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Sirtuin inhibition and anti-cancer activities of ethyl 2-benzimidazole-5-carboxylate derivatives†

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New benzimidazoles were synthesized based on the previously identified sirtuin inhibitor BZD9L1. The compounds were screened for their sirtuin (SIRT1, SIRT2 and SIRT3) inhibitory activities. Compound **BZD9Q1** was determined to be a pan-SIRT1–3 inhibitor. Furthermore, the proliferation of various cancer cells was inhibited by **BZD9Q1**. It was shown that **BZD9Q1** elicits a cytostatic effect by inducing cell cycle arrest at the G₂/M phase while also showing a prominent induction of apoptosis against oral cancer cells.

Introduction

Sirtuins are class III histone deacetylases that require NAD⁺ as a co-factor for their deacetylation activities.¹ Among the seven mammalian sirtuins which have been identified (SIRT1–7),² SIRT1–3 are the most studied. SIRT1–3 are believed to be implicated in cancer as their functions are frequently altered in cancer cells. Although the functions of sirtuins in cancer vary under different conditions, they have emerged as exciting targets for cancer therapy.^{3–6} Sirtuins controlled many downstream targets such as NF-κB,⁷ p53 (ref. 8) and FOXO proteins⁹ which are linked to the pathogenesis of cancer development.

We have previously identified benzimidazoles as a novel class of sirtuin inhibitors.^{10–12} In our previous studies, we have highlighted BZD9L1 as a promising anti-cancer agent.^{13,14} Following that, we would now like to report two new benzimidazoles bearing different substitutions at the R position (Fig. 1). The highlights of this study include the potent inhibition of **BZD9Q1** against SIRT1–3 enzymes and its strong antiproliferative activity against oral squamous cell carcinoma (OSCC) H103 cells.

Experimental

Synthesis

All chemicals were supplied by Sigma-Aldrich (USA), Merck Chemicals (Germany) and Acros Organics (USA). Elemental analyses were performed on a Perkin Elmer 2400 Series II CHN Elemental Analyzer and were within ±0.4% of the calculated values. ¹H and ¹³C NMR were performed on a Bruker Avance 300 or 500 spectrometer in either CD₃OD or DMSO-d₆. Mass spectra were recorded on a Varian 320-MS TQ LC/MS spectrometer in positive ESI mode.

Procedure for the preparation of compounds

The benzimidazole derivatives were synthesized according to a previously published protocol¹⁰ with modifications. Briefly, 4-fluoro-3-nitrobenzoic acid (5 g, 27 mmol) was refluxed in ethanol (50 mL) and concentrated H₂SO₄ (2 mL) for 8 hours to yield intermediate 1. To intermediate 1 (1 g, 4.6 mmol) which was dissolved in ethanol (10 mL) was added ammonium hydroxide (2 mL, excess) and the mixture was stirred overnight to yield intermediate 2. Intermediate 2 (1 mmol), ammonium formate (0.378 g, 6 mmol) and Pd/C (50 mg) were mixed in ethanol (10 mL) and the reaction mixture was refluxed for 1 hour to yield intermediate 3. Intermediate 3 and various sodium bisulfite adducts (1.5 mmol) were dissolved in DMF (5 mL). The reaction mixture was stirred at 90 °C under a N₂ atmosphere for 24–48 hours. After completion of the reaction, the reaction mixture was diluted in ethyl

Fig. 1 Compound BZD8A1. The R substitution is highlighted.

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acetate (25 mL) and washed with water (10 mL $\times$ 3). The organic layer was collected, dried over Na₂SO₄ and evaporated under reduced pressure to afford the desired final compounds.

