DR KENG YOON YEONG (Orcid ID : 0000-0003-0433-5070)

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Contrasting sirtuin and PARP activity of selected 2,4,6-trisubstituted benzimidazoles

Keng Yoon Yeong[a,b*], Soo Choon Tan[b], Chun-Wai Mai[c], Chee-Onn Leong[c,d], Felicia Fei-Lei Chung[d], Yean Kee Lee[e], Chin Fei Chee[e], Noorsaadah Abdul Rahman[e]

[a]*School of Science, Monash University Malaysia Campus, Jalan Lagoon Selatan, Bandar Sunway, 47500, Selangor, Malaysia.*

[b]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Minden, 11800, Penang, Malaysia.*

[c]*School of Pharmacy, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia*

[d]*Center for Cancer and Stem Cell Research, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.*

[e]*Department of Chemistry, University of Malaya, 50603 Kuala Lumpur, Malaysia.*

Abstract:

Both sirtuin and poly(ADP-ribose)polymerase (PARP) family of enzymes utilize NAD+ as co-substrate. Inhibitors of sirtuins and PARPs are important tools in drug discovery as they are reported to be linked to multiple diseases such as cancer. New potent sirtuin inhibitors (2,4,6-trisubstituted benzimidazole) were discovered from reported PARP inhibitor scaffold.

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Interestingly, the synthesized compounds have contrasting sirtuin and PARP-1 inhibitory activity. We showed that modification on benzimidazoles may alter their selectivity towards sirtuin or PARP-1 enzymes. This offers an opportunity for further discovery and development of new promising sirtuin inhibitors. Molecular docking studies were carried out to aid the rationalization of these observations. Preliminary antiproliferative studies of selected compounds against nasopharyngeal cancer cells also showed relatively promising results.

Keywords: Sirtuin, PARP, Benzimidazole, Anticancer, Nasopharyngeal, Molecular docking

Yeong Keng Yoon

[a]*School of Science, Monash University Malaysia Campus, Jalan Lagoon Selatan, Bandar Sunway, 47500, Selangor, Malaysia.*

[b]*Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Minden, 11800, Penang, Malaysia.*

Phone: +603 55145102

Fax: +603 55146184

Emails: Yeong.KengYoon@monash.edu

Introduction

The class III histone deacetylases (HDACs), or sirtuins include a group of proteins that are homologous with the yeast SIR2 family of proteins [1,2]. Unlike other classes of HDACs, the function of sirtuins is dependent on NAD+, which they use as a cofactor in the catalysis. To date, seven mammalian sirtuins have been identified (SIRT1-7) [3]. Even though all sirtuins

share a common catalytic domain, they each have unique additional N-terminal and/or C-terminal sequences of variable length, thus displaying different catalytic activities and substrate specificities [4]. Of the seven sirtuins, SIRT1 and SIRT2 have been linked to a plethora of diseases such as cancer [5,6], Alzheimer's [7] and Parkinson's [8] disease.

Both SIRT1 and SIRT2 are believed to have a role in the development of cancer as their functions are frequently altered in cancer cells. SIRT1 was shown to be upregulated in a spectrum of cancers, such as in leukemia [9] as well as prostate cancer [10]. Furthermore, since SIRT1 and SIRT2 both fulfill the same function of deacetylating p53, it has been reported that the simultaneous inhibition of SIRT1 and SIRT2 was beneficial against cancer by completely blocking p53 deacetylation, which led to cell death [11].

Several notable classes of small molecule sirtuin inhibitors which have been identified so far includes indoles (EX-527) [12], α-cyanopropenamide (AGK2) [13] and tenovins (Tenovin-6) [14]. Recently, compounds based on the benzimidazole scaffold have been demonstrated to be potent sirtuin inhibitors [15-18].

Sirtuins and poly(ADP-ribose) polymerases (PARPs) both are strongly conserved in eukaryotes and share the particularity of using NAD+ as a co-substrate [19,20]. Both PARPs and sirtuins cleave NAD+ into nicotinamide and ADP-ribose. Thus, it will be interesting to investigate if other benzimidazole motif such as 2,4-disubstituted benzimidazoles which have been reported as potent PARP inhibitors [21] share similar inhibitory potency for sirtuin enzymes. We hereby report on the synthesis of some 2,4-disubtituted and 2,4,6-trisubstituted benzimidazoles and subsequently evaluated their sirtuin inhibitory activities. In addition, antiproliferative activity of selected synthesized benzimidazole derivatives against c666-1 (nasopharyngeal) carcinoma cell line was also reported. Molecular docking was performed to rationalize the observed changes in activity.

