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Identification of a series of substituted 2-piperazinyl-5-pyrimidylhydroxamic acids as potent histone deacetylase inhibitors

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ABSTRACT

Pursuing our efforts in designing 5-pyrimidylhydroxamic acid anti-cancer agents, we have identified a new series of potent histone deacetylase (HDAC) inhibitors. These compounds exhibit enzymatic HDAC inhibiting properties with IC_{50} values in the nanomolar range and inhibit tumor cell proliferation at similar levels. Good solubility, moderate bioavailability, and promising in vivo activity in xenograft model made this series of compounds interesting starting points to design new potent HDAC inhibitors.

Two enzyme families play a crucial role in controlling the acetylation status of histones, thereby altering chromatin structure and impacting transcription processes: histones acetyl transferases (HATs) which catalyze the transfer of an acetyl group to lysine residues of chromatin and histone deacetylases (HDACs) which remove an acetyl group from those residues, thus leading to a changed chromatin structure impacting transcriptional activity.[1,2] Inhibiting the HDAC enzymes results in increasing the acetylation status of chromatin which has been linked to cell cycle arrest and apoptosis as well as down regulation of genes involved in tumor progression, invasion, and angiogenesis.[3–6] In the past decade, HDAC inhibition has emerged as an attractive target for the development of new anti-cancer agents,[7] resulting in the approval of the HDAC inhibitor (HDACi) vorinostat for Cutaneous T-Cell Lymphoma (CTCL),[8,9] and in the development of several other hydroxamic acids such as, for example, belinostat[10] or panobinostat.[11]

In recent years, a number of additional nonhistone HDAC substrates have also been identified adding growing evidence that HDACi exert their pleiotropic effect not only through epigenetic control.[12] Recent data have also demonstrated that HDACi could target novel therapeutic applications such as neurodegenerative diseases and inflammation.[13–15]

We have previously identified R306465 (Fig. 1) as a highly potent class I selective HDACi, showing oral anti-tumor activity in human xenograft-bearing athymic nude mice.[16]

In order to expand and optimize this first generation HDACi, we have explored a series of analogues on the basis that replacing the

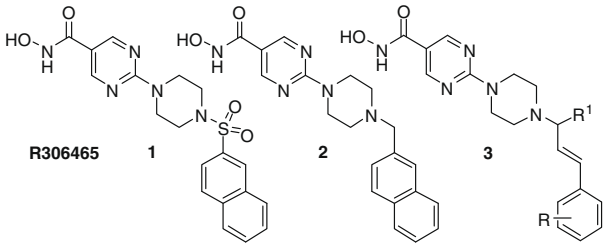

Figure 1. Johnson & Johnson first generation of HDACi: R306465 **1**, alkyl analogue **2**, and targeted compounds **3**.

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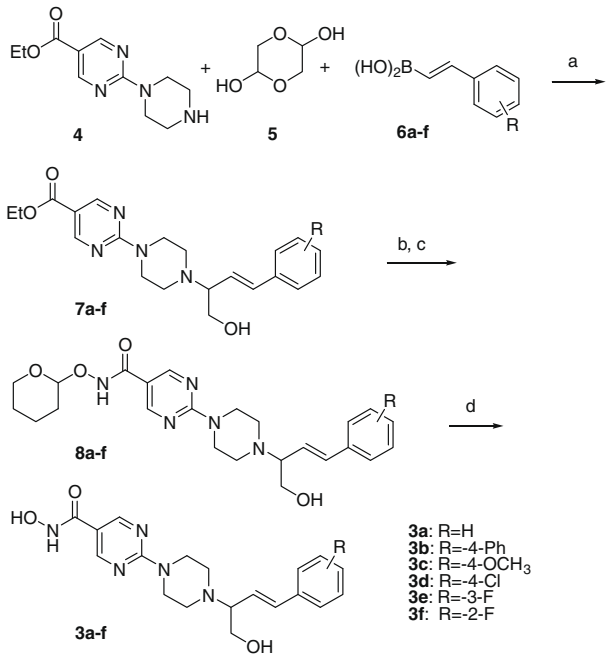
sulfonamide moiety would remove one key element hampering solubility. We first postulated that substituting the SO_2 moiety by a carbon atom would restore the basicity of the nitrogen atom and re-introduce the possibility of making salts, thus impacting compound solubility. Indeed replacement of the sulfonyl moiety by a CH_2 in **2** provided an HDACi of similar in vitro potency (see Table 1) and improved solubility (0.3 mg/mL at pH 4)[17] as compared to R306465 (<0.1 mg/mL at pH 4.8).

