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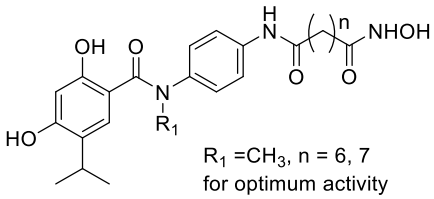
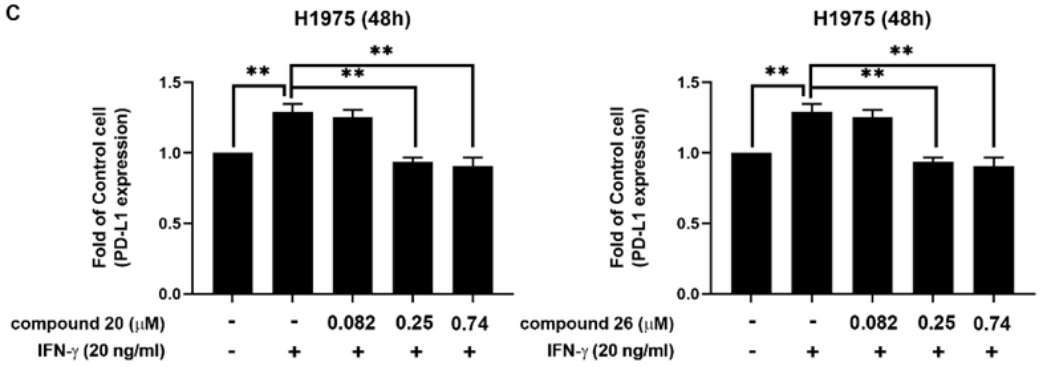
Graphical abstract



Dual HDAC/HSP90 inhibitor

20, HDAC IC_{50} = 194 nM, HSP90α IC_{50} = 153 nM.
26, HDAC IC_{50} = 360 nM, HSP90α IC_{50} = 77 nM.



Downregulation of PD-L1 expression in IFN-γ treated H1975 lung cancer cells.

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N-alkyl-hydroxybenzoyl anilide hydroxamates as dual inhibitors of HDAC and HSP90, downregulating IFN-γ induced PDL-1 expression

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Abstract: Novel dual inhibitors of histone deacetylase (HDAC) and heat-shock protein 90 (HSP90) are synthesized and evaluated. These compounds are endowed with potent HDAC and HSP90 inhibitory activities with IC_{50} values in nanomolar range with Compound **20** (HDAC IC_{50} = 194 nM; HSP90α IC_{50} = 153 nM) and compound **26** (HDAC IC_{50} = 360 nM; HSP90α IC_{50} = 77 nM) displaying most potent HDAC and HSP90α inhibitory activities. Both of these compounds induce HSP70 expression and down regulate HSP90 client proteins which play important roles in the regulation of survival and invasiveness in cancer cells. In addition, compounds **20** and **26** induce acetylation of α-tubulin and histone H3. Significantly, compounds **20** and **26** could effectively reduce programmed death-ligand 1 (PD-L1) expression in IFN-γ treated lung H1975 cells in a dose dependent manner. These findings suggest that dual inhibition of HDAC and HSP90 that can modulate immunosuppressive ability of tumor area may provide a better therapeutic strategy for cancer treatment in the future.

Key words: Histone deacetylase inhibitors, Dual inhibitors, Design Multiple Ligand, Lung Cancer, Heat shock protein, programmed death-ligand 1 (PD-L1)

1. Introduction

With the advancement in the knowledge of epigenetics it has become more evident that histone deacetylases (HDACs) are potential therapeutic targets with the ability to reverse aberrant epigenetic states associated with cancer. Various studies in cancer cell lines and tumor tissues revealed changes in the acetylation levels and the expression of the HDAC enzymes and it can be anticipated that HDAC inhibitors (HDIs) could be therapeutically useful as a single agent or in combination with other therapies, such as chemotherapy, immunotherapy or radiotherapy.[1-4] The approval of suberoylanilide hydroxamic acid (SAHA) by the FDA as first HDI in 2006 has accelerated the search for more potent and more selective HDIs. Many pharmaceutical companies have developed potent HDIs and till date four of these have been granted FDA approval (Figure 1). SAHA with simple chemical architect and easy synthesis has received much attention for further modifications. Many compounds based on SAHA with various heteroatoms as the surface recognition cap, exhibiting potent HDAC inhibitory activity have been reported including ACY-241 (**6**) (Figure 2).[5-9] Recently, much attention has been given to the designed multiple ligand (DML) approach for the development of compounds comprising two or more pharmacophores, multi-component ligands and multiple-ligands within the same molecule to achieve multi-target modulations. The deliberate use of dual inhibition for enzyme inhibition in cancer treatment is relatively new. Design of such multiple ligands is based on various interdependent intrinsic cellular pathways where one target could be a downstream target or client protein or co-chaperone of another or where inhibitors of two different targets have some synergistic effects. Since HDACs play crucial role in various cellular pathways, it is useful to evaluate HDIs not only in various combination therapies but also as compounds with dual or multitarget inhibition, achievable by modified chemical templates of HDIs. There have been some recent reports of dual inhibitors of inosine monophosphate dehydrogenase and histone deacetylase[10], potent multi-acting HDAC, EGFR, and HER2 inhibitors[11], dual inhibitors of histone deacetylase and Topoisomerase II[12] and Toposiomerase I and II[13.], histone deacetylase inhibitors equipped with

estrogen receptor modulation activity[14], Janus Kinase 2 (JAK2) and histone deacetylase bispecific inhibitors[15] and, c-Met/ HDIs based on pyridazinone derivatives.[16] Figure 3 illustrates the various examples of compounds that target dual or multiple signaling pathways.

HDIs increase acetylation of both histone and non-histone proteins, such as HSP 90. HSP90 is a reported downstream target of HDAC6 and inhibitors of HDAC6 lead to destabilization of the HSP90 chaperone function resulting in protein degradation.[17] Various HDIs have been used synergistically with HSP90 inhibitors (Figure 4) in studies like; HSP90 inhibitor NVP-AUY922 (**I**) + PXD101 (**3**) for anaplastic thyroid carcinoma[18], HSP90 inhibitor SNX5422 (**II**) + PXD101 (**3**)/SAHA (**1**)/Trichostatin A for treatment of anaplastic thyroid carcinoma[19], 17AAG (**III**) a HSP90 inhibitor + SAHA (**I**)/sodium butyrate to induce apoptosis in human leukemia cells[20] and, histone deacetylase inhibitor LBH589 (**4**) + HSP90 inhibitor 17-AAG for human CML-BC cells and AML cells with activating mutation of FLT-3.[21]

1.1 Design Rationale.

In the field of drug discovery, a single compound that simultaneously competes with multiple targets is an emerging paradigm. Multiple ligands designed in this way offer a cost-effective strategy over multi-drug combinations and are less likely to have drug-drug interactions. Reports have suggested that treatment with HDIs re-sensitizes resistant cells that have acquired resistance towards not only compound **III** but also towards other HSP90 inhibitors after treatment with **III**. This further supports the idea of using HSP90 and HDIs as a combination therapy.[22] Consequently, we used DML strategy to discover dual inhibitors of HSP90 and HDAC using SAHA (**1**) as a template with modified surface recognition cap using resorcinol (Figure 5). Due to better stability, resorcinol moiety has been preferably used for development of various HSP90 inhibitors (figure 4). Thus synthesized multiple ligands have potencies in the nanomolar range against various cancer cell lines,

downregulate various client proteins of HSP90 and HDAC, and are lead compounds for further modifications in the development of more potent dual inhibitors of HDAC and HSP90.

Results and discussion

2.1 Chemistry.

Syntheses of various *t*-butoxycarbamate anilines **(34-41**) are shown in Scheme 1**.** TEA (Triethylamine) and DMAP (4-Dimethylaminopyridine) were added to a solution of the appropriate nitroaniline (**28**-**30**) in Dichloromethane (DCM) to which was added Boc anhydride (Di-*tert*-butyl dicarbonate) dissolved in DCM. Thus obtained various *t*-Boc-nitroanilines were alkylated using sodium hydride and various alkyl halides at 0 ^{o}C in DMF. The resulting alkyl halides were reduced using Fe/NH_4Cl to give the corresponding anilines (**34**-**41**) in up to 80% yield.

Syntheses of various hydroxamates **(17–27**) are illustrated in Scheme 2. Esters were synthesized using HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), DIPEA (*N*,*N*-Diisopropylethylamine) and appropriate aliphatic acid (C-5 to C-8) added to a solution of the appropriate amine (**34**-**41**) in DMF. Amidation using 1,4-dibenzyloxy-5-isopropyl benzoic acid using HBTU and DIPEA provided corresponding amides (**42**-**52**) with yields of 49-65%. 1,4-dibenzyloxy-5-isopropyl benzoic acid was prepared following the published procedure[23].

A mixture of 1N LiOH (Lithium hydroxide) and **42**-**52** was stirred at 40 $^{\circ}$C for 2 h. Precipitates obtained after work up were filtered, dried, dissolved in DMF and amidation was accomplished with NH_2OBn using EDC•HCl (N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide HCl), HOBt (1-hydroxybenzotriazole) and NMM (N-methylmorpholine). De-protection of NH_2OBn was achieved using 10% Pd/C in methanol under H_2 at room temperature. The deprotected compound was purified to yield compounds **17**-**27** in 49-63% yield.

2.2 Biological evaluation.

2.2.1 *Inhibition of HeLa nuclear HDAC enzyme and HSP90α.*

Compounds **17**–**27** were evaluated for their HDAC inhibitory activity using HeLa nuclear extract as HDAC source. They were also examined for activity against HSP90α (Table 1). Compound **17**, in which R_1 = H and there is an alkyl chain of six carbons (C-6) attached to the *p*-position of the benzamide, displays HDAC inhibitory activity (IC_{50} = 415 nM) but does not exhibit activity against HSP90α (IC_{50} = >1000 nM). SAHA, a HDI, has IC_{50} = 98 nM against HDAC HeLa extract but no HSP90α inhibitory activity >1000 nM. On the other hand, 17-AAG, a HSP 90 inhibitor, exhibits potent HSP90α inhibition (IC_{50} = 98 nM) but no HDAC inhibitory activity (IC_{50} >1000 nM). To investigate the regiomeric effect of the alkyl chain with respect to the benzamide, compounds **18** (*meta*) and **19** (*ortho*) were synthesized and evaluated. Compound **18** has increased HDAC inhibitor activity (IC_{50} = 126 nM) but there was no change with respect to the activity against HSP90α (IC_{50} >1000 nM) as compared with **17**. Compound **19** fails to show any apparent potency against HDAC and HSP90α. To evaluate the effect of *N*-alkyl substitution of benzamide ($R_1 \neq H$), compounds **20** - **24** were synthesized. Compounds **20** (*para*, *N*-Me, whose IC50 with HSP90α is IC_{50} = 153 nM)**, 21** (*meta*, *N*-Me, IC_{50} = 302 nM), **22** (*para*, *N*-Et, IC_{50} = 186 nM) and **23** (*meta*, *N*-Et, IC_{50} = 145 nM) show significant improvement in their HSP90a inhibitory compared to that of their precursors. For the activity against HDAC of *para*-substituted compounds, the inhibitory activity of *N*-Me substitution (**20**, IC_{50} = 194 nM) was better than for the *N*-Et compound (**22**, IC_{50} = 461 nM). The reverse order was favored for *meta*-substituted compounds, and thus **23** (*N*-Et, IC_{50} = 126 nM) is less active than **21** (*N*-Me, IC_{50} = 211 nM). These results indicate the favorability of compounds containing an *N*-substituted benzamide group for dual inhibition of HDAC and HSP90α. The *N*-isopropylamide (**24**) displays diminished activity (HDAC and HSP90α IC_{50} >1000 nM) suggesting that a bulky group is not favored. To evaluate the effect of alkyl chain length on the activity of this series, compounds **25** (C-5), **26** (C-7) and **27** (C-8) were synthesized and evaluated. These compounds maintain HDAC inhibitory activity with compound **26** exhibiting the best HSP90α inhibition (IC_{50} = 77 nM) amongst all the compounds. The lower HDAC inhibitory activity of **27** (C-

8; IC_{50} = 671 nM) compared to its precursor **26** (C-7; IC_{50} = 360 nM) indicates that a chain length ≤ C-7 is optimum for maintaining activity.