1. MATERIALS AND METHODS

All other chemicals were purchased from Sigma-Aldrich or Merck and were of analytical grade. All melting points were taken on a Stuart melting point apparatus SMP30 (Staffordshire, UK). NMR spectra were obtained using Jeol FX-270 (270 MHz) (Jeol Ltd., USA) and Bruker Avance III HD (400 MHz) (Bruker Biospin Corp., Billerica, USA) NMR spectrometers with tetramethylsilane as the internal standard. All chemical shifts are reported in ppm. MS analysis was performed on an Agilent 6500 series accurate mass Q-TOF (Agilent Technologies, Santa Clara, CA, USA) Analytical thin-layer chromatography (TLC) was carried out on pre-coated aluminum silica gel sheets (Kieselgel 60 F254) from Merck (Darmstadt, Germany). Column chromatography was performed with silica gel 60 (230–400 mesh) from Merck.

1.1 General procedure for synthesis of compounds 1-10

To a solution of carboxylic acid ***b*** (1 mmol) in pyridine (1 mL) and DMF (1 mL) was added 1,1'-carbonyldiimidazole (CDI, 1.1 mmol) and the solution was heated at 50 ^{o}C for 2 h. Then diamine ***a*** (1 mmol) was added and the mixture was stirred at room temperature overnight. Solvents were removed and the residue heated in 2 mL of acetic acid at 100 ^{o}C for 1 h. After concentration, the residue was extracted with ethyl acetate and saturated sodium bicarbonate solution. The organic layers were washed brine, dried over $MgSO_4$ and evaporated. The residue was subjected to column chromatography (dichloromethane-methanol, 9:1) to afford the title compound as free base.

1.2 Molecular docking

The crystal structure of human SIRT2 (PDB code: 3ZGV) and PARP-1 (PDB code: 4HHZ) were taken from the Protein Data Bank. Both 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. Ligand was 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.

1.3 Cell lines and cytotoxic assay

All human cell lines were obtained from American Type Cell Culture (ATCC) unless specified. Human breast epithelial cells, MCF-10A were cultured in Dulbecco's modified Eagle's medium and Ham's (DMEM/F12) supplemented with 5% horse serum, 20ng/mL epidermal growth factor, 0.5µg/mL hydrocortisone, 10µg/mL insulin, 100IU/mL penicillin and 100 µg/mL streptomycin. Human colon epithelial cells, CCD 841 CoN was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum, 100IU/mL penicillin and 100 µg/mL streptomycin. NP460 cells were maintained in Keratinocyte-SFM containing epidermal growth factor and bovine pituitary extract (BPE) (Invitrogen, USA). Human endometrial fibroblasts (T-HESC) were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1.5g/L sodium bicarbonate, 500ng/mL puromycin and 1% ITS Premix (Corning, USA). Human nasopharyngeal carcinoma cells (c666-1) and human lung fibroblast (MRC5) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. All cells were kept in 37 ^{o}C incubator with 5% carbon dioxide.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell viability assay was used to determine the cytotoxicity of compounds on the human non-transformed cell lines as optimized in previous studies [22,23]. All data were reported as mean ± standard deviation (S.D) from a minimum of three independent experiments. Statistical significant difference between negative control and treated cells were analysed using student independent *t*-test. A *p*-value less than 0.05 ($p<0.05$) was considered significant different.

2. Results

2.1 Synthesis of compounds

The benzimidazoles were synthesized using reported method outlined in Figure 1 [21]. Different functional groups were placed at the $\mathbf{R^1}$ and $\mathbf{R^2}$ position of the corresponding diamine and carboxylic acid intermediates through *de novo* synthesis [24]. Direct coupling of diamine ***a*** with carboxylic acid ***b*** in the presence of 1,1'-carbonyldiimidazole (CDI) gave amide ***c***. Subsequent thermal cyclization of the amide ***c*** in acetic acid afforded benzimidazole ***d*** in two-steps (total yield = 38 ~ 75%). Compound characterization details can be found in the Supporting Information.

