Review

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SAHA Analogues Bearing Azaheterocycles in CAP Group and Variable Methylene Chain Length: Synthesis and HDAC Inhibitory Ability

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SAHA Analogues Bearing Azaheterocycles in CAP Group and Variable Methylene Chain Length: Synthesis and HDAC Inhibitory Ability

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Abstract: This review covers the last 25 years literature on analogs of suberoylanilide hydroxamic acid (SAHA, known also as Vorinostat) acting as HDAC inhibitor. In particular, the topic has been focused on the synthesis and biological activity of compounds where the phenyl group (the surface recognition moiety, CAP) of SAHA has been replaced by an azaheterocycle through a direct bond with amide nitrogen atom, and the methylene chain in the linker region is of variable length. Most of the compounds reported displayed good to excellent inhibitory activity against HDACs and in many cases showed anti-proliferative activity against human cancer cell lines.

Keywords: HDAC; SAHA; azaheterocycles; synthesis; anticancer

1. Introduction

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) catalyze, respectively, deacetylation and acetylation of specific lysine residues situated on the amino-terminal tails of histone proteins. These enzymes play a key role in gene transcription [1] since acetylation is associated with an open chromatin configuration resulting in enhancing transcription [2] whereas the deacetylation process induces condensed and transcriptionally inactive heterochromatin [3].

Normally, it exists a balance between histone acetylation and deacetylation in normal cells, however, it has also demonstrated that these two enzymes are not only involved in the regulation of chromatin structure and gene expression, but can also regulate cell-cycle progression and carcinogenic processes [4]. Inhibition of HDACs can lead to cell differentiation, apoptosis and cell-cycle arrest both in several cancer cell lines and *in vivo*, thus making HDAC inhibitors (HDACi) a very important class of anticancer agents [5,6]. Besides to their anticancer effects, some HDACi also exhibit valuable neuroprotective properties in brain injuries such as stroke [7] and ischemia [8]. Further, some studies have reported the potential of HDACi to treat chronic neurological disorders such as amyotrophic lateral sclerosis [9], and Alzheimer's disease [10].

The common classification of HDACs is based on molecular phylogenetic analysis of primary structure. They are grouped (based on homology to yeast enzymes [11] in distinct classes: class I (HDAC1, HDAC2, HDAC3 and HDAC8), class IIa (HDAC4, HDAC5, HDAC7 and HDAC9), class IIb (HDAC6 and HDAC10) and class IV (HDAC11), these classes contain zinc-dependent domains. The class III belongs to a structurally and mechanistically distinct class of NAD^+-dependent hydrolases (sirtuins, Sirt1–Sirt7) [12].

To date, several HDAC inhibitors (HDACis) were addressed for cancer treatment, many of them contain an amide-alkyl-hydroxamic acid framework, such as that present in suberoylanilide hydroxamic acid (SAHA), well-known as Vorinostat (Figure 1). SAHA was the first HDAC inhibitor

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approved by US Food and Drug Administration in 2006 for the treatment of cutaneous T cell lymphoma [13].

SAHA structure, as well as that of many HDACis, conforms to the well-known HDAC inhibition pharmacophore A-B-C, where A is a cap group (CAP) for protein surface interactions, C is a zinc coordinating group (ZBG) that repress the hydrolysis of acetyl group in the lysine residue, and B is a linker group that connects CAP with ZBG [14].

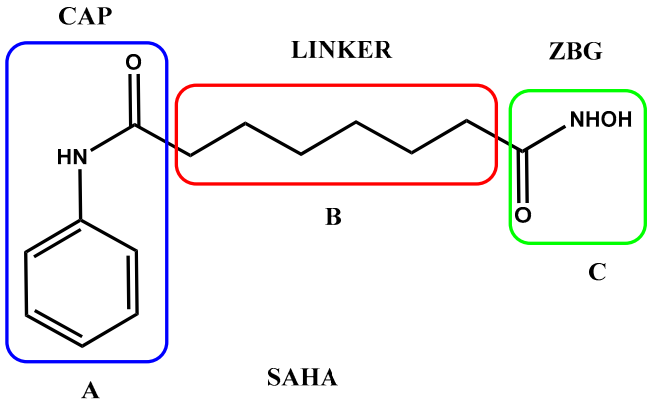

Figure 1. Structure of SAHA with indication of his specific CAP-LINKER-ZBG framework.

Along the years, many SAHA analogues have been synthesized and tested as HDACi. The present review reports the synthesis and biological activity of HDACi analogues of Vorinostat focusing attention on those bearing an aza-heteroaromatic instead of phenyl group in CAP fragment, a linear aliphatic chain of different length as linker, and carboxy-, ester-, or hydroxamic group as ZBG (Figure 2). This review, excluding patent literature, covers literature articles of the last 25 years.

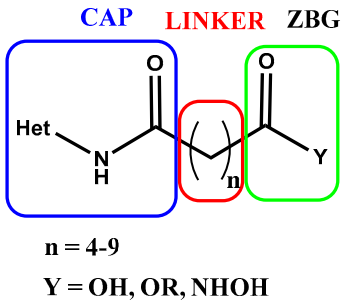

Figure 2. Structure of SAHA analogues HDACi reported in this review.

For the sake to make easier the reading of this contribute we divided the review in sub-headings, depending on the length chain of the aliphatic linker. In turn, each sub-heading has been structured based on the class of heterocycle bound to amide nitrogen atom of the CAP group.

2. Two-carbon linker chain

2.1. 2-amino-1,3,4-thiadiazoles in CAP group

The only reported HDACi bearing a C-2 alkyl chain are 2-amino-1,3,4-thiadiazole-based hydroxamates **4a** and **4b** [15]. They were synthesized as depicted in Scheme 1.

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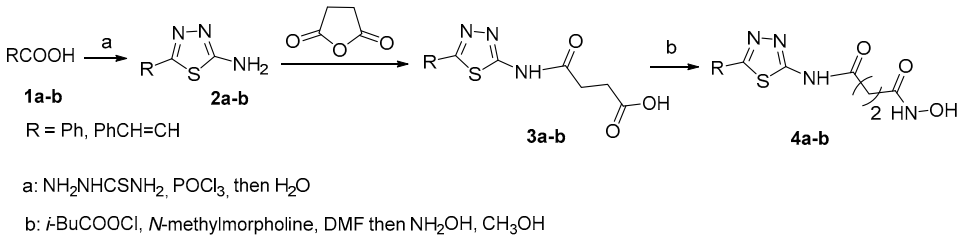


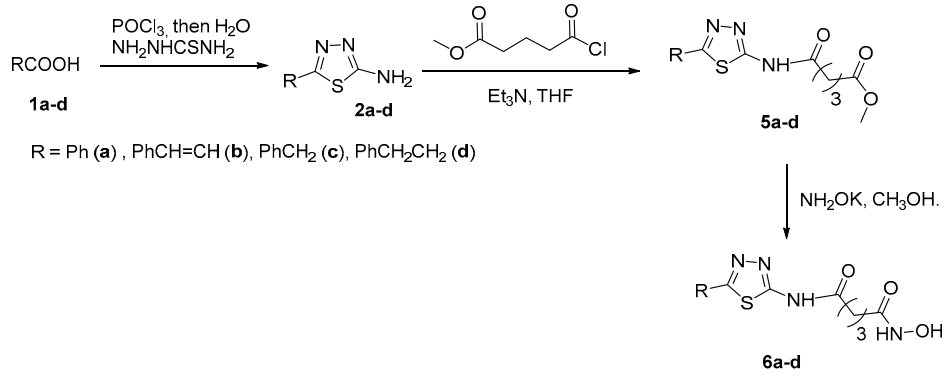
Scheme 1. Synthetic pathway to 1,3,4-thiadiazole derivatives **4a-b**.

HDAC inhibitory activity of compounds 4a and 4b was assessed by the Color de Lys assay and the results showed in both cases IC_{50} values > 5 μM, lower than that of SAHA (IC_{50} = 0.15 ± 0.02).

3. Three-carbon linker chain

3.1. 2-amino-1,3,4-thiadiazoles in CAP group

Hydroxamates bearing 2-amino-1,3,4-thiadiazole-derivatives in the CAP group and a C-3 linker chain were obtained as depicted in Scheme 2. Intermediates 5a-d were obtained by reaction between 2-amino-1,3,4-thiadiazoles 2a-d and methyl 5-chloro-5-oxopentanoate (in turn obtained from dimethyl glutarate after partial hydrolysis and treatment with $SOCl_2$). Treatment of 5a-d with NH_2OK in methanol gave compounds 6a-d [15].



Scheme 2. Synthetic route for preparation of compounds **6a-d**.

Among 6a-d, only 6b showed HDAC inhibition activity (IC_{50} =0.16 ± 0.03) close to that of SAHA (IC_{50} = 0.15 ± 0.02). Moreover, the effect of 6b on the cell viability in MDA-MB-231 breast cancer cells and K562 chronic myelogenous leukemia cells was evaluated, resulting in a IC_{50} value = 5.90 ± 2.75 and 6.75 ± 2.37, respectively.

