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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis, and biological evaluation of novel 2,4-thiazolidinedione derivatives as histone deacetylase inhibitors targeting liver cancer cell line

Rhea Mohan · Ajit K. Sharma · Sanjay Gupta · C. S. Ramaa

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Abstract As a part of an ongoing effort to find alternate chemotherapeutic agents for hepatocellular carcinoma, we herein, report the design and synthesis of two novel compounds targeting histone deacetylase (HDAC) with 2,4-thiazolidinedione as zinc chelating group. Further, we demonstrate that these compounds show cytotoxicity that parallels their ability to inhibit HDACs activity in human liver cancer cell line HepG2. The findings obtained in this study indicate that 2,4-thiazolidinedione group may be utilized successfully to inhibit HDAC activity with future potential for lead optimization by chemical derivatization of active compound, *N*-(6-(2,4-dioxothiazolidin-3- yl)hexyl) benzenesulfonamide.

Keywords Hepatocellular carcinoma cell line · HDAC inhibitors · 2,4-Thiazolidinedione · Histones

Introduction

Primary liver tumors, mainly hepatocellular carcinoma (HCC) and cholangiocarcinoma, constitute the fifth most frequent types of cancer, whereas in the rank of mortality, they are the third. The clinical relevance of liver tumor increases further if the number of deaths due to liver metastasis of cancers of extrahepatic origin is included (Marin *et al.*, 2008). HCC is often diagnosed at an advanced stage, when it is not amenable to curative therapies. Treatment of liver cancer is primarily limited by its refractivity to available chemotherapy and development of resistance during treatment (Avila *et al.*, 2006). Advances in cancer biology suggest that a limited number of pathways are responsible for initiating and maintaining dysregulated cell proliferation, which is the major cellular alteration responsible for the cancer phenotype (Roberts and Gores, 2005). New treatments in development target several of these critical pathways, one of them being the epigenetic histone deacetylation pathway. Rikimaru *et al.*, in their report, were the first to conclude that a high Histone Deacetylase 1 (HDAC1) expression might play an important role in the aggressiveness, cell dedifferentiation, and poor outcome in patients with HCC. A high HDAC1 expression was found to be an independent poor prognostic factor in patients with HCC. Normal hepatocytes did not express HDAC1; therefore HDAC1 in HCC may be a good molecular target for anticancer agents (Rikimaru *et al.*, 2007).

The last decade has seen an exponential growth in research addressing HDACs and their inhibitors, and a significant number of HDAC inhibitors have been identified and developed (Walkinshaw and Yang, 2008; Miller *et al.*, 2003; Paris *et al.*, 2008). The mode of action of HDAC inhibitors for anticancer activities is partially through the transcriptional regulation of genes involved in proliferation, differentiation, or apoptosis (Carew *et al.*, 2008). Normal cells are relatively resistant to HDAC inhibitor induced cell death. It may be speculated that cancer cells with multiple defects cannot reverse the critical effects of HDAC inhibitors as normal cells may do. It was previously reported that Trichostatin A (TSA) induces cell cycle arrest/apoptosis and hepatocyte differentiation in

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human liver cancer cell lines (Yamashita *et al.*, 2003). In addition, HDAC inhibitors like suberoyl anilide hydroxamic acid (SAHA) and sulfatase 1 enzyme (SULF1) were seen to inhibit HCC tumor in in vivo animal models (Venturelli *et al.*, 2007; Lai *et al.*, 2006). Thus, the current results are considered to provide important evidence of the future application of HDAC inhibitors in patients with HCC.

Herein we report the results of design, synthesis, antiproliferative and HDAC inhibitory activity of two novel compounds with 2,4-thiazolidinedione as zinc chelating group, which may be used as a lead for the development of new antitumor agents targeting liver cancer.

Results and discussions

Designing

The HDAC catalytic domain consists of a narrow, tube like pocket of a length equivalent to that of a 4–6 carbon straight chain. A zinc cation is positioned near the bottom of this enzyme pocket, which in co-operation with two His-Asp charge relay system facilitates the deacetylation catalysis (Finnin *et al.*, 1999).

Mai *et al.* have elaborated a structural model for Class I/II HDAC inhibition. All known natural or synthetic inhibitors possess an extremely variable cap group. This moiety contacts residues on the rim of the catalytic pocket and is connected to an electronegative group (X), that is able to interact by hydrogen bond with other residues. This in turn is bound to a hydrocarbon linker interacting with channel residues of the active site of the enzyme and finishing with the enzyme inhibiting group (EIG), that in many cases chelate the zinc cation near the bottom of the catalytic pocket (Mai *et al.*, 2005). Using this structural model as a scaffold, we designed and docked several molecules having the general structure shown in Fig. 1 into the active pocket of histone deacetylase-like protein (HDLP).