2.2 Sirtuin enzymatic assays

The *in vitro* enzymatic screening assay for SIRT1 and SIRT2 inhibitory activity were performed using fluorimetric sirtuin kits (AnaSpec, Fremont, CA) according to the manufacturer's protocol. EX-527 (SIRT1 selective inhibitor), AGK-2 (SIRT2 selective inhibitor) and Tenovin-6 (pan-SIRT1/2 inhibitor) were used as standard control while DMSO was used as the vehicle control. Experiments were performed in triplicates and standard deviations obtained from all experiments are less than 20%.

All the ten evaluated compounds demonstrated relatively higher selectivity for SIRT2 over SIRT1 (Table 1). The trend of the results is consistent with those observed previously for 1,2,5-trisubstituted benzimidazole derivatives [15-18]. Compounds **3-6**, with functional groups other than a phenyl/pyridinyl ring at the $\mathbf{R^2}$ were found to have poor activity. Neither electron donating ($-OCH_3$) nor electron withdrawing ($-CF_3$) showed significant activities. However, the sirtuin inhibitory activity remarkably improved when bulkier groups such as phenyl, phenyl(pyrrolidinyl) or pyridinyl(pyrrolidinyl) were attached to the $\mathbf{R^2}$ position. These observations implied that a relatively bulkier system at the $\mathbf{R^2}$ position is required to sufficiently occupy the active cavity of the sirtuin enzymes, especially the SIRT2 enzyme. Meanwhile, incorporating a methyl group at the $\mathbf{R^1}$ position of the benzimidazole ring has also greatly improved the sirtuin inhibitory activity. This was showcased by the increase in potency for those compounds which carry the methyl group across the whole series. The results shown here along with our earlier reports clearly demonstrated the potential of benzimidazoles acting as potent sirtuin inhibitors.

2.3 Molecular docking

Molecular docking for both compound **9** and **10** into the active site of SIRT2 (PDB entry code: 3ZGV, x-ray resolution = 2.30 Å) [25] and PAPR-1 (PDB entry code: 4HHZ, x-ray resolution = 2.72 Å) [26] were performed and compared in view of their observed significant difference in activity. The receptor and the drug candidate were optimized before actual docking in Autodock 4.2 using standard procedure of the software.

The region which controls the activity of sirtuins can generally be divided into three binding sites, namely the adenosine binding site (A pocket), ribose binding site (B pocket) and nicotinamide binding site (C pocket) [27]. Analysis of the top-ranked pose of compound **10**

within the SIRT2 active binding site demonstrated several plausible molecular interactions. Docking analysis reveals that compound **10** interacts with SIRT2 primarily due to hydrogen bonding. Strong hydrogen bond could be observed between the N-H group from the benzimidazole scaffold with Arg97. Meanwhile, the amide moiety from the compound could form intra-molecular bonding with the N atom from the imidazole ring. Moreover, it can also form hydrogen bond with Ile169 to stabilize the overall structure. Hydrogen bond interaction can also be observed between the C=O from the phenyl(pyrrolidinyl)methanone moiety and Glu288 (Figure 2). As shown through the *in vitro* assays, compound **10** (as of all the other compounds) displayed weaker SIRT1 inhibitory activity. It was found to dock in part of the C pocket (Ile347, Gly319 and Asp348) and loosely occupy part of the A pocket (Tyr280) and acetyl lysine channel (Phe414) of the active site (Figure 3).

Through the acquired *in vitro* assay results, it was noted that changes at the $\mathbf{R^1}$ position played important role towards the potency of the compounds. Therefore, docking for compound **9** (which lacks the methyl group at $\mathbf{R^1}$ and showed much weaker sirtuin inhibitory activity) was subsequently carried out to rationalize this observation. Poses for both compounds were then compared as shown in Figure 4. The docking analysis of compound **9** showed that part of the compound (-phenyl(pyrrolidinyl)methanone) shifted out from the A and B binding site of SIRT2 with different binding orientation, resulting in less favorable complexes. This could explain the weaker *in vitro* SIRT2 inhibitory activities observed.

In comparison, both compound **9** and **10** have weaker SIRT1 activity. Similarly, the effect of the methyl group on PARP-1 activity was investigated through molecular modeling. When the docking poses of both compound **9** and **10** were superimposed, it was observed that both compounds displayed different structural conformation (Figure 5). Notably, the important interactions between amide group of **10** with Trp200, His201 and Gly202 were lost, leading

to its weaker inhibitory activity as compared to **9**. The additional methyl group at the $\mathbf{R^1}$ position thus have a contrary effect on the activities of sirtuins and PARP-1.

