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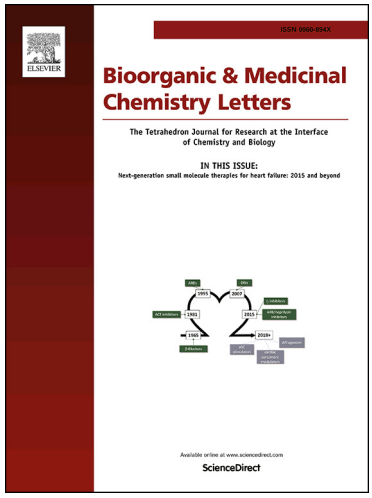

Synthesis of *N′*-propylhydrazide analogs of hydroxamic inhibitors of histone deacetylases (HDACs) and evaluation of their impact on activities of HDACs and replication of hepatitis C virus (HCV)

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Synthesis of *N′*-propylhydrazide analogs of hydroxamic inhibitors of histone deacetylases (HDACs) and evaluation of their impact on activities of HDACs and replication of hepatitis C virus (HCV).

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ABSTRACT

N′-Propylhydrazide analogs of hydroxamic inhibitors of histone deacetylases (HDACs), including tubastatin A, vorinostat and belinostat, were synthesized. All prepared compounds inhibited HDAC1/2/3, but not HDAC6, except for one hydrazide analog of HDAC4/5/7 inhibitor that was completely inactive. A novel 4-substituted derivative of *N′*-propylbenzohydrazide with extremely high anti-HCV activity was discovered.

Key words: HDAC; HCV; Inhibitor; Pharmacophore; Hydroxamic Acid; *N′*-Propylhydrazide

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Histone acetyltransferases (HATs) transfer the acetyl group of acetyl coenzyme A to lysine residues of protein substrates, while histone deacetylases (HDACs) catalyze the reverse process. Together, both groups of enzymes support the necessary level of acetylation of histones, transcription factors, and other nuclear and cytoplasmic proteins.

Zinc-dependent HDACs of class I, IIb and IV catalyze the hydrolysis of the acetamide bond in the ε-*N*-acetyl lysine residues of protein substrates, coordinating the oxygen atom of the carbonyl group by the zinc ion that is a part of the enzyme active site.[1] Histone deacetylases of these classes can be localized either only in nucleus (HDAC1/2/3 class I), or in the nucleus and cytoplasm (HDAC8 class I), or only in the cytoplasm (HDAC6/10 class IIb and HDAC11 class IV).[2] Note in particular that HDAC4/5/7/9 comprising class IIa do not have deacetylating activity but are capable of catalyzing detrifluoroacetylation of artificial substrates.[3]

The structure of the classic hydroxamic multipotent inhibitor of HDACs – vorinostat – contains a sequence of three main elements that form the pharmacophore: *cap*, *linker* and *zinc binding group* (*ZBG*) (Fig. 1). On the one hand, the *linker* is associated with *ZBG* and facilitates its penetration into the hydrophobic “tunnel” of the active site of HDACs, at the bottom of which chelation of the catalytic zinc ion takes place. On the other hand, the *linker* is associated with the

cap structure, most often aryl or heteroaryl radical which additionally interacts with the protein substrate binding cavity, affecting the strength and selectivity of inhibition.[4]

However, in the non-hydroxamic inhibitors of HDACs class I, one more element of the pharmacophore can be distinguished – the *leader* that is preceding *ZBG* and is the first to enter the hydrophobic "tunnel". Penetrating further into the acetate release channel, the *leader* ensures the necessary positioning of the *ZBG* relative to the catalytic zinc ion (Fig. 1). Choosing the right combination of *linker-ZBG-leader* makes it possible to control the selectivity of inhibition of isoform HDACs class I even in the case of minimum sizes of *cap*, as demonstrated for UFO10 and Cmpd4 or Cmpd60 (Fig. 1).

