

# **Discovery of Novel *N*-Benzylbenzamide Derivatives as Tubulin Polymerization Inhibitors with Potent Antitumor Activities**

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## **Abstract**

A series of novel *N*-benzylbenzamide derivatives were designed and synthesized as tubulin polymerization inhibitors. Among fifty-one target compounds, compound **20b** exhibited significant antiproliferative activities with IC<sub>50</sub> values ranging from 12 to 27 nM against several cancer cell lines, and possessed good plasma stability and satisfactory physicochemical properties. Mechanism studies demonstrated that **20b** bound to the colchicine binding site and displayed potent anti-vascular activity. Notably, the corresponding disodium phosphate **20b-P** exhibited an excellent safety profile with the LD<sub>50</sub> value of 599.7 mg/kg (i.v. injection), meanwhile, it significantly inhibited tumor growth and decreased microvessel density in liver cancer cell H22 allograft mouse model without obvious toxicity. Collectively, **20b** and **20b-P** are novel promising anti-tubulin agents with more druggable properties and deserve to be further investigated for cancer therapy.

**Keywords:** *N*-benzylbenzamide, Anti-vascular, Druggability, Tubulin inhibitors, Antitumor.

## **1. Introduction**

Microtubules are stiff, cylindrical polymers of  $\alpha$ ,  $\beta$ -tubulins, and the head-to-tail association of  $\alpha$ - $\beta$  heterodimers forms linear protofilaments [1, 2]. They play diverse roles in cells, such as protein transport, maintaining cell structure, chromosome segregation and mitosis [3-5]. Microtubule targeting agents (MTAs) such as paclitaxel or vincristine, have been widely used in clinics for the treatment of various cancers [6, 7].

Six binding sites for taxane, laulimalide, vinca alkaloid, pironetin, maytansine and colchicine have been identified in microtubules. Inhibitors targeting taxane and laulimalide binding sites can stabilize microtubules while those targeting other four binding sites can destabilize microtubules [6, 8]. Currently, all FDA approved drugs that disrupt microtubule polymerization for cancer treatment bind to either the taxane binding site or the vinca alkaloids binding site. In recent decades, colchicine binding

site inhibitors (CBSIs) have held great potential as a new generation of tubulin inhibitors, which can overcome the limitations of existing tubulin inhibitors such as structure complexity, poor aqueous solubility, narrow therapeutic index, wide inter-patient variability and high toxicity [9-18]. Notably, CBSIs exhibit promising ability to rapidly disrupt existing tumor blood vessels, leading to the collapse of blood vessels and tumor cell death [19, 20].

Several typical small-molecular inhibitors of tubulin targeting the colchicine binding site were evaluated in clinical trials as vascular disrupting agents (VDAs) (Figure 1A). Combretastatin A-4 (**1**, CA-4) is a natural *cis*-stilbene derivative isolated from the bark of the African willow tree *Combretum caffrum* [9], which was identified as a typical CBSI [21]. CA-4 displayed both potent antiproliferative activity against multiple human cancer cell lines [22] and anti-vascular activity [23]. The phosphate CA-4 (CA-4P) had been evaluated in clinical trials, however, it was discontinued due to the lack of a meaningful improvement in progression-free survival (PFS) and unfavorable partial response data [24]. Verubulin (**3**), also known as MPC-6827, was a very potent tubulin polymerization inhibitor and demonstrated low nanomolar (nM) potency against diverse cancer cell lines [25], and it was found to act as a VDA by inducing rapid shutdown of tumor blood flow [26]. Some verubulin derivatives have been reported to exhibit potent antitumor efficacy and improved therapeutic index [27-29]. Verubulin was also evaluated in clinical trials; however, it was discontinued due to cardiovascular toxicity revealed in phase I and phase II studies [30]. Plinabulin (**4**), developed from phenylahistin as a metabolite isolated from *Aspergillus ustus* [31], was another typical CBSI which attracted much attention [32]. Currently, a combination of plinabulin and docetaxel is being evaluated in clinical phase III trials for the treatment of non-small cell lung cancer [33].

3,4,5-Trimethoxyphenyl (TMP) is a common moiety which serves as ring A of classical CBSIs, such as colchicine, combretastatin A-4, podophyllotoxin. TMP is crucial for interacting with tubulin and producing the maximum antiproliferative activities [34]. Though modifications on the TMP moiety of CBSIs are usually reported to significantly reduced anticancer potency [35, 36], there are still many reports in

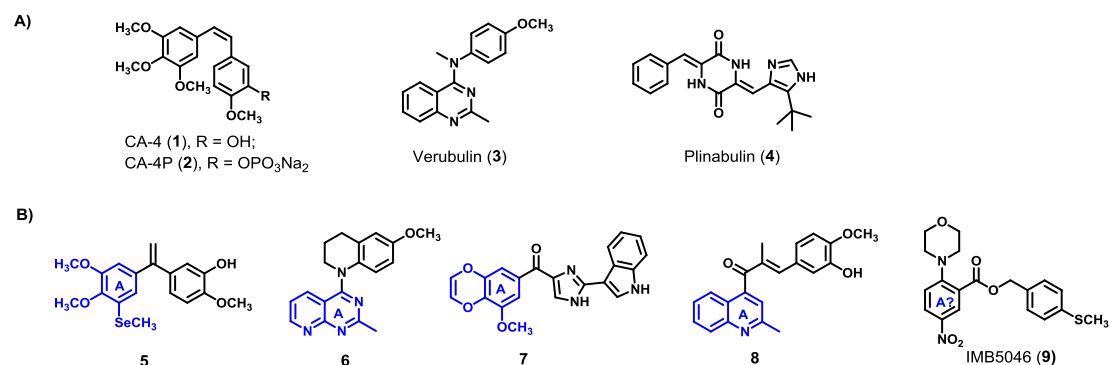
which modifying TMP or replacing TMP with other moieties led to the improvement of activity. In 2017, Pang *et al.* introduced a selenium atom to the TMP moiety of isocombretastatin A-4 (isoCA-4), the 3-methylseleno derivative (structure **5** in Figure 1B) displayed comparable *in vitro* antiproliferative activity to isoCA-4 while the *in vivo* antitumor activity was significantly improved [37]. The quinazoline of compound **6** was thought to be a surrogate of TMP moiety when binding to the colchicine site, and this was supported by its crystal structure complexed with tubulin [29]. In 2018, Wang *et al.* reported a modification on the TMP moiety of lead VERU-111, and compound **7** with a unique 3-methoxybenzo[4,5]-dioxene ring was found to be 2-8 times more potent than VERU-111 against three cancer cell lines [38]. Our group also discovered a quinoline-chalcone compound **8**, and its antiproliferative activity was improved by 8-10 fold when compared to its parent compound bearing a TMP moiety [39].

A novel nitrobenzoate IMB5046 (**9**, 2-morpholin-4-yl-5-nitrobenzoic acid 4-methylsulfanylbenzyl ester), which was reported by Zheng *et al.* in 2016, was found to display both potent *in vitro* and *in vivo* antitumor activities, and mechanism studies indicated that it bound to the colchicine binding site [40]. IMB5046 is distinct from any other known CBSIs; however, its structure-activity relationships (SARs) have not been investigated. Though IMB5046 had a remarkable antitumor activity in several mouse xenograft models, the ester bond, which may be unstable *in vivo*, and the nitro group, which is often associated with mutagenicity and genotoxicity [41], might complicate the further development of IMB5046. In addition, its docking study showed that the 4-methylthiophenyl moiety occupied the position where TMP of colchicine was located [40], which structurally differs from the SARs of classical CBSIs [14]. Thus, the binding mode of IMB5046 with tubulin should be further investigated.

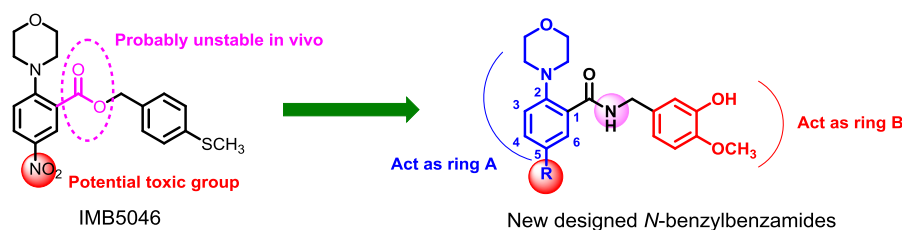
Based on our previous works on discovering and developing novel anticancer agents targeting the tubulin-microtubule system [39, 42-48], we further considered to design and synthesize novel *N*-benzylbenzamide derivatives based on IMB5046 with the wish to obtain promising anti-tubulin agents with more druggable properties (Figure 2). We set for ourselves the goal of designing new analogs with (a) equal or higher potency compared to IMB5046, (b) substantially low toxicity as tested in mouse models, and (c)

improved plasma stability.

Herein, we would like to report the synthesis of these *N*-benzylbenzamide derivatives, their SARs and mechanism studies, evaluation of preliminary druggability, *in vitro* and *in vivo* antitumor and anti-vascular activities as well as the illustration of the 2-morpholinophenyl moiety as a structurally novel ring A of CBSIs.



**Figure 1.** A) Typical CBSIs under clinical trials; B) Reported CBSIs by modifying TMP or replacing TMP moiety; The 4-methylthiophenyl moiety of IMB5046 was predicted to act as ring A in Zheng's work. However, in this study, we speculated that 2-morpholine-5-nitrophenyl moiety located in the position of TMP acting as ring A of known CBSIs according to our SARs and docking study below.



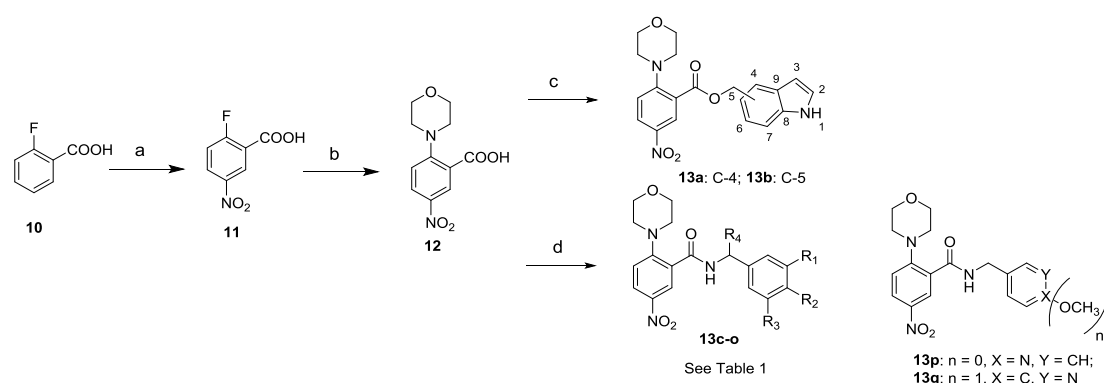
**Figure 2.** The designing concept for *N*-benzylbenzamide derivatives.

## 2. Results and discussion

### 2.1 Synthesis of Target *N*-benzylbenzamide Derivatives

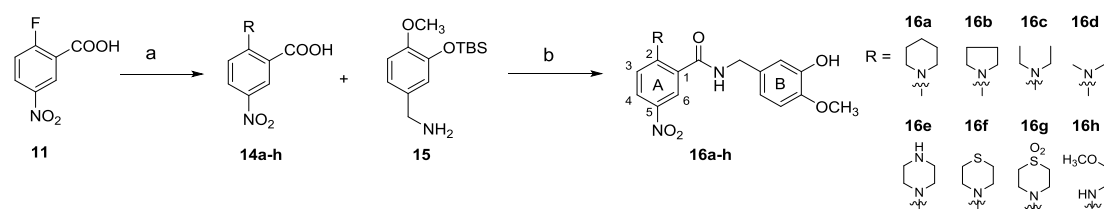
In our first-round modification, we investigated the SARs of ring B. Seventeen compounds **13a-q** including two indolyl benzoates **13a** and **13b** and fifteen *N*-benzylbenzamides **13c-q** were synthesized. As shown in Scheme 1, 2-fluoro-5-nitrobenzoic acid **11** was prepared by the nitration of the starting material 2-fluorobenzoic acid **10**, which underwent a nucleophilic attack by morpholine to give 2-morpholino-5-nitrobenzoic acid **12**. The esterification or amidation of intermediate **12**

with different indolylcarbinols or benzylamines yielded target compounds **13a-q**.



**Scheme 1.** Reagents and conditions: (a) con. HNO<sub>3</sub>, con. H<sub>2</sub>SO<sub>4</sub>, 0 °C to rt, 2 h, 71.2%; (b) morpholine, dioxane, rt, 2 h, yield 76.4%; (c) 4-indolylcarbinol or 5-indolylcarbinol, EDCI, DMAP (cat.), DCM, rt, 2 h, 60.3-67.8%; (d) i) various benzylamines, EDCI, HOBT, DMAP (cat.), DCM, rt, 2 h, 42.0-88.0%; ii) when R<sub>1</sub> or R<sub>2</sub> = OTBS, TBAF, THF, rt, 15 min, 61.5-81.1%.

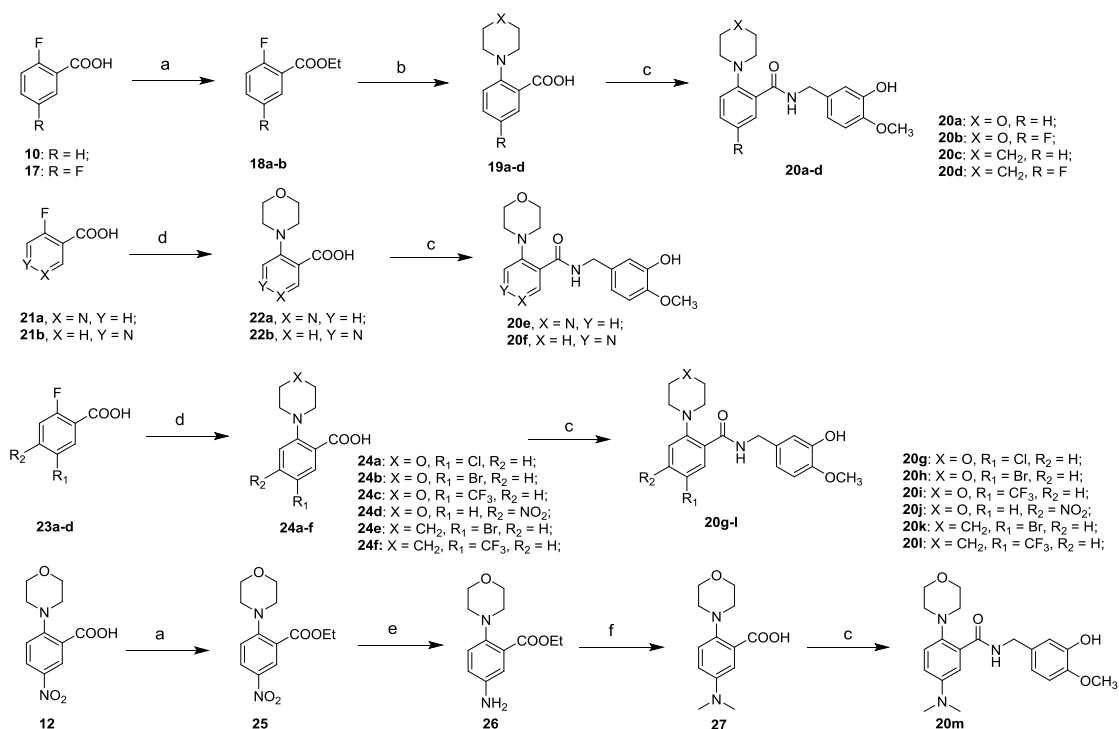
In the second-round modification, the SARs of C-2 position on ring A were investigated. Eight compounds **16a-h**, of which different amino groups were substituted at the C-2 position while ring B was maintained as an isovanillic ring, were synthesized as shown in Scheme 2. Intermediate **11** underwent nucleophilic substitutions by different amines to give **14a-h**, which were reacted with TBS-protected benzylamine **15** followed by deprotection of TBS to yield target compounds **16a-h**.



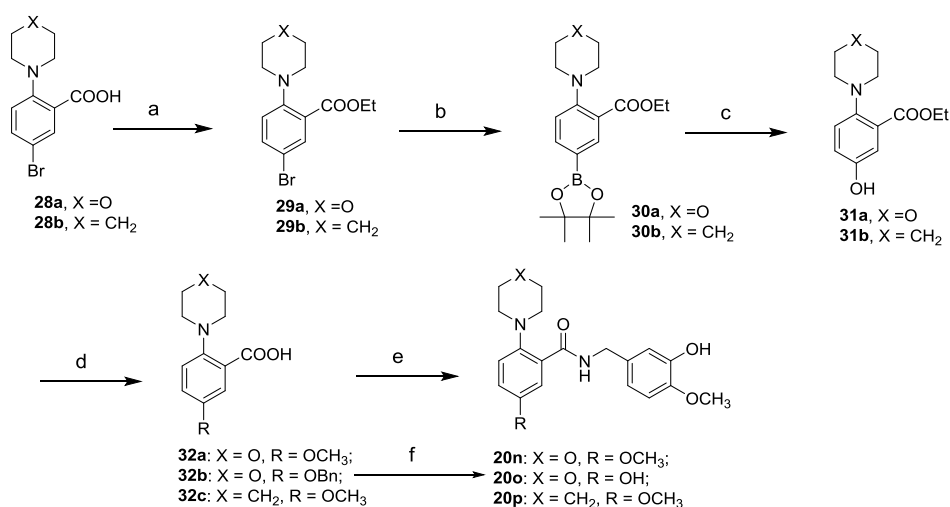
**Scheme 2.** Reagents and conditions: (a) various amines, dioxane, rt, 2 h, 70.3-90.3%; (b) i) EDCI, HOBT, DMAP (cat.), DCM, rt, 2 h; ii) TBAF, THF, rt, 15 min, 44.4-62.9% over 2 steps; when R was *N*-Boc piperazine, Boc was removed under condition: CF<sub>3</sub>COOH, DCM, rt, 2 h, 43.9% over 2 steps.

In the third-round modification, the effects of C-4 and C-5 positions on activity was further examined, thus, twenty-six compounds **20a-p** and **34a-j** were synthesized, of which ring B was an isovanillic ring and C-2 was substituted with morpholine or

piperidine. As illustrated in Scheme 3, benzoic acids **19a-d** in which R were hydrogen or fluorine were prepared by the reactions of their corresponding ethyl 2-fluorobenzoates **18a-b** with morpholine or piperidine and following hydrolysis of the ethyl ester. Benzoic acids **22a-b** and **24a-f** were prepared by refluxing their corresponding 2-fluorobenzoic acids with morpholine or piperidine in dioxane (Scheme 3). 5-(Dimethylamino)-2-morpholinobenzoic acid **27** was prepared by reductive amination of 5-amino-2-morpholinobenzoic acid **26** with paraformaldehyde followed by the hydrolysis of ethyl ester (Scheme 3). Benzoic acids **32a-c** in which R were methoxy or benzyloxy groups were prepared by a different method as shown in Scheme 4. Benzoic acid pinacol esters **30a-b** were prepared by Miyaura borylations of their corresponding bromobenzenes **29a-b**, followed by oxidation using hydrogen peroxide gave **31a-b**, and the intermediates **31a-b** underwent etherification and saponification to produce benzoic acids **32a-c**. Larger R groups were also introduced to the C-5 position of ring A. As shown in Scheme 5, benzoic acids **33a-c** and **33d-g** were prepared by reductive aminations and amidations of intermediate **26**, respectively, and **33h-j** were prepared by Suzuki coupling reactions of intermediate **30a**. All prepared benzoic acids reacted with TBS-protected benzylamine **15** followed by deprotection of TBS to afford their corresponding target compounds **20a-p** and **34a-j**.



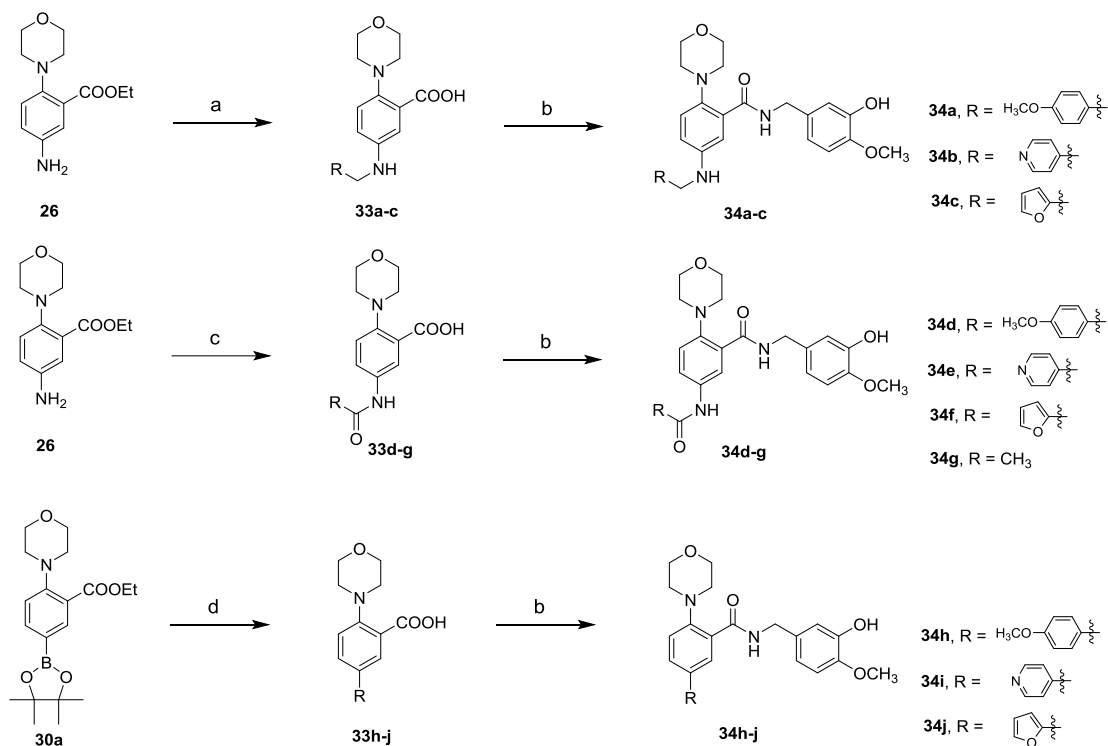
**Scheme 3.** Reagents and conditions: (a) Con. H<sub>2</sub>SO<sub>4</sub> (Cat.), EtOH, overnight, reflux; (b) i) morpholine or piperidine, DMSO, 120 °C, 6 h; ii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min; 44.9-55.4% over 2 steps; (c) method A: i) EDCI, HOBT, DMAP (cat.), DCM, rt, 2 h; ii) TBAF, THF, rt, 15 min, 40.7-59.0% over 2 steps; method B: i) HATU, Et<sub>3</sub>N, CH<sub>3</sub>CN, rt, 2 h; ii) TBAF, THF, rt, 15 min, 39.5-55.7% over 2 steps; (d) i) morpholine or piperidine, dioxane, reflux, 2 h-overnight, ii) neutralized by 10% HCl aqueous, 73.8-93.6% over 2 steps; (e) Fe, EtOH, AcOH, reflux, 30 min, 76.1%; (f) i) paraformaldehyde, NaBH(CN)<sub>3</sub>, AcOH, 30 min, rt; ii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min; 65.4% over 2 steps.



**Scheme 4.** Reagents and conditions: (a) Con. H<sub>2</sub>SO<sub>4</sub> (Cat.), EtOH, overnight, reflux; (b)



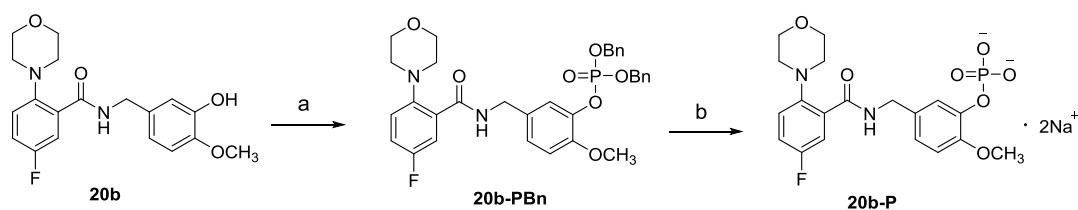
Bis(pinacolato)diboron, KOAc, PdCl<sub>2</sub>(CH<sub>3</sub>CN)<sub>2</sub>, dioxane, 80 °C, 3 h, 78.5-86.1%; (c) H<sub>2</sub>O<sub>2</sub>, CH<sub>3</sub>OH, rt, 30 min, 89.7-93.4%; (d) i) NaH, (CH<sub>3</sub>)<sub>2</sub>SO<sub>4</sub>, 0 °C to rt, 30 min; ii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min; 54.6-63.3% over 2 steps; (e) i) HATU, Et<sub>3</sub>N, CH<sub>3</sub>CN, rt, 2 h; ii) TBAF, THF, rt, 15 min; 39.5-54.5% over 2 steps; (f) i) HATU, Et<sub>3</sub>N, CH<sub>3</sub>CN, rt, 2 h ii) TBAF, THF, rt, 15 min; iii) Pd-C, H<sub>2</sub>, CH<sub>3</sub>OH, rt, 2 h, 16.8% over 3 steps.



