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Design and Synthesis of Novel HDAC8 Inhibitory 2,5-disubstituted-1,3,4-Oxadiazoles containing Glycine and Alanine hybrids with Anti Cancer Activity

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Short title: 2,5-Disubstituted-1,3,4-Oxadiazoles as HDAC8 inhibitors

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Abstract

Oxadiazole is a heterocyclic compound containing an oxygen atom and two nitrogen atoms in a five-membered ring. Of the four oxadiazoles known, 1,3,4-oxadiazole has become an important structural motif for the development of new drugs and the compounds containing 1,3,4-oxadiazole cores have a broad spectrum of biological activity. Herein, we describe the design, synthesis and biological evaluation of a series of novel 2,5-disubstituted 1, 3, 4-oxadiazoles (**10a**-**10j**) as class I histone deacetylase (HDAC) inhibitors. The compounds were designed and evaluated for HDAC8 selectivity using *in silico* docking software (Glide) and the top 10 compounds with high dock score and obeying Lipinski's rule were synthesized organically. Further the biological HDAC inhibitory and selectivity assays and anti-proliferative assays were carried out. In *in silico* and *in vitro* studies, all compounds (**10a**-**10j**) showed significant HDAC inhibition and exhibited HDAC8 selectivity. Among all tested compounds, **10b** showed substantial HDAC8 inhibitory activity and better anticancer activity which is comparable to the positive control, a FDA approved drug, vorinostat (SAHA). Structural activity relation is discussed with various substitutions in the benzene ring connected on 1,3,4- oxadizole and glycine/alanine. The study warranted further investigations to develop HDAC8-selective inhibitory molecule as a drug for neoplastic diseases. Novel 1,3,4- oxadizole substituted with glycine/alanine showed HDAC8 inhibition.

Key words

1, 3, 4-oxadiazole, Glycine, Alanine, HDAC inhibition activity, class selective, Anti-proliferative effect.

1. INTRODUCTION

Histone deacetylases (HDACs) are family of enzymes involved in removal of acetyl group of lysine residues on histone proteins leading to gene repression[1]. The 18 known human HDACs have been classified into four classes (Class I, II, III & IV) [2]. Overexpression of HDACs has been observed in many pathological conditions including cancer and neurodegenerative diseases [3]. Evidences indicate that HDACs are linked with several malignancies by promoting the repression of tumor suppressor genes. Thus, inhibition of HDACs has been proven to be a promising way to treat several cancers [4].

Several synthetic HDAC inhibitors such as hydroxamates, cyclic peptides, aliphatic acids and benzamides with anticancer activities have been developed and patented by many pharmaceutical industries and academic institutions [2, 5-10]. Some of them are already at various phases of clinical trials [11]. Trichostatin (TSA) is the first natural hydroxamate found to inhibit HDACs [12]. Vorinostat, a structural analog of TSA, is the first FDA approved HDAC inhibitor for the treatment of progressive and persistent cutaneous T-cell lymphoma (CTCL)[13]. However, due to the overlapping functions of the HDACs in different classes, many adverse effects are observed. Therefore development of class selective and isoforms-specific HDAC inhibitors has been the recent research focus [14].

Oxadiazoles are important class of organic molecules with wide range of medicinal applications [15]. Valente et al synthesized 1,3,4-oxadiazole-containing hydroxamates with HDAC1 selective histone deacetylase inhibitory activity [16]. Rajak et al., designed and synthesized 2,5-Disubstituted-1,3,4-oxadiazoles based HDAC inhibitors and demonstrated their *in vitro* and *in vivo* anticancer activity [17]. The study

suggested that 1,3,4 oxadiazole linking enhanced HDAC inhibition activity and raising hope of 1,3,4-oxadiazole skeleton for the development of novel HDAC inhibitors. In this point of view, we have designed a series of 2,5-disubstituted-1,3,4-oxadiazoles. Several substitutions were designed using chemically diverse groups including amino acid groups. During virtual screening using *in silico* docking approach, amino acids, specifically alanine and glycine showed good docking results. The 10 compounds with high docking score were synthesized with high yields and performed *in vitro* HDACs assays. Further, preliminary antiproliferative effects on MDA-MB 231 breast cancer cell line were also evaluated.

2. MATERIALS AND METHODS

2.1. *In silico* analysis

Taking 1,3,4-oxadiazole as a backbone, several compounds were designed by various substitutions. Crystal structures of HDAC1, 2, 3, 8 were retrieved from protein data bank and binding of the compounds to the active site was analyzed bioinformatically by docking studies using Glide software (Schrodinger). The top 10 compounds (10a-10j) showing good dock score with HDAC8 expressed in Kcal/mol and representing the binding affinity of ligand to protein were synthesized.

2.1.1. Druggable properties

Druggable properties of the **10(a-j)** compounds were calculated according to Lipinski`s rule of 5 using online software Molinspiration [18].

2.2. Chemistry

2.2.1. Material and methods

All chemicals used for the synthesis were of reagent grade and procured from Sigma-Aldrich, Bangalore, India. ^{1}H and ^{13}C NMR spectra were recorded on AS 400 MHz Varian NMR spectrometer using TMS as an internal standard. IR spectra were recorded by using Perkin-Elmer Spectrum 100 Series FT-IR spectrometer. Mass spectra were recorded on Agilent 1200 Series LC/MSD VL system. Melting points were determined by using Buchi melting point B-545 instrument and are uncorrected. All the reactions were monitored by thin layer chromatography (TLC) using percolated silica 60 F254, 0.25 mm aluminum plates (Merck). The crude compounds were purified by column chromatography using silica gel (100-200 mesh) and gradient (0-20%) ethyl acetate in hexane as the eluent system.