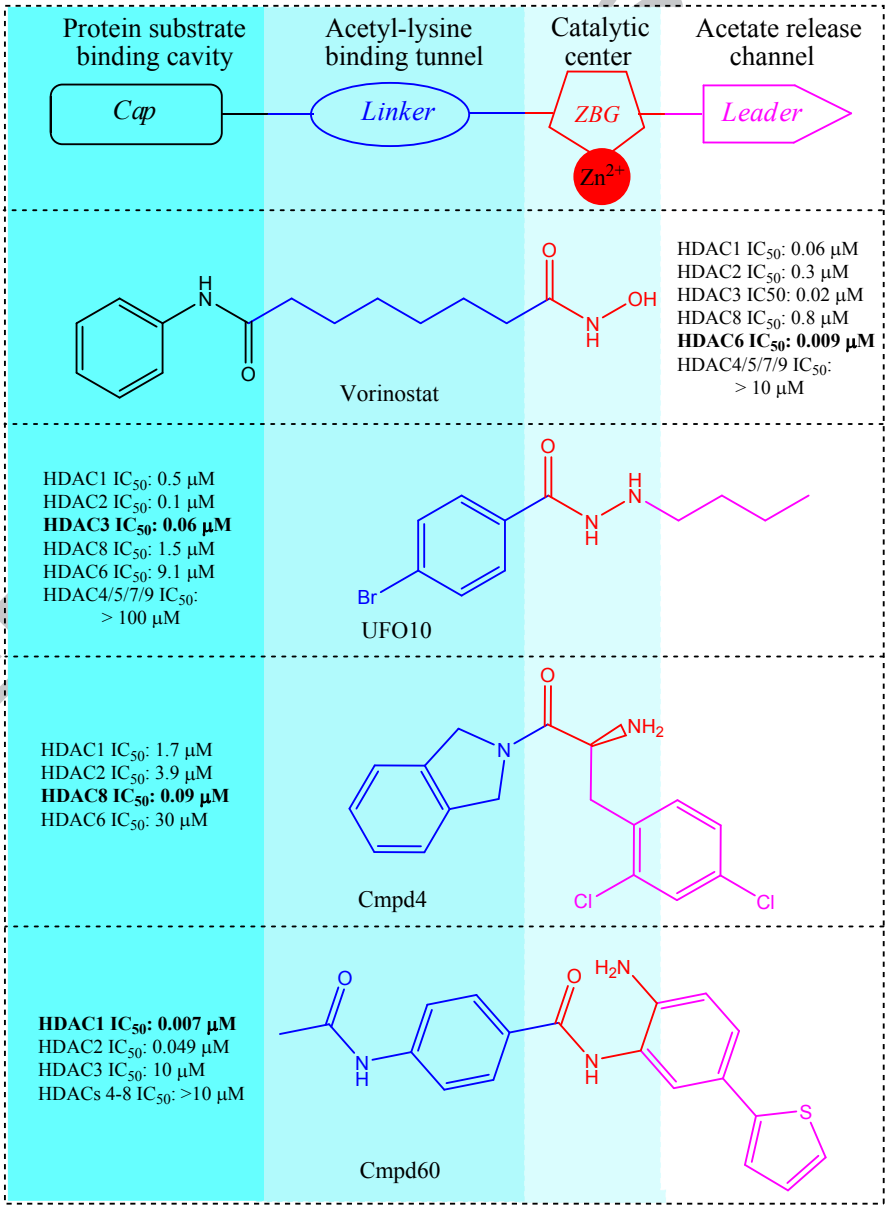


Fig. 1. Pharmacophore 2D model for HDAC inhibitors carrying different *zinc binding groups* (*ZBG*): Vorinostat (hydroxamate),[5] UFO10 (*N'*-butylhydrazide),[5] Cmpd4 (chiral α-amino-ketone)[6] and Cmpd60 (α-aminobenzamide).[7,8]

Hydroxamic inhibitors of histone deacetylases block the replication of the hepatitis C virus (HCV), however, whether it happens by HDACs-dependent mechanism or not is still not quite clear.[9-13] We conducted a comparative study of hydroxamic inhibitors of HDACs and their hydrazide analogues (Fig. 2) in order to find out how the introduction of a *ZBG-leader* fragment in the form of the N'-propylhydrazide residue will affect (i) the selectivity of the inhibition of HDACs, (ii) blocking of HCV replication, and (iii) how these processes are associated with each other.

