_3, DMF, 39%.



Scheme 3. Reagents and conditions: (a) Cs_2CO_3, DMF, 90%; (b) PPh_3, H_2O, THF, 80%; (c) hex-5-ynoic acid, EDC, DCM, 75%; (d) TFA, DCM, 11%; (e) NH_2OTHP, EDC, DCM; (f) HCl/Et_2O, DCM, 46% for 2 steps (e and f).

The synthesis of probe **3** started with phenol **9**, which was protected as a TBS ether **10** (Scheme 2).[26] Upon treatment with isopropyl magnesium bromide, the iodo functionality in compound **10** was converted into a Grignard reagent, which condensed with Weinreb amide **11**[27] to give ketone **12**. A Heck reaction performed on **12** afforded *tert*-butyl ester **13**, in which the TBS protective group was removed simultaneously to give a free phenol. Via this free phenol, an ethylene glycol-based spacer was subsequently attached. Mono-tosylate **16**,[28] which was needed for the spacer, was prepared from polyethylene glycol **14** via di-tosylate **15**.[28–30] Displacement of tosylate **16** by phenol **13** under basic conditions gave azide **15**, which was then reduced to primary amine **18** with PPh_3 (Scheme 3). After introduction of a terminal alkyne through an amide formation, *tert*-butyl ester **19** underwent a series of chemical transformations identical to those described for compound **4**, finally leading to probe **3**.

2.5. Anti-HCV activity and toxicity

We tested probe **3** for its anti-HCV activity (EC_{50}) in the HCV subgenomic replicon model and its cellular toxicity (CC_{50}) (Fig. 4 and Table 3). It possessed an EC_{50} value of 1.2 μM, leading to about 9-fold and 3-fold reduction in activity when compared with compound **1** and its truncated analog **4**, respectively. The TI value also decreased mainly due to an erosion of the anti-replicon activity. We also tested carboxylic acid **20**, which showed no anti-HCV activity at 10 μM, highlighting the crucial role of a hydroxamic acid functionality as observed in our initial SAR.[18] Furthermore, we examined whether biotin-azide, a key reagent used in the copper (I)-catalyzed click reaction during ABPP, has any activity in the subgenomic replicon. Biotin-azide showed negligible activity, eliminating its effect on the replicon model. Our preliminary studies demonstrated that probe **3** retained good anti-HCV activity and

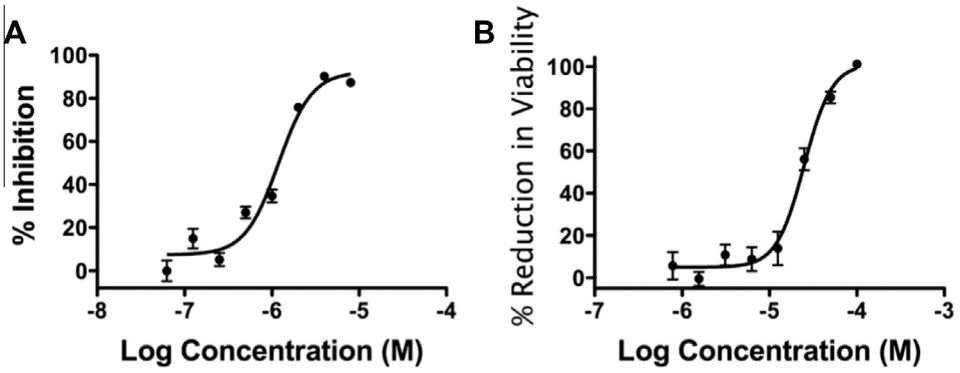


Figure 4. Dose–response curves of probe **3**. (A) Anti-HCV activity. (B) Viability.

selectivity, two attributes that made **3** a suitable probe for future ABPP experiments.

3. Conclusions

We discovered compound **1** as a potent and selective anti-HCV agent, whose anti-replicon activity was confirmed using RT-qPCR. Compound **1** also exhibited generally favorable physicochemical and in vitro ADME properties. However, our preliminary biochemical screening compound **1** suggested that unbiased identification of potential molecular targets was a challenging task that could be achieved by using ABPP. Therefore, we designed and synthesized probe **3**, whose good anti-HCV activity and TI, albeit decreased relative to compound **1**, indicated that it could serve as a suitable ABPP probe. It will be used in our efforts in identifying potential molecular targets of compound **1**. On the other hand, probe **3** showed reduced anti-HCV activity, indicating that a benzophenone-based probe like **3** needs further optimization. Design and synthesis of new ABPP probes are also in progress.