2.2.2. General Procedure for the synthesis of 9(a-j):

To the stirring mixture of substituted 1,3,4-oxadizole (1.0 eq) in DMF (10.0 T) was added HATU (1.2 eq) and DiPEA (2.5 eq) at R.T, stirred for 40.0 min then added Boc-protected glycine/alanine (1.0 eq). The above reaction mixture was stirred for 1.0 hr and checked on TLC. The completed reaction mixture was extracted with ethyl acetate. The obtained ethyl acetate layer was dried over sodium sulphate and distilled under vacuum to obtain crude 9(a-j), which was further purified by column chromatography using ethyl acetate and hexane mixture (80:20) and concentrated under vacuum to obtain compound 9(a-j) in 75 % yield.

General Procedure for the synthesis of 10(a-j):

To a solution of compound 9(a-j) (0.5 g) in DCM (5.0 ml), was added drop wise TFA (1.5 ml) and stirred for 16h. The obtained solid was collected by filtration and washed with ethyl acetate, dried under vacuum to obtain compounds **10 (a-j**) with good yields. The total yield and M.P for the compounds obtained is summarized in Table1.

Table 1: Yield and M.P

Compound	Yield %	M.P ^{0}C
10a	70	152 – 154
10b	60	215 – 219
10c	75	130 – 135
10d	66	220-224
10e	65	157 – 159
10f	53	222 – 225
10g	72	176-180
10h	61	210 – 212
10i	52	210-215
10j	45	212-214

2.2.3. Characterization of compounds data

*2-amino-N-((5-phenyl-1,3,4-oxadiazol-2-yl)methyl)acetamide***(10a):**Yield 70%; off white solid, m.p.152 - 154 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.70 (s, 2H), 4.71 (d, *J* = 4.0 Hz, 2H), 7.65(m, 3H, ArH), 7.98(d, 2H, *J* = 8.0 Hz, ArH), 8.10 (s, 1H,NH_2). 9.22 (s, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.2, 40.6, 123.7, 127.0 (2C), 130.0 (2C), 132.6, 164.2, 164.7, 167.1; IR (KBr) cm^{-1} : 3254, 3067, 2986,1691,1620, 1536,

1414,1199, 1170, 1109, 959, 929, 907, 828, 794; MS (ESI) m/z [M+H]+: 232.9; Anal. Calcd. for $C_{11}H_{12}N_4O_2$ C, 56.89; H, 5.21; N, 24.12. Found C, 57.10; H, 4.92; N,24.05

(*R)-2-amino-N-((5-phenyl-1,3,4-oxadiazol-2-yl)methyl)propanamide***(10b):**Yield60%; light yellow solid, m.p. 215 - 218 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.46 (d, 3H), 3.86 (q, 1H), 3.93 (d, *J* = 8.0 Hz, 2H), 7.58 (m, 3H, ArH), 7.92 (d, 2H, *J* = 8.0 Hz, ArH), 8.24 (s, 2H,NH_2). 8.84 (t, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 17.8, 40.9, 48.6, 127.9 (2C), 129.0 (2C), 132.3, 132.9, 166.7, 168.1, 170.3; ; IR (KBr) cm^{-1}: 3268, 3097, 2839, 1993, 1683, 1567, 1518, 1486, 1411, 1279, 1227, 1131, 1023, 972, 839, 753; MS (ESI) m/z [M+H]+: 264.9(H_2O aduct); Anal. Calcd. for $C_{12}H_{14}N_4O_2$; C, 58.53; H, 5.73; N, 22.75. Found C,58.20; H, 5.20; N,22.70.

*2-amino-N-((5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl)methyl)acetamide***(10c):**

Yield 75 %; off white solid, m.p.130 - 132 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.66 (s, 2H), 4.70 (d, *J* = 4.0 Hz, 2H), 7.49 (t, *J* = 8.0 Hz, ArH), 8.08 (t, 2H, *J* = 8.0 Hz, ArH), 9.20 (s, 1H,NH; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.2, 40.5, 117.1, 117.3, 120.3, 129.6, 163.3, 164.0, 164.2, 165.8, 167.2; IR (KBr) cm^{-1}: 3257, 3166, 2821, 1966, 1649, 1570, 1521, 1426, 1394,1327, 1269, 1158, 1132, 1091, 972, 875, 810; MS (ESI) m/z [M+H]+: 268.9(H_2O aduct); Anal. Calcd. for $C_{11}H_{11}FN_4O_2$: C, 52.80; H, 4.43; F, 7.59; N, 22.39. Found C,52.80; H, 4.20; N,22.70.

*(R)-2-amino-N-((5-(4-fluorophenyl)-1,3,4-oxadiazol-2-yl)methyl)propanamide***(10d):**

Yield 66 %; off white solid, m.p.220 - 223 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.36 (d, *J* = 4.0 Hz, 2H), 3.86 (s, 1H), 3.92 (s, 2H), 7.34 (t, 2H, ArH), 7.94 (t, 2H, ArH), 8.74 (s,

1H,NH2), 8.81 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 17.8, 40.8, 48.7, 115.8, 116.0, 129.3, 130.5, 130.6, 163.4, 164.9, 168.2, 170.83; IR (KBr) cm^{-1} :3271, 3149, 2842, 1992, 1684, 1602, 1500, 1401, 1328, 1278,1224, 1131, 1078, 906,813, 759; MS (ESI) m/z [M+H]+: 282.9(H_2O aduct); Anal. Calcd. for $C_{12}H_{13}FN_4O_2$ C, 54.54; H, 4.96; F, 7.19; N, 21.20. Found C,54.80; H, 4.82; N,21.43.