In addition, the piperazine secondary amine gave us the opportunity to use the Petasis multicomponent reaction[18] as an option to further introduce chemical diversity on the cap-part of this pyrimidyl-based hydroxamic acid series. First attempts were conducted using 2-phenyl-ethenylboronic acid **6a** in order to test the chemistry route as **6a** is known for its good reactivity in the Petasis reaction. Furthermore we postulate that the resulting double bond in **3** (Fig. 1) would mimic the first phenyl group of the naphtyl moiety in **2**. Finally, it enabled us to introduce a hydroxyl group to allow for further chemical modifications.

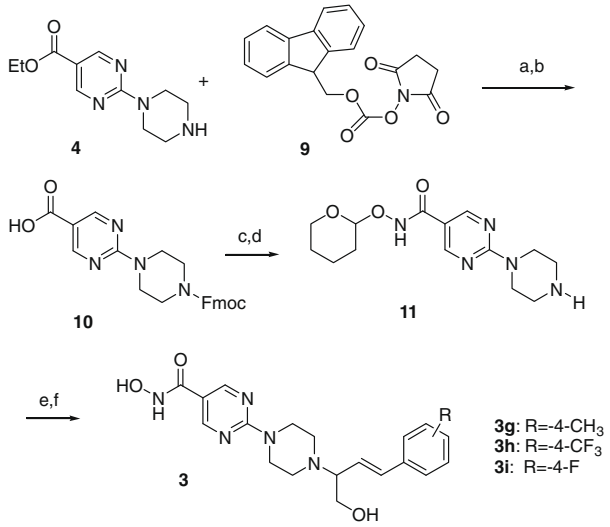
Reacting the 2-(piperazin-1-yl)pyrimidylethyl ester **4**,[19] with the glycolaldehyde dimer **5** and various boronic acids **6a–f** in ethanol at room temperature provided us with esters **7a–f**. Introduction of the hydroxamic acid moiety was then realized in three steps using O-(tetrahydro-2H-pyran-2yl)-hydroxylamine as hydroxylamine source. The first step encompasses the cleavage of the ester into acids or carboxylates, then a classical coupling reaction enabled us to introduce the protected hydroxyl amine. This allowed purification of the amides **8a–f** by chromatography on silica gel. This synthetic scheme was preferred from direct reaction of the hydroxylamine with the ester as reported previously for other HDACi,[20] as, in our hands, hydroxamic acids where difficult to separate from the corresponding acids in case of incomplete reaction. Tetrahydropyranyl (THP) protection was smoothly removed in acidic media using trifluoroacetic acid which allowed easy isolation, upon crystallization, of compounds **3a–f** as trifluoroacetates.

Enantiomeric separation of **3a** by liquid chromatography on chiral phase proved to be difficult. We therefore chose to separate the two enantiomers of the corresponding esters **7a1**, **7a2** and to introduce the hydroxamic acid moiety exactly as described above.

Alternatively, THP-protected hydroxamate **11** (Scheme 2) was prepared as a key intermediate to rapidly test piperazine substitution. In one pot, the 2-(piperazin-1-yl)pyrimidylethyl ester **4** was first saponified into its acid which was reacted with **9** to provide



Scheme 1. Reagents and conditions: (a) EtOH, rt, 42–64%; (b) LiOH or NaOH, THF, H_2O, rt, 89–96%; (c) H_2N–O–THP, 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC), 1-hydroxy-1,2,3-benzo-triazole (HOBT), NEt_3, THF, CH_2Cl_2, rt, 36–90%; (d) CF_3CO_2H, MeOH, rt, 72–84%.



