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Potent sirtuin inhibition with 1,2,5-trisubstituted benzimidazoles†

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Two series of compounds were synthesized based on the benzimidazole scaffold. The compounds were subsequently screened for their SIRT1, SIRT2 and SIRT3 activities. Three of the compounds showed good inhibitory activity against SIRT2 in this study with the most potent compound (5i) having an IC_{50} value of 2.9 µM. Molecular docking analysis performed demonstrated that 5i was able to inhibit SIRT2 by displacing the co-factor NAD^+ in the active site. This was further confirmed experimentally by ligand-NAD^+ competitive assay.

Introduction

Benzimidazole is a heterocycle which contained a phenyl ring fused to an imidazole ring. The benzimidazole core structure is an interesting platform for drug discovery[1] as they were shown to possess a wide spectrum of pharmacological activities such as antiviral[2], anti-inflammatory[3,4] and anticancer[5] properties. More applications for benzimidazoles or benzimidazole-containing compounds have recently been unraveled. Among the new discoveries was the potential use of benzimidazole analogues in inhibiting sirtuin enzymatic activity as recently reported by our group[6].

Sirtuins are NAD-positive-dependent class III HDACs that share extensive homologies with the yeast HDAC Sir2[7]. Seven human sirtuins have been identified (SIRT1-7) to date[8]. Collectively, they controlled many downstream targets such as NF-κB[9], p53[10] and FOXO proteins[11]. Sirtuins have recently been linked to age-related disorders such as cancer[12,13], Parkinson's[14], Alzheimer[15] and cardiovascular diseases. Owing to their broad involvement in various diseases, sirtuins were actively researched.To date, several classes of sirtuin inhibitors have been identified such as the nicotinamide,[16] hydroxynaphthyldehyde derivatives such as salermide and cambinol,[17,18] pyrimidine-based inhibitors,[19] splitomicin analogs[20] and tenovins.[21]

In our previous studies, we have shown that the reported scaffold (Figure 1) gave varying degree of inhibitory activity with changes in the R^1 and R^2 positions. In most cases, strong electron donating basic terminal at the R^2 position would enhance the sirtuin inhibitory activity[22,23]. It was also revealed that sirtuin inhibitors based on the benzimidazole scaffold showed that they mostly occupied the adenosine diphosphate (A pocket) and ribose (B pocket) binding sites.

In view of this, longer chains (> 6 atoms length) were applied at the R^1 position in the effort to increase the inhibitory potential of the compounds as this may allow the side chain to occupy the nicotinamide pocket (C pocket) of the active binding site. In the present study, the synthesis and sirtuin inhibitory activities of compounds with long chains at the R^1 position were reported. Molecular docking experiments were also carried out to provide insights into the binding mode of this series of benzimidazoles. Compounds **5a-g** were previously synthesized[24] and evaluated for their antimycobacterium activity following established protocol (Figure 2). However, since the compounds have a long and flexible chain substitution at R^1 which may be suitable for inhibiting sirtuins, the compounds were included in this study.

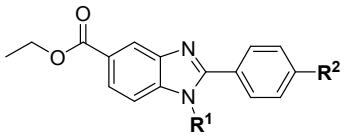

Figure 1. Benzimidazole-based core structure for sirtuin inhibition

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Experimental

Chemistry

All chemicals were supplied by Sigma-Aldrich (U.S.A.), Merck Chemicals (Germany) and Acros Organics (U.S.A.). Elemental analyses were measured on Perkin Elmer 2400 Series II CHN Elemental Analyzer and were within ± 0.4% of the calculated values. ^{1}H and ^{13}C NMR were performed on Bruker Avance 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz) spectrometer in $CDCl_3$. Mass spectra were recorded on Varian 320-MS TQ LC/MS in positive ESI mode.

Procedure for the preparation of *Ethyl-4-fluoro-3-nitrobenzoate*

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, the solvent was evaporated under reduced pressure. The aqueous layer was extracted with ethyl acetate (25 mL x 3). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to yield **1** as cream-coloured powder (75%).

