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Discovery of Highly Selective and Potent HDAC3 Inhibitors Based on a 2-Substituted Benzamide Zinc Binding Group

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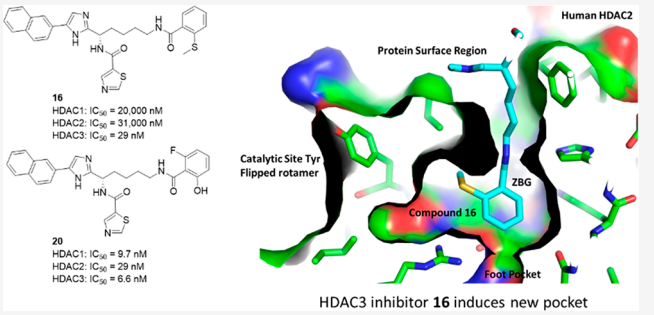
ABSTRACT: The selectivity of histone deacetylase inhibitors (HDACis) is greatly impacted by the zinc binding groups. In an effort to search for novel zinc binding groups, we applied a parallel medicinal chemistry (PMC) strategy to quickly synthesize substituted benzamide libraries. We discovered a series containing 2-substituted benzamides as the zinc binding group which afforded highly selective and potent HDAC3 inhibitors, exemplified by compound **16** with a 2-methylthiobenzamide. Compound **16** inhibited HDAC3 with an IC_{50} of 30 nM and with unprecedented selectivity of >300-fold over all other HDAC isoforms. Interestingly, a subtle change of the 2-methylthio to a 2-hydroxy benzamide in **20** retains HDAC3 potency but loses all selectivity over HDAC 1 and 2. This significant difference in selectivity was rationalized by X-ray crystal structures of HDACis **16** and **20** bound to HDAC2, revealing different binding modes to the catalytic zinc ion. This series of HDAC3 selective inhibitors served as tool compounds for investigating the minimal set of HDAC isoforms that must be inhibited for the HIV latency activation in a Jurkat 2C4 cell model and potentially as leads for selective HDAC3 inhibitors for other indications.



HDAC3 inhibitor **16** induces new pocket

KEYWORDS: *HDAC, HDAC3, inhibitors, 2-methylthiobenzamide, induced pocket*

Histone deacetylases (HDACs) and histone acetyl transferases (HATs) are two enzyme families which regulate histone acetylation level, causing structural changes to chromatin, hence controlling the transcription of DNA. Histone acetylation diminishes the charge−charge interaction between histones and DNA, and this relaxed conformation induces gene expression.[1,2] Eighteen unique isoforms of HDACs have been discovered and are separated into four distinct classes.[3] The Zn^{2+}-dependent isoforms are class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 7, 9, and 10), and class IV (HDAC11); the NAD^{+}-dependent isoforms are the class III Sirtuins (SirT1−7). HDACs have been the targets for various therapeutics. Small molecule HDAC inhibitors (HDACis) were reported to demonstrate capabilities to regulate gene transcription, regulate cell cycle progression, and induce differentiation and/or apoptosis in cancer cells with a few HDACis being approved for the treatment for oncology.[4−7] HDACis have also been broadly explored as HIV latency-reversing agents (LRAs) to activate the latently infected $CD4^{+}$ T cells. Clinically, several HDACi drugs approved for the treatment of cancer have been shown to induce HIV RNA transcription in resting $CD4^{+}$ T cells from the combination antiretroviral therapy (cART) suppressed HIV patients.[8−10] This HIV latency reversal activity by HDACis was thought to be driven by HDAC3 inhibition,[11,12] which led to the investigation of HDAC3 selective inhibitors.

In the attempt to search for HDAC isoform selective inhibitors, we recently reported two series of HDACs 1, 2, 3 inhibitors: the ethyl ketone series exemplified by compound **1** and the aryl ketone series exemplified by compound **2** (Figure 1).[13−15] These class I HDAC inhibitors showed efficacy for HIV latency activation in a Jurkat 2C4 cell model and induction of HIV *gag* p24 protein in patient latent $CD4^{+}$ T cells.[14] In the process of determining the required HDAC subtype inhibition for HIV latency activation, we demonstrated that inhibition of only HDAC1, 2 by compound **3**[16] did not show any cellular activity in the HIV latency activation.[13] We invested our effort to search for highly selective inhibitors of HDAC3. Due to the high homology between HDACs 1 and 2, it has proven very difficult to find a selective inhibitor that