2.2. Indazoles in CAP group

Among the series of indazole derivatives **16a-s** characterized by different spacer length and substituents on the heterocyclic ring, prepared as shown in Scheme 3, the only reported compound with a 3-carbon linker chain is **16a** (n =3, R = 3-methoxyphenyl) and related precursors [16].

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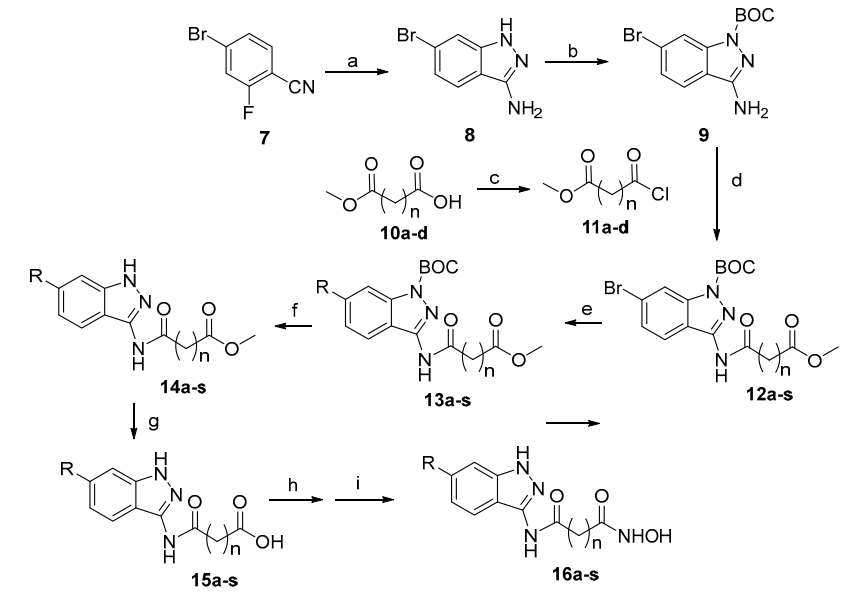

Scheme 3. Preparation of indazole-based series **16a-s**.

The biological activity as HDACi of compound **16a** was tested against HDAC1, HDAC2, and HDAC8 (IC_{50} = 76nM, 168nM, and 54 nM, respectively) and compared with that of SAHA (IC_{50} = 13nM, 70nM, and 44 nM, respectively). Moreover, **16a** was administered to solid cancer cell lines HCT116 (human colorectal cancer cells, IC_{50} > 50μM), MCF-7 (human breast cancer cells, IC_{50} > 41.5mM) and HeLa (human cervical cancer cells, IC_{50} > 50mM) but its activity was lower than that found for SAHA (IC_{50} = 4.9 mM, 0.8 mM, 5.0 μM for the three cell lines, respectively).

3. Four-carbon linker chain (4-C spacer)

3.1. 2-amino-1,3,4-thiadiazoles in CAP group

Compounds **17 a-d** (Figure 3) were obtained with the same synthetic sequence shown in Scheme 2, using in this case 6-chloro-6-oxohexanoic acid as acyl chloride [15].

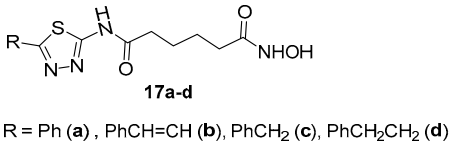

Figure 3. Thiadiazole hydroxamic acids with a four-methylene chain.

The HDAC inhibitory activity of the compounds was assayed: in all four cases it was lower compared with that SAHA chosen as positive control.

3.2. Indazoles in CAP group

With the synthetic sequence depicted in Scheme 3, when n=4 and R = 3-methoxyphenyl, indazole derivative **16b** (Figure 4) was obtained [16]. This compound showed activity towards HDAC1 (IC_{50} = 13 nM), HDAC2 (IC_{50} = 62 nM), and HDAC8 (IC_{50} = 41 nM) equal or a little better than that of SAHA (HDAC1 IC_{50} = 13 nM; HDAC2 IC_{50} = 70 nM; HDAC8 IC_{50} = 44 nM).

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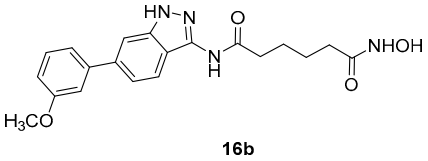
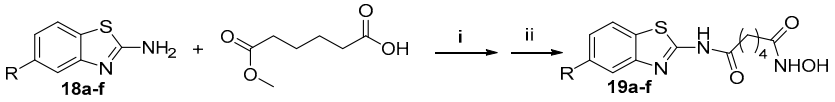


16b

Figure 4. Indazole derivative **16b**.

3.3. Benzothiazoles in CAP group

Benzothiazole derivatives **19a-f** [17] were obtained in good yields (from 70 to 90%) by reaction between 5-substituted 2-aminobenzothiazoles and adipic acid monomethyl ester in the presence of 1,1'-carbonyldiimidazole and triethylamine in THF followed by conversion of the ester intermediates to the corresponding hydroxamic acids (Scheme 4).



a, R = -H; **b**, R = -CH_3; **c**, R = -OCH_3
d, R = -OC_2H_5; **e**, R = -SO_2CH_3; **f**, R = -NO_2

(i) CDI, TEA, DMF, 4–5 h; (ii) NH_2OH HCl, NaOH, MeOH, 0 °C, 30 min to 1 h

Scheme 4. Synthesis of benzothiazolyl analogues of SAHA with C-4 methylene linker.

Cytotoxicity assays of compounds **19a-f** against five cancer cell lines, namely SW620, MCF-7, PC3, AsPC-1, and NCI-H460 revealed that compounds **19a–d** exhibited cytotoxicity against all tested cancer cell lines with IC_{50} values from 7.90 to 15.12 μg/ml whereas compounds **19e** and **19f** were not cytotoxic (IC_{50} of >30 μg/ml).

However, when the effect of **19a-f** on histone acetylation in SW620 cells was examined, the HDAC inhibition at concentration of 1 μg/mL was not significant.

3.4. 4-Anilinothieno[2,3-d]pyrimidine derivatives in CAP group

Thieno[2,3- d]pyrimidine-based HDACs inhibitors with different length of the spacer (n = 2, 3, 4) [18] were synthesized from the thieno[3,2-d]pyrimidin-4(3H)-one **20** after nitration in alpha position to thiophene ring, chlorination on the pyrimidine ring, treatment with different anilines and reduction of the nitro group to amino group. The latter was reacted with the acyl chloride $MeOCO(CH_2)_nCOCl$ (n = 4-6) to form the methyl ester intermediates **25** that were subjected to hydrolysis. Subsequent treatment of the obtained acid with hydroxylamine hydrochloride in the presence of BOP and DMAP afforded the targeted compounds **26** (Scheme 5).

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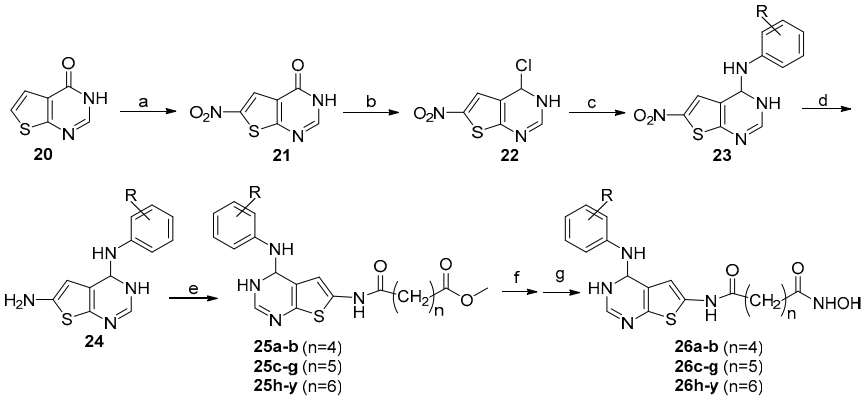


a: 3-Cl, 4-F; **b**: 3-CF_3, 4-Cl.
c: 3-Cl, 4-F; **d**: 3-CF_3, 4-Cl; **e**:H; **f**: 4-Me; **g**: 3-Me, 4-Me.
h: 3-Cl, 4-F; **i**: 3-CF_3, 4-Cl; **j**: 2-F, 4-F; **k**: 2-F, 4-Cl; **l**: 2-F, 4-Br; **m**: 2-F, 4-I; **n**: 2-F, 3-Cl;
o: 3-Cl; **p**: H; **q**: 4-Me; **r**: 3-Me, 4-Me; **s**: 4-N$(Et)_2$; **t**: 3-F, 4-N$(Et)_2$; **u**: 3-F, 4-N$(Me)_2$; **v**: 3-Cl, 4-$OCHCH_2OCH_2CH_2$-;
w: 3-F, 4-*N*-piperidinyl; **x**: 3-F, 4-*N*-pirrolidinyl; **y**: 4-*N*-pirrolidinyl.