Sirtuin *in vitro* enzymatic fluorescence assay

Sirtuin substrates were derived from human p53 sequences (SIRT1: amino acids 379–382 conjugated to 7-dimethylamino-4-methylcoumarin, AMC; SIRT2: amino acids 317–320 conjugated to 7-dimethylamino-4-methylcoumarin, AMC; SIRT3: amino acids 317–320 conjugated to 7-dimethylamino-4-methylcoumarin, AMC). Sirtuin substrate (125 μ M), NAD⁺ (3 mM), test compounds (50 μ M) and sirtuin human recombinant were incubated for 45 minutes at 37 °C. 50 μ L of stop solution consisting of nicotinamide and sirtuin developer was then added and the mixture was incubated for a further 30 minutes at 37 °C. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission) and the inhibition was calculated as the ratio of absorbance under each experimental condition to that of the control.

Western blot analysis

The H103 OSCC cell line was obtained from the European Collection of Cell Cultures (ECACC). H103 cells were seeded at a density of 5×10^5 in 6-well plates and treated with BZD9Q1 at 32 μ M (for 24 h) and 5.83 μ M (for 72 h). After the treatments, proteins were harvested and resolved by SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were then blocked in 5% w/v skimmed milk with specific primary antibodies overnight at 4 °C and then incubated with secondary antibodies for 1 h at room temperature. Enhanced chemiluminescence detection reagents (Thermo Scientific, MA, USA) were used to visualize the positive reactive protein band by exposure to chemiluminescence light film. The resulting images were quantified using ImageJ. All the antibodies were obtained from Santa Cruz (CA, USA).

Western blots were performed using the following antibodies: monoclonal acetylated α -tubulin (1:1000), monoclonal α -tubulin (1:1000), monoclonal acetylated p53 (1:500), monoclonal p53 (1:1000), and monoclonal β -actin (1:5000). Primary antibodies were detected using horseradish peroxidase linked to anti-mouse (1:5000) and anti-rabbit conjugates as appropriate (1:1000).

Molecular docking

The crystal structure of human SIRT2 (PDB code: 3ZGV) were taken from the Protein Data Bank. The enzyme and ligand were structurally optimized prior to the actual docking simulation. After removing the co-crystallized water molecules, hydrogen atoms were added to the protein structure. Ligands were energy minimized with Chem 3D Pro 13.0 using the MM2 forcefield. Docking was carried out using Autodock 4.2. The top-ranked pose for each ligand was retained and further analyzed using VMD 1.9.1 molecular graphics software.

Cell proliferation assay

CCRF-CEM, HCT-116 and MDA-MB-468 cell lines were obtained from the American Type Culture Collection (Rockville, MD). The H103 OSCC cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were seeded in 96-well plates at a density of 5×10^3 per well. The cells were treated with interested compounds (final concentration <1% DMSO) and allowed to adhere for 72 hours. Then, the proliferative activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to monitor the number of viable cells. After 4 hours of incubation at 37 °C in a humidified 5% CO₂ atmosphere, the medium–MTT solution was carefully removed and DMSO was added to dissolve the purple formazan crystals. The absorbance value was read using iControl software at 570 nm wavelength. All experiments were done in triplicate, and the proliferation rate was calculated as the ratio of absorbance under each experimental condition to that of the control.

Cell cycle analysis

H103 cells (5×10^5 cells per well) were seeded into a 6-well plate and allowed to adhere overnight. They were then treated with the indicated concentrations of BZD9Q1 for 24 h. The treated cells were trypsinized and washed with PBS. The cells were then fixed with ice-cold 70% ethanol for 30 min at 4 °C. The fixed cells were washed and re-suspended in 100 μ L of staining buffer containing 5 μ L of RNase and 15 μ L of 7-aminoactinomycin D (7-AAD) solution. Fluorescence was then detected using an Amnis ImageStream Flow Cytometer at 488 nm. Collected data were subsequently analyzed using the IDEAS 6.2 software (Merck, USA).