2.2.2 *In Vitro Cell Growth Inhibitory Activity.*

The anti-proliferative activity of the synthesized hydroxamates (**17**–**27**) against lung carcinoma A549 cells, human colon cancer HCT116 cells and human non-small cell lung EGFR-resistant cancer cells H1975 was evaluated (Table 1). Cancer cell growth inhibitory results are consistent with the HDAC and HSP90α inhibitory activities. Compounds **17**-**19** (R_1 = H); fail to show potent activity in cancer cell lines with exception of the human colon cancer HCT116 cells {**17** (GI_{50} = 5.43 µM), **18** (GI_{50} = 6.23 µM), **19** (GI_{50} = 4.92 µM)}. *N*-alkylated compounds (**20** – **23**) showed marked improvement in cancer cell growth inhibitory activities and their results are consistent with the HDAC and HSP90α inhibition. Compound **20** (*para*, *N*-Me) displayed potent activity in the sub-micromolar range (A549 GI_{50} = 0.77 µM; HCT116 GI_{50} = 0.83 µM and H1975 GI_{50} = 0.69 µM). Cell growth inhibitory activities followed the same trend as that of HDAC and HSP90α inhibition with a *para N*-Me compound **20** (A549 GI_{50} = 0.77 µM; HCT116 GI_{50}= 0.83 µM and H1975 GI_{50} = 0.69 µM) > **22** (A549 GI_{50} = 5.63 µM; HCT116 GI_{50}= 3.56 µM and H1975 GI_{50} = 1.38 µM) and the *N*-Et substituted *meta* derivative **23** (A549 GI_{50} = 1.59 µM; HCT116 GI_{50} = 1.12 µM and H1975 GI_{50} = 0.94 µM) > **21** (A549 GI_{50} = 2.30 µM; HCT116 GI_{50} = 1.17 µM and H1975 GI_{50} = 1.66 µM). Compound **24** was active against only HCT116 cells with IC_{50} = 6.23 µM. To explore the effect of alkyl side chain on the SAR, compounds **25** - **27** were synthesized and evaluated. Each of these compounds showed potent cell growth inhibition with **26** (A549 GI_{50} = 0.44 µM; HCT116 GI_{50} = 1.06 µM and H1975 GI_{50} = 0.40 µM) maintaining an appropriate balance between cell growth inhibitory activity and HDAC and HSP90α inhibitory activity.

2.2.3 *HDAC Isoform Inhibition.*

Compounds **20** and **26** were tested for enzymatic activities of HDAC isoforms, including HDAC isoform **1-11** and, SIRT1 (Table 2). The results indicated that both **20** and **26** decrease enzymatic activities of various HDAC isoforms with **20** exhibiting at least 22 times more selectivity for HDAC 6 isoform with an IC_{50} value of 0.04 μM.

2.2.4 *Effects of Test Compounds on HSP90-Regulated Client Proteins*

HSP 90 chaperone inhibitors reportedly cause pronounced induction of HSP70, and quantification of HSP70 levels is related to the potency of HSP90 inhibition. Thus, HSP70 induction can be a useful marker to predict the effects of HSP90 inhibitors.[24,25] We explored the protein levels of HSP70 and well-known HSP90 clients after drug treatment. Exposure to test compounds induces HSP70 expression and downregulates the protein levels of client proteins, such as EGFR, Src, FAK, and Rb. Figure 6 shows the HSP90 inhibitory activity of compounds **20** and **26**.

2.2.5 *Effect of α-Tubulin and Histone H3 Acetylation on Human Non-Small Cell Lung Cancer Cells.*

Compounds **20** and **26** were evaluated by western blot analysis for their ability to acetylate α-Tubulin and Histone H3. The expression of these HDAC-inhibition biomarkers was observed in a concentration-dependent fashion upon treatment with **20** or **26** (Figure 7). The results indicate that these compounds are potent inhibitors of HDACs in human non-small cell H1975 lung cancer cells.

2.2.6 *Effect of cell cycle distribution and cell death on Human Non-Small Cell Lung Cancer Cells.*

We evaluated cell cycle distribution alteration in response to **20** and **26** by Flow Cytometry. As shown in Figure 8A, treatment with higher concentrations of **20** and **26** induce severe subG1 phase cells accumulation, suggesting these two compounds generate dramatic cell death in H1975 cells. Further, **20** and **26** induce apoptosis by activating caspase 3,8,9, PARP, and γH2AX (Figure 8B). These results indicate **20** and **26** are potent inducers of apoptotic cell death in human non-small cell

H1975 lung cancer cells.

2.2.7 *Effect of IFN-γ induced PDL-1 expression in Human Non-small Cell Lung Cancer Cells.*

T cells, especially cytotoxic T cells, can infiltrate into the tumor area to eliminate tumor cells by releasing cytotoxins (perforins and granzymes) and inflammatory cytokines (IFN-γ and TNF-α)[26,27]. However, IFN-γ also triggers the expression of programmed death-ligand 1 (PD-L1) on the surface of many tumor cells, leading T-cell suppression and immune evasion of tumor cells from cellular cytotoxicity[28-30]. To investigate whether the Compounds **20** and **26** prevent the IFN-γ induced PD-L1 up-regulation in tumor cells, PD-L1 expression on human non-small cell lung cancer cells (H1975) was stimulated by 20 ng/ml of IFN-γ and these cells were co-treated with compounds **20** or **26**. The western blot analysis (Figure 9A) shows that compound **20** and **26** could effectively reduce PD-L1 expression in IFN-γ treated H1975 cells in a dose dependent manner. Moreover, the expression level of surface PD-L1 on IFN-γ treated H1975 cells could also be inhibited by compounds **20** and **26** with the concentrations above 0.25μM (Figure 9B, 9C).

3. Conclusion

We have designed a series of novel hydroxamic acids (**17-27**) as potent dual inhibitors of histone deacetylase and the HSP90 chaperone. Alkylated compounds (**20-23**) were more potent than the non-alkylated compounds (**17-19**). Compounds **20** (*para*, *N*-Me, C-6, HDAC IC_{50} = 194 nM; HSP90α IC_{50} = 153 nM) and **26** (*para*, *N*-Me C-7, HDAC IC_{50} = 360 nM; HSP90α IC_{50} = 77 nM) exhibited most potent activities while maintaining inhibition of HDAC and HSP90α. An alkyl side chain of 7 carbons was found to be the maximum tolerable length. Compounds **20** showed potent HDAC 6 inhibition with IC_{50} value of 0.04 μM and is 26 to 222 times more selective for HDAC 6 isoform compared to other synthetics. Upregulation of HSP70 and downregulation of well-known HSP90 client proteins such as EGFR, Src, FAK, and Rb further indicates the HSP90 inhibitory potential of

20 and **26**. They induce apoptosis by activating caspase 3,8,9, PARP, and γH2AX. Substantially, compounds **20** and **26** downregulate PD-L1 expression in IFN-γ treated lung H1975 cells in a dose dependent manner thus could be lead molecules for further development owing to their tumor growth and immunosuppression inhibitory activities.

4. Experimental section

4.1 Chemistry.

Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) spectra were obtained with a Bruker DRX-500 spectrometer (operating at 300 MHz and 500 MHz), with chemical shifts in parts per million (ppm, *δ*) downfield from TMS as an internal standard. High-resolution mass spectra (HRMS) were measured with a JEOL (JMS-700) electron impact (EI) mass spectrometer. Purity of the final compounds was determined using an Hitachi 2000 series HPLC system using C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μM. 4.6 mm × 150 mm) and were found to be ≥ 95%. Flash column chromatography was done using silica gel (Merck Kieselgel 60, No. 9385, 230-400 mesh ASTM). All reactions were carried out under an atmosphere of dry N_2.

***N*-4-Nitrophenyl-*t*-butylcarbamate (31).** A solution of di-*tert*-butyl dicarbonate (0.91 g, 4.20 mmol) in DCM was added dropwise to a solution of 4-nitroaniline (**28**, 0.5 g, 4.20 mmol), TEA (trimethylamine, 0.59 mL, 4.20 mmol) and DMAP (4-Dimethylaminopyridine, 0.25 g, 2.10 mmol) and the mixture was stirred for 3 h. The reaction was then quenched using H_2O, extracted with DCM (25 mL × 5) and purified by column chromatography to give **31** in 78% yield, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55 (s, 9H), 6.82 (bs, 1H), 7.53 (d, *J* = 9.3 Hz, 2H), 8.20 (d, *J* = 9.3 Hz, 2H).

N-4-Aminophenyl-*t*-butylcarbamate (34). 31 (1.1 g, 4.45 mmol) was dissolved in a mixture of IPA: H_2O (isopropyl alcohol: water. 4:1). Iron powder (Fe, 0.74 g, 13.35 mmol), NH_4Cl (ammonium chloride, 0.47 g, 8.89 mmol) were added and the mixture was refluxed for two hours. After cooling to room temperature, the reaction mixture was filtered through celite and extracted with EtOAc

(ethyl acetate, 25 mL × 3). The combined organic layer was dried over anhydrous $MgSO_4$, concentrated under reduced pressure and purified by column chromatography to give **34** as white solid in 91% yield, ^{1}H NMR (300 MHz, CD_3OD): δ 1.48 (s, 9H), 7.12. (d, *J* = 9.0 Hz, 2H), 7.15 (d, *J* = 9.3 Hz, 2H).

7-[4-(2,4-Bis-benzyloxy-5-isopropyl-benzoylamino)-phenylcarbamoyl]-heptanoic acid methyl ester (42). HBTU (1.82 g, 3.59 mmol), DIPEA (0.84 mL, 4.80 mmol) and monomethyl suberate (0.93 mL, 5.28 mmol) were added to a solution of **34** (1.0 g, 4.80 mmol) in DMF (10 mL) and the mixture was stirred for 12 h at rt. Then the reaction was quenched with H_2O and extracted using EtOAc, dried over $MgSO_4$ and passed through a filter column to give corresponding ester which was dissolved in the minimum amount of dioxane. H_2O with pH adjusted to 3 using 3N HCl was added and the reaction mixture was stirred at reflux overnight to yield the corresponding free amine. The reaction was basified and extracted using EtOAc, then dried, concentrated and passed through a filter column to give the free amine. To a solution of this free amine (1.0 g, 3.59 mmol) in DMF (10 mL) was added HBTU (1.36 g, 3.59 mmol), DIPEA (0.66 mL, 3.59 mmol) and 1,4-dibenzyloxy-5-isopropyl benzoic acid (0.76 g, 4.31 mmol) and the solution was stirred for 12 h at 80 ^oC . The reaction mixture was quenched with H_2O and extracted with EtOAc (25 mL × 3). The combined organic layer was collected, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a light yellow residue, which was purified by silica gel chromatography (EtOAc:n-hexane = 1: 1) to give **42** as a colorless liquid in 65% yield (overall from **34**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.27 (t, *J* = 6.9 Hz, 6H), 1.38- 1.41 (m, 4H), 1.62 – 1.69 (m, 4H), 2.32 - 2.38 (m, 4H), 3.65. (s, 3H),5.25 (s, 2H), 5.29 (s, 2H), 6.92 (s, 1H), 7.18(d, *J* = 9.0 Hz, 2H), 7.38 – 7.51 (m, 10 H), 7.56 – 7.58 (m, 2H), 7.98 (s, 1H).

Octanedioic acid [4-(2,4-dihydroxy-5-isopropyl-benzoylamino)phenyl]amide hydroxyamide (17). A mixture of 1N LiOH (7 mL) and **42** (1 g, 1.57 mmol) was stirred at 40 ^oC for 2 h. The reaction was concentrated under reduced pressure and then H_2O was added. The mixture was

acidified with 3N HCl to give an off-white liquid. The off-white liquid (0.07 g, 0.11 mmol) was dissolved in DMF (1 mL) and EDC•HCl (0.03 g, 0.16 mmol), HOBt (0.02 g, 0.16 mmol), and NMM (0.04 mL, 0.38 mmol) were added. After being stirred at room temperature for 30 min, NH_2OBn (0.02 g, 0.12 mmol) was added and the mixture was stirred for an additional 5 h. The reaction mixture was quenched with H_2O and was extracted with EtOAc (25 mL × 3). The combined organic layer was collected, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a light yellow residue, which was purified by silica gel chromatography (EtOAc:*n*-hexane = 1: 1) to give a colorless liquid. To a solution of the resulting product in methanol (10 mL) was added 10% Pd/C and the mixture was stirred at room temperature for 3 h under H_2. The reaction mixture was filtered through celite, concentrated under reduced pressure and purified by column to afford the desired compound (**17**), in 63% yield (from **42**); ^{1}H NMR (300 MHz, CD_3OD): δ 1.25 (d, *J* = 6.9 Hz, 6H), 1.39-1.42 (m, 4H), 1.62-1.72 (m, 4H), 2.12 (t, *J* = 7.5 Hz, 2H), 2.38 (t, *J* = 7.5 Hz, 2H), 3.17-3.26 (m, 1H), 6.35 (s, 1H), 7.53-7.60 (m, 4H), 7.75 (s, 1H). ^{13}C NMR (300 MHz, CD_3OD): 25.21, 25.37, 26.59, 28.44, 28.52, 36.44, 102.26, 107.54, 120.22, 121.84, 126.08, 127.30, 134.15, 135.00, 160.07, 173.13. LRMS *m*/*z*: calculated 480.49 found 480.2 (M+Na). HRMS (ESI) $C_{24}H_{32}N_3O_6$ ($M+H^+$) calcd, 458.2291; found, 458.2287.

N-3-Nitrophenyl-*t*-butylcarbamate (32). Compound **32** was synthesized using 3-nitroaniline (**29**, 0.5 g, 4.20 mmol), TEA (0.59 mL, 4.20 mmol), DMAP (0.25 g, 2.10 mmol) and di-*tert*-butyl dicarbonate (0.91 g, 4.20 mmol) following the method used for the synthesis of compound **31**, to give **32** in 81% yield, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.58 (s, 9H), 6.69 (bs, 1H), 7.72 (d, *J* = 7.8 Hz, 1H), 7.85 – 8.02 (m, 3H).

