Bioorganic & Medicinal Chemistry Letters 24 (2014) 349–352

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Evaluation of benzoic acid derivatives as sirtuin inhibitors

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ARTICLE INFO

Article history:
Received 4 September 2013
Revised 1 November 2013
Accepted 5 November 2013
Available online 12 November 2013

Keywords:
Sirtuin
Class III HDAC inhibitor
Marine-derived *Streptomyces* sp.
4-Dimethylaminobenzoic acid
4-*tert*-Butylbenzoic acid

ABSTRACT

Employing a genetically modified yeast strain as a screening tool, 4-dimethylaminobenzoic acid (**5**) was isolated from the marine sediment-derived *Streptomyces* sp. CP27-53 as a weak yeast sirtuin (Sir2p) inhibitor. Using this compound as a scaffold, a series of disubstituted benzene derivatives were evaluated to elucidate the structure activity relationships for Sir2p inhibition. The results suggested that 4-alkyl or 4-alkylaminobenzoic acid is the key structure motif for Sir2p inhibitory activity. The most potent Sir2p inhibitor, 4-*tert*-butylbenzoic acid (**20**), among the tested compounds in this study turned out to be a weak but selective SIRT1 inhibitor. The calculated binding free energies between the selected compounds and the catalytic domain of SIRT1 were well correlated to their measured SIRT1 inhibitory activities.

Sirtuins are a group of NAD^+-dependent histone deacetylases (HDACs) that are evolutionally conserved from bacteria to mammals.[1] This group of enzymes regulates the key biological functions including gene silencing and cell cycle by removing acetyl groups from lysine residues in the histones or non-histone substrates. To date, seven human isoforms (SIRT1–7) have been found and categorized as class III HDACs that are different from classical zinc-dependent class I/II/IV HDACs.[2] Recently, SIRT1 and SIRT2 have been considered as molecular targets for cancer chemotherapy. The physiological functions of SIRT1 with regard to tumorigenesis include the negative regulation of the tumor suppressor gene 53,[3] the positive regulation of the oncoprotein B-cell lymphoma 6 protein (BCL6),[4] and induction of the FOXO-1-dependent vascular growth factor-C (VEGF-C).[5] On the other hand, SIRT2 promotes cancer cell proliferation due to the enhanced stability of the Myc oncoproteins.[6] It has been reported that apoptosis caused by p53 accumulation in HeLa cells[7] and granulocytic differentiation in the acute promyelocytic leukemia (APL)[8] can be induced based on down-regulation and inhibition of SIRT2. Though SIRT1 and SIRT2 are also reported to show tumor-suppressing effects[9–11] and to be down-regulated in specific cancer types,[12] the tumor promoting effects listed above strongly suggest SIRT1/2 inhibitors have great potential to be anticancer drugs. Encouraging evidence includes significant cytotoxic properties of the two SIRT1/2 inhibitors, sirtinol (**1**: synthetic[13]) and toxoflavin (**2**: natural product[14]), against the MCF-7 and A549 cancer cell lines, respectively.[15,16] It is interesting that the potent and selective SIRT1 inhibitor, EX-527 (**3**: synthetic[17]), required at least 100 μM to effectively repress MCF-7 cell proliferation.[18] However, the selective SIRT2 inhibitor, AC-93253 (**4**: synthetic), exhibited potent cytotoxic effects (IC_{50} 10–100 nM) against the prostate (DU-145), lung (A549, NCI-H460) and pancreas (MiaPaCa2) cancer cell lines with a great therapeutic window (up to 200-fold).[19]

We have recently reported the HDAC screening method using the genetically modified yeast strain DMY2843 to identify SIRT1/2 inhibitors from marine-sediment derived actinomycetes.[20] The primary screening for the extract library using the yeast assay selected a total of 19 actinomycete strains that would produce yeast sirtuin (Sir2p) inhibitors. A new polyketide tetramic acid derivative designated as streptosetin A with weak Sir2p and SIRT1/2 inhibitory activities has been isolated from one of the active strains, *Streptomyces* sp. CP13-10.[20] Our continued search for sirtuin inhibitors from the remaining active strains led to the identification of 4-dimethylaminobenzoic acid (**5**) produced by the *Streptomyces* sp. CP27-53 strain as a Sir2p inhibitor. In this article, we report the identification of **5** and structure activity relationships (SARs) and SIRT inhibitory activities of benzoic acid derivatives and related analogues of **5**.

