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N-Acylhydrazone Derivatives as Potent Histone Deacetylase 6 Inhibitors

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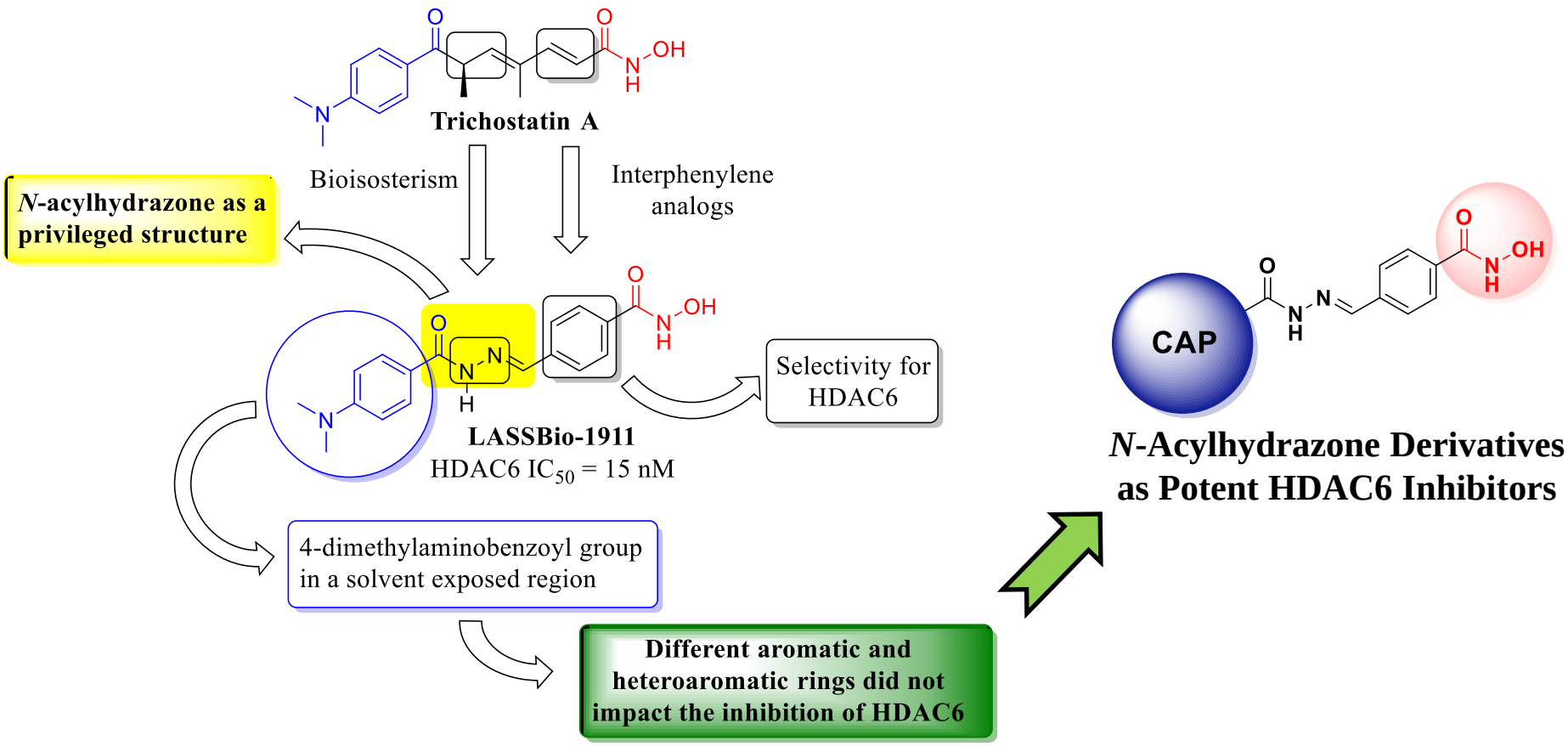
N-Acylhydrazone Derivatives as Potent Histone Deacetylase 6 Inhibitors

Graphical Abstract

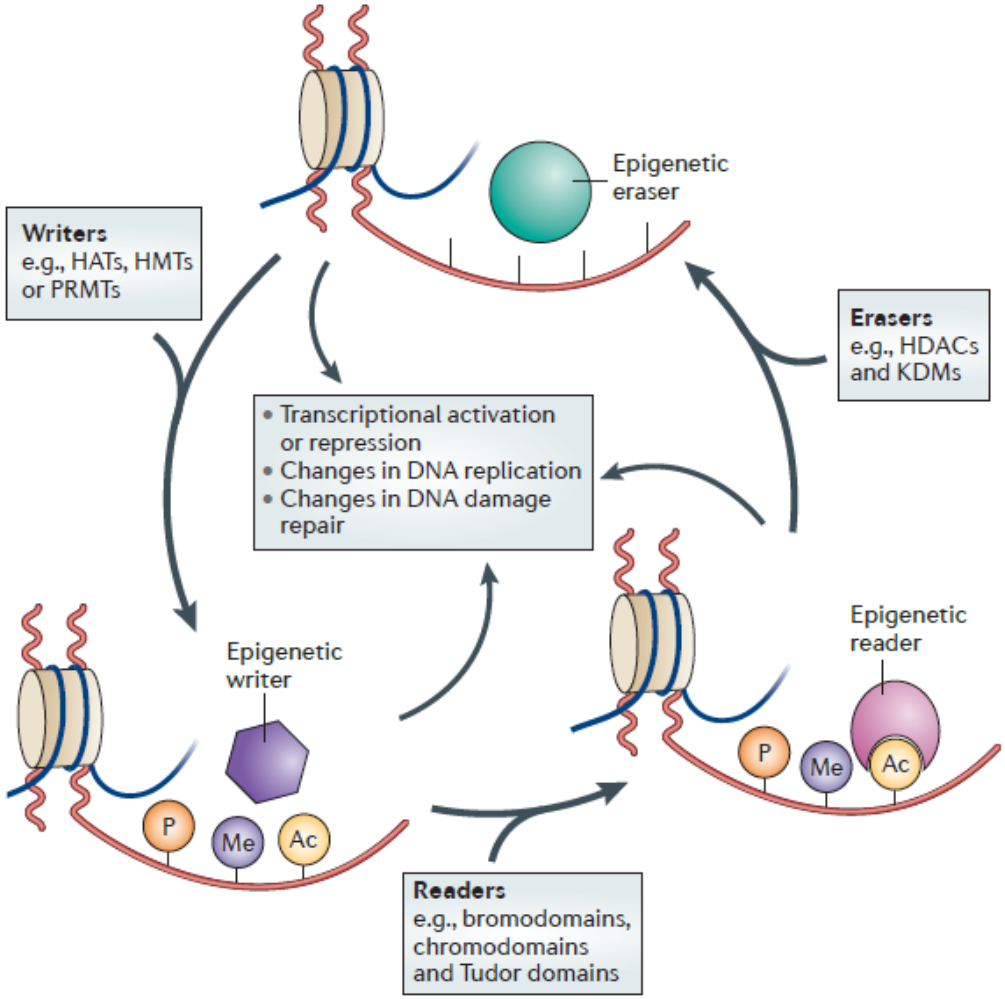


Abstract:

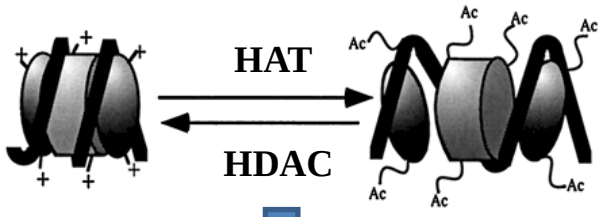
Histone deacetylase 6 (HDAC6) catalyses the removal of acetyl groups from the lysine residues of a series of non-histone proteins, e.g., α-tubulin, Hsp90 and cortactin. The design of selective inhibitors of HDAC6 is related with important outcomes in the oncological, immunological and neurological fields. Herein, we describe the design, synthesis and pharmacological evaluation of a series of *N*-acylhydrazones (NAH) designed from the trichostatin A as HDAC6 inhibitors. The use of the phenyl linker in the design of the compounds led to HDAC6 selectivity among the HDAC family. *Para*-substituted phenyl-hydroxamic acids presented a more potent inhibition of HDAC6 than their *meta*-substituted analogs. The *N*- and *C*- methylation of the NAH framework attached to *para*-substituted phenyl-hydroxamic unit was evaluated and the compound LASSBio-1911 was identified as a potent and selective HDAC6 inhibitor (IC_{50} = 15 nM). In the next step, we evaluated the influence of the cap group. We found that the use of different aromatic and heteroaromatic rings did not influence the inhibition of HDAC6. Some of these compounds were able to reduce significantly cell migration, corroborating their inhibitory profile against HDAC6. On the other hand, an analysis of their antiproliferative activity against different tumor cell lines showed that they can induce cell cycle arrest or induce apoptosis through caspase 3/7 activation, with particular relevance for hepatocellular carcinoma (HepG2) cells.

Keywords: HDAC, *N*-acylhydrazone, Cancer, HDAC6, HDAC6 inhibitor

Introduction – HDAC and the Epigenetic Landscape



HDAC
Zn^{2+}
Class I: HDAC1, 2, 3 e 8
Class IIa: HDAC4, 5, 7 e 9
Class IIb: HDAC6 e 10
Class IV: HDAC11
NAD^{+}
Class III: Sir1-7

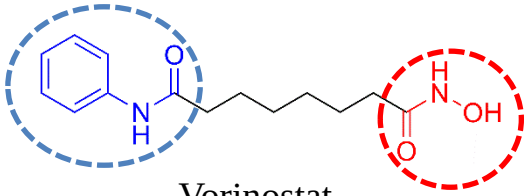


HDAC inhibition results in the growth arrest, differentiation and apoptosis of many transformed cells.

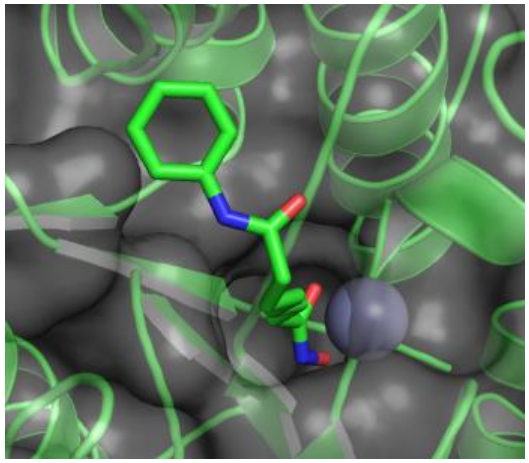
The epigenetic landscape: Epigenetic writers, readers and erasers, Copyright © 2014, Springer Nature.
Falkenberg, K. J.; Johnstone, R. W. *Nat. Rev. Drug Discov*, **2014**, 13, 673 – 691; Kouzarides, T. *Cell*, **2007**, 128, 693 – 705.; Witt, O. et al. *Cancer Letters*, **2009**, 277, 8 – 21.

Introduction - Chemical structures of FDA-approved HDAC inhibitors

Cap Linker ZBG



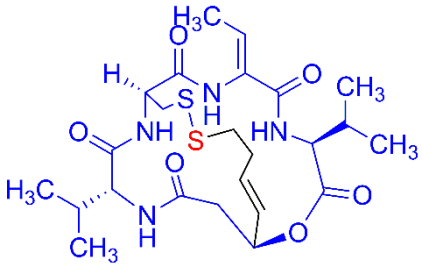
Vorinostat
(**Zolinza®**, Merck)
Cutaneous T-Cell Lymphomas (CTCL) (2006)



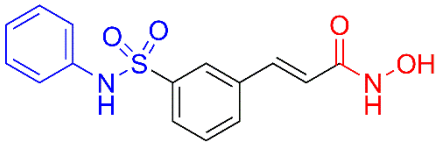
PDB: 4LXZ (Vorinostat in HDAC2)

HDACIs possess a well-known pharmacophore

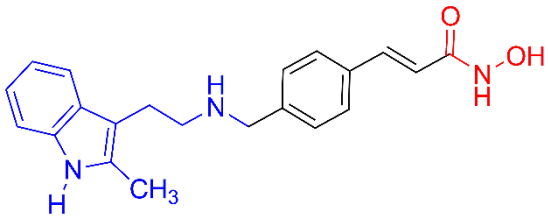
Notably, no selective HDACIs have been approved for clinical use



Romidepsin
(**Istodax®**, Celgene)
Cutaneous T-Cell Lymphomas (CTCL) (2009) and Peripheral T-Cell Lymphomas (PTCL) (2011)