Apoptosis assay

Cells were stained with Annexin V and propidium iodide (PI) according to the manufacturer's protocol (FITC Annexin V/PI apoptosis detection kit, BD Biosciences). Briefly, H103 cells (5×10^5 cells per well) were seeded into a 6-well plate and allowed to adhere overnight. The treated cells were then washed twice with ice-cold PBS and re-suspended in 100 μ L binding buffer (0.1 M Hepes/NaOH pH 7.4, 1.4 M NaCl, 25 mM CaCl₂). Then, 5 μ L of Annexin V-FITC and 5 μ L of 100 μ g mL⁻¹ PI solutions were added to the cells and incubated in the dark for 15 min. After incubation, 400 μ L of binding buffer were added to the stained cells before being analyzed using an Amnis ImageStream Flow Cytometer at 488 nm. The collected data were subsequently analyzed using INSPIRE and IDEAS 6.2 software (Merck, USA).

Results and discussion

Synthesis of compounds

The benzimidazole derivatives were synthesized according to the synthetic scheme shown in Fig. 2. The compounds were characterized using NMR, elemental and MS analyses.

Fig. 2 Synthetic scheme for the compounds tested in this study.

Enzymatic assays

The SIRT1, 2 and 3 inhibitory potential of the synthesized compounds was evaluated using commercially available fluorescence assay kits (Cayman Chemicals, Ann Arbor, MI). EX-527 (SIRT1 selective inhibitor), AGK-2 (SIRT2 selective inhibitor) and Tenovin-6 (pan-SIRT1, 2, and 3 inhibitor) were used as standard controls while DMSO was used as a vehicle control. Results showed that **BZD9Q1** and **BZD9V1** are pan-SIRT1–3 inhibitors and are active against all three sirtuins (Table 1). Among the synthesized compounds, **BZD9Q1** was found to be the most potent followed by **BZD9V1**. **BZD9Q1** is equipotent to AGK-2 for SIRT2 but possessed better inhibitory activity than Tenovin-6 for both SIRT2 and SIRT3. Meanwhile, **BZD9D1** has moderate activity against SIRT2 and poor activity against SIRT1 and SIRT3, making it a moderately active SIRT2 selective inhibitor. The other two compounds evaluated in this study, **BZD9H1** and **BZD9K1**, were not active against all three sirtuin enzymes. This is consistent with the previously reported trend where strong electron-withdrawing groups are most likely to generate poor sirtuin inhibitors.¹⁰

Cellular target engagement

Western blots were performed to investigate the cellular target engagement of **BZD9Q1**. The acetylation status of p53 and α -tubulin, which are well-established direct cellular bio-

Table 1 SIRT1, SIRT2 and SIRT3 inhibitory activities of the synthesized compounds

Compound	SIRT1 IC ₅₀ (μ M)	SIRT2 IC ₅₀ (μ M)	SIRT3 IC ₅₀ (μ M)
BZD9Q1	7.7 $\pm$ 1.4	5.6 $\pm$ 1.3	9.8 $\pm$ 2.0
BZD9V1	33.6 $\pm$ 2.8	30.5 $\pm$ 4.4	42.2 $\pm$ 5.0
BZD9D1	>50	45.8 $\pm$ 5.6	>50
BZD9H1	>50	>50	>50
BZD9K1	>50	>50	>50
EX-527	0.30 μ M	N.D.	N.D.
AGK-2	N.D.	8.34 μ M	N.D.
Tenovin-6	42.10 μ M	25.60 μ M	82.65 μ M

N.D., not determined

markers of SIRT1 and SIRT2, respectively, was monitored in H103 cells (Fig. 3A). An increased level of acetylated α -tubulin was observed when the treatment with **BZD9Q1** was prolonged to 72 h (Fig. 3B). However, there is no significant increase in the level of acetylated p53 in treated cells (Fig. 3C). It should be noted that H103 cells are p53 mutant. Tubulin plays an important role in regulating cell division. Hence, the inhibitory effect of **BZD9Q1** on the acetylation of α -tubulin may have attributed to the anti-proliferative effect of sirtuins in cancer cells.

