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Discovery of a small molecular compound simultaneously targeting RXR and HADC: Design, synthesis, molecular docking and bioassay

Guo-Liang Chen [a,†], Li-Hui Wang [b,c,†], Jian Wang [a], Kang Chen [b,c], Man Zhao [a], Zhao-Zhu Sun [a], Shuang Wang [b,c], Hong-Li Zheng [b,c], Jing-Yu Yang [b,c,*], Chun-Fu Wu [b,c,*]

[a] Key Laboratory of Structure-Based Drugs Design & Discovery of Ministry of Education, Shenyang Pharmaceutical University, 103 Wenhua Road, 110016 Shenyang, PR China
[b] Department of Pharmacology, Shenyang Pharmaceutical University, 103 Wenhua Road, 110016 Shenyang, PR China
[c] Benxi Institute of Pharmaceutical Research, Shenyang Pharmaceutical University, 103 Wenhua Road, 110016 Shenyang, PR China

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ABSTRACT

Retinoid X receptor (RXR) and Histone deacetylase (HDAC) are considered important targets for anti-cancer therapy due to their crucial roles in genetic or epigenetic regulations of cancer development and progression. Here, we have designed and synthesized a novel compound which targets both RXR and HADC. This dual-targeting agent is derived from bexarotene and suberoylanilide hydroxamic acid (SAHA), prototypical RXR agonist and HDAC inhibitor, respectively. Molecular docking studies demonstrate that this agent has a relatively strong affinity to RXR and HADC. Importantly, it presents the potentials of activation of RXR and inhibition of HDAC in both cell-free and whole-cell assays, and displays anti-proliferative effect on representative cancer cell lines and drug-resistant cancer cell lines.

Mechanism-based targeted therapies as a treatment for human cancers has been heralded as one of the major breakthroughs in human cancer research in the past three decades. However, the clinical effectiveness of such treatment is generally unsustainable in the long run and relapses are almost inevitable after the treatment.[1] One explanation is that the core hallmark capabilities of cancer are regulated by partially redundant signaling molecules. As such, a targeted therapeutic agent inhibiting only one key molecule in a tumor may not completely shut off the core hallmark capability, allowing some cancer cells to survive with residual function.[1] To address this problem, the next-generation of anticancer medicine will have to incorporate, within a single molecule, elements that simultaneously tackle multiple targets for cancer therapy.[2]

Retinoid X receptor (RXR) is a promising target for anti-cancer therapy. RXR belongs to a member of the nuclear receptor superfamily that regulates many physiological functions, including cell growth, differentiation and apoptosis.[3] RXR act as ligand-dependent transcription factor that functions as heterodimer with retinoid acid receprtor (RAR) to modulate the transcriptional activity of retinoid receptor target genes associated with cell growth and differentiation. Recently, RXR agonist has been identified as an important agent for cancer treatment.[3] A synthetic RXR agonist bexarotene has been approved by FDA for the treatment of cutaneous T-cell lymphoma (CTCL).[4] In addition, several clinical trials are ongoing to assess the potential of bexarotene for treatment of other tumors. However, a large number of the known RXR agonists have elicited only limited in vivo antitumor activities and have not progressed beyond clinical trials.[5,6] The RXR agonists that modulate the functions of additional intracellular targets, other than the RXR, may be able to ameliorate the shortcomings of current RXR agonists.

Histone deacetylase (HDAC), which deacetylate lysines on core histones and other cellular proteins,[7] plays the crucial roles in the epigenetic regulation of gene transcription and controlling other cellular functions, particularly on cancer.[8] HDAC inhibitors have been demonstrated to induce cell-cycle arrest, terminal differentiation and apoptosis in a broad spectrum of human tumor cell lines in vitro[8], and have antiangiogenic and antitumor activity in human xenograft models.[8,9] Two HDAC inhibitors, SAHA (vorinostat) and FK228 (romidepsin), have been approved by FDA for the treatment of CTCL. Preclinical data with numerous cancer cell lines has shown synergistic and additive effects when combining HDAC inhibitors with various antitumor therapies, suggesting HDAC might be an ideal target for combination.[10]

In theory, hyperacetylation of histone proteins induced by HDAC inhibitors could increase the accessibility of DNA within chromatin, loosen gene promoter and consequently potentiate the anticancer activities of RXR agonist. Moreover, recent research

* Corresponding authors. Tel./fax: +86 24 23986339 (C.-F.W.).
E-mail addresses: yangjingyu2006@gmail.com (J.-Y. Yang), wucf@syphu.edu.cn (C.-F. Wu).
† These two authors contributed equally to this work.

