Medicinal Chemistry
ISSN: 2161-0444

Kaliyaperumal et al., Med Chem (Los Angeles) 2018, 8:6
DOI: 10.4172/2161-0444.1000508

Research Article Open Access

Molecular Docking, Synthesis and Evaluation of Novel Hydroximic Acid Mimics as Anti-cancerous Histone Deacetylase Inhibitors

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Abstract

In the current study, development of novel hydroximate mimics for HDAC-8 inhibition, as an anticancer target was carried out. To design the novel hydroximate, we must find out which protein can be targeted in pathogenesis so that, molecular docking was carried out and human HDAC-8 was found to be a potential cancer drug target for hydroximates. Focusing on identifying a potential HDAC-8 inhibitor, the Ligand-1 (IUPAC name: (N1 (2-hydroxyethyl) N8-phenyloctanediamide) has been identified as a potential and novel lead molecule further, the Ligand-1 was synthesized and characterized followed by cytotoxicity study against the MG-63 cell lines, *in vitro* HDAC enzyme inhibition assay and *in vivo* EAC model was carried out. The acute toxicity study (OECD-425) for LD50 was found to be 550 mg/kg/i.p and from this the final dose selected was 1/10th of 550 mg/kg that is 55 mg/kg, by using this dose *in vivo* was carried out with haematological parameters such as Hgb, RBCs, WBCs, %ILS, Body weight analysis and it was observed that Ligand-1 is extremely significant ($P<0.05$), from these results we concluded that, the novel hydroximic acid mimic that is Ligand-1 elicited as a remarkable anti-cancer activity.

Keywords: Histone acetyl transferase (HAT); Histone deacetylase (HDAC); Histone deacetylase inhibitors; EAC; MG-63; Molecular docking

Introduction

Histone is a protein which is present in all cells (mainly nucleosomes part of cell), and it has 4 types H2a, H2b, H3 and H4, the combination of these four leads to octomer of histone. To this octomer of histone proteins DNA (146 base pairs) is wrapped so ultimately, the change in histone protein leads to change in gene transcription (activation or repression) the imbalance in HAT and HDAC enzyme of histone leads to cancer, mainly overexpression of Histone Deacetylase (HDAC) enzymes play a major role in causing cancer and dreadful human diseases [1-9].

The proper level of histone acetylation altered normal gene transcription regulation, which is optimized on HAT (Histone Acetyl Transferase) and HDAC (Histone Deacetylase). In contrast hypoacetylation caused by overexpression of HDAC optimized the gene transcription deregulation which is leads to play cancer and dreadful human diseases.

Histone deacetylase inhibitors (HDACIs) are a potential and novel class of compounds for the treatment of cancers. HDAC inhibitors to play the functions of the gene expression whereas not modifying the deoxyribonucleic acid sequence and bind to the HDAC in the deoxyribonucleic acid through histones thus preventing the transcription of varied growth suppressor genes [10,11]. Hence, HDACi is one of the potent inducers of growth arrest and programmed necrobiosis of reworking cells and differentiation by regulation the gene expression.

HDACIs are mainly comprise of four main structural classes in conjunction with hydroxamates, short-chain fatty acids, benzamides and cyclic tetrapeptides significantly have a broad family of chemical compounds and these compounds even have totally different affinities for varied HDACs beside the structural dissimilarities and among these hydroxamic acids are the largest class of HDACIs with great therapeutic potential [12-25].

The present work was focused on anti-cancerous histone deacetylase activity of newly designed hydroxamic acid mimic (Figure 1a and 1b). To demonstrate the utility of *in silico* design, we achieved the novel hydroxamic acid mimics; it is histone deacetylase inhibitors of a potential anticancer drug target. The work was focused on study of the best molecular interaction exhibited among any one of the molecules through binding and activation by using molecular docking and the drug targeting protein was Histone Deacetylases-8 and then followed by synthesis of most active compound in continuation with *in vitro* and *in vivo* screening of active compounds.

Materials and Methods

The *in silico* software's and databases including ChemsDraw, Discovery Studio-C Docker, Swiss PDB viewer and Data bases such as Protein Data Bank(PDB), Protein Information Recourse (PIR), Mole inspiration (Physicochemical Properties Prediction Tools) were used for this study and formerly chemicals were used for Ethyl acetate (Karnataka fine chem.), 8-ethoxy-8-oxooctanoic acid (Supreme Scientifics), Sodium sulphate (CDH), DMSO (Himedia), Dichloromethane (CDH), Pyridine (CDH), Toluene Karnataka fine chem.), N, N^1-Carbonyl imidazole (Supreme Scientifics), Aniline (Karnataka), Triethyl amine (CDH), Tetra hydro furan (CDH), Sodium hydride (Sigma), Thionyl chloride (CDH), Lithium hydroxide (Sigma), Amino ethanol (CDH), MTT (Thermo fisher scientific). All other chemicals used in this project were of analytical grade.

Molecular docking

The molecular docking and ligand-protein interaction [26] was carried out by using -C-Docker-Discovery studio 3.5 version with the aim of identifying the most active inhibitors for HDAC-8, as anticancer target. The HDAC-8 protein was retrieved from protein data

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Received June 14, 2018; **Accepted** June 20, 2018; **Published** June 25, 2018

Citation: Kaliyaperumal R, Biradar YB, Mudugal MP (2018) Molecular Docking, Synthesis and Evaluation of Novel Hydroximic Acid Mimics as Anti-cancerous Histone Deacetylase Inhibitors. Med Chem (Los Angeles) 8: 166-176. doi: 10.4172/2161-0444.1000508

bank (Figure 2) and the 10 structural modulators (hydroxamic acid mimics) were drawn and retrieved in a mol format from chem draw and all the modulators drug likeness screening was carried out from molinspiration, only the filtered results of molinspiration ligands (Table 1) further followed by molecular docking studied.

Synthesis

Step 1: The 8-ethoxy-8 oxo octanoic acid, (5 grams) dissolved in tetrahydrofuran (50 ml), cooled to 0°C, added thionyl chloride (3.6 ml) (generally from 2 equivalents) stirred well for about 3 hours followed by TLC (generally, non-polar compared to starting material).

Work up procedure: The reaction mass, distilled on rotavapor and dried well under high vacuum. Now washed with 20 ml of n-hexane twice, decant the hexane, dried well the compound obtained to get 5.4 grams (100% yield) of the desired compound. (Don't add water to the reaction mass, it will covert acid chloride to acid (reverse reaction will happen). work up only distillation followed by n-hexane washing).

Step 2: Then the Aniline (2.3 ml), dissolved in DCM (60 ml), added approximately 5 equivalents of pyridine (10 ml) and stirred well for 30 minutes, added step 1 product (5.4 grams), stirred well for about 2 hours followed by TLC. Work up procedure: To the reaction mass, added 100 ml of water and 300 ml of DCM, stirred well for one-hour, separated DCM layer, dried over sodium sulphate, concentrated to get desired title compound (5.43 grams, 80% yield) molecular weight 277 (Unreacted aniline can be removed by washing the reaction mass with aqueous 1N HCl).