N-3-Aminophenyl-*t*-butylcarbamate (38). Compound **38** was synthesized using **32** (1.1 g, 4.45 mmol) dissolved in a mixture of IPA:H_2O (4:1), iron powder (0.74 g, 13.35 mmol) and NH_4Cl (0.47 g, 8.89 mmol) following the method used for the synthesis of compound **34**, to give **38** as white solid in 79% yield, ^{1}H NMR (300 MHz, CD_3OD): δ 1.46 (s, 9H), 6.85 (d, *J* = 7.8 Hz, 1H), 7.07 – 7.13 (m,

2H), 7.20 (d, *J* = 8.1 Hz, 1H), 7.26 (s, 1H).

7-[3-(2,4-Bis-benzyloxy-5-isopropyl-benzoylamino)-phenylcarbamoyl]-heptanoic acid methyl ester (43). Compound **43** was synthesized using **38** (1.0 g, 4.80 mmol) in DMF (10 mL), HBTU (1.82 g, 3.59 mmol), DIPEA (0.84 mL, 4.80 mmol) and monomethyl suberate (0.93 mL, 5.28 mmol) following the method used for the synthesis of compound **42**, to give **43** as a colorless liquid in 62% yield (overall from **38**); ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.01-1.11 (m, 6H), 1.37-1.40 (m, 4H), 1.77-1.88 (m, 4H), 2.33 (t, *J* = 7.2 Hz , 2H), 2.41 – 2.42 (m, 2H), 3.27-3.36 (m, 1H), 3.64. (s, 3H), 5.05(s, 2H), 5.26 (s, 2H), 6.54 (s, 1H), 7.39 – 7.40 (m, 15 H), 7.82 (s, 1H).

Octanedioic acid [3-(2,4-dihydroxy-5-isopropyl-benzoylamino)-phenyl]-amide hydroxyamide (18). Compound **18** was synthesized using a mixture of 1N LiOH (1 mL) and **43** (0.1 g, 0.15 mmol) following the method used for the synthesis of compound **17,** to afford the desired compound **18** as an off white liquid, in 59% yield (from **43**); ^{1}H NMR (300 MHz, DMSO-d6): δ 1.18 (d, *J* = 6.9 Hz, 6H), 1.23-1.28 (m, 4H), 1.44-1.59 (m, 4H), 1.93 (t, *J* = 7.5 Hz, 2H), 2.29 (t, *J* = 7.5 Hz, 2H), 3.06-3.24 (m, 1H), 6.37 (s, 1H), 7.22-7.35 (m, 3H), 7.76 (s, 1H), 7.96 (s, 1H), 8.65 (s, 1H), 9.90 (s, 1H), 10.14 (d, *J* = 8.4 Hz, 2H), 10.33 (s, 1H). ^{13}C NMR (300 MHz, CD_3OD): 13.66, 21.98, 24.87, 25.18, 25.43, 26.48, 28.46, 28.52, 28.64, 28.66, 30.48, 32.37, 34.11, 34.51, 35.78, 36.01, 102.52, 107.50, 125.19, 125.38, 125.68, 126.18, 127.62, 130.01, 132.09, 132.14, 155.14, 159.23, 160.33, 163.54, 167.77, 167.90, 171.71, 174.39, 174.45. LRMS *m*/*z*: calculated 480.49 found 480.1 ($M+Na^+$). HRMS (ESI) $C_{24}H_{32}N_3O_6$ ($M+H^+$), calcd, 458.2288; found, 458.2286.

N-2-Nitrophenyl-*t*-butylcarbamate (33). Compound **33** was synthesized using 2-nitroaniline (**30**, 0.5 g, 4.20 mmol), TEA (0.59 mL, 4.20 mmol) and DMAP (0.25 g, 2.10 mmol) following the method used for the synthesis of compound **31**, to give **33** in 72% yield, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (s, 9H), 7.07-7.13 (m, 1H), 7.59 -7.76 (m, 1H), 8.20 (dd, *J* = 8.4, Hz *J* = 9.9 Hz, 1H), 8.57 (dd, *J* = 8.4, Hz *J* = 8.7 Hz, 1H), 9.68 (s, 1H).

N-2-Aminophenyl-*t*-butylcarbamate (41). Compound **41** was synthesized using **33** (1.0 g, 4.40

mmol) which was dissolved in a mixture of IPA:H_2O (4:1) and iron powder (0.69 g, 12.88 mmol), NH_4Cl (0.39 g, 8.72 mmol) following the method used for the synthesis of compound **34,** to give **41** as white solid in 77% yield, ^{1}H NMR (300 MHz, CD_3OD): δ 1.48 (s, 9H),7.14 - 7.24 (m, 2H) 7.35. (d, *J* = 8.1 Hz, 2H), 7.53 (d, *J* = 8.4 Hz, 2H).

7-[2-(2,4-Bis-benzyloxy-5-isopropyl-benzoylamino)-phenylcarbamoyl]-heptanoic acid methyl ester (44). Compound **44** was synthesized using **41** (1.0 g, 4.80 mmol) in DMF (10 mL), HBTU (1.82 g, 3.59 mmol), DIPEA (0.84 mL, 4.80 mmol) and monomethyl suberate (0.93 mL, 5.28 mmol) following the method used for the synthesis of compound **42,** to give **44** as a colorless liquid in 57% yield (overall from **41**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.11- 1.26 (m, 6H), 1.31-1.36 (m, 4H), 1.52-1.74 (m, 4H), 2.29-2.25 (m, 2H), 2.37-2.45 (m, 2H), 3.10-3.21 (m, 1H), 3.66. (s, 3H), 5.15(s, 2H), 5.31 (s, 2H), 6.78 (s, 1H), 7.14-7.22 (m, 2 H), 7.35-7.54 (s, 14H).

Octanedioic acid [2-(2,4-dihydroxy-5-isopropyl-benzoylamino)-phenyl]-amide hydroxyamide (19). Compound **19** was synthesized using 1N LiOH (1 mL) and **44** (0.1 g, 0.15 mmol) following the method used for the synthesis of compound **17,** to give **19** as a colorless liquid in 49% yield (from **44**); ^{1}H NMR (300 MHz, CD_3OD): δ 1.25 (d, *J* = 6.9 Hz, 7H), 1.29-1.34 (m, 4H), 1.49-1.58 (m, 2H), 1.65-1.72 (m, 2H) 2.02-2.04 (m, 2H), 2.44 (t, *J* = 7.5 Hz, 2H), 3.17-3.26 (m, 1H), 6.37 (s, 1H), 7.20-7.25 (m, 1H), 7.28-7.33 (m, 2H), 7.68 (s, 1H), 7.81 (d, *J* = 7.8 Hz, 1H). ^{13}C NMR (300 MHz, DMSO-d6): 23.13, 25.47, 25.55, 26.39, 28.87, 31.26, 32.71, 36.17, 36.26, 103.05, 108.48, 124.71, 124.91, 126.32, 126.86, 127.01, 130.30, 159.06, 160.08, 162.81, 166.60, 169.61, 172.87. LRMS *m*/*z*: calculated 480.49 found 480.2 (M+Na). HRMS (ESI) $C_{24}H_{32}N_3O_6$ ($M+H^+$) calcd, 458.2287; found, 458.2286

N-4-Aminophenyl-N-methyl-*t*-butylcarbamate (35). 60% NaH (0.36 g, 9 mmol) was added at 0 ^oC to a solution of **31** (1.4 g, 6 mmol) in DMF and the mixture was stirred for 5 min before adding CH_3I (0.75 mL, 9 mmol). The reaction mixture was stirred for another 30 min at rt, then quenched by slow addition of H_2O and extracted with EtOAc (25 mL × 3). The combined organic layer was dried over

anhydrous $MgSO_4$ and concentrated under reduced pressure. This dried product (1.6 g, 6.46 mmol) was dissolved in a mixture of IPA:H_2O (4:1) then iron powder (1.1 g, 19.38 mmol) and NH_4Cl (0.7 g, 12.92 mmol) were added following the procedure used for the synthesis of compound **34**, to give **35** as white solid in 79% yield (from **31**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.48 (s, 9H), 2.59 (s, 3H), 6.82. (d, *J* = 9.0 Hz, 2H), 7.15 (d, *J* = 8.7 Hz, 2H).

7-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-methyl-amino]-phenylcarbamoyl}-heptanoic acid methyl ester (45). Compound **45** was synthesized using **35** (1.0 g, 4.50 mmol) in DMF (10 mL), HBTU (1.71 g, 4.50 mmol), DIPEA (0.78 mL, 4.50 mmol) and monomethyl suberate (0.87 mL, 4.95 mmol), following the method used for synthesis of compound **42**, to **45** give as a colorless liquid in 61% yield (overall from **35**), mp: 138-140 ^{o}C. ^{1}H NMR (300 MHz, CD_3OD): δ 1.07 (bs, 6H), 1.31-1.38 (m, 4H), 1.63 – 1.68 (m, 4H), 2.32 - 2.34 (m, 4H), 3.12-3.20 (m, 1H), 3.59 (s, 3H), 3.64. (s, 3H), 4.95(bs, 4H), 6.46 (s, 1H), 6.93 – 6.97 (m, 3H), 7.33-7.47 (m, 12 H).

Octanedioic acid {4-[(2,4-dihydroxy-5-isopropyl-benzoyl)-methyl-amino]-phenyl}-amide hydroxyamide (20). Compound **20** was synthesized using 1N LiOH (7 mL) and **45** (1 g, 1.53 mmol) following the method used for synthesis of compound **17**, to give **20** as white solid, in 64% yield (from **45**); mp: 164-165 ^{o}C. ^{1}H NMR (300 MHz, CD_3OD): δ 0.82 (d, *J* = 6.6 Hz, 6H), 1.31-1.38 (m, 4H), 1.64-1.69 (m, 4H), 2.10 (t, *J* = 7.2 Hz, 2H), 2.36 (t, *J* = 7.2 Hz, 2H), 2.88-2.97 (m, 1H), 3.41 (s, 3H), 6.19 (s, 1H), 6.61 (s, 1H), 7.12 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 21.53, 25.21, 25.31, 25.48, 28.46, 32.32, 36.45, 37.71, 101.93, 109.02, 120.41, 125.61, 126.87, 128.03, 137.44, 141.09, 158.07, 158.33, 171.92, 173.19. LRMS *m*/*z*: calculated 494.52 found 494.2 (M+Na). HRMS (ESI) $C_{25}H_{34}N_3O_6$ ($M+H^+$) calcd, 472.2448; found, 472.2444.

N-3-Aminophenyl- N-methyl-*t*-butylcarbamate (39). Compound **39** was synthesized using **32** (1.4 g, 6 mmol) in DMF, 60% NaH (0.36 g, 9 mmol) and CH_3I (0.75 mL, 9 mmol) following the method used for the synthesis of compound **35**, to give **39** as white solid in 72% yield (from **32**), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 3.23 (s, 3H), 6.96-6.70. (m, 1H), 7.29 (t, J = 8.1 Hz, 1H), 7.35-

7.39 (m, 1H), 7.59 (t, J = 2.1 Hz, 1H).

7-{3-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-methyl-amino]-phenylcarbamoyl}-heptanoic acid methyl ester (46). Compound **46** was synthesized using **39** (1.0 g, 4.50 mmol) in DMF (10 mL), HBTU (1.71 g, 4.50 mmol), DIPEA (0.78 mL, 4.50 mmol) and monomethyl suberate (0.87 mL, 4.95 mmol) following the method used for synthesis of compound **42**, to give **46** as **a** colorless liquid in 56% yield (overall from **39**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.07-1.09 (m, 6H), 1.31-1.36 (m, 4H), 1.59-1.68 (m, 4H), 2.88-2.34 (m, 4H), 3.12-3.19 (m, 1H), 3.41 (s, 3H), 3.64. (s, 3H), 4.92-4.97 (m, 4H), 6.46 (s, 1H), 6.74 (s, 1H), 6.99-6.71 (m, 2H), 7.24 -7.39 (m, 10 H), 7.56 (s, 1H).

