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

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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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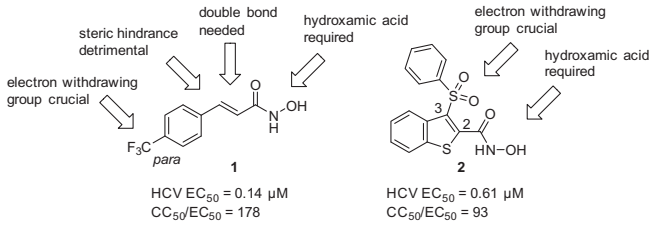

Figure 1. Lead hydroxamic acids and SAR trends.

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.

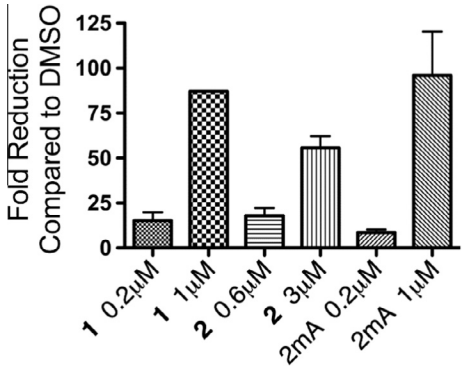

Figure 2. Fold reduction of HCV replicon RNA levels determined using RT-qPCR for cells treated with compounds **1**, **2** and 2 mA.

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				