Step 3: Then the product from step 2 (5.43 grams) was taken and added to sodium hydride (56%) 1.70 grams (2 equivalents), stirred well for about 30 minutes at 0°C, added 3 equivalents of amino methanol (2.8 ml) and 60 ml of toluene heated to reflux for about 3 hours followed by TLC. (While weighing the Sodium hydride, precautions should be taken, it is highly pyrophoric. So carefully weigh in clean dry glass bottle. Avoid moisture or water during reaction set up). Work up procedure: The reaction mass was taken and carefully added ice water in portions wise, extracted with ethyl acetate. Ethyl acetate layer dried over sodium sulphate, concentrated to get 3.6 grams of the desired title compound (52% yield); molecular weight 293.

In vitro cytotoxicity study-MTT assay

Cell culture: MG-63 cancer cell line was kept in increasing phase of growth in DMEM medium supplemented with heat-inactivated 10% fetal bovine serum, incubated in CO_2 incubator (5% CO_2/95% humidified air).

MTT assay: is a standard quantitative colorimetric assay for measuring cellular growth, cell survival and cell proliferation based on the ability of live cells. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials.

In this method, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (pale yellow) enters the cell and passes into the mitochondria of viable cell where mitochondrial dehydrogenase reduces MTT enzymatically to formazan crystals (dark blue) by cleaving the tetrazolium ring. The formazan crystals so formed are insoluble in aqueous solutions. The cells are then treated with an organic solvent, acid-isopropanol (0.04 N HCl in isopropanol) to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring between 490 to 600 nm wavelength using a multi well scanning spectrophotometer (ELISA reader). Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Procedure: 10 ml of the suspension cell culture was added to 15 ml tubes and were centrifuged at 2500 rpm up to 10 min, then the supernatant was castoff and the cell pellet was resuspended in 1 ml growth medium. The cell viability was checked by counting the numbers of viable cells in the above 1 ml suspension through hemocytometer and diluted the resuspended cells with growth medium to get required cell concentration. 1×104 exponentially growing cells were seeded in each well in 96 well plates. Cells were exposed to various concentrations (50, 100, 200 and 400 µm) of Ligand-1 (test). The plates which were incubated at 37°C in 5% CO_2/95% humidified air. After 24 hours of incubation, the plates were centrifuged at 2500 rpm for 10 min and the supernatant was discarded. 100 µl of growth medium and 10 µl of MTT was added (5 mg/ml) to each well of 96 well plates and plates were incubated at 37°C in 5% CO_2/95% humidified air for 4 hr. The plates were centrifuged at 2500 rpm for 10 min and the supernatant was discarded. The precipitated Formazan salt was dissolved to form a colored solution by adding 100 µl of acid-isopropanol (0.04 N HCl in isopropanol) into each well. The absorbance of this colored solution was measured at a wavelength of 492 nm using a multiwell scanning spectrophotometer (ELISA reader) [27-29].

In vitro HDAC enzyme inhibition assay

HDAC activity was measured using the Fluor de Lys activity assay (Cayman) using the manufacturer's protocol. To measure comprehensive HDAC inhibition, HeLa lysates (approximately 4 µg of total protein) were incubated with small molecule inhibitor or without small molecule inhibitor (Ligand-1) in 2% DMSO in HDAC

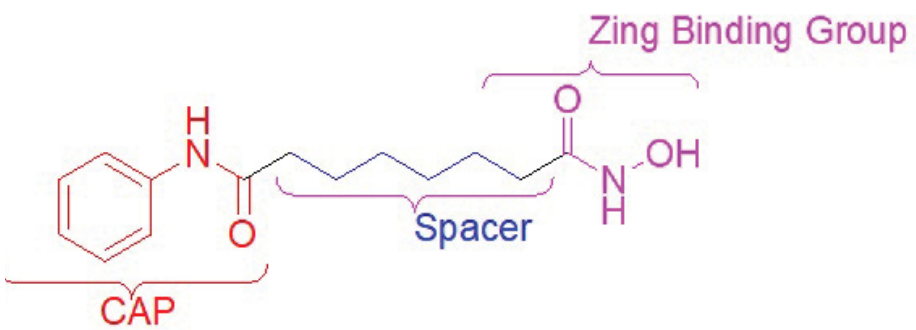


Figure 1a: General pharmacophore view of hydroximic acid.

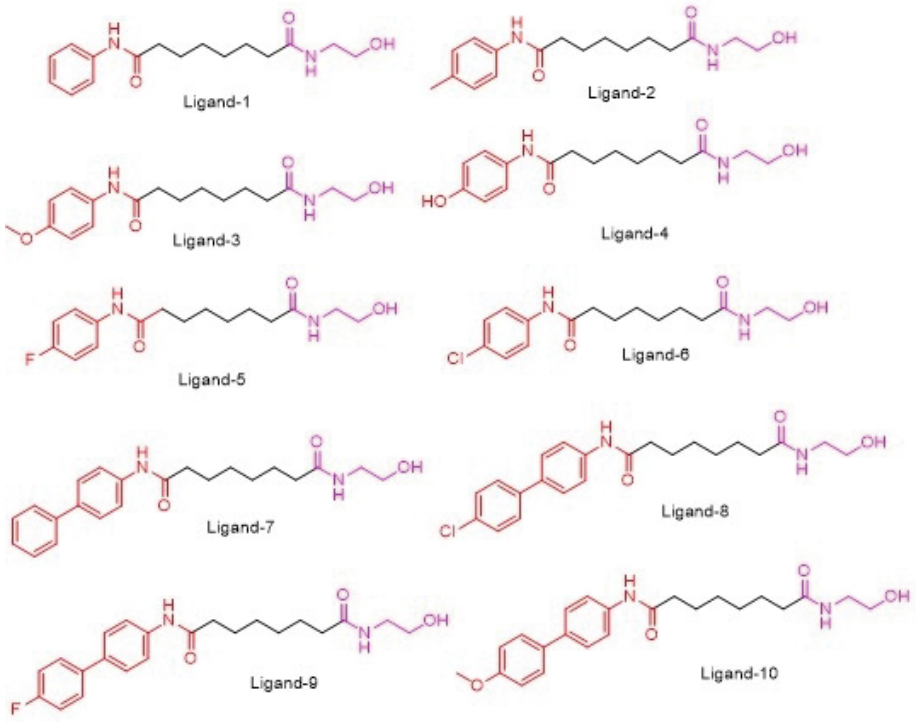


Figure 1b: Newly designed ten hydroximic acid mimics.

assay buffer (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$) in a final volume of 25 µL for 20 min at 23°C with 600 rpm shaking. Concentrations of small molecule (Ligand-1) between 50 nM, 100 N nM, 200 nM and 400 nM were used to determine IC50 values because the small molecules were stored in DMSO, dilution with HDAC buffer ensured that a maximum of 2% DMSO was present in the final reaction mixture. After the initial incubation, Fluor de Lys substrate in HDAC assay buffer (100 µM final concentrations) was added to make a total reaction volume of 50 µL. The reaction mixture was incubated at 30°C for 30 min with 600 rpm shaking. To quench the reaction and allow color development, Fluor de Lys developer (2.5 µL of 20X diluted up to 50 µL in HDAC assay buffer) was added to give a final 100 µL volume and incubated with shaking for 5 min at room temperature. The fluorescence intensity was determined using a Geniosplus Fluorimeter (Tecan) with excitation at 360 nm and emission at 465 nm.