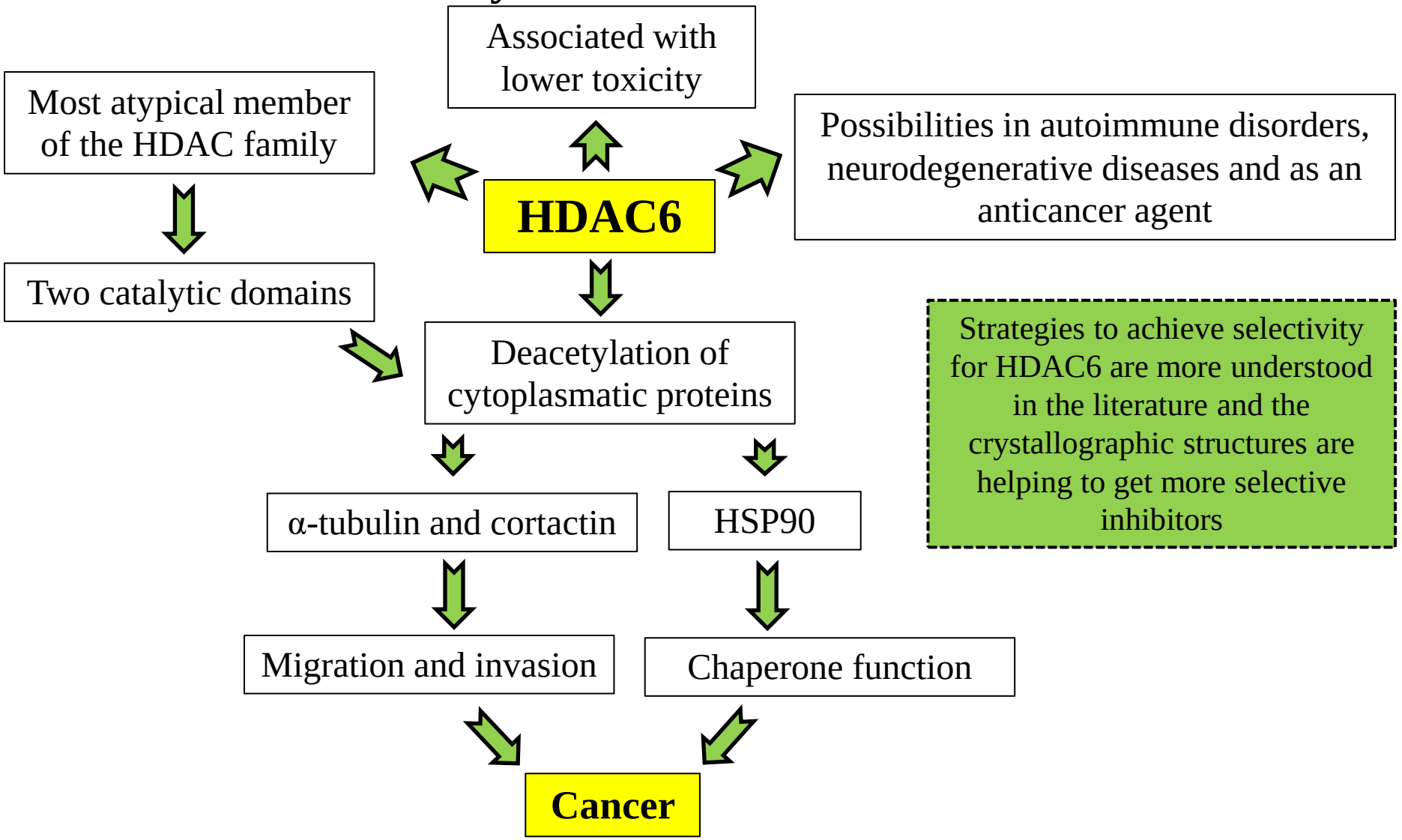
Belinostat
(**Beleodaq®**, Spectrum Pharmaceuticals)
Peripheral T-Cell Lymphomas (PTCL) (2014)



Panobinostat
(**Farydak®**, Novartis)
Multiple Myeloma (2015)

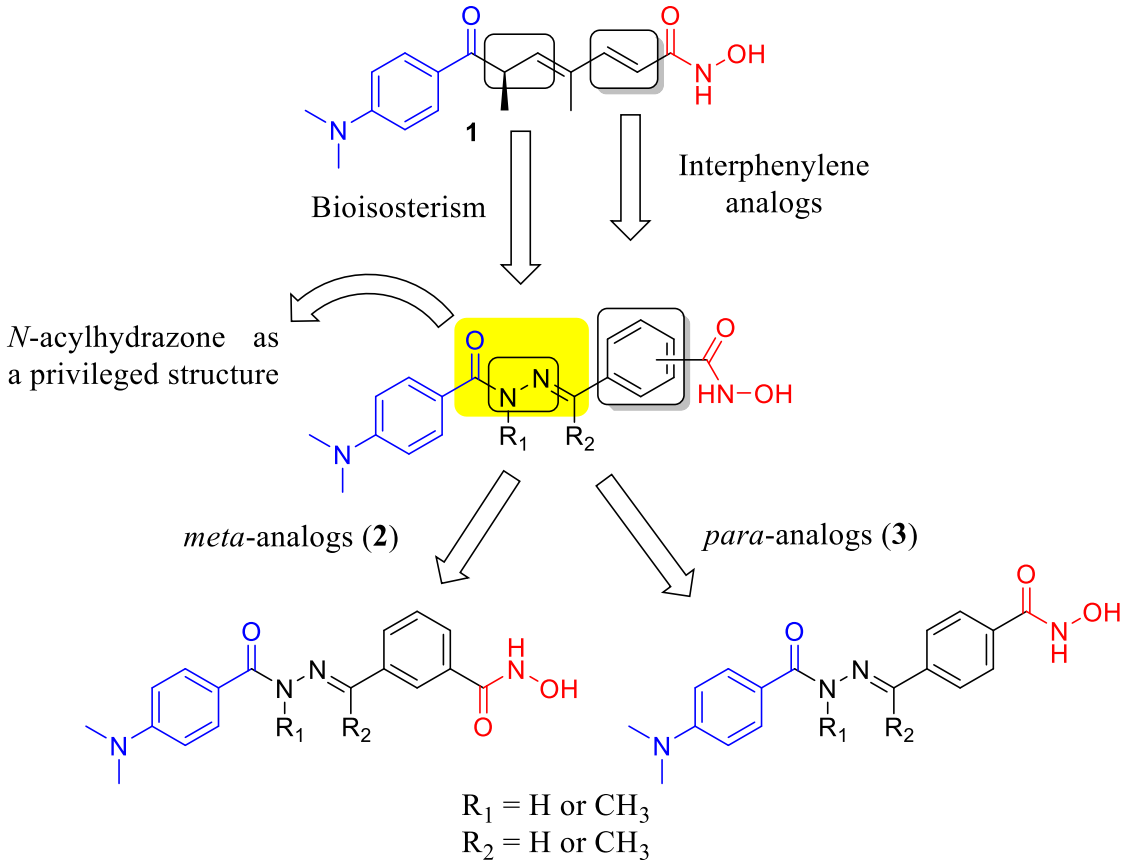
Falkenberg, K. J.; Johnstone, R. W. *Nat. Rev. Drug Discov*, **2014**, 13, 673 – 691. Rodrigues, D. A.; Thota, S.; Fraga, C. A. M. *Mini Rev Med Chem.*, **2016**, 16, 1175 – 1184.

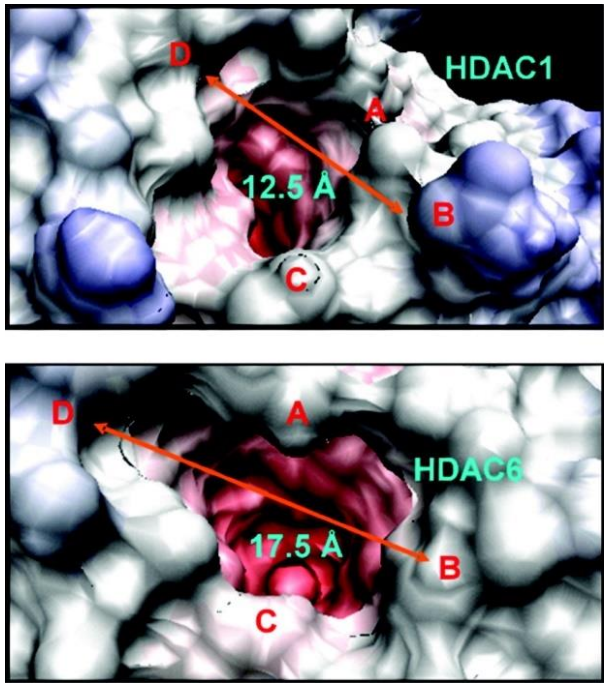
Introduction – Selectivity for HDAC6



Rodrigues, D. A.; Thota, S.; Fraga, C. A. M. *Mini Rev. Med. Chem.*, **2016**, 16, 1175 – 1184.