Scheme 2. Reagents and conditions: (a) (i) NaOH 1 N, THF, rt, 16 h then (ii), Na_2CO_3, H_2O, rt, 5 h, crystallized in CH_3CN, 40%; (c) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 68%; (d) Piperidine, CH_2Cl_2, rt, 59%. (e) EtOH, rt, 15–29%; (f) CF_3CO_2H, MeOH, rt, 64–81%.

the Fmoc-protected piperazine acid **10** with a moderate yield of 40%. THP-hydroxylamine was then introduced as in Scheme 1 and the Fmoc protection removed by reaction with piperazine in CH_2Cl_2 at room temperature to provide **11**. Petasis multicomponent reaction could then be performed on **11**, however with low yields, and removal of the THP by trifluoroacetic acid then gave compounds **3g–i**.

All compounds were tested for their ability to inhibit histone deacetylase activity of a mixture of HDAC enzyme obtained from a nuclear HeLa cell extract and to inhibit ovarian carcinoma (A2780) cell proliferation.[21,22]

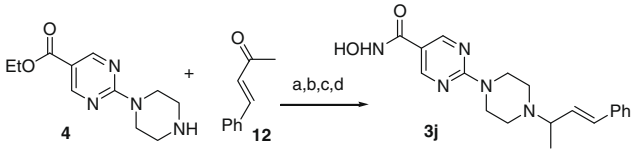
Table 1
Enzymatic and antiproliferative potency of compounds **3a–i**

Compounds	R	HDAC IC_{50}, nM[a] ± SD	Cell proliferation A2780 IC_{50}, nM[a] ± SD
1	NA	6 ± 2	39 ± 17
2	NA	1.5 ± 0.2	66 ± 8
3a	H	2.0 ± 0.1	25 ± 8
3b	–4–Ph	0.9 ± 0.1	29[b]
3c	–4–OCH_3	0.95 ± 0.1	18 ± 1
3d	–4–Cl	1.2 ± 0.2	17 ± 6
3e	–3–F	1.2 ± 0.3	36 ± 14
3f	–2–F	2[b]	<30[b]
3g	–4–CH_3	1.0 ± 0.1	18 ± 2
3h	–4–CF_3	2.1 ± 0.3	65 ± 18
3i	–4–F	1.2 ± 0.3	32 ± 12
3a1	H[c]	1.0 ± 0.1	40 ± 5
3a2	H[d]	1.8 ± 0.3	30 ± 7
3i1	–4–F[c]	1.05 ± 0.1	27 ± 13
3i2	–4–F[d]	1.2 ± 0.1	31 ± 12

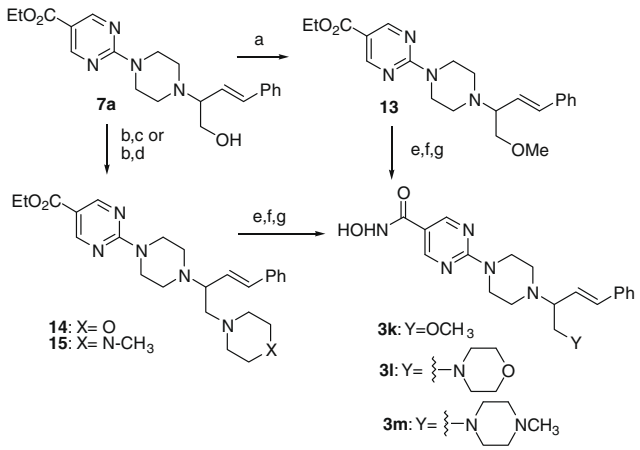
NA = not applicable.
[a] Mean results from experiments performed in triplicate.
[b] Single experiment.
[c] (+) enantiomer.
[d] (−) enantiomer.