Based on the scoring values, it was observed that compounds *N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)benzamide

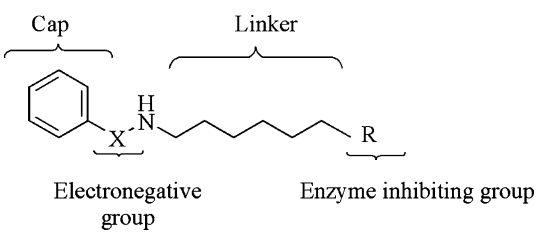


Fig. 1 General structure of designed histone deacetylase inhibitors

SRR1 (Andrew's p*K*i: 2.052, Predicted p*K*i: 5.765) and *N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl) benzenesulfonamide **SRR2** (Andrew's p*K*i: 0.9527, Predicted p*K*i: 6.602), both having 2,4-thiazolidinedione as the enzyme inhibiting group, showed better affinity for the receptor as compared to other molecules. SAHA was re-docked into active site of HDLP. The root mean square deviation (RMSD) between the heavy atom positions of bound and MOE conformation was 1.19 Å. Both conformations were quite well overlaid. Figure 2 depicts the docked poses of **SRR1** and **SRR2** with HDLP. These molecules also show high metal ligation values (**SRR1**: 0.206, **SRR2**: 0.275). The considerable difference in their values for Andrew's p*K*i and predicted p*K*i indicate that these molecules maybe specific to the receptor. These compounds were therefore selected for synthesis and detailed biological evaluation with respect to their anticancer and HDAC inhibiting potential.

Chemistry

Compounds **SRR1** and **SRR2** were synthesized by following the route outlined in Scheme 1. 1,6-Dibromohexane was refluxed with 2,4-thiazolidinedione in the presence of triethylamine (TEA) to obtain 3-(6-bromohexyl)-2,4-thiazolidinedione (a) (Aoki *et al.*, 2003). This intermediate was then heated under reflux in a solution of hexamine in chloroform which resulted in formation of a white precipitate of 1-[*N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)]-1-azonia-3,5,7-triazatricyclo decane bromide (b). This precipitate was filtered under vacuum and subjected to overnight hydrolysis in acidic ethanol at RT to obtain [*N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)] ammonium chloride (c) (Warmus *et al.*, 1993). This reaction gave a product which was highly unstable making its isolation difficult. Therefore, the final step was carried out in the same assembly without isolation of [*N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)] ammonium chloride using dichloromethane (DCM) as the solvent and TEA as base. The resultant final products were isolated by column chromatography and their structures were confirmed by various spectral analyses.

Biological evaluation

The biological activity of newly synthesized HDAC inhibitors having 2,4-thiazolidinedione as enzyme inhibiting group was evaluated using both cell proliferation assay and HDAC enzyme assay.

Antiproliferative testing

The antiproliferative activity was assessed on human liver cell lines, transformed (HepG2) and untransformed embryonic (WRL68) cell lines using dose-dependent and time-

