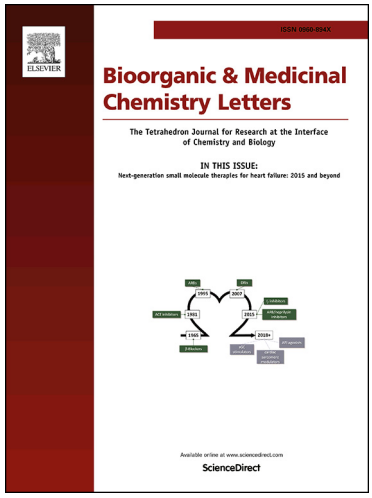
Accepted Manuscript



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Design, synthesis and biological evaluation of benzimidazole derivatives as novel human Pin1 inhibitors

Tianyi Ma, Min Huang, Aihua Li, Feng Zhao, Deyi Li, Dan Liu* and Linxiang Zhao*

Key Laboratory of Structure-based Drug Design and Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang 110016, P. R. China.

*Corresponding author:

Dan Liu: Tel: +86-024-43520218; E-mail: liudan@syphu.edu.cn

Linxiang Zhao: Tel: +86-024-43520221; E-mail: linxiang.zhao@vip.sina.com

ABSTRACT

In this work, a series of novel benzimidazole derivatives were designed and synthesized as Pin1 inhibitors. Protease-coupled assay was used to investigate the Pin1 inhibitory potency of all synthesized compounds. Thirteen of them showed preferable Pin1 inhibitory effects with IC_{50} values lower than 5 μM, and **12a**, **15b**, **15d** and **16c** exhibited the most promising Pin1 inhibitory activity at low micromolar level (0.33~1.00 μM) than the positive control compound Juglone. Flow cytometry results showed that treating PC-3 cells with **16c** caused slight cycle arrest in a concentration-dependent manner. The structure-activity relationships of R^1, R^2, R^3 and linker of the benzimidazole derivatives were analyzed in detail, which would help further exploration of new Pin1 inhibitors.

Keywords: Pin1 inhibitors, PPIase, 4-(1*H*-benzoimidazol-2-yl)-benzsulfamides, PC-3, Prostate cancer

Pin1 (Protein interaction with never in mitosis A1) is a kind of PPIase (peptidyl-prolyl cis-trans isomerase) which specifically catalyzed the isomerization of pSer/Thr-Pro peptide bonds in its substrate proteins to modulate their function and stability [1-3]. Abnormal expression of Pin1 often led to a variety of diseases especially cancer due to that pSer/pThr-Pro sequence existed in many functional proteins and regulated cell proliferation and differentiation [4-6]. Pin1 is overexpressed in various types of cancer, including prostate cancer, hepatic cancer, esophageal cancer and so on [7]. The expression of Pin1 correlates with malignant grade and poor prognosis in patients with cancer. Pin1 is reported to be involved in regulating many signaling processes such as cell cycle controllers, which was known to be regulated by Pin1 activity. In addition, Pin1 knockout mice developed normally to adulthood indicated that Pin1 was expected to become an ideal antitumor drug target [8].

There has been persistent interest for developing Pin1 inhibitors, and numbers of promising entities have been identified (**Figure 1**) [9-12]. Juglone was the first Pin1 inhibitor discovered by high-throughput screening, which was used as positive control widely at present [13]. The α, β-unsaturated ketone structure could interact with thiol of Cys113 in PPIase domain through Michael addition and played irreversible inhibition activity against Pin1. Peptidomimetics Pin1 inhibitors (PEP-3) exhibited strong enzyme inhibition activity at the molecular level [14-23], while it had the

poor stability and cell membrane permeability. Other small molecule inhibitors were designed based on PPIase activity pocket of Pin1, just like PFI-1 [24-27]. Unfortunately, most of these compounds did not show antitumor activity on cancer cells. A variety of strategies have been taken later to improve the activity of the compounds in cell level without breakthrough [28]. Our objective was to identify some small molecules as pin1 inhibitors with antiproliferative activity.

