Identification of Highly Selective and Potent Histone Deacetylase 3 Inhibitors Using Click Chemistry-Based Combinatorial Fragment Assembly

Takayoshi Suzuki\textsuperscript{1,2,*}, Yuki Kasuya\textsuperscript{3}, Yukihito Itoh\textsuperscript{1}, Yosuke Ota\textsuperscript{1}, Peng Zhan\textsuperscript{1}, Kaori Asamitsu\textsuperscript{4}, Hidehiko Nakagawa\textsuperscript{3}, Takashi Okamoto\textsuperscript{4}, Naoki Miyata\textsuperscript{2*}

\textsuperscript{1}Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan, \textsuperscript{2}PRESTO, Japan Science and Technology Agency (JST), Saitama, Japan, \textsuperscript{3}Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan, \textsuperscript{4}Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

Abstract

To find histone deacetylase 3 (HDAC3)-selective inhibitors, a series of 504 candidates was assembled using “click chemistry”, by reacting nine alkynes bearing a zinc-binding group with 56 azide building blocks in the presence of Cu(I) catalyst. Screening of the 504-member triazole library against HDAC3 and other HDAC isozymes led to the identification of potent and selective HDAC3 inhibitors T247 and T326. These compounds showed potent HDAC3 inhibition with submicromolar IC\textsubscript{50}s, whereas they did not strongly inhibit other isozymes. Compounds T247 and T326 also induced a dose-dependent selective increase of NF-kB acetylation in human colon cancer HCT116 cells, indicating selective inhibition of HDAC3 in the cells. In addition, these HDAC3-selective inhibitors induced growth inhibition of cancer cells, and activated HIV gene expression in latent HIV-infected cells. These findings indicate that HDAC3-selective inhibitors are promising candidates for anticancer drugs and antiviral agents. This work also suggests the usefulness of the click chemistry approach to find isozyme-selective HDAC inhibitors.

Introduction

Histone protein complexes associate with DNA to form higher-order structures called chromatin. Approximately 150 base pairs of DNA are wrapped twice around an octamer of histones to form a nucleosome, the basic unit of chromatin. Core histones with N-terminal tails extending from the compact nucleosomal core particles can be acetylated or deacetylated at the epsilon position of lysine residues, thereby modifying histone-DNA and histone-non-histone protein interactions. The acetylation status of histone and non-histone proteins is controlled by two enzyme classes with opposing activities; histone acetyltransferases and histone deacetylases (HDACs) [1–3]. HDACs are hydrolases that modulate epigenetic gene expression through deacetylation of the N-acetyl lysine residues of histone and non-histone proteins. There are currently 18 known HDACs that are organized into four classes: class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) and class IV HDAC (HDAC11) which are mainly localized to the nucleus; class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10) which shuttle between the nucleus and the cytoplasm; and class III HDACs (sirtuin 1–7), whose cellular localizations include various organelles [4]. Class I, II, IV HDACs are zinc-dependent enzymes, whereas class III HDACs are NAD\textsuperscript{+}-dependent enzymes [5–8].

Among the HDAC family members, HDAC3 is unique in that it is expressed in the nucleus, cytoplasm, or membrane, and it deacetylates histone and non-histone proteins such as NF-\textkappaB, myocyte enhancer factor 2, and Src kinase [9–16]. Furthermore, recent studies have indicated that HDAC3 is associated with several diseases including cancer, inflammation, and neurodegenerative disorders [17–20]. Therefore, HDAC3-selective inhibitors are of great interest not only as tools for probing the biological functions of HDAC3, but also as candidate therapeutic agents with potentially few side effects.

Although many efforts have been directed to the discovery of potent and selective HDAC inhibitors by numerous academic groups, as well as pharmaceutical companies, only a few HDAC3-selective inhibitors have been reported [4][21–26]. For example, HDAC3 is selectively inhibited by compounds 1 and 2 (Figure 1) [27–28], but their HDAC3-inhibitory activity and selectivity are insufficient for their development as candidate therapeutic agents. In addition, while this research was carried out, RGFP966, a novel HDAC3-selective inhibitor, was reported, although the details of the inhibitor are unclear [29]. Therefore, there is still a need to find HDAC3 inhibitors that are more potent and selective than compounds 1 and 2.

We recently described the identification of potent HDAC3-selective inhibitors from a triazole compound library generated by the use of Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), a
representative reaction in click chemistry [30–33]. Our results indicated that the click chemistry approach is useful for the discovery of isozyme-selective HDAC inhibitors. Following these findings, we performed a further click chemistry approach, seeking to find HDAC3-selective inhibitors more potent and selective than compounds 1 and 2. We describe here the rapid identification of potent and selective HDAC3 inhibitors via the use of click chemistry to generate a library of HDAC inhibitor candidates.