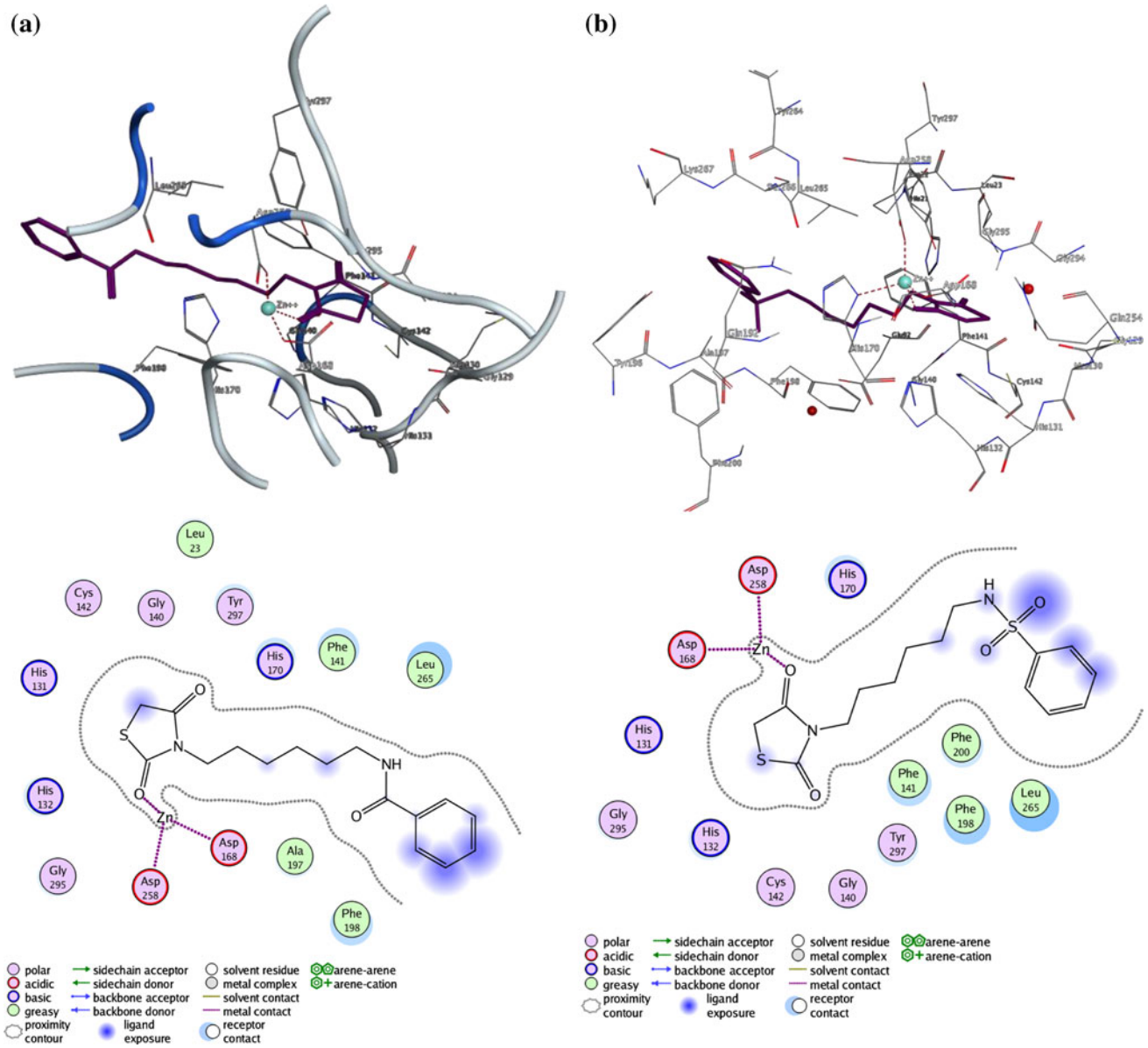
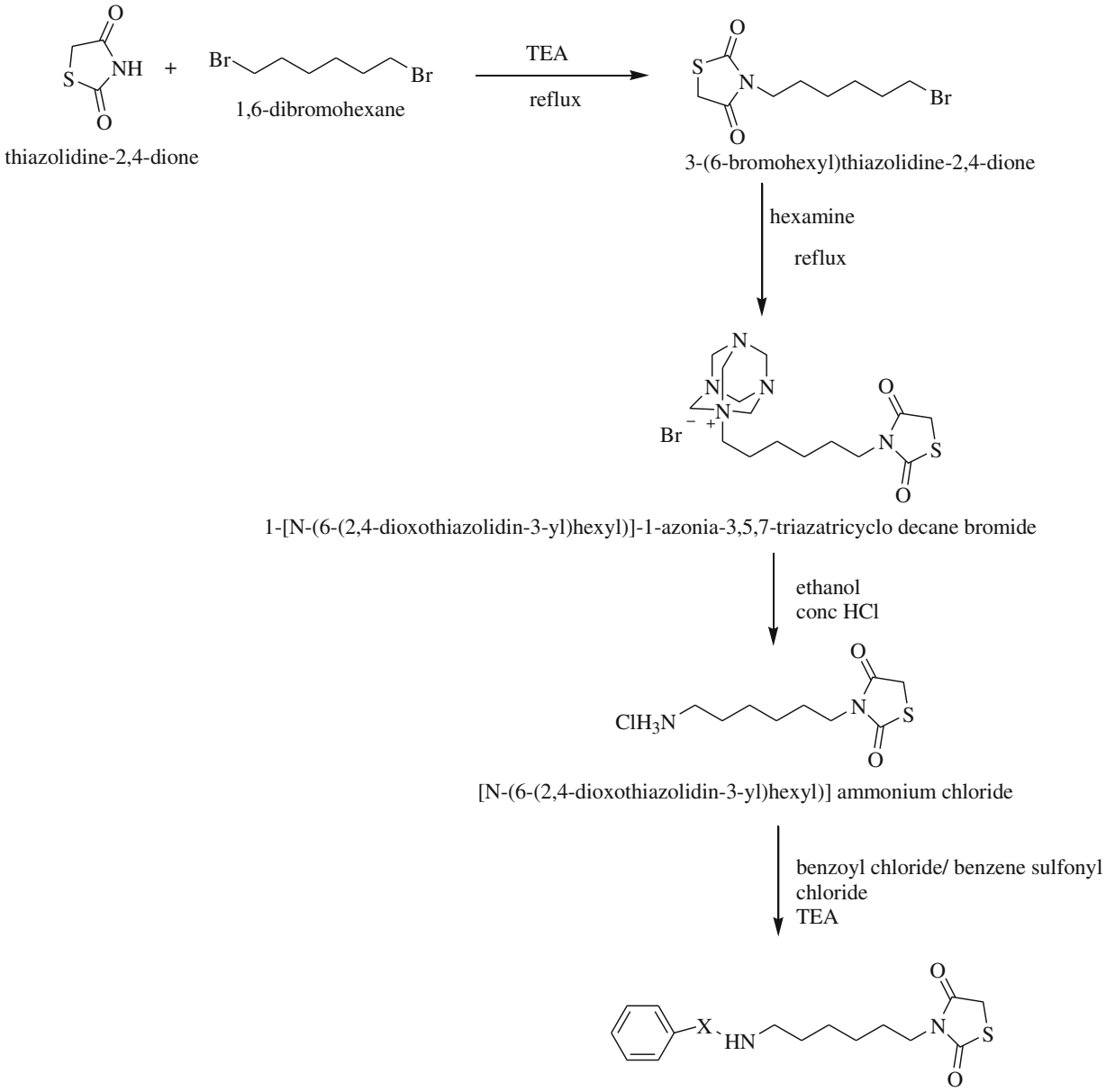


Fig. 2 Docked **SRR1** (**a**) and **SRR2** (**b**) showing zinc chelation (*dotted lines*) with HDLP

dependent MTT assay (spectrophotometric quantification of viable cells by cleavage of tetrazolium salt) (Mosmann, 1983). WRL68 cell line was used to determine the effect of test compounds on normal untransformed cells. The results obtained have been depicted in Figs. 3 and 4. In addition, cells treated with compounds **SRR1** and **SRR2** exhibited a more elongated shape than that of control HepG2 cells; this is similar to the morphological changes seen in sodium butyrate (SB) or other known HDAC inhibitor-treated cells (data not shown).