(Figure 1 should be listed here)

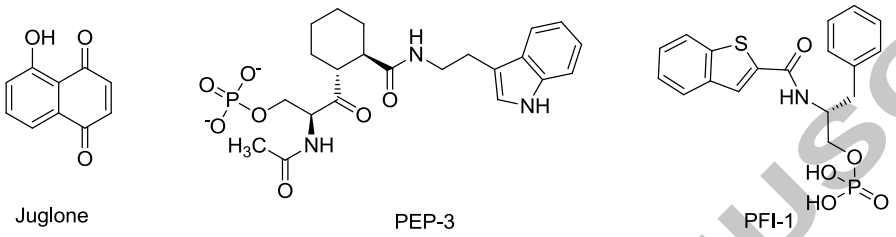


Figure 1. The structures of reported Pin1 inhibitors

Previously reported Pin1 inhibitors usually consisted of three fragments, forming necessary interactions with prolyl pocket, hydrophobic superficial area and basic amino acids enrichment region respectively. According to Jonathan D. Moore's study, the 3-(1*H*-benzo[d]imidazol-2-yl) propanoic acid moiety had been validated fitted well into the prolyl pocket and basic domain of Pin1. Furthermore, the docking result confirmed that an extra hydrophobic fragment at N1-position of benzimidazole occupied the shallow surface of Pin1 protein could enhance Pin1 inhibitory activity significantly (**Figure 2**). Therefore, hydrophobicity aromatic fragments were introduced to make the target compounds further combined with hydrophobic superficial PPIase domain. Besides, conformation qualified of compounds can reduce entropy penalty in the process of the ligand molecules binding to a receptor protein, and increase the stability of the complexes [29]. Therefore, we hoped to reduce polarity while maintain Pin1 inhibitory activity through replacing fatty acids with aromatic hydrophobic acids. As a result, the flexible propionic acid segments of benzimidazole C2-position were replaced with the rigid benzoic acids. Since the carboxyl performed to be hydrophilic excessively which was unfavorable to cell activity, we suspected that increasing hydrophobic moiety and replacing with bioisosteres will give Pin1 inhibitors with cell activity.

(Figure 2 should be listed here)

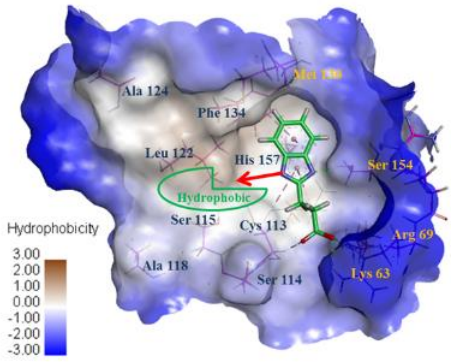
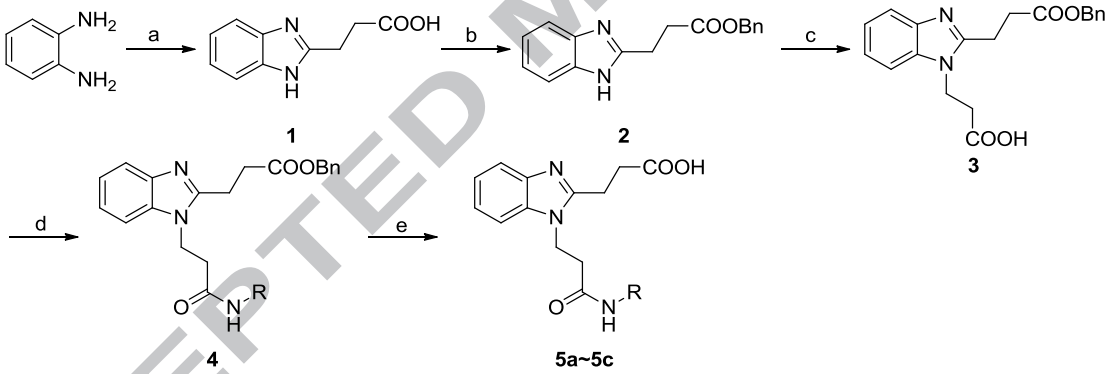


Figure 2. The predicated binding modes of 3-(1*H*-benzo[d]imidazol-2-yl) propanoic acid moiety

The synthetic route adopted for preparation of target compounds **5a~5c** was outlined in **Scheme 1**. The cyclization reaction of succinic anhydride and succinic anhydride afforded **1** easily in high yields, which was protected by a benzyl using benzyl chloride under basic conditions and then alkylated to give the key intermediate **3**. Subsequently, in the presence of EDCI, the coupling of **3** with differently substituted aniline yield **4**. An efficient deprotection was performed to afford compounds **5a~5c**.