**Results and Discussion**

**Enzyme Assays**

Most HDAC inhibitors reported so far fit a three-motif pharmacophoric model, namely, a zinc-binding group (ZBG), a linker, and a cap group [21–26]. For instance, vorinostat (3) (Figure 2) [34] [35], a clinically used HDAC inhibitor, consists of hydroxamic acid (ZBG), which chelates the zinc ion in the active site, anilide (cap), which interacts with amino acid residues on the rim of the active site, and alkyl chain (linker), which connects the cap group and ZBG with an appropriate separation. Based on the typical HDAC inhibitor structure, we previously designed a library of candidate HDAC inhibitors in which the cap group and the ZBG are connected by a triazole-containing linker (Figure 2), and we identified potent HDAC3-selective inhibitors through screening of the library [30]. Following these findings, we expanded the library by the design and preparation of new alkynes with a ZBG and azides with a cap structure to find potent and selective HDAC3 inhibitors. For the preparation of the triazole library in this work, we designed and synthesized three alkynes Ak1–Ak3 with o-aminoanilide as the ZBG and 14 azides Az1–Az14 with an aromatic cap structure as building blocks for HDAC inhibitor candidate synthesis via CuAAC reaction. In designing alkynes

![Figure 1. Previously reported HDAC3-selective inhibitors 1 and 2.](doi:10.1371/journal.pone.0068669.g001)

![Figure 2. Design of triazole-containing HDAC inhibitor candidates.](doi:10.1371/journal.pone.0068669.g002)
Ak1–Ak3, o-aminooanilide was selected as the ZBG because o-
aminooanilides tend to inhibit Class I HDACs [4]. Azides Az1–
Az14 bearing an aromatic ring were expected to interact with
aromatic amino acid residues such as Tyr and Phe which form the
HDAC3 active pocket [36].

The routes used for the synthesis of compounds Az1–Az14, and
Ak1–Ak3, which were prepared for this study, are shown in
Figures 3, 4, 5, 6. Figure 3 shows the preparation of aryl azides
Az1–Az5, Az7, and Az11. The coupling reaction of aryl iodides
4–10 with sodium azide was carried out in the presence of CuI/L-
proline catalyst to provide aryl azides Az1–Az5, Az7, and Az11 in
37–95% yield [37]. The routes for the synthesis of aryl azides Az6,
Az8–Az10, and Az12 are illustrated in Figure 4. Treatment of
anilines 11–15 with NaN3 under acidic conditions, followed by
NaN3 addition, yielded the desired aryl azides Az6, Az8–Az10,
and Az12. The preparation of alkyl azides Az13 and Az14 is
shown in Figure 5. Chlorides 16 and 17 were allowed to react with
NaN3 to afford alkyl azides Az13 and Az14. Figure 6 shows the
preparation of anilines Ak1–Ak3 bearing an o-aminooanilide
moiety. Condensation of phenylenediamine 21 with the appro-
priate carboxylic acid chloride 18–20 gave o-aminooanilide
derivatives Ak1–Ak3.

The CuAAC reaction between nine alkynes (newly prepared
Ak1–Ak3 and previously prepared Ak4–Ak9) and 56 azides
(newly prepared Az1–Az14 and previously prepared Az15–56)
allowed us to assemble a 504-member HDAC inhibitor candidate
library in microtiter plates [30–38]. Alkynes Ak1–Ak9 (1 eq) and
azides Az1–Az56 (1.4 eq) in the presence of CuSO4 (0.2 eq),
sodium ascorbate (1 eq), and tris[(1-benzyl-1,2,3-triazol-4-
ylimethyl)amine (TBTA) (0.2 eq) in a solvent mixture of
DMSO/H2O (1:1) afforded the 504-membered triazole library.

In all cases, disappearance of the alkynes and generation of the
triazoles were confirmed by TLC. The generated triazole-
containing HDAC inhibitor candidates T1–T504 are shown in
Figure 7.