General procedure for the preparation of *4-(2-substituted amino)-3-nitro-ethylbenzoate*

Ethyl-4-fluoro-3-nitrobenzoate, **1** (0.5 g, 2.34 mmol), primary amines [Series 5: N-(2-aminoethyl)piperazine, 0.15 mL, 1.16 mmol; Series 8: octylamine, 0.38 mL, 2.30 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, the reaction mixture was washed with water (10 mL x 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 brown oil (91%) or **6** as brown solid (86%).

General procedure for the preparation of *Ethyl-3-amino-4-(2-substituted amino)benzoate*

4-(2-substituted amino)-3-nitro-ethylbenzoate (1 mmol), ammonium formate (0.378 g, 6 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 *ethyl-3-amino-4-(2-substituted amino)benzoate***3** (70%) or **7** (79%).

General procedure for the preparation of sodium bisulfite addcuts of 4-substituted benzaldehyde

Appropriate benzaldehyde (10 mmol) was dissolved in ethanol (20 mL). Sodium metabisulfite (15 mmol) in 5 mL water was added in portion over 5 minutes. The reaction mixture was stirred at room temperature for 1 hour and subsequently stirred at 4°C overnight.The precipitate formed was filtered and dried to afford sodium bisulfite adducts (55%-90%).

General procedure for the preparation of 2-substituted benzimidazole derivatives (5a-j and 8a-j)

Ethyl-3-amino-4-(2-substituted amino)benzoate (1 mmol) and various sodium bisulfite adducts (1.5 mmol) were dissolved in DMF (5 mL). The reaction mixture was stirred at 90°C under N_2 atmosphere for 24-48 hours. After completion of reaction, the reaction mixture was diluted in ethyl acetate (25 mL) and washed with water (10 mL x 3). The organic layer was collected, dried over Na_2SO_4 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 µM), NAD+ (3 mM), test compounds (50 µM) and sirtuin human recombinant were incubated for 45 minutes at 37°C. 50 µL of stop solution consisting 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.

Molecular docking

The crystal structure of human SIRT1 (PDB code: 4IGI), SIRT2 (PDB code: 3ZGV; 4RMG; 5FYQ) and SIRT3 (PDB code: 4JSR) were taken from the Protein Data Bank. The enzymes and ligands 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. For each molecule, 10 docking runs were performed. The top-ranked pose for each ligand were retained and further analyzed with VMD 1.9.1 molecular graphics software.

Results and discussion

Synthesis of compounds

The benzimidazole derivatives were synthesized according to the synthetic scheme shown in Figure 2. Briefly, the synthetic scheme follows a four-step pathway. The first step involves the esterification of the starting material 4-fluoro-3-nitro benzoic acid. The second step involves amination while reduction of the nitro

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group to the amino follows in the third step. Finally, the last step involves cyclization and formation of the benzimidazole core. The sodium bisulfite adducts were attached with different substitution at the R^1 position to yield the desired final compounds.