Reaction conditions: (a) H_2SO_4/HNO_3, RT-90 °C, 1 h; (b) $POCl_3$, 110 °C or $POCl_3$, TEA, CH_3CN, 70 °C; (c) various substituted anilines, i-PrOH, 80 °C; (d) Fe, NH_4Cl(aq), EtOH, 45 °C; (e) $MeOCO(CH_2)_nCOCl$, TEA, THF, n = 4, 5, 6; (f) LiOH, MeOH-THF, RT; (g) $NH_2OH.HCl$, DMAP, BOP, DMF, RT

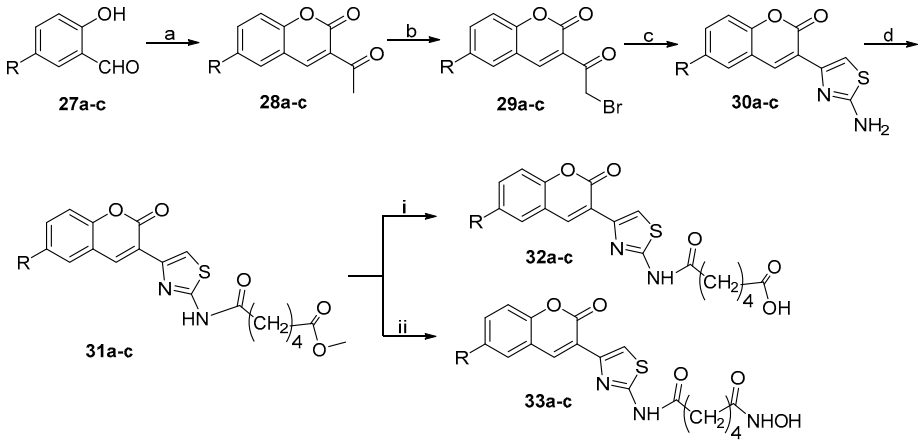
Scheme 5. Synthetic approach to thieno[2,3- d]pyrimidine-based HDACs inhibitors with different length of the spacer.

Focusing attention on the C-4 spacer, compounds **26a** (R = 3-Cl, 4-F) and **26b** (R = 3-CF_3, 4-Cl) their *in vitro* inhibitory activity against HDAC1, HDAC3, and HDAC6 was lower with respect to that of SAHA, except for **26a** towards HDAC3 (**26a** IC_{50} = 126.56 ± 9.04; SAHA IC_{50} = 158.17 ± 6.66).

3.5. Thiazolyl-coumarin derivatives in CAP group

The *in vitro* inhibitory activity against HDACs of thiazolyl-coumarins linked, through a C-6 alkyl spacer, to classic zinc binding groups, such as hydroxamic and carboxylic acid moieties, was evaluated [19].

In particular, compounds **32a-c** and **33a-c** were synthesized as shown in Scheme 6.



R = H (**a**); Br (**b**); OCH_3 (**c**)

(a) Ethyl acetoacetate, piperidine (drops), 50 °C, 0.5–2 h (depending on R); (b) Br_2, $CHCl_3/CH_3COOH$, 40–60 °C, 3h; (c) (c.1)$SC(NH_2)_2$, EtOH, 78 °C (reflux), 15 min,(c.2) CH_3COONa, H_2O, 50 °C; (d) $ClCO(CH_2)_4COOCH_3$, CH_3CN, TEA, 5h; (i) DMSO, HCl conc. 70 °C, 45 min; (ii) NH_2OH, MeOMeOH, 45 °C, 3h

Scheme 6. Synthetic route to thiazolyl derivatives **32** and **33**.

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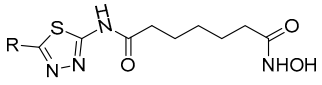
The first step was a Knoevenagel-type condensation between from salicyl aldehydes **27a-c** and ethyl acetoacetate. After bromination of the cumarin acetyl group, the Hantzsch synthesis gave the thiazole intermediates **30a-c** that was reacted with the acyl chloride derived from adipic acid methyl ester to give **31a-c**. From the latter, acids **32a-c** and hydroxamic acids **33a-c** were obtained. Compounds **33a-c** were the most active inhibitory compounds of HDACs towards HeLa cells

In addition, it has been shown that the expression and activity of distinct histone deacetylases (HDACs) are strongly correlated to the cardiac fibrosis (CF) development. In particular, HDAC1 and HDAC2 are mainly associated with the regulation of the biology of CFs in the heart; in this context, compound **33a** showed significant inhibition on CFs proliferation at 1 μM and also a decrease of procollagen type I and α–smooth muscle actin (α–SMA) expression levels.

4. Five-carbon linker chain (5-C spacer)

4.1. 2-amino-1,3,4-thiadiazoles in CAP group

Compounds **34 a-d** (Figure 5) were obtained as shown in Scheme 2, using in this case 7-chloro-7-oxohexanoic acid as acyl chloride [15].



34 a-y

R = Ph (**a**) , PhCH=CH (**b**), $PhCH_2$ (**c**), $PhCH_2CH_2$ (**d**)
R = 4-morpholino-Ph (**e**), 4-$N(CH_3)_2$-Ph (**f**), 4-OCH_3-Ph (**g**), 3,4-OCH_3-Ph (**h**), 4-CH_3-Ph (**i**), (1,1'-Biphenyl)-4-yl (**j**), 4-F-Ph (**k**), 4-Cl-Ph (**l**), 3-Cl-Ph (**m**), 2-Cl-Ph (**n**), 4-Br-Ph (**o**), 4-I-Ph (**p**), 4-SO_2CH_3-Ph (**q**), 4-NO_2-Ph (**r**), 4-CF_3-Ph (**s**), Naphthalen-1-yl (**t**), Naphthalen-2-yl (**u**), Pyridin-3-yl (**v**), Pyridin-4-yl (**w**), Furan-2-yl (**x**), Thiophen-2-yl (**y**).

Figure 5. 2-Amino-1,3,4-thiadiazoles connected to a C-5 spacer through amide bond.

The HDAC inhibitory activity evaluation of compound **34a** (R = Ph, IC_{50} = 0.089 ± 0.005 μM), resulted better than that of SAHA (IC_{50} = 0.15 ± 0.02 μM), similar in cases **34c** (R = $PhCH_2$, IC_{50} = 0.22 ± 0.04 μM) and **34d** (R = $PhCH_2CH_2$, IC_{50} =0.33 ± 0.05 μM), while for **34b** (R = PhCH=CH) was >5μM.

Other compounds bearing 1,3,4-thiadiazole ring as the surface recognition motif, obtained with a sequence very similar to that depicted in Scheme 2, were compounds **34e-y** (Figure 5) [20].

Among them, only **34g** (R = 4-OCH_3), **34i** (R = 4-CH_3), **34v** (R = pyridin-3-yl), **34w** (R = pyridin-4-yl), **34x** (furan-2-yl), **34y** (thiophen-2.yl) showed IC_{50} values IC_{50} of HDACs close to that of SAHA.

4.2. 4-Anilinothieno[2,3-d]pyrimidine derivatives in CAP group

The inhibitory activity of compounds **26c-g**, synthesized as depicted in Scheme 5, was tested for HDAC1, HDAC3, and HDAC6. In all cases it was higher than that of SAHA, as reported in Table 1 [18]

Table 1. '*In vitro*' inhibitory activity against HDACs of compounds **26c-26g.**

Compound	R	HDAC1 IC_{50} (nM)	HDAC3 IC_{50}(nM)	HDAC6 IC_{50}(nM)
26c	3-Cl, 4-F	35.89 ± 16.34	37.67 ± 1.61	23.99 ± 0.72
26d	3-CF_3, 4-Cl	40.84 ± 8.23	48.26 ± 1.78	30.00 ± 1.14
26e	H	11.77 ± 0.50	20.77 ± 0.64	26.99 ± 4.95
26f	4-CH_3	14.01 ± 1.32	9.33 ± 0.10	19.68 ± 1.96
26g	3-CH_3, 4-CH_3	29.82 ± 11.51	14.74 ± 0.03	16.87 ± 3.02
SAHA		93.34 ± 2.78	158.17 ± 6.66	78.98 ± 13.19

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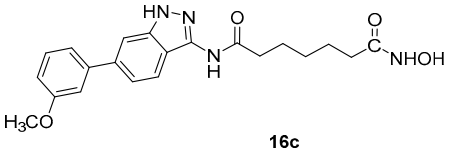
4.3. Indazole nucleus in CAP group

Indazolyl compound **16c** (Figure 6) was obtained through the synthetic sequence depicted in Scheme 3, when n=5 and R = 3-methoxyphenyl [16]

This compound showed activity towards HDAC1 (IC_{50} = 2.6 nM), HDAC2 (IC_{50} = 6.3 nM), and HDAC8 (IC_{50} = 4.5 nM) very higher than that observed for the corresponding homologues **16a** and **16b**, thus indicating a strong effect of the chain length in inducing biological activity.