*2-amino-N-((5-p-tolyl-1,3,4-oxadiazol-2-yl)methyl)acetamide***(10e):**

Yield 65%; off white solid, m.p.157 – 159 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.43 (s, 3H), 3.65 (s, 2H), 4.67 (d, *J* = 4.0 Hz, 2H), 7.41 (d, 2H, *J* = 8.0 Hz, ArH), 7.85 (d, 2H, *J* = 8.0 Hz, ArH), 8.01 (s, 2H,NH_2). 9.15 (t, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 21.5, 34.2, 41.0, 120.9, 126.9 (2C), 130.4 (2C), 142.7, 163.9, 164.7, 167.1; IR (KBr) cm^{-1} : 3242, 3073, 2842, 2013, 1708,1679, 1646, 1597, 1567, 1499, 1375, 1267, 1167, 1019,968, 871, 821, 798; MS (ESI) m/z [M+H]+: 246.9; Anal. Calcd. for $C_{12}H_{14}N_4O_2$ C, 58.53; H, 5.73; N, 22.75. Found C,58.20; H, 5.62; N,21.93.

*(R)-2-amino-N-((5-p-tolyl-1,3,4-oxadiazol-2-yl)methyl)propanamide***(10f):**

Yield 53%; off white solid, m.p 222 – 225 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.48 (d, *J* = 8.0 Hz, 3H), 3.93 (m, 3H), 4.70 (d, *J* = 4.0 Hz, 2H), 7.36 (dd, *J* = 8.0 Hz, 2H, ArH), 7.95 (dd, 2H, *J* = 4.0 Hz, ArH), 8.20 (s, 2H,NH2), 8.81 (s, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 17.5, 34.2, 48.6, 56.0, 115.4(2C), 116.0, 128.8(2C), 162.5, 163.5, 164.5, 170.6; IR (KBr) cm^{-1} : 3293, 3193, 2013, 1899, 1679, 1691, 1573, 1497, 1396, 1377, 1176, 1104, 957, 839, 760; MS (ESI) m/z [M+H]+: 278.9; Anal. Calcd. for $C_{13}H_{16}N_4O_2$ C, 59.99; H, 6.20; N, 21.52. Found C,59.79; H, 6.32; N,21.82.

*2-amino-N-((5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)methyl)acetamide***(10g):**

Yield 72 %; off white solid, m.p 180 – 183 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.43 (s, 3H), 3.65 (s, 2H), 4.67 (d, *J* = 4.0 Hz, 2H), 7.41 (d, 2H, *J* = 8.0 Hz, ArH), 7.85 (d, 2H, *J* = 8.0 Hz, ArH), 8.01 (s, 2H,NH_2). 9.15 (t, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.1, 41.1, 55.9, 115.3(2C), 115.9, 128.8(2C), 162.4, 163.5, 165.3, 168.2.17.5, 40.9, 48.6,124.1(2C), 129.6(2C), 138.5, 149.8, 164.3, 168.0, 170.3; IR (KBr) cm^{-1} : 3230, 3134, 2818, 2012, 1686, 1617, 1563, 1489, 1432, 1400, 1304, 1282, 1177, 1114, 1046, 962, 901, 844, 786, 701; MS (ESI) m/z [M+H]+: 262.9; Anal. Calcd. for $C_{12}H_{14}N_4O_3$ C, 54.96; H, 5.38; N, 21.36. Found C,54.80; H, 5.12; N,21.23.

*(R)-2-amino-N-((5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)methyl)propanamide***(10h):**

Yield 61 %; off white solid, m.p 210 – 212 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.65 (s, 2H), 3.86 (s, 3H), 4.66 (d, *J* = 4.0 Hz, 2H), 7.16 (d, *J* = 8.0 Hz, 2H, ArH), 7.92 (d, *J* = 8.0 Hz, 2H. ArH), 8.23 (s, 3H,NH_2). 9.33 (t, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 17.4, 34.2, 48.5, 55.9, 115.3(2C), 116.0, 128.7(2C), 162.5, 163.6, 164.5,170.4; IR (KBr) cm^{-1} : 3361, 3092, 2924, 2026, 1988, 1679, 1645, 1611, 1573, 1497, 1421, 1376, 1255, 1177, 1020, 971, 904, 839, 761; MS (ESI) m/z [M+H]+: 276.9; Anal. Calcd. for $C_{13}H_{16}N_4O_3$C, 56.51; H, 5.84; N, 20.28. Found C,56.83; H, 5.42; N,20.33.

*2-amino-N-((5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)methyl)acetamide***(10i):**

Yield 52 %; light brown solid, m.p.210 – 212 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.70 (s, 2H), 4.75 (s, 2H), 8.30 (d, 2H, *J* = 8.0 Hz, ArH), 8.47 (t, 2H, *J* = 8.0 Hz, ArH), 9.25 (s, 1H,NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 34.1, 41.0, 125.1(2C),

128.3(2C),129.1,149.7,163.3, 165.2, 167.2; IR(KBr)cm^{-1}:3200, 3069, 2921, 2012, 1924, 1805, 1679, 1589, 1469, 1433, 1321, 1276, 1165,1039,900,848,709;MS(ESI)m/z[M+H]+:277.8;Anal.Calcd.for $C_{11}H_{11}N_5O_4$C,47.66;H,4.00;N,25.26. Found C,47.79; H, 3.68; N,25.43

*(R)-2-amino-N-((5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)methyl)propanamide***(10j):**

Yield 45%; light brown solid, m.p.218 – 220 ^{0}C;^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.37 (d, *J* = 8.0 Hz, 3H), 3.91 (d, *J* = 4.0 Hz, 1H), 3.96 (d, *J* = 4.0 Hz, 1H), 8.10(d, 2H, *J* = 8.0 Hz, ArH), 8.35(d, 2H, *J* = 8.0 Hz, ArH), 8.78 (s, 1H,NH). 10.25 (s, 1H, NH);^{13}C NMR (100 MHz, DMSO-d_6) δ: 17.5, 40.9, 48.6,124.1(2C), 129.6(2C), 138.5, 149.8, 164.3, 168.0, 170.3;IR (KBr) cm^{-1} : 3280, 3043, 2839, 1950, 1687, 1601, 1568,1485, 1394, 1312, 1269, 1172, 1010, 970, 869, 778; MS (ESI) m/z [M+H]+: 291.8; Anal. Calcd. for $C_{12}H_{13}N_5O_4$ C, 49.48; H, 4.50; N, 24.04. Found C,49.80; H, 4.52; N,23.73.