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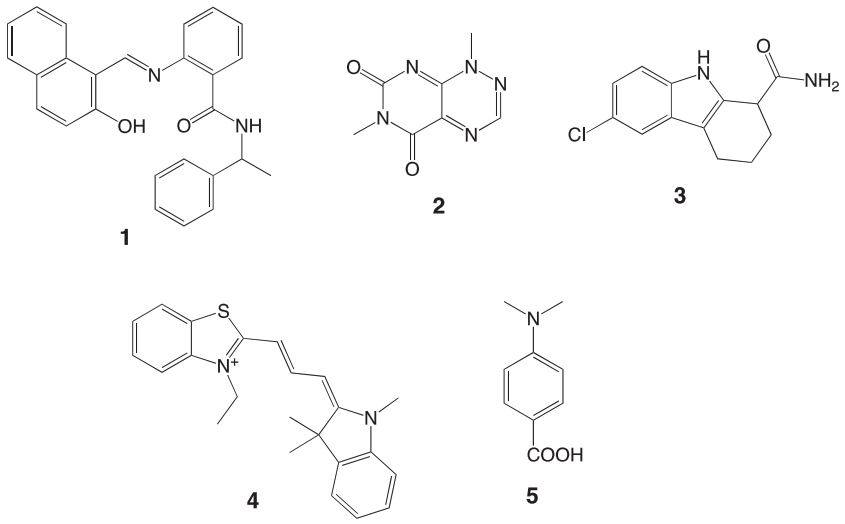
http://dx.doi.org/10.1016/j.bmcl.2013.11.004



The marine sediment-derived *Streptomyces* sp. CP27-53 was cultured in a liquid medium (15 L) containing soluble starch (1%), yeast extract (0.4%), peptone (0.2%), $CaCO_3$ (0.1%) and $FeSO_4{\cdot}7H_2O$ (40 mg) in artificial seawater adjusted to pH 7.4 for 10 days at 30 °C at 200 rpm. The culture was separated to broth and pellet by centrifugation. The broth was treated with HP20 to absorb organic compounds, which were eluted with MeOH, whereas the pellet was extracted with MeOH three times. The combined MeOH extract was cleaned by liquid–liquid partition between EtOAc and H_2O to give an organic extract. Yeast screening of the HPLC peak library created from the organic extract revealed that the compound eluting at 14.3 min in the HPLC chromatogram was responsible for the Sir2p inhibitory activity (Fig. S1). Furthermore, this active compound was purified by reversed-phase HPLC and identified as 4-dimethylaminobenzoic acid (**5**) based on the spectroscopic data (see Supplementary data). The structure was further confirmed by direct comparison of the ^{1}H and ^{13}C NMR data with those of an authentic sample. This compound showed Sir2p inhibitory activity with an MIC of 200 μM after 48 h against the yeast strain.

To elucidate the SARs for Sir2p inhibition by **5**, a series of substituted benzoic acid derivatives and related analogues of **5** were evaluated using the yeast strain DMY2843 (Table 1 and Fig. 1). The compounds in Group A (**6**–**9**) were inactive against the yeast strain, which suggested that the two functional groups, dimethylamino group and carboxylic acid, must be on *para*-positions to show Sir2p inhibitory activity. The MIC values of 400 and 800 μM for compounds **10** and **11** in Group B, respectively, indicated that more methyl groups on the nitrogen enhanced the Sir2p

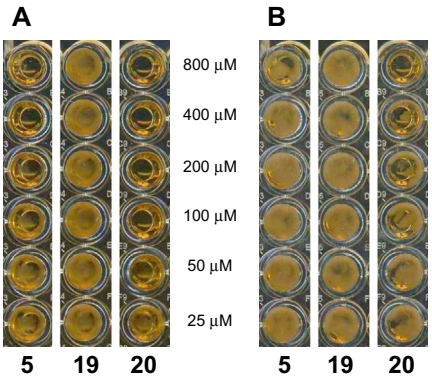


Figure 1. Yeast HDAC screening results (48 h) for **5**, **19** and **20**. (A) YPD media with 5-fluoro (5-FOA) and (B) YPD media. Sir2p inhibition was defined as selective activity when the tested compound resulted in the death of yeast cells (clear) only in the presence of 5-FOA. Cytotoxicity was observed as the death of yeast cells in both media A and B.