Figure 4. Scheme for the synthesis of Az6, Az8–Az10, and Az12.
Reagents and conditions: (a) i) NaN3, H2O, TFA, 0 °C; ii) NaN3, H2O, 0 °C
to room temp, 18–90%.
doi:10.1371/journal.pone.0068669.g004

Figure 3. Scheme for the synthesis of Az1–Az5, Az7, and Az11.
Reagents and conditions: (a) NaN3, CuI, L-Pro, NaOH, DMSO, 60 °C, 37–95%.
doi:10.1371/journal.pone.0068669.g003

Figure 5. Scheme for the synthesis of Ak1–Ak3. Reagents and
conditions: (a) EDCI, HOBr, DMF, room temp, 36–62%.
doi:10.1371/journal.pone.0068669.g006

Figure 6. Scheme for the synthesis of Ak1–Ak3. Reagents and
conditions: (a) EDCI, HOBr, DMF, room temp, 36–62%.
doi:10.1371/journal.pone.0068669.g006

Discovery of Histone Deacetylase 3 Inhibitors

inhibitory activity without further purification [30] [39–44]. Since
our final goal in this work is to identify compounds that selectively
inhibit HDAC3 in cells, it is desirable to carry out in vitro enzyme
assays in conditions similar to cellular environments. Because
HDAC3 forms a complex with NCOR1 in cells [43], we used
HDAC3/NCOR1 complex in in vitro HDAC3 assay. In addition,
it is more important to find inhibitors that discriminate HDAC3
from HDAC1 and HDAC2 in cells. Therefore, as a primary
in vitro screening for HDAC3 selectivity, we used total HDACs
from HeLa nuclear extracts, in which the combined deacetylase
activity of HDAC1 and HDAC2 is much higher than the activity
of HDAC3 [46]. Initially, o-aminooanilides T1–T336 (10 μM) and
hydroxamates T337–T504 (1 μM) were tested for inhibitory
activity against HDAC3. In our HDAC3 assay, the IC50 values of
compounds 1–3 were 19 μM, >100 μM, and 0.27 μM, respec-
tively. We therefore used compound 1 and vorinostat (3) as
reference compounds in this assay. As shown in Figure 7, 39 o-
aminooanilides inhibited HDAC3 deacetylase activity by more
than 90% at 10 μM, and 48 hydroxamates showed more than 60%
HDAC3 inhibition at 1 μM. Next, we evaluated these 107
Figure 7. Inhibition of HDAC3 in the presence of T1–T504 (10 μM for o-aminoanilides T1–T336; 1 μM for hydroxamates T337–T504).

o-Aminoanilides inhibiting more than 90% of HDAC3 activity and hydroxamates inhibiting more than 60% of HDAC3 activity are indicated in red. Vorinostat (3) (1 μM) and compound 1 (10 μM) inhibited 98% and 47% of HDAC3 activity, respectively.

doi:10.1371/journal.pone.0068669.g007
compounds for inhibitory activity against total HDACs from HeLa nuclear extracts, in which the deacetylase activity of HDAC1 and HDAC2 is much higher than that of HDAC3 [46]. While all of the hydroxamates displayed more than 70% inhibition of total HDACs at 1 μM (Figure 8), 11 ω-aminoanilides showed less than 10% inhibition at 10 μM (Figure 9) suggesting that these ω-aminoanilides exhibited HDAC3-selective inhibition. Furthermore, we investigated the HDAC3-inhibitory activity of these 11 ω-aminoanilides at 1 μM and 3 μM. Among them, T247 and T326 showed HDAC3 inhibition comparable to that of vorinostat (3) at both 1 μM and 3 μM (Table 1). These results indicated that T247 and T326 might be potent and selective HDAC3 inhibitors.

Figure 10 illustrates the resynthesis of triazoles T247 and T326. Cu-catalyzed coupling of alkyne Ak5 with Az23 and Ak6 with
Az46 provided triazoles T247 and T326, respectively. The resynthesized compounds T247 and T326 were purified by column chromatography and recrystallization. The pure T247 and T326 were then examined for inhibitory effects on total HDACs, HDAC1, HDAC4, HDAC6, and HDAC8. The results of the enzyme assays are shown in Table 2. Compounds T247 and T326 displayed potent HDAC3-inhibitory activity, greater than that of compound 1 and comparable to that of vorinostat (3) (IC50 of 1 19 μM, vorinostat (3) 0.27 μM, T247 0.24 μM, T326 0.26 μM). Furthermore, while vorinostat (3) inhibited total HDACs, HDAC1, HDAC6, and HDAC8, compounds T247 and T326 inhibited HDAC3 selectively over the other isozymes. Thus, T247 and T326 are potent and selective inhibitors of HDAC3.