2.3. *In vitro* biological studies

2.3.1. Compounds

A 10 mM stock solution of compounds was prepared in 100% Dimethyl sulfoxide (DMSO) and the final concentration of DMSO used in cell culture assays is 0.01%.

2.3.2. Human HDAC inhibition studies

2.3.2.1. HDAC8 purification

Full length human HDAC8 cDNA was cloned into pGEX4T1 bacterial expression vector and the recombinant protein was expressed in *E. coli* DH5α (DE3). The culture was then

harvested by centrifugation at 8000 rpm for 15 min. The harvested cell pellet was resuspended in bacteria lysis buffer [50mM Tris-HCl (pH 8.0), glycerol 10%, glucose 20% and 1x protease inhibitor cocktail (SIGMA ALDRICH)], followed by sonication. The cell lysate was then separated by centrifugation at 11000 rpm for 10 min at 4 °C and the soluble fraction was collected. Supernatant was incubated with glutathione sepharose beads for 90 min at 4 °C, followed by four 20 min washes with PBS. The HDAC8 protein was then eluted using 20 mM reduced glutathione. The purity of the recombinant HDAC8 protein was checked on SDS-PAGE.

2.3.2.2. Immunoprecipitation

Breast cancer cells, MDA-MB-231, were grown in DMEM medium supplemented with 10% FBS, and grown at 37°C, 5% CO_2 until 80% confluency. For immunoprecipitation, cells were lysed in lysis buffer [20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 1x protease inhibitor cocktail] and immunoprecipitation with the anti-HDAC antibody was carried out for overnight at 4°C. Using 500 ug of total protein and 1 ug of antibody, the HDAC1, HDAC2, HDAC3 and HDAC8 proteins were pull down using agarose A/G beads for 1hr at 4°C. The protein complex bound to the agarose beads were collected by brief centrifugation after washing in lysis buffer, the beads were suspended in HDAC assay buffer for activity assay.

2.3.2.3. HDAC8 activity assay

The deacetylation activity of recombinant protein (GST-HDAC8) and immunoprecipitated HDACs was measured using the Fluor-de-Lys® HDAC fluorometric activity assay kit (, ENZO Life Sciences, Cat No. BML-AK500-0001) according to

manufacturer's specifications. Purified GST-HDAC8 (histone deacetylase 8) or immunoprecipitated beads with HDACs along with assay buffer (50mM Tris pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1mg/ml BSA) was incubated with 25 µM Fluor-de-Lys substrate for 15 min at 37 °C, in the presence or absence of either 10 µM SAHA or test compounds. An equal volume of DMSO was used in control well without drug. After adding 50 µl of developer solution, the plate was incubated for 45 min at room temperature in dark and fluorescence was measured in a multimode fluorescence reader (BioTek) at excitation and emission wavelengths of 350 and 440 nm, respectively. The inhibition of HDAC activity by the test compounds was calculated as percentage of control.

2.3.3. Anticancer studies

2.3.3.1. Cell culture

MDA-MB231 breast cancer cells were grown in complete medium (DMEM medium, 10% heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2mM L-glutamine) at 37 °C and 5% CO_2. Cell viability was determined by trypan blue dye and cells were sub-cultured twice a week, seeding at a density of about 2×10^4 cells/ml.

2.3.3.2. MTT Assay

Cytotoxicity was evaluated by MTT assay [19]. Cells were seeded at a density of 5×10^3 cells/wells in a 96-well plate and incubated for 4 hr. Cells were then treated with compounds (0.001, 0.01, 0.1, 1, and 10 µM) for 24 h. 10 µl of 5 mg/ml MTT solution

was added and incubated for another 3 hr. The formazan crystals formed were dissolved in 50 µl DMSO and read at 570 nm. The cell viability was measured as % control (untreated cells) and the % inhibition of growth was plotted as graph.

2.4 Statistical analysis

For the activity assays, the mean and standard error of mean (SEM) was calculated from the data obtained from 3 independent experiments with triplicates in each experiment. IC_{50} was calculated using non-regression dose-response (inhibition) analysis in Graph Pad Prism software.

3. Results

3.3. *In silico* drug designing

Taking, 1,3,4-oxadiazole as backbone, several substitutions were designed using chemically diverse groups including amino acid groups. All the designed molecules were first filtered to eliminate non-drug like sub-structures through a careful visual assessment and application of Lipinsky`s rule. The selected molecules were then assessed for binding affinity towards HDAC8 crystal protein for its catalytic activity inhibition followed by docking with other class I enzymes, HDAC1, HDAC2, HDAC3, for selectivity (Table 2). Surprisingly, 1,3,4 oxadiazole ring lined with glycine/alanine (compounds **10a-10j**) showed high binding affinity and better selectivity towards HDAC8 protein than other HDACs (1,2&3) proteins. However, compound 10b showed a higher affinity to HDAC8 than other compounds including SAHA and was also showing least dock score to HDAC1 when compared to other compounds.