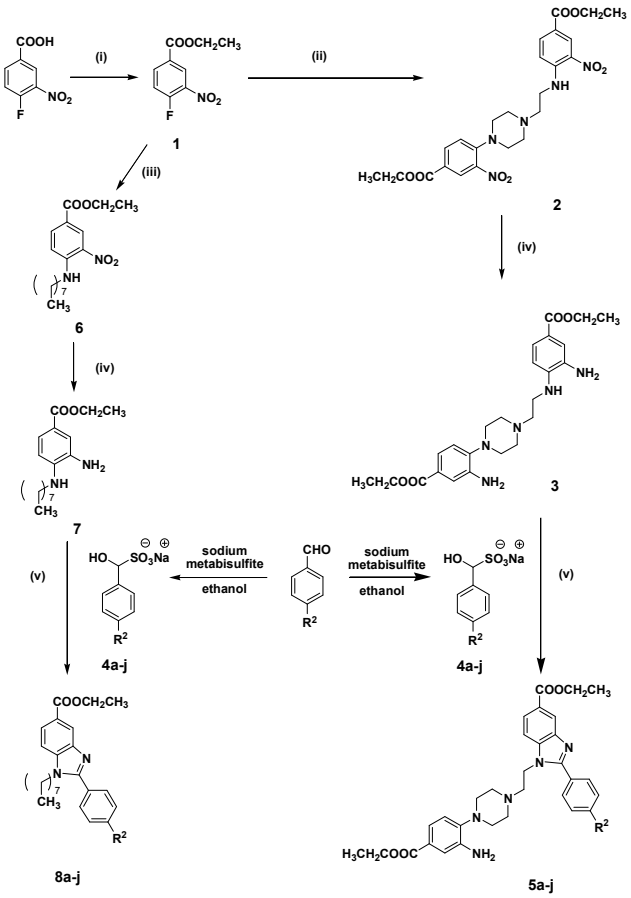

Figure 2. Synthesis protocol of compounds **5a-j** and **8a-j**. (i) ethanol/H_2SO_4, reflux, 8 h; (ii) N-(2-aminoethyl)piperazine, DIPEA, dichloromethane, room temperature, 18 h; (iii) octylamine, DIPEA, dichloromethane, room temperature, 18 h (iv) ammonium formate, Pd/C, ethanol, 60°C, 1h; (v) DMF, 90°C, 24-48 h.

Enzymatic Assays

The synthesized compounds were evaluated for their *in vitro* SIRT1, 2 and 3 inhibitory activities using commercially available fluorescent 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 control while DMSO was used as a vehicle control. IC_{50} values were determined for all compounds which showed over 50% inhibition at 50 µM. Experiments were performed in triplicates. Generally, the synthesized benzimidazole derivatives showed better inhibition against SIRT2 compared to SIRT1 or SIRT3 as shown in Table 1. It was also revealed that compounds from Series 5 showed better sirtuin inhibitory activity than compounds from Series 8.

As compounds from Series 5 were shown to have better sirtuin inhibitory activities, three novel derivatives with different substituent at R^2 position (**5h**: 2-methoxyphenol, **5i**: *N,N*-dimethylbenzeneamine, **5j**: phenylpiperidine) were synthesized following similar procedure and were subsequently evaluated for their *in vitro* SIRT1, 2, and 3 inhibition.Structure of the novel compounds were determined by NMR, elemental as well as MS analysis. The dimethylamino and piperidine functional groups were chosen mainly based on our previous results which showed potent sirtuin inhibitory activity when the R^2 position was substituted with amino functionalized groups[22,23]. The fact that **5c** and **5f** (which were doubly substituted on the phenyl ring) were found to be the two most potent inhibitors from this series also prompted us to synthesize and evaluate compound **5h**. Apart from that, analogous compounds **8h-j** were also synthesized and evaluated for comparison purposes. The sirtuin inhibitory results of the six novel compounds were tabulated in Table 2.

Compound **5i** was found to be the most potent inhibitor of the series (SIRT1 IC_{50} = 10.22 µM; SIRT2 IC_{50} = 2.92 µM; SIRT3 IC_{50} = 10.02 µM). It is gratifying to note that **5i** is more inhibitory than AGK2, one of the most potent SIRT2 inhibitors commercially available. In fact, **5i** is the most potent sirtuin inhibitor derived from the benzimidazole scaffold reported thus far. It was found to be a pan-SIRT1/2/3 inhibitor, albeit with a preference to inhibit SIRT2. Its selectivity for SIRT2 is more than 3-fold over both SIRT1 and SIRT3.

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

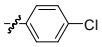
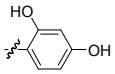
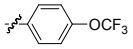
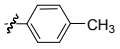
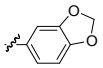
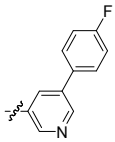
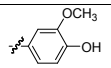
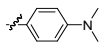
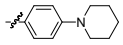
	R^2	SIRT1 IC_{50} (μM) or % of inhibition at 50 μM	SIRT2 IC_{50} (μM) or % of inhibition at 50 μM	SIRT3 IC_{50} (μM) or % of inhibition at 50 μM
5a		31.09 ± 4.23 %	26.77 ± 5.18 %	25.15 ± 3.03 %
8a		23.89 ± 4.16 %	30.02 ± 2.22 %	20.39 ± 2.87 %
5b		28.19 ± 4.64 %	43.30 ± 5.52 %	37.96 ± 7.38 %
8b		24.34 ± 5.61 %	38.34 ± 4.50 %	24.88 ± 2.29 %
5c		19.96 μM	7.87 μM	23.26 μM
8c		29.03 ± 3.63 %	45.94 ± 1.26 %	31.54 ± 1.82 %
5d		24.56 ± 3.09 %	37.16 ± 2.86 %	20.59 ± 3.28 %
8d		25.02 ± 1.94 %	35.59 ± 2.04 %	16.85 ± 1.03 %
5e		28.38 ± 5.40 %	42.98 ± 6.03 %	30.71 ± 2.46 %
8e		15.30 ± 2.77 %	30.33 ± 2.10 %	19.44 ± 3.29 %
5f		29.80 μM	16.12 μM	27.03 μM
8f		>50 μM	41.40 μM	>50 μM
5g		20.60 ± 6.99 %	20.92 ± 7.89 %	18.44 ± 4.65 %
8g		22.01 ± 2.30 %	13.96 ± 2.94 %	24.40 ± 3.05 %
EX-527	-	0.30 μM	N.D.	N.D.
AGK2	-	N.D.	8.34 μM	N.D.
Tenovin-6	-	42.10 μM	25.60 μM	82.65 μM