Molecular docking

To predict the interaction between **BZD9Q1** and SIRT2, molecular docking of **BZD9Q1** with SIRT2 enzyme (PDB ID: 3ZGV)¹⁵ was performed using Autodock 4.2 along with AutoDockTools (Scripps Research Institute, La Jolla, CA). The receptor and the drug candidates were optimized before actual docking in Autodock 4.2. Analysis of the top-ranked pose of compound **BZD9Q1** demonstrated several plausible molecular interactions between the ligand and the enzyme.

The groove at the interface between the two globular domains of the enzyme is the binding site for the substrate and co-substrate, acetylated protein and NAD⁺. Compound

Fig. 3 Western blot of α -tubulin and p53 protein expression and their acetylated forms identified in H103 OSCC cells treated with **BZD9Q1**. (A) The images are representative of two independent experiments. The relative ratios of (B) Ac- α -tubulin and (C) Ac-p53 were obtained by normalization first to the internal control (actin), then to the normalized total protein and last to the untreated group. Error bars represent SD ($n = 2$). Bars with asterisk (*) represent significant differences between control and treatment groups ($p < 0.05$).

BZD9Q1 was found to occupy the adenosine binding pocket (A pocket) in the active site. Fig. 4A shows the overlap of **BZD9Q1** on ADPr, indicating that **BZD9Q1** inhibits SIRT2 by preventing the co-factor NAD^+ from binding to the enzyme. The mode of action was corroborated by using competition analysis following a method adopted from Lai *et al.* and performed using the same SIRT2 fluorescence assay kit that was used for enzymatic screening.¹⁶ The inhibition of **BZD9Q1** was tested with increasing concentrations of NAD^+ . The competition analysis clearly showed that **BZD9Q1** competes with NAD^+ to occupy the same binding site in the receptor as increasing the concentration of NAD^+ resulted in a reduction of inhibitory activity ($\text{ESI}^\dagger$ data). The docking analysis reveals that **BZD9Q1** forms strong H-bonds with residues Thr262 and Arg97. Similarly, interaction between the ligand and residues Ser98, Lys287 and Cys324 could be observed. The planar benzimidazole moiety lined the bottom of the A pocket (Fig. 4B).

BZD9Q1 inhibits growth of cancer cells

As sirtuins are closely implicated in cancer progression, the anti-cancer potential of the synthesized compounds was subsequently investigated. The growth inhibitory activities of the synthesized compounds were initially screened against a panel of human cancer cells using the MTT assay after 72 h of incubation at 50 μM concentration (Fig. 5).

Similar to the sirtuin enzyme inhibition trend, **BZD9Q1** has better potency compared to **BZD9V1** against all four cancer cell lines tested (HCT116 – colorectal, CCRF-CEM – leukemia, MDA-MB-468 – breast and H103 – oral), implying the correlation between sirtuin inhibition and antiproliferation of cancer cells. Although SIRT1–3 have a dual role in cancer (tumor promotion and suppression depending on the different conditions), inhibition of SIRT1 and SIRT2 has been largely implicated in the suppression of colorectal^{17,18} and breast^{19,20} cancers as well as leukemia,^{21,22} while inhibition of SIRT3 has been reported to suppress OSCC.²³ In the preliminary screening, **BZD9Q1** was shown to potently inhibit the growth of H103 OSCC. Further studies determined that **BZD9Q1** is equipotent to cisplatin in inhibiting the growth of

Fig. 4 (A). **BZD9Q1** (red) docked into the active site of SIRT2. It was postulated to compete with NAD^+ as it was found to occupy the same binding site as ADPr (orange). (B) Compound **BZD9Q1** docked in human SIRT2 (PDB entry code: 3ZGV).

Fig. 5 Growth inhibitory effect of synthesized compounds against HCT-116, MDA-MB-468, CCRF-CEM and H103 cancer cells after 72 h of incubation. Results derived from triplicate experiments.