Results and Discussions - Design concept of a class of NAH derivatives for HDAC6 inhibition.





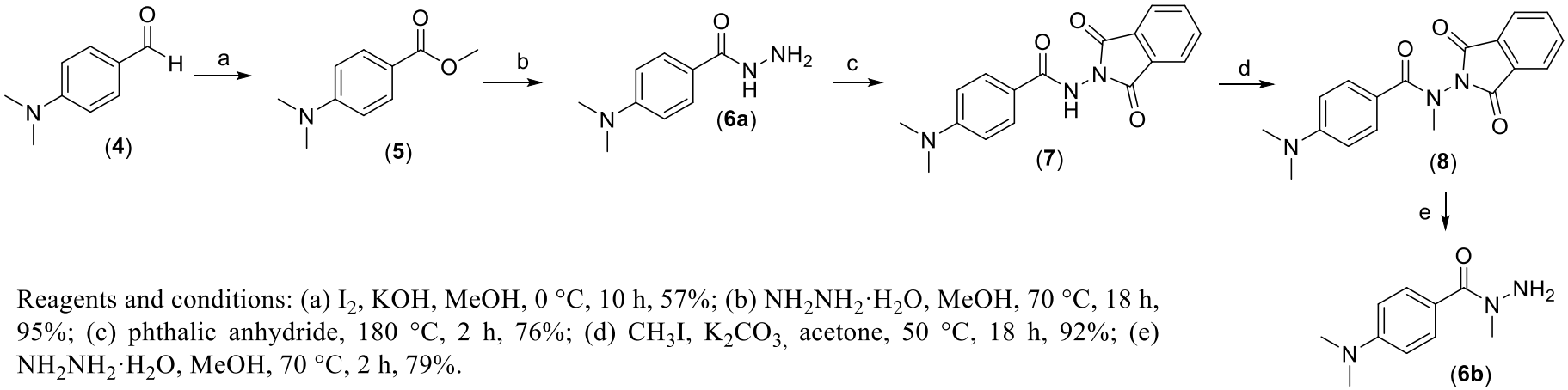
Differences in the dimensions of the catalytic site of HDAC1 e HDAC6. Reprinted with permission from BUTLER et al. *J. Am. Chem. Soc.* **2010**, 132, 10842-10846. Copyright © American Chemical Society.

Use of the phenyl linker confers selectivity for HDAC6.

Rodrigues, D. A.et al. *Med. Chem.*, **2016**, 59, 655-706.; Duarte, C. D.; Barreiro, E. J.; Fraga, C. A. M. *Mini Rev. Med. Chem.*, **2007**, 7, 1108 – 1119.; Thota, S. et al. *Bioorg. Med. Chem. Lett.*, **2018**, 28, 2797 – 2806.; Butler, K. V. et al. *J. Am. Chem. Soc.* **2010**, 132, 10842 – 10846.; Rodrigues, D. A.; Thota, S.; Fraga, C. A. M. *Mini Rev. Med. Chem.*, **2016**, 16, 1175 – 1184.

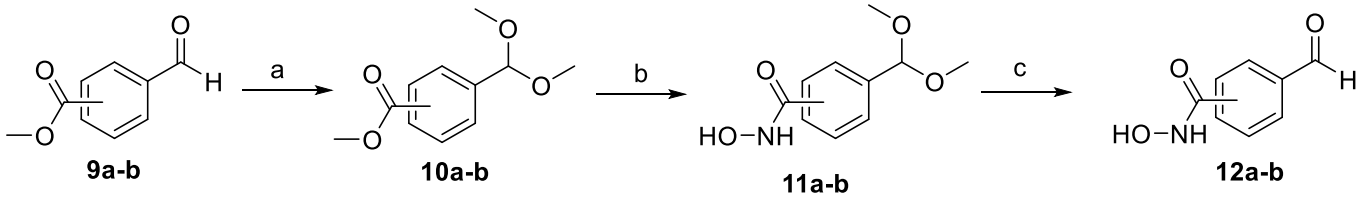
Results and Discussions – Synthesis of the key intermediates

1) Synthesis of hydrazides: 4-(dimethylamino)benzohydrazide (**6a**) and 4-(dimethylamino)-*N*-methylbenzohydrazide (**6b**).



Reagents and conditions: (a) I_2, KOH, MeOH, 0 °C, 10 h, 57%; (b) $NH_2NH_2 \cdot H_2O$, MeOH, 70 °C, 18 h, 95%; (c) phthalic anhydride, 180 °C, 2 h, 76%; (d) CH_3I, K_2CO_3, acetone, 50 °C, 18 h, 92%; (e) $NH_2NH_2 \cdot H_2O$, MeOH, 70 °C, 2 h, 79%.

2) Synthesis of aldehydes: 3-formyl-*N*-hydroxybenzamide (**12a**) and 4-formyl-*N*-hydroxybenzamide (**12b**).



Reagents and conditions: (a) 2,2-dimethoxypropane, $TsOH_{cat.}$, MeOH, r.t., 2 h, 82-85%; (b) $NH_2OH \cdot HCl$, KOH, MeOH, r.t., 4 h, 90-94%; (c) H_2SO_4 15% (w/v), acetone, r.t., 2 h, 87-91%.

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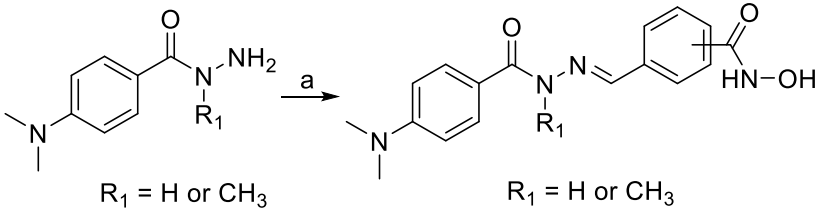
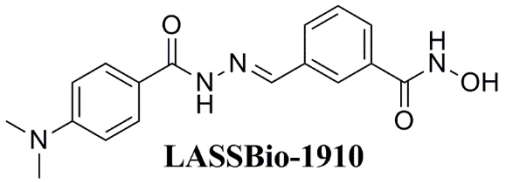
Results and Discussions – Synthesis and evaluation of *N*AH

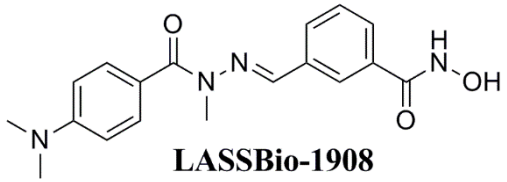
1) Synthesis of NAH:.

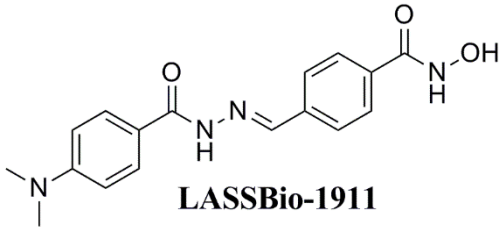


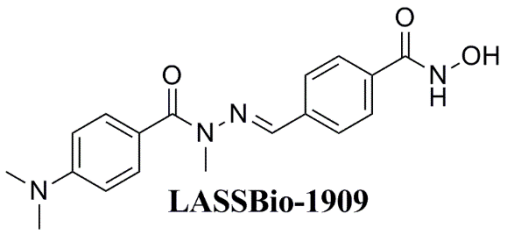
Reagents and conditions: (a) 3-formyl-*N*-hydroxybenzamide (**12a**) or 4-formyl-*N*-hydroxybenzamide (**12b**), $HCl_{cat.}$, EtOH, r.t., 2 h, 62-98%.

