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Synthesis and evaluation of novel benzimidazole derivatives as sirtuin inhibitors with antitumor activities

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ABSTRACT

A total of 15 novel benzimidazole derivatives were designed, synthesized and evaluated for their SIRT1 and SIRT2 inhibitory activity. All compounds showed better inhibition on SIRT2 as compared to SIRT1. Among these, compound **5j** displayed the best inhibitory activity for SIRT1 (IC_{50} = 58.43 μM) as well as for SIRT2 (IC_{50} = 45.12 μM). Cell cytotoxicity assays also showed that compound **5j** possesses good antitumor activity against two different cancer cell lines derived from breast cancer (MCF-7 and MDA-MB-468). A simple structure–activity-relationship (SAR) study of the newly synthesized benzimidazole derivatives was also discussed.

1. Introduction

Reversible protein acetylation is an important process that regulates the function of histones as well as many non-histone proteins. This modification is controlled by histone acetyltransferases and histone deacetylases (HDACs).[1] Sirtuins are class III HDACs which is NAD^+-dependant that catalyze the deacetylation of proteins.[2] They are involved in various cellular functions, such as longevity and metabolism.[3–5] Seven sirtuin members are found in human (SIRT1-7)[6] and among these, SIRT1 and SIRT2 are the most studied.[7,8] SIRT1 and SIRT2 are reportedly associated with diseases such as cancer[9] and neurodegenerative disorders.[10,11] Since sirtuins have been found upregulated in many tumor types, are able to inactivate some tumor suppressor proteins such as p53 at transcriptional and post-translational level and maintain chromosomal stability, the inhibitors of sirtuins have been proposed as potential anti-cancer agents.[12–14] Recently, a SIRT2 specific inhibitor (AGK-2) has also been proposed as a useful agent for protection against alpha-synuclein-induced toxicity in different models of Parkinson's disease.[15] Therefore, potent SIRT1 and SIRT2 modulators could be used as valuable tools to gain insight into the specific cellular functions of their effector proteins.

To date, several classes of sirtuin inhibitors have been identified such as the physiological inhibitor nicotinamide,[16] sirtinol and derivatives,[17,18] splitomycin analogs[19] and tenovins.[20] A large high-throughput screening effort led to the discovery of a series of indole compounds as interesting inhibitors of SIRT1, including one of the most potent compounds known so far, EX-527.[21] Since indole and benzimidazole share some structure similarities, we embark to synthesize and evaluate the potential of utilizing benzimidazoles as sirtuin inhibitors. The pharmacokinetics of benzimidazoles were also well studied, therefore they are a good starting point in developing new drugs. Herein we would like to report a new class of sirtuin inhibitors based on the benzimidazole scaffold. To the best of our knowledge, there has been no report on utilizing benzimidazole or their analogs as sirtuin inhibitors apart from those reported by Sirtris (now GSK) as sirtuin modulators.[22] In addition, anti-proliferative activity of the novel benzimidazole derivatives against two breast tumor cell lines (MCF-7 and MDA-MB-468) were also reported. Additionally, systematic structure–activity relationship (SAR) study was also performed and investigated with the 15 novel compounds.

2. Results and discussion

2.1. Chemistry

The procedure to synthesize benzimidazole derivatives was adopted and modified from previously published literature (Scheme 1).[23,24] Our synthetic study into novel benzimidazoles

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started with 4-fluoro-3-nitro benzoic acid which was esterified in the presence of catalytic sulfuric acid in ethanol by refluxing for 8 h to afford the ethyl ester **1** in 75% yield. Our research work takes into account the basic functional groups that are amendable to pro-drug design and strategy, including the ability to increase lipophilicity. This first step reaction to convert carboxylic acid group to ester group was also an attempt to mask potential undesirable drug properties such as low solubility in lipid membranes and chemical instability.

The ethylbenzoate **1** was then treated with ethanolamine and DIPEA in dry dichloromethane at room temperature yielded ethyl 4-(2-hydroxyethylamino)-3-nitrobenzoate **2**, which was then reduced to the amine **3** using ammonium formate and 10% Pd/C by refluxing for 3 h. The structure of ethyl 4-(2-hydroxyethylamino)-3-aminobenzoate **3** was confirmed by spectroscopic analysis.

Ethyl 4-(2-hydroxyethylamino)-3-aminobenzoate **3** was then refluxed with various substituted bisulfite adducts of aromatic aldehydes **4a**–**l** in DMF overnight to afford benzimidazole derivatives **5a**–**l** in good yields (75–95%).[25] The structure of the novel benzimidazoles were confirmed by spectroscopic analysis.

2.2. Enzymatic assays

The in vitro enzymatic screening assay for SIRT1 and SIRT2 inhibitory activity were performed using Sensolyte® fluorimetric drug discovery kits (AnaSpec, Fremont, CA) according to the manufacturer's protocol. Cambinol was used as standard control while DMSO was used as a vehicle control. IC_{50} values were determined for all compounds which showed over 50% inhibition for either SIRT1 or SIRT2 at 50 μM. Initial in vitro screening on 11 of the compounds (**5a**–**k**) showed that compounds with strong electron donating group such as dimethylamino at R_1 possessed the best sirtuin inhibitory activities. The inhibition activity was however, greatly affected when the substitution was replaced by weaker electron donating groups such as methyl or even hydroxyl and methoxyl groups. To explore the importance of the strong electron donating effect on the sirtuin inhibitory activity, we then proceed to synthesize a compound with two electron donating substituents on the phenyl ring (1,3-dioxole, **5l**). The inhibitory effect was enhanced when another electron donating group was added to the phenyl ring. Although knowing well the potential downside of the carboxylic acid derivatives of the benzimidazoles in terms of bioavailability, we find it is worth investigating the carboxylic acid derivatives of compounds **5j**, **5k** and **5l** (**6j**, **6k** and **6l**, respectively). The compounds were then synthesized and screened for their SIRT1/SIRT2 inhibitory activities as direct comparison to their ester derivatives. The screening results revealed that the carboxylic acid derivatives showed poorer inhibitory activity against both SIRT1 and SIRT2.