Performing the inhibitor screening assay, the 96 well plates were used. The three wells designated for Background Wells and the three wells designated for 100% Initial Activity and each three wells designated to 50 µM, 100 µM, 200 µM and 400 µM concentration of Ligand-1.

- **100% Initial activity:** Take 140 µl of Assay buffer, 10 µl of Hela Nuclear Extract (Human cervical cancer cell line containing HDAC1-8) and 10 µl of solvent (The same solvent used to dissolve inhibitors) to three wells.
- **Background wells (Blank):** Take 150 µl of assay buffer, 10 µl of solvent (The same solvent used to dissolve inhibitors) to three wells.
- **Inhibitors wells:** Take 140 µl of Assay buffer, 10 µl of Hela Nuclear Extract and 10 µl of each three wells designated to 50 µM, 100 µM, 200 µM and 400 µM concentration of Ligand-1, totally 54 wells.
- Initiate the reaction by adding 10 µl of HDAC substrate to all the wells to being used.
- Cover the plate with the plate cover and incubate on a shaker for 30 min at room temperature.
- Remove the cover and added 40 µl of Developer, then cover the plate with plate cover and incubate with 15 min at room temperature.
- Remove the plate cover and read the fluorescence using an excitation wave length of 360 nM and emitted wave length of 465 nM. It may be necessary to gain setting on the instrument to allow the measurements of all the samples. The development is stable for 30 min.
- The inhibitors are dissolved with assay buffer and dimethyl sulfoxide (DMSO). And should added the assay in a final volume of 10 µl of three wells of different concentration (50-400 µM) of Ligand-1.

Calculating the percentage of inhibition

- To determine the average fluorescence of each sample.
- To subtract the fluorescence of the background (Blank) wells from all wells on the plate.

The determination of percentage inhibition for each sample, to subtract each inhibitors value from the 100% Initial Activity sample value. Then divide the result from 100% initial activity and then multiply by 100 to give % inhibition [30].

In vivo Ehrlich ascites carcinoma model

Dose selection-Acute toxicity study: The oral acute toxicity study of Ligand-1 was carried out, based on OECD-425 guideline, the LD_{50} were identified in the dose of 550 mg/kg, from these the selected final dose was 50 mg/kg [31] and 20 mg of 5-flurouracil (Standard drug) was selected for screening anticancer activity against EAC induced cancer in mice.

Cancer cell count and induction: 0.1 ml of normal saline (0.9%) was injected intraperitoneally into donor mouse. After injecting saline, immediately 1 ml of ascites fluid was collected from peritoneal cavity and diluted with normal saline up to 10 ml. 10 µl ascites fluid from this was taken and placed on Neubauer's chamber and the number of cells appeared on chamber were calculated and concentration of 1×10^6 cells were injected to each mouse intraperitoneally.

Treatment protocol: Swiss albino mice were randomly divided into 4 groups of 8 mice each, groups-II, III and IV (Table 2) were induced with Ehrlich Ascites Carcinoma and where, group-I served as normal control, group-II served as cancer control, group-III Ligand-1 (50 mg/kg), group-IV 5-Flurouracil (20 mg/kg) the treatment protocol is given below [32].

Determination of hematological parameters: In order to know the effect of the Ligand-1 on hematological status of EAC cells bearing mice, a comparison between Group-I (Normal control), Group-II (Cancer control), Group-III (Ligand-1), Group IV (5-FU) was done. Blood was drawn from each mouse by retroorbital plexus method and was collected in 12 µl of EDTA tube, for the Hematological studies and this blood sample was subjected to Animal Blood Counter (blood all count) for RBC count, WBC count and the hemoglobin content [33,34].

Measurement of mean survival time (MST) and Percentage increase in life span (% ILS): The effect of Ligand-1 (50 mg/kg) and 5-FU (20 mg/kg) on tumor growth was monitored by recording the mortality rate daily until all the animals were dead and %ILS was calculated by using the formula,

$$\% \text{ ILS} = [\text{MST of treated group} / \text{MST of control group-1}] \times 100$$

Body weight analysis: All the mice were weighed weekly after tumor cell inoculation and the average increase in the body weight of the carcinoma induced mice was measured and the percentage decrease in the body weight was determined by using formula,

$$\text{Percentage decrease in the body weight} = (Gc - Gt)/Gc \times 100$$

Where, Gc=gain in the body weight of control group; Gt=gain in the body weight of treatment group.

Statistical analysis: All the data are expressed as Mean ± SEM and SD, the data analyzed by software GraphPad Prism 7 and the parameters were analyzed by one-way ANOVA followed by Dunnett's t-test for multiple comparisons and P<0.05(***) was taken as significant, SEM=Standard Error Mean (*in vivo*), SD=Standard Deviation (*in vitro*).

Results

The ten hydroxamic acid mimics has been made (Figure 1a and 1b), based on the slight structural modification of a pharmacophore view of SAHA, the cap group generally hydrophobicity linked with certain amide based (Hydroxamates) zinc bind groups which is mainly responsible for enzymatic cleavation.

The molecular docking reported that, the pi-pi interaction between ligand-1 cap group and the protein residue of ARG-A37 and TRP-A315 shown strong electrostatic and vander walls forces which indicates strongest hydrophobicity. The hydroxamates group of ligand-1 producing the hydrogen bonding between ARG-A37 and SER-A138 which indicating the strongest zinc binding, which mainly responsible for enzymatic cleavation. Hence, apart from the ten structural modulators, the ligand-1 was shown the most potential HDAC-8 inhibitor (Figure 3 and Table 3).

Effect of drugs on hemoglobin

The effect of Ligand-1 and standard 5FU on hemoglobin in EAC induced mice are given in Table 4 and Figure 4.

The result shown decrease in Hgb level in the cancer control, i.e., 5.35 ± 0.78 gm/dL. The Hgb of normal group was 13.5 ± 0.98 gm/dL. Test contributed to increase the Hgb level towards the normal. With the dose of 50 mg/kg it has raised Hgb 10.47 ± 0.82 gm/dL (P<0.001). With the 5-Flurouracil, the level of Hgb was found to be increased significantly (10.45 ± 1.15 gm/dL) (P<0.001).

n=6 and values were expressed as Mean ± SEM Data analyzed by one-way ANOVA followed Dunnett's t-test for multiple comparisons.

