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Benzohydroxamic acids as potent and selective anti-HCV agents

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ABSTRACT

A diverse collection of 40 derivatives of benzohydroxamic acid (BHAs) of various structural groups were synthesized and tested against hepatitis C virus (HCV) in full-genome replicon assay. Some of these compounds demonstrated an exceptional activity, suppressing viral replication at sub-micromolar concentrations. The compounds were inactive against key viral enzymes NS3, and NS5B in vitro assays, suggesting host cell inhibition target(s). The testing results were consistent with metal coordination by the BHAs hydroxamic group in complex with a target(s). Remarkably, this class of compounds did not suppress poliomyelitis virus (PV) propagation in RD cells indicating a specific antiviral activity of BHAs against HCV.

HCV is a major cause of chronic hepatitis, which affects three to four million people worldwide annually. Chronic hepatitis C is associated with a high risk for development of liver cirrhosis and hepatocellular carcinoma.[1] The generally accepted HCV therapy is treatment with a combination of interferon-alpha-2b and ribavirin. However, this treatment promotes a sustained viral response in only about 50% of the patients.[2] Therefore, new efficient antiviral therapies are in great demand.

HCV is a positive strand RNA virus whose genome consists of 9600 base pairs encoding 3 structural and 7 non-structural proteins.[3] Two key viral enzymes NS3 protease and NS5B polymerase remain the most popular targets for design of new anti-HCV drugs;[4] however, rapid resistance development to the agents targeting these enzymes justifies the search and characterization of new proteins involved in viral life cycle, that could be affected by small molecules.

In our previous work[5] we have proposed a binding mode for α,γ-diketo acid inhibitors (DKA, Fig. 1) in the active center of RNA-dependent RNA polymerase of HCV (NS5b). According to the model, DKA present an additional Mg^{2+} (or Mn^{2+}) ion into the active site of the enzyme, thereby interfering with nucleotidyl transfer catalysis.[5] Z-imide form[6] of salicylic hydroxamic acid (SHA, compound **1**) and *ortho*-anisic hydroxamic acid (*ortho*-OCH_3 BHA, compound **2**) seemed to mimic 'three-oxygen' metal-chelating system of DKA in enol form[7] consistently with high anti-HCV activity in full-genome HCV replicon assay (Fig. 1). However, these compounds failed to inhibit polymerase activity of NS5b as tested in an in vitro assay. Furthermore, benzohydroxamic acid (BHA, compound **3**), while structurally much less related to DKA, possessed significant antiviral activity as well (Fig. 1). It was established that compounds **1**–**3** were inactive against HCV protease/helicase (NS3) in vitro assays and did not interfere with stability of another viral protein (NS5a) that contains zinc finger domain.[8] These findings suggest an host cell inhibition target for BHA derivatives and grant further detailed investigation on their inhibitory mechanism. In the present research, we report the synthesis and structure–activity relationship (SAR) studies on HCV inhibition in a full-genome replicon assay for benzohydroxamic acid (BHA) derivatives. Some of the new compounds displayed exceptional antiviral activity and low toxicity for mammalian cells, which makes them promising candidates for drug development.

All tested hydroxamic acids were synthesized by four different methods depending on steric hindrance of carboxylic groups as well as on reactive ability of corresponding methylated hydroxylamines. The use of free acids and CDI as condensing agent[9,10] proved to be the most convenient route to BHAs with unhindered carbonyl group (method a). Compound **33** was obtained by treatment of corresponding methyl ester of 5-nitrosalicylic acid with hydroxylamine in aqueous DMSO under weakly basic conditions (method b). Methyl esters of benzoic acids derivatives with bulky groups in *ortho*-position readily reacted with hydroxylamine in methanol in the presence of double molar excess of potassium hydroxide yielding the desired hydroxamates (method c).[11] The chloroanhydrides of corresponding acids were useful for acylation of O- and N-methylated hydroxylamines as well as for preparation of extremely sterically hindered benzohydroxamate **37** (method d). All benzamidoxime derivatives were prepared from related

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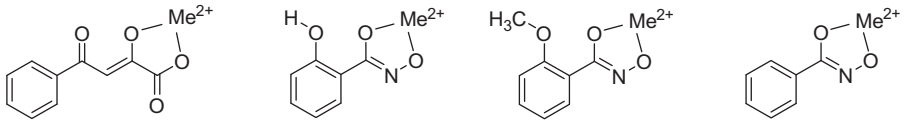
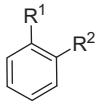
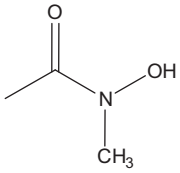
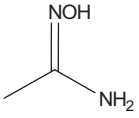
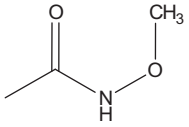
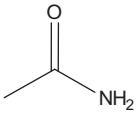


Figure 1. Molecular structures of DKA, SHA, *ortho*-OCH_3 BHA and BHA complexes with Me^{2+} ion (DKA is shown in enol form, SHA, *ortho*-OCH_3 BHA and BHA in Z-imide form) and values of anti HCV activity and cytotoxicity for Huh7 cells.