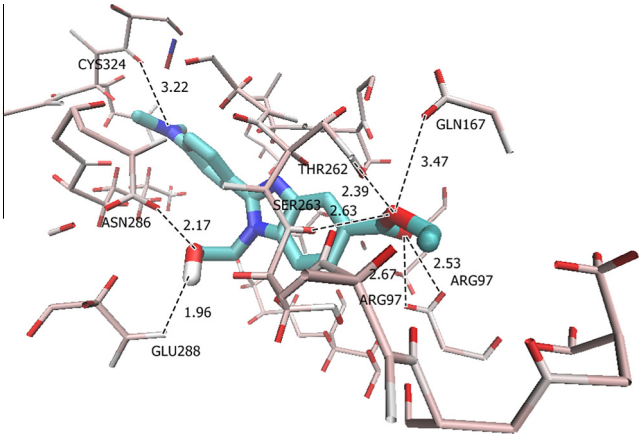


Figure 1. Hydrogen bonds between **5j** and amino acid within the NAD^+ cavity of SIRT2.

The in vitro screening of the total 15 novel benzimidazole derivatives led to the identification of three potent SIRT1/SIRT2 inhibitors (**5j**, **5k** and **5l**) and the results are shown in Table 1. Experiments were performed in triplicates. Standard deviation obtained for all experiments are less than 15%.

Basically, all the novel benzimidazole derivatives showed better inhibition on SIRT2 as compared to SIRT1. The most potent inhibitor for SIRT2 as well as SIRT1 was found to be **5j** (SIRT1 IC_{50} = 58.43 μM; SIRT2 IC_{50} = 45.12 μM). As shown in Table 1, compound **5l** also showed good SIRT2 inhibitory activity but its potency was slightly less that that of compound **5j**. Overall, compound **5j** showed better SIRT2 inhibitory activity compared to the standard control used (cambinol). However, none of the compounds screened in the present derivatives are found to be more potent than cambinol in inhibiting SIRT1.

2.3. Molecular docking

In an attempt to predict the binding mode of this novel chemical series, the most active compound (**5j**) was docked into the active site of human SIRT2. Since the X-ray crystal structure of SIRT2-substrate complex structure has recently been reported,[26]

Table 1
SIRT1 and SIRT2 inhibitory activities of novel benzimidazole derivatives

Compound	SIRT1 inhibition (%) at 50 μM	IC_{50} SIRT1 inhibition (μM)	SIRT2 inhibition (%) at 50 μM	IC_{50} SIRT2 inhibition (μM)
5a	12.73	N.D.	22.73	N.D.
5b	14.72	N.D.	22.49	N.D.
5c	5.32	N.D.	11.60	N.D.
5d	11.74	N.D.	19.23	N.D.
5e	23.22	N.D.	23.46	N.D.
5f	33.77	N.D.	36.29	N.D.
5g	24.94	N.D.	29.03	N.D.
5h	12.65	N.D.	15.32	N.D.
5i	17.79	N.D.	18.29	N.D.
5j	48.70	58.43	68.50	45.12
5k	43.33	74.52	64.19	60.34
5l	39.63	80.11	61.47	56.05
6j	19.65	N.D.	26.78	N.D.
6k	24.00	N.D.	25.70	N.D.
6l	13.97	N.D.	21.99	N.D.
Cambinol	70.77	47.90	68.32	52.89

N.D. = Not determined.

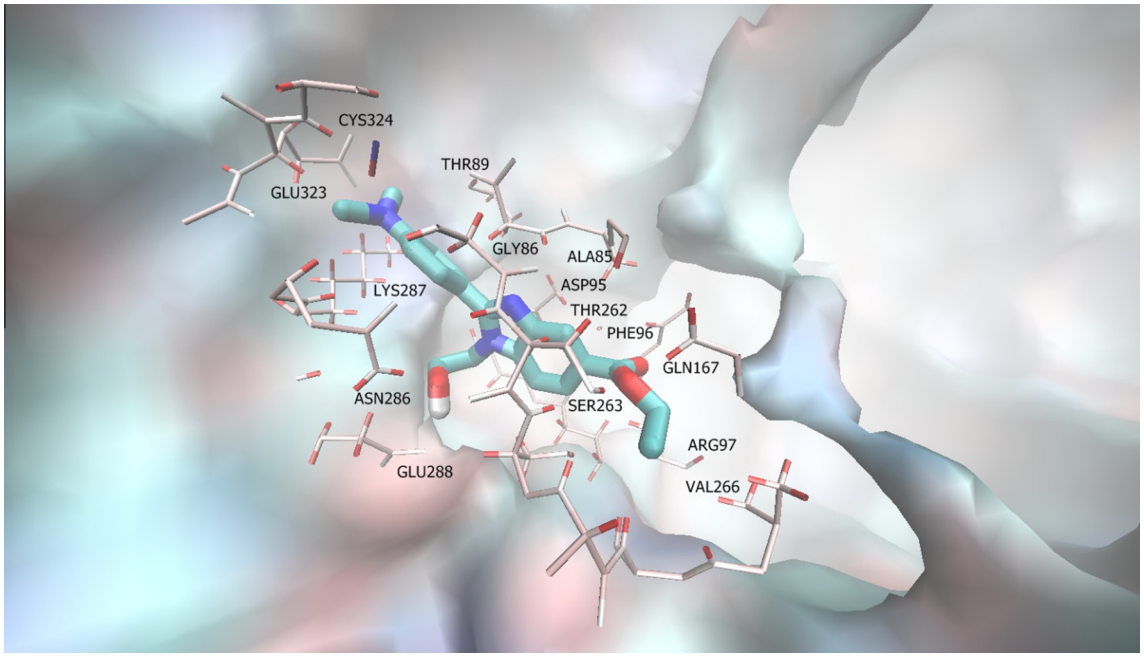


Figure 2. Molecular interactions between **5j** and SIRT2.

we used that X-ray crystal structure in this docking study (PDB entry code: 3ZGV, X-ray resolution = 2.30 Å) instead of the apo-structure. The receptor and the drug candidate were optimized before actual docking in Autodock 4.2 using standard procedure of the software.