**Scheme 5.** Reagents and conditions: (a) i) Various benzaldehydes, CH<sub>3</sub>OH, rt, 30 min; ii) NaBH<sub>4</sub>, CH<sub>3</sub>OH, rt, 15 min; iii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min; 49.8-66.9% over 3 steps; (b) i) HATU, Et<sub>3</sub>N, CH<sub>3</sub>CN, rt, 2 h; ii) TBAF, THF, rt, 15 min, 42.9-57.9% over 2 steps; (c) HATU, Et<sub>3</sub>N, CH<sub>3</sub>CN, rt, 2 h; ii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min; 42.5-64.5% over 2 steps; (d) i) Various bromobenzenes, K<sub>2</sub>CO<sub>3</sub>, Pd(PPh<sub>3</sub>)<sub>4</sub>, DMF, 90 °C, 2 h, ii) 10% NaOH aqueous, CH<sub>3</sub>OH, 80 °C, 30 min, 44.3-57.9% over 2 steps.

Finally, considering the excellent antiproliferative activity and satisfactory druggability of compound **20b**, its disodium phosphate **20b-P** was prepared as shown in Scheme 6. Dibenzyl phosphoryl chloride was prepared according to the literature method [49]. **20b-PBn** was prepared by the reaction of **20b** with newly prepared dibenzylphosphoryl chloride, which was then debenzylated and neutralized with NaOH

to afford the target disodium phosphate **20b-P**.



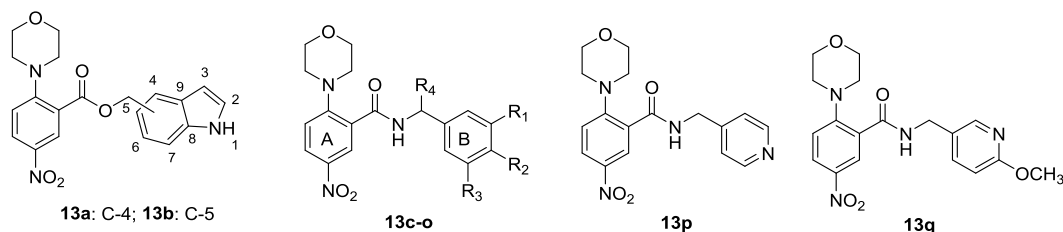
**Scheme 6.** Reagents and conditions: (a) NaH, dibenzylphosphoryl chloride, 0 °C to rt, 2 h, 63.8%; (b) i) Pd-C, H<sub>2</sub>, CH<sub>3</sub>OH, rt, 30 min; ii) NaOH, CH<sub>3</sub>OH, rt, 30 min, 89.6% over 2 steps.

## 2.2 Antiproliferative Activities and SARs

We first investigated the SARs of ring B. A431 epidermoid cancer cell line was chosen to test the antiproliferative activities since it was the most sensitive cell line in Zheng's work [40]. As the indole moiety has long been considered to be a privileged structure in ring B of CBSIs [50, 51], we first evaluated the activities of **13a** and **13b**, of which the indole moiety took the place of 4-methylthiophenyl moiety in IMB5046. However, they all displayed a significant decrease of activity ( $IC_{50} > 1 \mu M$ ) (Table 1). The following modifications led to fifteen *N*-benzylbenzamide derivatives **13c-q** by replacing the ester bond of IMB5046 with amide linkage. Unfortunately, the amide linkage replacement of ester bond led to about a 22-fold decrease in activity (**13c** vs. IMB5046). Compound **13d** in which R<sub>2</sub> was substituted with methoxy, was more potent than compounds in which R<sub>2</sub> was substituted with other groups such as ethoxy (**13e**), trifluoromethyl (**13f**), trifluoromethoxy (**13g**), dimethylamino (**13h**), chlorine (**13i**) and hydroxy (**13j**). The introduction of methyl into the linkage (**13k**) led to a roughly 2-fold drop of potency when compared with **13d**. Encouragingly, the introduction of fluorine (**13l**) and hydroxy (**13n**) increased the potency and **13n** was the most potent compound with an  $IC_{50}$  of 45 nM though it was 4.5-fold less potent than IMB5046 ( $IC_{50} = 10$  nM). Interestingly, the isovanillic ring, which can be found in numerous known CBSIs, was the most beneficial moiety as ring B of the *N*-benzylbenzamides, indicating that the 4-methylthiophenyl moiety of IMB5046 might act as ring B of CBSIs, which is contrary to its predicted binding mode [40]. Consequently, the decision was made to maintain

the isovanillic ring as ring B and focus on efforts to improve potency on other areas of *N*-benzylbenzamide derivatives.

**Table 1. SARs of Ring B**



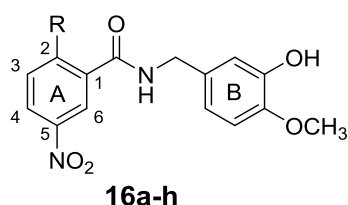
Compd.	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	A431 IC <sub>50</sub> values (μM) <sup>a</sup>	Compd.	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	A431 IC <sub>50</sub> values (μM) <sup>a</sup>
<b>13a</b>	-	-	-	-	> 1	<b>13j</b>	H	OH	H	H	> 1
<b>13b</b>	-	-	-	-	> 1	<b>13k</b>	H	OCH <sub>3</sub>	H	CH <sub>3</sub>	0.241 ± 0.015
<b>13c</b>	H	SCH <sub>3</sub>	H	H	0.221 ± 0.015	<b>13l</b>	F	OCH <sub>3</sub>	H	H	0.093 ± 0.007
<b>13d</b>	H	OCH <sub>3</sub>	H	H	0.127 ± 0.010	<b>13m</b>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	> 1
<b>13e</b>	H	OC <sub>2</sub> H <sub>5</sub>	H	H	0.143 ± 0.012	<b>13n</b>	OH	OCH <sub>3</sub>	H	H	0.045 ± 0.005
<b>13f</b>	H	CF <sub>3</sub>	H	H	> 1	<b>13o</b>	OCH <sub>3</sub>	OCH <sub>3</sub>	OCH <sub>3</sub>	H	> 1
<b>13g</b>	H	OCF <sub>3</sub>	H	H	> 1	<b>13p</b>	-	-	-	-	> 1
<b>13h</b>	H	N(CH <sub>3</sub> ) <sub>2</sub>	H	H	0.145 ± 0.015	<b>13q</b>	-	-	-	-	> 1
<b>13i</b>	H	Cl	H	H	> 1	<b>IMB5046</b>	-	-	-	-	0.010 ± 0.005

<sup>a</sup>IC<sub>50</sub> values are indicated as the mean ± SD of three independent experiments.

We further made our efforts to optimize C-2 position of ring A, which indicated that substitutions at this position exert great influences on antiproliferative activity (Table 2). When a piperidine moiety was introduced (**16a**), the activity was improved by about 4-fold when compared to the morpholine counterpart **13n**. The IC<sub>50</sub> of **16a** was 12 nM in A431 cells, which was comparable to that of IMB5046. However, the activity significantly decreased when the piperidine was changed to pyrrole (**16b**) or dimethylamine (**16d**), while the diethylamine compound **16c** still retained potency (IC<sub>50</sub> = 51 nM). The replacement of oxygen in morpholine by nitrogen (**16e**) and sulfone (**16g**)

led to loss of activity while **16f** bearing a thiomorpholine exhibited a slightly decreased activity ( $IC_{50} = 60$  nM). Furthermore, compound **16h** bearing a secondary amino group mimicking the structure of morpholine also displayed loss of activity.

**Table 2. SARs of C-2 Position of Ring A**



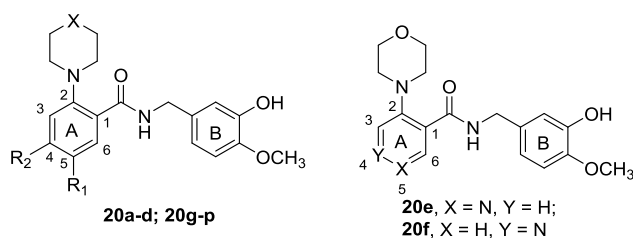
Compd.	R	A431 $IC_{50}$ values ( $\mu M$ ) <sup>a</sup>	Compd.	R	A431 $IC_{50}$ values ( $\mu M$ ) <sup>a</sup>
<b>13n</b>		$0.045 \pm 0.005$	<b>16e</b>		$> 1$
<b>16a</b>		$0.012 \pm 0.002$	<b>16f</b>		$0.060 \pm 0.005$
<b>16b</b>		$0.372 \pm 0.025$	<b>16g</b>		$> 1$
<b>16c</b>		$0.051 \pm 0.025$	<b>16h</b>		$> 1$
<b>16d</b>		$> 1$	<b>IMB5046</b>		$0.010 \pm 0.005$

<sup>a</sup> $IC_{50}$  values are indicated as the mean  $\pm$  SD of three independent experiments.

With the above SARs information in hand, we further investigated SARs of the C-4 and C-5 positions of ring A, and found that substituents at the C-5 position were well-tolerated while those at the C-4 position were not (Table 3). Unexpectedly, when  $R_1$  was hydrogen, fluorine, bromine, trifluoromethyl and methoxy groups, the morpholine derivatives were slightly more potent than the piperidine counterparts (**20a** vs **20c**, **20b** vs **20d**, **20h** vs **20j**, **20i** vs **20l** and **20n** vs **20p**). Moreover, in the case of morpholine derivatives, halogens (**20b**, **20g** and **20h**) substituted at the C-5 position was favorable for activity, while trifluoromethyl (**20i**), dimethylamino (**20m**) and hydroxy (**20o**) containing compounds was slightly less potent. Compound **20n** bearing a methoxy group also exhibited potent activity with an  $IC_{50}$  value of 28 nM. Incorporation of

nitrogen heterocycles as ring A (**20e** and **20f**) also led to a significant drop of potency. The C-4 position did not tolerate modifications as compound **20j** bearing a nitro group at C-4 position displayed much less potent activity compared to compound **13n**. We further introduced moieties with more steric hindrance to the C-5 position of ring B. As shown in Table 4, compounds **34a-j** all displayed decreased activity while **34h** with a biphenyl skeleton had moderate activity with an IC<sub>50</sub> value of 154 nM, indicating that there might exist an ample space where the C-5 position is located in the colchicine binding pocket.

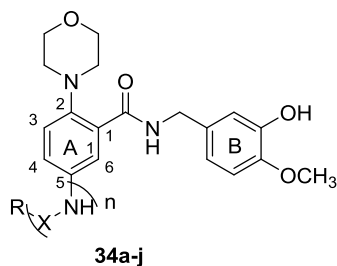
**Table 3. SARs of C-4 and C-5 Position of Ring A**



Compd.	R <sub>1</sub>	R <sub>2</sub>	X	A431 IC <sub>50</sub> values (μM) <sup>a</sup>	Compd.	R <sub>1</sub>	R <sub>2</sub>	X	A431 IC <sub>50</sub> values (μM) <sup>a</sup>
<b>13n</b>	NO <sub>2</sub>	H	O	0.045 ± 0.005	<b>20h</b>	Br	H	O	0.030 ± 0.003
<b>16a</b>	NO <sub>2</sub>	H	CH <sub>2</sub>	0.012 ± 0.002	<b>20i</b>	CF <sub>3</sub>	H	O	0.037 ± 0.005
<b>16f</b>	NO <sub>2</sub>	H	S	0.060 ± 0.005	<b>20j</b>	H	NO <sub>2</sub>	O	0.224 ± 0.018
<b>20a</b>	H	H	O	0.020 ± 0.002	<b>20k</b>	Br	H	CH <sub>2</sub>	0.092 ± 0.010
<b>20b</b>	F	H	O	<b>0.014 ± 0.003</b>	<b>20l</b>	CF <sub>3</sub>	H	CH <sub>2</sub>	0.097 ± 0.010
<b>20c</b>	H	H	CH <sub>2</sub>	0.030 ± 0.004	<b>20m</b>	N(CH <sub>3</sub> ) <sub>2</sub>	H	O	0.105 ± 0.009
<b>20d</b>	F	H	CH <sub>2</sub>	0.025 ± 0.004	<b>20n</b>	OCH <sub>3</sub>	H	O	0.028 ± 0.004
<b>20e</b>	-	-	-	0.977 ± 0.085	<b>20o</b>	OH	H	O	0.121 ± 0.008
<b>20f</b>	-	-	-	0.118 ± 0.020	<b>20p</b>	OCH <sub>3</sub>	H	CH <sub>2</sub>	0.057 ± 0.005
<b>20g</b>	Cl	H	O	0.025 ± 0.003	<b>IMB5046</b>	-	-	-	0.010 ± 0.005

<sup>a</sup>IC<sub>50</sub> values are indicated as the mean ± SD of three independent experiments.

**Table 4. Further SARs of C-5 Position of Ring A**



Compd.	R	X	n	A431 IC <sub>50</sub> values (μM) <sup>a</sup>	Compd.	R	X	n	A431 IC <sub>50</sub> values (μM) <sup>a</sup>
<b>34a</b>		CH <sub>2</sub>	1	0.306 ± 0.023	<b>34f</b>		C=O	1	0.478 ± 0.035
<b>34b</b>		CH <sub>2</sub>	1	1.080 ± 0.102	<b>34g</b>	CH <sub>3</sub>	C=O	1	0.220 ± 0.016
<b>34c</b>		CH <sub>2</sub>	1	0.315 ± 0.024	<b>34h</b>		-	0	0.154 ± 0.013
<b>34d</b>		C=O	1	> 1	<b>34i</b>		-	0	0.388 ± 0.022
<b>34e</b>		C=O	1	0.962 ± 0.085	<b>34j</b>		-	0	0.215 ± 0.015

<sup>a</sup>IC<sub>50</sub> values are indicated as the mean ± SD of three independent experiments.

Five additional cancer cell lines and one human normal hepatocytes L-O2 cell line were chosen for further evaluation of representative compounds. As shown in Table 5, all these selected compounds displayed potent activities against these five cancer cell lines in nanomolar ranges, and the SARs were generally consistent with their performances in A431 cells. All these compounds also had less potent cytotoxicity against L-O2 cells, which indicated that these *N*-benzylbenzamide derivatives might have low toxicity. Even though **16a** was the most active compound with IC<sub>50</sub> values ranging from 11 to 29 nM, the existence of an undesired nitro group in the structure of **16a** prompted us to choose another compound **20b**, which had comparable IC<sub>50</sub> values (19 to 27 nM) to those of **16a**, for further biological evaluation. The comprehensive SARs of the newly designed *N*-benzylbenzamide derivatives were summarized as shown in Figure 3.

**Table 5. Antiproliferative Activities of Selected Compounds Against Five Cancer Cell Lines**

**and Normal Human Liver Cells**

Compd.	IC <sub>50</sub> values (μM) <sup>a</sup>					
	HepG2	K562	HCT-8	MDA-MB-231	H22	L-O2
<b>13n</b>	0.061 ± 0.008	0.059 ± 0.005	0.060 ± 0.008	0.057 ± 0.003	0.050 ± 0.008	0.116 ± 0.015
<b>16a</b>	0.012 ± 0.002	0.019 ± 0.003	0.011 ± 0.002	0.029 ± 0.004	0.025 ± 0.003	0.120 ± 0.010
<b>16f</b>	0.058 ± 0.004	0.049 ± 0.003	0.060 ± 0.004	0.062 ± 0.005	0.055 ± 0.004	0.113 ± 0.011
<b>20a</b>	0.048 ± 0.003	0.030 ± 0.005	0.062 ± 0.008	0.055 ± 0.006	0.049 ± 0.007	0.105 ± 0.012
<b>20b</b>	0.025 ± 0.003	0.019 ± 0.004	0.020 ± 0.005	0.027 ± 0.004	0.024 ± 0.005	0.112 ± 0.012
<b>20c</b>	0.031 ± 0.004	0.030 ± 0.005	0.029 ± 0.003	0.029 ± 0.003	0.029 ± 0.005	0.097 ± 0.009
<b>20d</b>	0.026 ± 0.002	0.011 ± 0.001	0.029 ± 0.006	0.086 ± 0.009	0.032 ± 0.006	0.088 ± 0.012
<b>20g</b>	0.029 ± 0.004	0.032 ± 0.003	0.025 ± 0.002	0.030 ± 0.004	0.031 ± 0.005	0.095 ± 0.010
<b>20h</b>	0.031 ± 0.003	0.024 ± 0.002	0.030 ± 0.003	0.029 ± 0.005	0.024 ± 0.004	0.108 ± 0.012
<b>20i</b>	0.062 ± 0.007	0.040 ± 0.005	0.056 ± 0.006	0.057 ± 0.006	0.055 ± 0.007	0.103 ± 0.013
<b>20n</b>	0.029 ± 0.004	0.061 ± 0.005	0.027 ± 0.003	0.029 ± 0.003	0.032 ± 0.004	0.052 ± 0.005
<b>IMB5046</b>	0.033 ± 0.004	0.010 ± 0.001	0.027 ± 0.003	0.031 ± 0.006	0.025 ± 0.005	0.044 ± 0.008
<b>CA-4</b>	0.010 ± 0.001	0.007 ± 0.002	0.012 ± 0.004	0.015 ± 0.003	0.015 ± 0.003	0.046 ± 0.009

<sup>a</sup>IC<sub>50</sub> values are indicated as the mean ± SD of three independent experiments

Multidrug resistance (MDR) is one of the main reasons for tumor chemotherapy failure [52]. As IMB5046 was reported to overcome multidrug resistance [40], we investigated the antiproliferative efficacy of compound **20b** in the drug-resistant and parental sensitive cells by MTT assays, and the results were shown in Table 6. The drug-resistant indexes (DRIs) of **20b** were comparable to those of CA-4 and were slightly better than those of IMB5046. Except for the Adriamycin-resistant cell line Bel-7402/ADR, **20b** had DRIs of 5.3-10.8 while positive drugs paclitaxel and vincristine showed 92.5~241.0-fold resistances to the drug-resistant cells, indicating that **20b** had a moderate antiproliferative effect on drug resistant cancer cells.

**Table 6. The IC<sub>50</sub> Values of Compound 20b in Different Drug-Resistant Cancer Cells**

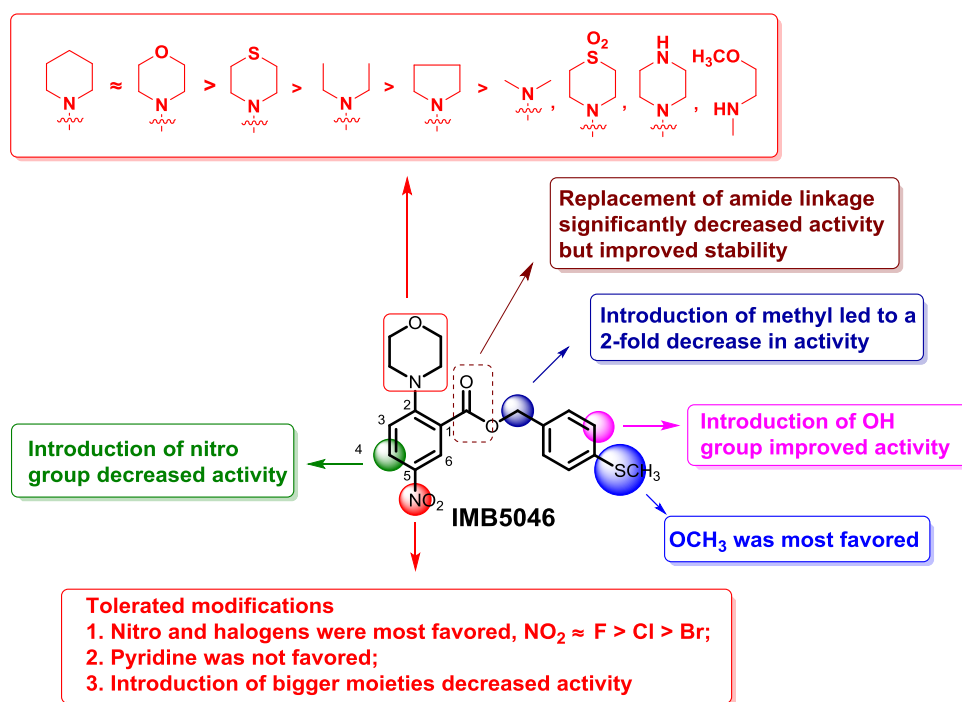
Compd.	IC <sub>50</sub> values (μM) <sup>a</sup>								
	Bel-7402	Bel-7402/ADR	DRI	HCT-8	HCT-8/Taxol	DRI	K562	K562/VCR	DRI
<b>20b</b>	0.027	0.104	3.9	0.020	0.106	5.3	0.019	0.205	10.8
	± 0.003	± 0.012		± 0.002	± 0.009		± 0.003	± 0.015	
IMB-5046	0.056	0.268	4.8	0.027	0.221	8.2	0.010	0.297	29.7
	± 0.004	± 0.022		± 0.002	± 0.023		± 0.001	± 0.031	
CA-4	0.011	0.058	5.3	0.011	0.087	7.9	0.007	0.057	8.1
	± 0.002	± 0.004		± 0.002	± 0.005		± 0.001	± 0.005	
ADR	0.274	1.732	6.3	ND	ND	-	ND	ND	-
	± 0.086	± 0.202							
Taxol	ND	ND	-	0.028 ±	2.590	92.5	ND	ND	-
				0.004	± 0.122				
VCR	ND	ND	-	ND	ND	-	0.021	5.060	241.0
				± 0.004	± 0.562				

<sup>a</sup>IC<sub>50</sub> values are indicated as the mean ± SD of three independent experiments

<sup>b</sup>DRI: drug-resistant index = (IC<sub>50</sub> of drug resistant cancer cell) / (IC<sub>50</sub> of parental cancer cell).

<sup>c</sup>ND: not detected.





**Figure 3.** The comprehensive SARs of newly designed *N*-benzylbenzamide derivatives.

### 2.3 Physicochemical and Drug-like Properties of **20b** and **20d**

Morpholine was a ubiquitous unit in drug discovery, such as Gefitinib and Linezolid. Aqueous solubility of lipophilic scaffolds is often improved by the attachment of a morpholine unit, and morpholine was reported to have improved solubility and reduced  $\log P$  when compared to piperidine [53]. Since compound **20b** with a morpholine unit and **20d** with a piperidine unit exhibited similar antiproliferative activities, their physicochemical and drug-like properties were further assessed with CA-4 as the reference. As shown in Table 7, the reference CA-4 showed low solubility ( $< 1 \mu\text{g/mL}$ ) both at pH 2.0 and pH 7.4. Notably, the morpholine derivative **20b** had aqueous solubility of  $323.2 \mu\text{g/mL}$  at pH 2.0 and  $76.6 \mu\text{g/mL}$  at pH 7.4, while the piperidine derivative **20d** showed low solubility ( $< 1 \mu\text{g/mL}$ ) at pH 7.4. Interestingly, the aqueous solubility of **20d** ( $1108.2 \mu\text{g/mL}$ ) under acidic conditions was much greater than **20b** in the same assay, which may be due to its more basic nature and ability to form a salt at pH 2.0. Compound **20b-P**, the disodium phosphate of **20b**, was soluble in water ( $> 1000 \mu\text{g/mL}$  at pH 2.0 and 7.4). Additionally, the lipophilicity of **20b** and **20d** were also

assessed by measuring their octanol/water (aqueous buffer pH 7.4) partition coefficients through the HPLC/UV method [54]. The  $\log D_{7.4}$  value of **20b** was lower than that of **20d**, which was consistent with its aqueous solubility (pH 7.4) and predicted  $\text{clog}P$ . Moreover, the predicted topological polar surface area (TPSA) values for **20b** and **20d** were in a desirable range (a criterion of  $< 140 \text{ \AA}^2$ ). Their drug-score values, which is a value used to judge a compound's overall potential to qualify for a drug, were also predicted by Osiris calculations. The results showed that **20b** had a higher drug-score value than that of **20d** and CA-4, indicating that **20b** might be more druggable than **20d** and CA-4.