2.4 Cellular Assay

Sirtuins has been previously implicated in cancer modulation and sirtuin inhibitors has been demonstrated to possess anticancer activity in the literature [28,29]. To investigate the effectiveness of the synthesized compounds as anticancer agents, the three most potent sirtuin inhibitory compounds (**2, 8** and **10**) were evaluated for their ability to impede the proliferation of metastatic human nasopharyngeal carcinoma cells (c666-1). Compounds **2, 8** and **10** were screened at 50 µM using MTT assay and incubated for 72 h. Interestingly, all three active compounds managed to significantly ($p < 0.05$) restrain the growth of c666-1 cancer cells (Figure 6). The tested compounds showed no significant cytotoxicity against a panel of normal cells comprising breast (MCF-10A), colon (CCD 841 CoN), nasopharyngeal (NP460), endometrial (T-HESC) and lung (MRC5) cells at 50 µM (with cell viability > 90%) unlike 5-FU which demonstrated widespread non-specific cytotoxicity [30,31].

3. Discussion

Crosstalk between sirtuins and PARP enzymes have previously been reported [19,20]. There were interest in the interplay of both classes of enzymes as they are involved in the same biological processes (competition for the common NAD+ co-substrate, transcriptional effects and post-translational modifications). Although sirtuins and PARPs both have NAD+ as co-substrate and cleave NAD+ into nicotinamide and ADP-ribose, the inhibitory effects of the synthesized benzimidazoles for sirtuin enzymes were interestingly in stark contrast to those

observed for PARP-1. Recently, Ekblad and Schüler have reported that the activity of sirtuins are unaffected by the most important PARP inhibitors such as rucaparib and veliparib due to the inaccessibility of PARP inhibitors getting into sirtuin cofactor site [32]. For this series of compounds, we found that those with methyl substitution at $\mathbf{R^1}$ such as **2**, **8** and **10** are poor PARP-1 inhibitors but are potent sirtuin (SIRT2) inhibitors while non-substituted compounds at $\mathbf{R^1}$ such as **1**, **7** and **9** are potent PARP-1 inhibitors but are poor sirtuin inhibitors. Compounds **3-6** were found to be poor inhibitors for both enzymes. Although only 10 compounds were evaluated in this study, the fact that novel potent sirtuin inhibitors were positively identified demonstrated the potential to similarly unearth sirtuin modulators by minor modification from other known NAD+ targeting scaffold.

One point to note was that there has not been any literature at drug discovery level linking nasopharyngeal cancer and SIRT2 although down-regulation of SIRT2 in nasopharyngeal carcinoma when treated with seliciclib was reported [33]. Latent membrane protein 1 (LMP1) is known to be expressed in the majority of nasopharyngeal carcinoma cells [34]. It was found that LMP1 up-regulates hypoxia-inducible factor-1α (HIF-1α) transcription of nasopharyngeal cancer cells [35]. Few studies have examined the relationship between sirtuin and HIF, and among the very few studies carried out, it was found that SIRT2 destabilizes HIF-1α [36]. The SIRT2 inhibitory compounds identified in this study may then have exerted their antiproliferative effect towards nasopharyngeal carcinomas by targeting HIF-1α via LMP1 down-regulation. This deserves to be further investigated as it may lead to a better understanding of sirtuin mediated effects on nasopharyngeal cancer.

Conclusion

In conclusion, we have discovered new potent sirtuin inhibitors from identified PARP inhibitor scaffold. We demonstrated that minor modification such as the introduction of a methyl group could alter the potency and selectivity of a compound towards either sirtuin or PARP-1 inhibition. Thus, this new general approach could be applied to aid the search for new promising sirtuin inhibitors from an existing class of compound. In the process, we have also showed the potential of 2,4,6-trisubstituted benzimidazole derivatives acting as potent sirtuin inhibitors. Moreover, these compounds possessed good anticancer activity against the c666-1 nasopharyngeal cancer cell line and showed no significant cytotoxicity against a panel of normal cell lines tested in this study.

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The authors declare no conflict of interest.

Figure Legends

Figure 1. General synthesis of benzimidazoles **1-10**. Reaction conditions: (i) CDI, pyridine, DMF, (ii) AcOH, 110 ^{o}C.

Figure 2. Compound **10** docked in the active site of SIRT2 (PDB: 3ZGV). Yellow dashed lines show hydrogen bonds.

Figure 3. Compound **10** docked in the active site of SIRT1 (PDB: 4I5I).