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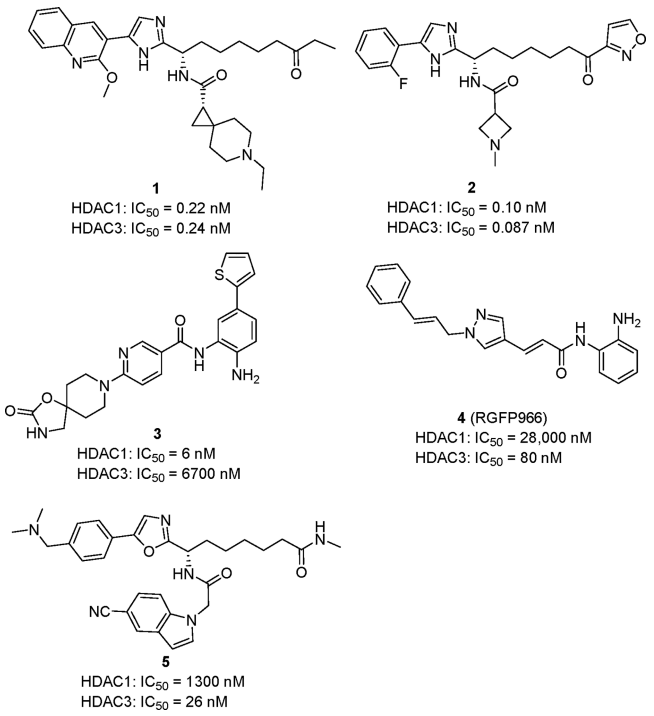


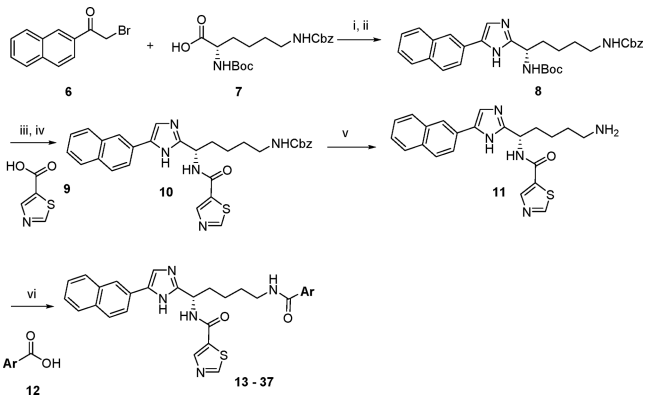
Figure 1. HDAC selective inhibitors with various zinc binding groups.

distinguishes between these two isoforms. However, there are many reports of HDAC3 selective inhibitors, especially the series of N-(2-aminophenyl)carboxamides represented by compound **4** (RGFP966).[17] A close analog **5** to our reported ethyl ketone and aryl ketone HDAC inhibitors with a methylcarboxamide was described to have improved HDAC3 inhibition compared to **4**, with 50-fold selectivity over HDAC1.[18] In this letter, we report our work, taking advantage of a parallel medicinal chemistry (PMC) strategy to quickly synthesize and screen a series of HDAC inhibitors. This effort resulted in the discovery of a series of highly potent and selective HDAC3 inhibitors based on 2-substituted benzamides as the zinc binding group.

Parallel medicinal chemistry (PMC) has been widely applied in drug discovery across the industry. Recent technology advancements in automation for synthesis and purification, data analysis, modeling, and in-silico prediction have driven the discovery chemistry effort in our company to become more data guided, knowledge based, and highly collaborative with extensive application of PMC. A PMC library was designed using the common intermediate **11** to carry out an amide coupling using selected carboxylic acids which also contain a potential second zinc binding group in addition to the amide carbonyl. We hypothesized that a zinc binding group capable of bidentate coordination with the HDAC zinc ion might enhance potency; however, it was less clear what, if any, role the zinc binding group may play in HDAC isoform selectivity.

Synthesis of the common intermediate **11** is depicted in Scheme 1, which started by reacting a bromoketone **6** and the protected lysine **7** to form a ketone ester intermediate in DMF with cesium carbonate as base. The imidazole ring in **8** was then prepared by condensation of this ketone ester intermediate with ammonium acetate in toluene under heating. The Boc protection in **8** was selectively removed by the treatment of trifluoroacetic acid in dichloromethane, and the freed amine was then coupled with thiazol-5-carboxylic acid **9**

Scheme 1[a]



[a]Reagents and conditions: (i) Cs_2CO_3, DMF, rt, 1 h, 97%; (ii) NH_4OAc, toluene, 110 °C, 18 h, 87%; (iii) TFA/DCM, rt, 99%; (iv) HATU, DIEA, DCM, rt, 16 h, 98%; (v) HBr/AcOH, rt, 16 h, 93%; (vi) HATU, TEA, DMF, rt, 16 h.

to provide an excellent yield of **10**. The benzylcarbamate protection was successfully removed after **10** was stirred in a solution of hydrobromide in acetic acid at room temperature overnight to generate the common intermediate **11**. The PMC focus library was designed using the common intermediate **11** with an amide coupling reaction to selected monomer of commercially available or readily prepared benzoic acids with 2-substituents which could potentially coordinate with the HDAC enzyme zinc ion as a bidentate chelation along with the amide carbonyl. These focus library sizes ranged from 24−100 monomers, and the optimization of the structure activity relationship (SAR) was carried out in six rounds of libraries with each round applying different substrates of substituted benzoic acids, pyridinyl carboxylic acids, and 5- and 6-member heteroaryl carboxylic acids.