4. Experimental section

4.1. General methods

All commercial reagents were used as provided unless otherwise indicated. An anhydrous solvent dispensing system (J.C. Meyer) using 2 packed columns of neutral alumina was used for drying THF, Et_2O, and CH_2Cl_2, while 2 packed columns of molecular sieves were used to dry DMF. Solvents were dispensed under argon. Flash chromatography was performed with Ultra Pure silica gel (Silicycle) or with RediSep® Rf silica gel columns on a Teledyne ISCO CombiFlash® R_f system using the solvents as indicated. Nuclear magnetic resonance spectra were recorded on a Varian 600 MHz with Me_4Si or signals from residual solvent as the internal standard for ^1H. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br s (broad singlet), and dd (double doublet). Values given for coupling constants are first order. High resolution mass spectra were recorded on an Agilent TOF II TOF/MS instrument equipped with a multi-mode source (ESI or APCI).

4.2. Chemical synthesis

4.2.1. *tert*-Butyl (*E*)-3-(4-benzoylphenyl)acrylate (6)

A mixture of 4-bromobenzophenone (**5**, 1 mmol), *t*-butyl acrylate (2 mmol), $Pd(OAc)_2$ (5% mmol), P(*o*-tolyl)$_3$ (10% mmol), and NEt_3 (2.0 mmol) in DMF (5 mL) was heated at 130 °C for 24 h and cooled to rt. The reaction mixture was diluted with EtOAc (20 mL) and the organic phase was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography (0–30% EtOAc/hexanes) to afford *t*-butyl ester **6** as a colorless oil (100 mg, 33%). ^1H NMR ($CDCl_3$, 600 MHz) δ 7.82–7.76 (m, 4H), 7.63–7.56 (m, 4H), 7.50–7.46 (m, 2H), 6.46 (d, J = 16.2 Hz, 1H), 1.54 (s, 9H). HRMS could not be obtained under varied conditions.

4.2.2. (*E*)-3-(4-Benzoylphenyl)acrylic acid (7)

A solution of *t*-butyl ester **6** (100 mg, 0.32 mmol) in TFA/DCM (2:1, 10 mL) was allowed to stir at rt for 1 h. After removal of the organic solvent, the residue was purified by flash column chromatography (0–15% MeOH/CH_2Cl_2) to afford *trans*-cinnamic acid **7** as a white solid (73 mg, 90%). ^1H NMR (DMSO-d_6, 600 MHz) δ 12.53 (br s, 1H), 7.90–7.84 (m, 2H), 7.78–7.64 (m, 6H), 7.62–7.56 (m, 2H), 6.68 (d, J = 16.2 Hz, 1H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ 195.6, 167.7, 143.0, 138.6, 138.4, 137.3, 133.3, 130.5, 130.0, 129.1, 128.7, 122.2. HRMS (ESI^-) calcd for $C_{16}H_{11}O_3$ $(M-H)^-$ 251.0714, found 251.0707.

4.2.3. (*E*)-3-(4-Benzoylphenyl)-*N*-((tetrahydro-2*H*-pyran-2-yl) oxy)acrylamide (8)

A solution of *trans*-cinnamic acid **7** (70 mg, 0.28 mmol), *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine (0.31 mmol) and EDC·HCl (0.31 mmol) in DCM (5 mL) was allowed to stir at rt overnight and the mixture was then washed with water and brine. After removal of the solvent, the residue was purified by flash column chromatography (0–15% MeOH/CH_2Cl_2) to afford **8** as a colorless oil (80 mg, 82%). ^1H NMR ($CDCl_3$, 600 MHz) δ 9.50 (s, 1H), 7.84–7.78 (m, 4H), 7.75 (d, J = 15.0 Hz, 1H), 7.66 (d, J = 7.8 Hz, 2H), 7.61 (dd, J = 7.5, 7.5 Hz, 1H), 7.54–7.46 (m, 2H), 7.37 (d, J = 15.0 Hz, 1H), 3.91 (t, J = 6.6 Hz, 1H), 2.35 (t, J = 6.6 Hz, 2H), 1.93–1.86 (m, 2H), 1.25–1.20 (m, 4H). HRMS (ESI^-) calcd for $C_{21}H_{20}NO_4$ $(M-H)^-$ 350.1398, found 350.1400

4.2.4. (*E*)-3-(4-Benzoylphenyl)-*N*-hydroxyacrylamide (4)