Octanedioic acid {3-[(2,4-dihydroxy-5-isopropyl-benzoyl)-methyl-amino]-phenyl}-amide hydroxyamide (21). Compound **21** was synthesized using 1N LiOH (1 mL) and **46** (0.1 g, 0.15 mmol) following the method used for the synthesis of compound **17**, to give **21**, in 61% yield (from **46**); mp: 173-174 ^{o}C ^{1}H NMR (300 MHz, DMSO-d6): δ 0.77 (d, *J* = 6.9 Hz, 6H), 1.23 (bs, 8H), 1.42-1.53 (m, 4H), 1.90-1.95 (m, 2H), 2.24 (t, *J* = 7.5 Hz, 2H), 2.79-2.86 (m, 1H), 3.29 (s, 3H), 6.19 (s, 1H), 6.60 (s, 1H), 6.86 (d, *J* = 7.8 Hz, 1H), 7.23 (t, *J* = 8.1 Hz, 1H), 7.41 (d, *J* = 8.1 Hz, 1H), 7.51 (s, 1H), 8.64 (s,1H), 9.72 (s,1H), 9.78 (s,1H), 10.31 (s, 1H), 10.70 (s, 1H). ^{13}C NMR (300 MHz, CD_3OD): 21.60, 25.13, 25.24, 25.32, 25.49, 28.46, 28.55, 28.71, 30.38, 32.37, 34.05, 35.00, 35.68, 36.51, 37.88, 102.03, 108.51, 117.95, 118.08, 121.84, 125.65, 128.17, 129.54, 139.92, 146.03, 158.29, 158.80, 163.52, 171.64, 171.92, 173.33. LRMS *m/z*: calculated 494.52 found 494.2 ($M+Na^+$). HRMS (ESI) $C_{25}H_{34}N_3O_6$ ($M+H^+$) calcd, 472.2444; found, 472.2442.

N-4-Aminophenyl-N-ethyl-*t*-butylcarbamate (36). Compound **36** was synthesized using **31** (0.93 g, 3.90 mmol) in DMF, 60% NaH (0.24 g, 5.85 mmol) and ethyl iodide (0.62 mL, 3.90 mmol) following the method used for the synthesis of compound **35,** to give **36** as white solid in 81% yield (from **31**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.23 (t, *J* = 7.2 Hz, 3H), 1.42 (s, 9H) 3.10-3.13 (m, 2H), 6.63. (d, *J* = 9.0 Hz, 2H), 7.28 (d, *J* = 9.0 Hz, 2H).

7-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-ethyl-amino]-phenylcarbamoyl}-heptanoic acid

methyl ester (47). Compound **47** was synthesized using **36** (1.0 g, 4.23 mmol) in DMF (10 mL), HBTU (1.60 g, 4.23 mmol), DIPEA (0.74 mL, 4.23 mmol) and monomethyl suberate (0.82 mL, 4.65 mmol) following the method used for the synthesis of compound **42**, to give **47 as a** colorless liquid in 54% yield (overall from **36**), ^{1}H NMR (300 MHz, CD_3OD): δ 0.78 (d, *J* = 7.2 Hz, 6H), 1.23 (t, *J* = 7.2 Hz, 3H), 1.22-1.27 (m, 4H), 1.31-1.41 (m, 4H), 2.30 - 2.34 (m, 4H), 3.06-3.08 (m, 1H), 3.09-3.14 (m, 2H), 3.66 (s, 3H), 5.15(s, 2H), 5.21 (s, 2H), 6.89 (s, 1H), 7.10 (d, *J* = 8.2 Hz, 2H), 7.33-7.52 (m, 13H).

Octanedioic acid {4-[(2,4-dihydroxy-5-isopropyl-benzoyl)-ethyl-amino]-phenyl}-amide hydroxyamide (22). Compound **22** was synthesized using 1N LiOH (7 mL) and **47** (1 g, 1.50 mmol) following the method used for the synthesis of compound **17**, to give **22**, in 59% yield (from **47**); ^{1}H NMR (300 MHz, CD_3OD): δ 0.82 (d, *J* = 6.9 Hz, 6H), 1.21 (t, *J* = 6.3 Hz, 3H), 1.38-1.40 (m, 4H), 1.62-1.70 (m, 4H), 2.11 (t, *J* = 6.9 Hz, 2H), 2.37 (t, *J* = 7.2 Hz, 2H), 2.90-2.97 (m, 1H), 3.89-3.96 (m, 2H), 6.19 (t, *J* = 0.9 Hz, 1H), 6.61 (s, 1H), 7.11 (d, *J* = 7.8 Hz, 2H), 7.57 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 11.78, 21.70, 25.05, 25.27, 25.37, 25.53, 28.48, 28.49, 28.60, 28.69, 32.41, 34.77, 35.79, 36.55, 45.50, 102.09, 109.16, 120.48, 120.58, 125.61, 127.85, 128.05, 137.56, 139.18, 158.01, 158.53, 171.44, 171.69, 173.29. LRMS (M+Na) *m*/*z*: calculated 508.55 found 508.2. HRMS (ESI) $C_{26}H_{36}N_3O_6$ ($M+H^+$) calcd, 486.2599; found, 486.2601.

N-3-Aminophenyl- N-ethyl-*t*-butylcarbamate (40). Compound **40** was synthesized using **32** (1.0 g, 3.98 mmol) in DMF, 60% NaH (0.25 g, 5.92 mmol) and C_2H_5I (0.66 mL, 4.10 mmol) following the method used for the synthesis of compound **35,** to give **40** as white solid in 74% yield (from **32**), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.15 (t, *J* = 7.2 Hz, 3H), 1.42 (s, 9H) 3.42-3.13 (m, 2H), 6.50-6.57. (m, 1H), 7.13-7.14 (m, 1H), 7.32-7.38 (m, 1H), 7.41 (t, *J* =2.1 Hz, 1H).

7-{3-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-ethyl-amino]-phenylcarbamoyl}-heptanoic acid methyl ester (48). Compound **48** was synthesized using **40** (1.0 g, 4.23 mmol) in DMF (10 mL), HBTU (1.60 g, 4.23 mmol), DIPEA (0.74 mL, 4.23 mmol) and monomethyl suberate (0.82 mL, 4.65

mmol) following the method used for synthesis of compound **42**, to give **48** as a colorless liquid in 49% yield (overall from **40**), ^{1}H NMR (300 MHz, DMSO-d6): δ 0.98-1.06 (m, 9H), 1.23-1.26 (m, 4H), 1.50- 1.52 (m, 4H), 2.21-2.30 (m, 4H), 3.02-3.10 (m, 1H), 3.56 (s, 3H), 3.72-3.80 (m, 2H), 5.01-5.14 (m, 4H), 6.64-6.65 (m, 2H), 6.86-6.88 (m, 1H), 7.07-7.46 (m, 1H), 7.23-7.46 (m, 13H), 7.57 (s, 1H), 9.81 (s, 1H).

Octanedioic acid {3-[(2,4-dihydroxy-5-isopropyl-benzoyl)-ethyl-amino]-phenyl}-amide hydroxyamide (23). Compound **23** was synthesized using 1N LiOH (7 mL) and **48** (1 g, 1.50 mmol) following the method used for the synthesis of compound **17**, to give **23** in 55% yield (from **48**); ^{1}H NMR (300 MHz, DMSO-d6): δ 0.75 (d, *J* = 6.9 Hz, 6H), 1.09 (t, *J* = 7.2 Hz, 3H), 1.23 (bs, 9H), 1.42-1.53 (m, 4H), 1.90-1.97 (m, 2H), 2.25 (t, *J* = 7.2 Hz, 2H), 2.78-2.85 (m, 1H), 3.74-3.81 (m, 2H), 6.18 (s, 1H), 6.57 (s, 1H), 6.83 (d, *J* = 9.0 Hz, 1H), 7.24 (t, *J* = 8.1 Hz, 1H), 7.44-7.49 (m, 2H), 8.64 (s, 1H), 9.72 (s, 1H), 9.90 (s, 1H), 10.32 (s, 1H), 10.84 (s, 1H). ^{13}C NMR (300 MHz, CD_3OD): 11.74, 21.59, 25.23, 25.31, 25.47, 28.45, 28.54, 32.36, 36.51, 45.58, 101.98, 108.62, 118.02, 118.87, 122.76, 125.55, 128.11, 129.45, 139.94, 144.31, 158.20, 159.02, 171.44, 171.64, 173.34. LRMS *m*/*z*: calculated 508.55 found 508.2 (M+Na). HRMS (ESI) $C_{26}H_{36}N_3O_6$ ($M+H^+$) calcd, 486.2599; found, 486.2601.

N-4-Aminophenyl- N-isopropyl-*t*-butylcarbamate (37). 60% NaH (0.25 g, 6.28 mmol) was added at 0 ^{o}C to a solution of **31** (1 g, 4.19 mmol) in DMF and stirred for 5 mins before adding C_2H_5I (0.62 mL, 3.90 mmol). The reaction mixture was stirred for another 30 min at rt and then heated at 50 ^{o}C for 5 h following the method used for synthesis of compound **35,** to give **37** as an off white solid in 69% yield (from **31**), ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.07 (d, *J* = 6.6 Hz, 6H), 1.38 (s, 9H), 4.44-4.45 (m, 1H), 6.88-6.95 (m, 4H).

7-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-isopropyl-amino]-phenylcarbamoyl}-heptanoic acid methyl ester (49). Compound **49** was synthesized using **37** (0.28 g, 1.12 mmol) in DMF (5 mL), HBTU (0.42 g, 1.12 mmol), DIPEA (0.19 mL, 1.12 mmol) and monomethyl suberate (0.22 mL, 1.23

mmol) following the method used for the synthesis of compound **42**, to give **49** as a colorless liquid in 49% yield (overall from **37**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.01-1.09 (m, 12H), 1.10-1.29 (m, 4H), 1.51-1.58 (m, 4H), 2.02-2.17 (m, 2H), 2.30-2.33 (m, 2H), 3.11-3.12 (m, 1H), 3.69 (s, 3H), 4.93(s, 3H), 4.96 (s, 2H), 6.44 (s, 1H), 6.87-6.92 (m, 3H), 7.30-7.45 (m, 12 H).

Octanedioic acid {4-[(2,4-dihydroxy-5-isopropyl-benzoyl)-isopropyl-amino]-phenyl}-amide hydroxyamide (24). Compound **24** was synthesized using 1N LiOH (1 mL) and **49** (0.1 g, 1.47 mmol) following the method used for the synthesis of compound **17**, to give **24** in 51% yield (from **49**); ^{1}H NMR (300 MHz, CD_3OD): δ 0.85 (d, *J* = 6.9 Hz, 6H), 1.18 (d, *J* = 6.9Hz, 6H), 1.36 (bs, 4H), 1.61-1.64 (m, 4H), 2.00-2.10 (m, 2H), 2.34 (t, *J* = 7.2 Hz, 2H), 2.85-2.98 (m, 1H), 4.93-4.97 (m, 1H), 6.13 (s, 1H), 6.58 (s, 1H), 7.07 (d, *J* = 8.7 Hz, 2H), 7.52 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 19.95, 21.67, 25.21, 25.31, 25.60, 28.46, 28.55, 36.49, 70.06, 101.87, 119.71, 125.46, 127.16, 130.29, 138.15, 157.17, 171.75, 173.24. LRMS (ESI) *m*/*z*: calcd, ($M+H^+$) 500.2 found 500.3. HRMS (ESI) $C_{27}H_{38}N_3O_6$ ($M+H^+$) calcd, 500.2757; found, 500.2755.

6-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-methyl-amino]-phenylcarbamoyl}-hexanoic acid ethyl ester (50). Compound **50** was synthesized using **35** (0.62 g, 2.78 mmol) in DMF (5 mL), HBTU (1.05 g, 2.78 mmol), DIPEA (0.48 mL, 2.78 mmol) and monoethyl pimelate (0.54 mL, 2.36 mmol) following the method used for the synthesis of compound **42**, to give **50** as a colorless liquid in 63% yield (overall from **35**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.11-1.26 (m, 9H), 1.62-1.77 (m, 4H), 2.30-2.38 (m, 4H), 2.37-2.45 (m, 2H), 3.01-3.13 (m, 1H), 3.16-3.32 (m, 2H), 3.42. (s, 3H), 4.08-4.15 (m, 2H), 5.15(s, 2H), 5.28 (s, 2H), 6.78 (s, 1H), 7.14 (d, *J* = 8.7 Hz, 2H), 7.55 (d, *J* = 8.7 Hz, 2H), 7.78-7.84 (m, 12H).

Heptanedioic acid [4-(2,4-dihydroxy-5-isopropyl-benzoylamino)-phenyl]-amide hydroxyamide (**25**). Compound **25** was synthesized using 1N LiOH (3 mL) and **50** (0.5 g, 0.84 mmol) following the method used for the synthesis of compound **17**, to give **25**, in 53% yield (from **50**); ^{1}H NMR (300 MHz, CD_3OD): δ 0.82 (d, *J* = 6.9 Hz, 6H), 1.36-1.44 (m, 2H), 1.61-1.75 (m, 4H), 2.11 (t, *J* = 7.2 Hz,

2H), 2.36 (t, *J* = 7.5 Hz, 2H), 2.88-2.97 (m, 1H), 3.40 (s, 3H), 6.20 (s, 1H), 6.62 (s, 1H), 7.11 (d, *J* = 9 Hz, 2H), 7.55 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 21.70, 25.08, 25.56, 32.27, 36.38, 37.93, 102.13, 109.16, 120.55, 125.73, 126.90, 128.09, 137.37, 141.04, 158.06, 158.21, 171.60, 171.92, 173.16. LRMS ($M+H^+$) m/z: calculated 458.2 found 458.2. HRMS (ESI) $C_{24}H_{33}N_3O_6$ ($M+H^+$) calcd, 458.2287; found, 458.2286.