Treatment of cells with test compounds resulted in cell line specific growth inhibition. Treatment of HepG2 cell line with 100 μM doses of test compounds **SRR1** and **SRR2** brought about 42.31% (Fig. 3a) and 57.27% (Fig. 3b) cell death, respectively, at the end of 48 h. The same dose for same time period caused 34.66% (**SRR1**) (Fig. 3a) and 37.5% (**SRR2**) (Fig. 3b) cell death in WRL68 cells. The dose-dependent studies indicate that test compounds are cytotoxic in both HepG2 as well as WRL68 cell lines although more selective toward transformed HepG2 cells. Also, the cellular effect of test compounds was further assessed for time-dependent differential growth inhibition in HepG2 and WRL 68 cell lines by MTT assay. The growth inhibition or cellular proliferation was measured at 48, 72, 96, and 120 h time points. The time-dependent studies indicate both the compounds retain their cytotoxicity at the end of 120 h causing 82.98% (**SRR1**) (Fig. 4a) and 69.45% (**SRR2**) (Fig. 4b) cell death in HepG2 cell line. The pattern



Scheme 1 Synthesis of 2,4-thiazolidinedione derivatives as HDAC inhibitors

of cell death in WRL 68 cell line shows a different pattern from that of HepG2. Even though cytotoxicity is seen, WRL 68 cells treated with compound **SRR2** recover since there is significant difference in the percent cell death in these cells (55.6%) as compared to the cell death in HepG2 cell line at the end of 120 h (Fig. 4b). Compound **SRR1** shows better cytotoxicity but it suffers from the drawback that it is equally active against transformed (82.98% cell death) and untransformed (92.34% cell death) cells (Fig. 4a).

In vitro HDAC assay

The HDAC inhibitory potential of synthesized molecules was assessed using HDAC enzyme assay. Nuclear extracts of treated and untreated HepG2 cells were analyzed for their HDAC activity at different time intervals ranging from 2 to 24 h. Maximum HDAC inhibition was seen in the 8th hour time interval (Fig. 5) wherein compounds **SRR1** and **SRR2** showed 42.11 and 56.85% HDAC inhibition as compared to positive controls SB (52.32% HDAC inhibition) and SAHA (57.89% HDAC inhibition).

Hyperacetylation of histones

Many studies have demonstrated that inhibition of HDAC activity by specific inhibitors induces hyperacetylation of histones in drug-treated samples. To investigate whether newly synthesized compounds inhibits HDAC activity in

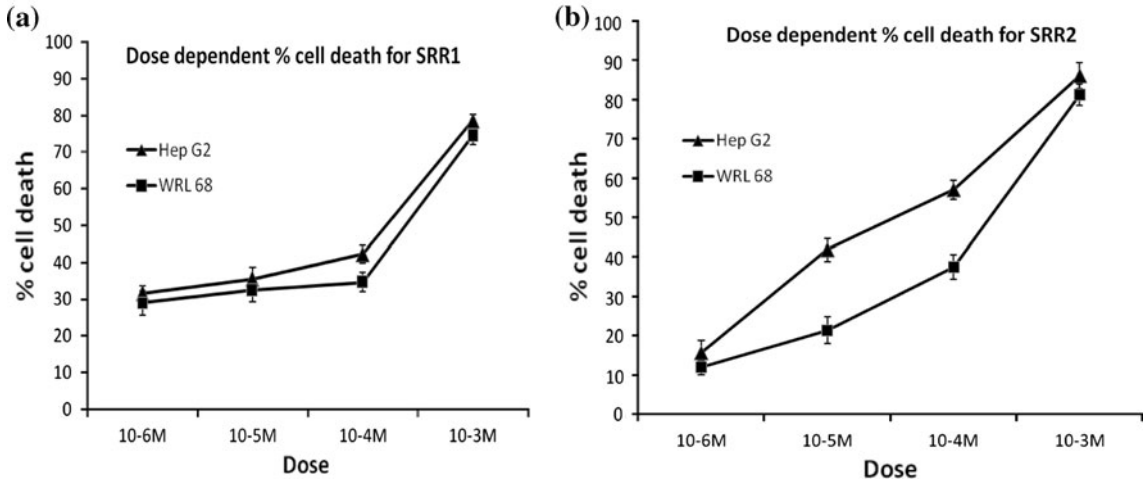


Fig. 3 Dose-dependent response curves of HDAC inhibitors. WRL 68 and HepG2 cells were treated with various concentrations of **SRR1** (**a**) and **SRR2** (**b**) as indicated, for 48 h. Cell viability was determined by MTT assay. Results are expressed as % of cell death as compared with control. Data represents mean values of at least three separate experiments