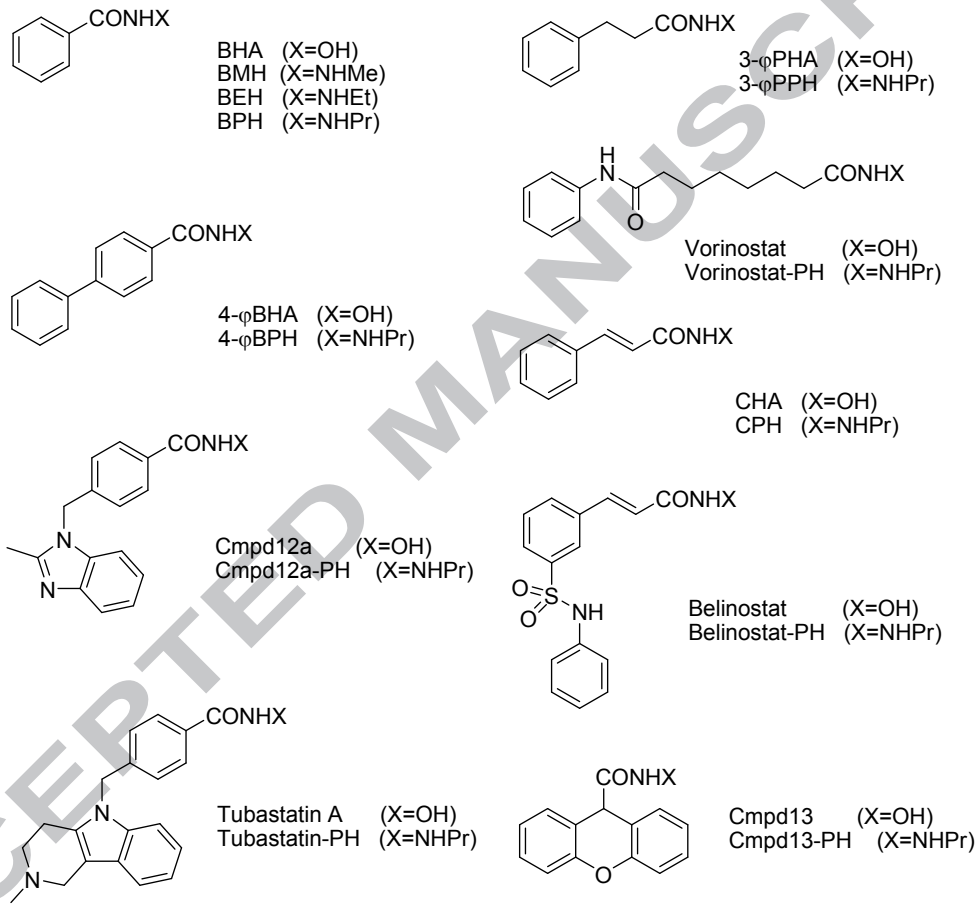
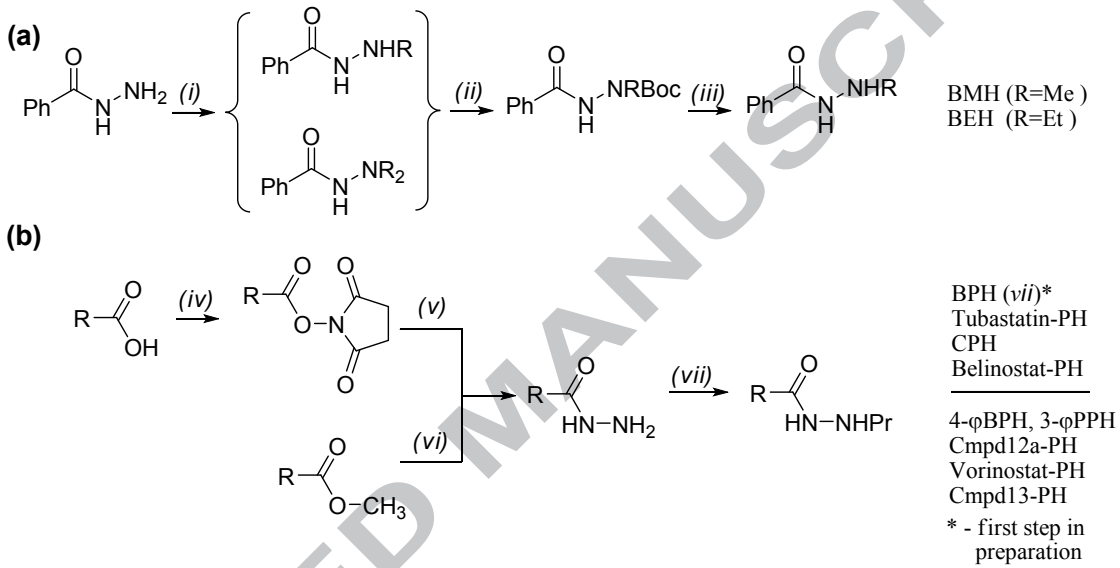