Analysis of the top-ranked pose of compound **5j** within the SIRT2-ADPr cofactor binding site demonstrated several plausible molecular interactions. The docking analysis reveals that the compound **5j** interact with receptor primarily due to hydrogen bonding as well as hydrophobic and mild polar interactions. The O–H group of compound **5j** is hydrogen bonded strongly to Glu288. Other hydrogen bonds which could be observe within 3.5 Å include interactions with ASN286, THR262, ARG97, SER263, GLN167 and CYS324 (Fig. 1). This is relatively consistent with the hydrogen bonds observed between SIRT2 and ADPr complex[26] as well as other SIRT2-inhibitor predictions including salermide and NF-675.[27] Hydrogen bonds between H from CYS324 and to a lesser extent H from LYS287 with N from dimethylamino group from the benzimidazole also helped to stabilized the binding complex.

Interactions between lone pair oxygen-π could also be observed through ASP95 and the imidazole ring as well as GLY86 and benzene ring. Apart from these, some hydrophobic and mild polar interactions could be observed between compound **5j** and THR89, ALA85, PHE96, GLU323 and VAL266 (Fig. 2).

As for compound **5k**, which also showed relatively good SIRT2 inhibition, the docking pose showed a different orientation as

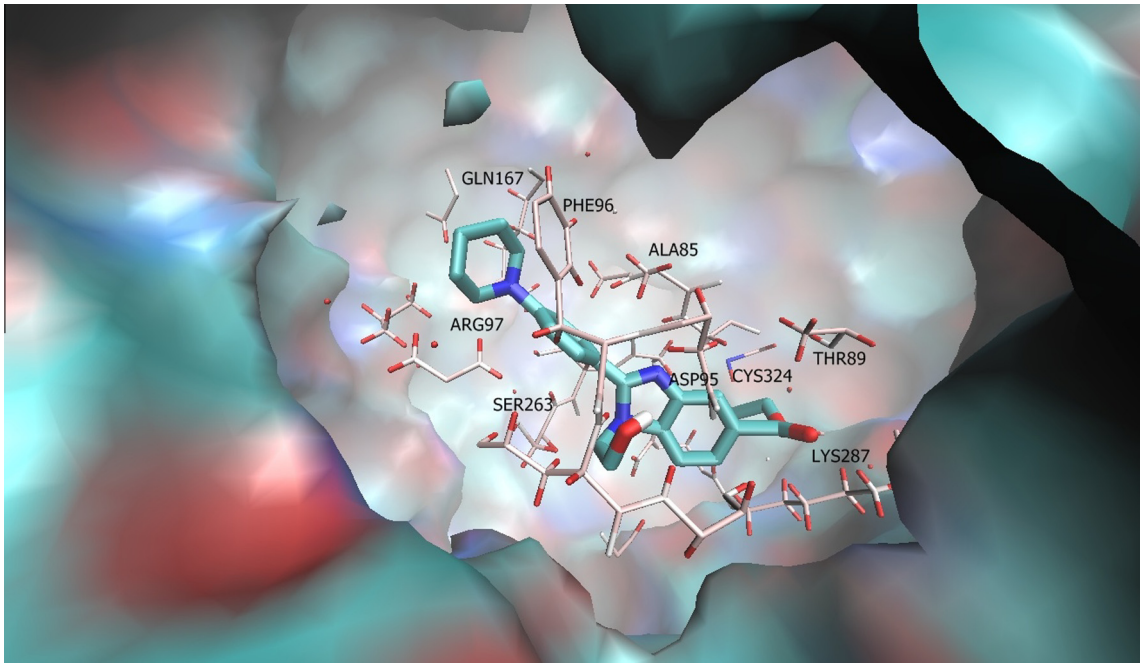
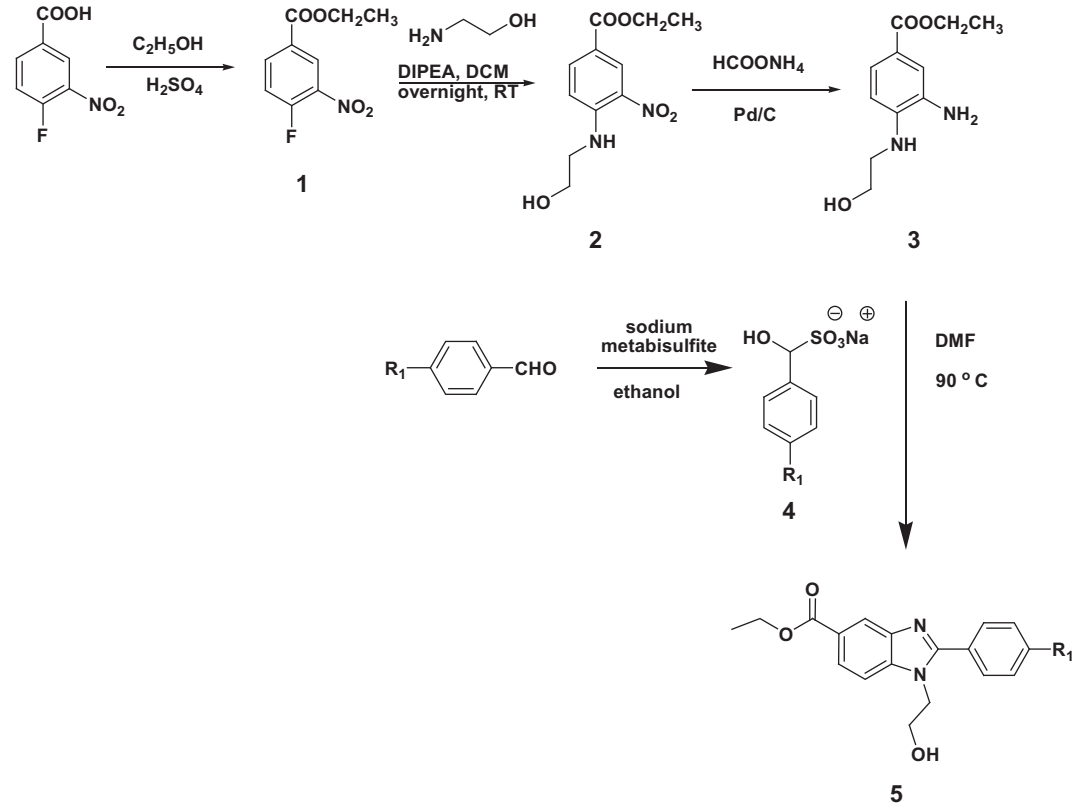


Figure 3. Molecular interactions between **5k** and SIRT2.