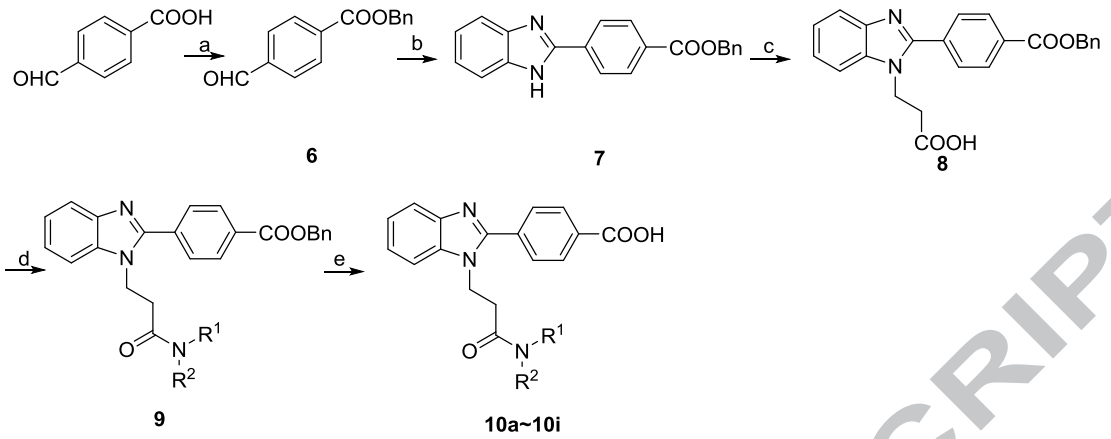
(Scheme 1 should be listed here)



Scheme 1. Synthesis of compounds **5a~5c.** Reagents and conditions: a) succinic anhydride, dioxane, 80 ^{o}C; b) BnCl, K_2CO_3, DMF, r.t.; c) 3-bromopropionic acid, K_2CO_3, acetone/DMF/H_2O, 70 ^{o}C; d) substituted aniline, EDCI, HOBt, CH_2Cl_2, r.t.; e) LiOH, THF/H_2O, 10 ^{o}C or H_2, 5% Pd/C, MeOH, r.t..

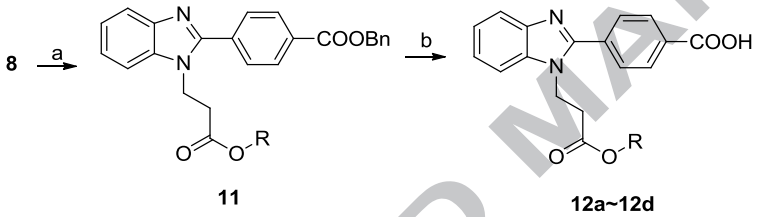
The synthetic route of target compounds **10a~10i, 12a~12d**, **15a~15d** and **16a~16c** was described in **Scheme 2~ Scheme 4**. Start material 4-carboxybenzaldehyde was protected by benzyl, then cyclization and alkylation were proceeded to provide **8**, which was condensed with different arylamines and debenzylation was performed to afford compounds **10a~10i**. The coupling of **8** with different phenol or naphthol and deprotection was performed to afford compounds **12a~12d**. Nucleophilic substitution and deprotection of key intermediate **7** underwent readily to give compounds **15a~15d**, which continued to react with differently substituted sulfonamide to afford compounds **16a~16c**.

(Scheme 2 should be listed here)



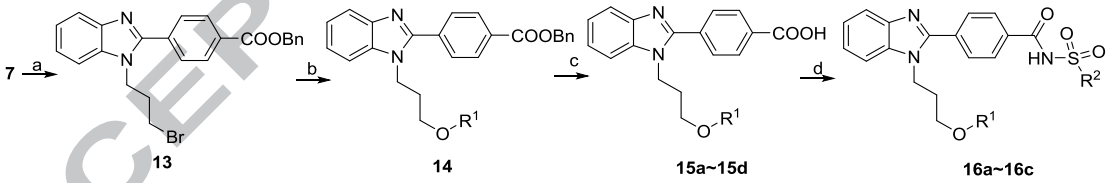
Scheme 2. Synthesis of compounds **10a~10i**. Reagents and conditions: a) BnBr, K_2CO_3, DMF, r.t.; b) 1,2-diaminobenzene, DMF/H_2O, 80°C; c) 3-bromopropionic acid, K_2CO_3, acetone/DMF/H_2O, 70 °C; d) substituted arylamine, EDCI, HOBt, 4-methylmorpholine, CH_2Cl_2, r.t. or substituted arylamine, $SOCl_2$, CH_2Cl_2, r.t.; e) LiOH, THF/H_2O, 10 °C or H_2, 5% Pd/C, MeOH, r.t..