Fig. 2. Structures of tested hydroxamic acids (HA) and *N′*-propylhydrazide analogs (PH).

The synthesis of compounds tested in this study is presented in Scheme 1. All *N′*-propylhydrazide analogs were obtained by reductive alkylation method, while *N′*-methyl- and *N′*-ethylbenzohydrazide (BMH and BEH) were synthesized by alkylation of benzohydrazide with the appropriate dialkyl sulfate with the subsequent product isolation through the carbamate derivative. Cinnamic acid hydrazides were prepared by hydrazinolysis of their NHS esters at room temperature to prevent the formation of pyrazolidin-3-one cycle.[14] Hydroxamates – 4-phenylbenzohydroxamic acid (4-φBHA), 3-phenylpropanhydroxamic acid (3-φPHA) and

Cmpd13 – were synthesized by activation of the corresponding carboxylic acid with carbonyldiimidazole and subsequent hydroxyaminolysis, as described earlier.[15]

The antiviral effect of the compounds was determined in a test system of a full-length replicon in cells of the Huh7 luc/neo line, whereas the efficiency of inhibition of HDAC1/2/3 (nuclear localization) and HDAC6 (cytoplasmic localization) was assessed using the Western blot analysis for the content of the acetylated forms of the corresponding substrate proteins – Ac-K9/K14 of histone H3 and Ac-K40 of α-tubulin in cell lysates.[16]



Scheme 1. Reagents and conditions: **(a)** (*i*) $(MeO)_2SO_4$ or $(EtO)_2SO_4$, 18h; (*ii*) Boc_2O, 1,2-DCE, 18h; (*iii*) TFA, 0.5h. **(b)** (*iv*) NHS, DCC, DMSO/CH_2Cl_2, 1h [Tubastatin-PH] or NHS, DCC, MeCN/THF, 1.5h [CPH and Belinostat-PH]; (*v*) $NH_2NH_2•H_2O$, DMSO/CH_2Cl_2/MeOH, 1h [Tubastatin-PH] or $NH_2NH_2•H_2O$, MeOH, 0.5h [CPH and Belinostat-PH]; (*vi*) $NH_2NH_2•H_2O$, EtOH, 3h [3-φPPH and Vorinostat-PH] or $NH_2NH_2•H_2O$, MeOH/EtOH, 70 °C, 5h [4-φBPH, Cmpd12a-PH and Cmpd13-PH]; (*vii*) CH_3CH_2CHO, MeOH, 4h, vacuum evaporation, $NaBH_4$, i-PrOH, 85 °C, 1.5h.