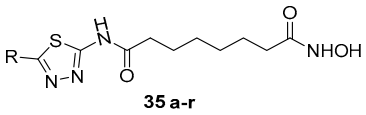
16c

Figure 6. Indazolyl SAHA analogue **16c**.

5. Six-carbon linker chain (6-C spacer)

5.1. 2-amino-1,3,4-thiadiazoles in CAP group

Compounds **35 a-d** (Figure 7) were obtained as shown in Scheme 2, using in this case 8-chloro-8-oxohexanoic acid as acyl chloride. [15] and compounds **35 e-r** were obtained in a similar manner [20].



35 a-r

R = Ph (**a**) , PhCH=CH (**b**), $PhCH_2$ (**c**), $PhCH_2CH_2$ (**d**)
R = 4-morpholino-Ph (**e**), (1,1'-Biphenyl)-4-yl(**g**), 3-Br-Ph (**h**),
2-Br-Ph (**i**), 4-I (**j**), 4-SO_2CH_3-Ph (**k**), 4-CF_3-Ph (**l**),
Naphthalen-1-yl (**m**), Naphthalen-2-yl (**n**),
Pyridin-3-yl (**o**), Pyridin-4-yl(**p**), Furan-2-yl (**q**), Thiophen-2-yl (**r**)

Figure 7. 2-amino-1,3,4-thiadiazoles connected to a C-6 spacer through amide bond, with hydroxamic acid as ZBG group.

Among them, only compounds **35o** (R = pyridin-3-yl), **35p** (R = pyridin-4-yl), and **35r** (R = thiophen-2-yl) showed an IC_{50} (referred to HDACs) value lower that that found for SAHA. This recalls the behavior found for **34v**, **34w** and **34y**, bearing the same CAP group but with a 5-C spacer, indicating the efficacy of pyridine and thiophene derivatives. Moreover, the viability of cancer cells MDA-MB-231, K562, and PC3 was measured by MTT assay for compounds **34v**, **34w**, **34y**, **35o**, **35p**, and **35r**. The results showed that **35r** is more efficacy than SAHA on all cell lines tested, whereas **35o** only on MDA-MB-231 cells.

Thiadiazole derivatives **40a-o** were synthesized as depicted in Scheme 7 starting from benzaldehyde **36a** (or differently substituted benzaldehydes **36b-o**) and thiosemicarbazide followed by cyclization to thiadiazole derivatives **38a-o**. The latter were reacted with 1,10-carbodiimidazole (CDI) and suberic monomethyl ester acid to obtain derivatives **39a-o**, from whose final hydroxamates **40a-o** were obtained [21].

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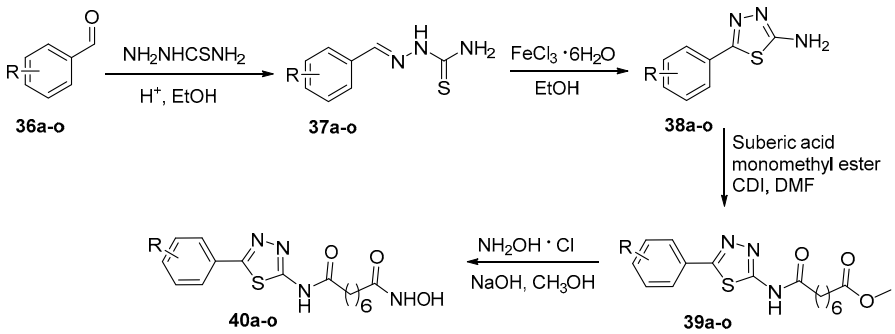

R = H (**a**) , 2-Cl (**b**), 3-Cl (**c**), 4-Cl (**d**), 4-F (**e**), 4-Br (**f**), 4-CH_3 (**g**), 4-OCH_3 (**h**), 4-$N(CH_3)_2$ (**i**),2-NO_2 (**j**), 4-NO_2 (**k**), 2,6-Cl_2 (**l**), 3,4-CH_2OCH_2- (**m**), 2,3,4-$(OCH_3)_3$ (**n**), 3,4,5-$(OCH_3)_3$ (**o**)

Scheme 7. Synthetic pathway for N1-hydroxy-N8-(5-substituted phenyl-1,3,4-thiadiazol-2-yl)octandiamides **40a–o**.

In this series, compounds **40b**, **40c** and **40d** were found to possess potent anticancer cytotoxicity and HDAC inhibition effects. They were generally two- to five-fold more potent in terms of cytotoxicity compared to SAHA against five cancer cell lines tested (SW620, colon cancer; MCF-7, breast cancer; PC3, prostate cancer; AsPC-1, pancreatic cancer; and NCI-H460, lung cancer). Docking studies revealed that these hydroxamic acids displayed higher affinities than SAHA toward HDAC8.

5.2. Thiazoles in CAP group

Phenylthiazole-bearing hydroxamates [22] *Ortho*- and *meta*-amino-substituted phenylthiazole derivatives **41-49** were synthesized starting from commercial 4-(2-nitrophenyl)thiazol-2-ylamine and 4-(3-nitrophenyl)thiazol-2-ylamine (Scheme 8).

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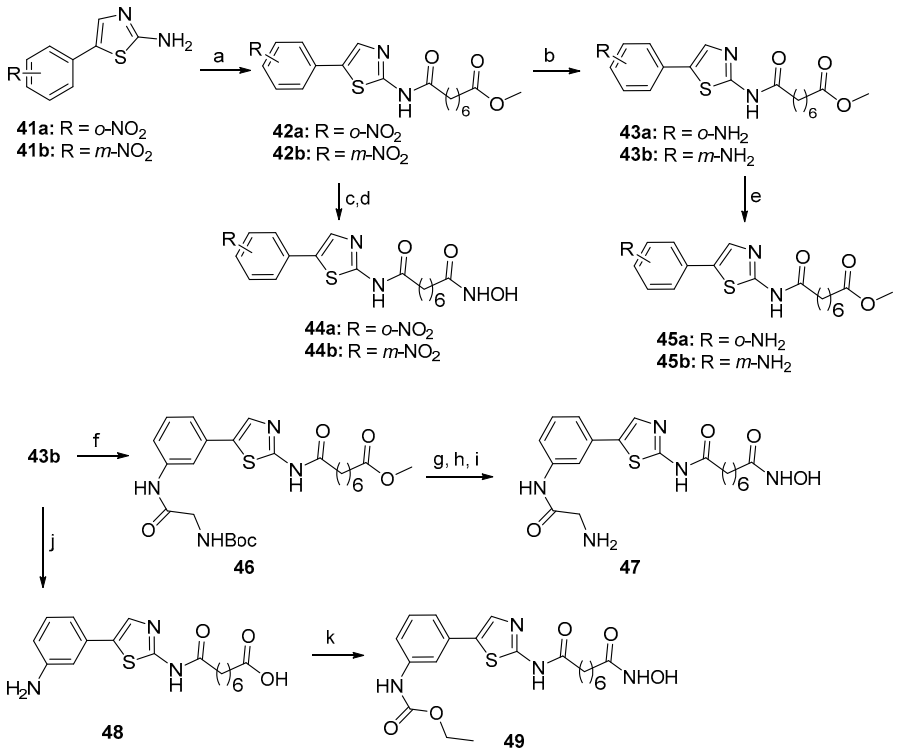

a) $POCl_3$, pyridine, suberic acid monomethyl ester, – 15 °C, 1 h; b) H_2, Pd/C, EtOH, AcOH, 50 °C, 2 h; c) LiOH; d) 1) isobutyl chloroformate, Et_3N, 0°C, 2) NH_2OH; e) NH_2OH, KOH, MeOH, room temperature, 1 h; f) EEDQ, Boc-Gly-OH; g) LiOH; h) $THPONH_2$, EDCI, HOBt, Et_3N, room temperature; i) TFA; j) LiOH; k) 1) ethyl chloroformate, Et_3N, 0°C, 2) NH_2OH.

Scheme 8. Synthetic sequence for preparation of phenylthiazole derivatives.

In the same paper, phenylthiazoles **50-55** bearing an amide or urethane residue on the benzene ring in linkage with a bulkier alkyl group have been reported. They were synthesized starting from compounds **43a** and **43b** through the sequence shown in Scheme 9.