Where, P<0.001(***), were taken as significant. SEM=Standard Error Mean.

Effect of drugs on RBCs

The effect of Ligand-1 and standard 5FU on red blood cells in EAC induced mice are given in Table 4 and Figure 5.

Treatment with Ligand-1 of (50 mg/kg) showed an extremely significant (P<0.05) increase (5.11 ± 1.37) in RBC count compared to EAC cancer control group (2.9 ± 0.55), while significant (P<0.001) increase in RBC count on administration of 5-FU (20 mg/kg) (5.51 ± 0.70).

n=6 and values were expressed as Mean ± SEM Data analyzed by one-way ANOVA followed Dunnett's t-test for multiple comparisons. Where, P<0.05 (*), P<0.001(***), were taken as significant.

Compound name	Log p	TPSA	n-atoms	Molecular weight	n-ON	n-OHNH	n-violations	n-rotatable bond	Volume
Ligand-1*	2.14	78.42	20	293.34	5	3	0	9	272.45
Ligand-2	2.59	78.42	21	292.38	5	3	0	9	289.01
Ligand-3	2.20	87.66	22	308.38	6	3	0	10	297.99
Ligand-4	1.06	98.65	21	294.35	6	4	0	9	280.46
Ligand-5	2.30	78.42	21	296.34	5	3	0	9	277.38
Ligand-6	2.82	78.42	21	312.80	5	3	0	9	285.98
Ligand-7	3.91	78.42	26	354.45	5	3	0	10	343.85
Ligand-8	4.59	78.42	27	388.89	5	3	0	10	357.39
Ligand-9	4.07	78.42	27	372.44	5	3	0	10	348.79
Ligand-10	3.97	87.66	28	384.38	6	3	0	11	369.40

Table 1: Physicochemical properties of ten structural modulators based on mole inspiration.

Groups	Treatment	Dose	No of animals
1	Normal Control	-	8
2	Cancer control	-	8
3	Test (Ligand-1)	50 mg/kg	8
4	Standard (5-FU)	20 mg/kg	8

Table 2: Treatment protocol.

Ligand Name	C-Docker Energy
Ligand-1	-24.7228
Ligand-2	-14.8014
Ligand-3	-11.7228
Ligand-4	-11.7044
Ligand-5	-11.4562
Ligand-6	-8.26753
Ligand-7	-8.26753
Ligand-8	-8.26753
Ligand-9	-7.60532
Ligand-10	-6.12596

Table 3: C-docker energy.

Groups	Hgb (g/dl)	RBC 1 × 10^6 /mm^3	WBC 1 × 10^3/mm^3
Normal Control	13.5 ± 0.98	8.45 ± 1.02	6.16 ± 0.70
Cancer Control	5.35 ± 0.78***	2.9 ± 0.55***	14.5 ± 1.57***
Ligand-1 (50 mg/kg)	10.47 ± 0.82***	5.11 ± 1.37*	10.7 ± 0.86***
5-FU (20 mg/kg)	10.45 ± 1.15***	5.51 ± 0.70***	9.5 ± 0.63***

Table 4: The effect of drugs on Hgb, RBCs, WBCs.

Groups	MST (days)	%ILS
Cancer Control	14.8 ± 0.30	0
Ligand-1	23.3 ± 0.49***	75.0
5-FU	25.5 ± 0.50***	95.0

Table 5: Increase in life span.

Groups	Gain in body weight (Mean ± sem)	%Decrease in body weight
Cancer Control	17.50 ± 1.02	0
Ligand-1	12.17 ± 0.58**	30.85
5-FU	13.42 ± 0.75***	23.42

Where, P<0.01(**), P<0.001(***)

Table 6: Body weight analysis.

Properties of ligand-1	
Solvent (solubility)	Methanol, Chloroform and DMSO
Color	White
Crystalline	Solid
Melting point ℃	250-252°C
Yield %	88%

Table 7: Physical and chemical properties of synthesized compound ligand-1.

SEM=Standard Error Mean.

Effect of drugs on WBCs

The effect of Ligand-1 and standard 5-FU on white blood cells in EAC induced mice are given in Table 4 and Figure 6.

The result shown that the level of WBC was increased in EAC cancer control mice (14.5 ± 1.57 × 10^3 /µl) when compared to the normal control mice (6.16 ± 0.70 × 10^3 /µl). On treatment with Ligand-1 of (50 mg/kg) showed an extremely significant (P<0.001) decrease (10.7 ± 0.86) of WBC count compared to EAC cancer control group, while significant (P<0.001) decrease (9.5 ± 0.63) of WBC count

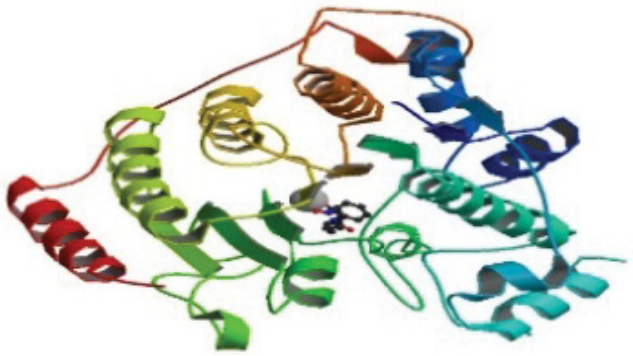


Figure 2: Crystal structure of HDAC8, PDB Id: 1T69 and complexed with TSA.

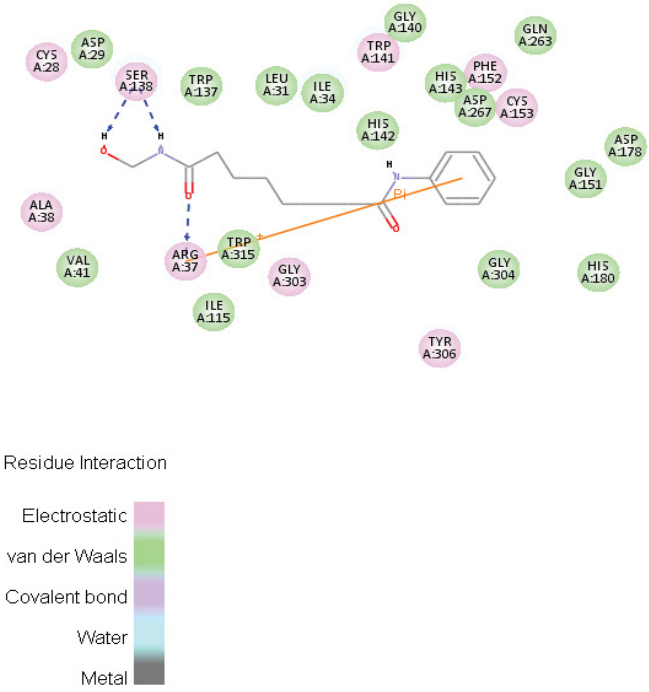


Figure 3: Ligand-Protein Interaction of the Most Active Ligand (Ligand-1) with HDAC-8 (PDB Id: 1T69).