All compounds from the focus library were tested in *in vitro* HDAC inhibition assays against HDACs 1, 2, 3, 6, or 8, while at the same time each compound was evaluated for Latency Reversal Activity (LRA) in the Jurkat 2C4 T-cell HIV latency model cell line.[19] The representative compounds are listed in Table 1. We discovered a series of highly selective HDAC3 inhibitors from the PMC libraries. Subtle variation of the substituents led to dramatic changes in subtype selectivity. The 2-methylamino benzamide **13** is a highly HDAC3 selective inhibitor with an IC_{50} of 41 nM toward HDAC3 and selectivity against other subtypes of over 366-fold (HDAC1/HDAC3). The HDAC3 selective inhibitor **13** showed no LRA activity in the 2C4 Jurkat T-cell model system. When the methyl on the aniline is absent, **14** as a 2-amino-6-fluorobenzamide became a HDAC1, 2, 3 inhibitor with only 4-fold selectivity between HDAC1 and HDAC3 inhibition activity. The HDAC1, 2, 3 inhibitor **14** demonstrated comparable LRA activity to that which we observed in the previous reported class I HDAC inhibitors. However, the 3-aminopyridinyl-2-carboxamide **15** dramatically reduced the inhibition of all HDAC enzymes except HDAC3 with still an IC_{50} of 1,100 nM. The selectivity of HDAC3 over HDAC1 for **15** is greater than 40-fold, although it is much less active. The most selective HDAC3 inhibitor discovered from this series is **16** with the 2-methylthiobenzamide. This compound afforded an IC_{50} of 29 nM toward HDAC3 and 690-fold selectivity over HDAC1. The removal of the methyl on the sulfur in **17** significantly reduced the HDAC3 inhibition; however, it also brought back

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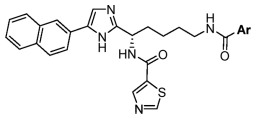
Table 1. PMC Library with Various 2-Substituted Benzamide Zinc Binding Groups



Ar Comp #	HDAC1 IC_{50} (nM)[a]	HDAC2 IC_{50} (nM)	HDAC3 IC_{50} (nM)	HDAC6 IC_{50} (nM)	HDAC8 IC_{50} (nM)	HDAC1/HDAC3 Selective folds	Cellular Assay EC_{50} (nM) % Act@Max dose
 **13**	15,000	>45,000	41	>45000	>45000	366	>40,000
 **14**	40	61	9.3	>45,000	>45,000	4	840 90%
 **15**	>45,000	>45,000	1,100	>45,000	>45,000	>40	>40,000
 **16**	20,000	31,000	29	>45,000	>45,000	690	>40,000
 **17**	15,000	18,000	1,200	NT[b]	NT	13	>40,000 8.1%
 **18**	6,600	7,700	150	>45,000	<45,000	44	>40,000
 **19**	8.9	29	12	>45,000	13,000	1	620 84%
 **20**	9.7	29	7.6	9,000	>45,000	1	324 67%
 **21**	29,000	40,000	420	>45,000	>45,000	69	>40,000
 **22**	>45,000	>45,000	810	>45,000	>45,000	>56	>40,000
 **23**	800	1,900	1,000	NT	NT	1	>40,000
 **24**	200	760	43	>45,000	>45,000	5	.40,000 13%
 **25**	>45,000	>45,000	24,000	NT	NT	>2	>40,000
 **26**	52	70	31	>45,000	3,400	1.7	22,000 73%

[a]Reported values are the average of ≥2 independent measurements with standard deviation less than 3-fold of the reported mean. [b]NT = not tested.

some inhibition activity toward HDAC1 and 2 for much less HDAC3 selectivity. The 2-methyl ether benzamide **18** also showed HDAC3 selectivity with 44-fold over HDAC1, although it is 5-fold less active than the methylthio benzamide