**Table 7. Physicochemical and Drug-like Properties of Compounds 20b and 20d**

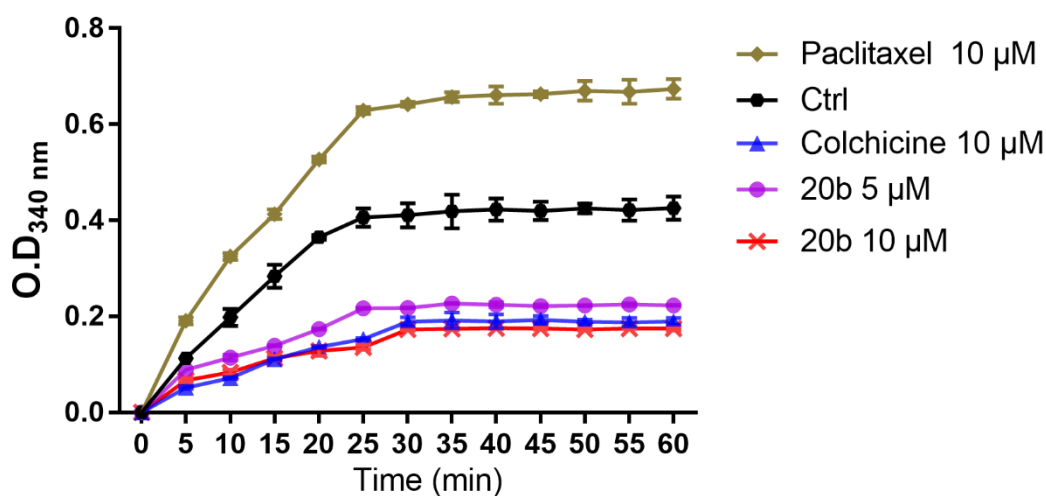
Compd.	MW <sup>a</sup>	$\text{clog}P^b$	TPSA <sup>c</sup>	Drug-Score	Aqueous Solubility <sup>e</sup> ( $\mu\text{g/mL}$ )		$\log D_{7.4}^f$
					pH 2.0	pH 7.4	
<b>20b</b>	360.0	1.87	71.03	0.82	323.2	76.6	1.61
<b>20d</b>	358.0	3.03	61.80	0.71	1108.2	< 1	2.97
CA-4	316.0	3.24	57.15	0.48	< 1	1 [55]	2.68
<b>20b-P</b>	-	-	-	-	> 1000	> 1000	-

<sup>a</sup>MW: molecular weight; <sup>b</sup> $\text{clog}P$ : calculated logarithm of the octanol-water partition coefficient; <sup>c</sup>TPSA: topological polar surface area, predicted by Osiris calculations in <https://www.organic-chemistry.org/prog/peo/>. <sup>e</sup>A general solubility guideline for human oral absorption is  $< 10 \mu\text{g/mL}$ , low;  $10\text{-}60 \mu\text{g/mL}$ , moderate;  $> 60 \mu\text{g/mL}$ , high. <sup>f</sup>Experimental logarithm of the n-octanol/water distribution coefficient at pH 7.4.

#### 2.4 Tubulin Polymerization by Binding to the Colchicine Binding Site

To investigate whether these *N*-benzylbenzamide derivatives are tubulin targeting agents, a representative compound **20b** was selected to undergo tubulin polymerization assay *in vitro*. In this assay, tubulin monomer was self-polymerized to microtubules, increasing light scattering at 340 nm. Microtubule-destabilizing agent (MDA) colchicine is known to inhibit self-polymerization of tubulin. On the contrary,

microtubule-stabilizing agent (MSA) paclitaxel promotes self-polymerization of tubulin. As shown in Figure 4, **20b** produced a significant inhibition of tubulin polymerization with a similar action to that of colchicine, indicating that **20b** acted as an MDA. The IC<sub>50</sub> values of **20b** in inhibiting tubulin polymerization were further determined as shown in Table 8. The results showed that **20b** exhibited a comparable activity to IMB5046 with an IC<sub>50</sub> value of 3.03 μM, which was slightly less potent than that of CA-4 (IC<sub>50</sub> = 2.64 μM). In addition, **20b** was examined for its potential inhibition of the binding of [3H]-colchicine to tubulin. The binding potency of **20b** to the colchicine binding site was 82.9% at 5 μM while those of IMB5046 and CA-4 were 85.7% and 90.3%, respectively, at the same concentration (Table 8), suggesting that **20b** bound to the colchicine binding site almost as well as IMB5046 and CA-4. Besides, the IC<sub>50</sub> value of **20b**-phosphate in inhibiting tubulin polymerization was 6.18 ± 0.50 μM.



**Figure 4.** Effects of **20b** on tubulin polymerization *in vitro*. After incubation of purified tubulin protein with tested compounds, polymerizations were followed by an increase in absorbance at 340 nm over a 60 min period at 37 °C. The experiments were performed three times.

**Table 8. Inhibition of Tubulin Polymerization<sup>a</sup> and Colchicine Binding to Tubulin<sup>b</sup>**

Compd.	Inhibition of tubulin polymerizaion	Inhibition of colchicine binding (%) inhibition ± SD
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	IC <sub>50</sub> ( $\mu$ M)	1 $\mu$ M	5 $\mu$ M
<b>20b</b>	3.03 $\pm$ 0.31	56.9 $\pm$ 4.4	82.9 $\pm$ 4.7
IMB5046	2.99 $\pm$ 0.23	58.0 $\pm$ 0.6	85.7 $\pm$ 2.2
CA-4	2.64 $\pm$ 0.22	80.2 $\pm$ 1.5	90.3 $\pm$ 3.4
<b>20b-P</b>	6.18 $\pm$ 0.50	ND <sup>c</sup>	ND <sup>c</sup>

<sup>a</sup>All Data are presented as the mean  $\pm$  SD from three independent experiments.

<sup>b</sup>Tubulin, 1  $\mu$ M; [<sup>3</sup>H]-colchicine, 5  $\mu$ M; and inhibitors, 1 or 5  $\mu$ M.

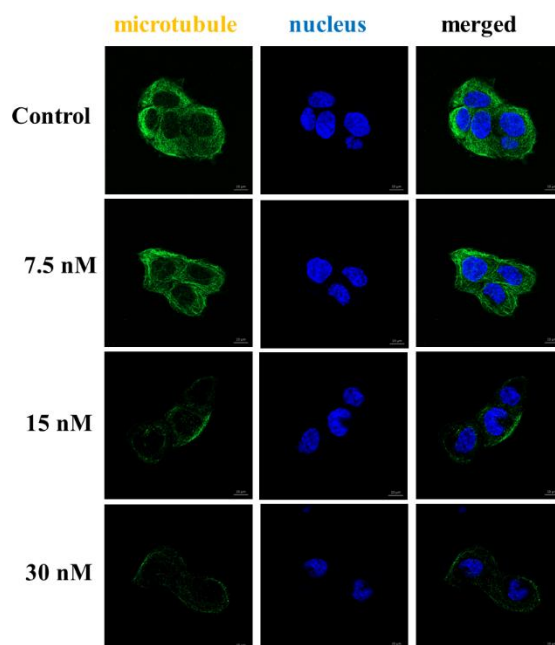
<sup>c</sup>ND = not determined.

### 2.5 Docking Profiles of Compound **20b** with Tubulin

In Zheng's work, the 4-methylthiophenyl moiety of IMB5046 was predicted to act as ring A resembling the TMP of colchicine [40]. However, our SARs have showed that the isovanillic ring was the most beneficial moiety when replacing the 4-methylthiophenyl of IMB5046, indicating that the 4-methylthiophenyl moiety might act as ring B of CBSIs. In addition, C-5 position on ring A of *N*-benzylbenzamide tolerated modifications with even more sterically demanding moieties introduced, indicating that there might exist an ample space where the C-5 position fits in the colchicine binding pocket. As reported before [56], a hydrophobic pocket, named as zone 3, was buried deeper in the  $\beta$ -subunit of tubulin, which could be occupied by some CBSIs [44, 57, 58]. Thus, we speculated that 4-methylthiophenyl of IMB5046 resembles the binding mode of isovanillic ring of ring B while the 2-morpholine-5-nitrophenyl moiety located in the position of TMP acting as ring A of known CBSIs. To further support this hypothesis, docking studies of compound **20b** and IMB5046 with tubulin were performed using the Glide module in the Schrodinger software. The original ligand CA-4 in the crystal structure (PDB:5lyj) [59] was first redocked into the colchicine site, which was quite close to that in the crystal structure (RMSD value = 0.72 Å), indicating the feasibility of our docking method. As shown in Figure 5A and 5C, the phenolic hydroxyl of **20b** and CA-4 formed hydrogen bonds with the residue Thr179. The binding mode of 5-fluorine-2-morpholine phenyl moiety was slightly



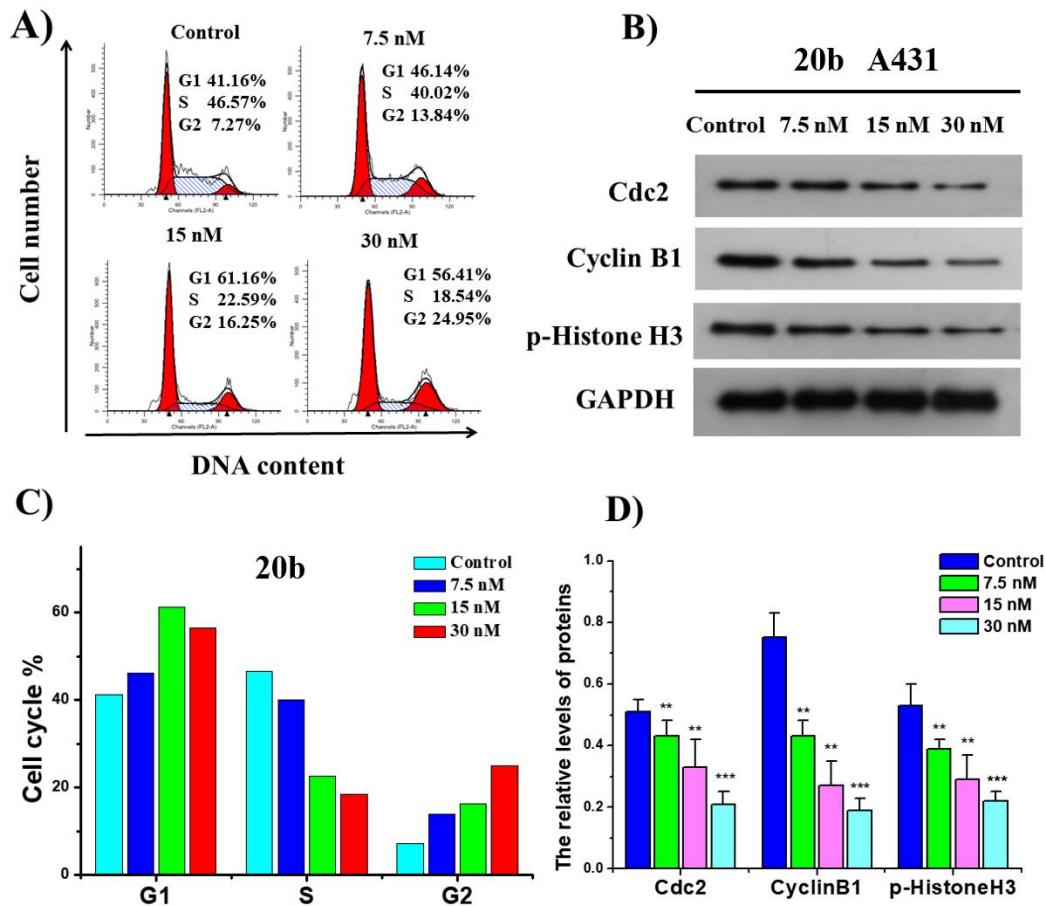
15 nM, and 30 nM) for 24 h resulted in significant disruption of microtubule networks, loss of cellular structure, and formation of cell membrane rounding. These results indicated that **20b** significantly caused microtubule network disruption at the cytological level.



**Figure 6.** Effects of **20b** on the cellular microtubule network visualized by immunofluorescence.

### 2.7 Cell Cycle Analysis

Most microtubule polymerization inhibitors interrupt the tubulin-microtubule balance thus causing mitotic arrest at the G2/M phase, and ultimately leading to apoptosis [60]. Therefore, the effects of **20b** on cell cycle progression of A431 cells were evaluated by flow cytometry. As illustrated in Figure 7A and 7C, treatment of **20b** at concentrations of 7.5, 15, and 30 nM caused a rise of cells in G2/M population from 7.27% (control) to 13.84%, 16.25%, and 24.95%, respectively, indicating that **20b** arrested A431 cell cycle at the G2/M phase. Effects of **20b** on the expression of cell cycle regulatory proteins were further tested and the results were shown in Figure 7B and 7D. It was found that **20b** decreased cdc2, cyclin B1 and p-histone H3 protein levels in a concentration-dependent manner, suggesting that the **20b**-induced G2/M arrest may be correlated with the regulation of cdc2, cyclin B1 and p-Histone H3 protein.



**Figure 7.** Compound **20b** induced G2/M arrest in A431 cancer cells. (A) The percentages of cells at different phases of the cell cycle after treatment with various concentrations of **20b**. (B) Western blotting analysis on the effect of **20b** on the G2/M regulatory proteins. (C) Histograms display the percentage of cell cycle distribution. (D) Histograms display the density ratios of cdc2, cdc25c and p-histone-H3 to GAPDH. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group.

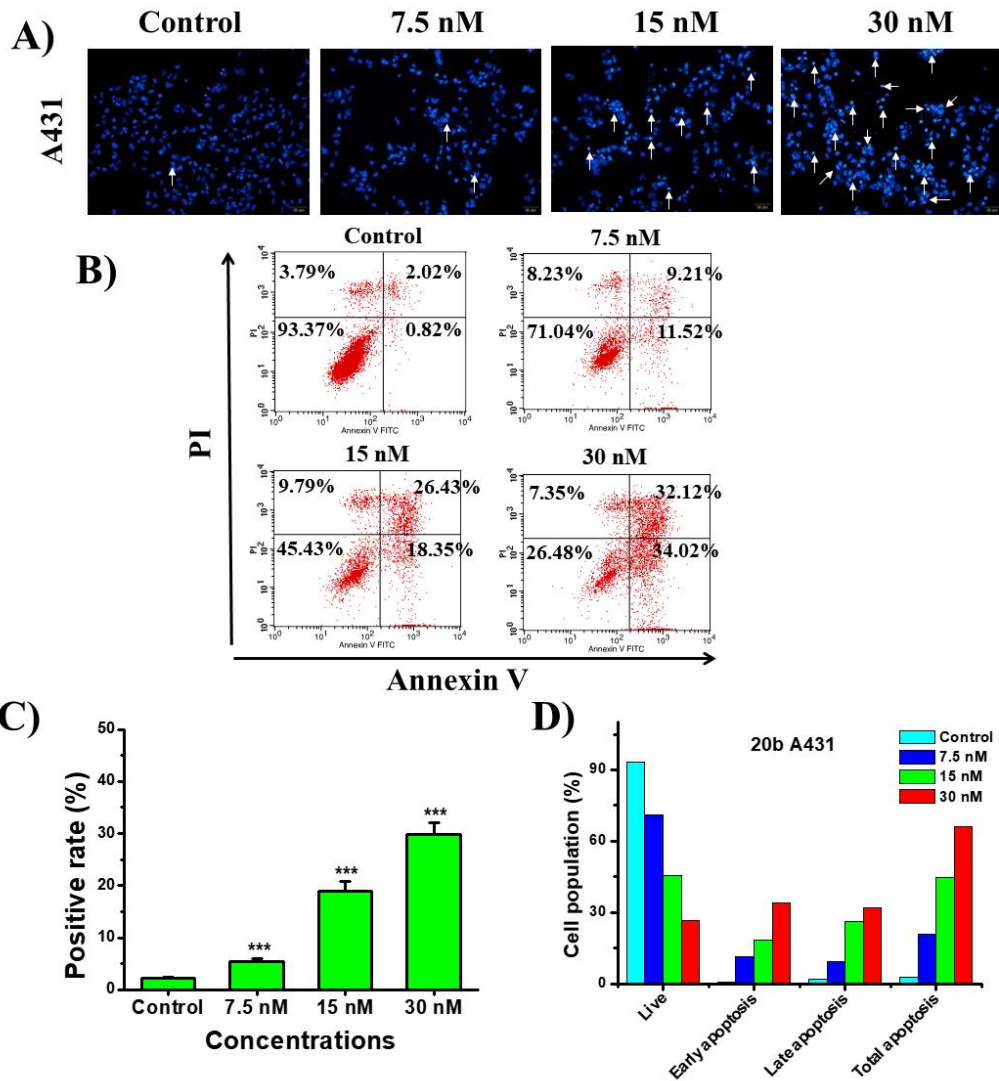
### 2.8 Cell Apoptosis Assay

Mitotic arrest of tumor cells by tubulin-targeted agents is generally associated with cellular apoptosis. Thus, the effects of **20b** on A431 cell apoptosis were examined by fluorescent microscopy in Hoechst 33342 assays. As shown in Figure 8A and 8C, A431 cells treated with **20b** (7.5 nM, 15 nM, and 30 nM) for 48 h displayed significant changes in cell morphology, such as nucleus fragmentation and chromatin condensation. The results of flow cytometry analysis in Annexin-V/PI assays also indicated that **20b** induced A431 cell apoptosis in a dose-dependent manner (Figure 8B and 8D). A431

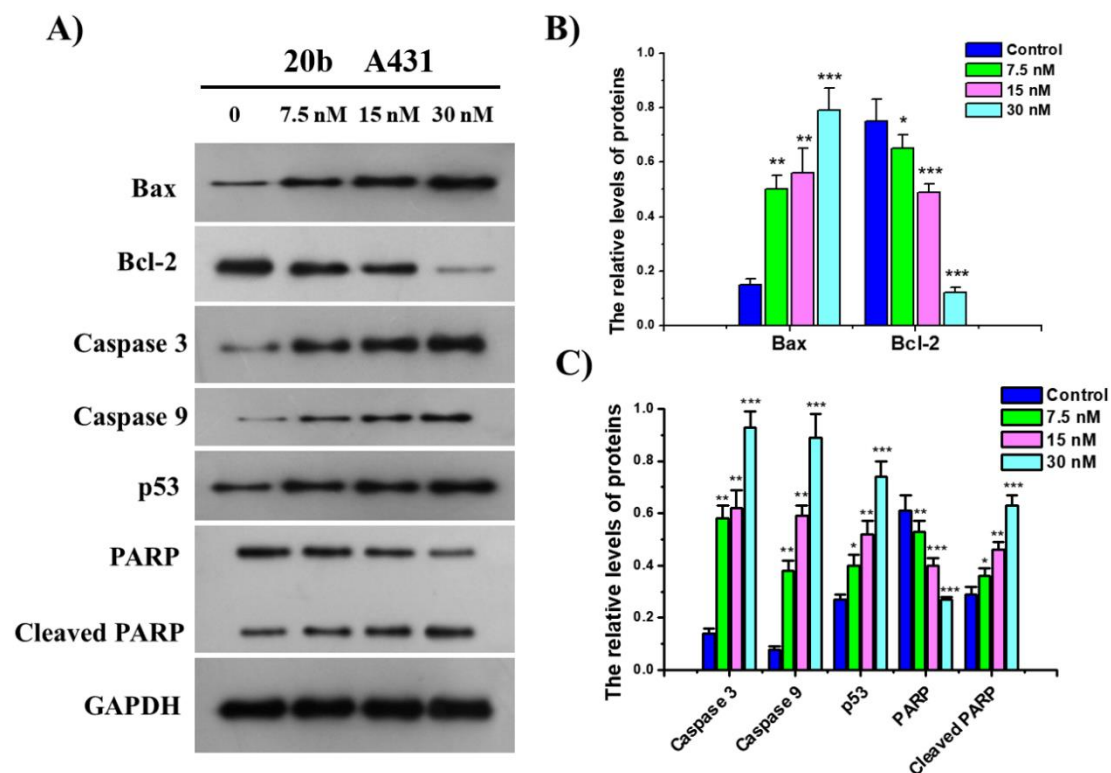
cells treated with **20b** at concentrations of 7.5, 15, and 30 nM showed an accumulation of apoptotic cells (Annexin-V+/PI<sup>-</sup> and Annexin-V+/PI<sup>+</sup>) from 2.84% (control) to 20.73%, 44.78%, and 66.14%, respectively.

As compound **20b** could effectively induced A431 cell apoptosis, we further evaluated its effects on the expression of apoptosis-related proteins by Western blot analysis. The Bcl-2 family has been considered as important proteins that control the mitochondrial pathway of apoptosis [61]. As shown in Figure 9A and 9B, **20b** upregulated the expression of the pro-apoptotic protein Bax and downregulated the expression of the anti-apoptotic protein Bcl-2 in a dose-dependent manner. The caspase families are considered to be markers for cells undergoing apoptosis and can facilitate cellular disassembly. Caspase-3 is one of the most important “executioner” caspases, which cleaves many important cellular substrates [62]. As shown in Figure 9A and 9C, **20b** produced a concentration-dependent activation of caspase-3. Furthermore, a significant increase of caspase-9 was also observed, which interacted with caspase-3 and activated apoptosis. The downstream substrate poly (ADP-ribose) polymerase (PARP) of caspase-3 was also observed to be cleaved after incubations with **20b**. Moreover, p53, which can act in the cytosol and mitochondria to promote apoptosis through transcription-independent mechanisms [63], was activated after treatments of **20b**. Taken together, **20b**-induced cell apoptosis may be correlated with upregulation of pro-apoptotic proteins, downregulation of anti-apoptotic proteins, caspase activation, cleaved PARP and p53 activation.





**Figure 8.** Compound **20b** induced apoptosis in A431 cancer cells. (A) Cell morphological alterations and nuclear changes (white arrows) associated with A431 cells after incubation with **20b**; (B) A431 cells stained with Annexin V/PI, followed by flow cytometric analysis; (C) Histograms display the percentage of positive cells; (D) Histograms display the percentage of cell distribution. \*\*\* $p < 0.001$  vs. control group.



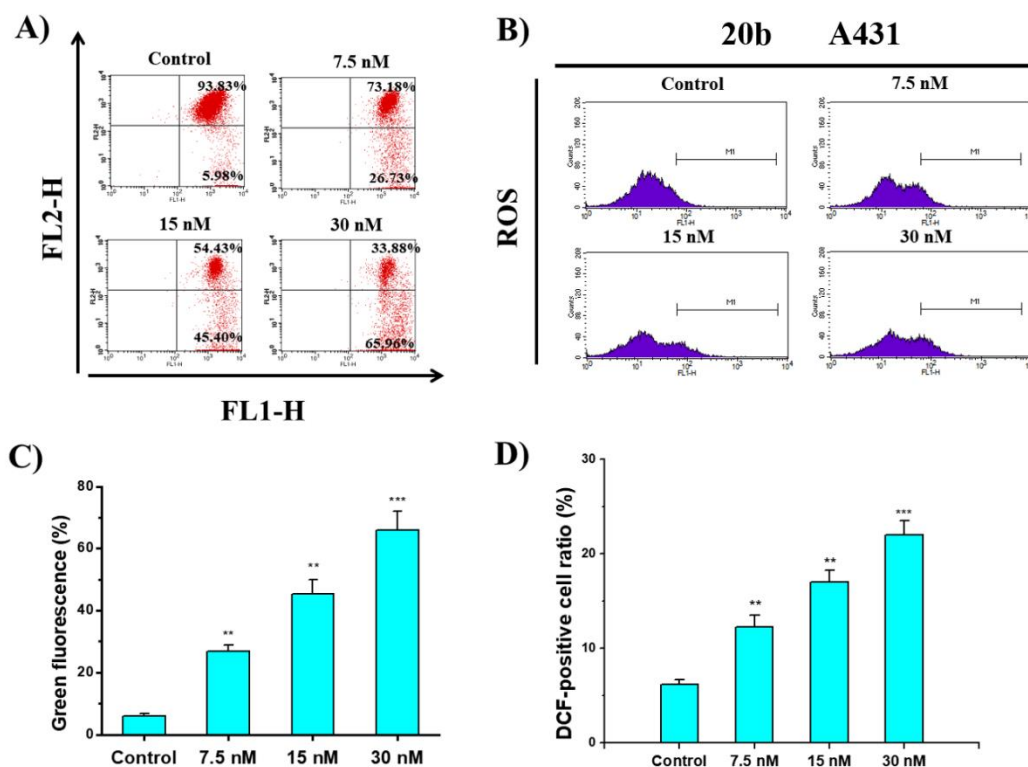
**Figure 9.** (A) Western blot analysis of apoptosis-related proteins. (B) Histograms display the density ratios of Bax and Bcl-2 to GAPDH. (C) Histograms display the density ratios of caspase 3, caspase 9, p53 and PARP to GAPDH. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group.

### 2.9 In Vitro Evaluation of Mitochondrial Membrane Potential and Reactive Oxygen Species (ROS) Generation

Mitochondrial membrane potential (MMP) reduction is considered an early event of apoptosis. The lipophilic cationic JC-1 staining method was used to determine the effect of compound **20b** on MMP of A431 cells. As presented in Figure 10A and 10C, with the concentration of **20b** increasing from 0 to 30 nM, the red fluorescence intensity decreased from 93.83% to 33.88%, and the green fluorescence intensity increased from 5.98% to 65.96%, indicating that **20b** decreased the MMP of A431 cells during the apoptosis process.

Mitochondria is an important source of ROS in cells [64]. To further explore the mechanism of **20b** for inhibiting tumor cells, the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA) was used to evaluate the intracellular ROS

levels after incubation with **20b**. As shown in Figure 10B and 10D, **20b** induced intracellular ROS generation in a dose-dependent manner. The ratio of DCF positive cells was increased from 6.17% in cells incubated with DMSO to 21.99% in cells incubated with 30 nM of **20b**. Collectively, **20b** was able to increase ROS levels of A431 cells and eventually triggered apoptotic cell death.