R_1: a = H
b = Br
c = Cl
d = OCF_3
e = CF_3
f = NO_2
g = CH_3
h = OH
i = OCH_3
j = dimethylamino
k = piperidine
l = 1,3-dioxole

Scheme 1. Protocol for synthesis of titled compounds.

compared to **5j** (Fig. 3). Docking simulation indicated that the dimethylamino group from compound **5j** was able to form a hydrogen bond to CYS324. However, **5k** has a different binding mode as the bulkier piperidine moiety was unable to fit into the cavity to form hydrogen to CYS324, which was located at the end of the pocket. It was able to form strong hydrogen bonds with ASP95, ARG97 and LYS287.

Weaker hydrogen bonds were also observed between H from GLN167 with N from piperidine moiety as well as THR89 and CYS324 with O the from ester chain. Apart from these, π–π stacking interaction was also indicated between the piperidine ring of **5k** and PHE96.

2.4. Competition analysis

Besides molecular modeling, the binding mode of compound **5j** towards SIRT2 was also analyzed using competition analysis following method adopted from Lai et al.[28] The inhibition of **5j** was tested with increasing concentration of NAD^+ while the other parameters of the assay were kept constant. Competition analysis revealed that compound **5j** is competitive with respect to NAD^+ which imply that the inhibitor competes with NAD^+ to occupy the same binding site in the receptor (see Supporting information). This is in agreement with our molecular docking prediction.

2.5. Cellular assays

The antitumor activity of various known sirtuin inhibitors has been previously demonstrated in the literature.[20,29] To investigate the effectiveness of our compounds as antitumor agents, they were evaluated for their ability to inhibit growth of tumor cells. Human breast cancer MCF-7 cells and MDA-MB-468 cells were used as tumor cells, because the inhibition of SIRT2 has been shown to completely inhibit MCF-7 cell proliferation and could also inhibit tumor cell growth in a mouse xenograft model of triple negative breast cancer.[30] Interestingly as shown in Figure 4, compounds **5j**, **5k** and **5l** exerted potent anti-proliferative activity against MCF-7 tumor cells compared to the other compounds tested. Similar growth inhibitory trend was observed against MDA-MB-468 tumor cells. This showed that not only the newly

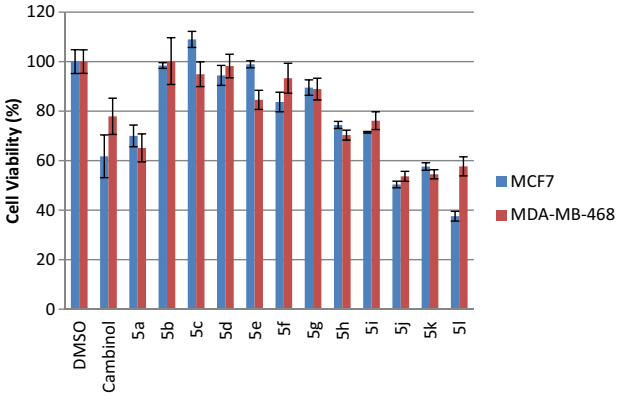


Figure 4. Inhibitory activity of compounds **5a–l** against MCF-7 and MDA-MB-468 cell line.

Table 2
Anti-proliferative activities of selected benzimidazole derivatives against MCF-7 and MDA-MB-468 cancer cell lines

Compound	Cell inhibition (%) at 50 μM	
	MCF-7	MDA-MB-468
5j	49.63	46.33
5k	42.37	45.51
5l	62.43	42.30
Cambinol	38.26	22.09

synthesized compounds are able to inhibit the luminal subtype breast cancer cells (MCF-7) but they are also able to inhibit the triple-negative breast cancer cells (MDA-MB-468) which target therapies currently do not exist. Taken together, these results suggested that sirtuin inhibitors especially SIRT1 and SIRT2 inhibitors bearing the 2-phenylbenzimidazole moiety with strong electron donating group on the *para*-position are potential antitumor agents. Results for the selected compounds which showed good anti-proliferative activity (**5j**, **5k**, **5l**) against both cancer cell lines are tabulated in Table 2.

SIRT2 could potentially play a more important role in inhibiting breast cancer MCF-7 and MDA-MB-468 cells as compared to SIRT1. This is due to the observation that compounds with good cytotoxic effect have potent SIRT2 inhibition. However, it should be noted that these compounds also exhibited inhibitory effects to SIRT1, albeit at higher concentrations. We cannot rule out the possibility that combinational inhibitory effects of SIRT1 and SIRT2 activity may contribute to the observed cytotoxicity as has been reported previously by Peck et al.[31]

3. Conclusion

In conclusion, we have discovered some novel benzimidazole derivatives which showed good SIRT1/SIRT2 inhibition activity with micromolar IC_{50} values. Moreover, they possess good antitumor activity against both MCF-7 (luminal) as well as MDA-MB-468 (basal-A subtype) breast cancer cell lines evaluated in this study. More importantly, we are able to correlate between in vitro SIRT2 (and to a lesser extent SIRT1) inhibition and cancer cell cytotoxicity using small molecule sirtuin inhibitors. Further studies to explore the mechanism of action of these potent small molecule sirtuin inhibitors on cancer cells are currently underway in our laboratory. Compounds with potent SIRT2 inhibition and which demonstrates cytotoxicity activities such as **5j** and **5l** are prime candidates for modifications to further improve their activities. An extension of the study in future may contribute to the development of useful anticancer agents in this series.