Molecular Modeling
The lowest energy conformation of T247, the most active HDAC3-selective inhibitor in this series, was obtained when it was docked into a model based on the crystal structure of HDAC3 (PDB code 4A69) [36], using the Molegro Virtual Docker software package. Inspection of the simulated HDAC3/T247 complex showed that the o-aminoanilide group coordinates to the Zn ion bidentately through its NH2 and CO groups, and also forms two hydrogen bonds with His 134 and Gly 143 (Figure 11). In addition, the phenyltriazole part of the inhibitor snugly fits the catalytic site. The phenyltriazole group of T247 lies in the hydrophobic tunnel formed by Phe 144, Phe 200, and Leu 266, where it can interact with the amino acid residues via hydrophobic interactions. There also appears to be a hydrophobic interaction of the thiophene ring of T247 with Pro 23 and Phe 144. The observed interactions between T247 and HDAC3 suggest the importance of the o-aminoanilide as a ZBG and a hydrogen-bond-forming group for high potency. They also suggest the significance of the lipophilic aromatic rings of T247 for hydrophobic interactions. The triazole ring appears to orient the ZBG and hydrophobic group into appropriate geometry.

Cell-based Assays
To examine whether compounds T247 and T326 selectively inhibit HDAC3 in cells, we performed a cellular assay using western blot analysis. Since HDAC3 is known to catalyze the deacetylation of NF-κB [13–14], we initially examined the effects of the inhibitors on the acetylation levels of NF-κB in HCT116 cells. As we expected, T247 and T326 induced a dose-dependent increase of NF-κB acetylation, and their effect was greater than that of compound 1 and comparable to that of vorinostat (3) (Figure 12). Although T247 and T326 caused NF-κB acetylation, it has also been reported that NF-κB is deacetylated by HDAC1 and HDAC2 [47]. To examine whether T247 and T326 can distinguish HDAC3 from HDAC1 in cells, we next analyzed the effects of T247 and T326 on the acetylation levels p53, a substrate protein of HDAC1 [48]. As can be seen in Figure 12, while vorinostat (3), a non-selective HDAC inhibitor, induced non-selective acetylation of NF-κB and p53, the levels of acetylated p53

Table 1. HDAC3 inhibition in the presence of vorinostat (3), compound 1, and 11 o-aminoanilides at 1 μM and 3 μM.

| Conc. | HDAC3 inhibition (%) |
|-------|-----------------------|
|       | 3  | T247 | T199 | T247 | T251 | T254 | T261 | T263 | T266 | T267 | T318 | T326 |
| 1 μM  | 83 | 96  | 55   | 59   | 89   | 75   | 55   | 75   | 74   | 73   | 80   | 77   | 86   |
| 3 μM  | 93 | 29  | 81   | 83   | 95   | 92   | 80   | 89   | 91   | 91   | 92   | 91   | 95   |

Values are means of two experiments.
doi:10.1371/journal.pone.0068669.t001

Figure 10. Scheme for the synthesis of T247 and T326. Reagents and conditions: (a) CuSO4, sodium ascorbate, EtOH, H2O, room temp, 65% for T247; 97% for T326.
doi:10.1371/journal.pone.0068669.g010
were not elevated in the presence of T247 and T326. These results indicate that T247 and T326 do not inhibit HDAC1 and selectively inhibit HDAC3 in the cells. In addition, T247 and T326 did not enhance the acetylation of α-tubulin, a substrate of HDAC6 [49] suggesting that T247 and T326 are HDAC3-selective inhibitors in cell-based assays.

Because it has been suggested that HDAC3 is highly expressed in human colon cancer cells and prostate cancer cells and is associated with the cancer cell growth [50–51], vorinostat (3), compound 1, T247, and T326 were tested in cell growth-inhibition assays using human colon cancer HCT116 and prostate cancer PC-3 cell lines. The results are shown in Table 3. HDAC3-selective inhibitors T247 and T326 showed clear growth-inhibitory effects on both HCT116 and PC-3 cell lines. In particular, the cell growth-inhibitory activity of compound T247 and T326 was much greater than that of compound 1 and comparable to that of vorinostat (3). These results suggest that HDAC3-selective inhibitors might be useful in the treatment of colon cancers and prostate cancers.

We also examined the effects of T247 and T326 on latent HIV-infected cells, because it has been suggested that HDAC3 represses the transcription of HIV type 1 (HIV-1) genes in such cells [52]. HIV-1-infected OM10.1 cells were treated with 0.1 μM, 1 μM, and 10 μM compound 1, vorinostat (3), T247, and T326. Although compound 1, a weak HDAC3 inhibitor, did not show any activity, vorinostat (3), T247, and T326 significantly stimulated HIV-1 expression at 1 μM and/or 10 μM (Figure 13). Compound T326 was less active at 10 μM due to cytotoxicity. These data suggest that the combination of HDAC3-selective inhibitor and other anti-HIV agents may be useful in the treatment of HIV infection [53–55].