(Scheme 3 should be listed here)



Scheme 3. Synthesis of compounds **12a~12d**. Reagents and conditions: a) $SOCl_2$, CH_2Cl_2, r.t., substituted phenol or naphthol, 5% NaOH, 10 °C; b) H_2, 5% Pd/C, MeOH, r.t..

(Scheme 4 should be listed here)

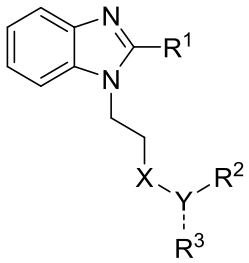


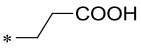
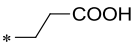
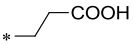
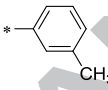
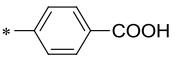
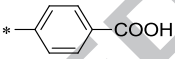
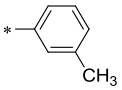
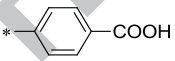
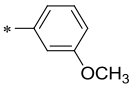
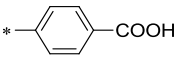
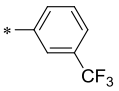
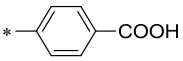
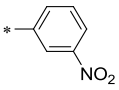
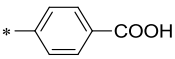
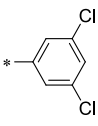
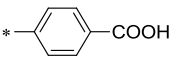
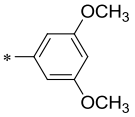
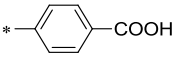
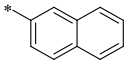
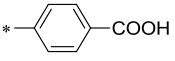
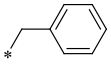
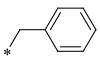
Scheme 4. Synthesis of compounds **15a~15d** and **16a~16c**. Reagents and conditions: a) 1,3-dibromopropane, K_2CO_3, MeCN, 82 °C; b) substituted phenol or naphthol, K_2CO_3, acetone, reflux; c) LiOH, THF/H_2O, r.t.; d) $R_2SO_2NH_2$, EDCI, DMAP, DMF, 0 °C to r.t..

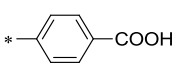
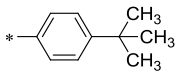
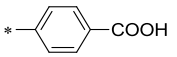
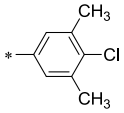
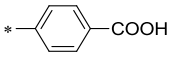
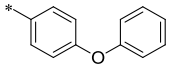
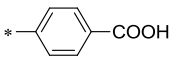
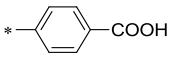
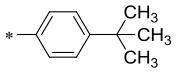
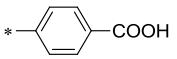
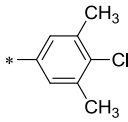
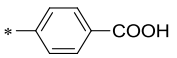
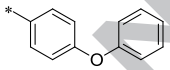
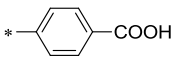
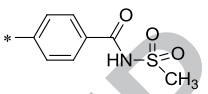
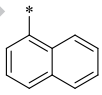
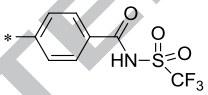
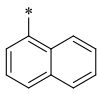
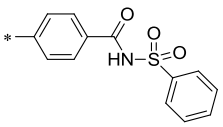
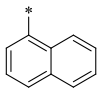
Protease-coupled assay was used to investigate the Pin1 inhibitory potency of all synthesized compounds. As shown in **Table 1**, compounds **5a~5c** which bearing hydrophobicity aromatic fragments accompanied using ethyl as linker possessed moderate Pin1 inhibitory activity (7.56~19.72 μM). Phenyl amide derivative **5a** displayed modest activity with IC_{50} value of 19.72 μM. The inhibitory activities of compounds **5b** and **5c** with monosubstituted phenyl of side chain were increased to 9.81 μM and 7.56 μM, respectively.

(Table 1 should be listed here)

Table 1. The structures and Pin1 inhibitory activities of all the target compounds.