4. Experimental

4.1. Chemistry

All general chemicals were supplied by Sigma–Aldrich (U.S.A) and Merck Chemicals (Germany). Cambinol was obtained from Cayman Chemicals (U.S.A). Thin layer chromatography (silica gel G) was ran in the solvent system chloroform–methanol (9:1). The spots were located under short (254 nm)/long (365 nm) UV light. Elemental analyses were performed on Perkin Elmer 2400 Series II CHN Elemental Analyzer and were within ±0.4% of the calculated values. ^1H and ^{13}C NMR were performed on Bruker Avance 300 spectrometer in $CDCl_3$ using TMS as internal standard. Mass spectra were recorded on Varian 320-MS TQ LC/MS using ESI mode. Column chromatography purification was done in solvent system chloroform–methanol (9:1) using Silica Gel 60 (0.063–0.200 mm).

4.1.1. Procedure for the preparation of ethyl-4-fluoro-3-nitro benzoate (1)

4-Fluoro-3-nitrobenzoic acid (5 g, 27 mmol) was refluxed in ethanol (50 mL) and concentrated H_2SO_4 (2 mL) for 8 hours. After completion of reaction (as evident from TLC), the solvent was evaporated under reduced pressure. The aqueous layer was extracted with ethyl acetate (25 mL × 3). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield **1** as cream-coloured powder (75%).

Data for **1**. Yield: 75%; ^1H NMR (300 MHz, $CDCl_3$): δ 1.44 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.45 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 7.41 (1H, d, J = 8.4 Hz, H arom.), 8.70 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 8.89 (1H, s, H arom.). ESI-MS: m/z 214.1 $[M+H]^+$. Anal. Calcd for $C_9H_8NO_4F$: C, 50.71; H, 3.78; N, 6.57. Found: C, 50.65%; H, 3.83%; N, 6.60.

4.1.2. Procedure for the preparation of ethyl 4-(2-hydroxyethy lamino)-3-nitrobenzoate (2)

Ethyl-4-fluoro-3-nitrobenzoate, **1** (0.5 g, 2.34 mmol), ethanolamine (2.58 mmol) and *N*,*N*-Diisopropylethylamine, DIPEA (0.49 mL, 2.78 mmol) were mixed in dichloromethane (10 mL). The reaction mixture was stirred overnight at room temperature. After completion of reaction (as evident from TLC), the reaction mixture was washed with water (10 mL × 2) followed by 10% Na_2CO_3 solution (10 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to afford **2** as yellow solid (89%).

Data for **2**. Yield: 89%; ^1H NMR (300 MHz, $CDCl_3$): δ 1.44 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 3.48 (2H, t, J = 5.7 Hz, –NCH_2–), 3.88 (2H, t, J = 5.7 Hz, –CH_2OH), 4.45 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.82 (1H, d, J = 8.4 Hz, H arom.), 8.25 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 8.71 (1H, s, H arom.). ESI-MS: m/z 255.2 $[M+H]^+$. Anal. Calcd for $C_{11}H_{14}N_2O_5$: C, 51.97; H, 5.55; N, 11.02%. Found: C, 51.95; H, 5.56; N, 11.05.

4.1.3. Procedure for the preparation of ethyl 4-(2-hydroxyethy lamino)-3-aminobenzoate (3)

Ethyl 4-(2-hydroxyethylamino)-3-nitrobenzoate, **2** (1 mmol), ammonium formate (3 mmol) and Pd/C (50 mg) were mixed in ethanol (10 mL). The reaction mixture was refluxed until completion (solution turned colourless). The reaction mixture was then filtered through Celite 545. The filtrate was evaporated under reduced pressure. It was resuspended in ethyl acetate and washed with water, dried over Na_2SO_4 and evaporated to dryness to yield **3** (75%).

Data for **3**. Yield: 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 3.48 (2H, t, J = 5.7 Hz, –NCH_2–), 3.88 (2H, t, J = 5.7 Hz, –CH_2OH), 4.44 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.56 (1H, d, J = 8.4 Hz, H arom.), 7.13 (1H, s, H arom.), 7.25 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.). ESI-MS: m/z 225.2 $[M+H]^+$. Anal. Calcd for $C_{11}H_{14}N_2O_5$: C, 58.91; H, 7.19; N, 12.49. Found: C, 58.78; H, 7.29; N, 12.42.

4.1.4. General procedure for the preparation of sodium bisulfite addicts of 4-substituted benzaldehyde (4)

Appropriate benzaldehyde (10 mmol) was dissolved in ethanol (20 mL). Sodium metabisulfite (15 mmol) in 5 mL water was added in portion over 5 min. The reaction mixture was stirred at room temperature for 1 h and subsequently stirred at 4 °C overnight. The precipitate formed was filtered and dried to afford sodium bisulfite adducts (85–98%).

Data for **4j**. Yield: 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.00 (3.00 (6H, s, –N(CH_3)$_2$), 6.87 (2H, d, J = 8.4 Hz, H arom.), 7.45 (2H, d, J = 8.4 Hz, H arom.). ESI-MS: m/z 253.2 $[M]^+$.

4.1.5. General procedure for the preparation of 2-substituted benzimidazole derivatives (5)

Ethyl 4-(2-hydroxyethylamino)-3-aminobenzoate, **3** (1 mmol) and various sodium bisulfite adducts, **4** (1.5 mmol) were dissolved in DMF (5 mL). The reaction mixture was stirred at 90 °C under N_2 atmosphere for 24–48 h. After completion of reaction (evident by TLC), the reaction mixture was diluted in ethyl acetate (25 mL) and washed with water (10 mL × 3). The organic layer was collected, dried over Na_2SO_4 and evaporated under reduced pressure to afford crude products. Final compounds **5**–**6** were obtained in 75–95% yields after recrystallisation from ethanol or column purification in solvent system chloroform–methanol (9:1) using Silica Gel 60 (0.063–0.200 mm).