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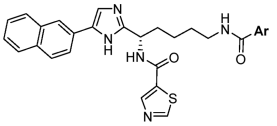
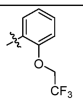
16. On the other hand, when this methyl ether is replaced by a hydroxyl, compound **19** became a HDAC1, 2, 3 inhibitor with high inhibition potencies without any HDAC3 selectivity. The 6-fluoro substituent did not have much impact on the potency and selectivity for both phenol (**20**) and methyl ether (**21**). Again interestingly, when the two ortho positions of the benzamide were substituted by hydroxy and methyl ether in **22**, it is a HDAC3 selective inhibitor like the methyl ether benzamide **18**. Both 4- and 5-methyl ether-2-hydroxybenzamides (**23**, **24**) exhibited the same inhibition pattern of 2-hydroxybenzamide **19** as HDAC1, 2, 3 inhibitors. The 3-methyl ether-2-hydroxybenzamide (**25**) lost all inhibition activity to all HDACs probably due to unfavorable interactions in the binding pocket. Unlike the aminopyridine compound **15**, the 3-hydroxypyridinyl-2-carboxamide compound **26** was a HDAC1, 2, 3 inhibitor. Consistently, all the HDAC3 selective inhibitors in Table 1 displayed no activity in the cellular assay to activate the HIV latency, while all HDAC1, 2, 3 inhibitors showed a certain level of potency and activation in the cellular assay.

The first round PMC focus library led to the discovery of a series of highly HDAC3 selective inhibitors with 2-substituted benzamide. We noticed that when the 2-substituents were good zinc binding groups such as hydroxy and amino, the compounds were mostly likely HDAC1, 2, 3 inhibitors. However, when the 2-substituents were not good zinc binders and even nitrogen, oxygen, and sulfur with substituents, the compounds were HDAC3 selective inhibitors with various potencies and selectivities. To further explore the SAR, PMC focus libraries were designed using benzamide with bulkier 2-substituents to investigate the impact on HDAC3 inhibition and selectivity. Representative compounds are listed in Table 2. HDAC5, 6 inhibition and cellular assay data were not listed since these compounds were not active against those isoforms. The 2-dimethylamino **27** and the 2-N-pyrolyl **28** were much less active in HDAC3 inhibition and less HDAC3 selective compared to the methylamino compound **13**. The larger 2-ethylthio substituent (**29**) also led to a 20-fold loss of HDAC3 inhibition potency relative to the methylthio compound **16**, though the HDAC3 selectivity is probably maintained since **29** was completely inactive in HDAC1, 2 inhibition. The electron-withdrawing and bigger substituents trifluoromethyl ether (**30**) and trifluoroethyl ether (**31**) led to a significant loss of HDAC3 inhibition activity compared to the methyl ether substituent (**18**), although **30** and **31** maintained the HDAC3 selectivity. The small electron-withdrawing substituents 2-chloro (**33**), cyano (**34**), and sulfinyl (**35**) also led to a loss of HDAC3 inhibition but still retain certain levels of HDAC3 selectivity. The 2-alkyl substituents such as 2-ethyl (**35**), 2-cyclopropyl (**36**), and the fused dihydroindene carboxamide (**37**) all demonstrated high HDAC3 selective inhibition. The 2-cyclopropylbenzamide **36** had an HDAC3 inhibition activity with an IC_{50} of 170 nM and 147-fold selectivity over HDAC1. The cyclopropyl (**36**) may have the shape fits best to the pocket compared to ethyl (**35**) and fused cyclopentyl (**37**). This 2-substituted benzamide SAR confirmed that installing nonzinc binding substituents on the 2-position of the benzamide led to HDAC3 selective inhibitors with the methylthio compound **16** as the most potent HDAC3 selective inhibitor.

We profiled the HDAC3 selective inhibitor **16** against all subtypes of histone deacetylases 1 to 11. Besides HDAC1 and 2, **16** also showed weak inhibition of HDAC10 (IC_{50} = 10,000 nM) and HDAC11 (IC_{50} = 2,000 nM), although this is 69-fold less active relative to HDAC3.

Table 2. PMC Library with Various 2-Substituted Benzamide Zinc Binding Groups



Ar Comp #	HDAC1 IC_{50}(nM)[a]	HDAC2 IC_{50} (nM)	HDAC3 IC_{50} (nM)	HDAC1/3 Selectivity
 **27**	25,000	>45,000	5,000	5
 **28**	26,000	>45,000	1,900	14
 **29**	>45,000	>45,000	670	>67
 **30**	>45,000	>45,000	3,900	>12
 **31**	>45,000	>45,000	12,000	>4
 **32**	>45,000	>45,000	760	>59
 **33**	>45,000	>45,000	7,300	>6
 **34**	25,000	43,000	7,300	3
 **35**	>45,000	>45,000	1,200	>37
 **36**	25,000	29,000	170	147
 **37**	>45,000	>45,000	1,800	>25

[a]Reported values are the average of ≥2 independent measurements with standard deviation less than 3-fold of the reported mean.