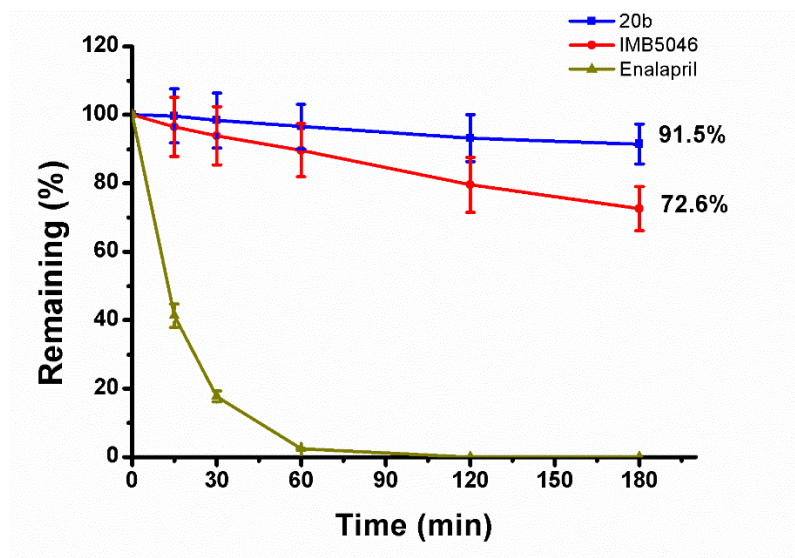


**Figure 10.** A) Effects of **20b** on the MMP of A431 cells; (B) The generation of ROS was measured using DCF-DA in combination with FACScan flow cytometry; (C) Histograms display the FACScan flow cytometry; (D) Histograms display the intracellular ROS contents in the absence or presence of **20b**. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control group.

### 2.10 Plasma Stability of Compound **20b**.

To assess whether compound **20b**, of which amide linkage was introduced, had improved stability, the *in vitro* assay using rat plasma was performed. As shown in Figure 11, the positive control Enalapril quickly degraded within one hour after incubation with rat plasma at 37 °C, while **20b** and IMB5046 were relatively stable in the same assay. Consistently, **20b** had an improved plasma stability profile compared

to IMB5046. After a 3-h incubation, there remained 91.5% of **20b** and only 72.6% of IMB5046. The unexpected stability of IMB5046, which bears an ester bond, is most likely attributable to the steric hindrance adjacent to the ester bond, which might hinder the esterase-catalyzed hydrolysis reaction. Nevertheless, the remarkable plasma stability of **20b** prompted us to further investigate its safety profile and *in vivo* antitumor efficacy.



**Figure 11.** Stability of **20b** and IMB5046 in rat plasma.

### 2.11 The Safety Profile of **20b** in Mice.

To understand the safety of **20b**, the acute toxicity of its disodium phosphate prodrug **20b-P** was assessed in ICR mice. **20b-P** dissolved into saline was intravenously injected at doses of 327.7, 409.6, 512.0, 640, and 800 mg/kg ( $n = 10$  per group) and mice were observed during two weeks. As shown in Table 10, treatment with **20b-P** at a dose of 327.7 mg/kg only caused one death in ten mice, while treatment with **20b-P** at 800 mg/kg killed eight mice. Finally, the median lethal dose ( $LD_{50}$ ) value of **20b-P** was calculated to be 599.7 mg/kg, implying the low toxicity of **20b-P**.

**Table 10.** Acute Toxicity of **20b-P** in Mice

dose (mg/kg)	num. of mice	total mortality	survival (%)	$LD_{50}$ (mg/kg) <sup>a</sup>
327.7	10	1	90	
409.6	10	2	80	

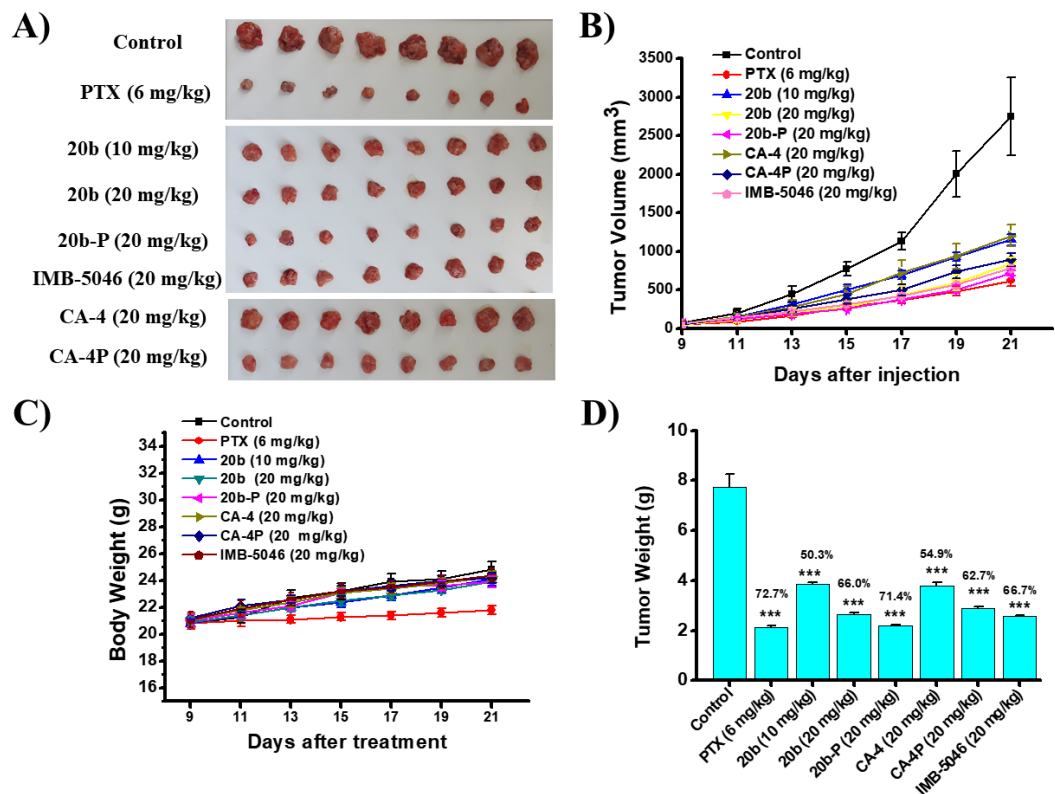
512.0	10	3	70	599.7
640.0	10	5	50	
800.0	10	8	20	

<sup>a</sup>The 95% confidence limits: 530.8-677.5 mg/kg.

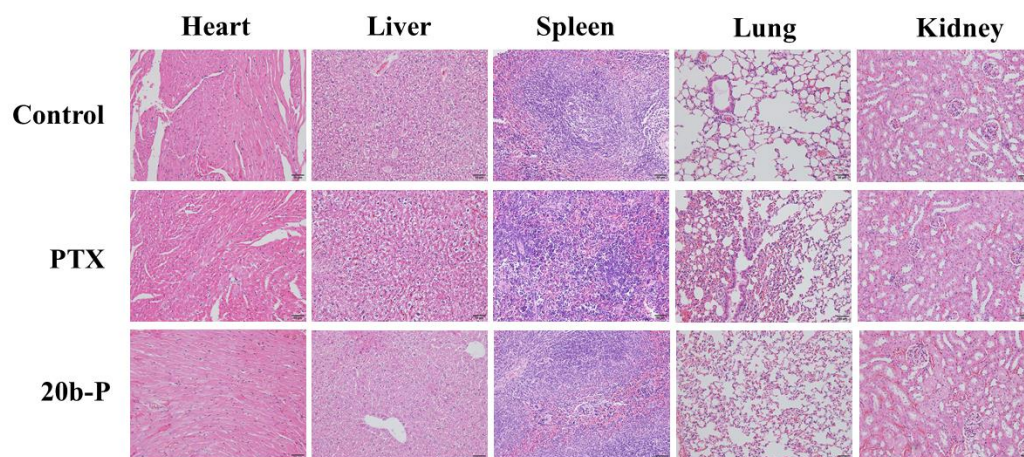
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### 2.12 *In Vivo* Anti-tumor Activity of Compound **20b** and **20b-P**

In order to verify the potential antitumor effect of **20b** and **20b-P** *in vivo*, a liver cancer allograft mouse model was established by subcutaneous injection of H22 cells into the right flank of mice. Paclitaxel (PTX), IMB5046, CA-4 and its disodium phosphate prodrug CA-4P were used as the positive controls. As shown in Fig. 12A and Fig. 12B, **20b** and **20b-P** had a significant inhibitory effect on tumor growth. PTX caused a reduction of tumor weight by 72.7% at a dose of 6 mg/kg every other day (i.v.) after three weeks as compared to vehicle, while **20b** and **20b-P** reduced tumor weights by 66.0% and 71.4% at a dose of 20 mg/kg per day (i.v.), respectively (Figure 12D). Particularly, **20b-P** (20 mg/kg) displayed more potent antitumor activity than CA-4P (62.7%) at the same dose. **20b** exhibited a comparable inhibitory rate to IMB5046 at the dose of 20 mg/kg while that of the disodium phosphate prodrug **20b-P** was much higher. Additionally, PTX caused a significant loss of body weight (Figure 12C) while **20b** and **20b-P** did not obviously affect body weight even at doses up to 20 mg/kg, indicating the low toxicities of **20b** and **20b-P** toward mice. Further H&E staining of some important organs including heart, liver, spleen, lung and kidney collected from the **20b-P**-treated group (20 mg/kg) also implied low toxicity of **20b-P** (Figure 13). Taking these results together, **20b** and **20b-P** were efficacious and safe in inhibiting the growth of cancer *in vivo*, and should be further investigated as potential anticancer agents.



**Figure 12.** **20b** and **20b-P** inhibited liver cancer xenograft growth *in vivo*. Mice were treated with vehicle, PTX (6 mg/kg every other day), **20b** (10 or 20 mg/kg per day), **20b-P** (20 mg/kg per day), IMB5046 (20 mg/kg per day), CA-4 (20 mg/kg per day), and CA-4P (20 mg/kg per day) for three weeks, and then sacrificed for analysis. All the groups were administered by intravenous injection. The treatments started the day after cell inoculum and the statistics started since the tumor grew out on the 9th day. (A) The images of tumors isolated from the mice at the end of the experiment; (B) Tumor volume changes of mice during treatment; (C) Body weight changes of mice during treatment; (D) The weight of the excised tumors of each group. \*\*\* $P < 0.001$  vs. control group.



**Figure 13.** HE staining of vital organs of mice including heart, liver, spleen, lung and kidney.

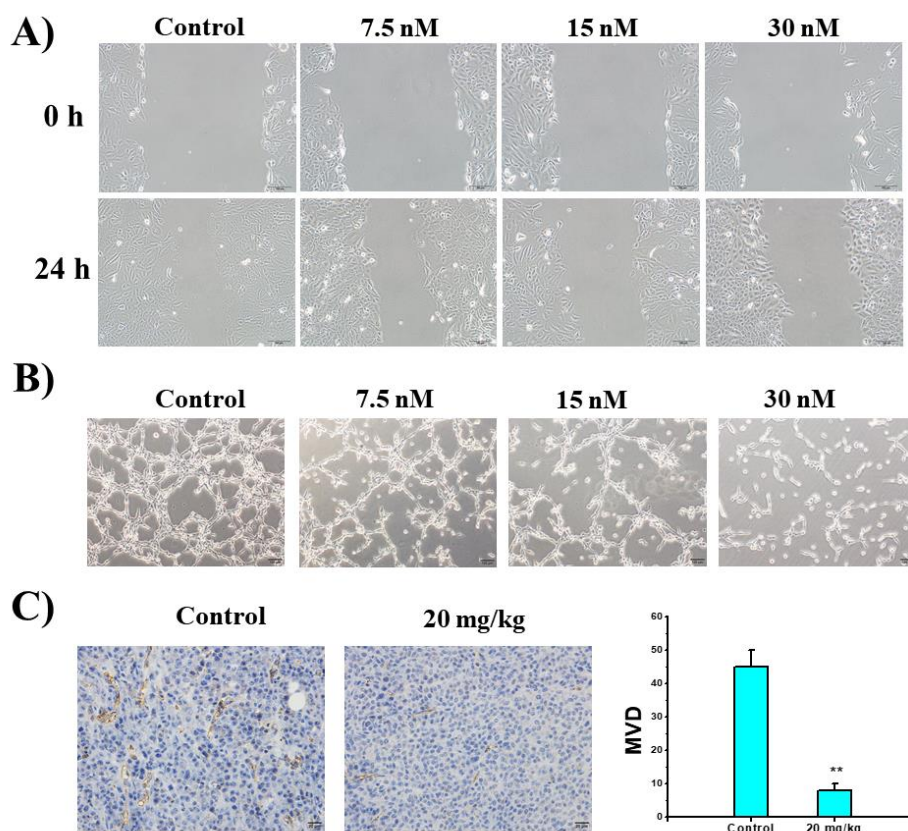
### 2.13 *In Vitro* and *In Vivo* Evaluation of Anti-vascular Activity

Some microtubule binding drugs possess vascular disrupting activity due to their disruption ability to microtubule dynamics of endothelial cells [65]. Thus, wound healing assays was performed to evaluate the anti-vascular activity of **20b** by using human umbilical vein endothelial cells (HUVECs). Standardized scratches (wounds) were made in confluent monolayers of HUVECs and then incubated with increasing concentrations (7.5, 15 and 30 nM) of **20b**. As shown in Figure 14A, **20b**-treatment resulted in significant inhibition of migration capacity of HUVECs especially at the dose of 30 nM, which indicated that **20b** had significant anti-vascular activity.

The formation of HUVEC tube is the key step in angiogenesis. Thus, we further evaluated the anti-vascular activity of **20b** by performing a tube formation assay. As shown in Figure 14B, HUVECs in the control group form capillary-like tubules with multicentric junctions, while treatment with **20b** at the concentration of 30 nM completely inhibited HUVEC cord formation. The  $IC_{50}$  value of **20b** against HUVECs after 6-h treatment was  $0.12 \pm 0.03 \mu M$ , indicating that the *in vitro* potent anti-vascular activity of **20b** was not caused by its cytotoxicity.

CD31 is commonly used as an endothelial cell marker to highlight tumor blood vessels reflecting the degree of angiogenesis [66]. To assess whether **20b** could exert anti-vascular effects *in vivo*, immunohistochemical staining against CD31 was performed to quantify the microvessel density (MVD) of tumors treated with vehicle

and **20b** (20 mg/kg). The **20b**-treated group exhibited significantly lower tumor microvessel density (MVD) as compared with the control group (Figure 14C), suggesting that **20b** exhibited potent anti-vascular activity *in vivo*.



**Figure 14.** *In vitro* and *in vivo* anti-vascular activity of **20b**. (A) Scratches were made with 200  $\mu$ L sterile pipette tips, and images were taken at 0 h and 24 h with a phase contrast microscopy after treatments with 0, 7.5, 15 and 30 nM of **20b**; (B) HUVEC capillary-like tubular network formation after treatments with 0, 7.5, 15 and 30 nM of **20b** for 6 h; (C) Immunohistochemical staining of CD31 in tumors treated with vehicle or **20b** (20 mg/kg). \*\* $P < 0.01$  vs. control group.

### 3. Conclusions

With the aim of discovering novel drug candidate with better druggable profiles as well as illustrating their binding modes with tubulin, we have performed a comprehensive SARs study of *N*-benzylbenzamide derivatives based on the structure of the lead compound IMB5046. These compounds have a new type of A ring segment with better drug-like properties, replacing the traditional 3,4,5-trimethoxyphenyl group. Due to the



simplicity in the structure, the target compounds can be obtained through a convenient and efficient synthetic route from cheap and easily available starting materials. As a result, the representative compound **20b** was eventually found to exhibit potent antiproliferative activity comparable to IMB5046 with the IC<sub>50</sub> values ranging from 12 to 27 nM against six cancer cell lines. Compound **20b** displayed a moderate selectivity for human normal hepatocytes LO2 cells, and its antiproliferative efficacies against three drug-resistant cancer cells were also evaluated with the DRIs comparable to those of CA-4 and slightly better than those of IMB5046. Compound **20b**, which has a morpholine unit, and compound **20d**, which has a piperidine unit, were further assessed for their physicochemical and drug-like properties. Our experiments showed that **20b** had satisfactory physicochemical parameters and its drug-score value was predicted to be higher than that of **20d** or CA-4.

In addition, compound **20b** significantly inhibited tubulin polymerization and disrupted microtubule networks of A431 cells, and it was further confirmed that **20b** binds to the colchicine binding pocket by colchicine competition inhibition assay and molecular docking studies through colchicine competition inhibition assay and molecular docking studies. Notably, our SARs and docking studies suggested that the binding mode of **20b** resembles that of classical CBSI CA-4 with the 5-fluorine-2-morpholinephenyl moiety proposed to be a novel ring A of CBSIs. Moreover, **20b** induced A431 cell cycle arrest at the G2/M phase by regulation of G2/M-related protein expression of cdc2, cyclin B1 and p-Histone H3 protein. **20b** also induced A431 cell apoptosis, which might be the resulting effects of upregulation of pro-apoptotic protein, downregulation of anti-apoptotic protein, caspase activation, cleaved PARP and p53 activation. Additionally, **20b** depolarized the mitochondria membrane potentials and induced ROS generation in A431 cells.

Furthermore, **20b** possessed good plasma stability (remaining = 91.5% after 3-h incubation) and safety profiles (LD<sub>50</sub> value of acute toxicity = 599.7 mg/kg). The antitumor effects of **20b** and its disodium phosphate **20b-P** were validated in liver cancer xenograft mouse model without obvious toxicity. Compounds **20b** and **20b-P** reduced tumor weights by 66.0% and 71.4% at a dose of 20 mg/kg per day (*iv.*),

respectively, which were more potent than the reference CA-4. Finally, the anti-vascular activity of **20b** was confirmed in *in vitro* HUVEC wound healing and tube formation assays as well as *in vivo* microvessel formation in xenograft tumor tissues. Therefore, **20b** and **20b-P** deserved further investigations as novel, safe and efficacious anti-tubulin drug candidates for the treatment of cancers.

## 4. Experimental

### 4.1 Chemistry

#### 4.1.1. General

All commercially available reagents were used without further purification. Solvents were dried through routine protocols. Flash column chromatography was carried out on 200-300 mesh silica gel (Qingdao Haiyang Chemical, China). Reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silicagel plates (GF254) and visualized under UV light. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded with a Bruker AV-300 spectrometer (Bruker Company, Germany) in the indicated solvents (CDCl<sub>3</sub> or DMSO-*d*<sub>6</sub>, TMS as internal standard): the values of the chemical shifts are expressed in  $\delta$  values (ppm) and the coupling constants (*J*) in Hz. Low- and high-resolution mass spectra (LRMS and HRMS) were measured on Finnigan MAT 95 spectrometer (Finnigan, Germany). Purity of all tested compounds was  $\geq 95\%$ , as estimated by HPLC (SHIMADZU Labsolutions, UV detection at  $\lambda = 254$  nm) analysis on the Agilent C18 column (4.6  $\times$  150 mm, 5  $\mu$ m) eluting at 1 mL/min of menthol and water (80:20), representative HPLC trace see Supporting Information.

#### 4.1.2. 2-Morpholino-5-nitrobenzoic acid (**12**).

To a solution of 2-fluorobenzoic acid (**10**) (5.0 g, 35.7 mmol) in 10 mL concentrated H<sub>2</sub>SO<sub>4</sub>, 10 mL concentrated HNO<sub>3</sub> was added dropwise at 0 °C. After stirring for 2 h at room temperature, the mixture was slowly poured into ice water. The resulting precipitate were collected by filtration, which were washed by water, and dried to give 4.7 g 2-fluoro-5-nitrobenzoic acid (**11**) as yellow solid, yield 71.2%; To the solution of **11** (500 mg, 2.7 mmol) in 10 mL dioxane, morpholine (1.2 mL, 13.5 mmol) was added in one portion. After stirring for 2 h at room temperature, the solvent and remaining morpholine were removed in vacuo. Then, the residues were dissolved into water and

neutralized by 10% HCl aqueous. The resulting precipitate were collected, washed by water, and dried to give 520 mg 2- morpholine-5-nitrobenzoic acid (**12**) as yellow solid, yield 76.4%. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 8.40 (d, *J* = 2.9 Hz, 1H), 8.20 (dd, *J* = 9.2, 2.9 Hz, 1H), 7.19 (d, *J* = 9.2 Hz, 1H), 3.72 (d, *J* = 4.8 Hz, 4H), 3.28 (d, *J* = 4.7 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.39, 155.09, 138.17, 127.42, 127.28, 120.86, 117.75, 65.70, 50.95; ESI-MS *m/z* 252.1 [M-H]<sup>-</sup> 251.1.

#### 4.1.3. General procedures for the preparation of **13a** and **13b**

To a solution of **12** (80 mg, 0.32 mmol) in 10 mL anhydrous DCM, indolylcarbinol (56 mg, 0.38 mmol), EDCI (296 mg, 1.6 mmol) and catalytic amount of DMAP were added. The mixtures were stirred for 2 h, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (4:1) to give target compounds **13a** and **13b**.

**(1H-indol-4-yl)methyl 2-morpholino-5-nitrobenzoate (13a)**. Yellow solid, 60.3%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.62 (d, *J* = 2.8 Hz, 1H), 8.36 (s, 1H), 8.20 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.43 (d, *J* = 7.6 Hz, 1H), 7.30 - 7.18 (m, 3H), 6.92 (d, *J* = 9.2 Hz, 1H), 6.73 (d, *J* = 2.9 Hz, 1H), 5.70 (s, 2H), 3.61 (t, *J* = 4.6 Hz, 4H), 3.09 (t, *J* = 4.7 Hz, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 166.32, 155.94, 139.75, 136.06, 128.47, 127.88, 127.12, 126.89, 124.94, 121.96, 121.33, 120.97, 117.23, 112.02, 100.92, 72.80, 66.28, 51.66; HR-MS (ESI) *m/z*: calcd for C<sub>20</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub> [M+Na]<sup>+</sup> 404.1217 found 404.1211.

**(1H-indol-5-yl)methyl 2-morpholino-5-nitrobenzoate (13b)**. Yellow solid, 67.8%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.59 (d, *J* = 2.8 Hz, 1H), 8.40 - 8.28 (m, 1H), 8.19 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.77 (d, *J* = 1.5 Hz, 1H), 7.41 (d, *J* = 8.3 Hz, 1H), 7.30 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.24 (t, *J* = 2.8 Hz, 1H), 6.91 (d, *J* = 9.2 Hz, 1H), 6.57 (ddd, *J* = 3.1, 2.1, 1.0 Hz, 1H), 5.47 (s, 2H), 3.69 - 3.63 (m, 4H), 3.13 - 3.07 (m, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 166.28, 155.78, 139.64, 135.79, 128.22, 127.89, 127.74, 126.59, 125.13, 123.38, 122.10, 121.41, 117.10, 111.28, 102.85, 68.60, 66.26, 51.57. HR-MS (ESI) *m/z*: calcd for C<sub>20</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub> [M+H]<sup>+</sup> 404.1217 found 404.1212.

#### 4.1.4. General procedures for the preparation of **13c-q**

To a solution of **12** (80 mg, 0.32 mmol) in 10 mL anhydrous DCM, various benzylamines (0.38 mmol), EDCI (296 mg, 1.6 mmol), HOBt (52 mg, 0.38 mmol) and catalytic amount of DMAP were added. The mixtures were stirred for 2 h, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (4:1) to give target compounds. For benzylamines that containing TBS-protected phenolic hydroxyl, the products obtained in above procedure were dissolved into 10 mL THF, and TBAF (1.2 e.q) was added. The mixtures were stirred for 15 min, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (2:1) to give target compounds **13j** and **13n**.

***N*-(4-methylthiobenzyl)-2-morpholino-5-nitrobenzamide (13c)**. Yellow solid (78 mg, yield 63.5%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.78 (dd, *J* = 2.9, 1.0 Hz, 1H), 8.60 (t, *J* = 5.7 Hz, 1H), 8.22 (dd, *J* = 8.9, 2.0 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 2H), 7.24 (d, *J* = 8.1 Hz, 2H), 7.15 (d, *J* = 8.9 Hz, 1H), 4.60 (d, *J* = 5.8 Hz, 2H), 3.58 (t, *J* = 4.6 Hz, 4H), 3.02 (t, *J* = 4.6 Hz, 4H), 2.48 (s, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 165.01, 155.49, 143.31, 138.60, 134.53, 128.93, 128.18, 127.35, 127.02, 127.00, 119.43, 66.47, 52.80, 43.64, 15.91; HR-MS (ESI) *m/z*: calcd for C<sub>19</sub>H<sub>22</sub>N<sub>3</sub>O<sub>4</sub>S [M+H]<sup>+</sup> 388.1326, found 388.1320.