Compound	X	Y	R^1	R^2	R^3	IC_{50} (μM) [a]
5a	CO	N			H	19.72
5b	CO	N			H	9.81
5c	CO	N			H	7.56
10a	CO	N			H	7.27
10b	CO	N			H	1.10
10c	CO	N			H	18.68
10d	CO	N			H	11.28
10e	CO	N			H	13.62
10f	CO	N			H	2.89
10g	CO	N			H	3.57
10h	CO	N				1.53
10i	CO	N				5.40

12a	CO	O			-	13.89
12b	CO	O			-	0.72
12c	CO	O			-	1.02
12d	CO	O			-	1.87
15a	CH_2	O			-	1.89
15b	CH_2	O			-	0.47
15c	CH_2	O			-	2.20
15d	CH_2	O			-	0.33
16a	CH_2	O			-	7.83
16b	CH_2	O			-	2.26
16c	CH_2	O			-	1.00
Juglone	-	-	-	-	-	10.81

[a]The IC_{50} value was measured at two independent experiments.

The inhibition data of compounds **10a** and **10b** with aromatic acids substitution verified the hypothesis that introduction of benzoic acid caused to significant improvement on pin1 inhibition (**10a** vs. **5b**, **10b** vs. **5c**). The compounds **10c**~**10g** with meta- electron-withdrawing or electron-donating substituents of phenyl were synthesised for SARs study. Among them, compounds **10f** and **10g** showed improved potency with IC_{50} of 2.89 μM and 3.57 μM, but other modifications were not tolerable. In addition, **10 h** (1.53 μM) and **10i** (5.40 μM) with bulkier and more lipophilic segments on nitrogen was unable to further enhance the pin1 inhibition potency. The reason for this phenomenon might be the stereo-hindrance effect from the interaction of two aromatic rings. Therefore, follow-up studies focused

on single hydrophobic aromatic ring. Meanwhile, other linkers such as ester bond or ether bond were investigated. In addition, compounds **16a~16c** were prepared to investigate effects of carboxylic acid bioisosteres [30].

Compounds **12a~12d** replacing amide with ester bond of linker at N1-position of benzimidazole exhibited different pin1 inhibition potency. Among them, compounds **12b~12d** displayed outstanding activity with IC_{50} value ranged from 0.72 μM to 1.87 μM, whereas **12a** are less active among them. Compounds **15a~15d** bearing ether bond linker were designed and synthesized. The results showed that **15a~15d** displayed excellent pin1 inhibition potency with IC_{50} value ranged from 0.33 μM to 2.20 μM, and **15d** (0.33 μM) was most potent. Taken together, ether bond between benzimidazole and substituention of N1-position were better. Compounds **16a** (7.83 μM), **16b** (2.26 μM), **16c** (1.00 μM) are sulfonamides bioisosteres synthesized from the corresponding carboxylic acid **15d**, compound **16c** with benzene sulfonamide formyl was the most excellent which showed 1.00 μM values against Pin1.

Pin1 inhibitors were anticipated to interfere with multiple cellular processes including cell-cycle regulation, we therefore tested whether **16c** which has the potential to be more cell-permeable interfered with cell cycle. **16c** was evaluated for the antiproliferative activity against PC-3 cell lines using MTT assay, and the results indicate that **16c** showed excellent activity with GI_{50} value of 9.0 μM. The impact to cell cycle of **16c** at 2.5 μM, 5 μM, and 10 μM were investigated through flow cytometry to verify the antiproliferative activity were correlated with inhibitory activity against Pin1 (**Figure 3**). The results showed that there was slight cycle arrest in a concentration-dependent manner in G_0/G_1 phase of PC-3 cells after treatment with **16c** for 24 h. Therefore, these data provided considerable evidence that the antiproliferative effects on PC-3 cells of **16c** were mediated, at least partly, via inhibition of Pin1.

(Figure 3 should be listed here.)