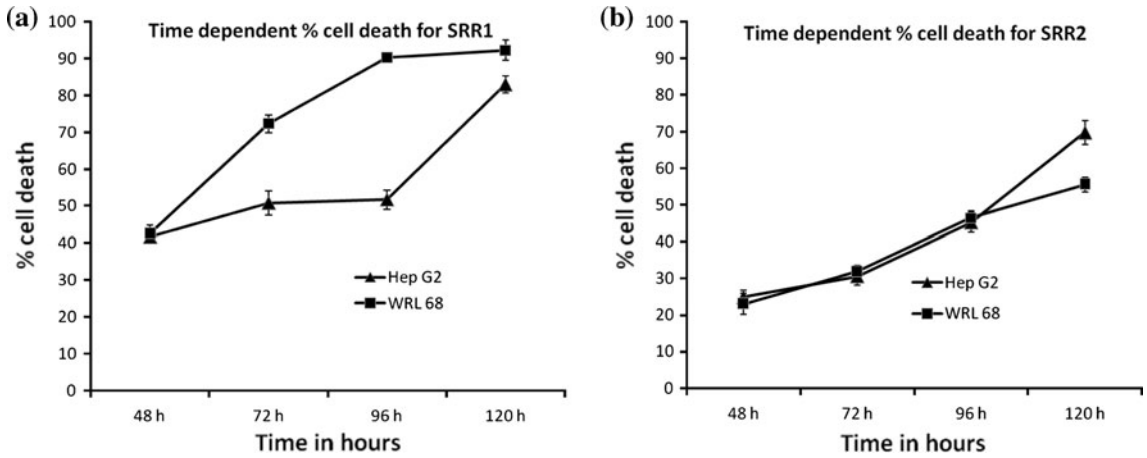


Fig. 4 Time-dependent response curves of HDAC inhibitors. WRL 68 and HepG2 cells were treated with **SRR1** (**a**) and **SRR2** (**b**) at various time intervals as indicated. Cell viability was determined by MTT assay. Results are expressed as % of cell death compared with control. Data represents mean values of at least three separate experiments

cells, the levels of post-translational modifications (PTMs) in histone was visualized using triton acid urea polyacrylamide gel electrophoresis (TAU PAGE) and antibodies specific for acetylated H3-histone (Abcam ab47915).

TAU PAGE is a commonly used method to separate histone and their post-translationally modified forms. The TAU PAGE separates proteins on the basis of mass, charge and hydrophobicity. The hyperacetylation induced by newly synthesized compounds peaks at 8th hour time point as shown by HDAC assay. Therefore, after 8 h, histones were extracted and subjected to TAU gel electrophoresis to determine changes, if any, in the PTM status of the various histones. TAU gel shows the presence of linker histone H1. Preliminary identification of core histones can be obtained in this gel system because core histones show histone-specific differences in their affinity for triton and thus a histone-specific reduction in gel mobility. Affinity for triton typically increases from low for histone H4, to moderate for H2B, to intermediate or high for H3, and to high or very high for H2A.

TAU gel electrophoresis of total histones purified from HepG2 cells, treated with SB, SAHA or newly synthesized compounds **SRR1** and **SRR2** shows modified forms of histone H3 and H4 and low levels of similar modifications in histone H2A and H2B in comparison to untreated cells (Fig. 6).

Western blotting

To further investigate whether newly synthesized compounds, **SRR1** and **SRR2** inhibits HDAC activity in HepG2 cells, the levels of hyperacetylated histone H3 was

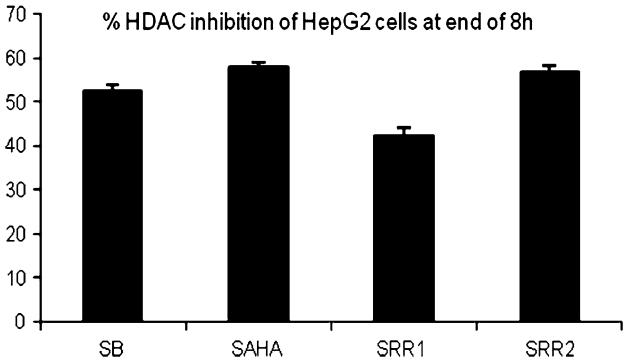


Fig. 5 HDAC activity of test compounds on HepG2 cell line. Eight hours post-treatment with sodium butyrate (SB), SAHA, **SRR1**, and **SRR2** cells were harvested for HDAC assay. SB and SAHA were used as positive control for the experiment. % HDAC activity is expressed on basis of OD at 400 nm. Data represents mean values of at least three separate experiments

studied by western blotting (Dickinson and Fowler, 2002). As shown in Fig. 7, test compounds increase the acetylation of lysine residue in the N-terminal tail of H3 histone.

Experimental

Designing

All computations for designing were carried out on a Pentium 2.67 GHz workstation, 256 MB memory with Windows operating system and Molecular Operating Environment (MOE 2006.08) as computational software. The crystal structure of an archbacterial HDAC homologue (HDAC like protein- HDLP) complexed with SAHA [1c3s] was obtained from Protein Data Bank library of MOE and used for docking studies.

All the designed molecules were built, energy minimized and used as ligands for the docking studies using MOE software. The protein was prepared for docking by addition of hydrogen atoms and energy minimization using AMBER99 forcefield. The active site was generated using atom selector wherein atoms involved in binding the inhibitor, SAHA, to HDLP were taken into consideration. These atoms were assigned as "binding pocket". The inhibitor was then deleted from the crystal structure.

The energy minimized ligands were then docked into the active site of HDLP. Docking was performed using Alpha triangle placement method with London dG scoring based on the free energy of binding of a ligand from a given pose. The alpha triangle placement method generates different ligand orientations by superposing ligand and receptor triplet points. These are the alpha spheres that represent tight packing of residues in the active site. The London dG scoring method evaluates the binding free energy for the poses generated.