8-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-methyl-amino]-phenylcarbamoyl}-octanoic acid methyl ester (51). Compound **51** was synthesized using **35** (0.57 g, 2.56 mmol) in DMF (5 mL), HBTU (0.97 g, 2.56 mmol), DIPEA (0.45 mL, 2.56 mmol) and monomethyl azelate (0.55 mL, 2.84 mmol) following the method used for the synthesis of compound **42**, to give colorless liquid as **51** in 63% yield (overall from **35**), ^{1}H NMR (300 MHz, CD_3OD): δ 1.52-1.53 (m, 4H), 1.76-1.81 (m, 6H), 2.03-2.12 (m, 4H), 2.73-2.78(m, 4H), 3.21-3.25 (m, 1H), 3.77 (m, 3H), 4.08 (s, 3H), 5.40 (bs, 4H), 6.91 (s, 1H), 7.39-7.42 (m, 2 H), 7.77-7.84 (m, 14H).

Nonanedioic acid [4-(2,4-dihydroxy-5-isopropyl-benzoylamino)-phenyl]-amide hydroxyamide (26). Compound **26** was synthesized using 1N LiOH (2 mL) and **51** (0.4 g, 0.58 mmol) following the method used for the synthesis of compound **17**, to give **26**, in 51% yield (from **51**); ^{1}H NMR (300 MHz, CD_3OD): δ 0.76 (d, *J* = 6.9 Hz, 6H), 1.25-1.32 (m, 6H), 1.55-1.65 (m, 4H), 2.04 (t, *J* = 7.5 Hz, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.83-2.92 (m, 1H), 3.36 (s, 3H), 6.15 (s, 1H), 6.56 (s, 1H), 7.07 (d, *J* = 8.7 Hz, 2H), 7.51 (d, *J* = 8.7 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 21.57, 25.32, 25.45, 25.50, 28.69, 28.68, 28.74, 37.76, 101.97, 120.42, 125.61, 126.88, 128.06, 141.08, 158.08. LRMS ($M+H^+$) *m*/*z*: calcd. 486.5 found 486.2. HRMS (ESI) $C_{26}H_{36}N_3O_6$ ($M+H^+$) calcd, 486.2599; found, 486.260.

9-{4-[(2,4-Bis-benzyloxy-5-isopropyl-benzoyl)-methyl-amino]-phenylcarbamoyl}-nonanoic acid methyl ester (52). Compound **52** was synthesized using **35** (0.6 g, 2.69 mmol) in DMF (5 mL), HBTU (1.02 g, 2.69 mmol), DIPEA (0.47 mL, 2.69 mmol) and monomethyl sebacate (0.64 g, 2.96 mmol) following the method used for the synthesis of compound **42,** to give **52** as a colorless liquid in 63% yield (overall from **35)**, ^{1}H NMR (300 MHz, CD_3OD): δ 1.06- 1.08 (m, 6H), 1.31-1.34 (s,

8H), 1.59- 1.68 (m, 4H), 2.28-2.36 (m, 4H), 3.11-3.22 (m, 1H), 3.64 (s, 3H), 4.94 (bs, 4H), 6.45 (s, 1H), 6.91-6.97 (m, 2H), 7.32-7.49 (m, 14H).

Decanedioic acid [4-(2,4-dihydroxy-5-isopropyl-benzoylamino)-phenyl]-amide hydroxyamide (27). Compound **27** was synthesized using 1N LiOH (2 mL) and **52** (0.3 g, 0.44 mmol) following the method used for the synthesis of compound **17**, to give **27**, in 53% yield (from **52**); ^{1}H NMR (300 MHz, CD_3OD): δ 0.82 (d, *J* = 6.6 Hz, 6H), 1.31-1.35 (m, 10H), 1.60-1.68 (m, 4H), 2.09 (t, *J* = 7.5 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.89-2.95 (m, 1H), 3.42 (s, 3H), 6.20 (s, 1H), 6.61 (s, 1H), 7.13 (d, *J* = 8.7 Hz, 2H), 7.56 (d, *J* = 9.0 Hz, 2H). ^{13}C NMR (300 MHz, CD_3OD): 21.64, 25.07, 25.38, 25.52, 28.73, 28.82, 28.85, 28.91, 32.44, 36.63, 37.87, 102.05, 108.93, 120.47, 125.64, 126.88, 128.10, 137.46, 141.05, 158.12, 158.44, 171.71, 171.91, 173.34. LRMS (ESI) m/z: calcd, ($M+H^+$) 500.2 found 500.3. HRMS (ESI) $C_{27}H_{38}N_3O_6$ ($M+H^+$) calcd, 500.2759; found, 500.2755

4.2 Biology

4.2.1 Tumor Cell Culture. All human cancer cells were maintained in RPMI 1640 medium supplemented with 10% FBS and penicillin (100 units/ml)/streptomycin (100 μg/ml)/amphotericin B (0.25 μg/ml). All cells were maintained in humidified air containing 5% CO_2 at 37°C and cultured every 2-3 days. All cells were cultured in tissue culture flasks in a humidified air containing 5% CO_2 and 95% air at 37 ° C and cultured every 2-3 days.

4.2.2 Sulforhodamine B Assays. Cells were seeded at a density of 5000 cells/well into 96-plate overnight. Basal cells were fixed with 10% trichloroacetic acid (TCA) to represent cell population at the time of compound addition (T_0). After additional incubation of DMSO (C) or different doses of test compounds (Tx) for 48 h, cells were fixed with 10 % TCA and stained with sulforhodamine B (SRB) at 0.4 % (w/v) in 1% AcOH (acetic acid). Unbound SRB was washed out using 1% AcOH and SRB-bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. The 50% growth inhibition (GI_{50}) was calculated as $100 - [(T_x - T_0) / (C -$

$T_0)] \times 100$.

4.2.3 HeLa Nuclear Extract HDAC Activity Assay. HDAC Fluorescent Activity Assay Kit (BioVision, CA) was used to detect HeLa nuclear extract HDAC activity according to manufacturer's instructions. Briefly, the HDAC fluorometric substrate and assay buffer were added to HeLa nuclear extracts in a 96-well format and incubated at 37°C for 30 min. The reaction was terminated by addition of lysine developer, and the mixture was incubated for another 30 min at 37°C. Additional negative controls included incubation without the nuclear extract, without the substrate, or without both. A fluorescence plate reader with excitation at 355 nm and emission at 460 nm was used to quantify HDAC activity.

4.2.4 HDAC Biochemical Assays. Enzyme inhibition assays (HDAC1-11 and SIRT1) were conducted by the Reaction Biology Corporation, Malvern, PA. (www.reactionbiology.com) Compounds were dissolved in DMSO and tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 10 μM. HDAC Control Compound trichostatin A was tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 10 μM as shown in supporting information.

4.2.5 Heat shock protein (HSP) 90α activity assay. An HSP90α activity assay kit (BPS Bioscience, San Diego, CA, USA) was used to determine the compound inhibitory effect of HSP90α activity according to manufacturer's instructions. Briefly, the reactions were conducted at room temperature for 3 h in a 100 μL mixture containing FITC-labeled geldanamycin, Hsp90α enzyme, and test compounds in assay buffer. A fluorescence polarization plate reader with excitation at 475-495 nm and emission at 518–538 nm was used to quantify HSP90α activity.

4.2.6 FACScan Flow Cytometric analysis. Cells were seeded in 6-well plates (2.5×10^5/well) and incubated overnight. Next day cells were treated with DMSO or indicated compounds at various concentrations for 48 h. Cells were washed with phosphate-buffered saline, fixed in ice-cold 70% ethanol at −20 °C overnight, and stained with propidium iodide (80 ug/ml) containing Triton X-100 (0.1%, v/v) and RNase A (100 ug/ml) in phosphate-buffered saline. DNA content was counted and

analyzed with the FACScan and CellQuest software (Becton Dickinson, Mountain View, CA, USA).

4.2.7 Western Blot Analysis. Cells were incubated with indicated compounds for 24 h and lysed with ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2.5 mM β-glycerolphosphate, 1 mM $Na_4P_2O_7$, 5 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail from Millipore) on ice for 30 min followed by centrifugation at 13000 rpm for 30 min. Protein concentrations were determined and equal amounts of protein were separated by 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to poly(vinylidene difluoride) (PVDF) membranes. Membranes were immunoblotted with specific antibodies overnight at 4°C and then applied to appropriate horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies for 1 h at room temperature. Signals were detected using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Primary antibodies against heat shock protein 70 (HSP70) and other proteins were purchased from Cell Signalling Technology (Danvers, MA, USA).

4.2.8 Western blot analysis of PD-L1 expression. Cell lysates of H1975 cells which were treated with DMSO, Compound 20, Compound 26, or IFN-γ at various concentrations were electrophoresed in 10% reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk (dissolved in phosphate-buffered saline containing 0.05% Tween 20 (PBST)) and incubated with mouse anti-PDL-1 antibody (Cat#AW5698;ABGENT, San Diego, CA, U.S.A.) or mouse anti-β-actin (Cat# A5416; Sigma-Aldrich, St. Louis, MO, U.S.A.) for 1h. HRP-conjugated goat anti-mouse IgG antibodies (1 μg/ml; Cat# 115-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) and enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific, Waltham, MA, U.S.A.) were used to detect protein signals.

4.2.9 Analysis of PD-L1 expression on cell surface by flow cytometry. H1975 cells were seeded in 6 well-plates (3.2×10^5 cells/well) and treated with DMSO, Compound **20**, **26**, or IFN-γ at various

concentrations for 48h. After removing supernatant, cells were suspended at 3 x 10^5 cells/tube in PBS buffer containing 0.05% (w/v) BSA (PBS/BSA) and then incubated with 5 µg/ml of mouse anti-PD-L1 antibody (Cat# 14-5983-82; Thermo Fisher Scientific) for 1h. Then cells were incubated with FITC-conjugated goat anti-mouse IgG antibody (Cat# 115-096-071; Jackson ImmunoResearch Laboratories) for 1h. After the removal of unbound antibodies by extensive washing with PBS/BSA, forward scatter (FSC) signal, side scatter (SSC) signal, and FITC fluorescence of the cells were measured by FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The data and mean fluorescence intensity (MFI) of FITC signal were analyzed by CellQuest software (BD Biosciences, San Jose, CA, USA).

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Table 1. Inhibition of HeLa Nuclear Extract HDAC Activity and Antiproliferative Activity (SRB assay) against Human Cancer Cell Lines by **17-27** and reference compounds **1** and **III**.

Compd	Cell type (GI_{50} ± SD, μM[a])			IC_{50} ± SD (nM[a])	
	Lung A549	Colorectal HCT116	Lung H1975	HDAC HeLa	HSP90α
17	>10	5.43 ± 0.90	>10	415.07 ± 77.6	>1000
18	>10	6.23 ± 0.58	>10	105.47 ± 14.3	>1000
19	>10	4.92 ± 0.68	>10	>1000	>1000
20	0.77 ± 0.08	0.83 ± 0.07	0.69 ± 0.06	194.55 ± 45.6	153.07 ± 14.7
21	2.3 ± 0.31	1.17 ± 0.45	1.66 ± 0.18	211.33 ± 56.7	302.29 ± 48.84
22	5.63 ± 0.16	3.56 ± 1.17	1.38 ± 0.14	461.7 ± 22.9	186.07 ± 21.08
23	1.59 ± 0.22	1.12 ± 0.41	0.94 ± 0.12	126.01 ± 38.5	145.8 ± 40.60
24	>10	>10	>10	>1000	>1000
25	2.83 ± 0.14	3.78 ± 0.31	0.69 ± 0.09	391.4 ± 35.7	169.34 ± 26.3
26	0.44 ± 0.19	1.06 ± 0.03	0.40 ± 0.04	360.82 ± 17.8	77.21 ± 4.27
27	1.08 ± 0.11	0.59 ± 0.16	0.21 ± 0.08	671.83 ± 56.4	ND
III	0.0075 ± 0.007	0.34 ± 0.06	0.16 ± 0.06	>1000	98.33 ± 14.96
1	1.02 ± 0.15	0.55 ± 0.1	2.61 ± 0.13	98.35 ± 10.06	>500

[a]SD: standard deviation. All experiments were independently performed at least three times. ND: not determined. GI_{50}: 50 % growth inhibition; IC_{50}: half maximal inhibitory concentration.

Table 2. Activities of **20** and **26** against HDAC Isoforms 1-11, and SIRT1.

	IC_{50} (μM)[a]	
isoform	**20**	**26**
HDAC1	1.31	6.06
HDAC2	1.19	4.91
HDAC3	2.06	1.44
HDAC4	>10	>10
HDAC5	7.86	5.41
HDAC6	0.04	0.316
HDAC7	>10	6.40
HDAC8	0.422	3.41
HDAC9	>10	>10
HDAC10	2.67	>10
HDAC11	3.61	1.89
SIRT1	>10	>10

[a]These assays were conducted by the Reaction Biology Corporation, Malvern, PA. All compounds were dissolved in DMSO and tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 10 μM. IC_{50}: half maximal inhibitory concentration.