***N*-(4-methoxybenzyl)-2-morpholino-5-nitrobenzamide (13d)**. Yellow solid (103 mg, yield 88.0%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.84 (d, *J* = 2.7 Hz, 1H), 8.50 (s, 1H), 8.24 (dd, *J* = 9.0, 2.7 Hz, 1H), 7.31 (d, *J* = 8.3 Hz, 2H), 7.16 (d, *J* = 8.9 Hz, 1H), 6.90 (d, *J* = 8.2 Hz, 2H), 4.58 (d, *J* = 5.6 Hz, 2H), 3.81 (s, 3H), 3.55 (t, *J* = 4.5 Hz, 4H), 3.01 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 164.82, 159.45, 155.50, 143.32, 129.84, 129.78, 128.28, 127.36, 126.93, 119.40, 114.40, 66.41, 55.43, 52.77, 43.57; HR-MS (ESI) *m/z*: calcd for C<sub>19</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub> [M+H]<sup>+</sup> 372.1554, found 372.1548.

***N*-(4-ethoxybenzyl)-2-morpholino-5-nitrobenzamide (13e)**. Yellow solid (80 mg, yield 77.9%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.85 (d, *J* = 2.8 Hz, 1H), 8.51 (s, 1H), 8.25

(dd,  $J = 8.9, 2.8$  Hz, 1H), 7.30 (d,  $J = 8.6$  Hz, 2H), 7.16 (d,  $J = 8.9$  Hz, 1H), 6.89 (d,  $J = 8.6$  Hz, 2H), 4.58 (d,  $J = 5.6$  Hz, 2H), 4.03 (q,  $J = 7.0$  Hz, 2H), 3.58 - 3.48 (m, 4H), 3.06 - 2.97 (m, 4H), 1.42 (t,  $J = 7.0$  Hz, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.69, 158.83, 155.51, 143.39, 129.77, 129.73, 128.41, 127.43, 126.90, 119.40, 114.96, 66.42, 63.61, 52.81, 43.59, 14.89; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  386.1710, found 386.1712.

**2-Morpholino-5-nitro-*N*-(4-(trifluoromethyl)benzyl)benzamide (13f).** Yellow solid (99 mg, yield 81.1%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.81 (d,  $J = 2.8$  Hz, 1H), 8.77 (t,  $J = 6.5$  Hz, 1H), 8.25 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.63 (d,  $J = 8.0$  Hz, 2H), 7.51 (d,  $J = 8.0$  Hz, 2H), 7.20 (d,  $J = 8.9$  Hz, 1H), 4.72 (d,  $J = 6.0$  Hz, 2H), 3.70 - 3.55 (m, 4H), 3.14 - 2.99 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.19, 155.56, 143.50, 142.09, 130.31 (q,  $J = 32.6$  Hz), 128.54, 128.07, 127.48, 127.18, 125.99 (q,  $J = 3.8$  Hz), 124.05 (q,  $J = 270.5$  Hz), 119.66, 66.58, 52.92, 43.51; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{19}\text{F}_3\text{N}_3\text{O}_4$   $[\text{M}+\text{H}]^+$  410.1322 found 410.1319.

**2-Morpholino-5-nitro-*N*-(4-(trifluoromethoxy)benzyl)benzamide (13g).** Yellow solid (79 mg, yield 58.6%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.86 (d,  $J = 2.8$  Hz, 1H), 8.67 (s, 1H), 8.27 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.43 (d,  $J = 8.6$  Hz, 2H), 7.23 (d,  $J = 8.6$  Hz, 2H), 7.20 (d,  $J = 9.0$  Hz, 1H), 4.67 (d,  $J = 5.9$  Hz, 2H), 3.62 - 3.54 (m, 4H), 3.06 - 2.98 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.04, 155.54, 148.93, 143.46, 136.88, 129.84, 128.17, 127.44, 127.10, 121.60, 120.51 (q,  $J = 255.8$  Hz), 119.59, 66.51, 52.89, 43.25; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{19}\text{F}_3\text{N}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  426.1271, found 426.1267.

***N*-(4-(dimethylamino)benzyl)-2-morpholino-5-nitrobenzamide (13h).** Yellow solid (99 mg, yield 81.2%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.86 (d,  $J = 2.8$  Hz, 1H), 8.37 (s, 1H), 8.25 (dd,  $J = 8.9, 2.9$  Hz, 1H), 7.24 (d,  $J = 9.4$  Hz, 2H), 7.15 (d,  $J = 8.9$  Hz, 1H), 6.71 (d,  $J = 8.5$  Hz, 2H), 4.55 (d,  $J = 5.5$  Hz, 2H), 3.56 (t,  $J = 4.6$  Hz, 4H), 3.08 - 2.99 (m, 4H), 2.95 (s, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.66, 155.49, 150.49, 143.37, 129.53, 128.57, 127.42, 126.83, 125.20, 119.26, 112.85, 66.44, 52.80, 43.76, 40.69; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{25}\text{N}_4\text{O}_4$   $[\text{M}+\text{H}]^+$  385.1870, found 385.1873.

***N*-(4-chlorobenzyl)-2-morpholino-5-nitrobenzamide (13i).** Yellow solid (91 mg, yield 82.4%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.85 (d,  $J = 2.8$  Hz, 1H), 8.66 (s, 1H), 8.27

(dd,  $J = 8.9, 2.8$  Hz, 1H), 7.34 (d,  $J = 1.8$  Hz, 4H), 7.19 (d,  $J = 8.9$  Hz, 1H), 4.63 (d,  $J = 5.8$  Hz, 2H), 3.65 - 3.53 (m, 4H), 3.09 - 2.98 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.16, 155.51, 143.33, 136.41, 133.94, 129.74, 129.20, 128.01, 127.36, 127.10, 119.53, 66.52, 52.83, 43.38; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{18}\text{H}_{19}\text{ClN}_3\text{O}_4$   $[\text{M}+\text{H}]^+$  376.1059 found 376.1058.

***N*-(4-hydroxybenzyl)-2-morpholino-5-nitrobenzamide (13j)**. Yellow solid (65 mg, yield 81.1% over 2 steps).  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.35 (s, 1H), 9.01 (t,  $J = 6.0$  Hz, 1H), 8.17 (dd,  $J = 9.0, 2.9$  Hz, 1H), 8.12 (d,  $J = 2.8$  Hz, 1H), 7.15 (t,  $J = 8.1$  Hz, 3H), 6.73 (d,  $J = 8.4$  Hz, 2H), 4.32 (d,  $J = 5.9$  Hz, 2H), 3.51 (t,  $J = 4.5$  Hz, 4H), 3.09 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  166.37, 156.47, 154.38, 139.00, 129.33, 129.15, 126.89, 126.14, 125.50, 117.56, 115.09, 65.53, 50.48, 42.33; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{18}\text{H}_{20}\text{N}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  358.1397 found 358.1396.

***N*-(1-(4-methoxyphenyl)ethyl)-2-morpholino-5-nitrobenzamide (13k)**. Yellow solid (99 mg, yield 81.1%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.82 (d,  $J = 2.8$  Hz, 1H), 8.43 (d,  $J = 7.7$  Hz, 1H), 8.24 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.34 (d,  $J = 8.7$  Hz, 2H), 7.16 (d,  $J = 8.9$  Hz, 1H), 6.92 (d,  $J = 8.7$  Hz, 2H), 5.36 - 5.27 (m, 1H), 3.82 (s, 3H), 3.64 - 3.43 (m, 4H), 3.00 (dd,  $J = 5.5, 3.9$  Hz, 4H), 1.61 (d,  $J = 6.9$  Hz, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.15, 159.34, 155.47, 143.29, 134.58, 128.46, 127.84, 127.35, 126.85, 119.39, 114.36, 66.32, 55.46, 52.75, 49.02, 21.47; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  343.1401 found 343.1399.

***N*-(3-fluoro-4-methoxybenzyl)-2-morpholino-5-nitrobenzamide (13l)**. Yellow solid (52 mg, yield 42.0%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.82 (s, 1H), 8.62 (s, 1H), 8.25 (d,  $J = 8.9$  Hz, 1H), 7.46 - 7.03 (m, 3H), 6.95 (t,  $J = 8.4$  Hz, 1H), 4.57 (d,  $J = 5.8$  Hz, 2H), 3.90 (s, 3H), 3.60 (s, 4H), 3.03 (s, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.01, 161.69, 160.45, 155.53, 154.18, 149.95 (d,  $J = 20.1$  Hz), 143.57, 130.81 (d,  $J = 7.1$  Hz), 128.25, 127.48, 127.10, 124.30 (d,  $J = 3.4$  Hz), 119.58, 116.33, 116.08, 113.76, 66.56, 56.47, 52.93, 43.28; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{21}\text{FN}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  390.1460, found 390.1467.

***N*-(3,4-dimethoxybenzyl)-2-morpholino-5-nitrobenzamide (13m)**. Yellow solid (85 mg, yield 66.9%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.85 (d,  $J = 2.8$  Hz, 1H), 8.55 (s,

1H), 8.26 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.17 (d,  $J = 8.9$  Hz, 1H), 6.93 (dt,  $J = 4.9, 2.0$  Hz, 2H), 6.86 (d,  $J = 8.6$  Hz, 1H), 4.59 (d,  $J = 5.7$  Hz, 2H), 3.89 (s, 3H), 3.88 (s, 3H), 3.61 - 3.51 (m, 4H), 3.07 - 2.97 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.84, 155.49, 149.44, 148.94, 143.39, 130.35, 128.27, 127.40, 126.97, 120.78, 119.43, 111.68, 111.35, 66.44, 56.09, 56.07, 52.83, 43.98; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_6$   $[\text{M}+\text{H}]^+$  402.1660, found 402.1658.

***N*-(3-hydroxy-4-methoxybenzyl)-2-morpholino-5-nitrobenzamide (13n)**. Yellow solid (75 mg, yield 61.5% over 2 steps).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.85 (d,  $J = 2.8$  Hz, 1H), 8.51 (s, 1H), 8.25 (dd,  $J = 8.9, 2.9$  Hz, 1H), 7.17 (d,  $J = 8.9$  Hz, 1H), 6.94 (d,  $J = 1.9$  Hz, 1H), 6.89 - 6.81 (m, 2H), 4.55 (d,  $J = 5.6$  Hz, 2H), 3.90 (s, 3H), 3.57 (t,  $J = 4.7$  Hz, 4H), 3.07 - 3.00 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ )  $\delta$  166.46, 154.37, 146.80, 146.44, 138.95, 131.56, 126.81, 126.17, 125.53, 118.70, 117.51, 115.40, 112.13, 65.57, 55.71, 50.50, 42.39; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{N}_3\text{O}_6$   $[\text{M}+\text{H}]^+$  388.1503 found 388.1502.

***N*-(3,4,5-trimethoxybenzyl)-2-morpholino-5-nitrobenzamide (13o)**. Yellow solid (63 mg, yield 46.1%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.85 (d,  $J = 2.8$  Hz, 1H), 8.55 (s, 1H), 8.27 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.18 (d,  $J = 8.9$  Hz, 1H), 6.61 (s, 2H), 4.58 (d,  $J = 5.8$  Hz, 2H), 3.86 (s, 6H), 3.84 (s, 3H), 3.64 - 3.56 (m, 4H), 3.08 - 2.98 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.94, 155.47, 153.74, 143.37, 137.94, 133.58, 128.26, 127.36, 126.99, 119.45, 105.58, 66.47, 61.03, 56.36, 52.85, 44.45; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{21}\text{H}_{26}\text{N}_3\text{O}_7$   $[\text{M}+\text{H}]^+$  432.1765, found 432.1768.

**2-Morpholino-5-nitro-*N*-(pyridin-4-ylmethyl)benzamide (13p)**. Yellow solid (89 mg, yield 82.4%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.97 - 8.76 (m, 2H), 8.60 (d,  $J = 6.1$  Hz, 2H), 8.29 (dd,  $J = 8.9, 2.8$  Hz, 1H), 7.29 (d,  $J = 6.1$  Hz, 2H), 7.23 (d,  $J = 8.9$  Hz, 1H), 4.68 (d,  $J = 6.0$  Hz, 2H), 3.76 - 3.63 (m, 4H), 3.15 - 3.03 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.45, 155.56, 150.36, 146.94, 143.43, 127.88, 127.45, 127.26, 122.79, 119.70, 66.67, 52.93, 42.85; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}_4$   $[\text{M}+\text{H}]^+$  343.1401 found 343.1399.

***N*-((6-methoxypyridin-3-yl)methyl)-2-morpholino-5-nitrobenzamide (13q)**. Yellow solid (79 mg, yield 58.6%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.85 (d,  $J = 2.8$  Hz,

1H), 8.58 (s, 1H), 8.27 (dd,  $J = 8.9, 2.8$  Hz, 1H), 8.17 (d,  $J = 2.4$  Hz, 1H), 7.65 (dd,  $J = 8.5, 2.5$  Hz, 1H), 7.19 (d,  $J = 8.9$  Hz, 1H), 6.79 - 6.73 (m, 1H), 4.59 (d,  $J = 5.8$  Hz, 2H), 3.94 (s, 3H), 3.65 - 3.59 (m, 4H), 3.07 - 3.01 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.07, 164.04, 155.46, 146.55, 143.29, 139.12, 128.09, 127.35, 127.03, 126.35, 119.44, 111.35, 66.55, 53.65, 52.78, 40.89; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{18}\text{H}_{21}\text{N}_4\text{O}_5$   $[\text{M}+\text{H}]^+$  373.1506, found 373.1509.

#### 4.1.5. General procedures for the preparation of intermediates **14a-h** and **22a-b**

The synthesis methods of intermediates **14a-h** and **22a-b** were the same as that of **12**.

**5-Nitro-2-(piperidin-1-yl)benzoic acid (14a)**. Yellow solid, yield 80.5%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  13.62 (s, 1H), 8.36 (d,  $J = 3.0$  Hz, 1H), 8.16 (dd,  $J = 9.2, 3.0$  Hz, 1H), 7.19 (d,  $J = 9.3$  Hz, 1H), 3.24 (t,  $J = 5.2$  Hz, 4H), 1.71 - 1.51 (m, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  167.49, 155.35, 137.66, 127.33, 127.19, 120.78, 117.94, 51.86, 25.14, 23.18; ESI-MS  $m/z$  250.1  $[\text{M}-\text{H}]^-$  249.1.

**5-Nitro-2-(pyrrolidin-1-yl)benzoic acid (14b)**. Yellow solid, yield 76.3%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  13.21 (s, 1H), 8.26 (d,  $J = 2.8$  Hz, 1H), 8.05 (dd,  $J = 9.4, 2.8$  Hz, 1H), 6.83 (d,  $J = 9.5$  Hz, 1H), 3.29 (t,  $J = 6.2$  Hz, 4H), 1.90 (t,  $J = 6.1$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  168.51, 151.04, 135.17, 127.59, 126.96, 117.24, 114.34, 51.42, 25.70; ESI-MS  $m/z$  236.1  $[\text{M}-\text{H}]^-$  235.1.

**2-(Diethylamino)-5-nitrobenzoic acid (14c)**. Yellow solid, yield 85.9%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.34 (d,  $J = 2.9$  Hz, 1H), 8.17 (dd,  $J = 9.4, 3.0$  Hz, 1H), 7.22 (d,  $J = 9.4$  Hz, 1H), 3.40 (q,  $J = 7.0$  Hz, 4H), 1.12 (t,  $J = 7.0$  Hz, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  168.40, 153.06, 137.84, 127.31, 127.26, 121.67, 118.01, 46.90, 12.32; ESI-MS  $m/z$  238.1  $[\text{M}-\text{H}]^-$  237.1.

**2-(Dimethylamino)-5-nitrobenzoic acid (14d)**. Yellow solid, yield 88.2%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  13.27 (s, 1H), 8.35 (d,  $J = 2.9$  Hz, 1H), 8.11 (dd,  $J = 9.5, 2.9$  Hz, 1H), 7.02 (d,  $J = 9.4$  Hz, 1H), 3.03 (s, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  168.26, 154.89, 136.06, 128.05, 127.15, 117.94, 115.35, 42.99; ESI-MS  $m/z$  210.1  $[\text{M}-\text{H}]^-$  209.1.

**2-(4-(Tert-butoxycarbonyl)piperazin-1-yl)-5-nitrobenzoic acid (14e)**. Yellow solid, yield 70.3%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.41 (d,  $J = 2.9$  Hz, 1H), 8.19 (dd,



$J = 9.2, 2.9$  Hz, 1H), 7.17 (d,  $J = 9.3$  Hz, 1H), 3.48 (t,  $J = 4.7$  Hz, 4H), 3.28 (dd,  $J = 6.8, 3.6$  Hz, 4H), 1.42 (s, 9H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  167.30, 155.06, 153.83, 138.06, 127.47, 127.22, 120.62, 117.84, 79.12, 50.22, 27.99; ESI-MS  $m/z$  251.1 [M-H] $^-$  250.1.

**5-Nitro-2-thiomorpholinobenzoic acid (14f).** Yellow solid, yield 76.5%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  8.38 (d,  $J = 3.0$  Hz, 1H), 8.19 (dd,  $J = 9.3, 3.0$  Hz, 1H), 7.21 (d,  $J = 9.3$  Hz, 1H), 3.52 (t,  $J = 2.5$  Hz, 4H), 2.75 (t, 4H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  167.37, 155.78, 138.26, 127.28, 127.15, 121.60, 118.79, 53.35, 26.31; ESI-MS  $m/z$  268.1 [M-H] $^-$  267.1.

**2-(1,1-Dioxidothiomorpholino)-5-nitrobenzoic acid (14g).** Yellow solid, yield 78.3%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  13.55 (s, 1H), 8.47 (d,  $J = 2.9$  Hz, 1H), 8.25 (dd,  $J = 9.2, 2.9$  Hz, 1H), 7.35 (d,  $J = 9.2$  Hz, 1H), 3.76 – 3.64 (m, 4H), 3.41 – 3.17 (m, 4H);  $^{13}\text{C}$  NMR (101 MHz, DMSO)  $\delta$  167.45, 155.44, 139.80, 127.89, 127.74, 122.26, 120.64, 51.39, 50.35; ESI-MS  $m/z$  300.0 [M-H] $^-$  209.0.

**2-((2-Methoxyethyl)amino)-5-nitrobenzoic acid (14h).** Yellow solid, yield 83.2%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  8.87 (s, 1H), 8.63 (d,  $J = 2.8$  Hz, 1H), 8.16 (dd,  $J = 9.4, 2.9$  Hz, 1H), 6.92 (d,  $J = 9.5$  Hz, 1H), 3.58 (t,  $J = 5.0$  Hz, 2H), 3.51 (t,  $J = 5.2$  Hz, 2H), 3.30 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  168.51, 154.63, 134.64, 129.29, 128.51, 111.67, 109.14, 69.90, 58.08, 41.99; ESI-MS  $m/z$  240.1 [M-H] $^-$  239.1.

**4-Morpholinonicotinic acid (22a).** Yellow solid, yield 88.2%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  10.00 (s, 1H), 8.60 (s, 1H), 8.40 (d,  $J = 7.2$  Hz, 1H), 7.48 (d,  $J = 7.3$  Hz, 1H), 3.75 (t,  $J = 4.5$  Hz, 4H), 3.59 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  165.90, 156.79, 143.29, 140.30, 115.45, 112.19, 63.49, 42.89; ESI-MS  $m/z$  208.1 [M-H] $^-$  207.1.

**3-Morpholinoisonicotinic acid (22b).** Yellow solid, yield 90.3%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  8.53 (d,  $J = 3.1$  Hz, 1H), 8.32 (d,  $J = 4.3$  Hz, 1H), 7.51 (d,  $J = 4.4$  Hz, 1H), 3.72 (t, 4H), 3.11 (t,  $J = 4.4$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  168.12, 144.98, 144.13, 142.79, 132.75, 123.43, 66.62, 52.04; ESI-MS  $m/z$  208.1 [M-H] $^-$  207.1.

#### 4.1.6. General procedures for the preparation of target compounds **16a-h**

To the solutions of **14a-h** (0.32 mmol) in 10 mL anhydrous DCM, **15** (93 mg, 0.64

mmol), EDCI (296 mg, 1.6 mmol), HOBt (52 mg, 0.38 mmol) and catalytic amount of DMAP were added. The mixtures were stirred for 2 h, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (4:1) to give products. Then, above obtained products were dissolved into 10 mL THF, and TBAF (1.2 e.q) was added. The mixtures were stirred for 15 min, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (2:1) to give target compounds **16a-d** and **16f-h**. For piperazine compound **16e**, the product obtained in above procedure was dissolved into 10 mL anhydrous DCM, and 2 mL CF<sub>3</sub>COOH was added. The mixture was stirred for 2 h, and neutralize with saturated NaHCO<sub>3</sub> aqueous. The mixture was then extracted with DCM (3 × 25 mL), and the combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with DCM/MeOH (40:1) to give target compound **16e**.

***N*-(3-hydroxy-4-methoxybenzyl)-5-nitro-2-(piperidin-1-yl)benzamide (16a).**  
Yellow solid, yield 44.4% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 9.00 (t, *J* = 6.0 Hz, 1H), 8.95 (s, 1H), 8.13 (q, *J* = 3.3 Hz, 2H), 7.16 - 7.08 (m, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 6.80 (d, *J* = 2.1 Hz, 1H), 6.73 (dd, *J* = 8.2, 2.1 Hz, 1H), 4.31 (d, *J* = 5.9 Hz, 2H), 3.74 (s, 3H), 3.13 (s, 4H), 1.50 (s, 6H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.56, 155.06, 146.74, 146.45, 138.05, 131.68, 126.24, 126.17, 125.74, 118.48, 117.56, 115.26, 112.11, 55.71, 51.51, 42.34, 25.08, 23.38; HR-MS (ESI) *m/z*: calcd for C<sub>20</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub> [M+H]<sup>+</sup> 386.1710 found 386.1710.

***N*-(3-hydroxy-4-methoxybenzyl)-5-nitro-2-(pyrrolidin-1-yl)benzamide (16b).**  
Yellow solid, yield 50.2% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 8.98 (s, 2H), 8.27 - 7.77 (m, 2H), 7.17 - 6.56 (m, 4H), 4.46 - 4.19 (m, 2H), 3.74 (s, 3H), 3.30 (s, 4H), 1.85 (s, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.85, 149.75, 146.75, 146.43, 138.62, 134.36, 131.62, 125.84, 121.48, 118.45, 115.25, 113.36, 112.10, 55.71, 49.85, 42.43,

25.15; HR-MS (ESI)  $m/z$ : calcd for  $C_{19}H_{22}N_3O_5$   $[M+H]^+$  372.1554 found 372.1551.

**2-(Diethylamino)-*N*-(3-hydroxy-4-methoxybenzyl)-5-nitrobenzamide (16c).**

Yellow solid, yield 55.3% over 2 steps.  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  9.09 (t,  $J = 5.4$  Hz, 1H), 8.85 (d,  $J = 2.9$  Hz, 1H), 8.19 (dd,  $J = 8.9, 2.9$  Hz, 1H), 7.16 (d,  $J = 9.0$  Hz, 1H), 6.97 - 6.88 (m, 1H), 6.83 (d,  $J = 2.2$  Hz, 2H), 5.86 (s, 1H), 4.53 (d,  $J = 5.4$  Hz, 2H), 3.88 (s, 3H), 3.12 (q,  $J = 7.1$  Hz, 4H), 0.98 (t,  $J = 7.1$  Hz, 6H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  165.07, 154.85, 146.25, 145.99, 142.78, 131.10, 129.04, 127.26, 126.22, 121.87, 119.80, 114.36, 110.92, 56.12, 47.60, 43.77, 11.85; HR-MS (ESI)  $m/z$ : calcd for  $C_{19}H_{24}N_3O_5$   $[M+H]^+$  374.1710 found 374.1714.

**2-(Dimethylamino)-*N*-(3-hydroxy-4-methoxybenzyl)-5-nitrobenzamide (16d).**

Yellow solid, yield 62.9% over 2 steps.  $^1H$  NMR (300 MHz,  $DMSO-d_6$ )  $\delta$  8.97 (d,  $J = 7.4$  Hz, 2H), 8.31 - 7.68 (m, 2H), 6.89 (t,  $J = 11.3$  Hz, 2H), 6.85 - 6.45 (m, 2H), 4.29 (d,  $J = 5.7$  Hz, 2H), 3.74 (s, 3H), 2.95 (s, 6H);  $^{13}C$  NMR (75 MHz,  $DMSO-d_6$ )  $\delta$  167.61, 153.46, 146.70, 146.41, 135.46, 131.66, 126.02, 125.79, 122.66, 118.38, 115.18, 114.32, 112.11, 55.67, 42.35, 41.89; HR-MS (ESI)  $m/z$ : calcd for  $C_{17}H_{20}N_3O_5$   $[M+H]^+$  346.1397 found 346.1393.