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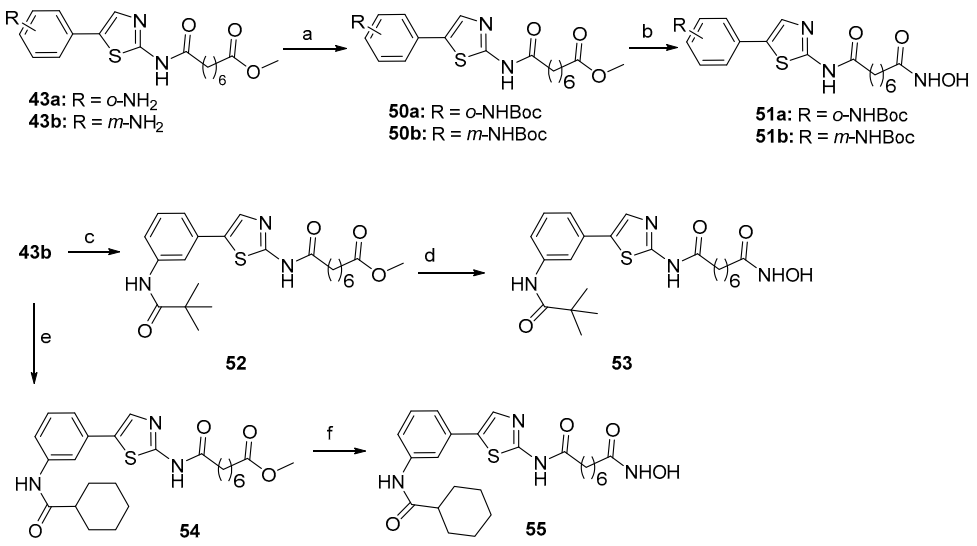

Scheme 9. Synthesis of compounds **50-55**.

The inhibitory activity of the above compounds has been tested towards HDAC1, HDAC2, HDAC3, HDAC8, HDAC10, and HDAC6. In comparison with the unsubstituted phenylthiazole analogue, the introduction of an amino group as in **45a** and **45b** or a glycineamide residue as in **47** do not produce significant changes in both, activity and isoform selectivity. The *ortho*-nitro compound **44a** is almost 10-fold less potent than the corresponding amine analogue **45a**. The meta-substituted ethyl carbamate **49** showed an activity against HDAC1 and HDAC2 very close to that of its amine analogue **45b**, but it showed a 3-fold improvement in its HDAC6 inhibitory activity. Ongoing from the ethyl- (**49**) to the *tert*-butyl- (**50b**) carbamate an increase in HDAC6 inhibitory activity was observed, but no changes in inhibitory activity toward HDAC1 and HDAC2. Moreover, the introduction of a Boc protecting group leads to an enhancement in the inhibitory activity toward HDAC6 (>15-fold in **51b** in comparison with **45b**). Interestingly, replacement of the *tert*-butyloxy group of **51b** by a cyclohexyl group as in **55** leads to subnanomolar potency against both HDAC2 and HDAC3 (IC_{50} values 200-fold increase against HDAC2 and >20-fold increase against HDAC3), while the IC_{50} value for HDAC6 was still below 0.2 nM. Compound **51a** showed a 2-fold decrease in activity toward HDAC1 and HDAC2, with similar inhibitory potency against HDAC6 relative to the unprotected *ortho*-NH_2 ligand **45a**. Also, conversion of **51b** to the pivaloyl derivative **53** produced a >10-fold decrease in HDAC6 inhibition. Inhibitory data of compound **44b** have been also reported in a previous work [23].

Compounds **44a**, **45a**, **45b**, and **49** have also been tested towards five pancreatic cancer cell lines and their antiproliferative activity was compared with that of SAHA and showed similar or improved potencies relative to SAHA. Among them, the *meta*-amino-substituted phenylthiazole **45b** gave the best IC_{50} value against the Mia Paca-2 cell line (IC_{50}=10 nM), while its carbamate analogue **49** showed the best overall inhibitory activity against all five pancreatic cancer cell lines.

In another study [24] the phenylthiazole-based probe **57**, with an azide group on the phenyl ring, was designed to mimic the scaffold of SAHA. The synthesis is shown in Scheme 10.

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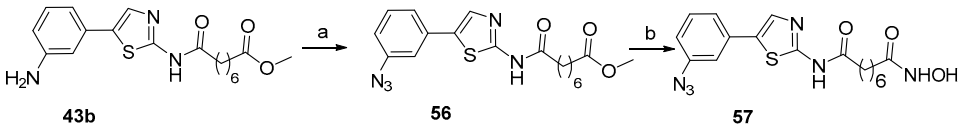

43b 56 57

(a) $NaNO_2$, AcOH/H_2O (9:1), 0 °C, 3 min, then NaN_3, 0 °C 30 min, 73%;
(b) NH_2OH 3 HCl, KOH, MeOH, 4 h, 0 °C to room temp, 28%.

Scheme 10. Synthesis of azide-containing compound **57**.

Compound **57** resulted 6.1-fold less active in inhibiting HDAC8 compared to SAHA and this behavior has been attributed to an increase of the lipophilic nature of the solvent exposed surface binding group that influences to the overall binding affinity.

Finally, hydroxamic derivatives bearing both unsubstituted- and *p-N*-pyrrolidinyl-substituted phenylthiazole amide functionality showed better HDAC potency and cellular activity (towards HT1080 and MDA435 cells) with respect to SAHA [25].

5.3. Pyrazole nucleus in CAP group

HDACi bearing pyrazole and isoxazole derivatives in CAP group have been synthesized and studied by Petukhov *et al.* [26,27].

In particular, compounds with pyrazole nucleus **61, 67** and **68a-d** have been synthesized according to Scheme 11, starting from commercially available 4-nitropyrazole (**58**). The synthetic strategy involves as first step an amide coupling between 4-aminopyrazole, obtained from **58** through hydrogenolysis, and monomethyl suberate to give (**59**). Alkylation of **59** with toluene-4-sulfonic acid 3-azido-5-azidomethylbenzyl ester in the presence of K_2CO_3 afforded compound **60**. Compounds **62** and **63** were obtained by alkylation of **59** with benzyl bromide or 4-nitrobenzyl bromide in the presence of NaH in DMF, respectively. Reduction of the nitro group of **63** gave aniline **64**, a key intermediate for compounds **65** and **66**. Diazotization of amino group of the aniline derivative **64** followed by azide displacement reaction with NaN_3 gave the corresponding azido compound **65**. Treatment of **64** with Boc anhydride furnished the carbamate **66**. Compound **67** was obtained by an amide coupling between **64** and 3-azido-5-azidomethylbenzoic acid followed by treatment with KOH/NH_2OH in MeOH. The same treatment on the methyl esters **60**, **62**, **63**, **65**, and **66** gave the corresponding hydroxamates **61** and **68a-d**, respectively.

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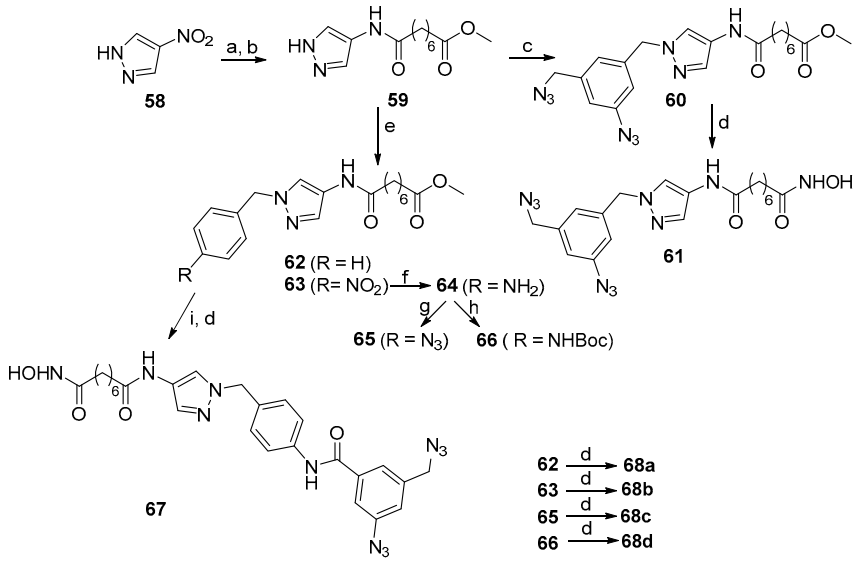

(a) H_2, Pd/C, MeOH, overnight; (b) monomethyl suberate, EDC, HOBt, DIPEA, CH_2Cl_2, 0°C-rt, 6 h;
(c) 3-azido-5-azidomethylbenzoic acid, K_2CO_3,acetone, reflux, 6 h;
(d) NH_2OH, KOH, 0°C-rt, 3 h; (e) BnBr or 4-nitrobenzyl bromide, NaH, DMF, 0 °C-rt, 4 h;
(f) $SnCl_2 \bullet 2H_2O$, MeOH, reflux,2 h;
(g) $NaNO_2$, AcOH-H_2O (9:1), 0 °C, 10 min, NaN_3, 0 °C-rt, 1 h; (h) $(Boc)_2O$, Et_3N, CH_2Cl_2, rt, 4 h;
(i) 3-Azido-5-azidomethylbenzoic Acid, EDC, HOBt, DIPEA, CH_2Cl_2, 0 °C-rt, 6 h.

Scheme 11. Multi-step route to SAHA analogues **61, 67** and **68a-d** with pyrazole nucleus in CAP group.