Properties of benzoic acid derivatives as anti-HCV agents are summarized in Table 1, whereas the results of the Western blot analysis are presented in Fig. 3. In agreement with previous data, BHA demonstrated selective inhibition of HDAC6 and moderate anti-HCV activity.[15,16] The lower analogues, BMH and BEH, were inactive in both tests. In contrast, BPH effectively inhibited HDAC1/2/3, but not HDAC6, and had antiviral activity even stronger than that of BHA.

Thus, among *N*′-alkyl-substituted benzohydrazide (alkyl = Me, Et and Pr), the inhibition of HDAC1/2/3 and the suppression of HCV replication were tightly associated with each other. It should be noted that BMH, BEH, and BPH are the closest homologues, and it is difficult to expect the existence of any additional antiviral target with the same profile of sensitivity to the

elongation of the alkyl radical. As previously shown, selective inhibition of HDAC3 with *N*-(*ortho*-aminophenyl)carboxamide derivative, RGFP966, led to the suppression of HCV replication.[17] The authors attributed the antiviral effect of RGFP966 to changes in the expression of *LEAP-1* and *Apo-A1* genes as a result of the hyperacetylation of histone H3 and transcription factors HIF1a and STAT3. It cannot be excluded that BPH blocks HCV replication in a similar way.

The benzohydroxamates that we tested – 4-φBHA, Cmpd12a and tubastatin A – have an aromatic or heterocyclic *cap* at the 4-position of *linker*'s benzene ring. In accordance with data from literature, Cmpd12a and tubastatin A demonstrated highly selective inhibition of HDAC6, which was ensured by their heterocyclic *caps*.[18,19] However, the hydrazide analogues – 4-φBPH, Cmpd12a-PH and tubastatin-PH – did not inhibit HDAC6, but only HDAC1/2/3. Thus, the *N*′-propylhydrazide *ZBG-leader* fragment completely changed the selectivity of inhibition, and the *caps* that were responsible for the selective inhibition of HDAC6 by hydroxamic precursors did not prevent this.