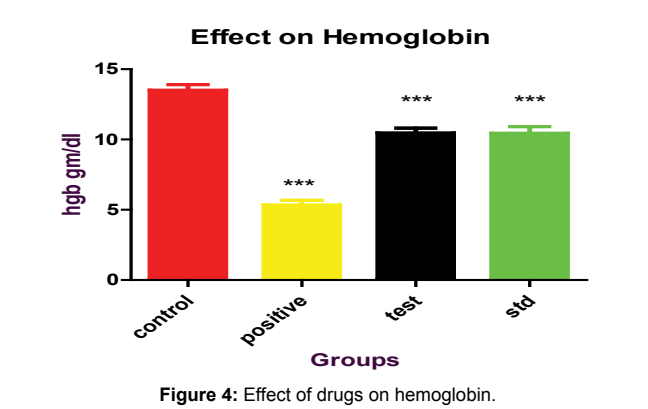


Figure 4: Effect of drugs on hemoglobin.

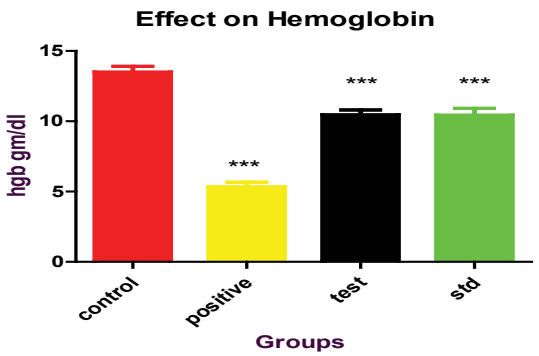


Figure 4: Effect of drugs on hemoglobin.

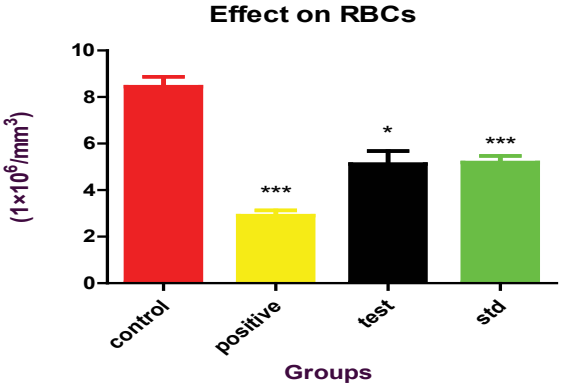


Figure 5: Effect of drug on RBCs.

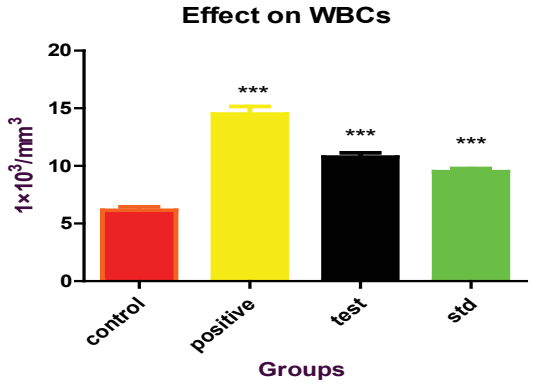


Figure 6: Effect of drugs on WBCs.

on administration of 5-FU (20 mg/kg).

n=6 and values were expressed as Mean ± SEM Data analyzed by one-way ANOVA followed Dunnett's t-test for multiple comparisons. Where, P<0.001(***), were taken as significant. SEM=Standard Error Mean.

Effect of drugs on life span (of mice)

The effect of Ligand-1 and standard 5-FU on mean survival time (MST) and percentage increase in life span (% ILS) in EAC induced mice are given in Table 5 and Figure 7.

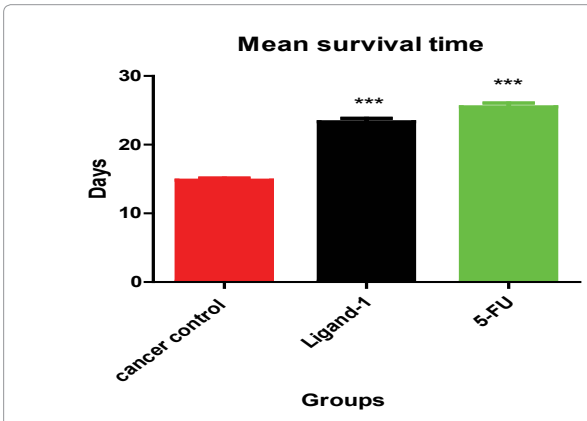


Figure 7: Mean survival time.

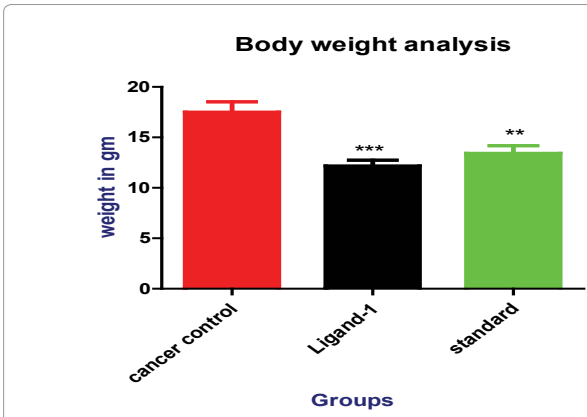


Figure 8: Body weight analysis.

The result shown that In EAC cancer control mice the mean survival time was 14.8 ± 0.30 days. Whereas, it was significantly increased on treatetment with test drug Ligand-1 (50 mg/kg) by (75.0%) (23.3 ± 0.49 days) (P<0.001) Whereas, comparision on treatment with standard drug 5-FU (20 mg/kg) increased the life span by (95.0%) and increased the mean survival time significantly (25.5 ± 0.50) (P<0.001).

n=6 and values were expressed as Mean ± SEM Data analyzed by one-way ANOVA followed Dunnett's t-test for multiple comparisons. where, P<0.001(***), were taken as significant. SEM=Standard Error Mean.

Effect of drugs on body weight (of mice)

The effect of Ligand-1 and standard 5-FU on body weight in EAC induced mice are given in Table 6 and Figure 8.

The result shown that In EAC cancer control mice the body weight was 17.50 ± 1.02 gm. Whereas, it was significantly decreased in body weight on treatetment with test drug Ligand-1 (50 mg/kg) by (30.85%) i.e., (P<0.001) Whereas, comparision on treatment with the standard drug 5-FU (20 mg/kg) decreased the body weight by (23.42%) and increased the (P<0.01).

n=6 and values were expressed as Mean ± SEM Data analyzed by one-way ANOVA followed Dunnett's t-test for multiple. Where P<0.01(**), P<0.001(***), were taken as significant. SEM=Standard Error Mean.