Results from Table 1 above clearly show that replacement of the naphtalene-2-sulfonyl moiety by phenylbutenol group provided compounds of similar in vitro potency. Substitution of the phenyl ring does not seem to have significant impact on enzymatic or anti-proliferative potency despite introduction of electron-withdrawing or electron-donating substituent. Since hydroxamic acids are very efficient Zn^{2+} chelators, this preliminary results may indicates that additional interactions generated by the phenyl ring, probably orientated towards the solvent region of the tube-shaped catalytic site described for a bacterial histone deacetylase by Finnin et al.,[23] are not key drivers of potency. It is also interesting to note that stereochemistry of the carbon linked to the piperazinyl moiety has virtually no influence on in vitro potency. Thermodynamic solubility of compound **3a** (>10 mg/mL at pH 4.6 and 1.1 mg/mL at pH 7.4) was greatly increased as compared to R306465 (<0.1 mg/mL at pH 4.8 and 7.7), thus indicating achievement of our first goal. This property was also confirmed with compounds **3i1**, **3i2**, and **21**, all showing a solubility >5 mg/mL at pH 4. As introduction of the hydroxyl group was not disturbing interaction with the catalytic site we were interested in further investigation structure–activity relationships (SAR) at that position and especially in testing introduction of solubility enhancing substituents. For the sake of comparison we replaced the hydroxyl by an hydrogen (compound **3j**) using reductive amination of **4** with commercially available butanone **12**, followed by introduction of the hydroxamic acid moiety in three steps as described before (Scheme 3). Compound **3j** showed a similar potency as **3a** (Table 2) confirming that the hydroxyl moiety is not making key interactions with the catalytic site.

Alkylation of ester **7a** with methyliodide provided methoxy intermediate **13** and further methoxy analogue **3k** showing a similar potency as **3a** (Scheme 4). Bulkier aminogroups were introduced (compounds **3l** and **3m**) by substitution of the methanesulfonic acid ester obtained by reaction of methanesulfonylchloride onto **7a**, followed by introduction of the hydroxamic acid moiety on the resulting esters **14** and **15** (Scheme 4). Compounds **3l** and **3m** showed ~10-fold drop in antiproliferative IC_{50} as compared with **3a** (Table 2).



Scheme 3. Reagents and conditions: (a) 4-phenylbut-3-ene-2-one, $Ti(OEt)_4$, 1,2-dichloroethane, rt, 8%; (b) NaOH, EtOH, H_2O, 80 °C; (c) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 70%; (d) CF_3CO_2H, MeOH, rt, 44%.

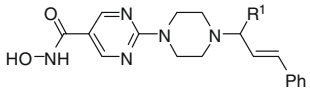


Scheme 4. Reagents and conditions: (a) NaH, CH_3I, THF, 0 °C rt, 34%; (b) MsCl, NEt_3, CH_3CN, 5 °C rt; (c) morpholine, K_2CO_3, CH_3CN, 80 °C, 84%; (d) *N*-methylpiperazine, K_2CO_3, CH_3CN, 80 °C, 33%; (e) LiOH, THF, H_2O, rt, 83%-quantitative; (f) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 35–89%; (g) CF_3CO_2H, MeOH, rt, 44–57%.

Petasis multicomponent reaction is also a powerful tool to prepare alpha-amino acids using glyoxalic acid and boronic acids. We could therefore rapidly access amides **3n** and **3o** using the pathway depicted in Scheme 5. However for these two compounds we also noticed a ~5-fold drop in antiproliferative activity. For compounds **3l–o**, enzymatic potency in inhibiting a mixture of HDAC enzyme, obtained from a nuclear HeLa cell extract, stayed comparable to **3a** with the exception of **3l** being slightly weaker (Table 2) but still in the nanomolar range.

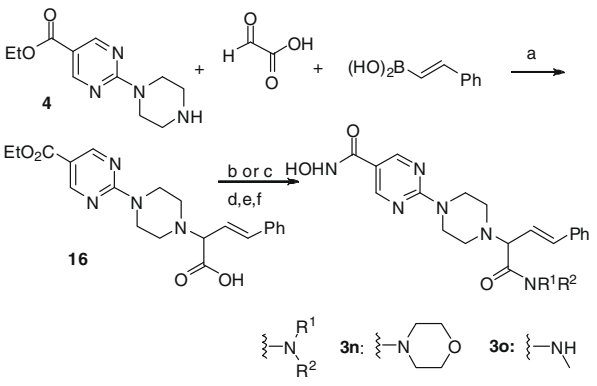
As cyclic amines such as **3l** and **3m** or amides were not suitable, we thought to replace the hydroxyl group of **3a** by a small, protonable amino moiety. We therefore prepared the phtalimide precursor **17** by Mitsunobu reaction on ester **7a** (Scheme 6). However reaction of **17** with hydrazine in the classical conditions did not give us the awaited 4-phenyl-2-piperazinyl-but-3-enylamine **18** but rather the 3-phenyl-1-(piperazinylmethyl)-allylamine derivative **19**. Formation of **19** might be the result of the rearrangement of a transient aziridinium similarly as already proposed on piperidines bearing beta-amino alcohol chain by Frizzle et al.[24] We took