Pyrazoles **61**, **67**, and **68a-d** were tested for inhibition of HDAC3 and HDAC8 isoforms. The inhibition of HDAC8 was measured using the fluorescent acetylated HDAC substrate Fluor de Lys and the commercially available recombinant human HDAC8, whereas the inhibition of HDAC3 was measured using the fluorescent HDAC substrate Boc-L-Lys(Ac)-AMC and the commercial recombinant human HDAC3/NCoR2.

The results are summarized in Table 2.

Table 2. Inhibition of HDAC3 and HDAC8 isoforms by pyrazoles **61**, **67**, and **68a-d** compared with that of SAHA.

Compound	HDAC3 IC_{50} ± SD (nM)	HDAC8 IC_{50} ± SD (nM)
SAHA	27± 1.0	440± 21
61	128 ± 9.8	17± 3
67	432± 52	487± 80
68a	44± 5.8	76± 5.0
68b	59± 1.0	82± 9.0
68c	22± 1.3	28± 3.0
68d	191± 18	147± 15

The simplest benzyl substituted pyrazole **68a** inhibited HDAC3 and HDAC8 with IC_{50s} of 44 and 76 nM, respectively. Introduction of a nitro group at the 4-position of the benzyl group of **68a** gave compound **68b** that showed slightly lower activity for both isoforms, whereas the corresponding azido compound **68c** exhibited a 2.0- and 2.7-fold better potency, being its IC_{50} values 22 and 28 nM for HDAC3 and HDAC8, respectively. Overall, compounds **68a-68c** exhibited an inhibitory activity against HDAC3 comparable to that of SAHA but a better double digit nanomolar activity against HDAC8. Introduction of a bulky Boc-protected amino group in **68d** decreased the HDAC activity by

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about 10-fold. Replacement of the Boc group with a lipophilic aromatic diazide as in **67** further decreased the activity for both HDAC3 and HDAC8 to 432 and 487 nM, respectively. Comparison of the activity data of **68b-68c** with **68d** and **67** shows that the presence of the bulky substituent in the *para* position of the terminal phenyl ring leads to the lower activities for both HDAC3 and HDAC8 isoforms. The replacement of the phenyl group with a 3-azido-5-azidomethyl phenyl group, resulting in **61** revealed that this compound was 8-fold more active toward HDAC8 than for HDAC3, with IC_{50s} equal to 17 and 128 nM, respectively. The activity of the methyl ester **60** towards HDAC8 was 36.0 ± 2.20 μM [27].

Compound **61**, also called SAHA diazide, was also tested against HDAC1 and HDAC4; compared with the activity of SAHA (*Ki* = 0.051 and >30 μM for HDAC1 and HDAC4, respectively), *Ki* values for **61** were *Ki* = 0.14 and 13.05 μM for HDAC1 and HDAC4, respectively.

5.4. Pyridine and pyrimidine nucleus in CAP group

The synthesis and biological activity of compounds **69 a-c**, bearing a pyridinyl substituent in the CAP group (Figure 8) have been reported, but their activity towards HDAC1 was much lower than that of SAHA [29].

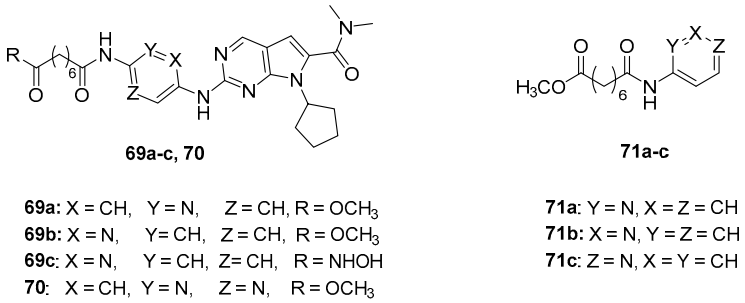

Figure 8. Pyridine derivatives tested against HDAC1.

Similar behavior was also found for the pyrimidine derivative **70**.

The more simple pyridinyl derivatives **71a-c** (Figure 8) were profiled partially purified HDAC enzyme obtained from H1299 cell lysate, in antiproliferative assays (towards H1299 and HCT116) and in a p21 promoter induction assay. [30]

In these cases, the activity towards enzyme was comparable to that of SAHA. The 2-pyridyl isomer **71a** was essentially equipotent to SAHA in the promoter assay, but 3-fold less potent in HCT116 growth inhibition, and >10 μM in H1299 growth inhibition. The 3- and 4-pyridyl isomers **71b** and **71c** were less potent than SAHA. The difference in cellular activity of these positional isomers has been hypothesized due to differences in cellular permeability or intracellular metabolism of the compounds.

5.5. Thienopyrimidine nucleus in CAP group

The biological activity of thienopyrimidine derivatives **26h-y**, synthesized as reported in Ref. 18 and depicted in above Scheme 5, have been tested as inhibitors of HDAC1, HDAC3, and HDAC6, and of proliferation of RMPI8226 and HCT-116 cancer cells. In all cases the activity found was comparable with that of SAHA.

In the same paper also the biological activity of compound **72** (Figure 9) was tested and the results showed poor inhibitory activity in many cases, suggesting that the presence of the 4-aniline fragment could increase the lipophilic interaction with HDACs to induce good inhibitory activities against them.

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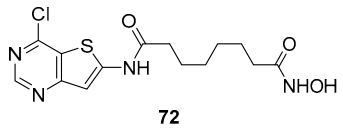
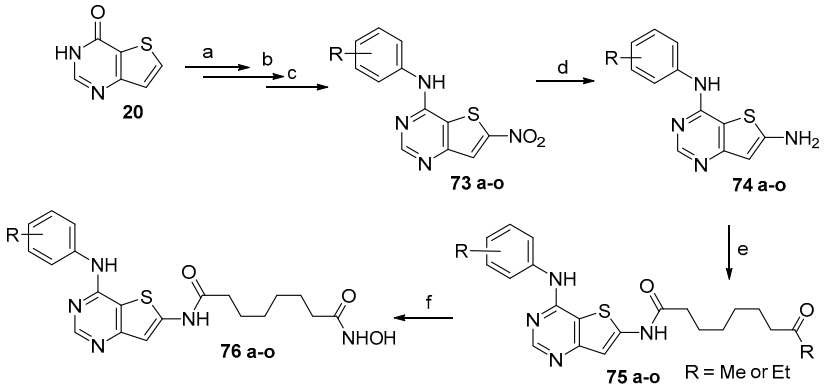


72

Figure 9. Structure of compound **72**.

The above cited paper was followed by a second [31], only focused on the C-6 spacer, in which the fifteen novel compounds **76 a-o**, bearing the thienopyrimidine fragment on the CAP group were synthesized from methyl 3-aminothiophene-2-carboxylate that, after cyclization with formamidine acetate under microwave conditions, gave the thieno[3,2-d]pyrimidin-4(3*H*)-one (**20)** in similar conditions to those already reported in Scheme 5. The latter was subjected to nitration and subsequent chlorination then coupled with a series of anilines to give compounds **73a-o**. Reduction of nitro group to amino group afforded the key precursors **74 a-o**. After treatment with acyl chlorides amides **75a-o** were obtained. Lastly, the target products **76 a-o** were obtained after reaction with hydroxylamine hydrochloride (Scheme 12).



a: 2-Me; **b**: 2-Me, 4-F; **c**: 2-F, 5-Me; **d**: 2-Me, 4-OMe; **e**: 3-Cl; **f:** 3-Br; **g**: 3-ethyl; **h**: 3-isopropyl; **i**: 3-tertbutyl; **j**: 3-ethynyl; **k**: 3-CN; **l**: 3-OCH_3; **m**: 4-ethyl; **n**: 4-isopropyl; **o**: 4-OCH_3

Reaction conditions: a) H_2SO_4/HNO_3, 0 °C-r.t; b) $POCl_3$, TEA, CH_3CN, 85 ° C; c) various substituted anilines, 1,4-dioxane, 85 °C; d) Fe, NH_4Cl(aq), EtOH, 50 °C, 2 h; e) RO-$CO(CH_2)_6COCl$, DIPEA, THF; f) $NH_2OH.HCl$, KOH, MeOH-THF, 0 ° C-r.t.

Scheme 12. SAHA analogues with thienopyrimidine nucleus in CAP group.

The ability of compounds **76 a-o** to inhibit recombinant human HDAC1, HDAC3, and HDAC6 isoforms and 'in vitro' activity against cancer cell lines RMPI 8226 and HCT 116 was tested. Most of them displayed good inhibitory and anticancer activities, particularly compound **76j** that showed IC_{50} values (29.81 ± 0.52 nM, 24.71 ± 1.16 nM, and 21.29 ± 0.32 nM for HDAC1, HDAC3, and HDAC6, respectively), much lower than those found for SAHA (195.00 ± 16.12 181.05 ± 28.92 105.10 ± 25.46). Moreover, the IC_{50} values of compound **76j** against RPMI 8226 and HCT 116 proliferation were 0.97 ± 0.072 mM and 1.01 ± 0.033 mM, respectively, and it up-regulated the level of histone H3 acetylation at the concentration of 0.3 mM.