Discussion

Molecular docking studies

The Ten Structural Modulators (Hydroximic acid mimics) of the HDAC inhibitors was made (Figure 1a and 1b), which is based on the general pharmacophore view of SAHA through slight structural modification of its cap and zinc binding group, The Docking studies and the ligand–protein interactions of the modulators were carried out with the aim of characteristic their malignant tumor activity in dysregulation of Human HDAC-8 through binding and activation by applying the program C-Docker with Discovery Studio-3.5 was used for studying the ligand–protein interactions. The studies were applied so as to spot an efficient, selective and effective anti-cancerous HDACi for the treatment of human cancers caused by the human HDAC-8 abnormality.

A molecular docking were performed for the studying and calculating the binding energy and ligand affinity with receptors and it was based on identified H-bond interaction, H-bond donor and acceptors, hydrophobicity and lipophobicity at the catalytic active site of HDAC-8 (PDB: 1T69) with all ten ligands from newly designed hydroxamic mimics were separately docked with HDAC-8 catalytic site, with the aim of identifying most active affinity modulators for targeting HDAC inhibitors as anti-cancer targets.

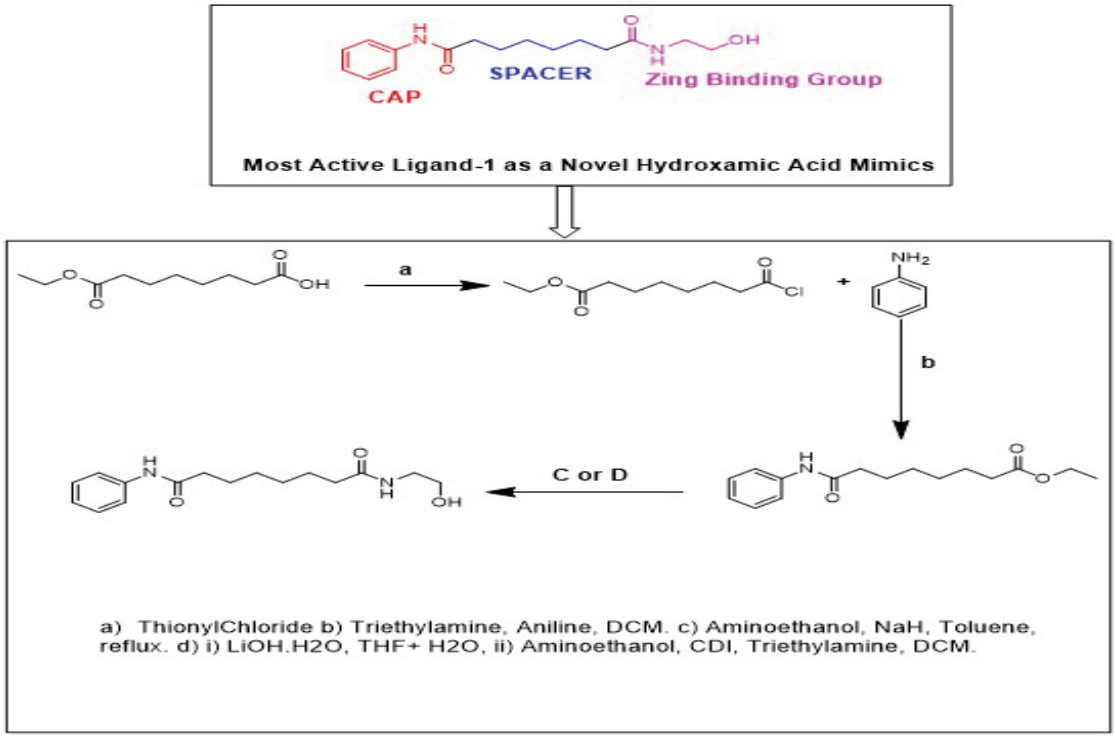
The study was gain functional and structural insight into the mechanism of most active compounds were obtained from the molecular docking simulation and it was performed by the aid of -C-Docker program of Discovery Studio. By performing the docking, the particular simulation and some feasible conformations and information's of the ligand within the protein/enzyme binding site were obtained. This information also was reflected the nature, quality and affinity of the ligand-Receptor interaction. In our study, the grid box for docking simulation was built with enough size to enable probing into the binding with HDAC-8 catalytic sites to expose the binding properties of newly designed ten HDAC inhibitors (Table 7). Based on the molecular docking studies the ligand-1((N^1 (hydroxyethyl) N^8-phenyloctanediamide)) were exhibit the best interaction and docking score -24.7228.

Synthesis

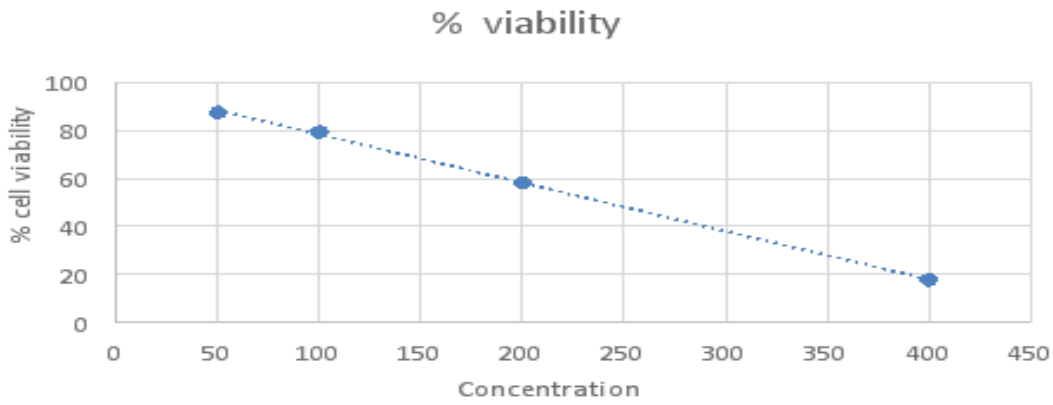
Considering the most active compound (ligand-1) IUPAC name; N^1-(2-hydroxyethyl), N^8-phenyloctanediamide, was needed to develop anticancer compound for HDAC-8 inhibition, the synthetic strategy (Scheme 1) was adequately decided according to the molecule. However, standard synthetic methods were used in order to achieve the final compound/inhibitor shown on Scheme 1, such as coupling reactions, nucleophilic substitutions, protection/deprotection steps, aromatic substitutions, acylation, additions etc. and the compound confirmed and characterized by ^{1}H-NMR, ^{13}C-NMR and Mass spectra.

In IR spectrum of Ligand-1 the stretching frequency at 3410 cm^{-1} of -OH and frequency observed at 1615 cm^{-1} is due to the -OH bending vibrations. Stretching frequency at 1748 and 1704 cm^{-1} are due to the >C=O groups. The aliphatic –CH stretching frequency observed at 2860, 2924 and 2961 cm^{-1}. The -NH stretching frequency appeared at 3207 cm^{-1}. The band observed at 1416 cm^{-1} is bending frequency of the aliphatic –CH. The aromatic -CH stretching frequency observed at 3093 cm^{-1}.