Table 2. SIRT1, SIRT2 and SIRT3 inhibitory activities of 5h-j and 8h-j.

	R^2	SIRT1 IC_{50} (μM)	SIRT2 IC_{50} (μM)	SIRT3 IC_{50} (μM)
5h		35.07 ± 2.42 μM	8.61 ± 1.29 μM	27.91 ± 2.21 μM
8h		>50 μM	46.95 ± 3.05 μM	>50 μM
5i		10.22 ± 0.98 μM	2.92 ± 0.33 μM	10.02 ± 1.11 μM
8i		>50 μM	38.10 ± 3.10 μM	>50 μM
5j		36.97 ± 1.82 μM	13.48 ± 1.75 μM	29.24 ± 2.48 μM
8j		>50 μM	36.76 ± 2.90 μM	>50 μM

Molecular docking

Based on the previous results, the reported scaffold with shorter substitution at the R^1 position were not able to effectively occupy the nicotinamide binding site (C pocket). Instead, the shorter substituents actually distorted the confirmation of the benzimidazole base structure when docked into the active site which resulted in weaker inhibitory activity (Supplementary data).

Therefore, in an attempt to rationalize the observed *in vitro* enzymatic activities, docking study of compound the most potent compound of the series (**5i**) into the active site of human SIRT2 was performed (PDB entry code: 3ZGV)[25]. The receptor and the drug candidates were optimized before actual docking in Autodock 4.2. Analysis of the top-ranked pose of compound **5i** demonstrated several plausible molecular interactions between the ligand and the enzyme.

Compound **5i** was found to occupy the location of the co-factor NAD^+ in the active site. The docking analysis reveals that **5i** interacts with residue Arg97 and Pro94 through hydrogen bonding. Hydrogen bond was also observed between the ester group from the benzimidazole structure with Ser263 and Ser98. In general, the planar benzimidazole scaffold occupied the adenosine binding site (A pocket) and ribose binding site (B pocket) of the active site while the *N,N*-dimethylbenzeneamine substituent was located at the gauge of the acetyl lysine channel. The importance of the *N,N*-dimethylbenzeneamine substituent was further highlighted through hydrogen bonding with Phe235. Lone pair oxygen-π interactions

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could also be observed between the nitrogen atom from the *N,N*-dimethylbenzeneamine group with benzene ring from Phe235. Moreover, the side chain of ethylpiperizinyl benzoate was located deep inside the nicotinamide binding site (C pocket) of the active site which was deemed important for controlling the activity of the enzyme (Figure 3). This was not seen In comparison, compound **8i** which has a linear octyl chain at the $\mathbf{R^1}$ position was found to have a different conformation when docked inside the active site of SIRT2 compared to compound **5i** (See supplementary data). In the process, it lost important interactions with some residues such as Arg97, making it a less potent inhibitor compared with compound **5i**.