***N*-(3-hydroxy-4-methoxybenzyl)-5-nitro-2-(piperazin-1-yl)benzamide (16e).**

Yellow solid, yield 43.9% over 3 steps.  $^1H$  NMR (300 MHz,  $DMSO-d_6$ )  $\delta$  9.04 (s, 2H), 8.15 (s, 2H), 7.15 (s, 1H), 6.82 (d,  $J = 25.3$  Hz, 2H), 5.76 (s, 1H), 4.30 (s, 2H), 4.10 - 3.56 (m, 3H), 3.15 (s, 4H), 2.80 (s, 4H);  $^{13}C$  NMR (75 MHz,  $DMSO-d_6$ )  $\delta$  166.49, 154.41, 146.74, 146.43, 138.71, 131.61, 126.54, 126.18, 125.64, 118.48, 117.70, 115.21, 112.12, 55.69, 50.13, 44.20, 42.36; HR-MS (ESI)  $m/z$ : calcd for  $C_{19}H_{23}N_4O_5$   $[M+H]^+$  387.1663 found 387.1664.

***N*-(3-hydroxy-4-methoxybenzyl)-5-nitro-2-thiomorpholinobenzamide (16f).**

Yellow solid, yield 56.0% over 2 steps.  $^1H$  NMR (300 MHz,  $DMSO-d_6$ )  $\delta$  9.02 (t,  $J = 6.0$  Hz, 1H), 8.97 (s, 1H), 8.15 (d,  $J = 9.3$  Hz, 2H), 7.17 (d,  $J = 8.9$  Hz, 1H), 6.88 (d,  $J = 8.1$  Hz, 1H), 6.80 (s, 1H), 6.73 (d,  $J = 8.1$  Hz, 1H), 4.31 (d,  $J = 6.0$  Hz, 2H), 3.74 (s, 3H), 3.49 - 3.38 (m, 4H), 2.60 (d,  $J = 4.7$  Hz, 4H);  $^{13}C$  NMR (75 MHz,  $DMSO-d_6$ )  $\delta$  166.42, 155.04, 146.76, 146.45, 138.94, 131.63, 127.23, 126.07, 125.54, 118.58, 118.50, 115.24, 112.21, 55.74, 52.96, 42.35, 26.22; HR-MS (ESI)  $m/z$ : calcd for  $C_{19}H_{22}N_3O_5S$

[M+H]<sup>+</sup> 404.1275 found 404.1277.

**2-(1,1-Dioxidothiomorpholino)-N-(3-hydroxy-4-methoxybenzyl)-5-nitrobenzamide (16g).** Yellow solid, yield 53.4% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 9.09 - 9.00 (m, 1H), 8.97 (s, 1H), 8.27 - 8.08 (m, 2H), 7.28 (d, *J* = 8.8 Hz, 1H), 6.88 (d, *J* = 8.2 Hz, 1H), 6.78 (d, *J* = 2.1 Hz, 1H), 6.73 (dd, *J* = 8.1, 2.1 Hz, 1H), 4.32 (d, *J* = 5.9 Hz, 2H), 3.74 (s, 3H), 3.65 - 3.48 (m, 4H), 3.11 (t, *J* = 5.1 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.27, 153.54, 146.79, 146.48, 139.92, 131.58, 127.65, 126.01, 125.41, 119.67, 118.39, 115.10, 112.28, 55.70, 50.61, 49.39, 42.39; HR-MS (ESI) *m/z*: calcd for C<sub>19</sub>H<sub>22</sub>N<sub>3</sub>O<sub>7</sub>S [M+H]<sup>+</sup> 436.1173 found 436.1166.

**N-(3-hydroxy-4-methoxybenzyl)-2-((2-methoxyethyl)amino)-5-nitrobenzamide (16h).** Yellow solid, yield 62.4% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 9.31 (s, 1H), 9.16 (s, 1H), 8.95 (s, 1H), 8.62 (s, 1H), 8.14 (d, *J* = 9.3 Hz, 1H), 6.77 (dd, *J* = 32.8, 16.3 Hz, 4H), 4.32 (s, 2H), 3.75 (s, 3H), 3.56 (s, 2H), 3.45 (s, 2H), 3.30 (s, 3H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.30, 154.08, 146.68, 146.44, 134.63, 131.83, 128.12, 125.51, 118.11, 114.86, 113.11, 112.20, 111.07, 70.00, 58.13, 55.71, 54.93, 42.05; HR-MS (ESI) *m/z*: calcd for C<sub>18</sub>H<sub>22</sub>N<sub>3</sub>O<sub>6</sub> [M+H]<sup>+</sup> 376.1503 found 376.1496.

#### 4.1.7. General procedures for the preparation of target compounds **20a-p**

Target compounds **20a-p** were synthesized by two methods. Method A: the same as the synthesis of **16a-h**. Method B: To the solutions of various benzoic acids (0.32 mmol) in 10 mL anhydrous CH<sub>3</sub>CN, **15** (93 mg, 0.64 mmol), HATU (145 mg, 0.38 mmol), and Et<sub>3</sub>N (53 μL, 0.38 mmol) were added. The mixtures were stirred for 2 h, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (4:1) to give products. Then, above obtained products were dissolved into 10 mL THF, and TBAF was added. The mixtures were stirred for 15 min, and extracted with DCM (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products, which were purified by column chromatography with petroleum/ethyl acetate (2:1) to give target compounds. For compound **20o**, the obtained product (56 mg, 0.13 mmol) in

above procedure was dissolved into 10 mL CH<sub>3</sub>OH, Pd-C (5 mg) was added. The reaction was stirred for 2 h under hydrogen atmosphere, then the mixture was filtered and the filtrate was concentrated in vacuo to provide the crude product, which were purified by column chromatography with petroleum/ethyl acetate (1:1) to give target compound **20o**.

**N-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20a)**. Method A, white solid, yield 54.9% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.04 (s, 1H), 8.22 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.44 (td, *J* = 7.7, 1.8 Hz, 1H), 7.26 (dd, *J* = 7.5, 1.4 Hz, 1H), 7.20 (td, *J* = 8.0, 1.2 Hz, 1H), 6.97 (d, *J* = 2.0 Hz, 1H), 6.87 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.82 (d, *J* = 8.2 Hz, 1H), 6.02 (s, 1H), 4.55 (d, *J* = 5.3 Hz, 2H), 3.88 (s, 3H), 3.61 - 3.44 (m, 4H), 2.98 - 2.85 (m, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 165.99, 150.82, 146.35, 146.13, 132.22, 131.87, 131.60, 127.77, 125.29, 120.50, 119.99, 114.70, 110.91, 66.88, 56.11, 53.47, 43.57; HR-MS (ESI) *m/z*: calcd for C<sub>19</sub>H<sub>23</sub>N<sub>2</sub>O<sub>4</sub> [M+H]<sup>+</sup> 343.1652 found 343.1645.

**5-Fluoro-N-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20b)**. Method B, white solid, yield 46.5% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.35 (t, *J* = 5.4 Hz, 1H), 7.96 (dd, *J* = 9.7, 3.0 Hz, 1H), 7.16 (m, 2H), 6.95 (s, 1H), 6.89 - 6.79 (m, 2H), 5.96 (s, 1H), 4.53 (d, *J* = 5.2 Hz, 2H), 3.89 (s, 3H), 3.50 (t, *J* = 4.4 Hz, 4H), 2.86 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 164.47, 160.16 (d, *J* = 243.8 Hz), 146.85 (d, *J* = 3.0 Hz), 146.38, 146.13, 131.37, 130.09 (d, *J* = 6.9 Hz), 122.93 (d, *J* = 7.9 Hz), 120.06, 118.83 (d, *J* = 22.4 Hz), 118.37 (d, *J* = 24.0 Hz), 114.67, 110.91, 66.86, 56.13, 53.70, 43.72; HR-MS (ESI) *m/z*: calcd for C<sub>19</sub>H<sub>22</sub>FN<sub>2</sub>O<sub>4</sub> [M+H]<sup>+</sup> 361.1558 found 361.1551.

**N-(3-hydroxy-4-methoxybenzyl)-2-(piperidin-1-yl)benzamide (20c)**. Method A, white solid, yield 50.5% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.61 (s, 1H), 8.24 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.40 (td, *J* = 7.6, 1.9 Hz, 1H), 7.20 (t, *J* = 7.7 Hz, 2H), 6.96 (d, *J* = 2.1 Hz, 1H), 6.86 - 6.78 (m, 2H), 5.99 (s, 1H), 4.55 (d, *J* = 5.4 Hz, 2H), 3.86 (s, 3H), 2.81 (d, *J* = 5.0 Hz, 4H), 1.44 (s, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 166.26, 152.69, 146.15, 146.00, 133.43, 131.98, 131.56, 127.66, 124.84, 120.98, 119.84, 114.68, 110.93, 56.16, 54.83, 43.49, 26.24, 23.72; HR-MS (ESI) *m/z*: calcd for C<sub>20</sub>H<sub>25</sub>N<sub>2</sub>O<sub>3</sub>

[M+H]<sup>+</sup> 341.1860 found 341.1862.

**5-Fluoro-*N*-(3-hydroxy-4-methoxybenzyl)-2-(piperidin-1-yl)benzamide (20d).**

Method B, white solid, yield 55.7% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.79 (s, 1H), 7.97 (dd, *J* = 9.9, 3.2 Hz, 1H), 7.19 (dd, *J* = 8.8, 4.9 Hz, 1H), 7.08 (ddd, *J* = 8.8, 7.2, 3.2 Hz, 1H), 6.95 (d, *J* = 2.0 Hz, 1H), 6.87 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.81 (d, *J* = 8.2 Hz, 1H), 5.62 (s, 1H), 4.54 (d, *J* = 5.3 Hz, 2H), 3.88 (s, 3H), 2.79 (d, *J* = 5.1 Hz, 4H), 1.51 - 1.42 (m, 6H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 169.92, 159.94 (d, *J* = 243.4 Hz), 148.69 (d, *J* = 2.8 Hz), 146.15, 145.98, 139.31, 131.78, 123.20 (d, *J* = 7.9 Hz), 119.94, 118.57 (d, *J* = 22.1 Hz), 117.96 (d, *J* = 24.0 Hz), 118.12, 117.80, 114.63, 110.90, 56.19, 55.07, 43.61, 26.24, 23.62; HR-MS (ESI) *m/z*: calcd for C<sub>20</sub>H<sub>24</sub>FN<sub>2</sub>O<sub>3</sub> [M+H]<sup>+</sup> 359.1765 found 359.1762.

***N*-(3-hydroxy-4-methoxybenzyl)-4-morpholinonicotinamide (20e).**

Method A, white solid, yield 40.7% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.96 (s, 1H), 8.52 (d, *J* = 5.6 Hz, 1H), 7.99 (s, 1H), 6.94 (d, *J* = 1.9 Hz, 1H), 6.88 (d, *J* = 5.6 Hz, 1H), 6.85 (d, *J* = 1.8 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 4.53 (d, *J* = 5.7 Hz, 2H), 3.90 (s, 3H), 3.59 - 3.47 (m, 4H), 3.04 - 2.93 (m, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 165.69, 156.51, 152.41, 151.94, 146.72, 146.38, 131.05, 122.44, 119.91, 114.78, 112.62, 111.04, 66.36, 56.11, 51.69, 43.52; HR-MS (ESI) *m/z*: calcd for C<sub>18</sub>H<sub>22</sub>N<sub>3</sub>O<sub>4</sub> [M+H]<sup>+</sup> 344.1605 found 344.1599.

***N*-(3-hydroxy-4-methoxybenzyl)-3-morpholinoisonicotinamide (20f).**

Method A, white solid, yield 56.1% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 9.60 (t, *J* = 5.5 Hz, 1H), 8.53 (d, *J* = 4.3 Hz, 2H), 7.98 (d, *J* = 5.0 Hz, 1H), 6.97 (s, 1H), 6.85 (s, 2H), 4.54 (d, *J* = 5.5 Hz, 2H), 3.88 (s, 3H), 3.51 (t, *J* = 4.4 Hz, 4H), 2.99 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 164.00, 146.77, 146.68, 146.32, 145.05, 143.09, 134.62, 130.73, 124.40, 119.92, 114.83, 111.04, 66.62, 56.07, 53.12, 43.65; HR-MS (ESI) *m/z*: calcd for C<sub>18</sub>H<sub>22</sub>N<sub>3</sub>O<sub>4</sub> [M+H]<sup>+</sup> 344.1605 found 344.1604.

**5-Chloro-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20g).**

Method A, white solid, yield 53.2% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 9.93 (d, *J* = 6.6 Hz, 1H), 8.19 (d, *J* = 2.7 Hz, 1H), 7.39 (dd, *J* = 8.5, 2.8 Hz, 1H), 7.13 (d, *J* = 8.6 Hz, 1H), 6.95 (d, *J* = 2.0 Hz, 1H), 6.90 - 6.80 (m, 2H), 5.88 (s, 1H), 4.53 (d, *J* =

5.3 Hz, 2H), 3.89 (s, 3H), 3.50 (t,  $J = 4.5$  Hz, 4H), 2.87 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.62, 149.28, 146.40, 146.15, 131.99, 131.78, 131.37, 131.15, 129.48, 122.17, 120.06, 114.66, 110.94, 66.79, 56.15, 53.51, 43.70; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{ClN}_2\text{O}_4$   $[\text{M}+\text{H}]^+$  377.1263 found 377.1266.

**5-Bromo-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20h).**

Method A, white solid, yield 59.0% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.86 (s, 1H), 8.33 (d,  $J = 2.5$  Hz, 1H), 7.54 (dd,  $J = 8.5, 2.5$  Hz, 1H), 7.06 (d,  $J = 8.6$  Hz, 1H), 6.94 (s, 1H), 6.90 - 6.79 (m, 2H), 5.84 (s, 1H), 4.53 (d,  $J = 5.3$  Hz, 2H), 3.89 (s, 3H), 3.50 (t,  $J = 4.5$  Hz, 4H), 2.87 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.56, 149.78, 146.41, 146.15, 134.96, 134.70, 131.35, 129.68, 122.40, 120.04, 118.78, 114.66, 110.94, 66.75, 56.14, 53.42, 43.69; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{BrN}_2\text{O}_4$   $[\text{M}+\text{H}]^+$  421.0757 found 421.0752.

***N*-(3-hydroxy-4-methoxybenzyl)-2-morpholino-5-(trifluoromethyl)benzamide (20i).**

Method A, white solid, yield 46.6% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.39 (s, 1H), 8.43 (d,  $J = 2.3$  Hz, 1H), 7.68 (dd,  $J = 8.4, 2.4$  Hz, 1H), 7.24 (d,  $J = 8.4$  Hz, 1H), 6.95 (d,  $J = 1.9$  Hz, 1H), 6.93 - 6.85 (m, 1H), 6.83 (d,  $J = 8.2$  Hz, 1H), 5.83 (s, 1H), 4.55 (d,  $J = 5.4$  Hz, 2H), 3.89 (s, 3H), 3.68 - 3.36 (m, 4H), 2.95 (dd,  $J = 5.5, 3.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.92, 153.51, 146.40, 146.13, 131.25, 129.35 (q,  $J = 3.7$  Hz), 128.90 (q,  $J = 3.6$  Hz), 128.40, 126.99 (q,  $J = 33.2$  Hz), 123.92 (q,  $J = 270.5$  Hz), 120.31, 120.11, 114.61, 110.92, 66.69, 56.15, 53.29, 43.70; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{22}\text{F}_3\text{N}_2\text{O}_4$   $[\text{M}+\text{H}]^+$  411.1526 found 411.1519.

***N*-(3-hydroxy-4-methoxybenzyl)-2-morpholino-4-nitrobenzamide (20j).**

Method A, white solid, yield 56.5% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.34 (s, 1H), 8.31 (d,  $J = 8.6$  Hz, 1H), 8.06 (dd,  $J = 8.6, 2.1$  Hz, 1H), 8.00 (d,  $J = 2.2$  Hz, 1H), 6.96 (d,  $J = 1.9$  Hz, 1H), 6.91 - 6.80 (m, 2H), 5.73 (s, 1H), 4.56 (d,  $J = 5.5$  Hz, 2H), 3.91 (s, 3H), 3.54 (t,  $J = 4.6$  Hz, 4H), 3.02 - 2.91 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.30, 151.56, 149.91, 146.49, 146.18, 133.50, 133.14, 130.92, 120.13, 119.43, 115.21, 114.62, 110.93, 66.55, 56.15, 53.33, 43.77; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{22}\text{N}_3\text{O}_6$   $[\text{M}+\text{H}]^+$  388.1503 found 388.1498.

**5-Bromo-*N*-(3-hydroxy-4-methoxybenzyl)-2-(piperidin-1-yl)benzamide (20k).**

Method A, white solid, yield 46.3% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.34 (s, 1H), 8.36 (d,  $J = 2.6$  Hz, 1H), 7.50 (dd,  $J = 8.5, 2.6$  Hz, 1H), 7.06 (d,  $J = 8.5$  Hz, 1H), 6.94 (d,  $J = 2.0$  Hz, 1H), 6.86 (dd,  $J = 8.2, 2.0$  Hz, 1H), 6.81 (d,  $J = 8.2$  Hz, 1H), 5.62 (s, 1H), 4.54 (d,  $J = 5.4$  Hz, 2H), 3.88 (s, 3H), 2.80 (d,  $J = 5.2$  Hz, 4H), 1.50 - 1.44 (m, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  164.82, 151.61, 146.16, 145.97, 134.75, 134.40, 131.70, 129.58, 122.90, 119.91, 118.32, 114.59, 110.89, 56.19, 54.83, 43.59, 26.15, 23.61; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{20}\text{H}_{24}\text{BrN}_2\text{O}_3$   $[\text{M}+\text{H}]^+$  419.0965 found 419.0961.

***N*-(3-hydroxy-4-methoxybenzyl)-2-(piperidin-1-yl)-5-(trifluoromethyl)benzamide (20l)**. Method A, white solid, yield 57.7% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.86 (d,  $J = 6.9$  Hz, 1H), 8.47 (d,  $J = 2.4$  Hz, 1H), 7.63 (dd,  $J = 8.5, 2.4$  Hz, 1H), 7.23 (s, 1H), 6.94 (d,  $J = 2.0$  Hz, 1H), 6.87 (dd,  $J = 8.2, 2.0$  Hz, 1H), 6.81 (d,  $J = 8.2$  Hz, 1H), 5.60 (s, 1H), 4.56 (d,  $J = 5.5$  Hz, 2H), 3.88 (s, 3H), 2.88 (t,  $J = 4.7$  Hz, 4H), 1.51 (d,  $J = 12.4$  Hz, 6H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.12, 155.37, 146.21, 146.00, 131.56, 129.07 (q,  $J = 3.9$  Hz), 128.65 (q,  $J = 3.8$  Hz), 128.25, 126.29, 120.87, 119.93, 114.57, 110.91, 56.17, 54.73, 43.61, 26.09, 23.60; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{21}\text{H}_{24}\text{F}_3\text{N}_2\text{O}_3$   $[\text{M}+\text{H}]^+$  409.1734 found 409.1731.

**5-(Dimethylamino)-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20m)**. Method B, white solid, yield 46.3% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.84 (s, 1H), 7.70 (d,  $J = 3.2$  Hz, 1H), 7.13 (d,  $J = 8.8$  Hz, 1H), 6.96 (d,  $J = 2.0$  Hz, 1H), 6.89 - 6.76 (m, 3H), 5.91 (s, 1H), 4.54 (d,  $J = 5.2$  Hz, 2H), 3.88 (s, 3H), 3.47 (d,  $J = 5.1$  Hz, 4H), 2.97 (s, 6H), 2.82 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  166.21, 148.43, 146.23, 146.06, 140.19, 131.90, 128.07, 122.42, 119.99, 115.70, 115.08, 114.69, 110.86, 67.13, 56.14, 53.73, 43.65, 40.81; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{21}\text{H}_{28}\text{N}_3\text{O}_4$   $[\text{M}+\text{H}]^+$  386.2074 found 386.2074.

***N*-(3-hydroxy-4-methoxybenzyl)-5-methoxy-2-morpholinobenzamide (20n)**. Method B, white solid, yield 54.9% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.67 (s, 1H), 7.84 (d,  $J = 3.2$  Hz, 1H), 7.17 (d,  $J = 8.8$  Hz, 1H), 7.03 - 6.94 (m, 2H), 6.87 (dd,  $J = 8.3, 2.0$  Hz, 1H), 6.82 (d,  $J = 8.2$  Hz, 1H), 5.87 (s, 1H), 4.54 (d,  $J = 5.2$  Hz, 2H), 3.89 (s, 3H), 3.84 (s, 3H), 3.49 (t,  $J = 4.5$  Hz, 4H), 2.85 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.47, 157.18, 146.29, 146.10, 143.97, 131.68, 128.93, 122.72,



120.02, 118.88, 115.11, 114.68, 110.88, 67.01, 56.14, 55.74, 53.72, 43.69; HR-MS (ESI)  $m/z$ : calcd for  $C_{20}H_{25}N_2O_5$   $[M+H]^+$  373.1758 found 373.1748.

**5-Hydroxy-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (20o)**

Method B, white solid, yield 16.8% over 3 steps.  $^1H$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  10.32 (s, 1H), 9.50 (s, 1H), 8.96 (s, 1H), 7.37 (s, 1H), 7.20 (s, 1H), 6.88 - 6.74 (m, 4H), 4.35 (s, 2H), 3.74 (s, 3H), 3.43 (s, 4H), 2.75 (s, 4H);  $^{13}C$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  164.98, 154.38, 146.86, 146.61, 142.56, 131.62, 129.42, 122.92, 118.65, 118.36, 116.46, 115.28, 112.25, 66.18, 55.73, 53.26, 42.38; HR-MS (ESI)  $m/z$ : calcd for  $C_{19}H_{28}N_2O_5$   $[M+H]^+$  359.1601 found 359.1597.

***N*-(3-hydroxy-4-methoxybenzyl)-5-methoxy-2-(piperidin-1-yl)benzamide (20p)**

Method B, white solid, yield 39.5% over 2 steps.  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  11.08 (s, 1H), 7.85 (d,  $J = 3.2$  Hz, 1H), 7.16 (d,  $J = 8.8$  Hz, 1H), 6.98 - 6.92 (m, 2H), 6.87 (dd,  $J = 8.2, 2.1$  Hz, 1H), 6.81 (d,  $J = 8.2$  Hz, 1H), 5.61 (s, 1H), 4.55 (d,  $J = 5.3$  Hz, 2H), 3.88 (s, 3H), 3.83 (s, 3H), 2.78 (d,  $J = 5.2$  Hz, 4H), 1.44 (s, 6H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  165.82, 156.82, 146.08, 145.95, 145.86, 132.07, 128.72, 122.95, 119.88, 118.78, 116.32, 114.78, 114.64, 110.88, 56.19, 55.70, 55.02, 43.55, 26.35, 23.70; HR-MS (ESI)  $m/z$ : calcd for  $C_{21}H_{27}N_2O_4$   $[M+H]^+$  371.1965 found 371.1958.

**4.1.8 The general procedures for the preparation of intermediates 19a-d**

To the solutions of 2-fluorobenzoic acid (**10**) or 2,5-difluorobenzoic acid (**17**) (35.7 mmol) in 20 mL EtOH, a catalytic amount of concentrated  $H_2SO_4$  was added, and the mixtures were refluxed overnight. The solvents were then removed and the residues were extracted with DCM ( $3 \times 50$  mL). The combined organic layers were then washed with brine, dried over anhydrous  $Na_2SO_4$ , and concentrated in vacuo to provide **18a** or **18b**. To the solutions of intermediates **18a** or **18b** (29.1 mmol) in 20 mL DMSO, morpholine (14.3 mL, 145.7 mmol) or piperidine (13.3 mL, 145.7 mmol) were added. The mixtures were stirred at 120 °C for 6 h, and then diluted with 50 mL EtOAc, washed with water (20 mL  $\times$  3), saturated brine, dried over anhydrous  $Na_2SO_4$ , and concentrated in vacuo to afford crude products. To the solutions of the above crude products in 10 mL  $CH_3OH$ , 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min.  $CH_3OH$  was then removed in vacuo and acidized with 10% HCl

aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford **19a-d**.

**2-Morpholinobenzoic acid (19a)**. White solid, yield 55.4%. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 8.02 - 7.93 (m, 1H), 7.64 (d, *J* = 2.9 Hz, 2H), 7.37 (td, *J* = 5.4, 2.5 Hz, 1H), 3.80 (t, *J* = 4.6 Hz, 4H), 3.06 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.29, 150.95, 134.12, 131.36, 126.58, 125.40, 123.04, 66.79, 53.06; ESI-MS *m/z* 207.1 [M-H]<sup>-</sup> 206.1.

**5-Fluoro-2-morpholinobenzoic acid (19b)**. White solid, yield 44.9%. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.75 - 7.68 (m, 1H), 7.68 - 7.59 (m, 1H), 7.52 - 7.39 (m, 1H), 3.76 (t, *J* = 4.4 Hz, 4H), 3.03 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.02, 160.09 (d, *J* = 243.0 Hz), 146.97 (d, *J* = 3.0 Hz), 127.82 (d, *J* = 7.5 Hz), 125.85 (d, *J* = 8.3 Hz), 120.95 (d, *J* = 21.8 Hz), 117.14 (d, *J* = 23.3 Hz), 66.74, 53.17; ESI-MS *m/z* 225.1 [M-H]<sup>-</sup> 224.1.