The HDAC3 selective inhibitor **16** was characterized in a global counter screen and pharmacokinetic (PK) tests (Table 3). Compound **16** showed very strong activity toward the potassium ion channel with an hERG IC_{50} of 16 nM.[20] In the pharmacokinetic profiling when dosed at 0.05 mg/kg intravenously (IV) in rats, **16** did not show any drug concentration above the detectable level, presumably due to its high clearance. A similar SAR strategy to the other imidazole core HDAC inhibitors we reported[13−15,21] was applied here to the

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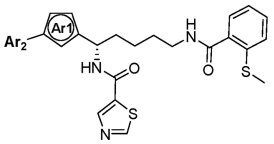
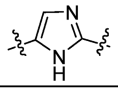
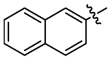
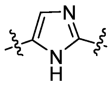
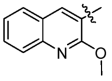
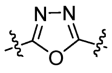
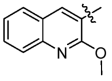
Table 3. HDAC Screen for HDAC3 Selective Inhibitor 16

Comp#	HDAC1 IC_{50} (nM)	HDAC2 IC_{50} (nM)	HDAC3 IC_{50} (nM)	HDAC4 IC_{50} (nM)	HDAC5 IC_{50} (nM)	HDAC6 IC_{50} (nM)	HDAC7 IC_{50} (nM)	HDAC8 IC_{50} (nM)	HDAC9 IC_{50} (nM)	HDAC10 IC_{50} (nM)	HDAC11 IC_{50} (nM)
16	20,000	31,000	29	>40,000	>40,000	>45,000	>40,000	0.45,000	>40,000	10,000	2,000

Table 4. SAR of HDAC3 Selective Inhibitors to Improve hERG Inhibition and Pharmacokinetic Profile



Comp#	Ar1	Ar2	HDAC1 IC_{50} (nM)	HDAC2 IC_{50} (nM)	HDAC3 IC_{50} (nM)	hERG IC_{50} (nM)	IV PK[a] (0.05 mg/kg)
16			20,000	31,000	29	16	BLQ[b]
38			>45,000	>45,000	114	1,329	AUC = 0.049 μM*h Cl = 29 mL/min/kg Vd = 0.86 L/kg $T_{1/2}$ = 0.32 h
39			>45,000	>45,000	857	13,000	Cmax = 0.029 μM*h Cl = 49 mL/min/kg Vd = 1.3 L/kg $T_{1/2}$ = 0.35 h

[a]Winstar Han rats were the PK test species, dosing vehicle = DMSO/PEG400/water: 20/60/20. [b]BLQ = Below the lower limit of quantification of 0.00220 μM (i.e., 1.00 ng/mL).

HDAC3 selective inhibitor series, to improve the off-target selectivity and PK profile (Table 4). Replacing the 2-naphthalenyl with 2-methoxy-3-quinolinyl in compound **38** retained the HDAC3 selectivity and inhibition and at the same time, significantly reduced the hERG activity[22] with an IC_{50} of 1,329 nM. HDAC3 inhibitor **38** also showed limited drug exposure of AUC 0.049 μM·h in the PK test[23] when dosed IV at 0.05 mg/kg. Upon further modification by replacing the imidazole with a 1,3,4-oxodiazole in **39**, the HDAC3 inhibition was weaker with an IC_{50} of 857 nM. The hERG off target activity is also much weaker for **39**. Compound **39** also demonstrated a similar PK profile to **38** in the IV PK test in rats. The SAR here proved that this HDAC3 inhibitor lead can be optimized to improve the off-target selectivity and the pharmacokinetic profile.