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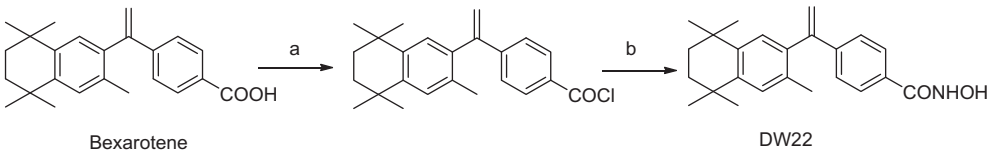


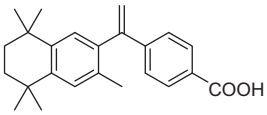
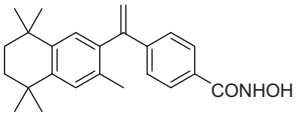
Scheme 1. Reagents and condition: (a) $SOCl_2$, CH_2Cl_2, 89%; (b) $NH_2OH{\cdot}HCL{\cdot}KOH$, MeOH, 92%.

has shown that the combination of RXR agonist and HDAC inhibitor synergistically induced apoptosis and inhibited the invasion of colon cancer cells.[11] More importantly, clinical trials demonstrated that HDAC inhibitor could augment the antitumor effect of RXR agonist in CTCL patients.[12] This evidence suggests that simultaneous RXR activation and HDAC inhibition could be a promising approach in cancer therapy.

Here, we disclosed a small molecular with dual acting RXR activation and HDAC inhibition. In addition, the compound exhibited potent anti-proliferative activity across a broader range of human cancer cell lines.

Hydroxamic acid derivatives are key HADC inhibitors due to their powerful zinc-binding ability. Taking bexarotene as a lead compound, we designed bexarotene hydroxamic acid (DW22) by incorporating the hydroxamic acid moiety into its structure in order to obtain a molecular with dual-target on RXR and HDAC. The preparation of bexarotene hydroxamic acid (DW22) is shown in Scheme 1.[13]

Table 1
The structures and binding free energies of bexarotene and DW22

Compd	Structure	Binding free energy (kcal/mol) HDAC-2(3MAX)	Binding free energy (kcal/mol) RXR-α (3H0A)
Bexarotene		−8.74	−12.93
DW22		−10.47	−13.07

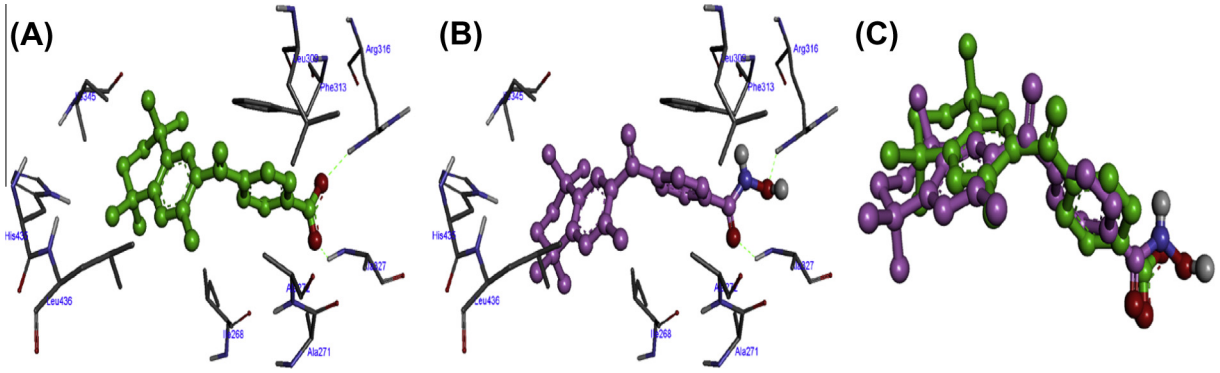


Figure 1. Binding modes of compound bexarotene (A), displayed with ball and stick with green color) and bexarotene_hydroxamic (B), displayed with ball and stick with pink color) with RXR and their bioactive-conformational alignments (C) within the receptor site.