Figure Caption

Figure 1. Examples of histone deacetylase inhibitors

Figure 2. Structures of SAHA based potent inhibitors of HDAC

Figure 3. Compounds with dual inhibitory potential

Figure 4. Examples of various HSP90 inhibitors

Figure 5. Dual inhibitors of HDAC and HSP90

Figure 6. **20** and **26** triggered induction of HSP70 and degradation of multiple HSP90 client proteins in a concentration and time-dependent manner. (A, C) H1975 cells were treated with DMSO (Control; C) or 0.1-3 μM of test compounds (**20** or **26** and 0.1 μM of compound **V**) for 24 h. Cells were harvested and subjected to Western blot analysis for the detection of various HSP90 client proteins. (B, D) H1975 cells were treated with 1 μM of **20** or **26** for the indicated times. Whole cell lysates were collected, and each protein expression was detected by immunoblotting. GAPDH was used as the internal control and all experiments were repeated at least three independent experiments.

Figure 7. HDAC inhibitory activity of compounds **20** and **26** in H1975 cells via acetylation of α-Tubulin and Histone H3. (A, B) The effect of α-Tubulin and Histone H3 acetylation changes in human non-small cell lung cancer cells. Cells were cultured for 24 h with **20**, **26** or compound **1** at indicated concentrations. Whole cell lysates were detected by immunoblotting. All experiments were repeated at least three times.

Figure 8. Compounds **20** and **26** induce accumulation of a sub-G1 phase population and significant cell apoptosis in H1975 cells. (A) Compound **20** (left panel) and **26** (right panel) induced sub-G1 phase cells accumulation. (B) Compound **20** (left panel) and **26** (right panel) induced cell apoptotic death in H1975 cells. Cells were cultured indicated concentrations with **20** or **26** for 48 h and cell lysates were detected by indicated antibodies.

Figure 9. Downregulation of PD-L1 expression by Compounds **20** and **26** in IFN-r treated H1975 lung cancer cells. (A) Western blot analysis of PD-L1 expression in cells treated with IFN-γ alone (20 ng/ml) or in combination with Compounds **20** and **26** at the indicated concentrations for 48 h. β-actin was used as a loading control. (B and C) Flow cytometry analysis of PD-L1 surface expression in cells treated with IFN-γ alone (20 ng/ml) or in combination with Compounds **20** and **26** at the indicated concentrations for 48 h. (C) The MFI of treated cells is presented relative as compare with the MFI of non-treated cells. The data are presented as mean ± S.D. obtained from three independent experiments. $^{**}P < 0.01$ compared with the IFN-r alone group.

Table 1. Inhibition of HeLa Nuclear Extract HDAC Activity and Antiproliferative Activity (SRB assay) against Human Cancer Cell Lines by **17-27** and reference compounds **1** and **III**.

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18	>10	6.23 ± 0.58	>10	105.47 ± 14.3	>1000
19	>10	4.92 ± 0.68	>10	>1000	>1000
20	0.77 ± 0.08	0.83 ± 0.07	0.69 ± 0.06	194.55 ± 45.6	153.07 ± 14.7
21	2.3 ± 0.31	1.17 ± 0.45	1.66 ± 0.18	211.33 ± 56.7	302.29 ± 48.84
22	5.63 ± 0.16	3.56 ± 1.17	1.38 ± 0.14	461.7 ± 22.9	186.07 ± 21.08
23	1.59 ± 0.22	1.12 ± 0.41	0.94 ± 0.12	126.01 ± 38.5	145.8 ± 40.60
24	>10	>10	>10	>1000	>1000
25	2.83 ± 0.14	3.78 ± 0.31	0.69 ± 0.09	391.4 ± 35.7	169.34 ± 26.3
26	0.44 ± 0.19	1.06 ± 0.03	0.40 ± 0.04	360.82 ± 17.8	77.21 ± 4.27
27	1.08 ± 0.11	0.59 ± 0.16	0.21 ± 0.08	671.83 ± 56.4	ND
III	0.0075 ± 0.007	0.34 ± 0.06	0.16 ± 0.06	>1000	98.33 ± 14.96
1	1.02 ± 0.15	0.55 ± 0.1	2.61 ± 0.13	98.35 ± 10.06	>500

[a]SD: standard deviation. All experiments were independently performed at least three times. ND: not determined. GI_{50}: 50 % growth inhibition; IC_{50}: half maximal inhibitory concentration.

Table 2. Activities of **20** and **26** against HDAC Isoforms 1-11, and SIRT1.

	IC_{50} (μM)[a]	
isoform	**20**	**26**
HDAC1	1.31	6.06
HDAC2	1.19	4.91
HDAC3	2.06	1.44
HDAC4	>10	>10
HDAC5	7.86	5.41
HDAC6	0.04	0.316
HDAC7	>10	6.40
HDAC8	0.422	3.41
HDAC9	>10	>10
HDAC10	2.67	>10
HDAC11	3.61	1.89
SIRT1	>10	>10

[a]These assays were conducted by the Reaction Biology Corporation, Malvern, PA. All compounds were dissolved in DMSO and tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 10 μM. IC_{50}: half maximal inhibitory concentration.

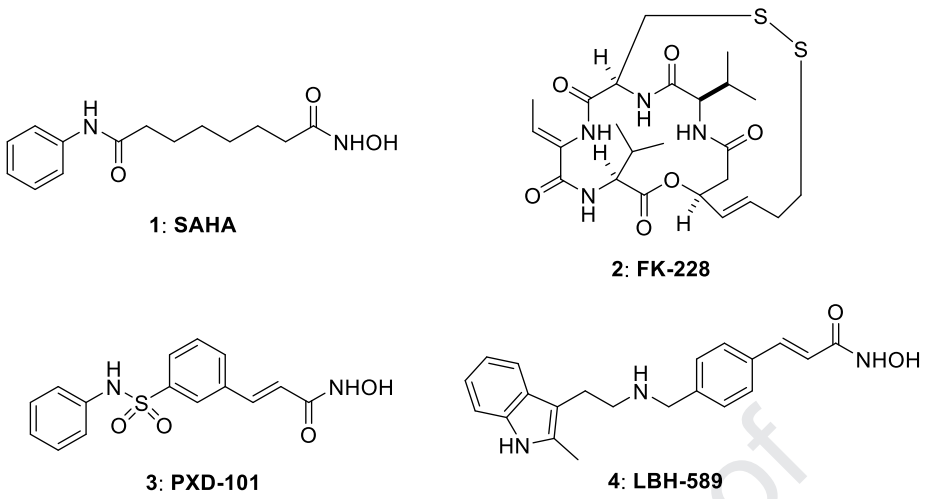


Figure 1. Examples of histone deacetylase inhibitors (HDIs)

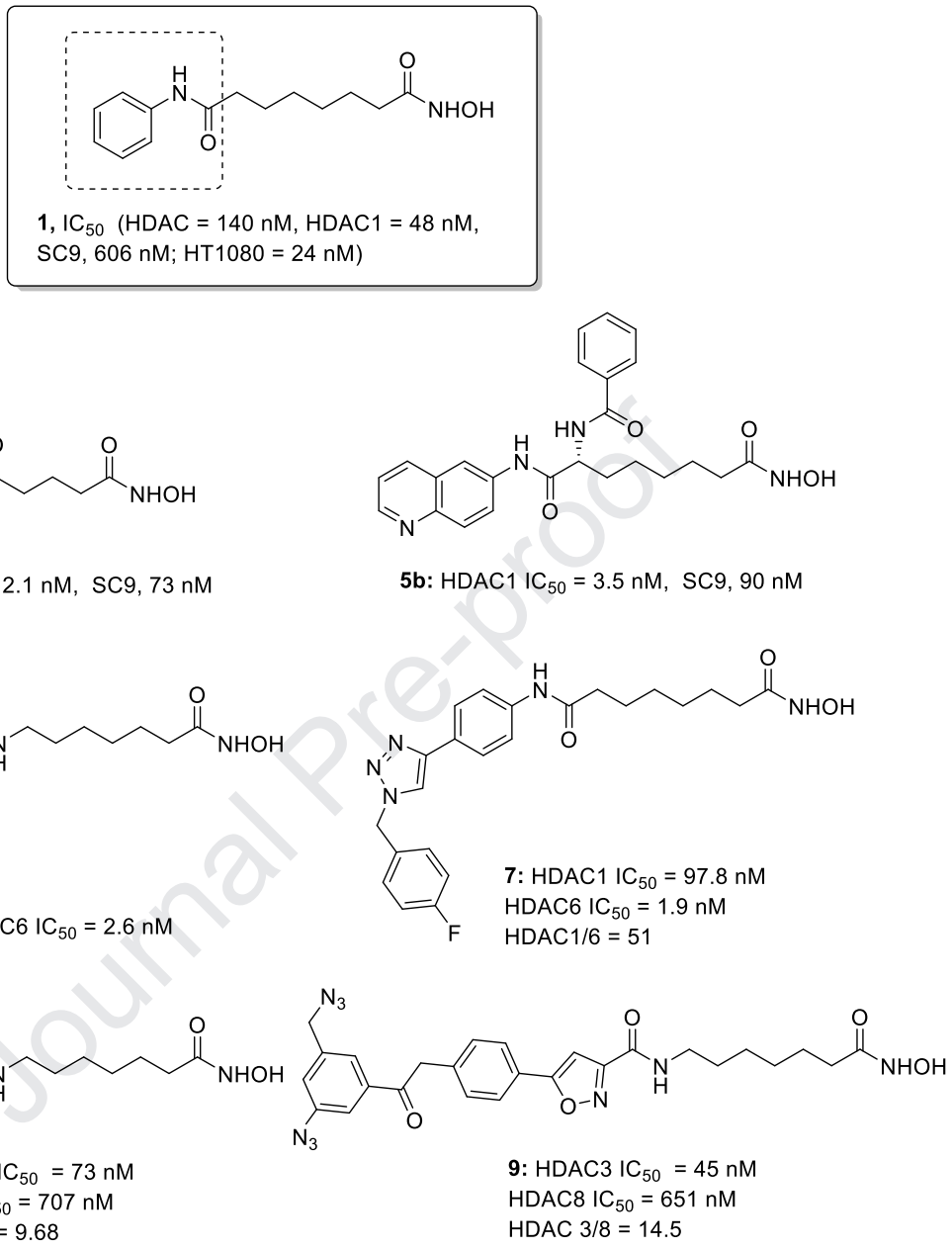
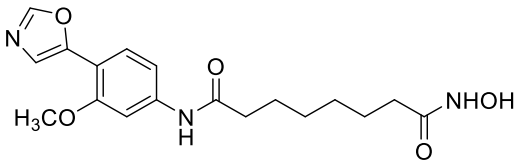


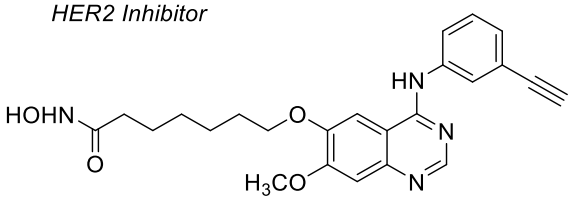
Figure 2: Structures of SAHA based potent inhibitors of HDAC

Dual Inhibitorof Inosine Monophosphate Dehydrogenase and Histone Deacetylases



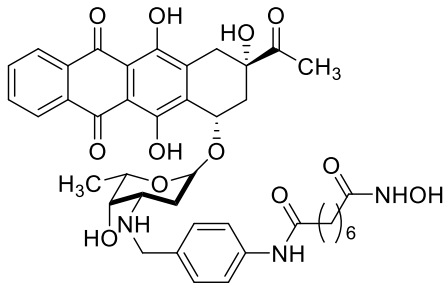
10, HDAC IC_{50}: 0.06 μM
EGFR IC_{50}: 0.29 μM

Potent Multi-Acting HDAC, EGFR, and HER2 Inhibitor

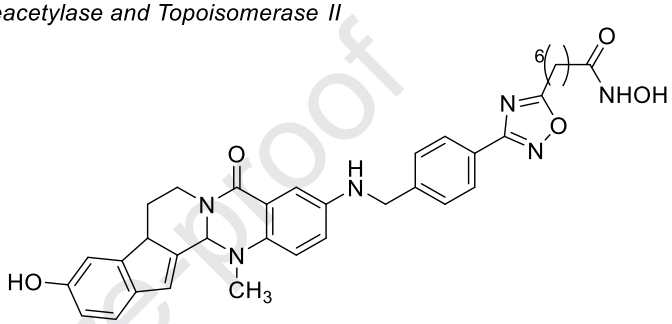


11, HDAC IC_{50}: 4.4 nM, HER IC_{50}: 15.7 nM
EGFR IC_{50}: 2.4 nM

Dual inhibitor of histone deacetylase and Topoisomerase II

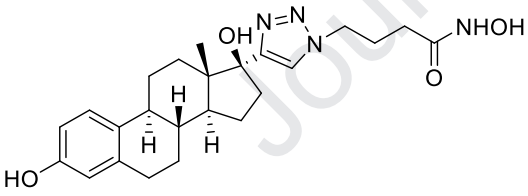


12, HDAC1 IC_{50}: 47 nM, HDAC1 IC_{50}: 220nM
HDAC6 IC_{50}: 27 nM, DU-145 IC_{50} 0.13 μM



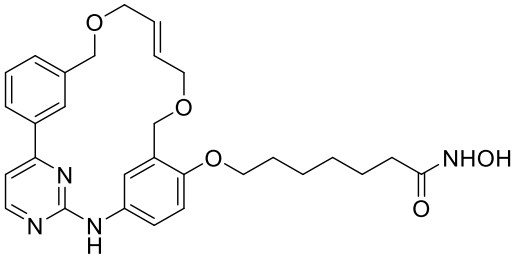
13, HDAC1/6/8 IC_{50}: 24nM/13nM/8.8 uM

Histone deacetylase inhibitors with estrogen receptor modulation activity



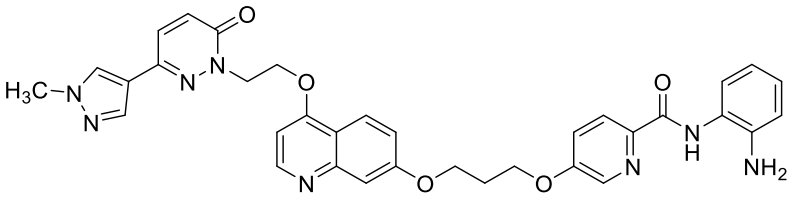
14, HDAC1 IC_{50}: 15 nM
HDAC6 IC_{50}: 8nM