Chemical Structure and LASSBio label of the compounds.









2) Evaluation against HDAC

Table. HDAC inhibition, as assessed in rat liver HDACs, and aqueous solubility of each NAH compound.

Compound	% inhibition of HDAC (1 µM)[a]	HDAC rat liver inhibition, IC_{50} (µM)[b]	Aqueous solubility (µM)[c]
LASSBio-1908	33.4	N.D.	> 70
LASSBio-1909	90.4	0.018	> 64
LASSBio-1910	66.0	1.2	> 67
LASSBio-1911	90.4	0.021	15

[a]The values presented are the average of two experiments. The data are shown as % inhibition of HDAC. [b]The values are the average of two experiments are shown as IC_{50} values in µM. The compounds were examined through a six-point enzyme assay with a three-fold serial dilution starting from 3 µM for LASSBio-1910 and 1 µM for LASSBio-1911 and LASSBio-1909. [c]Determined using the spectrophotometric method. N.D. = not determined.

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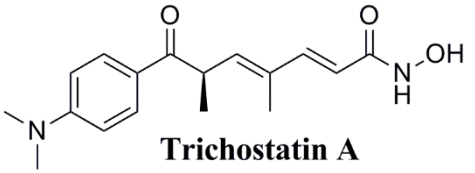
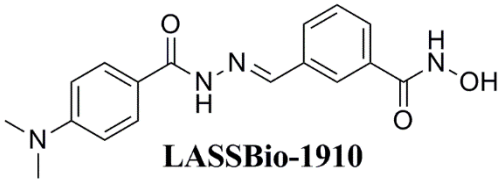
Results and Discussions – Selectivity profile of the evaluated *N*AH.

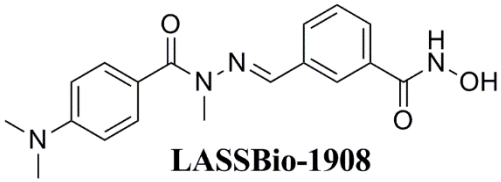
Table. Inhibitory profile of NAH derivatives and trichostatin A against human HDAC 1, 2, 6 and 8.

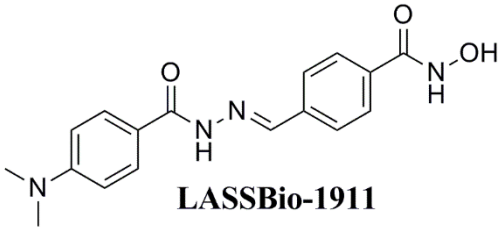
Compounds	Inhibition of human HDAC isoforms, IC_{50} (μM)[a]			
	HDAC 1	HDAC 2	HDAC6	HDAC8
Trichostatin A	0.0085	0.052	0.009	0.36
LASSBio-1910	>10.0	>10.0	0.39	2.2
LASSBio-1908	>10.0	>10.0	2.5	2.0
LASSBio-1911	>3.0	>3.0	0.015	0.23
LASSBio-1909	>3.0	>3.0	0.027	0.13

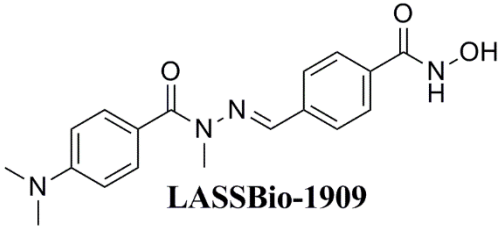
[a]The values are the average of three experiments and are shown as IC_{50} values in μM. The compounds were examined through a seven-point enzyme assay with a three-fold serial dilution starting from 10 μM for LASSBio-1910 and LASSBio-1908 and 3 μM for LASSBio-1911 and LASSBio-1909.











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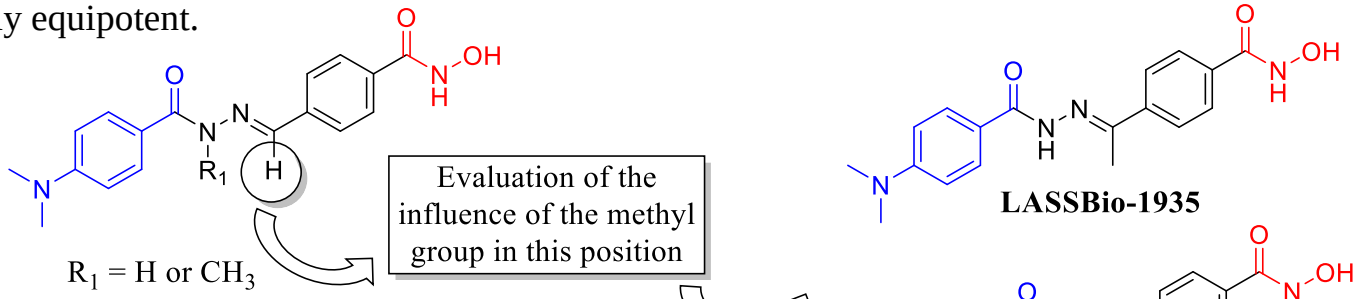
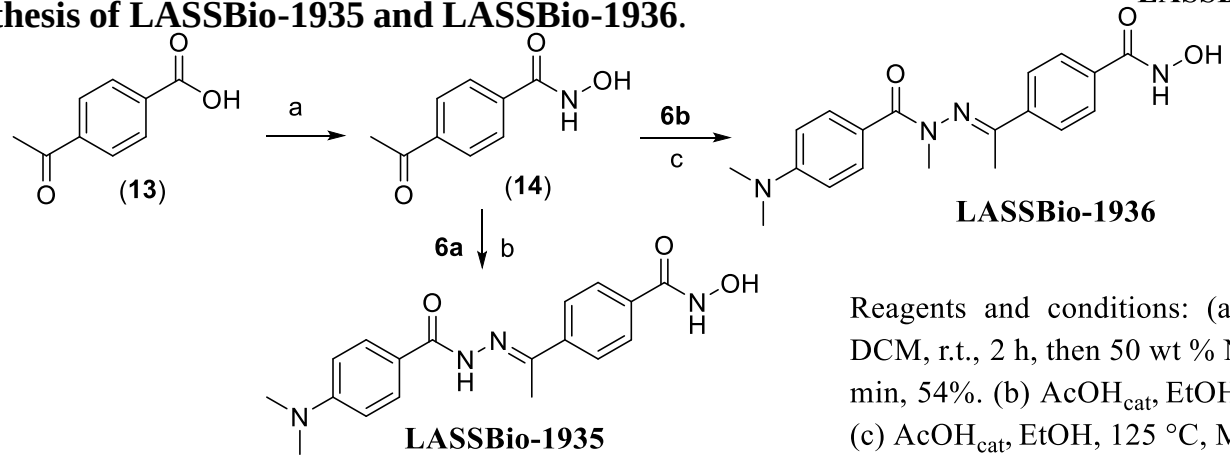
Results and Discussions – The Effect of the *C*-methylation

LASSBio-1911 and **LASSBio-1909** were extremely potent for HDAC6 inhibition, with IC_{50} values in the low nanomolar range. For this family of compounds, the substituents in the *para* position generated more potent compounds. The *N*-methylation of the *para* analogs series decreases their ability to inhibit HDAC6, although these compounds can be considered nearly equipotent.



para-analogs generated more potents compounds for the HDAC6 inhibition

1) Synthesis of LASSBio-1935 and LASSBio-1936.