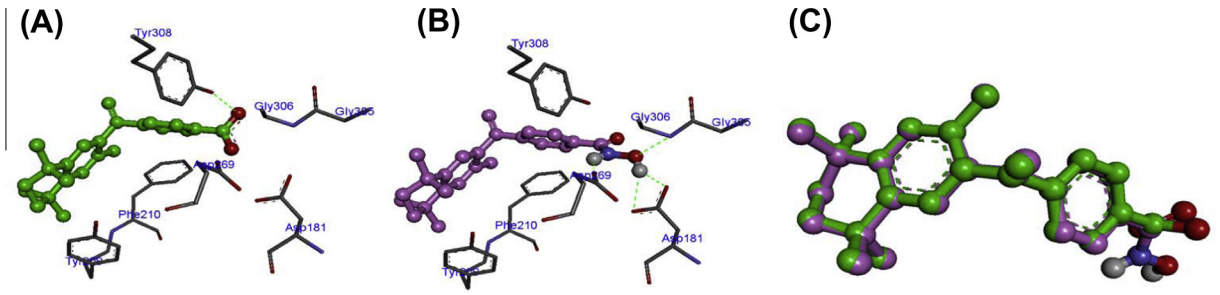


Figure 2. Binding modes of compound bexarotene (A), displayed with ball and stick with green color) and bexarotene_hydroxamic (B), displayed with ball and stick with pink color) with HDAC-2 and their bioactive-conformational alignments (C) within the receptor site.

Molecular docking studies were performed to investigate the binding affinities and interaction modes for the dual-targeting compound using AutoDock 3.05.[14] The ligand structures were built with the Sybyl software package[15] and minimized using the Tripos force field and assigned charges using the Gasteiger–Hückel method. The crystal structures of RXR (PDB code: 3H0A[16]) and HDAC-2 (PDB code: 3MAX[17]) were selected as the receptor. Polar hydrogens were added and partial charges were loaded for protein using the Kollman United Atom charges. Atomic solvation parameters and fragmental volumes for the proteins were assigned using the addsol utility in the program package. A grid map of dimensions 22.5 × 22.5 × 22.5 Å, with a grid spacing of 0.375 Å was generated for falcipain-2. Affinity grid fields were generated using the auxiliary program AutoGrid3.0.

The Lamarckian genetic algorithm (LGA) was used to find the appropriate binding positions, orientations, and conformations of the ligands. The resulting data was taken from docking experiments in which the lowest total docking energy was obtained. Ten LGA runs, each with 300 individuals in the population, were performed. Results differing by less than 2 Å in a positional root mean square deviation (rmsd) were grouped together. In each group, the lowest binding energy configuration with the highest% frequency was selected as the group representative.

The predicted binding free energies for the compounds bexarotene and DW22 were −8.74 and −10.47 kcal/mol for HDAC-2, and −12.93 and −13.07 kcal/mol for RXR, respectively (Table 1). Within the RXR binding site, both bexarotene and DW22 formed hydrogen bonds with Arg 316 and Ala 327, sharing a similar binding mode (Fig. 1). Within the HDAC-2 binding site, bexarotene formed hydrogen bonds with Tyr308, while compound Asp181, Asp269 and Gly306. They also bound with similar poses (Fig. 2).

To further understand the activities of DW22 against RXR-involved transcriptions, we carried out cell-based transactivation assays by cotransfection of RXRα with its specific response element reporter.[18] As shown in Figure 3, DW22 activated RXR reporter with EC_{50} values 19.5 ± 3.6 nmol/L, which is lower than that of bexarotene (EC_{50} = 149.0 ± 2.9 nmol/L). These results demonstrate that DW22 has improved biological activity on RXR activation as compared to bexarotene.

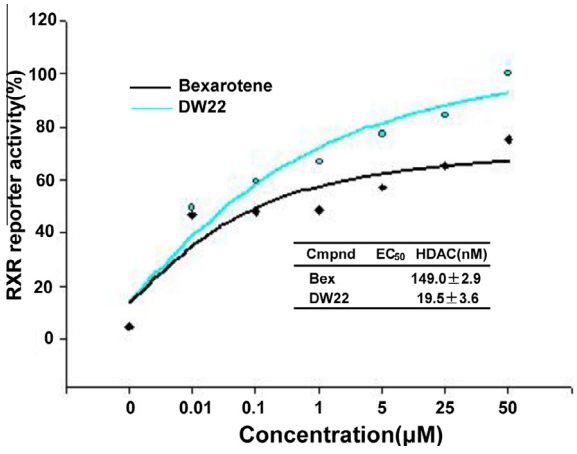


Figure 3. The effects of DW22 and bexarotene on RXR activation.