In summary, we have designed a 504-membered triazole-containing HDAC inhibitor candidate library and prepared it by means of CuAAC reaction between nine alkynes and 56 azides. Two compounds, T247 and T326, were hit as HDAC3-selective inhibitors by screening of the 504 library compounds. Compounds T247 and T326 showed potent inhibition of HDAC3 with IC50 values of 0.24 μM and 0.26 μM, respectively, but did not inhibit other HDAC isozymes even at 100 μM. The molecular modeling study of T247 with HDAC3 suggested the importance of the o-

| Table 2. HDAC-Inhibitory Activity of vorinostat (3), compound 1, T247, and T326. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound       | IC50 (μM)       | class I         | class IIa       | class IIb       |
|                | HDAC1 | HDAC3 | HDAC8 | HDAC4 | HDAC6 |
| vorinostat (3) | 0.073 | 0.39  | 0.27  | 0.66  | >10   | 0.34  |
| 1               | >100  | >100  | 19    | >100  | >100  | >100  |
| T247            | >100  | 19    | 0.24  | >100  | >100  | >100  |
| T326            | >100  | >100  | 0.26  | >100  | >100  | >100  |

*aValues are means of at least three experiments.*

doi:10.1371/journal.pone.0068669.t002

Figure 11. Binding mode of T247. (A) View of the conformation of T247 (tube) docked in the HDAC3 catalytic core. Compound T247 was docked into a model based on the crystal structure of HDAC3 (PDB code 4A69) using the Molegro Virtual Docker software package. Residues around T247 are displayed as wires. (B) The same view as A. The narrow and long tunnel of the active site is displayed as a green mesh. (C) Schematic diagram of T247-binding to the catalytic site.

doi:10.1371/journal.pone.0068669.g011
aminoanilide as a ZBG and a hydrogen-bond-forming group, and of the lipophilic part having three aromatic rings for hydrophobic interactions. In cellular assays, T247 and T326 induced a selective increase of acetylated NF-κB, suggesting that they are cellulary active HDAC3-selective inhibitors. T247 and T326 also inhibited the growth of colon cancer HCT116 and prostate cancer PC-3 cell lines, and stimulated HIV-1 gene expression in latent HIV-1-infected OM10.1 cells. We believe that T247 and T326 are the most potent HDAC3-selective inhibitors reported so far. The findings presented here should provide a basis for constructing new tools to probe the biology of HDAC3 and for developing new strategies to treat cancer and HIV-1 infection.

Many groups have ongoing research programs to find selective inhibitors of HDAC isozymes, however, there has been no reported isozyme-selective inhibitors of HDAC1, 2, 5, 7, 9, 10, and 11, although the isozymes have been reported to be crucial for biological events and be responsible for several disease states [4].

Our methodology using click chemistry could be used to find not only HDAC3- and HDAC6-selective inhibitors, but also other isozyme-selective inhibitors. We believe that selective inhibitors against the HDAC isozymes will be discovered using this click approach.

Table 3. Growth inhibition of colon cancer HCT116 cells and prostate cancer PC3 cells by vorinostat (3), compound 1, T247, and T326.

| Cell line                | Gl50 (µM) |
|--------------------------|-----------|
|                          | 3 1 T247  | T326      |
| HCT116 (colon cancer)    | 1.3 81    | 1.9 0.94  |
| PC3 (prostate cancer)    | 1.6 >100  | 1.4 1.0   |

*Values are means of at least three experiments.
doi:10.1371/journal.pone.0068669.g003

Figure 12. Western blot detection of acetylated NF-κB, p53, and α-tubulin levels in HCT116 cells after 8 h treatment with vorinostat (3), compound 1, T247, and T326.
doi:10.1371/journal.pone.0068669.g012

Figure 13. Induction of viral replication from OM10.1 cells latently infected with HIV-1. Cells were incubated with compound 1, vorinostat (3), T247, and T326 for 48 h. HIV-1 p24 antigen in the cell culture supernatant was measured using ELISA. Experiments were performed in triplicate, and the means ± S.D. are indicated. **P<0.01, *P<0.05; Student’s t test results indicated differences between DMSO and inhibitors.
doi:10.1371/journal.pone.0068669.g013
chemistry approach in the near future.