Table 2: Molecular docking studies of oxadiazole linked with glycine/alanine

Compound	Dock score (-Kcal/mol)			
	HDAC1 (PDB id: 4BKX)	HDAC2 (PDB id: 3MAX)	HDAC3 (PDB id: 4A69)	HDAC8 (PDB id: 1T64)
10a	6.112	6.282	6.331	7.028
10b	2.61	5.991	6.191	7.918
10c	6.153	6.482	6.12	7.233
10d	6.281	6.648	6.031	7.446
10e	6.227	6.287	6.212	7.251
10f	5.32	6.553	6.051	7.557
10g	5.612	6.326	6.173	7.153
10h	5.656	6.163	6.377	6.652
10i	5.326	5.94	6.462	7.131
10j	5.982	6.327	6.563	7.482
SAHA	6.39	6.51	6.13	6.42

Since class I HDACs are Zinc-dependent enzymes, any molecules targeting Zn moiety along with amino acids around Zn in catalytic domain are proven to be effective inhibitors. The binding site residues important for ligand binding in HDAC1 crystal structure (PDB ID: 4BKX) are Asp 264, Asp 176 and His 178. In the present study the compound 10b did not show any interaction with any of these residues and therefore the docking scores were less (-2.61 Kcal/mol). The crystal structure chosen for HDAC2 was 3MAX and the binding site residues in crystal structure are His 145, His 146, Gly 154 and Tyr 308. Compound 10b showed hydrogen bonding only with Gly 154 along with covalent bonding with Zn and showed a docking score of -5.991 Kcal/mol. HDAC3 binding site residues (PDB ID: 4A69) are Asp 170, Asp 172 and Asp 259. Compound 10b showed interactions with Gly 143 and Phe 144 along with covalent bonding with Zn and the binding score was -6.191 kcal/mol. HDAC8 binding site residues in crystal structure (1T64) were Asp 178, His 180, Asp 267, Gly 151, Phe 152 and Tyr 306.

Compound 10b was interacting with Tyr 306, gly 151, phe 152 along with Zn and the binding score was -7.918 kcal/mol. Based on these observations, it was hypothesized that compound 10b might be selective towards HDAC8 (Fig. 1).

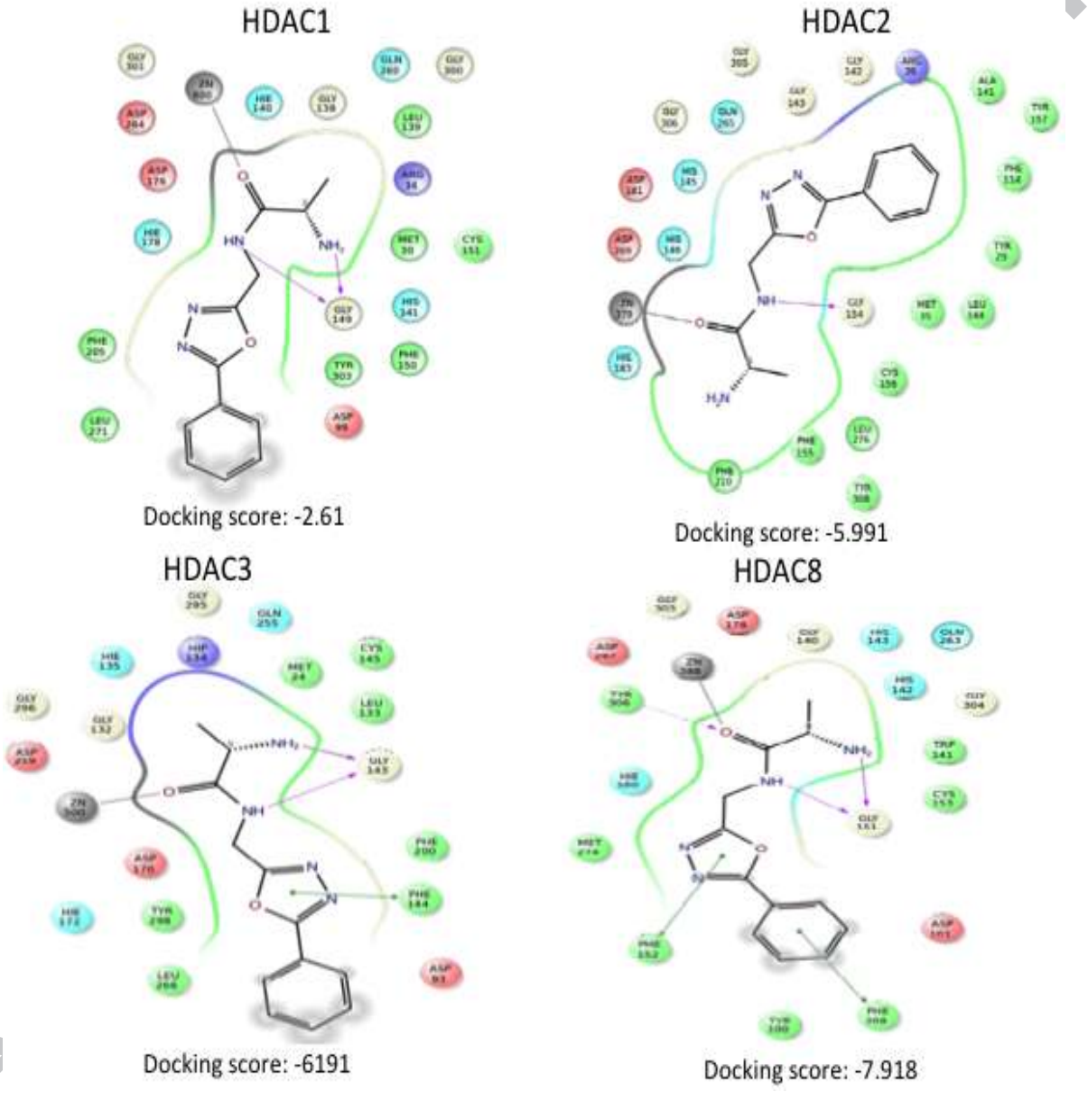


Fig. 1: Docking images of compound 10b showing the interactions with the active site residues and Zn in the active site of class I HDACs .