Table 2
Evaluation of the importance of the hydroxyl moiety (R = H)

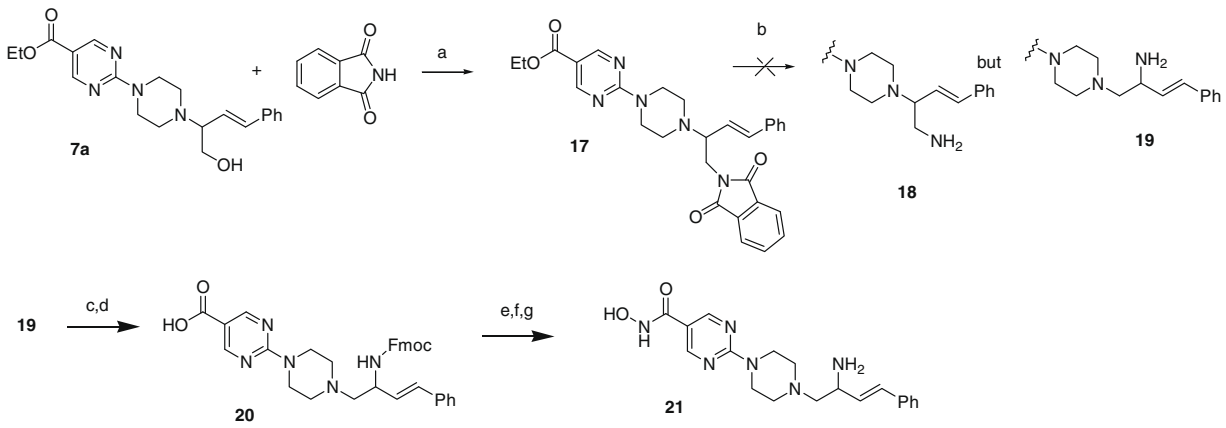


Compounds	R^1	HDAC IC_{50}, nM[a] ± SD	Cell proliferation A2780 IC_{50}, nM[a]
3j	$-CH_3$	1.7 ± 0.2	20[b]
3a	$-CH_2OH$	2 ± 0.1	25 ± 8
3k	$-CH_2OCH_3$	1.5 ± 0.2	45 ± 11
3l	$-CH_2$–1-morpholino	11 ± 2	315 ± 154
3m	$-CH_2$–4-methylpiperazin–1yl	6.9 ± 0.4	392 ± 189
3n	–C(O)-morpholine	7 ± 1	124 ± 26
3o	–C(O)NHMe	3.3 ± 0.1	159 ± 11
21	NA	3.8 ± 0.7	281 ± 137

NA = not applicable.
[a] See Table 1.
[b] Single experiment.



Scheme 5. Reagents and conditions: (a) glyoxalic acid, 2-phenyl-ethenylboronic acid, EtOH, 60 °C, 87%; (b) morpholine, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 38%; (c) CH_3NH_2·HCl, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 57%; (d) LiOH or NaOH, THF, H_2O, rt, 71–73%; (f) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 71–74%; (g) CF_3CO_2H, MeOH, rt, 75–87% .