Table 1
Minimum inhibition concentration (MIC) values of disubstituted benzene derivatives based on Sir2p inhibition against the yeast strain DMY2844

Structural group		R^1	R^2	Location	MIC (μM)
A	**5**	$(CH_3)_2N$–	–COOH	*p*	200
	6	$(CH_3)_2N$–	–COOH	*m*	NA[a]
	7	$(CH_3)_2N$–	–COOH	*o*	NA
	8	$(CH_3)_2N$–	None		NA
	9	None	–COOH		NA
B	**10**	CH_3NH–	–COOH	*p*	400
	11	NH_2–	–COOH	*p*	800
C	**12**	$(CH_3)_2N$–	$-COOCH_3$	*p*	400
	13	$(CH_3)_2N$–	$-CONH_2$	*p*	NA
	14	$(CH_3)_2N$–	$-SO_3H$	*p*	NA
	15	$(CH_3)_2N$–	–Br	*p*	NA
	16	$(CH_3)_2N$–	$-NO_2$	*p*	NA
D	**17**	CH_3–	–COOH	*p*	800
	18	$(CH_3)_2CH$–	–COOH	*p*	200
E	**19**	$(CH_3)_3N^+$–	$-COO^-$	*p*	NA
	20	$(CH_3)_3C$–	–COOH	*p*	50

[a] Not active at 800 μM.

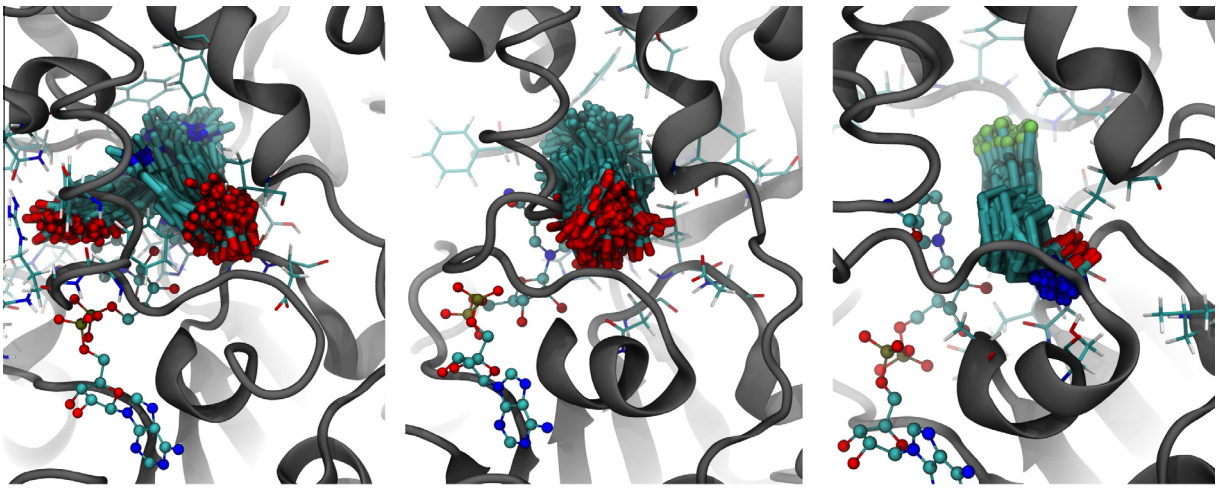
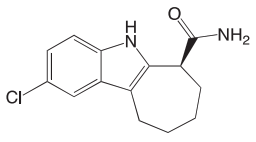


Figure 2. The representative conformational snapshots of compounds **5**, **20** and **21** in the catalytic domain of SIRT1 produced by 0.5 μs explicit solvent MD simulations.

activities. Next, the activity data for Group C including the weaker activity for compound **12** with methyl ester than that of compound **5** and the lack of activity for compounds **13**–**16** indicated that the carboxylic acid appeared to be required for inhibition activity. It is interesting that sulfonic acid **14** was inactive despite the similarity between the functional groups. In Group D, compound **17**, in which the dimethylamino group was replaced by a methyl group, showed diminished activity whereas compound **18**, in which the dimethylamino group was replaced by a similarly sized and shaped isopropyl group, showed similar inhibitory activity to that of compound **5**. The results indicated that the size of the substituent is very important for inhibitory activity. Therefore, we evaluated the two derivatives in Group E, compounds **19** and **20** with trimethylammonium and *tert*-butyl groups as respective substituents. Compound **20** showed the best activity (MIC 50 μM) among the derivatives tested, which supported the importance of the size of the substituent as suggested above. The lack of activity seen in compound **19** suggested that bulky but not charged groups are required for enhanced inhibitory activity for benzoic acid derivatives.