To a solution of **8** (65 mg, 0.18 mmol) in DCM (5 mL) was added 2 M HCl in Et_2O (2 mL). The reaction mixture was stirred at rt for 2 h. The white solid precipitate was filtrated and washed with DCM to give hydroxamic acid **4** as a white solid (38 mg, 77%). ^1H NMR (DMSO-d_6, 600 MHz) δ 7.80–7.72 (m, 6H), 7.69 (dd, J = 7.2, 7.2 Hz, 1H), 7.61–7.32 (m, 3H), 6.62 (d, J = 15.6 Hz, 1H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ 195.6, 162.6, 139.3, 137.7, 137.5, 137.4, 133.2, 130.7, 130.0, 129.1, 128.0, 122.2. HRMS (ESI^-) calcd for $C_{16}H_{12}NO_3$ $(M-H)^-$ 266.0823, found 266.0815.

4.2.5. *tert*-Butyl(4-iodophenoxy)dimethylsilane (10)[26]

To a solution of 4-iodophenol (**9**, 880 mg, 4.0 mmol) and TBSCl (723 mg, 4.8 mmol) in DMF (30 mL) was added imidazole (354 mg, 5.2 mmol). The reaction mixture was allowed to stir at 40 °C for 12 h and cooled to rt. After diluted with EtOAc (100 mL), the organic phase was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography (0–30% EtOAc/hexanes) to afford **10** as a colorless oil (1.2 g, 90%). ^1H NMR ($CDCl_3$, 600 MHz) δ 7.50 (d, J = 9.0 Hz, 2H), 6.61 (d, J = 9.0 Hz, 2H), 0.97 (s, 9H), 0.18 (s, 6H). HRMS could not be obtained under varied conditions.

4.2.6. 4-Bromo-*N*-methoxy-*N*-methylbenzamide (11)[27]

A solution of 4-bromobenzoic acid (1.0 mg, 5.0 mmol), *N*,*O*-dimethylhydroxylamine hydrochloride (536 mg, 5.5 mmol), EDC·HCl (1.05 g, 5.5 mmol), and NEt_3 (556 mg, 5.5 mmol) in DCM (30 mL) was allowed to stir at rt for 12 h. The reaction mixture was diluted with EtOAc (100 mL), and the organic phase was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography (0–30% EtOAc/hexanes) to afford **11** as a colorless oil (1.0 g, 81%). ^1H NMR ($CDCl_3$, 600 MHz) δ 7.59 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H), 3.54 (s, 3H), 3.36 (s, 3H). HRMS (ESI^+) calcd for $C_9H_{11}BrNO_2$ $(M+H)^+$ 243.9968, found 243.9970.

4.2.7. (4-Bromophenyl)(4-((*tert*-butyldimethylsilyl)oxy)phenyl) methanone (12)

To a solution of **10** (959 mg, 2.87 mmol) and LiCl (158 mg, 3.73 mmol) in THF (10 mL) at 0 °C was added isopropylmagnesium chloride (1.5 M in THF, 3.73 mmol). After 40 min, **11** (700 mg, 2.87 mmol) in THF (5 mL) was added and the reaction mixture was stirred at rt for 12 h and then quenched with aq NH_4Cl. The aqueous phase was extracted with EtOAc and the combined organic phase was dried over Na_2SO_4. After removal of the solvents, the residue was purified by flash column chromatography (0–30% EtOAc/hexanes) to afford **12** (1.0 g, 86%) as a light yellow oil. ^1H NMR ($CDCl_3$, 600 MHz) δ 7.74 (d, J = 9.0 Hz, 2H), 7.63

(d, J = 8.4 Hz, 2H), 7.60 (d, J = 7.8 Hz, 2H), 6.90 (d, J = 9.0 Hz, 2H), 1.00 (s, 9H), 0.25 (s, 6H). HRMS could not be obtained under varied conditions.

4.2.8. (*E*)-*tert*-Butyl 3-(4-(4-hydroxybenzoyl)phenyl)acrylate (13)

In a manner similar to that described for the preparation of compound **6**, bromide **12** (1.0 g, 2.47 mmol) underwent a Heck reaction to give *t*-butyl ester **13** as a yellow oil (700 mg, 87%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.79–7.72 (m, 4H), 7.62 (d, J = 16.2 Hz, 1H), 7.59 (d, J = 8.4 Hz, 2H), 6.94 (d, J = 8.4 Hz, 2H), 6.46 (d, J = 16.2 Hz, 1H), 1.55 (s, 9H). HRMS (ESI$^-$) calcd for $C_{20}H_{19}O_4$ $(M-H)^-$ 323.1289, found 323.1295.