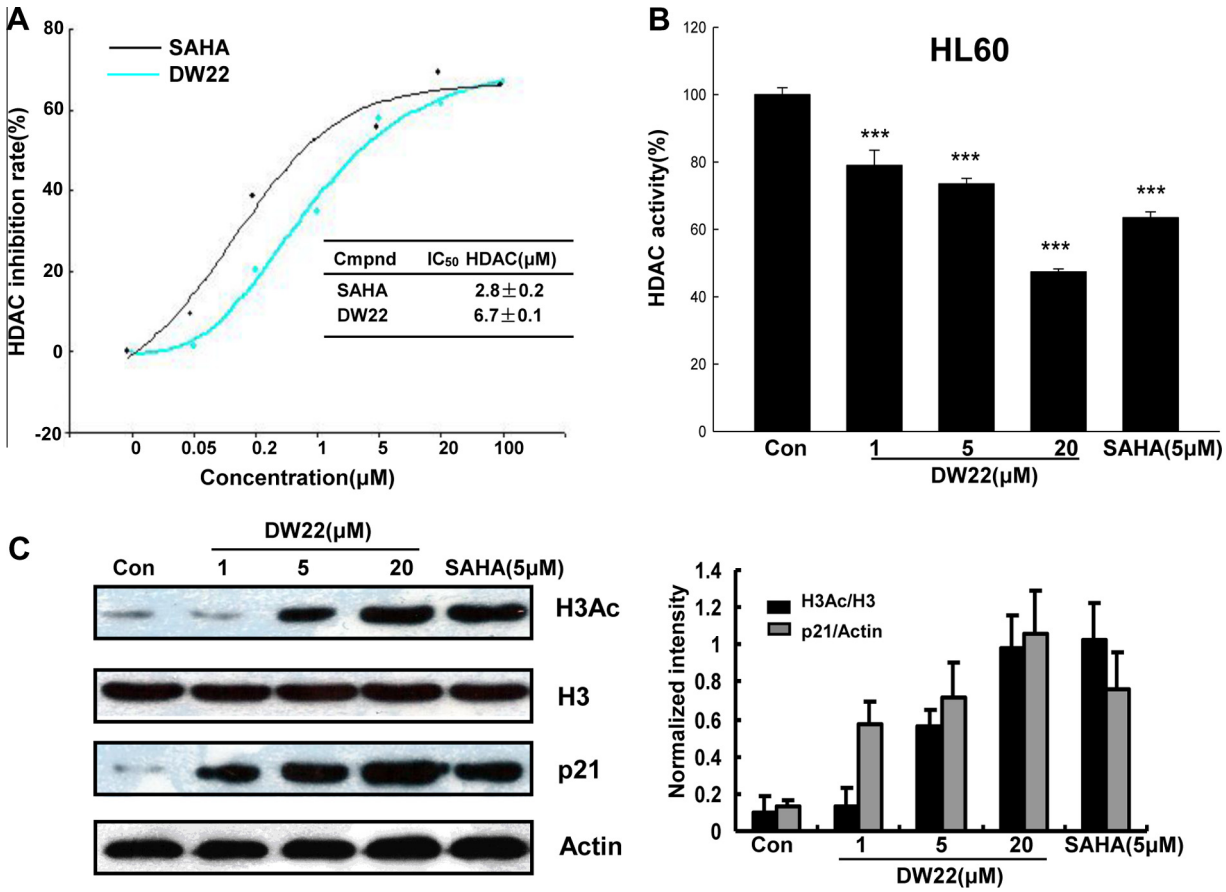


Figure 4. The effects of DW22 and SAHA on HDAC inhibition in cell free assay (A), HL60 cell assay (B), acetylated H3 and $p21^{WAF1/CIP1}$ (C). Histone 3 and β-actin were used as loading controls, respectively, and protein expression was quantified by densitometry relative to control. Quantification of the effect of exposure to increasing concentrations of DW22 on acetylated H3 and $p21^{WAF1/CIP1}$ expression is shown column diagram.

We subsequently tested the HDAC inhibition activity of DW22 against crude HeLa cell nuclear extract HDACs using a cell free assay.[19] DW22 showed a significant inhibition against HeLa cell nuclear extract HDACs (IC_{50} = 6.7 ± 0.1 μmol/L), which are comparable to that of the standard SAHA (IC_{50} = 2.8 ± 0.2 μmol/L) (Fig. 4**A**). More importantly, DW22 also exhibited HDAC inhibitory activity in a concentration-dependent manner in HL60 cells.[20] In the same concentration (5 μmol/L), DW22 displayed the similar ability to inhibit HDAC as compared with SAHA in HL60 cells (Fig. 4B).