To further investigate the observed HDAC isoform selectivity of 2-substituted benzamide HDACis, we determined the X-ray crystal structures of human HDAC2 bound to compounds **16** and **20** at resolutions of 2.7 and 1.3 Å, respectively (Figure 2).[24] Crystals of the wild-type human HDAC2 were used for inhibitor **20**, while crystals of human HDAC2 with a single amino acid insertion of residue Tyr205, designed to better reflect the amino acid insertion in HDAC3, were used for inhibitor **16**. Interestingly, the interactions of **16** and **20** with HDAC2 are quite similar on the surface and both inhibitors bind with their hydrophobic alkyl linkers in the same conformation, consistent with the interactions previously reported for the crystal structures of HDAC2 bound to **1** and **2**.[13,14] However, there are significant differences in the binding mode of the 2-substituted benzamides with HDAC2 at the zinc binding site. The HDAC2 enzyme with compound **20** has a similar conformation as reported before for other HDAC1, 2, 3 inhibitors.[13−15] The phenol oxygen and the benzamide carbonyl chelate the zinc ion in a bidentate mode. Unexpectedly, the HDAC3 selective inhibitor **16** bound to HDAC2 with the amide carbonyl making a monodentate contact to the zinc ion, and the methylthiophenyl group rotated in a manner that induced a rotamer change of the conserved catalytic site tyrosine (Tyr305) from an "in" to "out" position, significantly expanding the volume of the inhibitor binding pocket (Figure 2B). The energetic cost of the conformational rearrangement of the HDAC2 binding pocket to accommodate inhibitor **16** with only monodentate zinc binding may explain why the methylthiobenzamide is much less active against HDAC2 and HDAC1. The structural data therefore suggests that inhibitor **16** maintains potency against HDAC3 either by allowing a high affinity bidentate zinc binding mode in the HDAC3 active site or possibly by other structural features of HDAC3 that would stabilize the flipped out conformation of the catalytic site tyrosine. Interestingly, we note the previously reported HDAC3 crystal structure (Figure 2C) was determined as a complex with inositol tetraphosphate (IP4) and the DAD domain of the SMRT corepressor (PDB 4A69).[25] The catalytic pocket of HDAC3 is quite similar to HDAC2 with the equivalent tyrosine side chain phenol at the "in" position. However, the HDAC3 crystal structure also showed the proximity of Tyr298 and the nearby Arg265 to IP4. If compound **16** binds HDAC3 in the monodentate zinc binding mode, the induced conformation of Tyr298 and concomitant changes to Arg265 would be expected to be influenced by the presence of IP4 in the HDAC3/IP4/SMRT complex. While a crystal structure of HDAC3 bound to inhibitor **16** will be required to resolve these possibilities, these results nonetheless reveal unexpected conformational plasticity and an induced subpocket dependent on a rotamer change of the catalytic site tyrosine that is exploited by exquisitely

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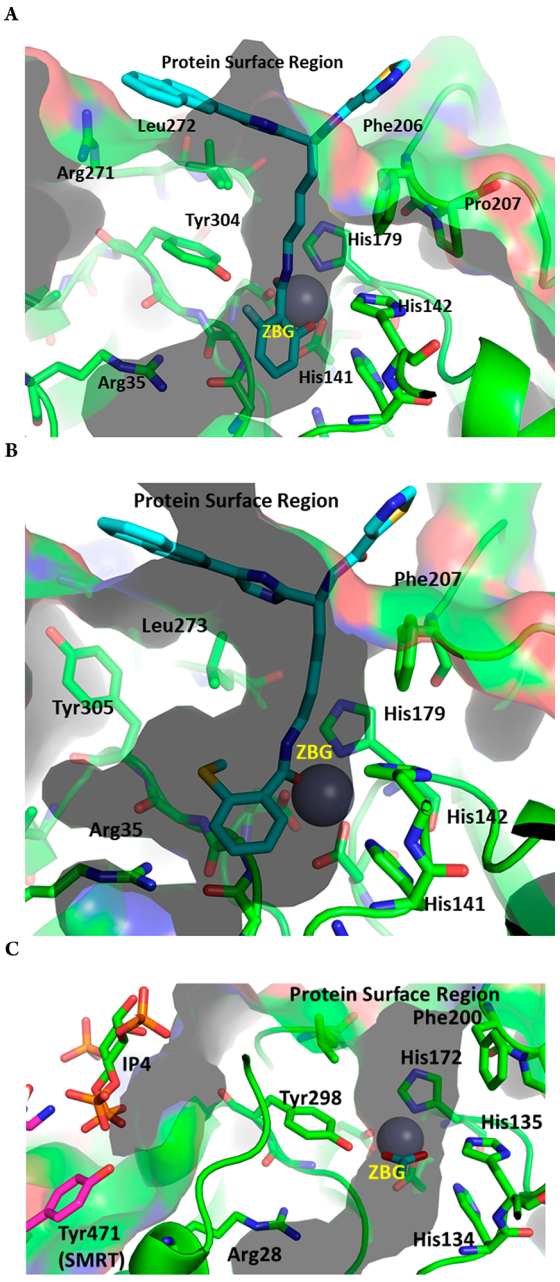

Figure 2. X-ray crystal structures of HDAC inhibitors binding to the HDAC2 enzyme with the pocket shown in gray: (A) zinc binding region of HDAC1, 2, 3 selective inhibitors **20**; (B) zinc binding region of HDAC3 selective inhibitor **16**; (C) HDAC3 X-ray crystal structure as a complex with the IP4/SMRT catalytic pocket (PDB code 4a69).

selective HDAC3 inhibitors. In the meantime, SAR of 2-substitution on the benzamide demonstrated that several substituents unable to coordinate the zinc ion, such as cyclopropyl (**36**), remain as potent HDAC3 selective inhibitors. Compound **36** must be monodentate and therefore should prefer the induced pocket to fit the 2-substituents. The SAR is more consistent with and supports the hypothesis that this series of 2-substituted benzamide HDAC3 selective inhibitors bind to zinc in a monodentate fashion in the induced pocket.