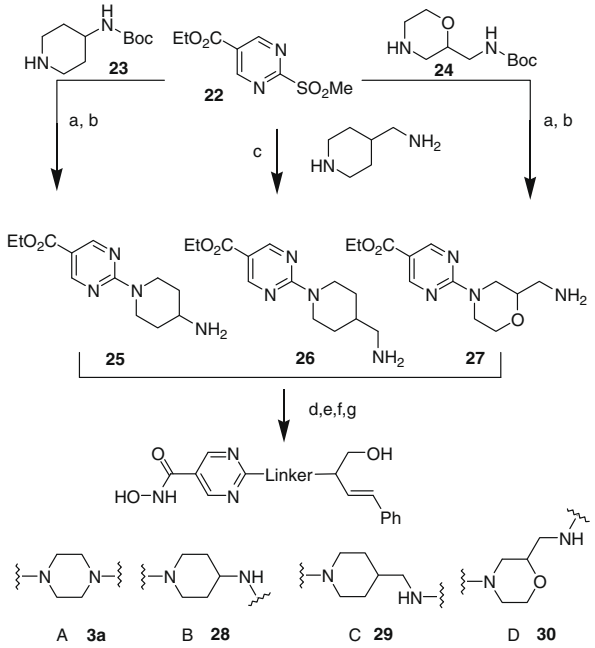


Scheme 6. Reagents and conditions: (a) 1*H*-isoindole-1,3(2*H*)-dione, P(*n*Bu)$_3$, DIAD, CH_2Cl_2, rt, 52%; (b) NH_2NH_2·HBr, EtOH, rt, 98%; (c) LiOH, THF, H_2O, rt; (d) 1[[(9*H*-fluoren-9-ylmethoxy)carbonyl]-oxy]-2,5-pyrrolidinedione, THF, rt, 82%; (e) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 76%; (f) piperidine, CH_2Cl_2, rt, 56%; (g) CF_3CO_2H, MeOH, rt, 87%.

this opportunity to prepare hydroxamic acid **21**, first protecting the amino moiety by Fmoc and then introducing THP-protected hydroxylamine after saponification of the ester function. Compound **21** displayed similar HDAC inhibiting potency on enzyme as **3a** showing that variation of linker size could be tolerated. This observation prompted us to examine the influence of longer diamine linkers (Scheme 7 and Table 3).

Esters **25**, **26**, and **27** were, respectively, prepared by condensation of boc-protected 4-aminopiperidine, 4-(aminomethyl)piperidine, and boc-protected-2-amino-methylmorpholine onto 2-methanesulfonylpyrimidine-5-carboxylic acid ethyl ester **22**.[19] Then Petasis reaction was performed onto **25–27** and hydroxamic acid functionality was introduced as described in Scheme 1 (Scheme 7).

All ligands were found to be equipotent with regard to enzymatic activity whereas Linker B (4-aminopiperidine) and linker D (2-methylamino-morpholine) were slightly less potent in the cellular assay.



Scheme 7. Reagents and conditions: (a) **23** or **24**, K_2CO_3, CH_3CN, rt, 53–75%; (b) CF_3CO_2H, CH_2Cl_2, rt, 35–96%; (c) 4-aminomethyl-piperidine, K_2CO_3, CH_3CN, rt, 33%; (d) **5** + **6a**, EtOH, rt, 47–73%; (e) NaOH or LiOH, THF, H_2O, rt; (f) H_2N–O–THP, EDC, HOBT, NEt_3, THF, CH_2Cl_2, rt, 4–54%; (g) CF_3CO_2H, MeOH, rt, 72–85%.

Table 3
Evaluation of several linker moieties (R = H)

Compounds	Linker	HDAC IC_{50}, nM[a]	Cell proliferation A2780 IC_{50}, nM[a]
3a	A	6 ± 2	25 ± 8
28	B	7 ± 5	219[b]
29	C	1.5 ± 0.2	47[b]
30	D	8[b]	295[b]

[a] See Table 1.
[b] Single experiment.

As demonstrated by the results from Tables 1–3, a wide range of Petasis substitution on the 2-piperazinyl-5-pyrimidyl-hydroxamic acid moiety gave birth to a novel series of potent in vitro HDAC inhibitors. Compounds **3a** and **3i2** showed an acceptable pharmacokinetic profile (see Table 4) with a bioavailability in rat of 19–22%, similar to the bioavailability of **1** (R306465).

Compound **3a**, **3i1**, and **3i2** were evaluated in an animal model allowing noninvasive real-time evaluation of the response to HDAC inhibitors using whole-body imaging.[25] Human A2780 ovarian carcinoma cells were engineered to express ZsGreen fluorescent protein under control of the $p21^{waf1,cip1}$ promoter, sub-cutaneously grafted to athymic nude mice, and induction of fluorescence in vivo was measured after 3 days of treatment. Results are reported as increased fluorescence intensity as compared to R306465 tested in same conditions and normalized to 1 (Fig. 2). This allowed rapid comparison of compounds **3a**, **3i1**, and **3i2** with R306465 as regards to their anti-tumor potency. Preliminary results indicate that the three compounds may possess an in vivo efficacy in that model superior to R306465. Compounds **3i1** and **3i2** showed also similar increase in fluorescence intensity (3–4-fold compared to R305465) confirming that their stereochemistry had no influence. **21** was chosen as a representative of compounds possessing decreased antiproliferative activity as compared to R306465 and showed reduced fluorescence than R306465.