In order to verify the HDAC inhibition activity of DW22, the effect of DW22 on the level of acetylated histone protein in the HL60 cells was examined by Western blot analysis using the specific antibodies against acetylated H3.[21] DW22 treatment significantly increased the levels of acetylated H3 in the HL60 cells (Fig. 4C) in a concentration-dependent manner. Recent studies have demonstrated that the HDAC inhibitor-mediated induction of $p21^{WAF1/CIP1}$ is the result of increased H3 acetylation associated with the $p21^{WAF1/CIP1}$ gene promoter[22]. Therefore, we further measured the levels of $p21^{WAF1/CIP1}$ expression to confirm the effect of DW22 on the HDAC. The result showed that DW22 could increase the expression of $p21^{WAF1/CIP1}$ in a concentration-dependent manner (Fig. 4C). These results suggest that DW22 might be an inhibitor of HDAC.

Further evaluation of their anti-proliferative activities was carried out in a broader range of human cancer cell lines representing gastric (HGC-27), gallbladder (GBC-SD), prostate (DU-145) and liver (Hep-3B) cancer by MTT assay with bexarotene and SAHA as positive control (Table 2).[23] The positive control compounds bexarotene and SAHA inhibit the proliferation of these tumor cell lines, respectively. Bifunctional compound DW22 decreased the viability of HGC-27 the most, while they showed the least cytotoxicity against GBC-SD. Although a little bit lower than that seen with SAHA, DW22 displays enhanced cytotoxicity as compared with bexarotene. The similar anti-proliferative activities between DW22 (Mean IC_{50} = 16.1 μmol/L) and SAHA (Mean IC_{50} = 13.2 μmol/L) suggest that HDAC inhibition is the dominating mode of antiproliferative activities of this compound. In addition, to demonstrate the advantage of the bifunctional compound, we also detected the effects of DW22 on bexarotene-resistant human oral squamous carcinoma KB cell line (KB/ bexarotene) and SAHA-resistant human lung cancer A549 cell line (A549/SAHA).[23] Our data indicated that DW22 inhibited KB/bexarotene and A549/SAHA cell growth with IC_{50} of 16.8 ± 2.4 and 3.8 ± 1.3 μmol/L, respectively. Compared with bexarotene with IC_{50} of 46.3 ± 0.6 μmol/L for KB/bexarotene cell and SAHA with IC_{50} of 80.3 ± 5.2 μmol/L for A549/SAHA cell(Table 3), the bifunctional compound has significant activity against bexarotene-resistant and SAHA-resistant cancer cell growth.

Table 2
Anti-proliferative activities of DW22 on human cancer cell lines

Compd	IC_{50}(μM)				
	HGC-27	GBC-SD	DU-145	Hep-3B	Mean
DW22	8.6 ± 1.2	24.1 ± 0.5	14.1 ± 1.3	17.4 ± 0.9	16.1
SAHA	5.4 ± 1.1	14.8 ± 1.2	27.2 ± 0.3	5.4 ± 0.2	13.2
Bexarotene	32.7 ± 0.5	73.3 ± 1.6	61.6 ± 2.6	191.8 ± 3.5	89.9

Table 3
Anti-proliferative activities of DW22 on bexarotene-resistant and SAHA-resistant cell lines.

Compd	IC_{50}(μM)			
	KB	KB/bexarotene	A549	A549/SAHA
DW22	6.1 ± 0.2	16.8 ± 2.4	9.4 ± 1.5	3.8 ± 1.3
SAHA	—	—	17.9 ± 0.2	80.3 ± 5.2
Bexarotene	8.8 ± 1.1	46.3 ± 0.6	—	—

In conclusion, we designed and synthesized a bifunctional compound DW22 which displayed an enhanced binding ability to both RXRα and HDAC-2 in molecular docking analysis. In addition, DW22 exhibited an improved biological activity on RXR activation than bexarotene, and as similar biological activity on HDAC inhibition as SAHA. On the other hand, DW22 showed a strong anti-tumor effect in several solid cancer cell lines and drug-resistant cancer cell lines. Thus, DW22 can be considered as an appropriate lead bifunctional compound to developing more potent anti-tumor agent for cancer therapy.