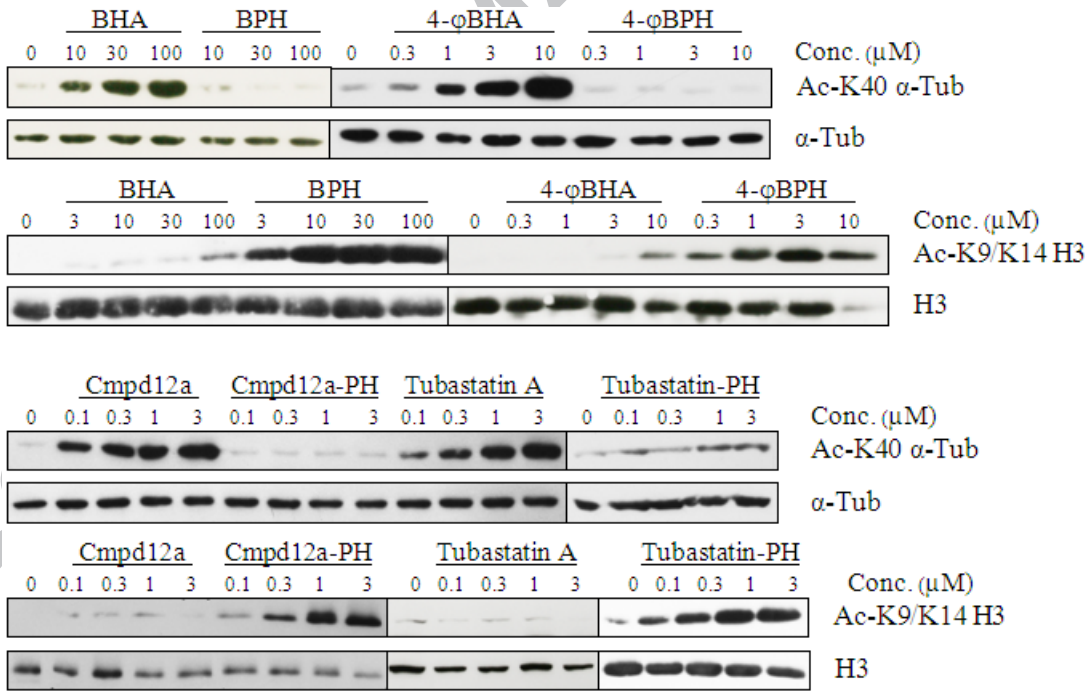


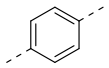
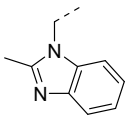
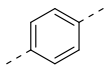
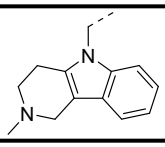
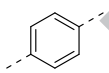
Fig. 3. Accumulation of acetylated α-tubulin and histone H3 in Huh7-luc/neo cells treated with benzohydroxamic acids and their *N*′-propylbenzohydrazide analogs for 24 h.

The approximately equal antiviral potency of benzohydroxamates – 4-φBHA, Cmpd12a and tubastatin A – changed in opposite directions upon the transition to benzohydrazide analogues – 4-φBHP, Cmpd12a-PH or tubastatin-PH. Accordingly, the value of EC_{50} either increased 3-fold or fell 11-fold, or remained almost unchanged. Interestingly, Cmpd12a-PH

showed an excellent antiviral activity with an EC_{50} value of 0.025 μM, but only slightly inhibited HDAC1/2/3 at a concentration of 0.1 μM. It is very likely that the suppression of HCV replication by this compound occurred via the HDAC-independent mechanism.

Table 1

SAR studies of benzohydroxamic acids and their *N*′-propylbenzohydrazide analogs

Compound	*Cap*	*Linker*	*ZBG*	*Leader*	*Inhibition of HDACs*	*EC_{50} (μM)*	*CC_{50} (μM)*	*TI*
BHA	none		HA	none	6>>1/2/3	15[a]	210[a]	14[a]
BMH			MH	Me	NA	NA	>1000	–
BEH			EH	Et	NA	NA	>1000	–
BPH			PH	Pr	1/2/3	5.5	120	22
4-φBHA			HA	none	6>>1/2/3	0.40	30	75
4-φBPH			PH	Pr	1/2/3	1.5	5.9	3.9
Cmpd12a			HA	none	6	0.27	7.9	29
Cmpd12a-PH			PH	Pr	1/2/3	0.025	9.9	400
Tubastatin A			HA	none	6	0.30[b]	11[b]	37[b]
Tubastatin-PH			PH	Pr	1/2/3>>6	0.24	5.3	22

[a] See Ref. 15

[b] See Ref. 10

In the literature, the hydrazide-based class I selective HDAC inhibitors are described, with *linker* structure in the form of a -$(CH_2)_n$- fragment of ε-amino carboxylic acids (n=6-8) or in the form of a residue of para-substituted cinnamic acid.[20] The potent inhibitors of HDACs – vorinostat and belinostat – possess almost identical *linker* structures (Fig. 1 and 2). According to the literature, both of these compounds suppressed HCV replication in the subgenomic replicon system, however, with low values of therapeutic index.[11] We thought it interesting to compare vorinostat and belinostat with their *N*′-propylhydrazide analogs, both as inhibitors of HDACs and as anti-HCV agents. In this series of experiments, hydroxamic and *N*′-propylhydrazide derivatives of 3-phenylpropanoic acid (3-φPHA and 3-φPPH) and cinnamic acid (CHA and CPH) played the role of reference compounds that do not possess a *cap* structure.