All compounds obeyed Lipinskys druggability rules (Table 3).

Table 3: Drug-like properties of compounds according to Lipinski's rule

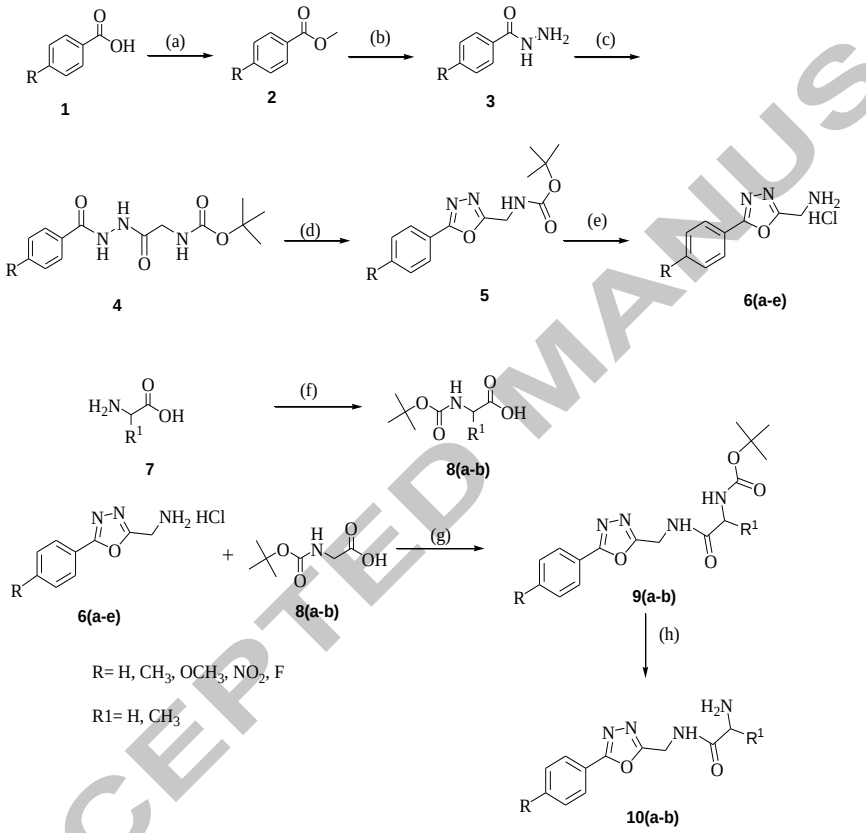
Compound	cLOGP	H bond donors <= 5 n-OHNH	H bond acceptors <= 10 n-OH	MW
10a	-1.1742	3	6	232.24
10b	-0.8652	3	6	246.27
10c	-1.0015	3	6	250.23
10d	-0.692	3	6	264.26
10e	-0.675201	3	6	246.27
10f	-0.3662	3	6	260.29
10g	-1.10217	3	7	262.26
10h	-0.793166	3	7	276.12
10i	-1.36755			277.24
10j	-1.05855			291.26

3.4. Organic synthesis

Chemical synthesis of designed oxadiazoles linked with glycine/alanine (**10a** – **10j**): Using convergent synthesis coupled with 1,3,4-oxadiazolylmethylamine, synthesis of designed 1,3,4-oxadiazoles linked with glycine/alanine compound was performed. 1,3,4-oxadiazolylmethylamine react with boc-protected glycine/alanine in presence of HATU and followed by de-protection by using TFA. The synthesis of 1,3,4-oxadiazolylmethylamine was started with aromatic acid to react with MeOH and catalytic amount of H_2SO_4 converted in to aromatic ester, which were reacted with hydrazine hydrate solvent as ETOH to give hydrazide, which was further coupled with Boc-protected glycine/alanine to give desired compounds **(10 a-j)** (Scheme 1).

All the tested compounds **(10 a-j)** were characterized by ^{1}H, ^{13}C nuclear magnetic resonance (NMR), infra red (IR) and liquid chromatography mass spectrometry (LC-MS) analysis.

Scheme 1



Compound	R	R^1
10a	R=H	R^1=H
10b	R=H	R^1= CH_3
10c	R=F	R^1=H
10d	R= F	R^1= CH_3
10e	R= CH_3	R^1=H
10f	R= CH_3	R^1= CH_3
10g	R= OCH_3	R^1=H
10h	R= OCH_3	R^1= CH_3
10i	R= NO_2	R^1=H
10j	R = NO_2	R^1= CH_3

(a) MeOH, H_2SO_4 Reflux (b) $NH_2NH_2.H_2O$ MeOH, rt (c) Bocglycine HATU, DMF DiPEA, rt

(d) PPh_3,CCl_4 DMF,TEA, rt (e) TFA DCM, rt, EtOAc in HCl (f) Boc anhydride,$CHCl_3$

(g) HATU, DMF, DiPEA, rt (h) TFA DCM, rt

3.3 *In vitro* HDAC inhibition activity

To further confirm the HDAC8 inhibitory activity of the synthesized Oxadiazoles linked with glycine/alanine, activity assay using recombinant purified HDAC8 and HDAC

fluorophore substrate was carried out. The results clearly demonstrated that all compounds showed significant HDAC8 inhibitory activities (Table 4). Similar to the *in silico* results, **10b** showed substantial HDAC8 inhibitory activity with an IC_{50} of 98 nM when compared with other compounds including SAHA (Table 4). **10b** showed comparable or more potency and better HDAC8 selectivity than SAHA. The strong interactions of **10b** with active site amino acids of HDAC8 are responsible for its HDAC8 inhibition activity (Fig. 1).