Table 1
Anti-HCV activity and cytotoxicity of methylated benzohydroxamates, benzamidoximes and benzamides



Compd	R_1	R_2	EC_{50}(μM)	CC_{50}(μM)	No.	R_1	R_2	EC_{50}(μM)	CC_{50}(μM)
4[d]		–H	65	400	**10**[e]		–H	270	>800
5[c]		–OH	>300	>800	**11**[e]		–OH	140	500
6[a]		$-OCH_3$	>300	>800	**12**[e]		$-OCH_3$	>300	>800
7[d]		–H	>300	>800					
8[d]		–OH	>300	>800	**13**		–OH	>300	>800
9[a]		$-OCH_3$	>300	>800	**14**		$-OCH_3$	>300	>800

[a,c,d,e]Method of preparation.

nitriles through treatment with aqueous EtOH solutions of hydroxylamine under neutral conditions (method e).[12] More than one stage syntheses of BHAs **5**, **8**, **30**, **32**, **38** and **40** were carried out as shown in Supplementary data.

To address the role of hydroxamic moiety in anti-HCV activity of BHAs compounds their modified analogs (compounds **4**–**14**) were prepared and tested in replicon assay (Table 1). All used compounds of this group representing derivatives of benzoic, salicylic and *ortho*-anisic acids can be divided into four groups based on the structure of carbonyl group radicals: *N*-hydroxy-*N*-methylamides **4-6**, *N*-methoxyamides **7**–**9**, amidoximes **10**–**12** as well as amides **13** and **14**. In comparison with dianionic bidentate ligand that hydroxamic residue represents, the chelating ability of the mentioned amide ligands is impaired because of reduction of their negative charge and (or) the number of metal-coordinating groups.

As seen from Table 1, only compounds **4**, **10** and **11** bearing monoanionic bidentate carbonyl residues showed detectable antiviral activity, whereas all remaining compounds were inactive. Remarkably, the presence of an extra coordination center (oxygen atom in *ortho*-position) in SHA derivatives **5**, **8** and **11** did not suppress the negative effect of hydroxamic group substitutions on antiviral activity. These results point to a very precise mode of metal coordination by the BHAs hydroxamic group in complex with a target(s).

Next SHA (compound **1**) and *ortho*-OCH_3 BHA (compound **2**) were tested in independent quantitative RT-PCR based assay to find how the luciferase activity in replicon assay correlated with the amount of viral RNA (Fig. 2). Compared to the EC_{50} values of SHA and *ortho*-OCH_3 BHA obtained by replicon assay (15 and 12 μM) the EC_{50} values as measured by qRT-PCR assay were somewhat lower (8 and 7 μM). This relatively small difference might be attributed to prolongation the life span of the reporter protein by hydroxamates or to enhanced degradation of the HCV RNA.

To test the specificity of the BHAs antiviral activity in regard to various viruses we analyzed the effect of the representative compounds *ortho*-OCH_3 BHA (active) and analog **5** (not active) on poliovirus (PV) infection of RD cells. The rational for this choice was that PV (as well as HCV) is single stranded RNA viruses with a genome of positive polarity. However, despite of similarity in genome organization and in replication cycle, these species differ drastically in many other biological aspects and virulence.[13]

The control treatment of non-infected RD cells with BHAs was essentially similar to that of Huh7 cells, except higher inhibitors concentration (twofold) and prolonged incubation time (additional 7 h) were used. Additional incubation time was equal to that of the PV reproduction cycle. No visible difference in the density of the cell monolayers compared to that of non-drug treated control cells was observed (Fig. 3A, C and E). There were also no overt morphological signs of cell death or changes in cell morphology as determined by fluorescence microscopy after staining of the nuclear DNA with Hoechst33342 (Fig. 3B, D and F). The above observations were consistent with CC_{50} values obtained by MTT assay for *ortho*-OCH_3 BHA (330μM) and compound **5** (>800 μM).

In the next set of experiments naive and drug-treated RD cells were infected with poliovirus (PV) at a multiplicity of infection (MOI) of ~20 or ~600 plaque forming units (PFU)/cell. Virus and drug induced alterations of the cell monolayer were examined right after infection or after 7 h, the time period of the PV reproduction cycle. As expected, PV infection resulted in destruction of RD cell monolayer and in nuclear alterations of productively of the infected cells, as determined by light microscopy of the cells and fluorescence microscopy of Hoechst stained nuclei (data not