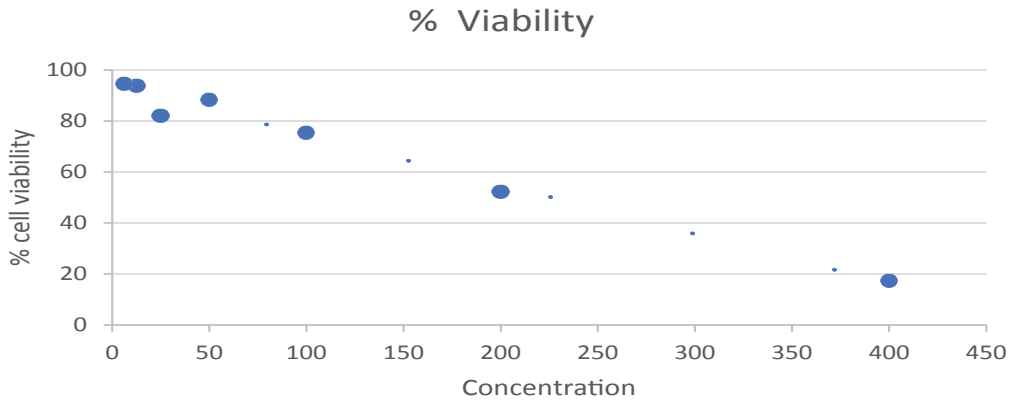
In ^{1}H NMR spectrum of ligand 1 the proton signal at 4.59 ppm is due to the -OH proton. The aliphatic protons (axial and equatorial protons) are observed on the range of 0.92-3.58 ppm. The two NH protons are appeared at 4.87 and 9.94 ppm. The aromatic protons are appeared in the range of 6.70-9.30 ppm.



Scheme 1: Synthesis schematic diagram of ligand-1.



Graph 1: %inhibition (MG-63 cancer cell) vs concentration plot of ligand-1(Test).



Graph 2: %inhibition(MG-63 cancer cell) vs concentration plot of 5-FU (Std).

In the ^{13}C NMR spectrum of ligand-1, the two carbonyl (>C=O) carbons are appeared at 169.94 and 178.24 ppm. Aliphatic carbons are appeared in the range of 22.71-43.61 ppm. Aromatic carbons are appeared in the range of 119.87-140.40 ppm.

Generally, in electron impact or chemical ionization mass spectroscopy, the carrying gas (methane gas) is ionized by electron impact. This in turn produced the primary ions followed by secondary ions. The secondary ions usually react with the organic molecule under study thereby produce the ions characteristic for the molecule and its fragments. In the mass spectrum of ligand-1 the molecular ion peak is observed at 293.34 due to the M+1 peak by mass per ratio.

Cytotoxicity study *in vitro* MTT assay

The Ligand-1 was assayed against selected cancer cell lines such as MG-63 (osteosarcoma) to determine its efficiency to inhibit the human osteosarcoma cancer cells viability, in the presence of different concentrations of the inhibitor (50, 100, 200 and 400 nM). In this, Ligand-1 shown on good inhibitory effect on MG-63 cancer cell line, the concentration of the inhibitor results in 50% inhibition (IC_{50}) was determined by plotting the percent inhibition and concentration of the inhibitors (Graphs 1 and 2).

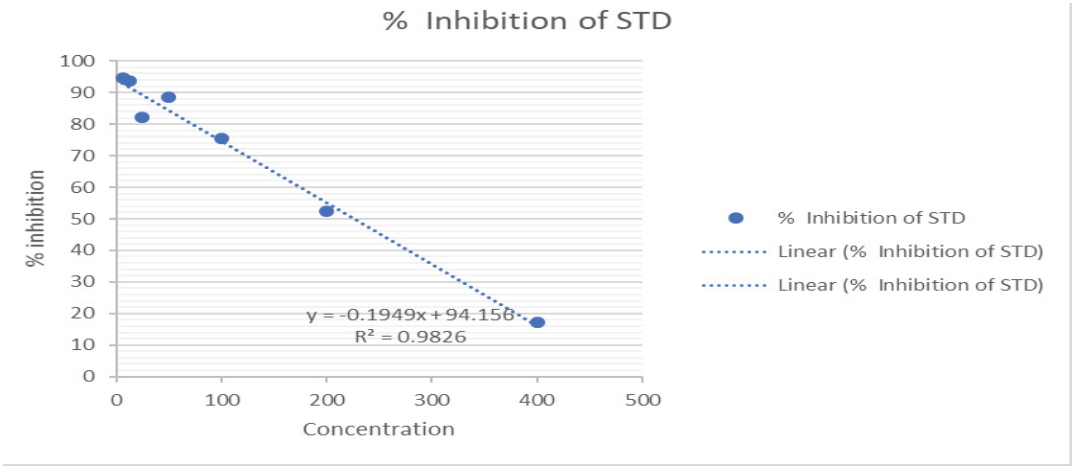
The results are summarized in Table 8 The Ligand-1 showing equipotent (IC_{50} of 58.22 ± 0.75) compared to the standard compound 5-FU (IC_{50} of 62.56 ± 0.37).

The IC_{50} values of test compound Ligand-1 showed significantly ($p<0.05$) lower when compared with 5FU. It indicates that Ligand-1 having most significant Anti-cancer activity.

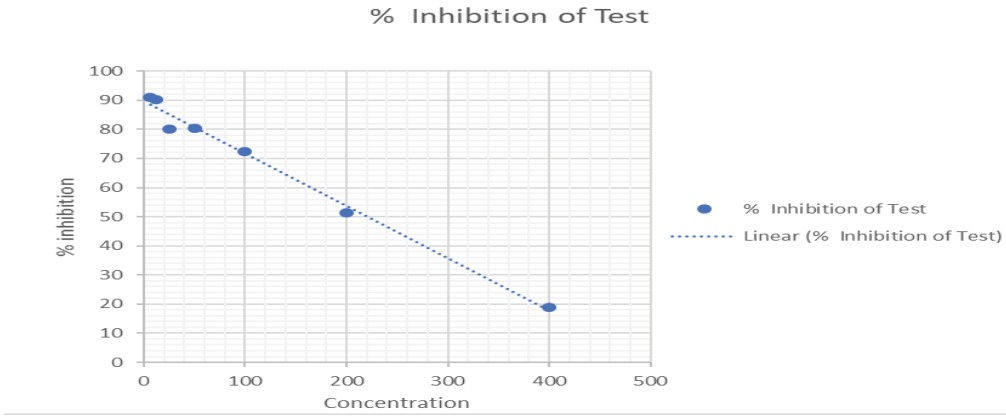
In vitro HDAC enzyme inhibition assay

A number of HDAC inhibitors have been potential targets as chemotherapeutic drugs due to that the over expression activity of HDAC, which leads cancer diseases, as discussed (Introduction) Our study is also focused on progress of novel HDAC inhibitors based on hydroxamates. Therefore, hydroxamic acid mimics (ligand-1) were screened and compared to Trichostatin A as a standard drug by using a Fluorescence high-throughput assay. The deacetylase activity was measured using the Fluor de Lys activity assay (Cayman) using the manufacturer's protocol.