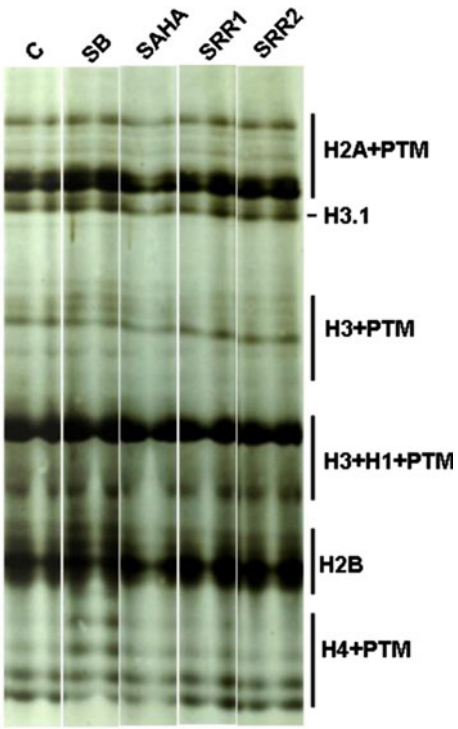


Fig. 6 AUT gel analysis of total nuclear histones extracted from HepG2 cells with or without (control) treatment with sodium butyrate, SAHA, SRR1, SRR2. 5 μg of histones were resolved on TAU PAGE. Gel was stained using ammoniacal silver nitrate staining method. Mobility of different histones are marked in the figure. Charge modified forms are marked as PTM

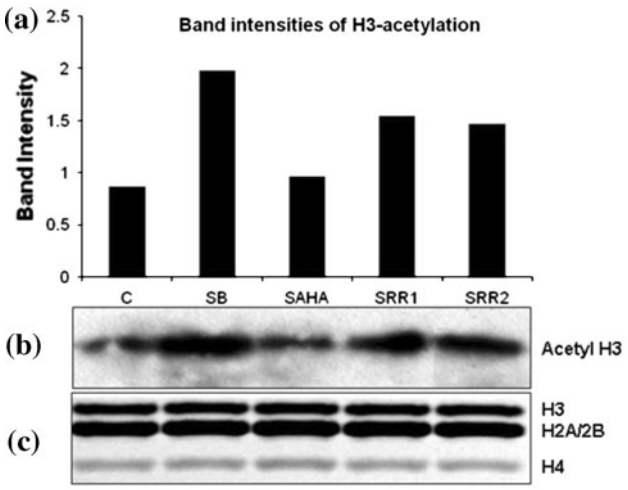


Fig. 7 Western blot analysis for hyperacetylation of histone H3. HepG2 cells were treated with compounds sodium butyrate, SAHA, SRR1, and SRR2 for 8 h. Cells were harvested and histones were acid extracted. **a** Histogram plotted after densitometer scanning, **b** immunoblot with antibody specific for acetylated H3 histones, and **c** silver-stained core histones to confirm the equal loading on the blot

Poses were further filtered using a pharmacophore query. Metal ligation was the most critical parameter that determined the inhibitory effect of designed molecule. It

was therefore decided to give prominence to this feature. Hydrophobicity of the linker chain was also included in the pharmacophore query. The entire system was further energy minimized.

The final models were evaluated for binding orientations using scoring svl functionality available in MOE which is used to visualize intermolecular contacts like direct hydrogen bonds, water-mediated hydrogen bonds, transition metal interactions, and hydrophobic interactions (Joshi *et al.*, 2006). It also computes predicted p*K*i of the ligand. Predicted p*K*i is an estimate of the affinity of a ligand for a specific receptor.

Chemistry

IR spectra were recorded as KBr discs, using Shimadzu 8400S FTIR spectrophotometer. ^{1}H NMR spectrums and ^{13}C NMR spectrums were recorded on Varian Mercury Plus spectrometer at 300 and 75 MHz, respectively. The synthesized compounds were dissolved in deuterated chloroform, $CDCl_3$, for this purpose and trimethylsilane (TMS) was used as an internal standard. Chemical shift values have been reported in ppm (δ). The elemental analysis (C, H, N, S, O) were performed using the Thermo Fisher FLASH EA 1112 analyzer and were within $\pm$0.4% of theoretical values. All reactions as well as column chromatography were followed by TLC using Merck pre-coated silica gel 60 F_{254} plates and spots were visualized by observing in UV cabinet under short UV. All reagents were used as received unless otherwise stated.

Synthesis of 3-(6-bromohexyl)-2,4-thiazolidinedione

36.6 g (22.8 ml, 0.15 mol) of 1,6-dibromohexane, 5.9 g (0.063 mol) of 2,4 thiazolidinedione, and 7.1 g of TEA (9.8 ml, 0.07 mol) were added to 125 ml of acetonitrile (ACN) and mixture was heated with stirring under reflux for 8 h. After cooling to room temperature (RT), solvent was evaporated under reduced pressure and the residue was poured into water. Mixture was extracted with DCM–water and organic layer was washed with saturated brine and dried over sodium sulfate. After solvent was evaporated under reduced pressure, residue was purified by column chromatography using chloroform as eluent to obtain title compound as pale yellow oil. This reaction gave a good yield of 92% of the title compound.