Compound **3i2**, has been tested towards a broad panel of recombinant HDAC enzymes in vitro and compared to compound **1** (R306465) (see Table 5).

With the exception of HDAC4, 10 and 11 where **3i2** showed a 10–50-fold drop in inhibiting potency compared to R306465, the overall profile of both compounds are very similar, even if **3i2** seems slightly less potent (1–3-fold). Compound **3i2** showed activity towards all HDAC enzyme tested with some specificity for class I inhibition, a class of HDACs where activity is key for uncontrolled proliferation of cancer cells.[26] Lowest in vitro potency was observed towards HDAC4, 6, and 7.

Table 4
Rat DMPK properties of compounds **3a** and **3i2**

Compounds	$AUC_{0\text{-}inf}$[a] (ng h/mL) (IV)	CL[a] (L/h/kg) (IV)	Vd_{ss}[a] (L/kg) (IV)	$T_{1/2}$ (h) 2 h to 8 h[a] (IV)	$AUC_{0\text{-}inf}$[b] (ng h/mL) (po)	*F* (%)
1	2654	0.8	0.6	1.5	3381	20
3a	413	5.9	5.8	2.6	316	19
3i2	521	4.6	1.6	0.8[c]	466	22

[a] Study in male Sprague-Dawley rats dosed at 2.5 mg/kg in water at pH 4.4.
[b] Study in male Sprague-Dawley rats dosed at 10 mg/kg in water at pH 4.5.
[c] Half life determined from 1 h till 4 h post dose.

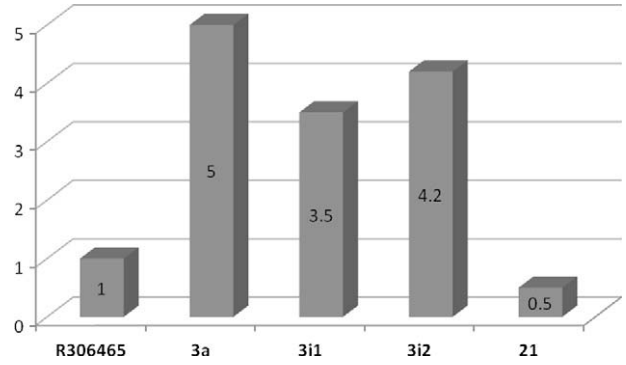


Figure 2. Folds increase of fluorescence for compounds **3a**, **3i1** and **3i2** as compared to R306465 (normalized at 1) following 3 days dosing po at 40 mg/kg in athymic nude mice bearing s.c. A2780-p21^{waf1,cip1}ZsGreen ovarian xenograft.

Table 5
Comparison of activity towards all HDAC enzymes[21]

Class	HDAC	R306465 IC_{50}, nM[a] ± SD	**3i2** IC_{50}, nM[a] ± SD
I	1	2.5 ± 0.4	7.1 ± 05
I	2	9 ± 3	17.9 ± 2.5
I	3	9 ± 3	30 ± 3
I	8	27 ± 13	30 ± 2
IIa	4	4.6 ± 0.35	207 ± 23
IIa	5	7.1 ± 2.3	21.8 ± 2.6
IIa	7	46 ± 8	102 ± 11.5
IIa	9	28 ± 1	40.7 ± 3.2
IIb	6	66 ± 4	224 ± 33
IIb	10	4.3 ± 1.1	42.6 ± 1.5
IV	11	2.1 ± 0.6	20.7 ± 1.4

[a] Mean data from experiments performed in triplicate.

In conclusion, we have identified a series of novel 2-piperazinyl-5-pyrimidyl-hydroxamic acids HDACi that are characterized by high potency on HDAC HeLa cellular extract and cellular antiproliferation assay and have demonstrated improved solubility as compared to our starting molecule **1**. First in vivo evaluation of leads **3a** and **3i2** indicates superior potential as compared to R306465. These results warrants more in depth evaluation of this series.

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