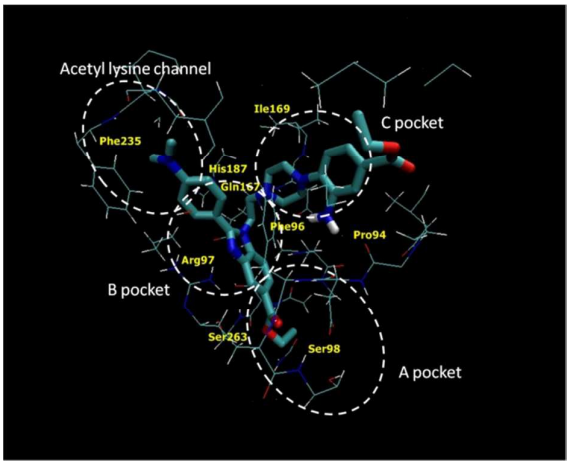

Figure 3. Compound **5i** docked in human SIRT2. (PDB entry code: 3ZGV)

When compared against other available SIRT2 structures [SIRT2-SirReal2 (extended C-pocket) PDB: 4RMG; SIRT2-Ran TFAcK37–13-mer peptide (lysine channel) PDB: 5FYQ], it was found that **5i** adopt different conformations. In the extended C-pocket (4RMG), **5i** was highly distorted and did not have strong contact with Tyr139 and Phe190 as reported by Jung *et al*[26]. Therefore, the exceptional SIRT2 selectivity shown by the SirReal2 ligand was not observed here. On the other hand, using the crystal structure of 5FYQ[27], it was found that only part of **5i** was docked in the lysine channel, with the benzimidazole moiety located out from the active site. This is plausible as **5i** is not a peptidomimetic-based inhibitor. Weaker binding of **5i** in both sites were indicated from their higher overall free-binding energy, further supporting the docking of **5i** in the NAD^+ binding site (Supplementary data).

Besides SIRT2, **5i** was also docked into the active site of human SIRT1 (PDB entry code: 4I5I)[28] and SIRT3 (PDB entry code: 4JSR)[29] to determine its interaction with both receptors. The mode of interaction with SIRT1 was unlike those observed for SIRT2 as **5i** was oriented differently in the active site of SIRT1. Docking simulation indicated that the *N,N*-dimethylbenzeneamine group was inverted "top-down" and now occupy part of the A pocket (Figure 4) while the ester group attached to the benzimidazole scaffold was located at the opening of the acetyl lysine channel formed by Phe414, His363 and Phe297. Hydrogen bonds were formed between the amino group of 5i with Ile347 and Asp348 in the C pocket.

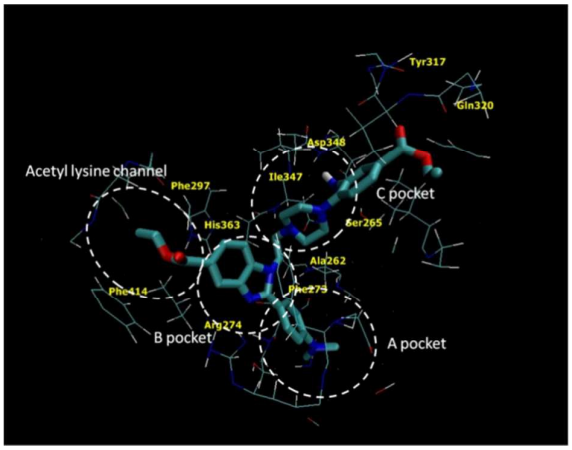

Figure 4. Compound **5i** docked in human SIRT1. (PDB entry code: 4I5I)

As for SIRT3, **5i** has strong interactions with residues primarily in the acetyl lysine channel and the C pocket of the active site (Figure 5). The conformation was supported by the strong hydrogen bonds formed with Asp231 and Gly295. Weaker interactions were also observed with Phe157, Leu298 and Glu296. Residue His248 which is critical for deacetylation activity of the enzyme[30,31] was also weakly bonded to the NH_2 group of the ligand.