Table 4: HDAC inhibition activities of oxadiazoles linked with glycine/alanine

Compounds	*In vitro* HDAC8 activity
	IC_{50} ± SEM (nM)
10a	155 ± 9.5
10b	98 ± 6.0
10c	135 ± 8.1
10d	116 ± 9.5
10e	139 ± 4.2
10f	121 ± 2.1
10g	142 ± 5.6
10h	153 ± 11.6
10i	134 ± 3.1
10j	127 ± 4.5
SAHA	1480 ± 5.6

Further, to assess the selectivity of **10b**, class I HDAC enzymes were immunoprecipitated from MDA-MB-231 cell lysates and activity assay was carried out using the immunoprecipitated proteins as enzyme source. The results clearly showed that **10b** was moderately selective in inhibiting HDAC8 (53% inhibition) than HDAC1 (30%), HDAC2 (19%) and HDAC3 (0%) at 100 nM concentration. A selectivity ratio was expressed as the ratio of IC_{50} for HDAC8 to that for HDAC1, 2, or 3 (Table 5) that showed compound 10b was moderately selective to HDAC8 than other class I HDACs.

Table 5: Selectivity ratio of Compound 10b for class I HDACs.

HDAC	**IC_{50} (nM)**	**Selectivity ratio (IC_{50} HDAC8/IC_{50} HDAC)**
HDAC1	260	0.38
HDAC2	135	0.74
HDAC3	>10,000	ND
HDAC8	100	1

3.4 ***In vitro*** **anti-cancer activity**

Anticancer activity of oxadiazoles linked with glycine/alanine **(10 a-j)** was evaluated against breast cancer MDA-MB-231 cells by MTT assay. All compounds showed significant anticancer activity. Among the tested compounds, compound **10b** has emerged as most active compound against MDA-MB-231 cancer cells (IC_{50} = 230 nM) (Table.6).

Table 6: Anti-proliferative effects of compounds

Compounds	*In vitro* anticancer activity against MDA-MB231breast cancer cells
	IC_{50} ± SEM (nM)
10a	283 ± 12.3
10b	230 ± 11.6
10c	254 ± 16.9
10d	247 ± 12.6
10e	260 ± 9.8
10f	254 ± 13.4
10g	276 ± 14.8
10h	295 ± 12.4
10i	276 ± 22.4
10j	261 ± 19.4
SAHA	6000 ± 10.8

Discussion

Development of class-selective and isoform-specific inhibitor of HDACs is currently a major focus of HDAC inhibitor design [20]. Selective HDAC inhibitors, for instance tubacin, HDAC6 selective inhibitor, not only can elucidate the function of individual HDAC but also provide candidates for the treatment of cancer or other diseases with less side effects [21]. Weiping et al., (2011) designed and discovered HDAC8-selective inhibitors from a novel collection of compounds [22]. However, only limited progress towards the discovery of HDAC8 selective inhibitors has been made to date.

Oxadiazole is an important pharmacophore, which has been reported to possess various bioactivities including HDAC inhibition [16]. In the present study, we have designed a number of molecules on a basic oxadizole core moiety. Surprisingly, glycine

and alanine linking to oxadizole moiety showed better selectivity towards HDAC8 than other isoforms of class I HDACs in both *in silico* and *in vitro* studies. Similar selectivity of oxadiazole-containing hydoxamates towards HDAC6, a class II isoform, has been reported recently [23].

The structure activity relationship studies showed that, glycine/alanine compounds, particularly L isomer, exhibited selective HDAC8 inhibition among other class I HDACs. Methyl substitution in R1 group and H substitution in R group of compound **10b** caused good HDAC8 inhibition and better selectivity. **10b** exhibited more HDAC8 inhibition and moderate selectivity than SAHA (a FDA approved drug). Further anticancer studies were performed on HDAC inhibitory compounds since HDACs are involved in pathophysiology of cancer. Among all compounds, HDAC8 inhibitory **10b** exhibited anti-proliferative potential against MDA-MB-231 breast cancer cells. Further detailed investigations are under progress to demonstrate the molecular mechanism involved in its anti-proliferative activity.

4. Conclusions

A series of HDAC8-selective oxadiazoles linked with glycine/alanine **(10 a-j)** were designed using *in silico* tools and chemically synthesized with high yields. *In vitro* HDAC and anticancer studies demonstrated that oxadiazoles linked with glycine/alanine (10 a-j) showed significant class I HDAC inhibition and anticancer activities. Among all compounds, **10b** showed moderate selectivity towards HDAC8 as well as better anticancer activity than SAHA (positive control). The results of the present study

warranted further studies on **10b** to develop as a potential candidate drug for the treatment of HDAC8-overexpressed cancers.

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References

[1] J. Park, S. Thomas, P.N. Munster, Epigenetic modulation with histone deacetylase inhibitors in combination with immunotherapy, Epigenomics 7(4) (2015) 641-52.

[2] M. New, H. Olzscha, N.B. La Thangue, HDAC inhibitor-based therapies: can we interpret the code?, Molecular oncology 6(6) (2012) 637-56.

[3] S. Ropero, M. Esteller, The role of histone deacetylases (HDACs) in human cancer, Molecular oncology 1(1) (2007) 19-25.