We also investigated binding kinetics for the two representative compounds—HDAC1, 2, 3 inhibitor **20** and HDAC3 selective inhibitor **16**. We applied the global progress curve analysis (GPCA) method[26] to measure the on rate for both inhibitors to associate with HDAC3. Interestingly, the HDAC3 selective inhibitor **16** had a fast on rate, although it needs to induce a pocket with quick conformational change of the protein, with a k_{on} of 1.5×10^4 M^{-1} S^{-1} while the HDAC1, 2, 3 inhibitor **20** also had a fast on rate, but relatively slower than **16** with k_{on} of 1.7×10^3 M^{-1} S^{-1} (detailed measurement can be found in the Supporting Information). The k_{off} of both compounds can be calculated based on the enzyme binding equation $K_D = k_{off}/k_{on}$, in which K_D is usually the same as the IC_{50} of the inhibitors against HDAC3. k_{off} for the HDAC1, 2, 3 inhibitor **20** is calculated to be 4.0×10^{-6} s^{-1} which results in a calculated residence time = $1/k_{off}$ of 69.4 h. However, the calculated k_{off} for the HDAC3 selective inhibitor **16** is 4.4×10^{-4} s^{-1} which results in a much shorter calculated residence time of 38 min. Furthermore, this significant difference of off rate and residence time for **20** and **16** was also confirmed by a jump dilution assay of both compounds in HDAC3 (Figure 3).

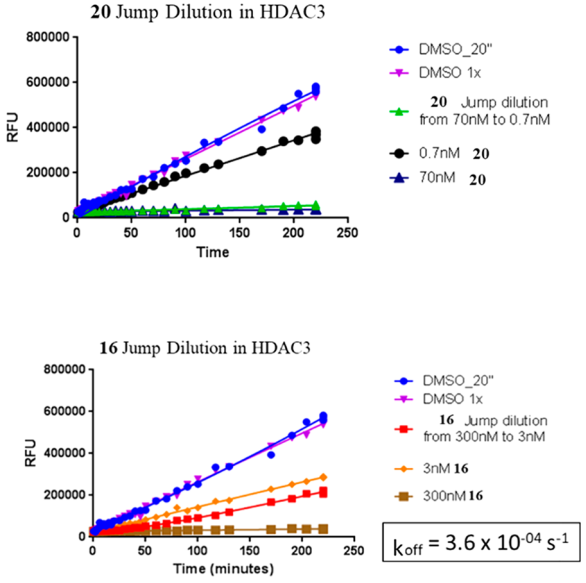

Figure 3. Jump dilution assays to measure off rates: (A) jump dilution for HDAC1, 2, 3 inhibitor **20**; (B) jump dilution for HDAC3 inhibitor **16**. RFU = relative fluorescence units.

The HDAC inhibitors with a concentration of $10 \times IC_{50}$, $0.1 \times IC_{50}$, and DMSO were treated with HDAC3 enzyme and the enzyme activity was monitored by the relative fluorescence units. The jump dilution of the assay solutions by 100 folds were performed on the $10 \times IC_{50}$ assay solution which resulted in the inhibitor concentration to $0.1 \times IC_{50}$ concentration. The HDAC1, 2, 3 inhibitor **20** had a very slow off rate with quite long residence time. Although the inhibitor concentration is diluted to a $0.1 \times IC_{50}$ concentration of 0.7 nM, the inhibitor still binds to the HDAC3 enzyme within the experimental time, which resulted in the continuous inhibition of enzyme activity. However, the HDAC3 selective inhibitor **16** has a fast-off rate, when jump dilution was performed on the assay solution with $10 \times IC_{50}$ concentration to $0.1 \times IC_{50}$ concentration of 3 nM, inhibitor **16** was released off the HDAC3 enzyme. Thus, the HDAC3 enzyme activity is recovered to a similar rate to that of the assay with 3 nM inhibitor. The off rate for **16** was measured from this jump dilution to be $k_{off} = 3.6 \times 10^{-04}$ s^{-1} which matches very well

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with that calculated from the assay with the GPCA method. Since the off rate of **20** is very slow, the k_{off} could not be obtained from the jump dilution assays. This also confirmed the calculated slow off rate for **20** from data with the GPCA method.