Dual Inhibitors of Janus Kinase 2 (JAK2) and Histone Deacetlyase



15, JAK2 IC_{50}: 1.4 nM, HDAC2 IC_{50}: 49nM
HDAC6 IC_{50}: 2.1 nM,HDAC10 IC_{50}: 80nM

Dual inhibitors of c-Met/Histone deacetylase



16, c-Met IC_{50}: 0.71 nm
HDAC1 IC_{50}: 38nm

Figure 3: Compounds with dual inhibitory potential

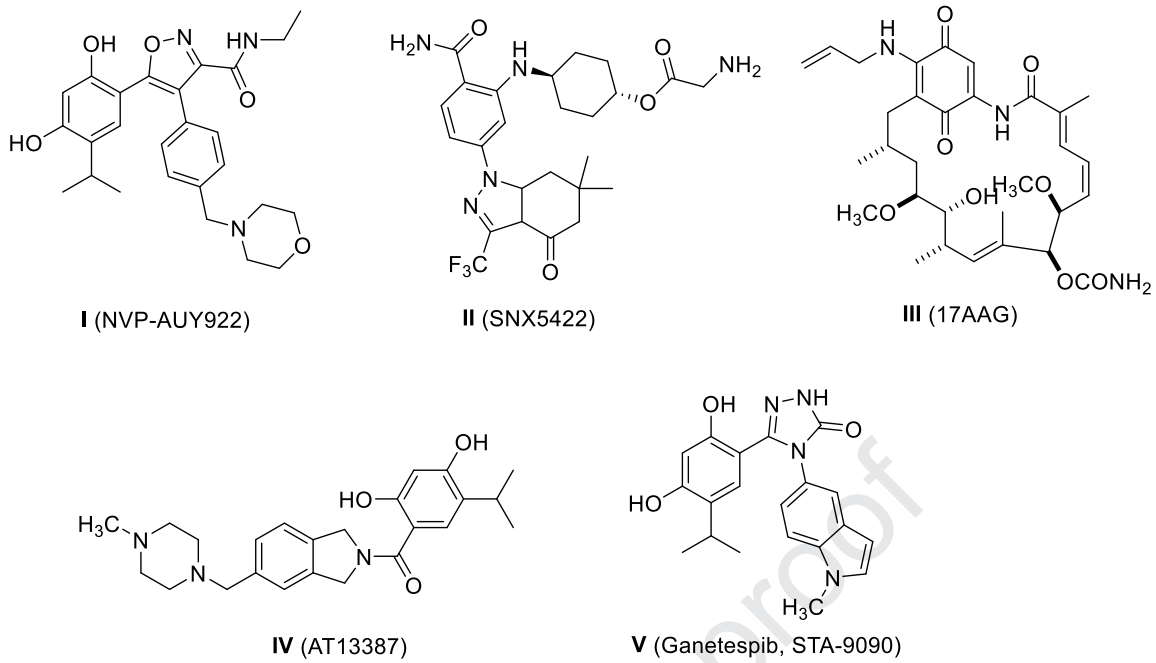
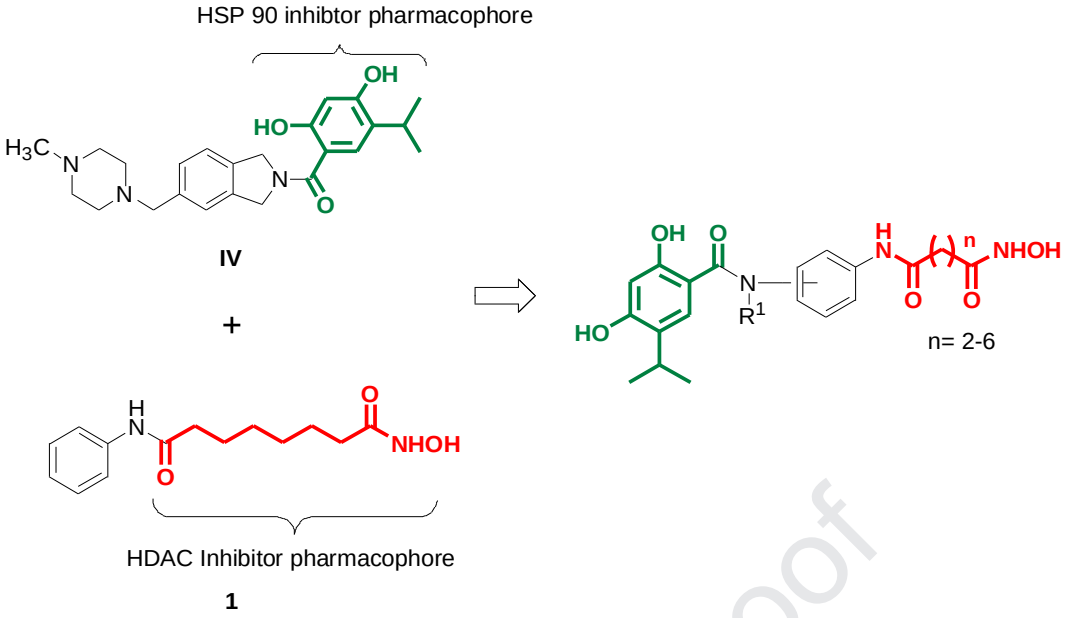


Figure 4: Examples of various HSP90 inhibitors



Compound	Position	R^1	n
17	Para	H	6
18	Meta	H	6
19	Ortho	H	6
20	Para	CH_3	6
21	Meta	CH_3	6
22	Para	C_2H_5	6
23	Meta	C_2H_5	6
24	Para	*i*-C_3H_7	6
25	Para	CH_3	5
26	Para	CH_3	7
27	Para	CH_3	8

Figure 5. Dual inhibitors of HDAC and HSP90

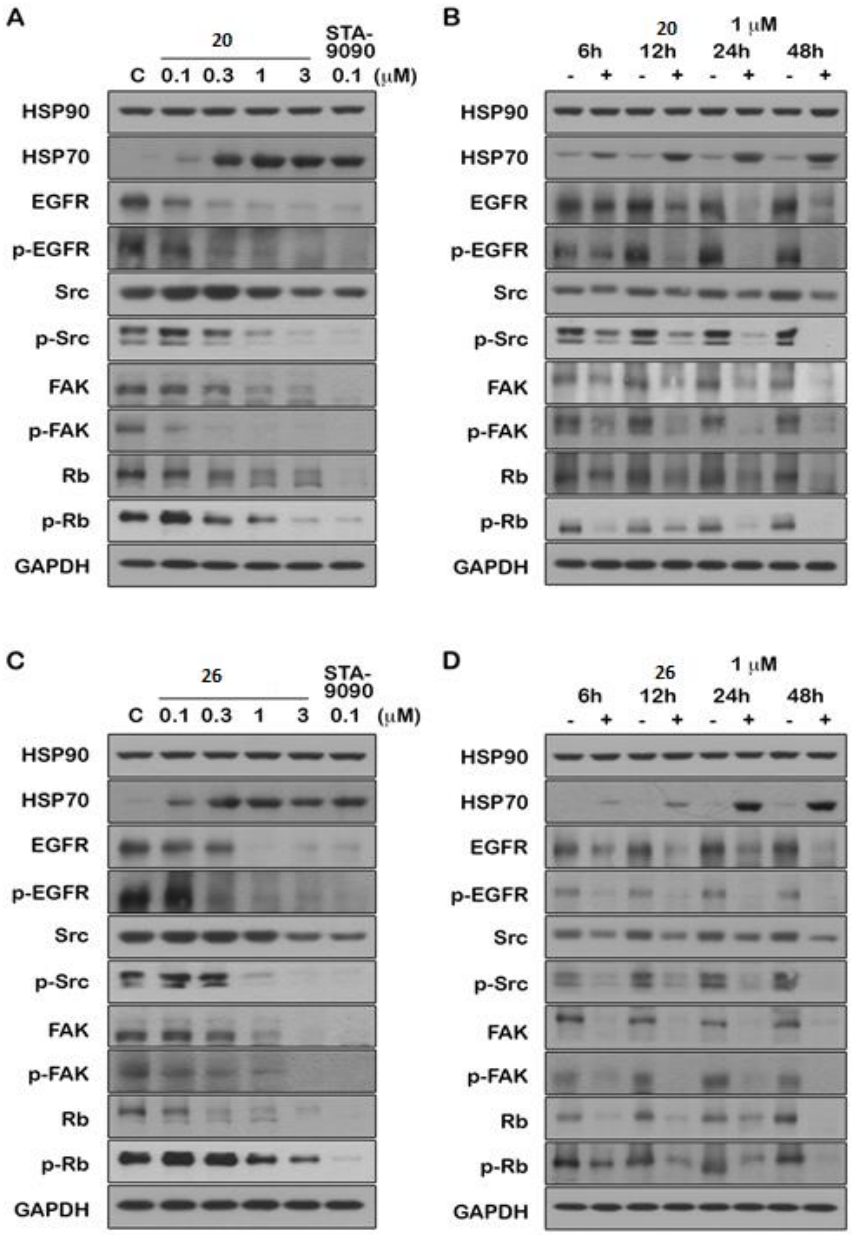


Figure 6. Compounds **2**0 and **26** triggered induction of HSP70 and degradation of multiple HSP90 client proteins in a concentration and time-dependent manner. (A, C) H1975 cells were treated with DMSO (Control; C) or 0.1-3 μM of test compounds (**20** or **26** and 0.1 μM of compound **V**) for 24 h. Cells were harvested and subjected to Western blot analysis for the detection of various HSP90 client proteins. (B, D) H1975 cells were treated with 1 μM of **20** or **26** for the indicated times. Whole cell lysates were collected, and each protein expression was detected by immunoblotting. GAPDH was used as the internal control and all experiments were repeated at least three independent experiments.

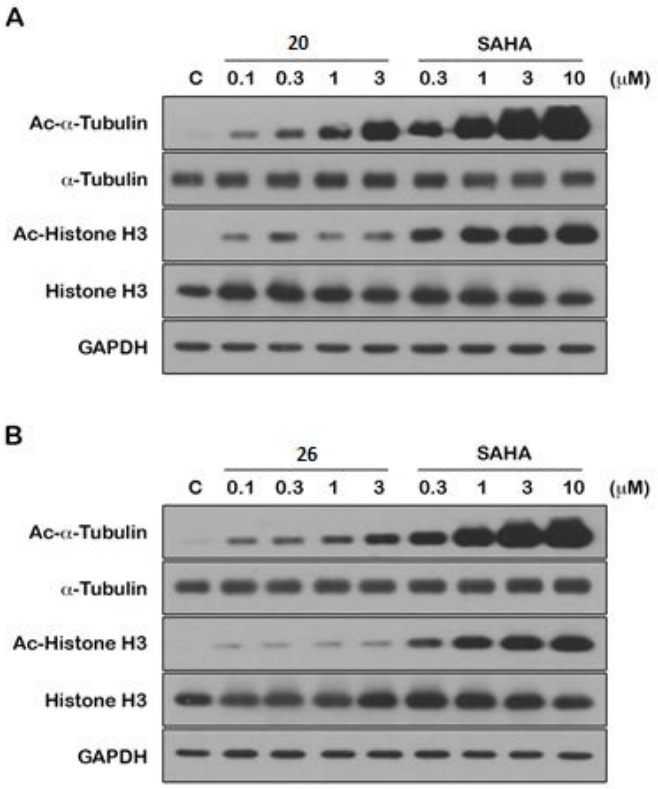


Figure 7. HDAC inhibitory activity of compounds **20** and **26** in H1975 cells via acetylation of α-Tubulin and Histone H3. (A, B) The effect of α-Tubulin and Histone H3 acetylation changes in human non-small cell lung cancer cells. Cells were cultured for 24 h with **20**, **26** or compound **1** at indicated concentrations. Whole cell lysates were detected by immunoblotting. All experiments were repeated at least three times.