[4] D. Mottet, V. Castronovo, Histone deacetylases: target enzymes for cancer therapy, Clinical & experimental metastasis 25(2) (2008) 183-9.

[5] Y.C. Tseng, S.K. Kulp, I.L. Lai, E.C. Hsu, W.A. He, D.E. Frankhouser, P.S. Yan, X. Mo, M. Bloomston, G.B. Lesinski, G. Marcucci, D.C. Guttridge, T. Bekaii-Saab, C.S. Chen, Preclinical Investigation of the Novel Histone Deacetylase Inhibitor AR-42 in the Treatment of Cancer-Induced Cachexia, Journal of the National Cancer Institute 107(12) (2015).

[6] M. Duvic, Histone Deacetylase Inhibitors for Cutaneous T-Cell Lymphoma, Dermatologic clinics 33(4) (2015) 757-64.

[7] J.J. Sung, K. Ververis, T.C. Karagiannis, Histone deacetylase inhibitors potentiate photochemotherapy in cutaneous T-cell lymphoma MyLa cells, Journal of photochemistry and photobiology. B, Biology 131 (2014) 104-12.

[8] C. Tiffon, J. Adams, L. van der Fits, S. Wen, P. Townsend, A. Ganesan, E. Hodges, M. Vermeer, G. Packham, The histone deacetylase inhibitors vorinostat and romidepsin downmodulate IL-10 expression in cutaneous T-cell lymphoma cells, British journal of pharmacology 162(7) (2011) 1590-602.

[9] K.B. Hymes, The role of histone deacetylase inhibitors in the treatment of patients with cutaneous T-cell lymphoma, Clinical lymphoma, myeloma & leukemia 10(2) (2010) 98-109.

[10] T. Suzuki, N. Miyata, Non-hydroxamate histone deacetylase inhibitors, Current medicinal chemistry 12(24) (2005) 2867-80.

[11] H. Wang, B.W. Dymock, New patented histone deacetylase inhibitors, Expert opinion on therapeutic patents 19(12) (2009) 1727-57.

[12] G. Bouchain, D. Delorme, Novel hydroxamate and anilide derivatives as potent histone deacetylase inhibitors: synthesis and antiproliferative evaluation, Current medicinal chemistry 10(22) (2003) 2359-72.

[13] S.M. Kavanaugh, L.A. White, J.M. Kolesar, Vorinostat: A novel therapy for the treatment of cutaneous T-cell lymphoma, American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists 67(10) (2010) 793-7.

[14] P. Bose, Y. Dai, S. Grant, Histone deacetylase inhibitor (HDACI) mechanisms of action: emerging insights, Pharmacology & therapeutics 143(3) (2014) 323-36.

[15] J. Sun, J.A. Makawana, H.L. Zhu, 1,3,4-oxadiazole derivatives as potential biological agents, Mini reviews in medicinal chemistry 13(12) (2013) 1725-43.

[16] S. Valente, D. Trisciuoglio, T. De Luca, A. Nebbioso, D. Labella, A. Lenoci, C. Bigogno, G. Dondio, M. Miceli, G. Brosch, D. Del Bufalo, L. Altucci, A. Mai, 1,3,4-Oxadiazole-containing histone deacetylase inhibitors: anticancer activities in cancer cells, Journal of medicinal chemistry 57(14) (2014) 6259-65.

[17] H. Rajak, A. Agarawal, P. Parmar, B.S. Thakur, R. Veerasamy, P.C. Sharma, M.D. Kharya, 2,5-Disubstituted-1,3,4-oxadiazoles/thiadiazole as surface recognition moiety: design and synthesis of novel hydroxamic acid based histone deacetylase inhibitors, Bioorganic & medicinal chemistry letters 21(19) (2011) 5735-8.

[18] C.A. Lipinski, Lead- and drug-like compounds: the rule-of-five revolution, Drug discovery today. Technologies 1(4) (2004) 337-41.

[19] A.M. Kalle, A. Mallika, J. Badiger, Alinakhi, P. Talukdar, Sachchidanand, Inhibition of SIRT1 by a small molecule induces apoptosis in breast cancer cells, Biochemical and biophysical research communications 401(1) (2010) 13-9.

[20] P. Zhan, Y. Itoh, T. Suzuki, X. Liu, Strategies for the Discovery of Target-Specific or Isoform-Selective Modulators, Journal of medicinal chemistry 58(19) (2015) 7611-33.

[21] S.J. Haggarty, K.M. Koeller, J.C. Wong, C.M. Grozinger, S.L. Schreiber, Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation, Proceedings of the National Academy of Sciences of the United States of America 100(8) (2003) 4389-94.

[22] W. Tang, T. Luo, E.F. Greenberg, J.E. Bradner, S.L. Schreiber, Discovery of histone deacetylase 8 selective inhibitors, Bioorganic & medicinal chemistry letters 21(9) (2011) 2601-5.

[23] J. Senger, J. Melesina, M. Marek, C. Romier, I. Oehme, O. Witt, W. Sippl, M. Jung, Synthesis and Biological Investigation of Oxazole Hydroxamates as Highly Selective Histone Deacetylase 6 (HDAC6) Inhibitors, Journal of medicinal chemistry (2015).

Highlights

- Synthesis of 2,5-disubstituted 1, 3, 4-oxadiazoles - glycine/alanine hybrids **(10a-10j).**
- *In silico* and *in vitro* evaluation of **(10a-10j)** as class I HDAC inhibitors.
- All compounds showed cytotoxic effects on breast cancer cell line, MDA-MB-231.
- Compound **10b** showed high binding affinity and moderate selectivity to HDAC8.
- Compound **10b** showed potent anti-proliferative effects on MDA-MB-231 cells.

Graphical Abstract