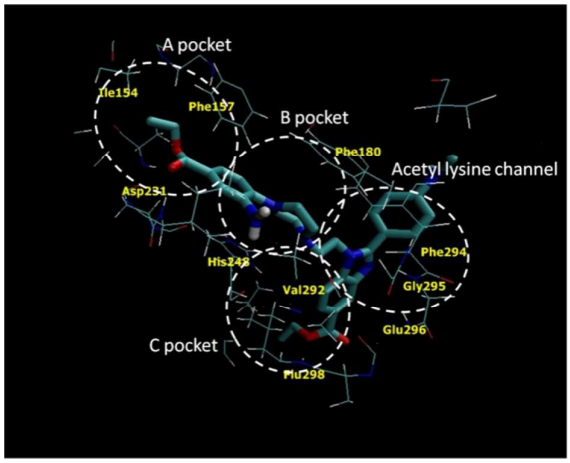

Figure 5. Compound **5i** docked in human SIRT3. (PDB entry code: 4JSR)

To test the hypothesis that the binding mode of **5i** towards SIRT2 was by displacing the co-factor, a ligand-NAD^+ competitive assay was carried out following previously reported method[32]. The inhibition of **5i** was tested with increasing concentration of NAD^+

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while the other parameters of the assay were kept constant (Figure 6).

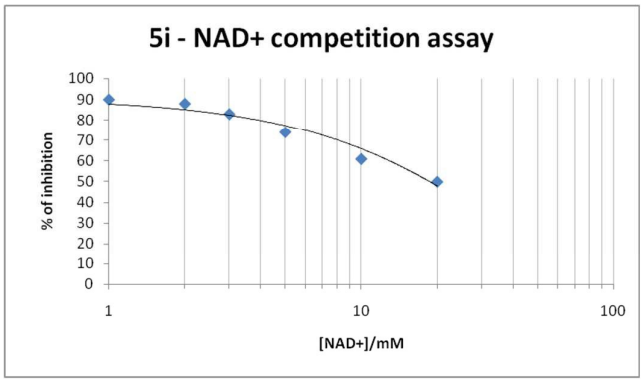

Figure 5. Decreasing SIRT2 inhibition by **5i** with increasing concentrations of NAD^+

Competition analysis revealed that compound **5i** is competitive with respect to NAD^+ which implies that the inhibitor competes with NAD^+ to occupy the same binding site in the receptor. This is in agreement with our molecular docking prediction. The competition analysis of **5i** with the peptide substrate revealed that the percentage of inhibition plateau at approximately 65% even with increments of peptide substrate concentration, thus indicating the inability of the peptide substrate to further dislodge **5i** from binding to the enzyme's active site (Supplementary data). Altough this is not a full kinetic study, the preliminary data shown here supported the molecular docking prediction.

Conclusions

In conclusion, we have discovered several novel benzimidazole derivatives which showed potent sirtuin inhibition activity, with preference towards SIRT2. The most potent compound found in this study, **5i**, showed better SIRT2 inhibitory activity compared to the standard controls used. It is also the most potent sirtuin inhibitor from the 1,2,5-trisubstituted benzimidazole scaffold reported to date. Molecular docking analysis performed demonstrated that **5i** was able to inhibit SIRT2 by occupy the position of co-factor NAD^+ in the active site. This was further confirmed experimentally by ligand-NAD^+ competitive assay. The importance of a long and flexible chain at R^1 position was highlighted with the increment of inhibitory potency as the compounds were able to occupy part of the C pocket in the active site.

Acknowledgements

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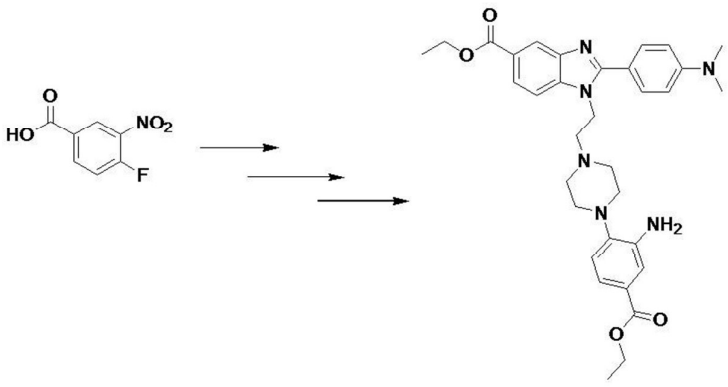
GRAPHICAL ABSTRACT



5i

SIRT1 IC_{50} = 10.22 uM
SIRT2 IC_{50} = 2.92 uM
SIRT3 IC_{50} = 10.02 uM

Benzimidazole analogs were previously shown to inhibit sirtuin activity. Novel compound **5i** was found to be a potent SIRT2 inhibitor with an IC50 value of 2.92 µM.