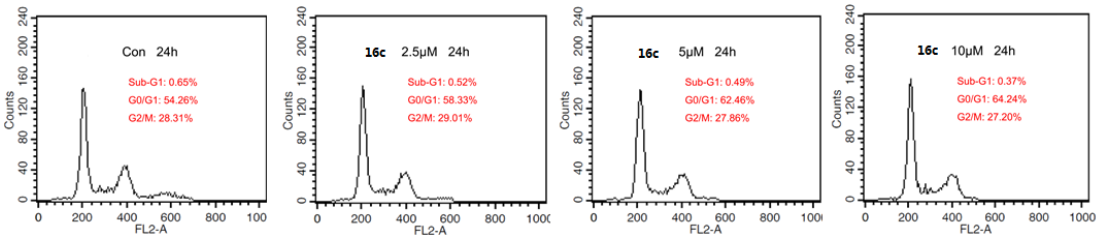


Figure 3. The regulation of cell cycle in PC-3 cells treated with **16c** for 24 h.

Molecular docking simulation studies were carried out to explore the binding modes of target compound **16c** with Pin1 protein. The results in **Figure 4** indicated that **16c** retained the key interaction

in the PPIase domain. The nitrogen at 3-position of imidazole ring formed hydrogen bond with Ser154, and sulfonyl of benzene sulfonamide formyl participated in hydrogen bond formation with Arg69 mimic carboxyl. In addition, the naphthalene of benzimidazole at C2-position formed hydrophobic interaction with the surrounding nonpolar amino acid. By analyzing the interactions mentioned above, it was cogent that the benzene sulfonamide formyl and naphthalene moiety were greatly helpful to enhance the Pin1 inhibition ability.

(Figure 4 should be listed here.)

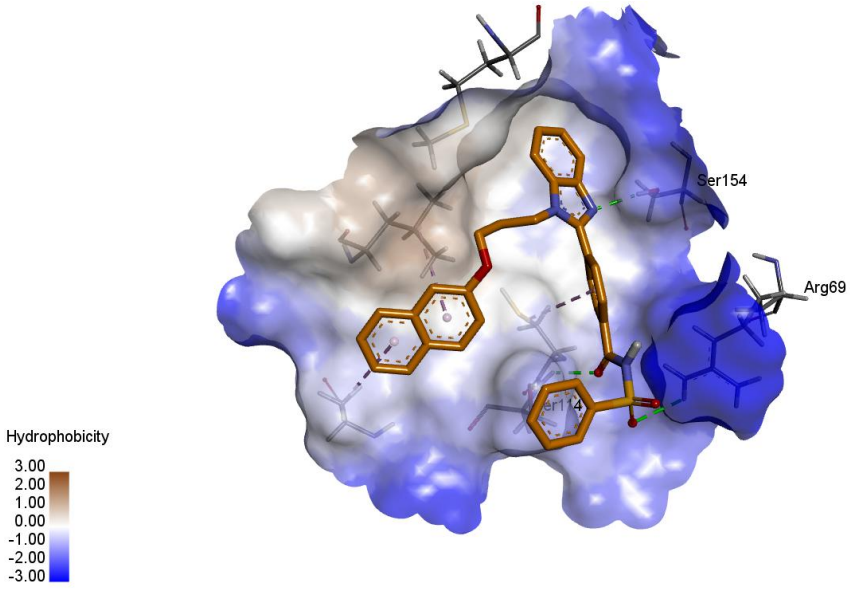


Figure 4. The predicated binding modes of **16c** with Pin1 PPIase domain

In summary, we synthesized a series novel 1,2-bis-substituted benzimidazoles derivatives as Pin1 inhibitors. The structure-activity relationship of these small-molecule inhibitors was discussed. Compound **15d** was the most potent compound with an IC_{50} value of 0.33 μM targeting Pin1. Flow cytometry results indicated **16c** induced slight cycle arrest in a concentration-dependent manner in G_0/G_1 phase against PC-3 cells. The docking studies with pin1 protein showed that the benzimidazole scaffold, benzene sulfonamide formyl moieties and naphthalene were necessary for target compounds to possess potent antitumor activities. This study provides a different class of Pin1 inhibitors bearing benzimidazole scaffold, which deserves further research.

Acknowledgment

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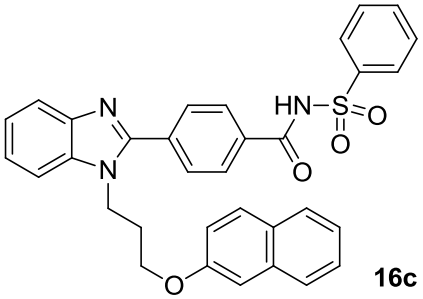
Graphical abstract



16c

Pin1: IC_{50} = 1.0 μM

PC-3 : GI_{50} = 9.0 μM