Reagents and conditions: (a) Oxalyl chloride, $DMF_{cat.}$, DCM, r.t., 2 h, then 50 wt % NH_2OH, TEA, THF, 0 °C, 30 min, 54%. (b) $AcOH_{cat}$, EtOH, 80 °C, MW, 30 min, 45%; (c) $AcOH_{cat}$, EtOH, 125 °C, MW, 4 h, 38%;.

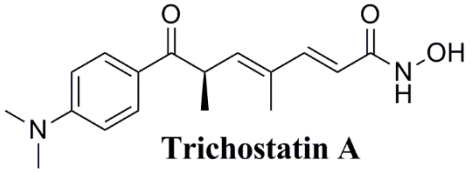
Rodrigues, D. A.et al. *Med. Chem.*, **2016**, 59, 655-706.

Results and Discussions – The Effect of the *C*-methylation

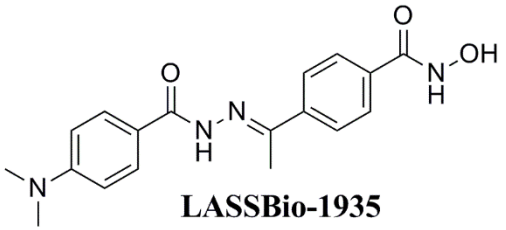
Table. Inhibitory profile of NAH derivatives and trichostatin A against human HDAC 1, 2, 6 and 8.

Inhibition of human HDAC isoforms, IC_{50} (μM)[a]				
Compounds	HDAC 1	HDAC 2	HDAC6	HDAC8
Trichostatin A	0.0085	0.052	0.009	0.36
LASSBio-1935	>10.0	>10.0	0.056	0.11
LASSBio-1936	>10.0	>10.0	0.097	0.054

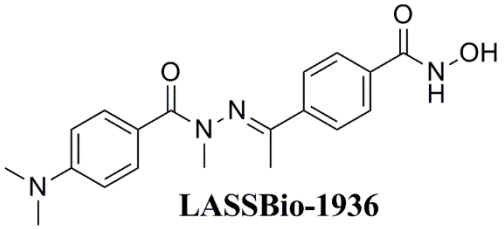
[a]The values are the average of three experiments and are shown as IC_{50} values in μM. The compounds were examined through a seven-point enzyme assay with a three-fold serial dilution starting from 3 μM for LASSBio-1935 and LASSBio-1936.



Trichostatin A

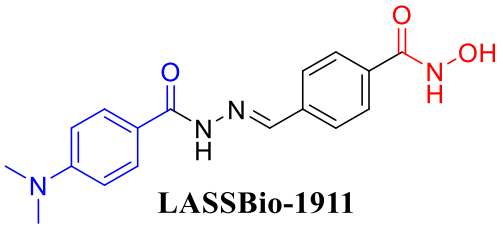


LASSBio-1935



LASSBio-1936

A methyl group in the R_2 position decreased the potency for HDAC6 inhibition, whereas the presence of this moiety increased the HDAC8 inhibitory ability. LASSBio-1911 was identified as the most potent for HDAC6 inhibition, IC_{50} = 15 nM



LASSBio-1911

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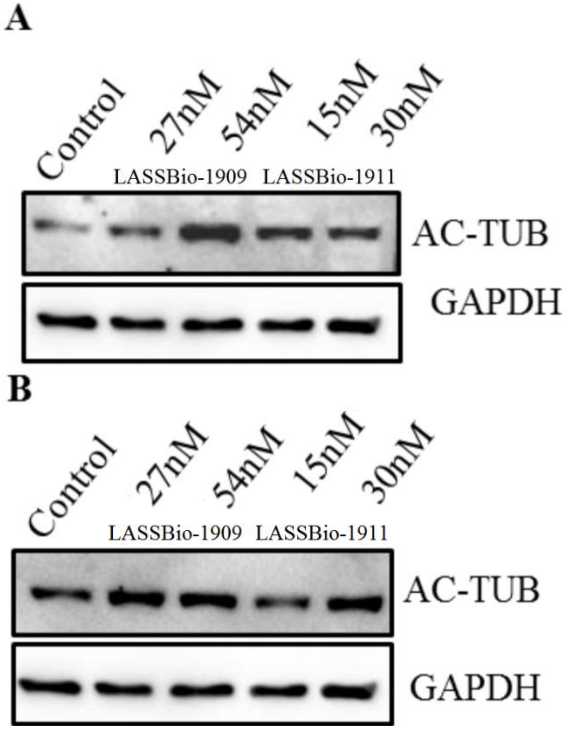
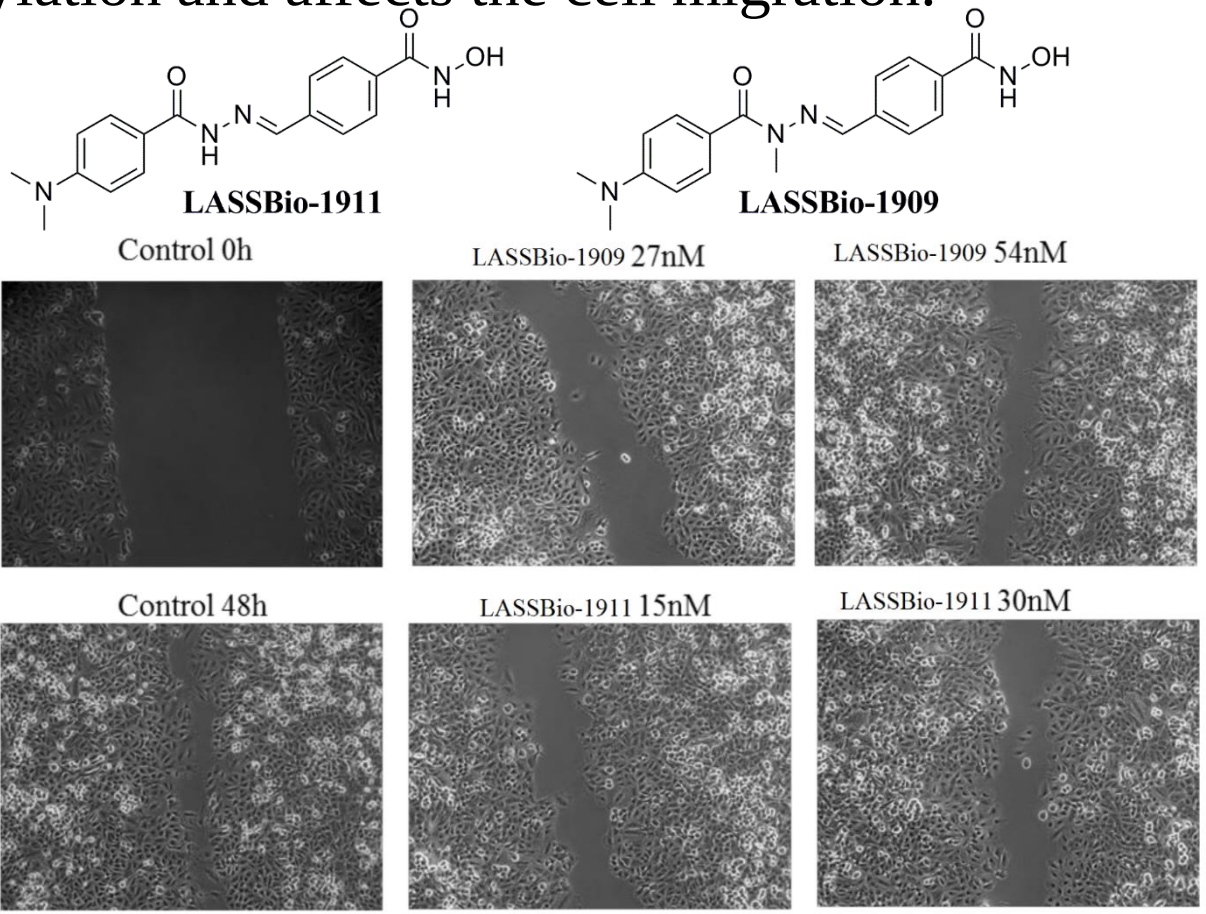
Results and Discussions – LASSBio-1911 and **LASSBio-1909** enhances the tubulin acetylation and affects the cell migration.