Materials and Methods

Chemistry

General. Melting points were determined using a Yanagimoto micro melting point apparatus or a Buchi 545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance spectra (1H NMR), carbon nuclear magnetic resonance spectra (13C NMR), electron impact mass spectra (EI/MS) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL JNM-LA500, JEOL JNM-A500 or BRUKER AVANCE600 spectrometer in the indicated solvents. Chemical shifts (δ) are reported in parts per million relative to the internal standard tetramethylsilane. Elemental analysis was performed by a Yanaco CHON CORDER NT-5 analyzer, and all values were within ±0.4% of the calculated values. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer. GC-MS analyses were performed on a Shimadzu GCMS-QP2010 IR spectrometers. A solution of Na2SO4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/hexane = 1/2) gave 1.0 g (97%) of compound Az1 as a yellow oil. 1H NMR (DMSO-d6, 500 MHz, δ ppm) 7.06 (2H, d, J = 8.8 Hz), 6.98 (2H, d, J = 8.8 Hz), 3.74 (1H, s). FTIR (neat, cm⁻¹) 2106. MS (EI) m/z 147 (M++). Compounds Az2–Az5, Az7 and Az11 were prepared from an appropriate aniline (11–15) and Na2SO4 using the procedure described for Az1.

1-Azido-4-iodobenzene (Az6). To a solution of 4-iodoaniline (11, 1.07 g, 4.87 mmol) in TFA (10 mL) was added a solution of NaNO2 (1.43 g, 21.0 mmol) in water (10 mL) at 0°C. The mixture was stirred at 0°C for 10 min and a solution of NaN3 (5.2 g, 49.2 mmol) in water (10 mL) was added. The reaction mixture was diluted with AcOEt, washed with water and brine, and dried over Na2SO4. Filtration and concentration in vacuo, and recrystallization from AcOEt gave 1.0 g (90%) of compound Az6 as a black solid. 1H NMR (DMSO-d6, 500 MHz, δ ppm) 7.73 (2H, d, J = 8.5 Hz), 6.95 (2H, d, J = 8.5 Hz). FTIR (neat, cm⁻¹) 2096. MS (EI) m/z 245 (M+). Compounds Az8–Az10 and Az12 were prepared from an appropriate aniline (11–15) using the procedure described for Az6.

4-Azidobenzene (Az8). Yield 80%; yellow solid; 1H NMR (DMSO-d6, 500 MHz, δ ppm) 8.24 (2H, d, J = 9.0 Hz), 7.35 (2H, d, J = 9.0 Hz). FTIR (neat, cm⁻¹) 2121. MS (EI) m/z 164 (M+).

4-Azidophenol (Az9). Yield 18%; black solid; 1H NMR (DMSO-d6, 500 MHz, δ ppm) 9.55 (1H, s), 6.91 (2H, d, J = 9.0 Hz), 6.78 (2H, d, J = 9.0 Hz); FTIR (CHCl3, cm⁻¹) 2114; MS (EI) m/z 135 (M+).

2-Azidobenzonitrile (Az10). Yield 87%; yellow oil; 1H NMR (DMSO-d6, 500 MHz, δ ppm) 7.50–7.40 (5H, m), 7.37 (3H, t, J = 8.0 Hz), (1H, t, J = 7.3 Hz). FTIR (CHCl3, cm⁻¹) 2125. MS (EI) m/z 167 (M+-N2).

1-Azidonaphthalene (Az11). Yield 43%; brown oil; 1H NMR (DMSO-d6, 500 MHz, δ ppm) 7.94 (1H, d, J = 8.0 Hz), 7.87 (1H, d, J = 8.0 Hz), 7.68 (1H, d, J = 8.0 Hz), 7.53–7.44 (3H, m), 7.36 (1H, d, J = 7.5 Hz). FTIR (neat, cm⁻¹) 2110. MS (EI) m/z 169 (M+).

2-Azido-N-(2,6-dimethylphenyl)acetamide (Az12). To a solution of 0.5 M NaNX (16 mmol) in DMSO (32 mL) was added 2-chloro-N-(2,6-dimethylphenyl)acetamide (16, 1.0 g, 5.3 mmol), and the mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with AcOEt, washed with water and brine, and dried over Na2SO4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/hexane = 1/2) gave 1.0 g (97%) of compound Az12 as a brown solid. 1H NMR (DMSO-d6, 500 MHz, δ ppm) 7.60–7.55 (2H, m), 7.19–7.12 (2H, m), 4.03 (2H, s). FTIR (neat, cm⁻¹) 3102. MS (EI) m/z 194 (M+).

Compound Az14 was prepared from 2-chloro-N-(2,6-dimethylphenyl)acetamide 17 and Na2SO4 using the procedure described for Az13.