Data for **5a**. Yield: 95%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.14 (2H, t, J = 5.7 Hz, –NCH_2–), 4.29 (2H, t, J = 5.7 Hz, –CH_2OH), 4.42 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.70–7.60 (6H, m, H arom.), 7.81 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.93 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.57, 47.31, 60.39, 60.50, 115.21, 116.79, 119.72, 120.45, 121.25, 121.75, 121.84, 124.30, 131.75, 137.13, 139.38, 152.79, 166.00. ESI-MS: m/z 311.2 $[M+H]^+$. Anal. Calcd for $C_{18}H_{18}N_2O_3$: C, 69.66; H, 5.85; N, 9.03. Found: C, 69.56; H, 5.90; N, 9.02.

Data for **5b**. Yield: 82%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.16 (2H, t, J = 5.7 Hz, –NCH_2–), 4.30 (2H, t, J = 5.7 Hz, –CH_2OH), 4.46 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.82 (1H, d, J = 8.4 Hz, H aromatic), 6.94 (1H, d, J = 8.4 Hz, H aromatic), 7.35 (1H, d, J = 8.4 Hz, H aromatic), 7.60 (1H, d, J = 8.4 Hz, H aromatic), 7.73 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.76 (1H, d, J = 8.4 Hz, H aromatic), 7.97 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.50, 47.57, 60.31, 60.67, 108.94, 111.85, 111.95, 122.65, 123.66, 126.19, 127.80, 128.10, 129.82, 130.56, 139.11, 142.03, 156.25, 166.54. ESI-MS: m/z 389.4 $[M+H]^+$. Anal. Calcd for $C_{18}H_{17}N_2O_3Br$: C, 55.54; H, 4.40; N, 7.20. Found: C, 55.59; H, 5.26; N, 7.27.

Data for **5c**. Yield: 86%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.18 (2H, t, J = 5.7 Hz, –NCH_2–), 4.30 (2H, t, J = 5.7 Hz, –CH_2OH), 4.47 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.85 (1H, d, J = 8.4 Hz, H aromatic), 6.95 (1H, d, J = 8.4 Hz, H aromatic), 7.36 (1H, d, J = 8.4 Hz, H aromatic), 7.69 (1H, d, J = 8.4 Hz, H aromatic), 7.75 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.80 (1H, d, J = 8.4 Hz, H aromatic), 8.01 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.54, 47.60, 60.30, 60.72, 109.46, 111.90, 112.07, 122.65, 123.68, 126.19, 127.87, 128.10, 130.82, 133.02, 139.11, 142.03, 156.25, 166.57. ESI-MS: m/z 345.2 $[M+H]^+$. Anal. Calcd for $C_{18}H_{17}N_2O_3Cl$: C, 62.70; H, 4.97; N, 8.12. Found: C, 62.87; H, 5.14; N, 8.19.

Data for **5d**. Yield: 89%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.17 (2H, t, J = 5.7 Hz, –NCH_2–), 4.32 (2H, t, J = 5.7 Hz, –CH_2OH), 4.45 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.86 (1H, d, J = 8.4 Hz, H aromatic), 6.95 (1H, d, J = 8.4 Hz, H aromatic), 7.36 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, d, J = 8.4 Hz, H aromatic), 7.75 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.78 (1H, d, J = 8.4 Hz, H aromatic), 8.00 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.54, 47.58, 60.34, 60.72, 109.45, 111.92, 112.07, 122.66, 122.80, 126.19, 127.87, 129.15, 130.82, 133.02, 139.11, 142.03, 156.25, 159.60, 166.79. ESI-MS: m/z 395.3 $[M+H]^+$. Anal. Calcd for $C_{19}H_{17}N_2O_4F_3$: C, 57.87; H, 4.35; N, 7.10. Found: C, 57.69; H, 4.19; N, 7.14.

Data for **5e**. Yield: 91%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.18 (2H, t, J = 5.7 Hz, –NCH_2–), 4.34 (2H, t, J = 5.7 Hz, –CH_2OH), 4.45 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.88 (1H, d, J = 8.4 Hz, H aromatic), 6.95 (1H, d, J = 8.4 Hz, H aromatic), 7.36 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, d, J = 8.4 Hz, H aromatic), 7.75 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.79 (1H, d, J = 8.4 Hz, H aromatic), 8.05 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.55, 47.60, 60.31, 60.72, 110.15, 111.93, 112.71, 123.10, 123.80, 126.19, 127.87, 129.33, 130.82, 131.88, 133.02, 139.11, 142.09, 157.43, 166.81. ESI-MS: m/z 378.3 $[M+H]^+$. Anal. Calcd for $C_{19}H_{17}N_2O_3F_3$: C, 60.32; H, 4.53; N, 7.40. Found: C, 60.35; H, 4.55; N, 7.38.

Data for **5f**. Yield: 80%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.20 (2H, t, J = 5.7 Hz, –NCH_2–), 4.34 (2H, t, J = 5.7 Hz, –CH_2OH), 4.48 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.88 (1H, d, J = 8.4 Hz, H aromatic), 6.95 (1H, d, J = 8.4 Hz, H aromatic), 7.36 (1H, d, J = 8.4 Hz, H aromatic), 7.73 (1H, d, J = 8.4 Hz, H aromatic), 7.75 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.80 (1H, d, J = 8.4 Hz, H aromatic), 8.06 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.53, 47.56, 60.31, 60.72, 110.10, 111.93, 112.71, 123.10, 123.69, 126.15, 126.97, 128.94, 131.88, 133.02, 139.11, 142.08, 149.86, 157.34, 166.80. ESI-MS: m/z 356.2 $[M+H]^+$. Anal. Calcd for $C_{18}H_{17}N_3O_5$: C, 60.84; H, 4.82; N, 11.83. Found: C, 60.91; H, 4.78; N, 11.83.