4.2.9. (Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl)bis(4-methylbenzenesulfonate) (15)[28–30]

To a solution of **14** (4.5 g, 30.0 mmol) and TsCl (12.59 g, 66.0 mmol) in DCM (100 mL) was added NEt_3 (6.68 g, 66.0 mmol). The reaction mixture was stirred at rt for 12 h and washed with water. The organic phase was dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography (0–40% EtOAc/hexanes) to afford **15** as a white solid (12.0 g, 87%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.78 (d, J = 7.8 Hz, 4H), 7.34 (d, J = 7.8 Hz, 4H), 4.19–4.10 (m, 4H), 3.70–3.62 (m, 4H), 3.57–3.48 (m, 4H), 2.44 (s, 6H). HRMS (ESI$^+$) calcd for $C_{20}H_{26}NaO_8S_2$ $(M+Na)^+$ 481.0961, found 481.0963.

4.2.10. 2-(2-(2-Azidoethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (16)[28]

A mixture of **15** (10.0 g, 21.8 mmol) and NaN_3 (609 mg, 9.37 mmol) in DMF (50 mL) was allowed to stir at rt for 24 h. The reaction mixture was washed with water and the organic phase was dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by flash column chromatography (0–50% EtOAc/hexanes) to afford **16** as a light yellow oil (3.0 g, 39%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.78 (d, J = 7.8 Hz, 4H), 7.34 (d, J = 7.8 Hz, 4H), 4.19–4.10 (m, 4H), 3.70–3.62 (m, 4H), 3.57–3.48 (m, 4H), 2.44 (s, 6H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ 144.8, 132.9, 129.8, 127.9, 70.7, 70.5, 70.0, 69.3, 68.7, 50.6, 21.6. HRMS (ESI$^+$) calcd for $C_{13}H_{19}NaO_5S$ $(M+Na)^+$ 352.0938, found 352.0937.

4.2.11. (*E*)-*tert*-Butyl 3-(4-(4-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)benzoyl)phenyl)acrylate (17)

To a solution of **13** (324 mg, 1.0 mmol) and **16** (362 mg, 1.0 mmol) in DMF (20 mL) was added $CsCO_3$ (390 mg, 1.2 mmol) and the resulting mixture was stirred at 50 °C for 12 h. After diluted with EtOAc (100 mL), the organic phase was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvents, the residue was purified by flash column chromatography (0–60% EtOAc/hexanes) to afford **17** as a light yellow oil (530 mg, 90%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.82–7.72 (m, 4H), 7.65–7.59 (m, 2H), 7.36–7.33 (m, 1H), 7.02–6.98 (m, 2H), 6.48 (d, J = 14.4 Hz, 1H), 4.24–4.20 (m, 2H), 3.93–3.88 (m, 2H), 3.80–3.65 (m, 6H), 3.41–3.34 (m, 2H), 1.55 (s, 9H). HRMS (ESI$^+$) calcd for $C_{26}H_{32}N_3O_6$ $(M+H)^+$ 482.2286, found 482.2282.

4.2.12. (*E*)-*tert*-Butyl 3-(4-(4-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)benzoyl)phenyl)acrylate (18)

A mixture of **17** (530 mg, 1.0 mmol), PPh_3 (866 mg, 3.3 mmol), and H_2O (200 μL, 11.0 mmol) in THF (15 mL) was stirred at 50 °C for 4 h. The reaction mixture was diluted with EtOAc (100 mL), and the organic phase was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvents, the residue was purified by flash column chromatography (0–60% EtOAc/hexanes) to afford **18** as a light yellow oil (370 mg, 80%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.84–7.75 (m, 4H), 7.59 (d, J = 8.4 Hz, 2H), 7.40–7.36 (m, 1H), 7.05–6.97 (m, 2H), 6.46 (d, J = 15.6 Hz, 1H), 4.26–4.20 (m, 2H), 3.91–3.86 (m, 2H), 3.80–3.65 (m, 6H), 3.20–3.15 (m, 2H), 1.55 (s, 9H). HRMS (ESI$^+$) calcd for $C_{26}H_{34}NO_6$ $(M+H)^+$ 456.2381, found 456.2388.