HDACI enhances tubulin acetylation. Calu-1 (A) and A549 (B) cells were treated with compounds **LASSBio-1911** and **LASSBio-1909** for 24 h, and the levels of acetylated tubulin were assessed by western blotting. GAPDH was used as a loading control. One representative of three independent experiments is shown. Cells treated with vehicle (DMSO) were used as controls.



HDAC6 inhibition abrogates cell migration. Calu-1 cells were treated with compounds **LASSBio-1911** and **LASSBio-1909** for 48 h, and their migration was determined using the scratch test. One representative of three independent experiments is shown. Cells treated with vehicle (DMSO) were used as controls.

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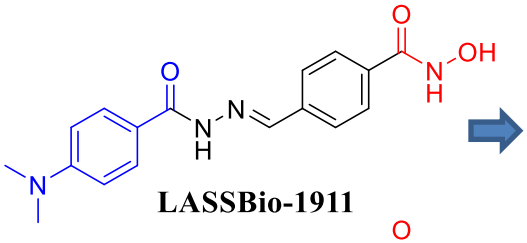
Results and Discussions – Antiproliferative activity of the NAH.

All of the tested compounds (**LASSBio-1908, LASSBio-1909, LASSBio-1910, LASSBio-1911, LASSBio-1935 and LASSBio-1936**) significantly reduced the viability of MCF-7, HT-144, and HepG2 cells in culture; however, the HT-144 and HepG2 cell lines were the most responsive to compounds **LASSBio-1909, LASSBio-1911, LASSBio-1935 and LASSBio-1936**.

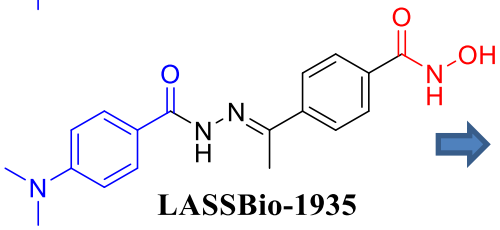
Table. IC_{50} values (μM) determined from data obtained through the MTS assay.

	Cell lines	
Compounds	**HepG2**	**HT144**
LASSBio-1911	9.48 ± 0.73	21.18 ± 0.69
LASSBio-1909	24.12 ± 0.87	36.10 ± 0.52
LASSBio-1935	11.52 ± 0.71	19.99 ± 0.64
LASSBio-1936	30.29 ± 1.38	51.64 ± 4.19



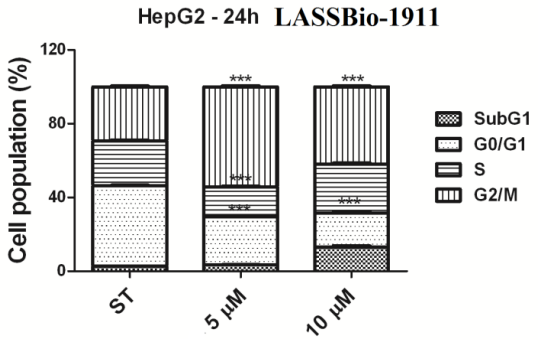
LASSBio-1911

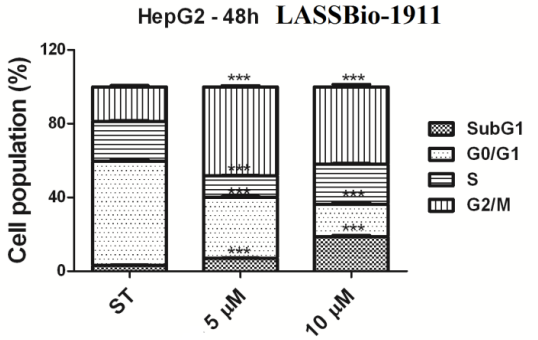
Cell cycle arrest!

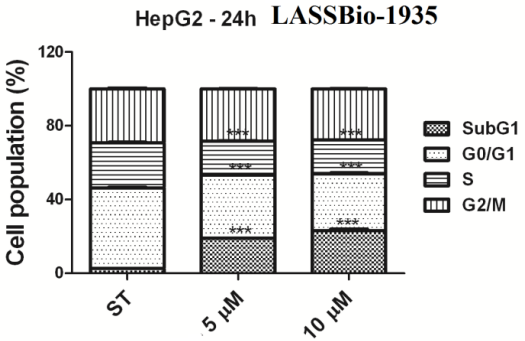


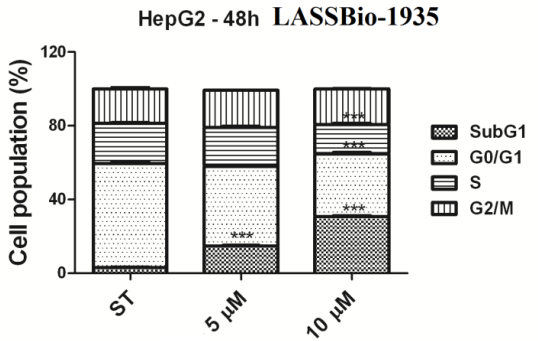
LASSBio-1935

Apoptosis – activation of caspase 3/7





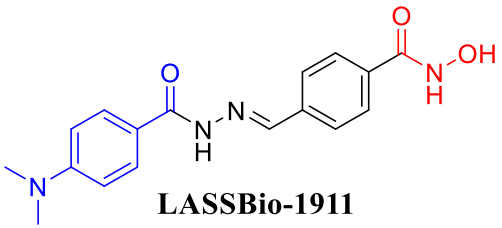




Cell cycle analysis of HepG2 cells treated with compounds **3c** and **3g** at concentrations of 5 and 10 μM for 24 and 48 h. The significance of the differences compared with the control results were determined by ANOVA followed by Tukey's post-test. *** $p<0.001$.