Synthesis of 1-[N-(6-(2,4-dioxothiazolidin-3-yl)hexyl)]-1-azonia-3,5,7-triazatricyclo decane bromide

To a solution of 1 g hexamethylene tetramine/hexamine (0.0072 mol) in 15 ml $CHCl_3$, was added 2 g of 3-(6-bromohexyl)-2,4-thiazolidinedione (0.0072 mol) obtained in the previous step. This mixture was heated under reflux for 4 h, after which reaction mixture was cooled and filtered under vacuum to give 1-[*N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)]-1-azonia-3,5,7-triazatricyclo decane bromide as a white precipitate. The yield was found to be 74%.

Synthesis of [N-(6-(2,4-dioxothiazolidin-3-yl)hexyl)] ammonium chloride

In a flask were mixed 4 ml of 95% ethanol and 0.7 ml conc. HCl. To the still warm solution was added 0.7 g (0.0023 mol) of 1-[*N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)]-1-azonia-3,5,7-triazatricyclo decane bromide in one portion. The reaction mixture was allowed to stir at RT for 18 h, cooled to 0°C and filtered to remove the precipitated ammonium chloride. Filtrate was concentrated on rotary evaporator to obtain the product as a yellow solid which was immediately taken to the next step, i.e., benzoylation.

*Synthesis of N-(6-(2,4-dioxothiazolidin-3-yl) hexyl)benzamide (**SRR1**)*

[*N*-(6-(2,4-Dioxothiazolidin-3-yl)hexyl)] ammonium chloride obtained in the previous step (0.0016 mol, 0.3 g) was dissolved in dichloromethane (10 ml). To this was added 0.6 ml (0.00417 mol) of TEA. This mixture was cooled to 2–5°C and then 0.2 ml (0.0016 mol) of benzoyl chloride was added dropwise. This mixture was stirred at RT for one and a half hours.

At the end of this time period, the mixture was given DCM-water (3 × 30 ml) washings. The organic layer was then subjected to dilute hydrochloric acid (HCl) washing to remove excess of TEA. The organic layer was again washed with water and dried over anhydrous sodium sulfate. This was then concentrated. The residue was given ether washings (2 × 20 ml). The precipitate was collected and subjected to column chromatography using chloroform: ethyl acetate (3:2) as the eluent.

Yield: 47%; R_f: 0.71 (4:1, chloroform/ethyl acetate); IR (KBr, cm^{-1}): 3311.55 (NH stretching vibration of amide), 1751.24 and 1668.31 (C=O stretching vibrations of cyclic imides), 1633.59 (C=O stretching vibration of amide), 1257.50 (C–N stretching vibration of amide); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.5 (m, 8H, aliphatic protons), 3.4 (q, 2H, –CH_2–NH), 3.6 (t, 2H, –CH_2 adjacent to thiazolidinedione ring), 4.0 (s, 2H, thiazolidinedione ring protons), 6.3 (bs, 1H, NH), 7.7 (m, 5H, aromatic protons). ^{13}C NMR: (75 MHz, $CDCl_3$) δ (ppm): 26.148, 26.239, 27.392, 29.415, 33.765, 39.763, 41.796, 126.899, 128.528, 131.330, 134.759, 167.554 (C=O), 171.560 (C=O), 171.954 (C=O); Anal. Calc. for $C_{16}H_{20}N_2O_3S$, %: C, 59.98; H, 6.29; N, 8.74; S, 10.01; O, 14.98. Found, %: C, 59.76; H, 6.32; N, 8.72; S, 9.87; O, 15.21.

*Synthesis of N-(6-(2,4-dioxothiazolidin-3-yl) hexyl)benzenesulfonamide (**SRR2**)*

The procedure followed for the synthesis of *N*-(6-(2,4-dioxothiazolidin-3-yl)hexyl)benzenesulfonamide was the same as described for synthesis of **SRR1**, wherein the benzoyl chloride was substituted with benzene sulfonyl chloride (0.0016 mol).

Yield: 43%; R_f: 0.68 (4:1, chloroform/ethyl acetate); IR (KBr, cm^{-1}): 3288.40 (NH stretching vibration of sulfonamide), 1751.24 and 1664.10 (C=O stretching vibrations of cyclic imides), 1325.01 and 1159.14 (C=O stretching vibrations of sulfonamide); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.5 (m, 8H, aliphatic protons), 2.9 (q, 2H, $–CH_2–$NH), 3.6 (t, 2H, $–CH_2$ adjacent to thiazolidinedione ring), 4.0 (s, 2H, thiazolidinedione ring protons), 5.0 (t, 1H, NH), 7.8 (m, 5H, aromatic protons). ^{13}C NMR: (75 MHz, $CDCl_3$) δ (ppm): 25.834, 25.986, 27.331, 29.354, 33.795, 39.763, 41.786, 43.000, 127.071, 129.155, 132.645, 140.050, 171.590 (C=O), 171.924 (C=O); Anal. Calc. for $C_{15}H_{20}N_2O_4S_2$, %: C, 50.54; H, 5.66; N, 7.86; S, 17.99; O, 17.95. Found, %: C, 50.36; H, 5.24; N, 8.14; S, 17.65; O, 18.13.