In the HIV latency in the Jurkat 2C4 cell model, we reported that HDAC1, 2, 3 inhibitors such as compounds **1** and **2** can induce HIV transcription.[13,14] However, the HDAC1, 2 selective inhibitor **3** and the HDAC3 selective inhibitor **16** are all inactive in this cellular assay. To further check if the inhibition of all HDAC1, 2, and 3 are required for the activation of the cellular HIV latency model, we carried out a test with the combinations of HDAC3 selective inhibitor **16** and HDAC1, 2 selective inhibitor **3** (Figure 4). Three titration

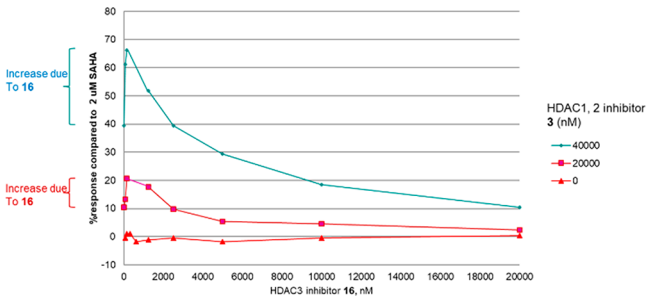

Figure 4. Cellular 2C4 activation by combinations of **16** (HDAC3 inhibitor) and **3** (HDAC1, 2 inhibitor).

curves were obtained with a constant concentration of HDAC1, 2 selective inhibitor **3** at 0 (red), 20000 (purple), and 40000 nM (green), combining with various concentrations of HDAC3 selective inhibitor **16** at 0, 78, 156, 1250, 2500, 5000, 10000, and 20000 nM. No cellular activity was observed when there is no HDAC1, 2 inhibitor **3** in the test. There is enhanced activation with a combination of **16** at low concentration, especially at 156 nM of **16** when the activation percentage reached the maximum. However, with an increasing concentration of **16**, the percentage of activation dropped due to the cytotoxicity of the combination of high concentrations of both **3** and **16**. This experiment confirmed again that inhibition of HDAC1, 2, and 3 is sufficient to cause activation of HIV latency in the Jurkat 2C4 cell model. The combination of the HDAC1, 2 inhibitor (inactive in 2C4 cells alone) and HDAC3 inhibitor (inactive in 2C4 cells alone) can lead to HIV latency activation. These data demonstrate that HDAC1/2 and 3 inhibition is required for HIV latency reversal and that inhibition of HDAC3 alone is not sufficient.

In summary, taking advantage of a parallel medicinal chemistry strategy, we discovered a series of HDAC inhibitors with 2-substituted benzamide as the zinc binding group. When the 2-substituents are involved in the zinc bidentate coordination, the inhibitors are HDAC1, 2, and 3 selective represented by **20**. However, if the 2-substituents do not chelate with the zinc ion, it could induce a binding pocket by flipping the Tyr305 side chain from an "in" to an "out" conformation which was observed in the X-ray crystal structure of **16** bound to the HDAC2 enzyme. The selectivity of **16** with inhibition to HDAC3 is rationalized by this induced pocket protein conformation of HDAC3 which is speculated to be stabilized by the HDAC3/IP4/SMRT complex while no comparable stabilization exists for HDAC1 and 2. This highly HDAC3 selective inhibitor **16** served as the tool compound to confirm that inhibition alone on HDAC3 does not activate HIV latency in the Jurkat 2C4 cells, but combination of **16** with HDAC1,2 inhibitor **3** is sufficient to activate HIV latency. The HDAC3 selective inhibitor **16** and HDAC1,2,3 inhibitor **20** demonstrated different binding kinetic with dramatically distinctive length of residence time. The off target and pharmacokinetic profile of this series of HDAC3 inhibitors can be optimized by SAR of the surface binding groups. Overall, we discovered a series of highly HDAC3 selective inhibitors with 2-substituted benzamides as the zinc binding groups which can serve as tool compounds to explore therapeutic indications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00462.

Synthetic methods, characterization of key compounds, biology assay protocols, and data (PDF)

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Notes

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■ ABREVIATIONS

HDAC, histone deacetylase; HATs, histone acetyl transferases; HDACi, histone deacetylase inhibitor; HIV, human immunodeficiency viruses; LRA, latency reversing agency; PMC, parallel medicinal chemistry; cART, combined antiretroviral therapy; SAR, structure activity relationship; DMF, dimethylformaldehyde; THF, tetrahydrofuran; DCM, dichloromethane; HATU, (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-*b*]pyridinium 3-oxide hexafluorophosphate; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; hERG, human ether-à-go-go-related gene; GPCA, global progress curve analysis; PK, pharmacokinetic.

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(22) hERG activity is measured by the inhibition of the compounds to the binding of MK-499 to potassium ion channel (IKr).

(23) *All pharmacokinetic studies in preclinical species were conducted according to the highest ethical standards in accordance with and using procedures approved by the Institutional Animal Care and Use*; Committee of Merck and Co., Inc, West Point, PA, USA.

(24) The data of X-ray crystal structures for HDAC3 inhibitor **16** and HDAC1, 2, 3 inhibitor **20** is uploaded to the Protein Data Bank (www.pdb.org) with codes 7KBH and 7KBG, respectively. The detailed procedure for crystallization, data collecting, and processing can be found in the Supporting Information.

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