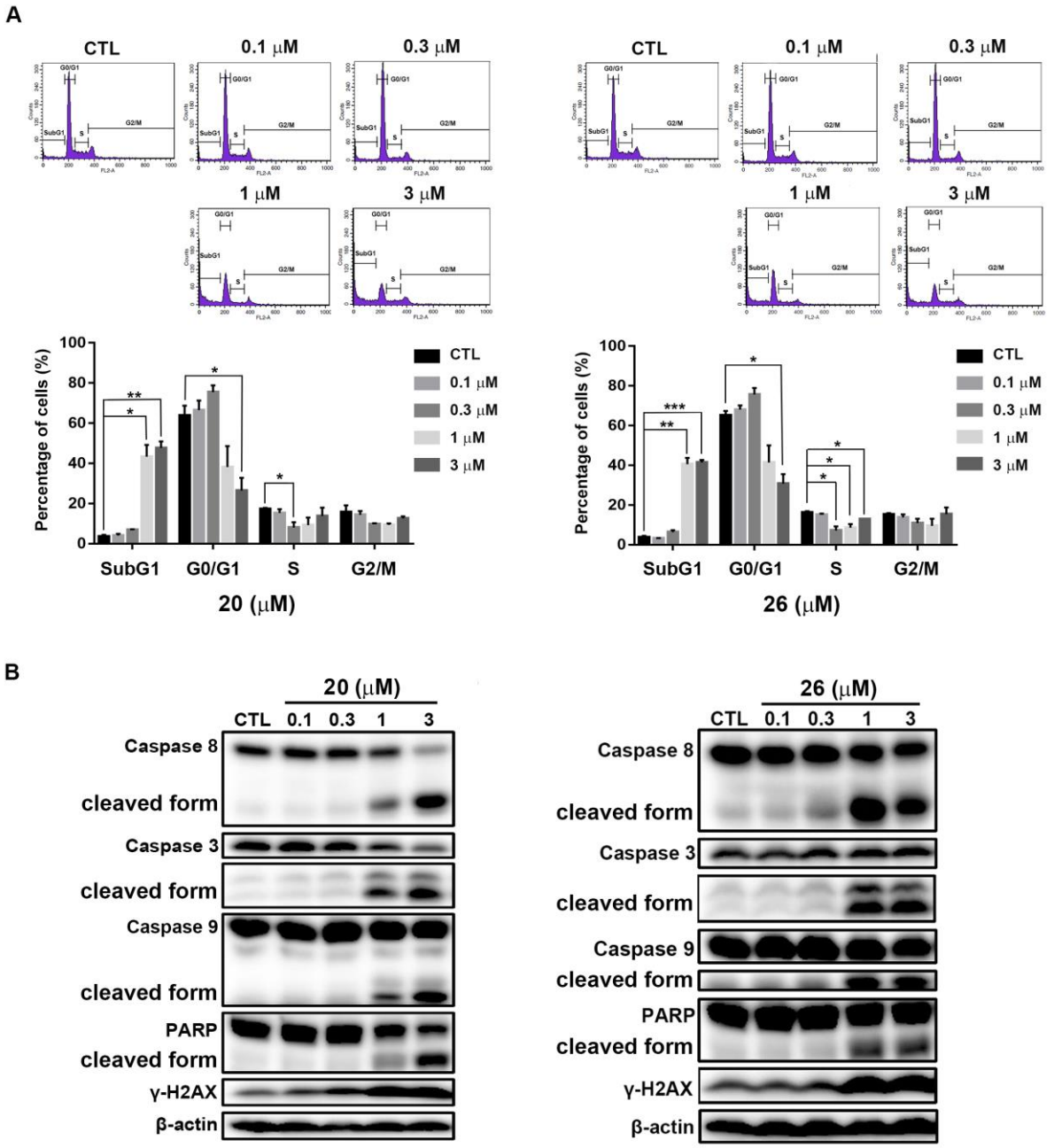


Figure 8. Compounds **20** and **26** induce accumulation of a sub-G1 phase population and significant cell apoptosis in H1975 cells. (A) Compound **20** (left panel) and **26** (right panel) induced sub-G1 phase cells accumulation. (B) Compound **20** (left panel) and **26** (right panel) induced cell apoptotic death in H1975 cells. Cells were cultured indicated concentrations with **20** or **26** for 48 h and cell lysates were detected by indicated antibodies.

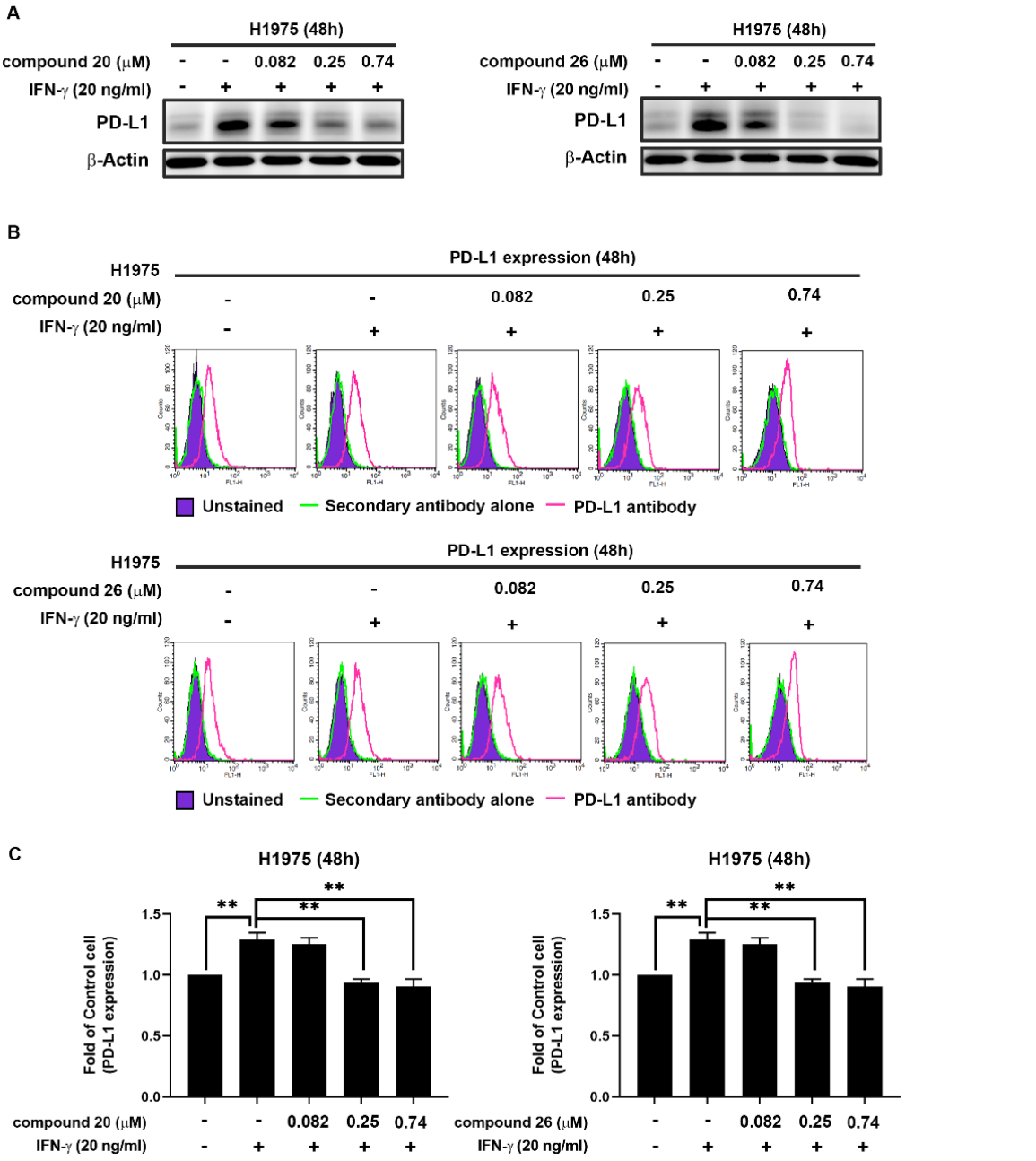
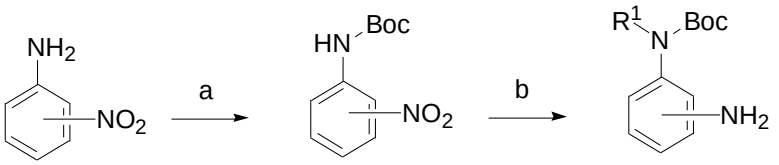


Figure 9. Downregulation of PD-L1 expression by Compounds **20** and **26** in IFN-γ treated H1975 lung cancer cells. (A) Western blot analysis of PD-L1 expression in cells treated with IFN-γ alone (20 ng/ml) or in combination with Compounds **20** and **26** at the indicated concentrations for 48 h. β-actin was used as a loading control. (B and C) Flow cytometry analysis of PD-L1 surface expression in cells treated with IFN-γ alone (20 ng/ml) or in combination with Compounds **20** and **26** at the indicated concentrations for 48 h. (C) The MFI of treated cells is presented relative as compare with the MFI of non-treated cells. The data are presented as mean ± S.D. obtained from three independent experiments. $^{**}P < 0.01$ compared with the IFN-r alone group.

Scheme 1. Synthetic approaches to compounds **34** to **41**[a]



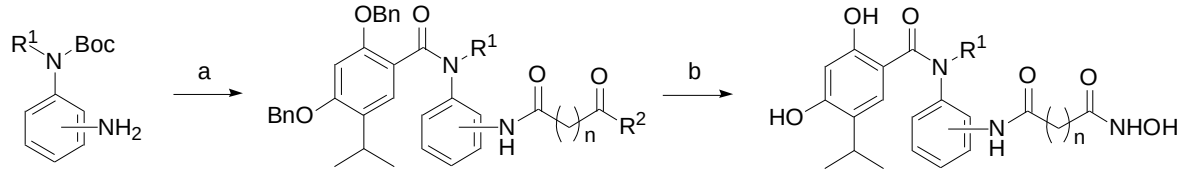
Position
28: Para
29: Meta
30: ortho

Position
31: Para
32: Meta
33: Ortho

Position	**R^1**
34: Para	H
35: Para	CH_3
36: Para	C_2H_5
37: Para	$CH(CH_3)_2$
38: Meta	H
39: Meta	CH_3
40: Meta	C_2H_5
41: Ortho	H

[a]Reagents and conditions: a) Boc anhydride, DMAP, TEA, DCM, rt, 72-81%; b) (i) alkyl iodides, NaH, DMF, 0 ^oC to rt; (ii) NH_4Cl, Fe, IPA: H_2O (4:1), reflux 69-91%.

Scheme 2. Synthetic routes to compounds **17** to **27**[a.]



Position	R^1
34: Para	H
35: Para	CH_3
36: Para	C_2H_5
37: Para	$CH(CH_3)_2$
38: Meta	H
39: Meta	CH_3
40: Meta	C_2H_5
41: Ortho	H

Position	R^1, n, R^2
42: Para	H, 6, OCH_3
43: Meta	H, 6, OCH_3
44: Ortho	H, 6, OCH_3
45: Para	CH_3, 6, OCH_3
46: Meta	CH_3, 6, OCH_3
47: Para	C_2H_5, 6, OCH_3
48: Meta	C_2H_5, 6, OCH_3
49: Para	$CH(CH_3)_2$, 6, OCH_3
50: Para	CH_3, 5, OC_2H_5
51: Para	CH_3, 7, OCH_3
52: Para	CH_3, 8, OCH_3

Position	R^1, n
17: Para	H, 6
18: Meta	H, 6
19: Ortho	H, 6
20: Para	CH_3, 6
21: Meta	CH_3, 6
22: Para	C_2H_5, 6
23: Meta	C_2H_5, 6
24: Para	$CH(CH_3)_2$, 6
25: Para	CH_3, 5
26: Para	CH_3, 7
27: Para	CH_3, 8

[a]Reagents and conditions: a) (i) appropriate long chain acids, HBTU, DIPEA, DMF; (ii) H_2O, dioxane, reflux; (iii) 2,4-dibenzyloxy-5-isopropyl benzoic acid, HOBt, DIPEA, DMF, 49-79%; b) (i) 1M LiOH (aq), dioxane, 40 ^oC; (ii) NH_2OBn, EDC, HOBt, NMM, DMF, rt; (iii) Pd/C, methanol, H_2 atmosphere 49-64%.

Research highlights

1. *N*-alkyl-hydroxybenzoyl anilide hydroxamates show potent dual inhibition of HDAC and HSP90a.

2. Lead compounds **20** and **26** induce HSP70 expression and down regulate HSP90 client proteins which play important roles in the regulation of survival and invasiveness in cancer cells.

3. Compounds **20** and **26** displayed their HDAC inhibitory effects due to increased acetylated α-tubulin and histone H3 levels.

4. Compounds **20** and **26** could effectively reduce programmed death-ligand 1 (PD-L1) expression in IFN-γ treated lung H1975 cells in a dose dependent manner.