Data for **5g**. Yield: 75%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 2.35 (3H, s, –CH_3 arom.), 4.16 (2H, t, J = 5.7 Hz, –NCH_2–), 4.29 (2H, t, J = 5.7 Hz, –CH_2OH), 4.40 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.75 (1H, d, J = 8.4 Hz, H aromatic), 6.80 (1H, d, J = 8.4 Hz, H aromatic), 7.26 (1H, d, J = 8.4 Hz, H aromatic), 7.60 (1H, d, J = 8.4 Hz, H aromatic), 7.69 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.77 (1H, d, J = 8.4 Hz, H aromatic), 7.89 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.48, 25.34, 47.59, 60.59, 60.70, 108.14, 109.77, 111.48, 120.91, 123.22, 125.05, 129.69, 131.50, 138.03, 138.24, 141.86, 157.39, 166.81. ESI-MS: m/z 325.2 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_3$: C, 70.35; H, 6.21; N, 8.64. Found: C, 70.13; H, 6.45; N, 8.46.

Data for **5h**. Yield: 77%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.15 (2H, t, J = 5.7 Hz, –NCH_2–), 4.29 (2H, t, J = 5.7 Hz, –CH_2OH), 4.40 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.73 (1H, d, J = 8.4 Hz, H aromatic), 6.78 (1H, d, J = 8.4 Hz, H aromatic), 7.26 (1H, d, J = 8.4 Hz, H aromatic), 7.61 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.74 (1H, d, J = 8.4 Hz, H aromatic), 7.90 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.47, 47.60, 60.61, 60.69, 109.25, 110.06, 111.48, 120.35, 123.78, 124.05, 125.69, 131.50, 132.03, 138.24, 141.86, 157.39, 159.85, 166.79. ESI-MS: m/z 327.2 $[M+H]^+$. Anal. Calcd for $C_{18}H_{18}N_2O_4$: C, 66.25; H, 5.56; N, 8.58. Found: C, 66.19; H, 5.77; N, 8.50.

Data for **5i**. Yield: 84%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 3.70 (3H, s, –OCH_3), 4.14 (2H, t, J = 5.7 Hz, –NCH_2–), 4.28 (2H, t, J = 5.7 Hz, –CH_2OH), 4.40 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.74 (1H, d, J = 8.4 Hz, H aromatic), 6.78 (1H, d, J = 8.4 Hz, H aromatic), 7.25 (1H, d, J = 8.4 Hz, H aromatic), 7.61 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.76 (1H, d, J = 8.4 Hz, H aromatic), 7.92 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.47, 47.60, 56.90, 60.62, 60.71, 109.25, 110.19, 111.48, 120.46, 123.78, 124.05, 125.69, 131.50, 132.17, 138.24, 141.86, 157.39, 160.75, 166.80. ESI-MS: m/z 341.2 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_2O_4$: C, 67.05; H, 5.92; N, 8.23. Found: C, 67.18; H, 5.76; N, 8.33.

Data for **5j**. Yield: 92%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 3.00 (6H, s, –N(CH_3)$_2$), 4.23 (2H, t, J = 5.7 Hz, –NCH_2–), 4.30 (2H, t, J = 5.7 Hz, –CH_2OH), 4.40 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.56 (1H, d, J = 8.4 Hz, H aromatic), 6.70 (1H, d, J = 8.4 Hz, H aromatic), 7.22 (1H, d, J = 8.4 Hz, H aromatic), 7.67 (1H, d, J = 8.4 Hz, H aromatic), 7.72 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.73 (1H, d, J = 8.4 Hz, H aromatic), 7.87 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.46, 40.02, 47.19, 56.90, 60.62, 60.69, 109.22, 111.01, 111.54, 120.55, 123.42, 124.44, 125.19, 131.10, 131.98, 138.31, 141.39, 151.07, 156.45, 166.80. ESI-MS: m/z 354.1 $[M+H]^+$. Anal. Calcd for $C_{20}H_{23}N_3O_3$: C, 67.97; H, 6.56; N, 11.89%. Found: C, 68.06; H, 6.55; N, 11.90.

Data for **5k**. Yield: 87%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.44 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 1.65 (2H, t, J = 5.7 Hz, H piperidine), 1.73 (4H, m, H piperidine), 3.32 (4H, t, J = 5.7 Hz, H piperidine), 4.04 (2H, t, J = 5.7 Hz, –NCH_2–), 4.26 (2H, t, J = 5.7 Hz, –CH_2OH), 4.40 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 7.00 (1H, d, J = 8.4 Hz, H aromatic), 7.28 (1H, d, J = 8.4 Hz, H aromatic), 7.31 (1H, d, J = 8.4 Hz, H aromatic), 7.65 (1H, d, J = 8.4 Hz, H aromatic), 7.79 (1H, d, J = 8.4 Hz, H aromatic), 7.85 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.90 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.42, 24.31, 25.55, 47.55, 48.28, 49.04, 60.32, 60.86, 109.10, 114.81, 119.90, 121.59, 123.95, 124.31, 124.65, 131.41, 136.20, 142.00, 144.88, 152.89, 166.55. ESI-MS: m/z 394.2 $[M+H]^+$. Anal. Calcd for $C_{23}H_{27}N_3O_3$: C, 70.21; H, 6.92; N, 10.68. Found: C, 70.12; H, 6.89; N, 10.78.

Data for **5l**. Yield: 90%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.47 (3H, t, J = 7.2 Hz, CH_3CH_2O–), 4.27-4.33 (4H, m, –OCH_2CH_2OH), 4.42 (2H, q, J = 7.2 Hz, CH_3CH_2O–), 6.02 (2H, s, –OCH_2O–), 6.72 (1H, d, J = 8.4 Hz, H aromatic), 7.19 (1H, d, J = 8.4 Hz, H aromatic), 7.32 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.40 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, dd, J = 1.5 Hz, 8.4 Hz, H aromatic), 7.74 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 14.46, 47.07, 60.25, 60.77, 101.54, 108.14, 109.27, 110.44, 120.78, 122.37, 123.78, 124.85, 125.15, 137.62, 141.03, 147.48, 149.13, 155.37, 166.49. ESI-MS: m/z 355.2 $[M+H]^+$. Anal. Calcd for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91. Found: C, 64.43; H, 5.20; N, 7.89.