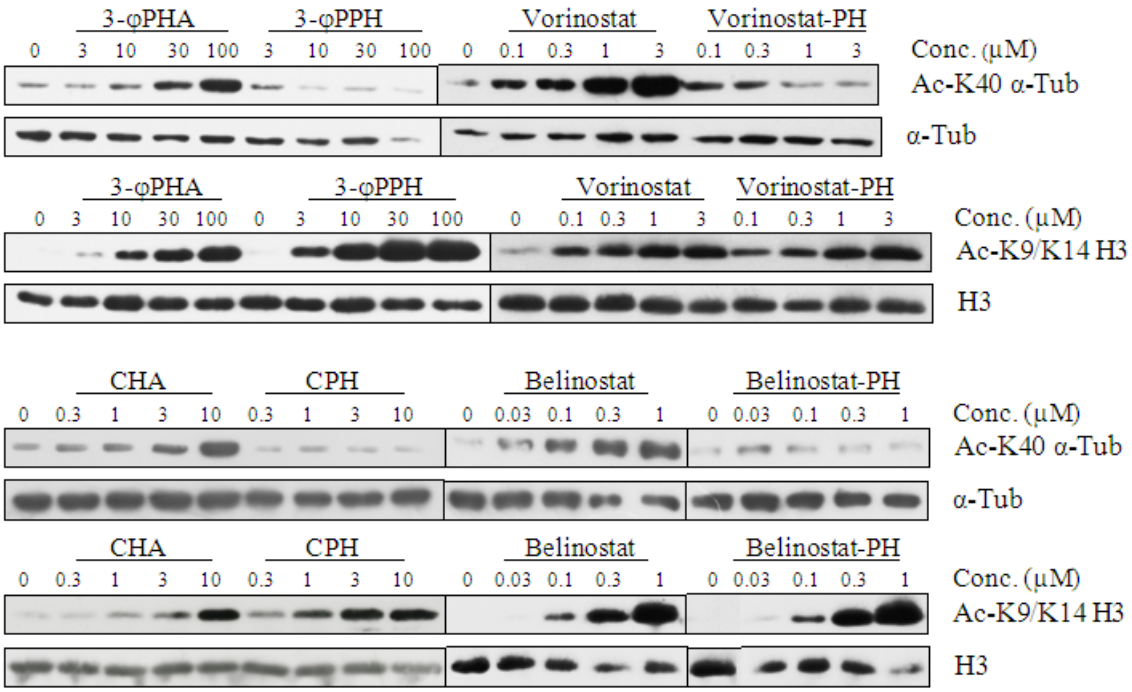


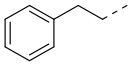
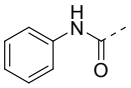
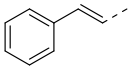
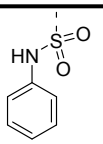
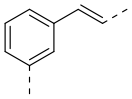
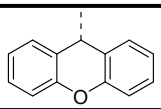
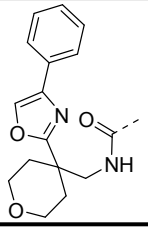
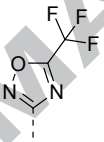
Fig. 4. Accumulation of acetylated α-tubulin and histone H3 in Huh7-luc/neo cells treated with aliphatic and cinnamic hydroxamic acids and their *N′*-propylbenzohydrazide analogs for 24 h.

As can be seen from the results presented in Fig. 4, all four of the tested hydroxamic inhibitors of HDACs had a multipotent effect, while all four of their hydrazide analogs inhibited only HDAC1/2/3. Thus, "fixation" of the new selectivity with the help of the *N′*-propylhydrazide *ZBG-leader* fragment occurred both in the absence and in the presence of *cap* structures, in full agreement with the results obtained for benzohydrazide inhibitors (Fig. 3).

Antiviral properties of hydroxamates and hydrazides of aliphatic and cinnamic acids are summarized in Table 2. Note that the obtained EC_{50} and CC_{50} values for vorinostat and CHA were slightly different from those previously published, however, in the case of belinostat, the discrepancies were minimal.[11] The loss of inhibitory effect on HDAC6 by the hydrazides 3-φPPH и vorinostat-PH was accompanied by a change in antiviral activity in opposite directions – for instance, the EC_{50} value decreased by a factor of 1.4 or increased by a factor of 2.3 when compared with the corresponding values for 3-φPHA and vorinostat. Suddenly, vorinostat-PH was 11 times less cytotoxic than its hydroxamic precursor. Interestingly, cinnamic acid hydrazides – CPH and belinostat-PH – lost antiviral activity compared to CHA and belinostat, but retained the strength of the cytotoxic effect. In the case of belinostat-PH, this meant a complete loss of selective suppression of HCV replication.