H103 cancer cells (**BZD9Q1** GI_{50} = 5.83 μM vs. cisplatin GI_{50} = 5.35 μM). The inhibition of SIRT3 may have played a role in the potent antiproliferative activity of **BZD9Q1** on H103 OSCC. SIRT3 was consistently found overexpressed in several human oral cancer cells²³ and downregulation of SIRT3 in these cells inhibited OSCC cell growth and enhanced drug chemosensitivity and therapy. Although the SIRT3 inhibitor has been shown to inhibit the cell growth and proliferation of OSCC,²⁴ the effect of a SIRT3 inhibitor (or any sirtuin inhibitor) on H103 OSCC specifically has not been reported. Thus, the preliminary results reported here expand the sirtuin–cancer paradigm.

BZD9Q1 affects the cell cycle distribution and induces apoptosis in H103 cells

Regulation of the cell cycle through the G_1/S and G_2/M checkpoints is a crucial event for cell proliferation and the checkpoints are potential targets for many anticancer drugs. Checkpoints that regulate different phases of cell cycle progression help ensure accurate DNA replication and proliferation of eukaryotic cells.²⁵ However, uncontrolled proliferation in cancer is well known as a consequence of genetic mutations that compromise cell cycle checkpoints. Therefore, one of the emerging trends in drug discovery is to seek

Fig. 6 **BZD9Q1** affects the cell cycle distribution. Flow cytometry analysis of 7-AAD stained cellular DNA in H103 cells after 24 h. (A) Vehicle control; (B) **BZD9Q1**.

Fig. 7 BZD9Q1 induces apoptosis in H103 cells. Flow cytometry analysis after 24 h. (A) Vehicle control; (B) cisplatin; (C) BZD9Q1.

therapeutic agents that target cell cycle checkpoints that regulate improper progression of cancers.^{26,27} Cancer chemotherapy and radiotherapy are designed to kill cancer cells mostly by inducing DNA damage. A defect in the G₂/M checkpoint in cancer may allow the damaged cells to enter mitosis but the massive damage eventually triggers apoptosis. An intervention that enhances G₂/M arrest could increase the cytotoxicity of chemotherapy/radiotherapy.²⁸

The mechanisms underlying the reduction of H103 cell viability by BZD9Q1 was investigated by analyzing the cell cycle distribution using flow cytometry with 7-AAD staining. For the control (sans treatment), 38.8% of H103 cells contained a 2 N amount of DNA (G₁ phase) and 36.5% with 4 N of DNA (G₂/M phase), while only 18.0% of cells were synthesizing their DNA (S phase). Treatment with BZD9Q1 significantly decreased H103 cells with the G₁ phase (8.1%), which was accompanied by an increase in the proportion of cells with the G₂/M phase (43.2%). The results thus imply that the compound induced G₂/M arrest in H103 oral cancer cells (Fig. 6).

Apoptosis induction was analyzed using flow cytometry with Annexin V/propidium iodide (PI) staining. Cells treated with cisplatin were analyzed at the same time as the positive control. In this study, we observed a slight decrease of early apoptotic cells after a 24 h treatment with cisplatin and BZD9Q1 (0.52% and 0.04%, respectively) compared to the control group (6.60%). BZD9Q1 induced late apoptosis cell death (7.82%) as compared to control where only 0.42% of control cells were detected to undergo late apoptosis (Fig. 6). The percentage of H103 cells which underwent necrosis also rose significantly compared to the control (11.30% vs. 1.96%). However, the highest levels of late apoptosis and necrosis were observed in cisplatin-treated H103 cells (24.2% and 17.9%, respectively) (Fig. 7). Taken together, these results suggested that the viability of H103 cells was affected by BZD9Q1 through induction of late apoptosis and a mild level of necrosis.

Conclusions

In conclusion, five novel benzimidazole derivatives were synthesized based on a previously reported scaffold. BZD9Q1 demonstrated potent SIRT1–3 inhibitory activity with a good anti-cancer profile especially against H103 oral cancer cells. It was found to arrest H103 cells at the G₂/M checkpoint and induced mainly apoptotic cell death in H103 cancer cells.

Conflicts of interest

The authors hereby declare no conflict of interests.

Note added after first publication

This article replaces the version published on 18th November 2019, which contained errors in Fig. 2.

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