Figure 4. Part of compound **9** (purple) was located out from the active site of SIRT2 when docked while compound **10** (green) fits well into the groove (PDB: 3ZGV).

Figure 5. In the PARP-1 active site, important interactions with Gly202, His201 and Trp200 were lost with compound **10** (yellow) when the phenyl(pyrrolidinyl)methanone side chain was oriented pointing in a different direction (PDB: 4HHZ).

Figure 6. Human nasopharyngeal cancer cells (c666-1), and a panel of human non-cancers cells were treated with 50μM of compound **2, 8, 10** and 5-FU for 72 hours. MTT assay was used to quantify cell viability. Results were the mean ± S.D. from at least 3 independent experiment. Symbol "*" indicates statistical significant ($p<0.05$) of treated cells compared to negative control (0.1% DMSO) using on student independent *t*-test.

Supporting Information contents:

1. Compound characterization details
2. NMR spectra
3. Sirt1 *in vitro* assay
4. Sirt2 *in vitro* assay
5. Cytotoxicity assay protocol

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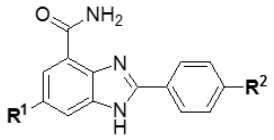
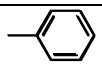
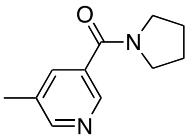
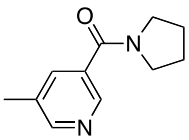
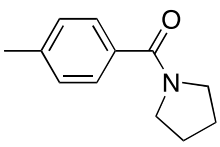
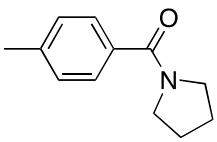
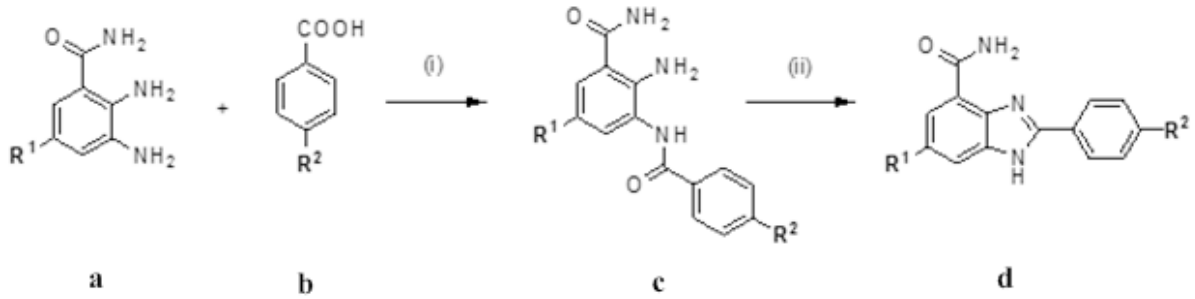
Table 1. SIRT1 and SIRT2 inhibitory activities of benzimidazoles **1-10**.



Compound	R^1	R^2	SIRT1 IC_{50} (μM) ± S.D. or % inhibition at 20μM	SIRT2 IC_{50} (μM) ± S.D. or % inhibition at 20μM	PARP-1 IC_{50} (μM) or % inhibition at 10 μM*
1	-H		19.1 %	36.5 %	0.71
2	$-CH_3$		54.8 ± 2.30 μM	9.2 ± 0.75 μM	28%
3	-H	$-OCH_3$	10.6 %	26.3 %	NA
4	$-CH_3$	$-OCH_3$	13.8 %	29.7 %	NA
5	-H	$-CF_3$	6.4 %	16.8 %	NA
6	$-CH_3$	$-CF_3$	8.5 %	10.2 %	NA
7	-H		29.9 %	45.0 %	0.029
8	$-CH_3$		44.8 ± 1.91 μM	10.9 ± 1.10 μM	NA
9	-H		23.3 %	46.4 %	0.012
10	$-CH_3$		34.6 ± 1.16 μM	6.5 ± 0.97 μM	17%
EX-527	-	-	0.30 ± 0.10 μM	N.D.	N.D.
AGK2	-	-	N.D.	8.34 ± 2.05 μM	N.D.
Tenovin-6	-	-	42.1 ± 3.39 μM	25.6 ± 3.08 μM	N.D.

N.D. Not determined; N.A. Not active (less than 15% inhibition at 10 μM). * Reported in [24]



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