Acknowledgements

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13. The synthesis of DW22 was carried out in a straightforward two-step synthesis from 4-(1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)vinyl)benzoic acid (bexarotene). Sequential treatment of the prepared bexarotene with refluxing thionyl chloride and dry dichloromethane (CH_2Cl_2) to afford the intermediate chloride in 89% yield, which was converted to DW22 by a freshly prepared hydroxylamine in 92% yield, DW22: white solid, total yield 82%. ^{1}H NMR (DMSO-d_6, 300 MHz): δ(ppm) 11.17 (1H, s, CONHO*H*), 8.99 (1H, s, CON*H*OH), 7.71 (2H, d, *J* = 8.4 Hz, –CH=CH–), 7.29 (2H, d, *J* = 8.4 Hz, –CH=CH–), 7.14 (1H, s, Ar-H), 7.06 (1H, s, Ar-H), 5.88 (1H, s, =CH_2), 5.22 (1H, s, =CH_2), 1.91 (3H, s, Ar-CH_3), 1.66 (4H, s, CH_2), 1.25 (12H, d, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.79, 148.03, 143.58, 142.62, 141.70, 137.80, 131.95, 131.72, 127.72, 127.14, 127.03, 125.92, 116.43, 34.53, 33.53, 33.39, 31.55, 31.52, 19.37. MS (ESI) *m*/*z* 364 $[M+H]^+$, 362 $[M-H]^-$.
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18. The 293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. The cells were cotransfected with CRBPII-tk-luc (a gift from makishima.makoto, Nihon University School of Medicine, JAPAN), pCMX–hRXR and pRL Renilla Luciferase Reporter Vector (Promega Corporation). The

ratio of these three plasmids is 10:3:2. 5 h after transfection, the cells were treated with different concentrations of DW22 and bexarotene. After 48 h transfection, the RXR activity was detected with Dual-Luciferase Reporter Assay System (Promega Corporation). RXR activity is presented as the means ± SD of three determinants.

19. The HeLa nuclear protein concentration was measured by BCA protein assay (Beyontime, CHN). The in vitro HDAC assay was performed with an HDAC fluorescent activity assay kit (Biovison, USA). Briefly, proteins were incubated with different concentrations of DW22 and SAHA (Sigma, USA) at 37 °C for 30 min in the presence of an HDAC fluorimetric substrate. The HDAC assay developer (which produces a fluorophore in reaction mixture) was added, and the fluorescence was measured using a microplate reader (Molecular Devices). HDAC activity is presented as the means ± SD of three determinants.
20. The HL60 cell was treated with different concentrations DW22 and SAHA for 24 h before assays. Proteins were isolated by using cell lysis buffer (Beyontime, CHN). The protein concentration was measured by BCA protein assay (Beyontime, CHN). The HDAC activity was measured with a HDAC fluorescent activity assay kit (Biovison, USA). In brief, cell lysates were incubated with the HDAC substrate Boc-Lys(Ac)-pNA (10 mM) in HDAC assay buffer. After 90 min at 37 °C, reactions were stopped by adding 10 μL of Lysine Developer and further incubated for 30 min at 37 °C. Absorbance was measured using a microplate reader (Molecular Devices). HDAC activity is presented as the means ± SD of three determinants.
21. About 1×10^7 HL60 cells were gathered after pre-treatment DW22 and SAHA for 48 h. Nuclear protein was prepared by a commercial kit (Thermo Scientific). In brief, an equal amount of total protein extracts from cultured cells were fractionated by 10–15% SDS–PAGE, then electrically transferred onto polyvinylidene difluoride (PVDF) membranes and probed with antibodies against acetylated H3 (Cell Signaling, MA, USA), histone H3 (Cell Signaling), $p21^{WAF1/CIP1}$ (Cell Signaling) or β-actin antibody (Sigma). The bound secondary antibodies on the PVDF membrane were reacted with ECL detection reagents (Thermo Scientific) and exposed to X-ray films.
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23. The anti-proliferative activity of the compounds was tested on a panel of human cancer cell lines. The bexarotene and SAHA-resistant cell sublines were generated by step-wise exposures of the KB cell line and the A549 cell line to increasing concentrations of either bexarotene or SAHA, starting with 2 μM for bexarotene or 5 μM for SAHA. Cells were seeded in 96-well plates at a density of 3000 cell/well and incubated with diluted DW22, SAHA and bexarotene for 48(for drug-resistant cells) or 72 h. The final DMSO concentration in the assay was 0.1%. The final number of cells per well was assessed using the MTT dye (Sigma, M5655) following the manufacturer instructions.