Biological evaluation

Materials and methods

The HDAC inhibitor, SB and the tetrazolium salt, MTT were procured from Sigma-Aldrich. Compounds **SRR1** and **SRR3** were synthesized in our laboratory with purities exceeding 99%, as is evident from NMR. For in vitro studies, stock solutions of inhibitors were prepared in dimethyl sulfoxide (DMSO) and stored frozen prior to use. At the time of compound addition, an aliquot of frozen concentrate was thawed and diluted to the desired final test concentration in 10% serum-containing medium for treatment of cells (final concentration of DMSO, <0.1%). The transformed liver cancer cell line HepG2 and untransformed normal embryonic liver cell line WRL68 were purchased from National Centre for Cell Science (NCCS), Pune. Culture media, RPMI 1640 and Dulbecco's Modified Eagle's Medium, DMEM, were obtained from Sigma-Aldrich. HDAC assay kit was purchased from Biovision. PVDF membrane and chemiluminescence kit were purchased from Millipore. Acetylated histone H3 antibody was procured from Abcam.

Antiproliferative testing

HepG2 cells were grown in monolayer cultures in RPMI 1640 medium and WRL68 cells in DMEM medium, both supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics and maintained at 37°C in a 5% CO_2/95% air atmosphere with 95% humidity. For testing, cells were harvested by trypsinization and plated (5,000 cells/well) in 96 well plates and incubated for 24 h at 37°C in the incubator.

Dose-dependent MTT assay

After incubating the plates for 24 h, the cytotoxic effects of synthesized compounds were tested in vitro against the cell lines at tenfold dilutions of four concentrations ranging from 10^{-3} to 10^{-6} M. The percentage growth was evaluated spectrophotometrically versus negative control not treated with test agents and positive control, treated with SB (1 mM), a proven HDAC inhibitor. At the end of 48 h, the medium from each well was removed by aspiration and the cells washed with Dulbecco's phosphate buffered saline (PBS). 20 µl of MTT solution (stock 5 mg/ml) was added to each well and plate was incubated at 37°C for 4 h. The media was then removed from each well and 100 µl of DMSO was added. Plates were then transferred to plate reader and absorbance was measured at 540 nm with reference absorbance at 690 nm (Mosmann, 1983).

Time-dependent MTT assay

For the time-dependent assay, the growth inhibition or cellular proliferation was measured at 48, 72, 96, and 120 h time points. The dose of synthesized molecules selected for this assay was 100 µM as determined from the results obtained for the dose-dependent assay. The procedure followed was the same as reported in dose-dependent assay.

In vitro HDAC assay

HepG2 cells were plated and treated with synthesized compounds (100 µM), SB (1 mM) and SAHA (250 nm) for different time periods ranging from 2 to 24 h. HDAC activity was assayed using 10 µg of nuclear extract prepared from control and drug-treated samples. Commercially available colorimetric HDAC assay kit (Biovision K331-100) was used to determine the HDAC inhibitory potential of synthesized compounds. The HDAC assay was carried out in accordance to the instructions provided by the manufacturer along with the kit. This method required two steps, both performed on the same microtiter plate. First, the HDAC colorimetric substrate, which comprises of an acetylated lysine side chain, was incubated with nuclear extract. Deacetylation of the substrate sensitized the substrate, so that, in the second step, treatment with lysine developer produced a chromophore. The chromophore was analysed using an ELISA plate reader at 400 nm.

Hyperacetylation of histones

TAU PAGE HepG2 cell line cells were plated and treated with test compounds, **SRR1** and **SRR2** (100 μM), SB (1 mM), and SAHA (250 nM). At the end of 8 h incubation, histones were isolated from these cells. Total histones were resolved on TAU PAGE according to method described by Waterborg (Waterborg, 2002). Five microgram histones were loaded in each well of the vertical slab gel (Macrokin, Technosource). Histone samples were electrophoresed at constant voltage of 250 V till methylene blue dye front reached bottom of gel. Amido black-silver staining was carried out as described by Mold *et al.* (1983). After electrophoresis, gel was immersed in 10 volumes of 1% amido black in 40% methanol and 7% acetic acid for 3 h at room temperature on shaker. Gel was destained overnight in excess of 32% ethanol and 8% acetic acid. After destaining, gel was stained by ammoniacal silver method (Khare *et al.*, 2009).

Western blotting HepG2 cell line cells were cultured and treated with test compounds, **SRR1** and **SRR2** (100 μM), SB (1 mM) and SAHA (250 nM) for 8 h. After treatment, cells were cultured and harvested by centrifugation. Histones were extracted and isolated histones were subjected to 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Membrane was immunoblotted with antibodies against acetylated H3 histones. Acetylated histones were determined using chemiluminescence kit (Dickinson and Fowler, 2002).

Conclusion

In summary, we have successfully performed docking studies of molecules with alternate zinc-binding heterocyclic groups to determine their effectiveness as HDAC inhibitors. Based on their scoring, two novel compounds containing 2,4-thiazolidinedione as enzyme inhibiting group were synthesized and subjected to antiproliferative and HDAC inhibition studies on liver cancer cell line HepG2. Although both the compounds showed good cytotoxicity and HDAC inhibition as confirmed by H3 acetylation status, compound **SRR2** proved to be the better candidate for future consideration on account of relative selectivity toward transformed cells. Thus, the experimental validation of 2,4-thiazolidinedione as a hitherto unexplored enzyme inhibiting group for HDAC will advance further efforts for lead optimization by chemical derivatization of newly synthesized active compound **SRR2**.

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Conflict of interest Authors have no conflict of interest regarding the work reported in this manuscript.

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