**2-(Piperidin-1-yl)benzoic acid (19c)**. White solid, yield 49.3%. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 8.01 (d, *J* = 7.8 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.66 - 7.59 (m, 1H), 7.45 - 7.36 (m, 1H), 3.01 (t, *J* = 5.2 Hz, 4H), 1.79 - 1.68 (m, 4H), 1.65 - 1.50 (m, 2H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.14, 150.97, 134.08, 131.20, 127.76, 125.34, 123.47, 54.15, 26.07, 22.68; ESI-MS *m/z* 205.1 [M-H]<sup>-</sup> 204.1.

**5-Fluoro-2-(piperidin-1-yl)benzoic acid (19d)**. White solid, yield 53.6%. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 18.54 (s, 1H), 7.85 (dd, *J* = 8.9, 4.8 Hz, 1H), 7.73 (dd, *J* = 9.1, 3.2 Hz, 1H), 7.55 (td, *J* = 8.5, 3.2 Hz, 1H), 3.09 (t, *J* = 5.4 Hz, 4H), 1.76 (p, *J* = 5.5 Hz, 4H), 1.68 - 1.58 (m, 2H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.01, 160.73 (d, *J* = 243.8 Hz), 146.59, 128.05 (d, *J* = 7.5 Hz), 126.05 (d, *J* = 8.3 Hz), 120.92 (d, *J* = 22.5 Hz), 116.92 (d, *J* = 23.3 Hz), 54.20, 25.95, 22.48; ESI-MS *m/z* 223.1 [M-H]<sup>-</sup> 222.1.

#### 4.1.9 The general procedures for the preparation of intermediates **24a-f**

To the solutions of various 2-fluorobenzoic acids **23a-d** (2.7 mmol) in 20 mL dioxane, morpholine (1.2 mL, 13.5 mmol) or piperidine (1.1 mL, 13.5 mmol) was added in one portion. The reactions were stirred at refluxing temperature, after the reactions completed, solvents and remaining morpholine or piperidine were removed in vacuo. Then, the residues were dissolved into water and neutralized by 10% HCl aqueous. The

resulting precipitate were collected, washed by water, and dried to give intermediates **24a-f**.

**5-Chloro-2-morpholinobenzoic acid (24a)**. White solid, yield 84.5%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  15.54 (s, 1H), 7.78 (d,  $J = 2.7$  Hz, 1H), 7.62 (dd,  $J = 8.8, 2.6$  Hz, 1H), 7.50 (d,  $J = 8.7$  Hz, 1H), 3.73 (t,  $J = 4.5$  Hz, 4H), 3.01 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  166.62, 149.77, 133.35, 130.48, 129.50, 127.30, 124.33, 66.65, 52.78; ESI-MS  $m/z$  241.1  $[\text{M-H}]^-$  240.1.

**5-Bromo-2-morpholinobenzoic acid (24b)**. White solid, yield 73.8%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  15.47 (s, 1H), 7.96 (d,  $J = 2.6$  Hz, 1H), 7.76 (dd,  $J = 8.6, 2.6$  Hz, 1H), 7.46 (d,  $J = 8.7$  Hz, 1H), 3.77 (t,  $J = 4.4$  Hz, 4H), 3.05 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  166.54, 150.22, 136.23, 133.47, 127.43, 124.45, 117.34, 66.64, 52.73; ESI-MS  $m/z$  285.0  $[\text{M-H}]^-$  284.0.

**2-Morpholino-5-(trifluoromethyl)benzoic acid (24c)**. White solid, yield 79.6%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  13.91 (s, 1H), 7.96 (d,  $J = 2.4$  Hz, 1H), 7.80 (dd,  $J = 8.7, 2.4$  Hz, 1H), 7.36 (d,  $J = 8.6$  Hz, 1H), 3.76 (t,  $J = 4.5$  Hz, 4H), 3.13 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  167.89, 154.15, 129.61 (d,  $J = 3.8$  Hz), 128.34 (d,  $J = 3.8$  Hz), 126.36, 124.33, 121.97 (q,  $J = 37.0$  Hz), 120.26, 66.43, 52.00; ESI-MS  $m/z$  275.0  $[\text{M-H}]^-$  274.0.

**2-Morpholino-4-nitrobenzoic acid (24d)**. Yellow solid, yield 93.4%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.81 (s, 1H), 7.79 (s, 2H), 3.69 (t,  $J = 4.6$  Hz, 4H), 3.06 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  168.09, 151.66, 150.04, 132.17, 131.10, 116.60, 114.08, 66.44, 52.02; ESI-MS  $m/z$  252.1  $[\text{M-H}]^-$  251.1.

**5-Bromo-2-(piperidin-1-yl)benzoic acid (24e)**. White solid, yield 86.8%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  17.90 (s, 1H), 8.08 (d,  $J = 2.6$  Hz, 1H), 7.85 (dd,  $J = 8.6, 2.6$  Hz, 1H), 7.69 (d,  $J = 8.6$  Hz, 1H), 3.07 (t,  $J = 5.2$  Hz, 4H), 1.73 (q,  $J = 5.5$  Hz, 4H), 1.68 - 1.56 (m, 2H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  166.00, 149.89, 136.54, 133.38, 127.62, 125.77, 119.95, 53.94, 25.93, 22.63; ESI-MS  $m/z$  283.0  $[\text{M-H}]^-$  282.0.

**2-(Piperidin-1-yl)-5-(trifluoromethyl)benzoic acid (24f)**. White solid, yield 83.5%.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  15.90 (s, 1H), 8.03 (d,  $J = 2.5$  Hz, 1H), 7.84 (dd,  $J = 8.6, 2.5$  Hz, 1H), 7.59 (d,  $J = 8.6$  Hz, 1H), 3.06 (t,  $J = 5.1$  Hz, 4H), 1.71 - 1.62 (m, 4H),

1.61 - 1.53 (m, 2H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  167.09, 154.41, 129.99 (d,  $J = 3.0$  Hz), 127.99 (d,  $J = 3.8$  Hz), 126.15, 125.21, 124.32 (q,  $J = 32.5$  Hz), 122.47, 53.37, 25.83, 23.16; ESI-MS  $m/z$  273.0 [M-H] $^-$  272.0.

#### 4.1.10 Ethyl 5-amino-2-morpholinobenzoate (**26**)

To the solutions of **12** (39.7 mmol) in 20 mL EtOH, a catalytic amount of concentrated H<sub>2</sub>SO<sub>4</sub> was added, and the mixtures were refluxed overnight. The solvents were then removed and the residues were extracted with DCM (3  $\times$  50 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide **25**. Ethyl 2-morpholino-5-nitrobenzoate (**25**) (5 g, 17.9 mmol) was dissolved into the mixture solvent of 20 mL EtOH and 20 mL AcOH, Fe powder (6 g, 107.2 mmol) was added, and the mixture was stirred at 65 °C for 30 min. Then, the solvent was removed in vacuo, and the residue was neutralized by saturated NaHCO<sub>3</sub> aqueous. The mixture was filtrated, and the filtrates was extracted with EtOAc (3  $\times$  50 mL), the combined organic layers were then washed with saturated brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the crude product, which was purified by column chromatography with petroleum/ethyl acetate (2:1) to give 3.5 g **26** as yellow solid, yield 76.1%.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  6.90 (d,  $J = 8.7$  Hz, 1H), 6.71 (s, 1H), 6.67 - 6.57 (m, 1H), 5.02 (s, 2H), 4.17 (q,  $J = 8.7$  Hz, 2H), 3.59 (s, 4H), 2.73 (s, 4H), 1.34 - 1.14 (m, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  168.39, 145.05, 141.21, 129.00, 121.79, 117.56, 114.83, 67.09, 60.71, 53.77, 14.66; ESI-MS  $m/z$  250.1 [M+H] $^+$  251.1.

#### 4.1.11 5-(Dimethylamino)-2-morpholinobenzoic acid (**27**)

To a solution of **26** (200 mg, 0.8 mmol) in 5 mL AcOH, NaBH<sub>3</sub>CN (200 mg, 3.2 mmol) was added. The mixture was stirred at room temperature for 30 min. Then, the solvent was removed in vacuo and the residue was neutralized by saturated NaHCO<sub>3</sub> aqueous. The mixture was filtrated, and the filtrates was extracted with EtOAc (3  $\times$  25 mL), the combined organic layers were then washed with saturated brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the crude product. To the solution of the above crude product in 10 mL CH<sub>3</sub>OH, 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min. CH<sub>3</sub>OH was then removed in vacuo and

acidized with 10% HCl aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford 154 mg **27** as grey solid, yield 65.4% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 17.78 (s, 1H), 7.61 (dd, *J* = 8.9, 4.5 Hz, 1H), 7.34 (d, *J* = 1.8 Hz, 0H), 7.04 (d, 1H), 3.83 (t, *J* = 4.8 Hz, 4H), 3.05 (t, *J* = 4.8 Hz, 4H), 2.99 (s, 2H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.39, 149.47, 139.07, 125.52, 124.47, 117.61, 113.10, 66.88, 53.38, 40.50; ESI-MS *m/z* 250.1 [M-H]<sup>-</sup> 249.1.

#### 4.1.12 Ethyl 2-morpholino-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (**30a**)

To the solutions of **28a** (35.0 mmol) in 20 mL EtOH, a catalytic amount of concentrated H<sub>2</sub>SO<sub>4</sub> was added, and the mixtures were refluxed overnight. The solvents were then removed and the residues were extracted with DCM (3 × 50 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide **29a**. To a solution of intermediate **29a** (500 mg, 1.59 mmol) in 20 mL dioxane, PdCl<sub>2</sub>(CH<sub>3</sub>CN)<sub>2</sub> (110 mg, 0.16 mmol), bis(pinacolato)diboron (613 mg, 2.39 mmol), and KOAc (470 mg, 4.77 mmol) were added. The mixture was stirred at 80 °C for 3 h. Then, the mixture was filtrated and the filtrate was concentrated in vacuo to afford the crude product, which was purified by column chromatography with petroleum/ethyl acetate (20:1) to give 495 mg **30a** as colorless oil, yield 86.1%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.14 (d, *J* = 1.9 Hz, 1H), 7.89 - 7.81 (m, 1H), 6.98 (dd, *J* = 8.3, 1.6 Hz, 1H), 4.35 (q, *J* = 6.6, 6.0 Hz, 2H), 3.86 (t, *J* = 3.9 Hz, 4H), 3.10 (t, *J* = 3.4 Hz, 4H), 1.40 (t, *J* = 7.1 Hz, 3H), 1.33 (s, 12H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.19, 154.03, 139.08, 138.18, 123.21, 117.36, 83.80, 66.91, 60.94, 52.25, 25.01, 24.83, 24.54, 14.37; ESI-MS *m/z* 261.2 [M+H]<sup>+</sup> 262.2.

The synthetic methods of intermediates **29b** and **30b** were the same as methods for the preparation of **29a** and **30a**.

**Ethyl 5-bromo-2-(piperidin-1-yl)benzoate (29b)**, colorless oil, yield 89.5%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.81 (d, *J* = 2.5 Hz, 1H), 7.48 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.92 (d, *J* = 8.8 Hz, 1H), 4.40 (q, *J* = 7.1 Hz, 2H), 3.09 – 2.88 (m, 4H), 1.85 – 1.67 (m, 4H), 1.65 – 1.55 (m, 2H), 1.43 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 167.02, 152.15, 134.92, 133.75, 125.89, 120.57, 112.75, 61.21, 53.72, 26.04, 24.10, 14.32; ESI-MS *m/z*

311.1 [M+H]<sup>+</sup> 312.1.

**Ethyl 2-(piperidin-1-yl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (30b).** colorless oil, yield 85.4%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.10 (d, *J* = 1.6 Hz, 1H), 7.81 (dd, *J* = 8.2, 1.7 Hz, 1H), 6.99 (d, *J* = 8.3 Hz, 1H), 4.39 (q, *J* = 7.1 Hz, 2H), 3.15 – 3.04 (m, 4H), 1.74 (p, *J* = 5.6 Hz, 4H), 1.67 – 1.56 (m, 2H), 1.43 (t, *J* = 7.1 Hz, 3H), 1.37 (s, 12H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 168.77, 154.82, 138.69, 137.96, 122.95, 117.36, 83.62, 60.80, 53.16, 25.97, 24.85, 24.25, 14.38; ESI-MS *m/z* 359.2 [M+H]<sup>+</sup> 360.2.

#### 4.1.13 Ethyl 5-hydroxy-2-morpholinobenzoate (31a)

To a solution of **30a** (320 mg, 0.89 mmol) in 20 mL CH<sub>3</sub>OH, 2 mL 30% H<sub>2</sub>O<sub>2</sub> aqueous was added dropwise. The mixture was stirred at room temperature for 30 min. Then, the CH<sub>3</sub>OH was removed in vacuo. The residue was extracted with EtOAc (3 × 25 mL), and the combined organic layers were then washed with saturated brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the crude product, which was purified by column chromatography with petroleum/ethyl acetate (5:1) to give 208 mg **31a** as white solid, yield 93.4%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.19 (d, *J* = 2.9 Hz, 1H), 7.00 (d, *J* = 8.7 Hz, 1H), 6.95 - 6.90 (m, 1H), 4.35 (q, *J* = 7.1 Hz, 2H), 3.85 (t, 4H), 2.96 (t, 4H), 1.38 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 167.93, 151.77, 145.21, 127.55, 121.30, 119.44, 117.41, 67.31, 61.23, 53.49, 24.79; ESI-MS *m/z* 251.1 [M+H]<sup>+</sup> 252.1.

The synthesis method of intermediate **31b** was the same as that of **31a**.

**Ethyl 5-hydroxy-2-(piperidin-1-yl)benzoate (31b).** white solid, yield 89.7%. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.21 (dd, *J* = 9.1, 2.9 Hz, 1H), 7.04 – 6.97 (m, 1H), 6.97 – 6.90 (m, 1H), 4.38 (p, *J* = 7.0 Hz, 2H), 3.92 (s, 1H), 3.01 – 2.71 (m, 4H), 1.73 (p, *J* = 5.6 Hz, 4H), 1.57 (q, *J* = 5.6, 5.1 Hz, 2H), 1.42 (t, *J* = 7.1 Hz, 3H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 168.66, 150.72, 146.81, 126.95, 121.05, 119.32, 117.28, 61.28, 54.70, 26.35, 24.16, 14.28; ESI-MS *m/z* 249.1 [M+H]<sup>+</sup> 250.1.

#### 4.1.14 The general procedures for the preparation of intermediates 32a-c

To the solutions of **31a-b** (0.48 mmol) in anhydrous THF, NaH (29 mg, 0.72 mmol, 60%) was added and stirred at 0 °C under nitrogen atmosphere for 15 min. Then,

(CH<sub>3</sub>)<sub>2</sub>SO<sub>4</sub> (0.45 mL, 0.53 mmol) or benzyl bromide (63 μL, 0.53 mmol) were added, and the mixtures were stirred at 0 °C for another 30 min. The mixtures were extracted with EtOAc (3 × 25 mL), and the combined organic layers were washed with saturated brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the crude products. To the solutions of the above crude products in 10 mL CH<sub>3</sub>OH, 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min. CH<sub>3</sub>OH was then removed in vacuo and acidized with 10% HCl aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford **32a-c**.

**Ethyl 5-methoxy-2-morpholinobenzoate (32a)**. White solid, yield 57.5% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.68 (d, *J* = 8.8 Hz, 1H), 7.47 (d, *J* = 3.1 Hz, 1H), 7.20 (dd, *J* = 8.9, 3.1 Hz, 1H), 3.78 (s, 3H), 3.75 (s, 4H), 3.02 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.65, 158.13, 143.39, 126.61, 125.32, 120.39, 114.79, 66.83, 56.03, 53.29; ESI-MS *m/z* 237.1 [M-H]<sup>-</sup> 236.1.

**Ethyl 5-(benzyloxy)-2-morpholinobenzoate (32b)**. White solid, yield 63.3% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.42 (s, 1H), 7.69 (d, *J* = 8.9 Hz, 1H), 7.58 (d, *J* = 3.1 Hz, 1H), 7.54 - 7.42 (m, 2H), 7.40 (s, 1H), 7.37 (d, *J* = 6.0 Hz, 1H), 7.31 (dd, *J* = 8.2, 5.0 Hz, 2H), 5.17 (s, 2H), 3.78 (t, *J* = 4.8 Hz, 4H), 3.04 (t, *J* = 5.0 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.71, 157.12, 143.58, 137.14, 128.93, 128.38, 128.08, 125.22, 120.98, 115.87, 70.06, 66.83, 53.28; ESI-MS *m/z* 313.1 [M-H]<sup>-</sup> 312.1.

**Ethyl 5-methoxy-2-(piperidin-1-yl)benzoate (32c)**. White solid, yield 54.6% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 18.73 (s, 1H), 7.69 (dd, *J* = 8.9, 3.6 Hz, 1H), 7.58 - 7.47 (m, 1H), 7.22 (d, *J* = 8.9 Hz, 1H), 3.80 (s, 3H), 3.04 (t, *J* = 5.2 Hz, 4H), 1.87 - 1.69 (m, 4H), 1.62 (s, 2H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.99, 158.36, 143.12, 126.76, 124.75, 120.01, 114.52, 55.99, 54.24, 26.02, 22.47; ESI-MS *m/z* 235.1 [M-H]<sup>-</sup> 234.1.

#### 4.1.15 The general procedures for the preparation of intermediates **33a-c**

To the solutions of intermediate **26** (200 mg, 0.8 mmol) in 10 mL CH<sub>3</sub>OH, various aldehydes (0.96 mmol) were added and stirred for 30 min, then NaBH<sub>4</sub> (46 mg, 1.2 mmol) was added and stirred for another 15 min. The reactions were quenched with saturated NH<sub>4</sub>Cl aqueous and extracted with EtOAc (3 × 25 mL), and the combined

organic layers were washed with saturated brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to afford the crude products. To the solutions of the above crude products in 10 mL CH<sub>3</sub>OH, 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min. CH<sub>3</sub>OH was then removed in vacuo and acidized with 10% HCl aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford **33a-c**.

**5-((4-Methoxybenzyl)amino)-2-morpholinobenzoic acid (33a)**. Grey solid, yield 66.9% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.39 (d, *J* = 8.5 Hz, 1H), 7.23 (d, *J* = 8.7 Hz, 2H), 6.85 (d, *J* = 8.2 Hz, 2H), 6.81 - 6.75 (m, 1H), 6.61 (d, *J* = 5.9 Hz, 1H), 4.19 (s, 2H), 3.75 - 3.70 (m, 4H), 3.68 (s, 3H), 2.93 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.34, 158.63, 148.08, 138.74, 131.82, 128.75, 125.64, 124.38, 117.31, 114.25, 113.47, 66.87, 55.46, 53.38, 46.19; ESI-MS *m/z* 342.2 [M-H]<sup>-</sup> 341.2

**2-Morpholino-5-((pyridin-4-ylmethyl)amino)benzoic acid (33b)**. Grey solid, yield 54.4% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 17.73 (s, 1H), 8.48 (d, *J* = 5.1 Hz, 2H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.33 (d, *J* = 5.2 Hz, 2H), 7.21 (d, *J* = 2.9 Hz, 1H), 6.82 (s, 1H), 6.78 (dd, *J* = 8.7, 3.0 Hz, 1H), 4.35 (d, *J* = 4.1 Hz, 2H), 3.75 (t, 4H), 2.95 (t, *J* = 4.4 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.25, 149.99, 149.63, 147.69, 139.16, 125.78, 124.57, 122.61, 117.31, 113.53, 66.85, 53.35, 45.69; ESI-MS *m/z* 313.1 [M-H]<sup>-</sup> 312.1.

**5-((Furan-2-ylmethyl)amino)-2-morpholinobenzoic acid (33c)**. Grey solid, yield 49.8% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 7.54 (s, 1H), 7.44 (d, *J* = 8.7 Hz, 1H), 7.27 (d, *J* = 2.7 Hz, 1H), 6.96 - 6.82 (m, 1H), 6.53 (t, *J* = 6.1 Hz, 1H), 6.35 (s, 1H), 6.25 (d, *J* = 3.1 Hz, 1H), 4.24 (d, *J* = 5.9 Hz, 2H), 3.74 (t, *J* = 4.5 Hz, 4H), 2.95 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.30, 153.25, 147.74, 142.54, 139.09, 125.67, 124.43, 117.34, 113.55, 110.84, 107.45, 66.88, 53.38; ESI-MS *m/z* 302.1 [M-H]<sup>-</sup> 301.1.

#### 4.1.16 The general procedures for the preparation of intermediates **33d-g**

To the solutions of intermediate **26** (200 mg, 0.8 mmol) in 10 mL CH<sub>3</sub>CN, various benzoic acids (0.96 mmol), HATU (366 mg, 0.96 mmol), and Et<sub>3</sub>N (134 μL, 0.96 mmol) were added. The mixtures were stirred for 2 h, and extracted with DCM (3 × 25 mL).



The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude products. To the solutions of the above products in 10 mL CH<sub>3</sub>OH, 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min. CH<sub>3</sub>OH was then removed in vacuo and acidized with 10% HCl aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford **33d-f**. For intermediate **33g**, intermediate **26** (200 mg, 0.8 mmol) in 10 mL CH<sub>3</sub>CN, acetic anhydride (91 μL, 0.96 mmol), and Et<sub>3</sub>N (134 μL, 0.96 mmol) were added. The mixtures were stirred for 2 h, and extracted with EtOAc (3 × 25 mL). The combined organic layers were then washed with brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo to provide the crude product, which was further hydrolyzed in above procedure to afford **33g**.

**5-(4-Methoxybenzamido)-2-morpholinobenzoic acid (33d)**. White solid, yield 42.5% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 10.30 (s, 1H), 8.39 (s, 1H), 8.09 (d, *J* = 8.7 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 1H), 7.86 (d, *J* = 8.3 Hz, 2H), 7.04 (d, *J* = 8.3 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 2H), 3.81 (s, 3H), 3.79 (s, 4H), 3.03 (s, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.44, 163.27, 138.41, 138.41, 131.78, 130.17, 125.61, 123.89, 123.44, 122.60, 114.24, 114.10, 66.82, 55.87, 53.24; ESI-MS *m/z* 356.1 [M-H]<sup>-</sup> 355.1.

**5-(Isonicotinamido)-2-morpholinobenzoic acid (33e)**. Grey solid, yield 46.8% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 16.83 (s, 1H), 10.69 (s, 1H), 8.77 (s, 2H), 8.38 (s, 1H), 8.07 (d, *J* = 8.7 Hz, 1H), 7.85 (s, 2H), 7.69 (d, *J* = 8.7 Hz, 1H), 3.77 (s, 4H), 3.04 (s, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 172.36, 166.90, 164.54, 150.80, 146.49, 141.84, 137.37, 125.72, 123.93, 122.88, 122.01, 66.83, 53.24; ESI-MS *m/z* 327.1 [M-H]<sup>-</sup> 326.1.

**5-(Furan-2-carboxamido)-2-morpholinobenzoic acid (33f)**. Grey solid, yield 50.9% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 16.84 (s, 1H), 10.34 (s, 1H), 8.31 (d, *J* = 2.6 Hz, 1H), 7.98 (dd, *J* = 8.8, 2.7 Hz, 1H), 7.86 (d, *J* = 1.6 Hz, 1H), 7.59 (d, *J* = 8.8 Hz, 1H), 7.30 (d, *J* = 3.5 Hz, 1H), 6.62 (dd, *J* = 3.5, 1.7 Hz, 1H), 3.71 (t, 4H), 2.96 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 166.87, 161.45, 156.75, 147.59, 146.45, 146.05, 137.51, 125.70, 123.98, 122.60, 115.57, 112.67, 66.81, 53.19; ESI-MS *m/z* 316.1 [M-H]<sup>-</sup> 315.1.

**5-Acetamido-2-morpholinobenzoic acid (33g).** White solid, yield 64.5% over 2 steps.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  10.14 (s, 1H), 8.17 (d,  $J = 2.6$  Hz, 1H), 7.86 (dd,  $J = 8.8, 2.6$  Hz, 1H), 7.61 (d,  $J = 8.7$  Hz, 1H), 3.76 (t,  $J = 4.5$  Hz, 4H), 3.00 (t,  $J = 4.5$  Hz, 4H), 2.02 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  168.99, 166.82, 145.37, 138.34, 125.70, 124.32, 124.12, 121.16, 66.80, 53.21, 24.40; ESI-MS  $m/z$  264.1  $[\text{M-H}]^-$  263.1.

*4.1.17 The general procedures for the preparation of intermediates 33h-j*

To a solution of **30a** (200 mg, 0.55 mmol) in 20 mL DMF, various bromobenzenes (0.66 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (60 mg, 0.06 mmol), and K<sub>2</sub>CO<sub>3</sub> (152 mg, 1.1 mmol) were added. The mixture was stirred at 90 °C for 2 h. Then, the mixtures were filtered and the filtrates were concentrated in vacuo to afford the crude products, which were dissolved into 10 mL CH<sub>3</sub>OH, 10 mL 10% NaOH aqueous was added and the mixtures were stirred at 80 °C for 30 min. CH<sub>3</sub>OH was then removed in vacuo and acidized with 10% HCl aqueous to pH 3. The precipitates were collected by filtration, washed with water and dried to afford **33h-j**.