4.2.13. (*E*)-*tert*-Butyl 3-(4-(4-(2-(2-(2-(Hex-5-ynamido)ethoxy)ethoxy)ethoxy)benzoyl)phenyl)acrylate (19)

A solution of **18** (370 mg, 0.8 mmol), 5-hexynoic acid (1.3 mmol) and EDC·HCl (1.3 mmol) in DCM (15 mL) was allowed to stir at rt overnight and the organic phase was then washed with water and brine. After removal of the solvent, the residue was purified by flash column chromatography (0–20% MeOH/CH_2Cl_2) to afford **19** as a colorless oil (330 mg, 75%). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.84–7.79 (m, 2H), 7.78–7.72 (m, 2H), 7.64–7.58 (m, 2H), 7.40–7.36 (m, 1H), 7.02–6.97 (m, 2H), 6.47 (d, J = 15.6 Hz, 1H), 6.04 (br s, 1H), 4.26–4.21 (m, 2H), 3.91 (t, J = 4.8 Hz, 2H), 3.74 (t, J = 4.2 Hz, 2H), 3.67 (t, J = 4.2 Hz, 2H), 3.58 (t, J = 4.8 Hz, 2H), 3.50–3.45 (m, 2H), 3.35 (s, 1H), 2.30 (t, J = 7.2 Hz, 2H), 2.24 (dt, J = 6.6, 2.4 Hz, 2H), 1.88–1.82 (m, 2H), 1.59 (s, 9H). HRMS (ESI$^+$) calcd for $C_{32}H_{40}NO_7$ $(M+H)^+$ 550.2799, found 550.2796.

4.2.14. (*E*)-3-(4-(4-(2-(2-(2-(Hex-5-ynamido)ethoxy)ethoxy)ethoxy)benzoyl)phenyl)acrylic acid (20)

A solution of *t*-butyl ester **19** (330 mg, 0.55 mmol) in TFA/DCM (2:1, 10 mL) was allowed to stir at rt for 1 h. After removal of the organic solvent, the residue was purified by flash column chromatography (0–12% MeOH/CH_2Cl_2) to afford *trans*-cinnamic acid **20** as a white solid (30 mg, 11%). ^{1}H NMR (DMSO-d_6, 600 MHz) δ 12.55 (s, 1H), 7.88–7.81 (m, 3H), 7.73 (d, J = 8.4 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 16.2 Hz, 1H), 7.09 (d, J = 9.0 Hz, 2H), 6.65 (d, J = 16.8 Hz, 1H), 4.19 (t, J = 4.5 Hz, 2H), 3.76 (t, J = 4.5 Hz, 2H), 3.61–3.56 (m, 2H), 3.54–3.50 (m, 2H), 3.39 (t, J = 6.0 Hz, 2H), 3.17 (q, J = 6.0 Hz, 2H), 2.74 (t, J = 2.7 Hz, 1H), 2.15–2.09 (m, 4H), 1.65–1.59 (m, 2H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ 177.8, 163.0, 159.6, 143.3, 139.3, 137.7, 137.4, 132.2, 129.8, 129.7, 127.6, 120.5, 114.0, 82.1, 70.4, 69.9, 69.2, 69.2, 68.8, 67.5, 38.9, 34.4, 24.5, 17.2. HRMS (ESI$^+$) calcd for $C_{28}H_{30}NO_7$ $(M-H)^-$ 492.2022, found 492.2027.

4.2.15. (*E*)-*N*-(2-(2-(2-(4-(4-(3-(Hydroxyamino)-3-oxoprop-1-en-1-yl)benzoyl)phenoxy)ethoxy)ethoxy)ethyl)hex-5-ynamide (3)

In a manner similar to that described for the preparation of compound **4**, carboxylic acid **20** (30 mg, 0.06 mmol) was converted into THP-protected hydroxylamine **21**, which was then deprotected with 2 M HCl in Et_2O (2 mL) to give hydroxamic acid **3** as a gray solid (14 mg, 46% for 2 steps). ^{1}H NMR (CD_3OD, 600 MHz) δ 7.80 (d, J = 9.0 Hz, 2H), 7.75 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 7.64 (d, J = 16.2 Hz, 1H), 7.08 (d, J = 8.4 Hz, 2H), 6.61 (d, J = 15.0 Hz, 1H), 4.27–4.22 (m, 2H), 3.91–3.87 (m, 2H), 3.74–3.70 (m, 2H), 3.66–3.62 (m, 2H), 3.55 (t, J = 5.4 Hz, 2H), 3.36 (t, J = 5.4 Hz, 2H), 2.29 (t, J = 7.5 Hz, 2H), 2.24–2.21 (m, 1H), 2.19 (dt, J = 7.2, 2.4 Hz, 2H), 1.81–1.75 (m, 2H). ^{13}C NMR (DMSO-d_6, 150 MHz) δ 171.9, 163.0, 162.7, 139.3, 137.7, 137.5, 137.4, 132.6, 130.4, 129.8, 127.9, 121.9, 114.8, 80.5, 71.9, 70.3, 70.0, 69.6, 69.2, 68.0, 38.9, 34.5, 24.7, 17.8. HRMS (ESI$^-$) calcd for $C_{28}H_{31}N_2O_7$ $(M-H)^-$ 507.2131, found 507.2132.