To quantify the global HDAC inhibition, HeLa lysates, which contains a mixture of HDAC1-8(rich in cervical cancer cell line), were incubated with or without Inhibitors (Test sample Ligand-1, with the concentration of 50 nM, 100 nM, 200 nM and 400 nM) in HDAC assay buffer solution. Later than initial incubation, Fluor de Lys substrate in HDAC assay buffer was added to the reaction. The peptidic substrates consisted of a ε-acetylated lysine residue and a 4-methylcoumarin-7-amide at the carboxy terminal unit. In the reaction catalyzed by HDACs, the acetylated lysine residue of the substrate was deacetylated, while acetylated lysine would stay in the reaction when inhibited by



Graph 3: Showing the HDAC IC50 values of TCS (Std).



Graph 4: Showing the HDAC IC_{50} values of Ligand-1 (Test).

Concentration (µM)	%viability of test (Ligand-1)	%viability of standard (5-FU).
400	17.77	19.89
200	58.22	62.56
100	79.37	85.38
50	87.45	92.44

Table 8: MTT assay shown the percentage cell viability of MG-63 cancer cell in different concentration.

Concentration (nM)	%Inhibition of STD (TSA)	%Inhibition of Test (Ligand-1)
400	17.23	10.78
200	52.39	51.42
100	75.38	72.37
50	88.56	80.45
25	82.29	80.18
12.5	93.78	90.1
6.25	94.75	91.08

Table 9: HDAC enzyme inhibition assay result.

Compound	Human HDAC8 IC_{50} (nM)
TSA (STD)	52.39 ± 0.37
Ligand-1 (Test)	51.42 ± 0.75

Table 10: HDAC Inhibition, IC_{50} Values of TSA (STD) and Ligand-1 (Test).

the inhibitors. To extinguish the reaction and allow color development, Fluor de Lys developer was added to the reaction mixture. In this reaction, the only deacetylated peptidic lysine substrates containing the methylcoumarinamide were cleaved by trypsin to liberate the fluorescence molecule, methyl coumarin. In other word, the acetylated lysine substrate present when the reaction was inhibited by the inhibitor did not result in quantifiable cleavage by trypsin and did not release the fluorescence molecule (no fluorescence activity). As a result, the high level of deacetylated activity of the substrates indicated low inhibitory activity of the inhibitors. The fluorescence intensity was determined using a Genioplasty Fluorimeter (Tecan) with excitation at 360 nm and emission at 465 nm.

The percent inhibition was determined by applied procedure showed in experimental procedures. The concentration of the inhibitor results in 50% inhibition (IC_{50}) was determined by plotting the percent inhibition and concentration of the inhibitors.

The results are summarized in Tables 9 and 10; Graphs 3 and 4. The Ligand-1 showing equipotent (IC_{50} of 51.42 ± 0.75) compared to the standard compound TSA (IC_{50} of 52.39 ± 0.37).

The IC_{50} values of Ligand-1 showed significantly ($p<0.05$) lower

when compared with TSA. It indicates that Ligand-1 is a potential lead compound having better HDAC activity.

In vivo Ehrlich ascites carcinoma model

Acute toxicity testing: The acute cytotoxicity study involved in estimating LD50 (Median lethal dose) value of Ligand-1 was found to be 550 mg/kg/i.p., and from this the final dose selected was $1/10^{th}$ of 550 mg/kg that is 55 mg/kg.

Parameters

In *In vivo* studies, the parameters checked are Hgb, RBCs, WBCs (Table 4 and Figures 4-6), MST (Table 5 and Figure 7), %ILS, Body weight analysis (Table 6 and Figure 8).

From *in vivo* studies we got that, when cancer is induced to mice (Groups II, III, IV) there is decrease in RBCs, Hgb, life span and a increase in WBCs value, body weight. The decrease in Hgb and RBCs may be due to anemia and The anemia encountered in tumor bearing mice is mainly due to reduction in RBC and hemoglobin percentage and this may occur either due to iron deficiency and haemolytic anemia (that is, Lymphoma affects the blood and bone marrow which damages the production of red blood cells and thereby causes haemolytic anaemia).

A hormone called erythropoietin, tells the body when to make more red blood cells which is present in bone or bone marrow but cancers affect the bone marrow directly or cancers that have spread to the bone (or bone marrow) lowers the production of erythropoietin contributing anaemia and Hemoglobin also falls as RBC count drops. Upon treatment with ligand-1 (50 mg/kg) and 5-FU (20 mg/kg) we saw that, there is decrease in WBCs value, body weight and increase in RBCs, Hgb and life span. Hence, we concluded that Ligand-1 shows an potentail anti-cancer activity.

Conclusion

In conclusion of the present work designed ten structural hydroxamic acid mimics, based on the slight structural modification of cap and zinc binding groups of a scaffold of hydroxamic acid to generate leads against the HDAC-8 complex protein. Finally, the ligand-1 was a lead molecule was identified as probable ligand for inhibition of HDAC-8 protein. These ligands were synthesized and screened against the HDAC complex, through molecular docking followed by *In vitro* cytotoxicity study, HDAC enzyme inhibition assay and *in vivo* EAC mice model, these results were compared with standard compound Tricostatin-A, it is a first derived natural compound of hydroximate derivatives of histone deacetylase inhibitors and 5-FU.

Hence, the new compound of Ligand-1 (IUPAC name: [N^1-(2-hydroxyethyl) N^8-phenyloctanediamide]) is a novel lead molecule for anti-cancerous HDAC inhibitors for disruption of Human HDAC-8.

The results were confirmed; by three times of repeated experiments and IC_{50} values were calculated by using graph pad software and ANOVA statistical analysis. The new compounds of ligand-1 showed equipotent activity, when compared with Trichostatin A. So, the compound Ligand-1 were found to be the most significant ($p<0.05$) free binding energy of around -24.7228 and IC_{50} value of Ligand-1 was 58.22 ± 0.75 and compared with standard 5-FU 62.56 ± 0.37 against MG-63 cancer cell line and HDAC activity of Ligand-1 was found to be 51.42 ± 0.75 Which were conformed from the standard drug TSA(Trico statin A) 52.39 ± 0.37 and continuation with the *in vivo* EAC mice model shown significant value with increase in MST, Hgb and RBCs and decrease in body weight and WBCs using ligand-1, and these values were compared with 5-FU. Hence, the results showing the ligand-1 is a potential HDAC inhibitor for anticancer.

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