5.6. Indazole nucleus in CAP group

In Figure 10 are shown indazolyl derivatives **16d-s**, synthesized through the approach above depicted in Scheme 3 [16].

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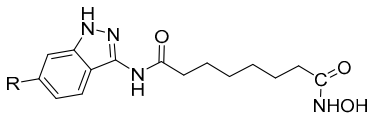


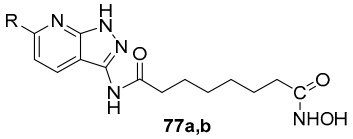
16d-s

d: R = phenyl; **e**: R = 3-methoxyphenyl; **f**: R = 3-ethoxyphenyl;
g: R = 3-chlorophenyl; **h**: R = 2-chlorophenyl; **i**: R = 3,4-dimethoxyphenyl;
j: R = 2-fluoro,5-methoxyphenyl; **k**: R = 2-fluoro,3-methoxyphenyl;
l: R = 2-fluorophenyl; **m**: R = 3-methoxyphenyl; **n**: R = 4-methoxyphenyl;
o: R = 3-trifluoromethylphenyl; **p**: R = 4-chlorophenyl;
q: R = 3,5-dimethoxyphenyl;**r**: R = 4-pyridinyl; **s**: R = 2-thiophenyl

Figure 10. Indazolyl SAHA analogues **16d-s**.

Among compounds **16d-s**, compounds **16n** and **16p** emerged as excellent inhibitors of HDAC1 (IC_{50} = 2.7 nM and IC_{50} = 3.1 nM), HDAC2 (IC_{50} = 4.2 nM and IC_{50} = 3.6 nM) and HDAC8 (IC_{50} = 3.6 nM and IC_{50} = 3.3 nM). Antiproliferation assays revealed that these compounds also showed anti-proliferative activities against HCT-116 and HeLa cells better than SAHA. Moreover, compounds **16n** and **16p** up-regulated the level of acetylated α-tubulin and histone H3 and promote cell apoptosis.

According to a similar synthetic route similar to that of Scheme 3, 1*H*-pyrazolo [3,4-b] pyridine derivatives **77a-b** (Figure 11), bioisosters of compounds **16e** and **16n**, respectively, were obtained from 2,6-dichloronicotinonitrile through a multistep sequence [16].



77a,b

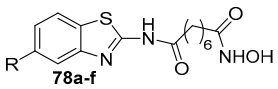
a: R = 3-methoxyphenyl; **b**: R = 4-methoxyphenyl;

Figure 11. structure of compounds **77a,b**.

The inhibitory activities of **77a** and **77b** towards HDACs slightly decreased indicating that the presence of the 6-phenyl-1*H*-indazole scaffold is important to affect the biological activity.

5.7. Benzothiazole moiety in CAP group

Compounds **78a-f** (Figure 12) were obtained from 2-aminobenzothiazole derivatives with the sequence depicted in Scheme 4, with the difference to use suberic acid monomethyl ester instead of adipic acid monomethyl ester [17].



78a-f

a, R = -H; **b**, R = $-CH_3$; **c**, R = $-OCH_3$
d, R = $-OC_2H_5$; **e**, R = $-SO_2CH_3$; **f**, R = $-NO_2$

Figure 12. SAHA analogues with benzothiazolyl scaffold in CAP group.

It was observed that several compounds showed good inhibition against HDAC3 and HDAC4. The amount of enhanced acetylation of histone-H3 and -H4 in SW620 cells by **78a–c**, and **78f** was similar to that found for SAHA.

Moreover, all six compounds displayed cytotoxicity against five cancer cell lines (SW620, colon cancer; MCF-7, breast cancer; PC3, prostate cancer; AsPC-1, pancreatic cancer; NCI-H460, lung cancer), with average IC_{50} values range from 0.59 to 11.08μg/mL

Homologues 4C-bridged compounds showed slight or no increase in histone acetylation, suggesting that the linker length between the benzothiazol and hydroxamic moieties required for

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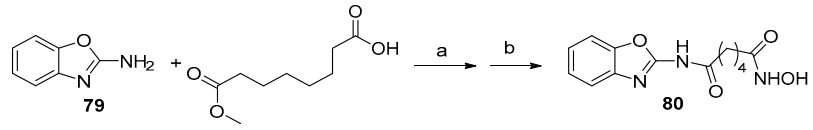
good HDAC inhibition of this compound series was similar to that of SAHA. In addition, the size of the 6-substituents on the benzene ring rather than their electronic effects was important for HDAC binding, for example, **78d** and **78e** bearing relatively larger substituents (–OC_2H_5 and –SO_2CH_3) compared to the other compounds in the series did not inhibit HDAC activity. Actually, compounds **78c** (bearing –OCH_3, an electron-donating group) and **78f** (bearing –NO_2, an electron-withdrawing group) showed similar HDAC inhibitor power and were almost equally cytotoxic.

5.8. Benzoxazole moiety in CAP group

From the reaction between 2-aminobenzoxazole and suberic acid monomethyl ester and subsequent transformation of the methyl ester to hydroxamic group, compound **80**, that can be considered a bioisoster of **78a**, was obtained [32].



(a) EDC.HCl, Et_3N, HOBt, CH_2Cl_2; (b) NH_2OH.HCl, EtONa, EtOH, r.t

Scheme 13. Benzoxazole analogue of SAHA from 2-aminobenzoxazole.

Compound **80** was an inhibitor of human HDAC1, HDAC2 more potent than Vorinostat and comparable as an inhibitor of HDAC6. It was a slightly more potent inhibitor than Vorinostat of the growth of A549, Caco-2 and SF268 cells and was chosen for further studies against two colon cancer cell lines HCT116 GNAS R201C/+ and LS174T cells, that genetically resemble PMP tumor cells and it proved to be a more potent antiproliferative compound than Vorinostat in both cases.

5.9. Isoquinoline moiety in CAP group

Novel HDACi bearing isoquinoline fragment in CAP group have been synthesized starting from 2-methyl-5-nitrobenzoic acid (**81**) [33]. After esterification to **82** followed by treatment with DMA-DMF and cyclisation with 3,4-dimethoxylbenzylamine intermediate **84** was obtained. The latter was deprotected to **85** then chlorinated to **86** which was coupled with a series of anilines to generate compounds **87**. Reduction of the nitro group followed by reaction with 8-methoxy-8-oxooctanoic acid afforded the amides **89** which, after treatment with freshly prepared hydroxylamine gave compounds **90a–h**. (Scheme 14) [33].

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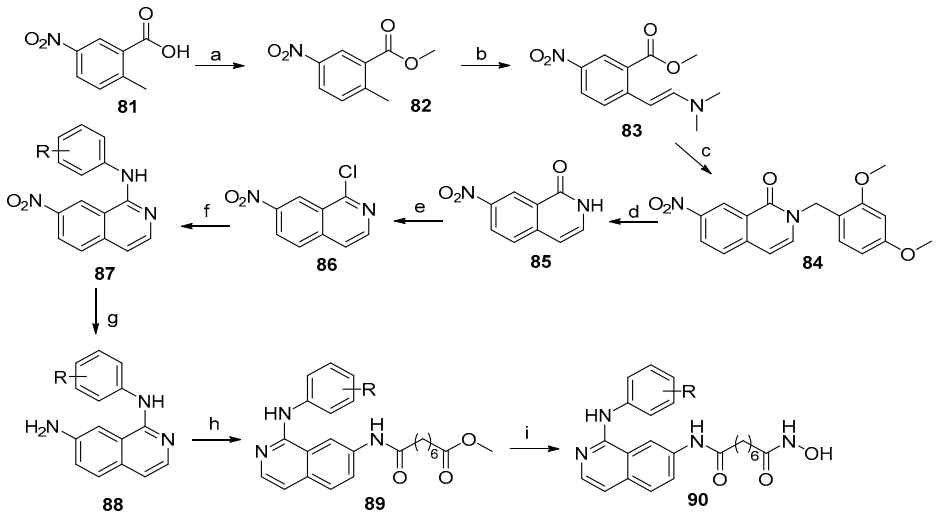

90a: R = 2-F, 4-Br; **90b**: R = 2-F, 3-Cl; **90c**: R = 4-CH_3; **90d**: R = 3, 4-Ph;
90e: R = 2-F, 4-CH_3; **90f**: R = 2-Cl, 4-CH_3; **90g**: R = 2-Br, 4-CH_3; **90h**: R = 2-F

(a) CH_3I, K_2CO_3, DMF, rt; (b) DMA-DMF, 100 °C; (c) 3,4-dimethoxylbenzylamine, toluene, 125 °C;
(d) CF_3COOH, 85 °C; (e) $POCl_3$, 100 °C;(f) Substituted aromatic amines, concd HCl, *n*-BuOH;
(g) Fe, NH_4Cl, EtOH, 50 °C; (h) EDCI, HOBT, DMF, $HOOC(CH_2)_6COOCH_3$, rt; (i) NH_2OH, MeOH, THF, 0 °C to rt.