Rodrigues, D. A.et al. *Med. Chem.*, **2016**, 59, 655-706.

Results and Discussions – Evaluation of the Capping group



LASSBio-1911

The 4-dimethylaminobenzoyl group is in a solvent exposed region

Different groups in the Cap group should not impact the potency towards HDAC6.

NAH derivatives as potent HDAC6 inhibitors.

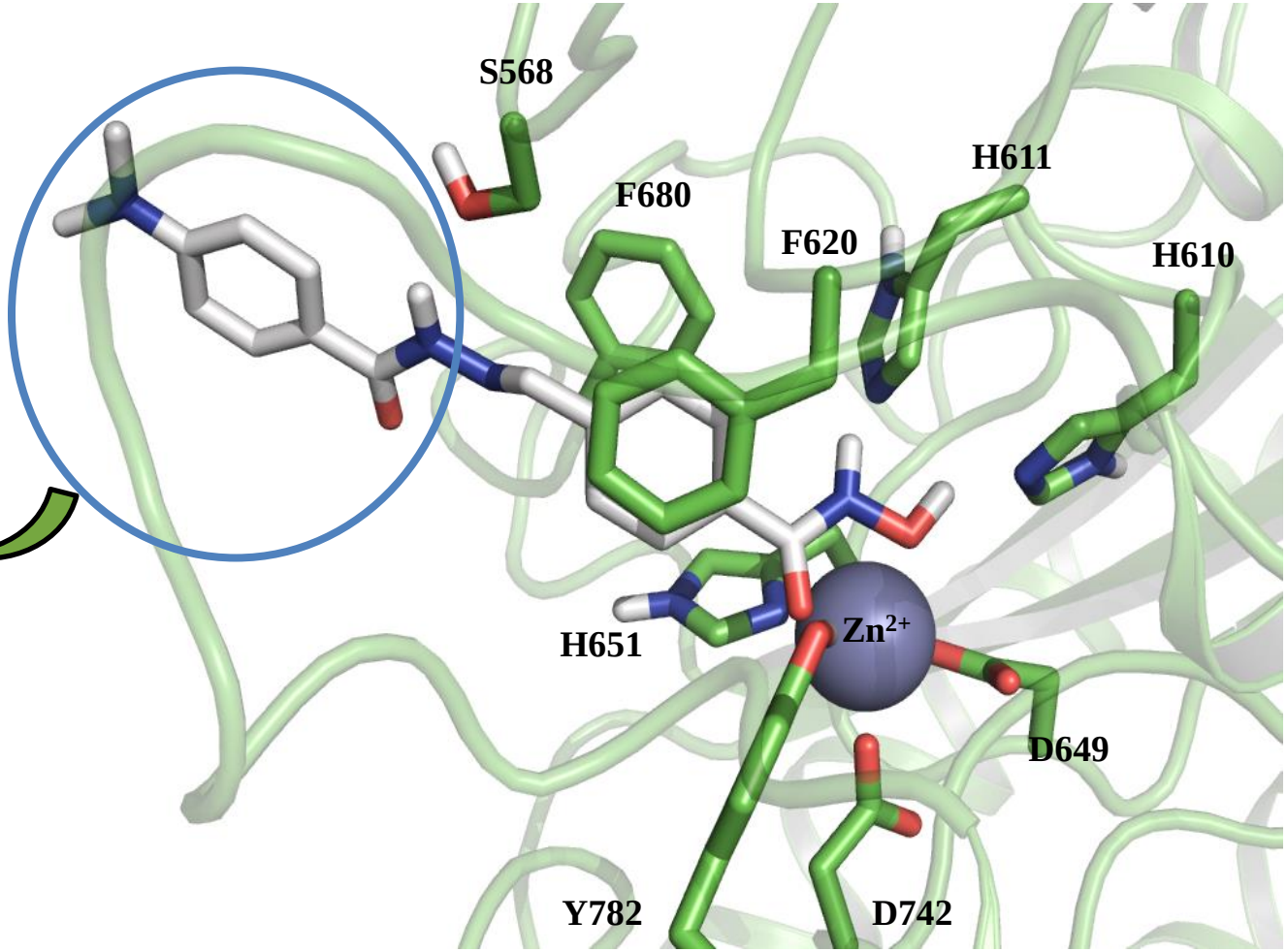
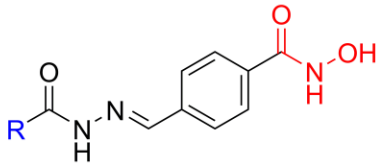


Figure. Predicted binding models of LASSBio-1911 in HDAC6 in the catalytic domain 2 (PDB: 5EDU). Docking studies were performed by using the program GOLD 5.2.

Pinheiro, P. S. M.; Rodrigues, D. A.; Sant'Anna, C. M. R.; Fraga, C. A. M. *Int. J. Quantum Chem.*, **2018**, 118, e25720.

Results and Discussions – Evaluation of the Capping group



R =

LASSBio-2132
IC_{50} = 18.4 nM

LASSBio-2133
IC_{50} = 27.5 nM

LASSBio-2134
IC_{50} = 30.6 nM

LASSBio-2135
IC_{50} = 25 nM

LASSBio-2136
IC_{50} = 25.1 nM

LASSBio-2143
IC_{50} = 23.5 nM

LASSBio-2142
IC_{50} = 35.6 nM

LASSBio-2137
IC_{50} = 26.5 nM

LASSBio-2138
IC_{50} = 25.3 nM

LASSBio-2147
IC_{50} = 32 nM

LASSBio-2148
IC_{50} = 45.5 nM

LASSBio-2151
IC_{50} = 52.4 nM

LASSBio-1911
IC_{50} = 18.7 nM

The use of different groups in the Cap has low impact the potency towards HDAC6, reinforcing the predicted mode of LASSBio-1911 in HDAC6.

Additional data about these compounds will be published in due course.

The compounds were tested in singlet 10-dose IC_{50} mode with 3-fold serial dilution starting at 1 μM against HDAC6. HDAC reference compound Trichostatin A (TSA) was tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 10 μM (IC_{50} = 3.2 nM).

Conclusions

- In this work the use of bioisosteric replacement in the natural product trichostatin A (**1**) lead us to characterize the privileged *N*-acylhydrazone moiety, allowing us to obtain novel potent and selective HDAC6 inhibitors.
- Compound **LASSBio-1911** primarily induced cell cycle arrest in the G2/M phase and eventual cell death in HepG2 cells, whereas compound **LASSBio-1935** effectively induced apoptosis through caspase 3/7 activation.
- The predicted interaction mode of LASSBio-1911 shows that the substituent in the acyl position is in a solvent exposed region, which allows to glimpse the use of other substituents without great impact in the potency.

Acknowledgments

- INCT-INOFAR (BR, Grant No. 465.249/2014-0);
- This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001;
- CNPq and FAPERJ (Grant/Award Number: E-26/201.954/2017) for the financial support provided and the fellowships awarded.