2-Azido-N-(2,6-dimethylphenyl)acetamide (Az14). Yield 64%; white solid; 1H NMR (DMSO-d6, 500 MHz, δ ppm) 9.51 (1H, s), 7.09 (3H, m), 4.09 (2H, s). FTIR (neat, cm⁻¹) 2094. MS (EI) m/z 176 (M+-N2).

Pent-4-ynoic acid (2-aminophenyl)amide (Ak1). A mixture of 4-pentoic acid (18, 437 mg, 4.45 mmol), 1,2-phenylenediamine (21, 407 mg, 3.76 mmol), EDCI (874 mg, 4.56 mmol), and HOBr/H2O (629 mg, 4.65 mmol) in dry DMF was stirred at room temperature for 6 h. The reaction mixture was diluted with AcOEt, washed with water and brine, and dried over Na2SO4. Filtration, concentration in vacuo, and purification by silica gel flash column chromatography (AcOEt/hexane = 1/1) gave 400 mg (56%) of compound Ak1 as a white solid. 1H NMR (CD3OD, 500 MHz, δ ppm) 7.07 (1H, d, J = 8.0 Hz), 7.02 (1H, t, J = 7.5 Hz), 6.83 (1H, d, J = 7.8 Hz), 6.70 (1H, t, J = 7.5 Hz), 2.63–2.57 (4H, m), 2.34–2.33 (1H, m). MS (EI) m/z 188 (M+). Compounds Ak2 and Ak3 were prepared from an appropriate carboxylic acid (19 or 20) and 1,2-phenylenediamine 21 using the procedure described for Ak1.
Hex-5-ynoic acid (2-aminophenyl)amide (Ak2). Yield 36%; pink solid; 1H NMR (CD3OD, 500 MHz, δ, ppm) 7.08 (1H, d, J = 7.8 Hz), 7.02 (1H, t, J = 7.5 Hz), 6.84 (1H, d, J = 8.0 Hz), 6.71 (1H, t, J = 7.5 Hz), 2.53 (2H, t, J = 7.5 Hz), 2.92–2.27 (3H, m), 1.91 (2H, quintet, J = 7.0 Hz). MS (EI) m/z 202 (M+).

Hept-6-ynoic acid (2-aminophenyl)amide (Ak3). Yield 62%; pink solid; 1H NMR (CD3OD, 500 MHz, δ, ppm) 7.07 (1H, d, J = 7.8 Hz), 7.02 (1H, t, J = 7.8 Hz), 6.84 (1H, d, J = 8.3 Hz), 6.71 (1H, t, J = 7.8 Hz), 2.44 (2H, t, J = 7.5 Hz), 2.28–2.24 (3H, m), 1.83 (2H, quintet, J = 7.5 Hz), 1.82 (2H, quintet, J = 7.5 Hz). MS (EI) m/z 216 (M+).

Construction of Triazole Library (T1-T504). To a solution of alkynes (25 mM, 20 μL), azide (35 mM, 20 μL), and TBTA (10 mM, 10 μL) in DMSO was added an aqueous solution of CuSO4·5H2O (4 mM, 25 μL) on a 96-well plate. To the resulting mixture was added an aqueous solution of sodium ascorbate (78 mg, 0.51 mmol), CuSO4·5H2O (4 mmol, 25 μL), and sodium ascorbate (21.8 mg, 0.11 mmol) in water and EtOH (v/v = 1/1) was stirred vigorously for 15 h at room temperature. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was washed with brine, and dried over Na2SO4. Filtration, concentration for enzyme assays by adding DMSO.

-N-(2-Aminophenyl)-4-[1-[2-thiophen-3-yethyl]-1H-[1,2,3]triazol-4-y]benzamide (T247). A mixture of Az23 (78 mg, 0.51 mmol), Ak5 (65 mg, 0.28 mmol), CuSO4·5H2O (13.7 mg, 0.055 mmol), and sodium ascorbate (21.8 mg, 0.11 mmol) in water and EtOH (v/v = 1/1) was stirred vigorously for 15 h at room temperature. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was washed with brine, and dried over Na2SO4. Filtration, concentration in vacuo, and purification by silica gel column chromatography (AcOEt/n-hexane = 5/1) gave 70 mg (63%) of T247 as a crude solid. The solid was recrystallized from water and MeOH to give 58 mg of T247 as colorless crystals. mp 194–195°C. 1H NMR (DMSO-d6, 500 MHz, δ, ppm) 9.70 (1H), s, 8.66 (1H, s), 8.06 (2H, d, J = 8.0 Hz), 7.94 (2H, d, J = 8.5 Hz), 7.48 (1H, t, J = 3.0 Hz), 7.25 (1H, s), 7.17 (1H, d, J = 8.0 Hz), 7.02–6.95 (2H, m), 6.78 (1H, d, J = 8.0 Hz), 6.60 (1H, t, J = 8.0 Hz), 4.91 (2H, s), 4.68 (2H, t, J = 7.5 Hz), 3.25 (2H, t, J = 7.0 Hz). 13C NMR (DMSO-d6, 150 MHz, δ, ppm) 164.87, 145.42, 143.19, 137.76, 133.65, 128.53, 128.24, 127.00, 126.74, 126.53, 126.26, 125.49, 124.73, 122.19, 122.15, 116.27, 116.14, 50.09, 30.19. MS (FAB) m/z 390 (MH+). Anal. (C21H19N5OS) C, H, N.