Table 2

SAR studies of aliphatic, cinnamic and hydroxamic acids and their N'-propylbenzohydrazide analogs and TMP269

Compound	*Cap*	*Linker*	*ZBG*	*Leader*	*Kind of inhibited HDACs*	*EC_{50} (μM)*	*CC_{50} (μM)*	*TI*
3-φPHA	none		HA	none	1/2/3/6	5.2	110	21
3-φPPH			PH	Pr	1/2/3	3.8	96	25
Vorinostat		-$(CH_2)_6$-	HA	none	1/2/3/6	0.56 *0.36*[a]	0.64 *1.9*[a]	1.1 *5.3*[a]
Vorinostat-PH			PH	Pr	1/2/3	1.3	7.0	5.4
CHA	none		HA	none	1/2/3/6	2.0 *0.86*[a]	20 *39*[a]	10 *45*[a]
CPH			PH	Pr	1/2/3	5.4	18	3.3
Belinostat			HA	none	1/2/3/6	0.19 *0.12*[a]	0.65 *0.68*[a]	3.4 *5.7*[a]
Belinostat-PH			PH	Pr	1/2/3	0.55	0.68	1.2
Cmpd13			HA	none	3/5/7[b]	0.75	300	400
Cmpd13-PH			PH	Pr	none	NA	>300	–
TMP269				none	3/5/7/9[c]	4.5	83	18

[a] See Ref. 11
[b] See Ref. 22
[c] See Ref. 23

As previously shown, HCV infection enhanced the expression of HDAC9, which in turn led to an increase in the expression of gluconeogenic genes via deacetylation of the transcription factor FoxO1 together with HDAC3.[21] The properties of two selective inhibitors of HDACs class IIa, TMP269 and Cmpd13, as anti-HCV agents are listed in Table 2. Although both compounds according to the literature had a similar profile of inhibition of HDACs,[22,23] Cmpd13 was many times more efficient than TMP269 both in antiviral activity and in selectivity of action. So, we demonstrated for the first time effective suppression of HCV replication with selective HDAC class IIa inhibitors and their potential usefulness for treating HCV-induced abnormal glucose homeostasis.[21]

The hydrazide analog Cmpd13-PH was inactive in both luciferase and MTT tests and did not inhibit either HDAC1/2/3 or HDAC6 (Table 2). To explain the resistance of HDAC1/2/3 to the *N'*-propylhydrazide *ZBG-leader* fragment of Cmpd13-PH, it should be recalled that the catalytic pocket of HDACs class IIa is capable of accommodating more bulky molecules than other HDACs.[3] It appears that the 9*H*-xanthene group of Cmpd13-PH simply cannot be correctly

oriented inside the active site of HDAC1/2/3. In addition, for some unknown reason, Cmpd13-PH probably does not inhibit HDAC4/5/7/9, as indicated by the complete absence of antiviral activity of this compound.

In conclusion, a comparative study of the hydroxamic inhibitors of HDACs and their hydrazide analogues demonstrated that the *N′*-propylhydrazide *ZBG-leader* fragment ensured a selective effect on HDAC1/2/3 regardless of the structure of the *cap-linker* fragment of its precursor, a hydroxamic inhibitor. An important exception to this rule was the complete inactivity of the hydrazide analog of HDACs class IIa hydroxamic inhibitor carrying a bulky *cap-linker* group. No consistent patterns in antiviral activity changes during the transition from hydroxamic inhibitors of HDACs of class I and IIb to their hydrazide analogs were found, however, among *N′*-alkyl-substituted benzohydrazides, the suppression of HCV replication was associated with the inhibition of HDAC1/2/3. Our results clearly demonstrated that different HDAC inhibitors targeting HDACs class I, IIa and IIb effectively suppress HCV replication. Therefore, the use of broad-spectrum inhibitors of HDACs for the treatment of HCV infection deserves closer consideration.

Acknowledgments

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