Scheme 14. Synthetic way to SAHA analogues **89** and **90** with isoquinoline scaffold in CAP group.

Compounds **90a-h** were tested against HDAC1, HDAC3, and HDAC6 and all showed better activity than SAHA, used as positive control. The best active compound resulted to be **90c**, showing IC_{50} values 4.17 nM, 4.00 nM and 3.77 nM against HDAC1, HDAC3, and HDAC6, respectively. Furthermore, the antiproliferative activity of compounds **90a-h** against multiple myeloma cell line RPMI 8226 was tested and the more active were **90a**, **90f, 90g** with IC_{50} values 0.46 μM, 0.52 μM and 0.47 μM, respectively.

When intermediate **86** was reacted with aliphatic amines under microwaves conditions, after reduction of the nitro group to amino group and subsequent treatment as reported in steps h and i of Scheme 14, isoquinolines **91a-d** (Figure 13) were obtained.

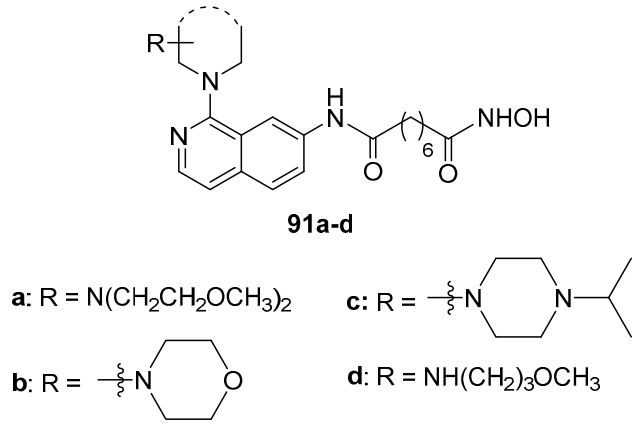

Figure 13. Isoquinoline derivatives **91a-d**.

Compound **91a** with a large substituent at C-1 position of the isoquinoline ring significantly decreased with respect to **91b-d** the inhibitory activities against HDACs as well as the proliferation of RPMI 8226 cells. Compounds **91b–d** displayed similar enzymatic activities suggesting that small aliphatic amines at the C-1 position do not significantly affect the inhibitory activities against HDACs

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enzyme *in vitro* and the proliferation of the cancer cells with respect to compounds **90a-h**, bearing an aromatic substituent at C-1 position.

Finally, to test the effects of spatial orientation of the *N*-substituents, compound **83** depicted in Scheme 14 was reacted with different aliphatic amines in toluene at 110 °C and the obtained intermediate subjected to steps g, h, and i (reported Scheme 14), thus obtaining isoquinoline-1(2*H*)-one derivatives **92a-d** (Figure 14)

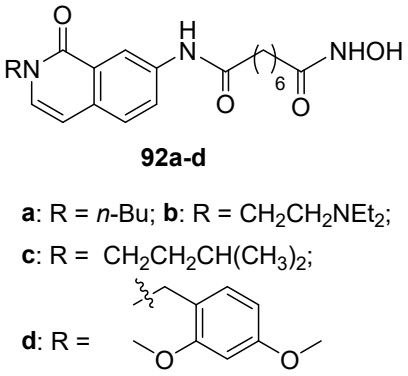

Figure 14. Isoquinoline-1(2H)-one derivatives **92a-d.**

The inhibitory activity of the series **92a-d** towards HDAC1, 3, 6 isoforms and cancer cell proliferation were evaluated. These compounds exhibited weaker inhibitory activities against HDACs indicating that the binding affinity between *N*-substituent isoquinoline-1-one scaffold and HDACs surface was decreased with respect to **91a-d** series.

In a paper focused on the study of the influence of substitution of the phenyl SAHA capping group with various substituents [34], two compounds bearing heterocyclic rings have been reported, one (**93**) with a isoquinolinyl group and the other (**94**)with a pyrimidin-2(1*H*)-one moiety (Figure 15) but both displayed a very weak antiproliferative and histone deacetylation activities.

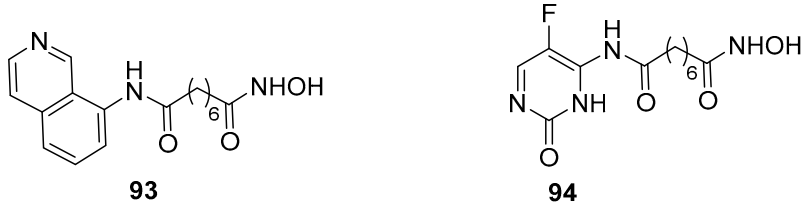

Figure 15. Isoquinoline and pyrimidinone derivatives **93** and **94**.

5.10. Quinazoline moiety in CAP group

Taking into account the known role of hydroxamic acids as HDAC inhibitors and that of quinazolines as EGFR/HER2 inhibitors, some authors designed to synthesize compounds bearing both functionalities, in order to find efficient multitarget inhibitors [35]. Thus, among various compounds, they prepared quinazoline derivative **101**, starting from **95** with the multistep procedure depicted in Scheme 15.

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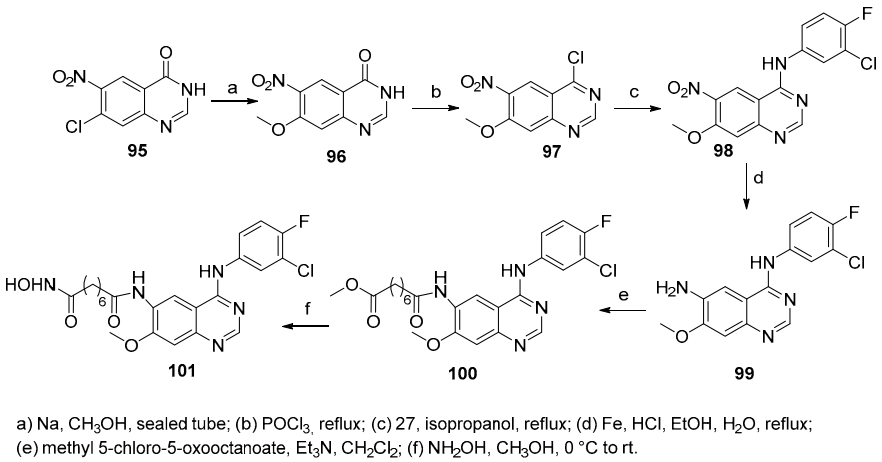


a) Na, CH_3OH, sealed tube; (b) $POCl_3$, reflux; (c) 27, isopropanol, reflux; (d) Fe, HCl, EtOH, H_2O, reflux; (e) methyl 5-chloro-5-oxooctanoate, Et_3N, CH_2Cl_2; (f) NH_2OH, CH_3OH, 0 °C to rt.

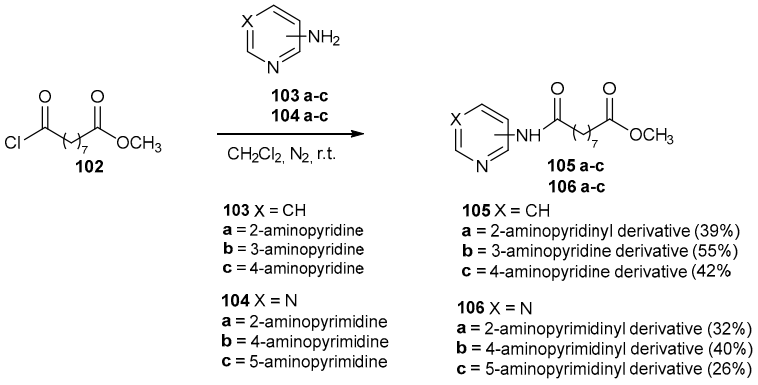
Scheme 15. Synthetic scheme to quinazoline derivative **101**.

The HDAC inhibitory activity of the quinazoline SAHA analogue **101** was determined using the Biomol Color de Lys system and the IC_{50} value was 15.3 nM. This compound showed to possess also EGFR and HER2 kinase activity.

6. Seven-carbon linker chain (7-C spacer)

6.1. Pyridine and pyrimidine moiety in CAP group

A series of compounds bearing pyridine or pyrimidine moiety bound to an azelayl scaffold through Schotten-Bauman-like reaction was synthesized as reported in Scheme 16 [36]. The series was subjected to biological screening on a panel of tumor cell lines: noticeably, none of the compounds induced cytotoxicity in the normal fibroblast cell line, while only osteosarcoma cells (U2OS) appeared to be sensitive to compound **106a**.



Scheme 16. Synthesis of pyridine and pyrimidine derivatives **105** and **106**.

Compound **106a** was studied 'in silico', by using histone deacetylases as molecular target, which revealed that it is able to interact with HDAC 7, in agreement with studies which have disclosed an unexpected function for HDAC7 in osteoclasts.

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