4.3. Biological assays

4.3.1. RT-qPCR assay and biochemical assays

RT-qPCR experiments were performed as described previously.[18] The biochemical assays against human HDAC1–11, MMP1–3, MMP7–10, MMP12–14, and TACE were performed by the Reaction Biology Corp. (RBC) (Malvern, PA, USA, http://www.reactionbiology.com) as described previously.[18] The biochemical

assay against HCV NS3/4A was performed using the HCV protease assay kit (P9001-01) from Protein One (Rockville, MD, USA) according to the manufacturer's instructions. HCV NS5B polymerase assay was carried out as described previously.[18]

4.3.2. HCV replicon and cellular toxicity assays

These assays were carried out as described previously.[18]

4.3.3. Thermodynamic solubility assay

The aqueous solubility of a test compound was determined in DPBS (pH = 7.4) under thermodynamic solubility conditions. Briefly, a saturated solution was made by adding DPBS to the solid compound. The mixture was shaken at 200 rpm for 72 h in a MaxQ 6000 orbital shaker at ambient temperature to allow equilibrium between the solid and dissolved compound. The suspension was then filtered through a 0.45 μm PVDF syringe filter and the filtrate was collected for analysis using UV spectrometry.

4.3.4. Plasma stability assay

The plasma stability assay was performed in triplicate by incubating a compound in normal mouse and human plasma at 37 °C. At 0, 1, 3, 6, and 24 h, aliquots of the plasma mixture were taken and quenched with 3 volumes of acetonitrile containing an appropriate internal standard and 0.1% formic acid. The samples were then vortexed and centrifuged at 14,000 rpm for 5 min. The supernatants were collected and analyzed by LC–MS/MS to determine the half-life time ($t_{1/2}$).

4.3.5. Microsomal stability assay

The in vitro microsomal stability assay was conducted in triplicate in mouse and human liver microsomal systems, which were supplemented with either nicotinamide adenine dinucleotide phosphate (NADPH) or uridine 5′-diphosphoglucuronic acid (UDPGA) as a cofactor for phase I or phase II metabolism, respectively. In a typical phase I microsomal incubation, a compound (typically 1 μM final concentration) was spiked into the reaction mixture containing 0.5 mg/mL of liver microsomal protein and 1 mM of NADPH in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C. In a typical phase II microsomal incubation, a compound (typically 1 μM final concentration) was spiked into the reaction mixture containing 0.5 mg/mL of liver microsomal protein, 50 μg/mg protein of alamethicin, 1 mM of $MgCl_2$ and 5 mM of UDPGA in 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C.

At various time points during either incubation, 1 volume of reaction aliquot was taken and quenched with 3 volumes of acetonitrile containing an appropriate internal standard and 0.1% formic acid. The samples were then vortexed and centrifuged at 14,000 rpm for 5 min. The supernatants were collected and analyzed by LC–MS/MS to determine the in vitro metabolic half-life ($t_{1/2}$). Verapamil and 7-hydroxycoumarin were used as a positive control for phase I and phase II metabolism, respectively.

4.3.6. LC–MS/MS bioanalysis

Quantification and analysis of compounds in biological samples were carried out on an AB Sciex QTrap 5500 mass spectrometer coupled with an Agilent 1260 Infinity HPLC. The chromatographic separation of compounds was achieved using a Phenomenex Kinetex C18 column (50 × 2.1 mm, 2.6 μm), and MS/MS analysis was conducted using an ESI ion source with MRM detection at negative mode. The MS/MS detection parameters including declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized for each compound.

Acknowledgments

This research was supported by the Center for Drug Design in the Academic Health Center of the University of Minnesota. We thank Riccardo Petrelli (University of Camerino, Italy) for providing the 2 mA.

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