In light of their Sir2p inhibitory activities, compounds **5** and **20** were further evaluated for SIRTs inhibitory activities. Compounds **5** inhibited SIRT1 and SIRT2 with 25.3% and 30.3% at 1.6 mM whereas compound **20** inhibited SIRT1 and SIRT2 with 54.8% and 28.0% at 1.6 mM, respectively. These results suggested that compound **20** was a weak but selective SIRT1 inhibitor (IC_{50} 1.0 mM). Furthermore, the SIRT1 inhibitory activity was enhanced twofold by replacing the dimethylamino group with *tert*-butyl group, which supported the SAR pattern described above.

The crystal structure of the SIRT1 catalytic domain with NAD^+ and an EX-527 analogue [(*S*)-2-chloro-5,6,7,8,9,10-hexahydrocyclohepta[*b*]indole-6-carboxamide (**21**)] has recently been reported.[21] Based on the SIRT1/NAD^+/**21** coordinates, we constructed SIRT1/NAD^+/**5** and SIRT1/NAD^+/**20** complexes to evaluate the binding free energies (ΔG_{bind}) of compounds **5** and **20** relative to compound **21**. The ΔG_{bind} energies were calculated by using the MM-GBSA approach for post processing of explicit solvent molecular dynamics (MD) trajectories. This approach has been proven to provide absolute free energies in good agreement with experimental data.[22–28] The potent and selective SIRT1 inhibitor **21** (IC_{50} 60–100 nM[17]) showed a binding energy of −37.5 kcal/mol whereas compounds **5** and **20** were found to have binding energies of −26.8 and −31.6 kcal/mol, respectively. Conformational ensembles for compounds **5**, **20** and **21** complexes produced by MD are depicted in Figure 2. The more favorable binding energy for compound **21** can be explained by its tighter conformational ensemble observed for this ligand in the binding domain than those of compounds **5** and **20**. For compound **5**, MD simulations produced two distinct conformational families, as clearly seen by the position of the carbonyl group. One conformational family was similar to those of compounds **20** and **21**, in which the polar functional groups positioned towards the pocket entrance. In the second conformational family, compound **5** rotated by approximately 90 degrees. This rotation placed the carbonyl group adjacent to the nicotinamide group of the NAD^+, resulting in slight displacement of the NAD^+ from the active site and thus creating a possible escape route from the binding pocket. This behavior was observed after first 100 ns of simulation, and could also support weaker binding of **5** than **20**. This conformational flexibility resulted in the less favorable binding energy of this ligand as compared to **20** and **21**. The smaller size of compound **20** (as compared to **21**) allowed it more freedom to move inside the binding pocket than compound **21**. Based on this conformational analysis, it is possible to propose that increase in conformational flexibility of the compound in the SIRT1 binding pocket leads to decrease in binding affinity. The results obtained from the MD calculations suggest that bulkier aromatic acid derivatives may be better SIRT1 inhibitors by complementing the size and hydrophobic environment of the enzyme binding pocket, and we plan to test such derivatives in the near future.



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In this study, 4-*tert*-butylbenzoic acid (**20**) was discovered as a new Sir2p inhibitor based on the SAR study on the naturally occurring weak Sir2p inhibitor, 4-dimethylaminobenzoic acid (**5**), isolated from the *Streptomyces* sp. CP27-53. Compound **20** also showed a weak but selective inhibitory activity against SIRT1. It is quite interesting that the structure of **5** was identical to the capping group of the potent class I/II HDAC inhibitor trichostatin A.[29] This study also demonstrated a reasonable correlation between the calculated binding energy and potency of SIRT1 inhibition activity, suggesting that it would be possible to establish a SIRT1 virtual screening method by collecting more data points. The SAR study and MD

calculations implied that the size of the substituent in benzoic acid appears to be important for enhanced activity and we thus plan to evaluate large aromatic acid derivatives to identify superior sirtuin inhibitors.

Acknowledgments

This investigation was supported by the Grants from the National Institutes of Health, SC2GM088057 (T.A.), SC2GM095448 (A.B.G.) and SC1GM095419 (W.W.) and the Beckman Scholarship (J.T.B.). The Cell and Molecular Image Center (CMIC) at the College of Science and Engineering, San Francisco State University was funded by the Grant (P20MD000544) from the National Center on Minority Health and Health Disparities.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.11.004.

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