Compound T326 was prepared from Az46 and Ak6 using the procedure described for T247.

Cell growth inhibition assay. The cells were plated at the initial density of 5,000 cells/well (50 μL/well) in 96-well plates in medium culture and exposed to inhibitors for 48 h in an incubator at 37°C in 5% CO2 in air. A solution (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added (10 μL/well) and incubation was continued for 3 h. The solubilized dye was quantified by colorimetric reading at 570 nm. The absorbance values of control wells (C) and test wells (T) were measured. The absorbance of the test wells (T0) was also measured at time 0 (addition of compounds). Using these measurements, cell growth inhibition (percentage of growth) by a test inhibitor at each concentration used was calculated as: % growth = 100 x [(T − T0)/ (C − T0)].

|       | T0 | C          | T         | % growth |
|-------|----|------------|-----------|----------|
| 0.00  | 8.00 | 10.00      | 0.00      |          |
| 0.25  | 7.75 | 10.00      | 2.75      | 95.8     |
| 0.50  | 7.50 | 10.00      | 2.50      | 95.8     |
| 1.00  | 7.00 | 10.00      | 3.00      | 93.8     |

Western Blot Analysis

HCT116 human colon cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in McCoy’s 5A culture medium containing penicillin and streptomycin, which was supplemented with fetal bovine serum as described in the ATCC instructions. HCT116 cells (1.0 x 105) were treated for 8 h with 20 μM etoposide and samples at the indicated concentrations in McCoy’s 5A medium, then collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using a Bradford protein assay kit (Bio-Rad Laboratories); equivalent amounts of proteins from each lysate were resolved in AnyK DSD-polycrylamide gels and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After having been blocked for 30 min with Tris-buffered saline (TBS) containing 3% skimmed milk, the transblotted membranes were incubated overnight at 4°C with acetyl NF-kB antibody (Sigma) (1:2000 dilution), α-tubulin antibody (Sigma) (1:2000 dilution), acetyl α-tubulin antibody (Sigma) (1:2000 dilution), acetyl p53 antibody (CALBIOCHEM) (1:500 dilution) in TBS containing 3% skimmed milk. The membrane was probed with the primary antibody, then washed twice with TBS, incubated with sheep anti-rabbit IgG-horseradish peroxidase conjugates (diluted 1:1000 for acetyl NF-kB, 1:2000 for acetyl α-tubulin, 1:5000 for acetyl p53) or donkey anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:5000 for acetyl α-tubulin, 1:5000 for acetyl p53, 1:500 for p53 antibody) for 1.5 h at room temperature, and again washed twice with TBS and once with TBS-tween 20 (TBS-T). The immunoblots were visualized by enhanced chemiluminescence.
**Viral p24 antigen assay.** The p24 antigen level in the cell culture supernatant was measured by p24 antigen capture ELISA assay using a commercial kit (RETRO-TEK HIV-1 p24 Antigen ELISA kit; Zepeto Metrox, Buffalo, NY, USA) according to the method reported in ref [54].

**Molecular modeling.** The X-ray structures of HDAC3 and HDAC8 (PDB code 4A69 and 1T6L, respectively) were used as the target structures for docking. Protein preparation, receptor grid generation and ligand docking were performed using the Molegro Virtual Docker software package. Compound T247 was docked into the active site of the protein and was located in a position where the amino group of T247 can interact with the zinc ion. The standard precision mode of Molegro Virtual Docker was used to determine favorable binding poses, which allowed the ligand conformation to be flexibly explored while holding the protein as a rigid structure during docking.

**Author Contributions**

Conceived and designed the experiments: TS TO NM. Performed the experiments: TS YK YI PZ YO KA HN. Analyzed the data: TS TO NM. Conceived and designed the experiments: TS TO NM. Performed the

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