4.1.6. General procedure for the preparation of 5-carboxylic acid-2-substituted benzimidazole derivatives (6)

Compounds **6j–6l** were synthesized as according to procedure 4.1.2 to 4.1.5 above. The esterification step of 4.1.1 was omitted.

Data for **6j**. Yield: 86%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.00 (6H, s, –N(CH_3)$_2$), 4.23 (2H, t, J = 5.7 Hz, –NCH_2–), 4.39 (2H, t, J = 5.7 Hz, –CH_2OH), 6.56 (1H, d, J = 8.4 Hz, H aromatic), 6.92 (1H, d, J = 8.4 Hz, H aromatic), 7.42 (1H, d, J = 8.4 Hz, H aromatic), 7.70 (1H, d, J = 8.4 Hz, H aromatic), 7.81 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.85 (1H, d, J = 8.4 Hz, H aromatic), 8.00 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 40.04, 47.19, 60.62, 60.69, 109.22, 111.01, 111.54, 120.55, 123.42, 124.44, 125.19, 132.10, 132.98, 138.87, 142.19, 151.32, 156.94, 167.47. ESI-MS: m/z 325.1 $[M+H]^+$. Anal. Calcd for $C_{18}H_{19}N_3O_3$: C, 66.45; H, 5.89; N, 12.91. Found: C, 66.30; H, 5.97; N, 12.99.

Data for **5k**. Yield: 90%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.65 (2H, t, J = 5.7 Hz, H piperidine), 1.73 (4H, m, H piperidine), 3.32 (4H, t, J = 5.7 Hz, H piperidine), 4.04 (2H, t, J = 5.7 Hz, –NCH_2–), 4.26 (2H, t, J = 5.7 Hz, –CH_2OH), 7.11 (1H, d, J = 8.4 Hz, H aromatic), 7.35 (1H, d, J = 8.4 Hz, H aromatic), 7.41 (1H, d, J = 8.4 Hz, H aromatic), 7.77 (1H, d, J = 8.4 Hz, H aromatic), 7.86 (1H, d, J = 8.4 Hz, H aromatic), 7.94 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 8.03 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 24.33, 25.55, 47.55, 48.28, 49.04, 60.86, 109.10, 114.81, 119.90, 121.59, 123.95, 124.31, 124.65, 131.41, 136.54, 142.39, 145.98, 153.02, 167.98. ESI-MS: m/z 366.2 $[M+H]^+$. Anal. Calcd for $C_{21}H_{23}N_3O_3$: C, 69.02; H, 6.34; N, 11.50. Found: C, 69.12; H, 6.55; N, 11.38.

Data for **5l**. Yield: 91%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.27–4.33 (4H, m, –OCH_2CH_2OH), 6.02 (2H, s, –OCH_2O–), 6.88 (1H, d, J = 8.4 Hz, H aromatic), 7.32 (1H, d, J = 8.4 Hz, H aromatic), 7.40 (1H, dd, J = 1.5 Hz, 8.4 Hz, H arom.), 7.55 (1H, d, J = 8.4 Hz, H aromatic), 7.79 (1H, dd, J = 1.5 Hz, 8.4 Hz, H aromatic), 7.89 (1H, s, H arom.). ^{13}C NMR (75 MHz, $CDCl_3$): 47.07, 60.77, 101.54, 108.14, 109.27, 110.44, 120.78, 122.37, 123.78, 124.85, 125.15, 138.75, 143.03, 147.32, 150.28, 155.39, 167.84. ESI-MS: m/z 327.2 $[M+H]^+$. Anal. Calcd for $C_{17}H_{14}N_2O_5$: C, 62.57; H, 4.32; N, 8.59. Found: C, 62.55; H, 4.28; N, 8.60.

4.2. Biology

Fluorescent optical density for in vitro assay was measured on Tecan Infinite M200. Optical density for cell proliferative assay was measured with Thermo Scientific MultiSkan FC microplate reader.

4.2.1. SIRT1 in vitro assay

3.3 μM of SIRT1 substrate derived from human p53 sequences, 66.7 μM NAD^+, 50 μM of interested compounds (all final concentration) and 0.5 μg of SIRT1 human recombinant (GenBank Accession #: NM_012238) with 193–741 amino acids and GST tag at its N-terminal, were incubated for 45 min at 37 °C. 50 μL of stop solution consisting nicotinamide and SIRT1 developer was then added and the mixture was incubated for a further 10 min at 37 °C. Fluorescence was measured at 490 nm (excitation) and 520 nm (emission) and the inhibition was calculated as the ratio of absorbance under each experimental condition to that of the control.

4.2.2. SIRT2 in vitro assay

6.7 μM of SIRT2 substrate derived from human p53 sequences, 333 μM NAD^+, 50 μM of interested compounds (all final concentration) and 0.5 μg of SIRT2 human recombinant (GenBank Accession #: NM_030593) with 13–319 amino acids and His tag at its C-terminal, were incubated for 45 min at 37 °C. 50 μL of stop solution consisting nicotinamide and SIRT1 developer was then added and the mixture was incubated for a further 10 min at 37 °C. Fluorescence was measured at 490 nm (excitation) and 520 nm (emission) and the inhibition was calculated as the ratio of absorbance under each experimental condition to that of the control.

4.2.3. Cell proliferation assay

All cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded in 96-well plates at a density of 5×10^3 per well. The cells were treated with 50 μM of interested compounds and allowed to adhere for 72 h. Then, the proliferative activity was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) to monitor the number of viable cells according to the manufacturer's instructions. Briefly, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt solu tion was added at 20 μL/well, and after 1 h of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the conversion of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt to formazan was measured in a plate reader at 490 nm. 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 nontransfectant.

4.2.4. Autofluorescence

Compounds in DMSO at 50 μM concentration (100 μL) were pipetted into Nunc Microwell 96-wells plate. DMSO was used as control well. Excitation wavelength was set at 490 nm and emission wavelength at 520 nm. The criteria for a compound being considered autofluorescent was defined as having >50% fluores-

cence of the control wells. None of the analyzed compounds were found to be autofluorescent.

5. Conflict of interest

The authors hereby declare there is no conflict of interests.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.029.

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