**4'-Methoxy-4-morpholino-[1,1'-biphenyl]-3-carboxylic acid (33h).** White solid, yield 57.9% over 2 steps.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  8.11 (s, 1H), 7.82 (s, 1H), 7.59 (s, 2H), 7.00 (s, 2H), 3.77 (s, 7H), 3.05 (s, 4H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  167.40, 166.41, 159.64, 149.46, 137.85, 131.46, 128.56, 128.21, 125.88, 123.46, 114.97, 66.80, 55.64, 53.03; ESI-MS  $m/z$  313.1  $[\text{M-H}]^-$  312.1.

**2-Morpholino-5-(pyridin-4-yl)benzoic acid (33i).** Grey solid, yield 44.3% over 2 steps.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  15.71 (s, 1H), 8.10 (s, 2H), 7.89 (d,  $J = 8.4$  Hz, 2H), 7.79 - 7.38 (m, 3H), 3.77 (s, 4H), 3.07 (s, 6H);  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  167.54, 156.64, 150.41, 146.73, 135.80, 131.70, 128.98, 125.89, 123.16, 116.74, 66.75, 53.35, 52.88; ESI-MS  $m/z$  282.1  $[\text{M-H}]^-$  281.1.

**5-(Furan-2-yl)-2-morpholinobenzoic acid (33j).** White solid, yield 55.4% over 2 steps.  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  15.93 (s, 1H), 8.23 (d,  $J = 2.2$  Hz, 1H), 7.91 (dd,  $J = 8.5, 2.3$  Hz, 1H), 7.78 (d,  $J = 1.7$  Hz, 1H), 7.59 (d,  $J = 8.5$  Hz, 1H), 7.03 (d,  $J = 3.4$  Hz, 1H), 6.62 (dd,  $J = 3.4, 1.8$  Hz, 1H), 3.88 - 3.69 (m, 4H), 3.16 - 2.98 (m, 4H);

<sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 167.36, 152.19, 149.77, 143.76, 128.56, 127.92, 125.90, 125.78, 123.13, 112.70, 106.94, 66.71, 52.85; ESI-MS *m/z* 273.1 [M-H]<sup>-</sup> 272.1.

#### 4.1.18 The general procedures for the preparation of target compounds **34a-j**

The synthetic methods of **34a-j** were the same as method B for the preparation of **20a-p**.

***N*-(3-hydroxy-4-methoxybenzyl)-5-((4-methoxybenzyl)amino)-2-morpholinobenzamide (34a)**. White solid, yield 66.9% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.84 (d, *J* = 5.4 Hz, 1H), 7.62 (d, *J* = 3.0 Hz, 1H), 7.32 - 7.25 (m, 2H), 7.05 (d, *J* = 8.6 Hz, 1H), 6.96 (d, *J* = 1.9 Hz, 1H), 6.92 - 6.74 (m, 4H), 6.65 (dd, *J* = 8.5, 3.0 Hz, 1H), 5.95 (s, 1H), 4.52 (d, *J* = 5.1 Hz, 2H), 4.27 (s, 2H), 3.87 (s, 3H), 3.79 (s, 3H), 3.46 (s, 4H), 2.79 (d, *J* = 4.7 Hz, 4H); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 165.92, 158.94, 146.27, 146.08, 146.00, 140.98, 131.80, 131.19, 128.88, 128.44, 122.73, 119.99, 115.62, 115.55, 114.72, 114.13, 110.86, 67.08, 56.12, 55.39, 53.75, 47.87, 43.64; HR-MS (ESI) *m/z*: calcd for C<sub>27</sub>H<sub>32</sub>N<sub>3</sub>O<sub>5</sub> [M+H]<sup>+</sup> 478.2336 found 478.2333.

***N*-(3-hydroxy-4-methoxybenzyl)-2-morpholino-5-((pyridin-4-ylmethyl)amino)benzamide (34b)**. White solid, yield 50.2% over 2 steps. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>) δ 10.45 (d, *J* = 6.3 Hz, 1H), 8.95 (d, *J* = 3.0 Hz, 1H), 8.56 - 8.42 (m, 2H), 7.38 - 7.29 (m, 2H), 7.24 (d, *J* = 2.9 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 1H), 6.86 (d, *J* = 8.2 Hz, 1H), 6.79 (s, 1H), 6.74 (dd, *J* = 8.1, 2.1 Hz, 1H), 6.62 (dd, *J* = 8.7, 3.0 Hz, 1H), 6.53 (d, *J* = 6.9 Hz, 1H), 4.39 - 4.26 (m, 4H), 3.73 (s, 3H), 3.40 (dd, *J* = 5.9, 3.2 Hz, 4H), 2.70 (t, *J* = 4.6 Hz, 4H); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>) δ 165.23, 149.56, 146.83, 146.59, 146.45, 140.22, 131.66, 128.78, 122.71, 122.18, 118.62, 115.27, 115.17, 114.83, 113.92, 112.23, 66.22, 55.71, 53.28, 45.47, 42.37; HR-MS (ESI) *m/z*: calcd for C<sub>25</sub>H<sub>29</sub>N<sub>4</sub>O<sub>4</sub> [M+H]<sup>+</sup> 449.2183 found 449.2184.

**5-((Furan-2-ylmethyl)amino)-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (34c)**. White solid, yield 54.2% over 2 steps. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 10.84 (t, *J* = 5.3 Hz, 1H), 7.63 (d, *J* = 3.0 Hz, 1H), 7.35 (d, *J* = 1.8 Hz, 1H), 7.08 (d, *J* = 8.6 Hz, 1H), 6.95 (d, *J* = 1.9 Hz, 1H), 6.88 - 6.80 (m, 2H), 6.73 (dd, *J* = 8.6, 3.0 Hz, 1H), 6.31 (dd, *J* = 3.2, 1.8 Hz, 1H), 6.25 (d, *J* = 3.2 Hz, 1H), 6.01 (s, 1H), 4.52 (d, *J* = 5.2 Hz, 2H), 4.33 (s, 2H), 4.23 (s, 1H), 3.88 (s, 3H), 3.45 (d, *J* = 4.8 Hz, 4H), 2.80 (t, *J*

= 4.6 Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.84, 152.46, 146.28, 146.07, 145.41, 142.04, 141.45, 131.70, 128.38, 122.70, 119.94, 116.10, 115.65, 114.66, 110.88, 110.43, 107.26, 67.02, 56.11, 53.70, 43.59, 41.45; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{24}\text{H}_{28}\text{N}_3\text{O}_5$   $[\text{M}+\text{H}]^+$  438.2023 found 438.2022.

***N*-(3-hydroxy-4-methoxybenzyl)-5-(4-methoxybenzamido)-2-morpholinobenzamide (34d)**. White solid, yield 46.8% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.49 (s, 1H), 8.48 (s, 1H), 8.41 - 8.34 (m, 1H), 8.09 (s, 1H), 7.89 (d,  $J = 8.5$  Hz, 2H), 7.23 (s, 1H), 6.96 (s, 1H), 6.93 (s, 2H), 6.81 (d,  $J = 1.2$  Hz, 2H), 5.94 (s, 1H), 4.46 (d,  $J = 5.2$  Hz, 2H), 3.89 (s, 3H), 3.85 (s, 3H), 3.49 (s, 4H), 2.88 (s, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ )  $\delta$  165.48, 164.71, 161.93, 146.84, 146.58, 146.08, 135.66, 131.70, 129.61, 128.76, 126.70, 123.24, 122.24, 120.86, 118.65, 115.35, 113.60, 112.22, 66.04, 55.72, 55.44, 52.91, 42.38; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{27}\text{H}_{30}\text{N}_3\text{O}_6$   $[\text{M}+\text{H}]^+$  492.2129 found 492.2126.

***N*-(3-((3-hydroxy-4-methoxybenzyl)carbamoyl)-4-morpholinophenyl)isonicotinamide (34e)**. White solid, yield 44.4% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.57 (s, 1H), 9.77 (t,  $J = 5.8$  Hz, 1H), 8.94 (s, 1H), 8.83 - 8.70 (m, 2H), 8.19 (d,  $J = 2.6$  Hz, 1H), 7.95 (dd,  $J = 8.7, 2.7$  Hz, 1H), 7.92 - 7.79 (m, 2H), 7.29 (d,  $J = 8.8$  Hz, 1H), 6.88 (d,  $J = 8.2$  Hz, 1H), 6.83 (d,  $J = 2.0$  Hz, 1H), 6.77 (dd,  $J = 8.2, 2.0$  Hz, 1H), 4.38 (d,  $J = 5.7$  Hz, 2H), 3.74 (s, 3H), 3.48 (t,  $J = 4.2$  Hz, 4H), 2.83 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO}-d_6$ )  $\delta$  165.42, 163.74, 150.30, 146.84, 146.72, 146.56, 141.65, 134.74, 131.67, 128.91, 123.34, 122.41, 121.57, 120.88, 118.67, 115.35, 112.20, 66.00, 55.72, 52.82, 42.38; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{25}\text{H}_{27}\text{N}_4\text{O}_5$   $[\text{M}+\text{H}]^+$  463.1976 found 463.1970.

***N*-(3-((3-hydroxy-4-methoxybenzyl)carbamoyl)-4-morpholinophenyl)furan-2-carboxamide (34f)**. White solid, yield 42.9% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.35 (t,  $J = 5.4$  Hz, 1H), 8.32 (m, 2H), 8.02 (d,  $J = 2.8$  Hz, 1H), 7.53 (s, 1H), 7.24 (d,  $J = 4.4$  Hz, 1H), 6.97 (s, 1H), 6.83 (t,  $J = 6.1$  Hz, 2H), 6.56 (s, 1H), 5.89 (s, 1H), 4.55 (d,  $J = 5.2$  Hz, 2H), 3.89 (s, 3H), 3.55 - 3.44 (m, 4H), 2.89 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.20, 156.35, 147.70, 147.04, 146.43, 146.19, 144.60, 135.07, 131.47, 128.33, 123.87, 122.73, 122.19, 120.02, 115.56, 114.75, 112.71, 110.96,

66.91, 56.16, 53.62, 43.72; HR-MS (ESI)  $m/z$ : calcd for  $C_{24}H_{25}N_2O_6$   $[M+H]^+$  452.1816 found 452.1817.

**5-Acetamido-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (34g).** White solid, yield 43.3% over 2 steps.  $^1H$  NMR (300 MHz, DMSO- $d_6$ )  $\delta$  10.02 (s, 1H), 9.89 (d,  $J = 5.9$  Hz, 1H), 8.96 (s, 1H), 7.98 (s, 1H), 7.77 (d,  $J = 8.6$  Hz, 1H), 7.22 (d,  $J = 8.7$  Hz, 1H), 6.87 (d,  $J = 8.2$  Hz, 1H), 6.82 (s, 1H), 6.76 (d,  $J = 8.1$  Hz, 1H), 4.36 (s, 2H), 3.74 (s, 3H), 3.45 (t,  $J = 4.2$  Hz, 4H), 2.96 - 2.67 (m, 4H), 2.03 (s, 3H);  $^{13}C$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  168.22, 165.34, 146.79, 146.57, 145.74, 135.73, 131.66, 128.82, 121.99, 121.18, 120.76, 118.68, 115.33, 112.22, 66.04, 55.73, 52.92, 42.38, 23.91; HR-MS (ESI)  $m/z$ : calcd for  $C_{21}H_{26}N_3O_5$   $[M+H]^+$  400.1867 found 400.1868.

***N*-(3-hydroxy-4-methoxybenzyl)-4'-methoxy-4-morpholino-[1,1'-biphenyl]-3-carboxamide (34h).** White solid, yield 57.9% over 2 steps.  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  10.06 (d,  $J = 4.8$  Hz, 1H), 8.44 (d,  $J = 2.4$  Hz, 1H), 7.62 (dd,  $J = 8.3, 2.5$  Hz, 1H), 7.60 - 7.51 (m, 2H), 7.22 (d,  $J = 8.3$  Hz, 1H), 7.05 - 6.90 (m, 3H), 6.88 (dd,  $J = 8.2, 2.0$  Hz, 1H), 6.83 (d,  $J = 8.2$  Hz, 1H), 5.88 (s, 1H), 4.57 (d,  $J = 5.3$  Hz, 2H), 3.88 (s, 3H), 3.85 (s, 3H), 3.52 (t,  $J = 4.4$  Hz, 4H), 2.92 (t,  $J = 4.6$  Hz, 4H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  166.00, 159.37, 149.36, 146.31, 146.10, 137.67, 132.33, 131.68, 129.95, 129.89, 128.09, 127.98, 121.00, 120.04, 114.68, 114.35, 110.90, 66.94, 56.14, 55.46, 53.54, 43.64; HR-MS (ESI)  $m/z$ : calcd for  $C_{26}H_{29}N_2O_5$   $[M+H]^+$  449.2071 found 449.2069.

***N*-(3-hydroxy-4-methoxybenzyl)-2-morpholino-5-(pyridin-4-yl)benzamide (34i).** White solid, yield 53.4% over 2 steps.  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  9.74 (t,  $J = 5.4$  Hz, 1H), 8.66 (d,  $J = 5.1$  Hz, 2H), 8.51 (d,  $J = 2.3$  Hz, 1H), 7.73 (dd,  $J = 8.4, 2.3$  Hz, 1H), 7.55 (d,  $J = 5.1$  Hz, 2H), 7.30 (s, 1H), 6.98 (s, 1H), 6.86 (q,  $J = 8.3$  Hz, 2H), 6.15 (s, 1H), 4.58 (d,  $J = 5.3$  Hz, 2H), 3.90 (s, 3H), 3.55 (t,  $J = 4.4$  Hz, 4H), 2.96 (t,  $J = 4.5$  Hz, 4H);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ )  $\delta$  165.61, 151.52, 150.24, 147.11, 146.52, 146.28, 134.44, 131.44, 130.54, 130.32, 128.47, 121.46, 121.01, 119.98, 114.75, 110.99, 66.81, 56.14, 53.44, 43.68; HR-MS (ESI)  $m/z$ : calcd for  $C_{23}H_{26}N_3O_4$   $[M+H]^+$  420.1918 found 420.1910.

**5-(Furan-2-yl)-*N*-(3-hydroxy-4-methoxybenzyl)-2-morpholinobenzamide (34j).**

White solid, yield 56.9% over 2 steps.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  9.94 (s, 1H), 8.49 (d,  $J = 2.3$  Hz, 1H), 7.75 (dd,  $J = 8.4, 2.4$  Hz, 1H), 7.47 (d,  $J = 1.8$  Hz, 1H), 7.20 (d,  $J = 8.4$  Hz, 1H), 6.97 (d,  $J = 2.1$  Hz, 1H), 6.94 - 6.78 (m, 2H), 6.71 (d,  $J = 3.4$  Hz, 1H), 6.48 (dd,  $J = 3.5, 1.8$  Hz, 1H), 5.74 (s, 1H), 4.57 (d,  $J = 5.4$  Hz, 2H), 3.90 (s, 3H), 3.66 - 3.39 (m, 4H), 2.92 (t,  $J = 4.6$  Hz, 4H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  165.75, 153.01, 149.58, 146.33, 146.12, 142.31, 131.56, 128.03, 128.01, 127.34, 127.03, 120.84, 120.02, 114.69, 111.89, 110.92, 105.59, 66.86, 56.12, 53.44, 43.64; HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{23}\text{H}_{25}\text{N}_2\text{O}_5$   $[\text{M}+\text{H}]^+$  409.1758 found 409.1752.

#### 4.1.19 5-((5-fluoro-2-morpholinobenzamido)methyl)-2-methoxyphenyl phosphate disodium (**20b-P**)

To a solution of **20b** (1.0 g, 2.77 mmol) in anhydrous THF, NaH (133 mg, 3.33 mmol, 60%) was added and stirred at 0 °C under nitrogen atmosphere for 15 min. Then, newly prepared dibenzylphosphoryl chloride (988 mg, 3.33 mmol) was added, and the mixture was stirred at room temperature for 2 h. The mixture was extracted with EtOAc (3  $\times$  25 mL), and the combined organic layers were washed with saturated brine, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated in vacuo to afford the crude product, which was purified by column chromatography with petroleum/ethyl acetate (5:1) to give 1.1 g **20b-Bn** as white solid, yield 63.8%. **20b-Bn** (1.1 g, 2.77 mmol) was dissolved into 20 mL  $\text{CH}_3\text{OH}$ , Pd-C (100 mg) was added. The reaction was stirred for 2 h under hydrogen atmosphere, then the mixture was filtered and the filtrate was concentrated in vacuo to provide 750 mg crude product as white solid, which was dissolved into 20 mL  $\text{CH}_3\text{OH}$ , and NaOH (129 mg, 3.24 mmol) was added. After stirring for 30 min, the mixture was concentrated in vacuo. The residue was solidified and washed with EtOAc to afford 769 mg **20b-P** as white solid, yield 89.6% over 2 steps.  $^1\text{H}$  NMR (300 MHz, MeOD)  $\delta$  7.71 (d,  $J = 1.7$  Hz, 1H), 7.62 (dd,  $J = 9.5, 3.1$  Hz, 1H), 7.32 (dd,  $J = 8.9, 4.9$  Hz, 1H), 7.20 (ddd,  $J = 8.9, 7.7, 3.2$  Hz, 1H), 6.97 - 6.91 (m, 2H), 4.51 (s, 2H), 3.85 (s, 3H), 3.53 - 3.46 (m, 4H), 2.89 - 2.84 (m, 4H);  $^{13}\text{C}$  NMR (75 MHz, MeOD)  $\delta$  165.83, 161.09, 157.87, 149.52 (d,  $J = 3.0$  Hz), 147.29, 144.43, 130.37 (d,  $J = 17.3$  Hz), 122.77 (d,  $J = 7.5$  Hz), 120.71, 119.97, 118.07 (d,  $J = 21.8$  Hz), 116.45 (d,  $J = 24.0$  Hz), 111.51, 66.49, 55.23, 53.09, 43.24. HR-MS (ESI)  $m/z$ : calcd for  $\text{C}_{19}\text{H}_{21}\text{FN}_2\text{Na}_2\text{O}_7\text{P}$   $[\text{M}-\text{Na}]^-$  461.0895

found 461.0861.

## 4.2 Biology.

### 4.2.1 Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), propidium iodide (PI), Hoechst33342, the Annexin V-FITC apoptosis detection kit, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1) were purchased from Nanjing KeyGen Biotech Co. Ltd. (Nanjing, China). Goat antimouse IgG/Alexa-Fluor 488 antibody was purchased from Jackson ImmunoResearch in USA. Primary antibodies against cdc2, cyclin B1, p-histone-H3, Bax, Bcl-2, caspase 3, caspase 9, p53, and PARP were purchased from Beyotime (Jiangsu, China). The purified tubulin polymerization kit was purchased from Cytoskeleton Inc. (Denver, USA). Rat plasma and Enalapril were purchased from Tripod (Jiangsu, China). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

### 4.2.2 Cell Lines and Cell Culture

Human chronic myelogenous leukemia cells (K562), hepatocellular carcinoma cells (HepG2), human colon cancer cells (HCT-8), and human breast cancer cells (MDA-MB-231), mouse liver cancer cells (H22) were grown in RPMI 1640 medium (KeyGen Biotech Co. Ltd., Nanjing, China). Human umbilical vein endothelial cells (HUVECs) and human normal hepatocytes LO2 cells were grown in F12K medium (KeyGen Biotech Co. Ltd., Nanjing, China). The medium for all cell lines were supplemented with 10% fetal bovine serum (Life Technologies, USA), 100 µg/mL streptomycin (Life Technologies, USA), and 100 U/mL penicillin (Life Technologies, USA) and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### 4.2.3 MTT Assay

The overall growth of human cancer cell lines was determined using the colorimetric MTT assay. Briefly, the cell lines were incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 24 h in 96-well plates prior to the experiments. K562, HepG2, H22, HCT-8, MDA-MB-231 and L-O2 cells were seeded at a density of  $5 \times 10^3$  cells/well. After

removal of medium, 100  $\mu\text{L}$  of fresh medium containing the test compounds at different concentrations was added to each well and incubated at 37 °C for 72 h (or 6 h). The percentage of DMSO in the medium did not exceed 0.25%. The number of living cells after 72 h (or 6 h) of culture in the presence (or absence, as in the case of the control) of the various compounds was directly proportional to the intensity of the blue color of media, which is quantitatively measured by spectrophotometry (Biorad, Nazareth, Belgium) at a 570 nm wavelength. The experiment was performed in quadruplicate and repeated three times.

#### *4.2.4 In Vitro Tubulin Polymerization Inhibitory Assay*

The tubulin reaction mix in 100  $\mu\text{L}$  PEM buffer contained 2 mg/mL tubulin (Cytoskeleton), 80 mM piperazine-N,N'-bis(2-ethanesulfonic acid) sequisodium salt PIPES (pH 6.9), 0.5 mM EGTA, 2 mM  $\text{MgCl}_2$ , and 15% glycerol. Then, the mixture was preincubated with compounds or vehicle DMSO on ice. PEG containing GTP was added to the final concentration of 3 mg/mL before detecting the tubulin polymerization reaction. After 30 min, the absorbance was detected by a spectrophotometer at 340 nm at 37 °C every 2 min for 60 min. The area under the curve was used to determine the concentration that inhibited tubulin polymerization by 50% ( $\text{IC}_{50}$ ), which was calculated with GraphPad Prism Software version 5.02.

#### *4.2.5 Competitive Inhibition Assays*

The competitive binding activity of inhibitors was evaluated using a radiolabeled [3H]-colchicine competition scintillation proximity (SPA) assay. In brief, 0.08  $\mu\text{M}$  [3H]-colchicine was mixed with **20b** (1  $\mu\text{M}$ , 5  $\mu\text{M}$ ) or CA-4 (1  $\mu\text{M}$ , 5  $\mu\text{M}$ ) and biotinylated porcine tubulin (0.5  $\mu\text{g}$ ) in a buffer of 100  $\mu\text{L}$  containing 80 mM PIPES (pH 6.8), 1mM EGTA, 10% glycerol, 1mM  $\text{MgCl}_2$ , and 1mM GTP for 2 h at 37 °C. Then streptavidin-labeled SPA beads (80  $\mu\text{g}$ ) were added to each mixture. The radioactive counts were measured directly with a scintillation counter.

#### *4.2.6 Immunofluorescent Staining*

A431 cells were seeded into 6-well plates and then treated with vehicle control 0.1% DMSO, and **20b** (7.5 nM, 15 nM, 30 nM). The cells were fixed with 4% paraformaldehyde and then washed three times with PBS. After blocking for 20 min by



adding 50-100  $\mu\text{L}$  goat serum albumin at room temperature, cells were incubated with a monoclonal antibody (anti- $\alpha$ -tubulin) at 37 °C for 2 h. Then the cells were washed three times by PBS following staining by fluorescence secondary antibody and labeling of nuclei by 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized on a Zeiss LSM 570 laser scanning confocal microscope (Carl Zeiss, Germany).

#### *4.2.7 Molecular Modeling*

The 2D structures of **20b**, IMB5046, and CA-4 were generated on ChemBioDraw Ultra 12.0. Ligprep module implemented in Schrödinger was used to generate energy minimized 3D structures. The X-ray structure of CA-4- $\alpha,\beta$ -tubulin complex was downloaded from the Protein Data Bank (PDB code 5lyj) and prepared for docking using Protein Preparation Wizard. Default settings were used for all the remaining parameters and no constraints were specified separately. All ligand conformers were docked to each of the receptor grid structures using Glide extra precision (XP) mode. The 2D interactions between ligands and tubulin were generated by Ligand Interaction Diagram module, and the structural image was obtained using PyMOL software.

#### *4.2.8 Cell Cycle Analysis*

A431 cells were seeded into 6-well plates and incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 24 h, and then treated with or without **20b** at indicated concentrations (7.5 nM, 15 nM, 30 nM) for another 48 h. The collected cells were fixed by adding 70% ethanol at 4 °C for 12 h. Subsequently, the cells were resuspended in PBS containing 100 mL RNase A and 400 mL of propidium iodide for 30 min. The DNA content of the cells was measured using a FACS Calibur flow cytometer (Bectone Dickinson, San Jose, CA, USA).

#### *4.2.9 Hoechst 33342 Staining*

Approximately  $5 \times 10^4$  cells per well were plated in six-well plates, and the cells were then incubated with **20b** (7.5 nM, 15 nM, 30 nM) for 48 h. After incubation, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min, and then stained with 20  $\mu\text{g}/\text{mL}$  Hoechst 33342 for 15 min at room temperature in the dark. Cells were then assessed by fluorescence microscopy for morphological changes after **20b** treatment.

#### *4.2.10 Cell Apoptosis Analysis*

After treatment with or without **20b** at indicated concentrations (7.5 nM, 15 nM, 30 nM) for 48 h, A431 cells were washed twice in PBS, centrifuged and resuspended in 500  $\mu$ L AnnexinV binding buffer. The cells were then harvested, washed and stained with 5 mL Annexin V-APC and 5 mL 7-AAD in the dark for 15 min. Apoptosis was analyzed using a FACS Calibur flow cytometer (Bectone Dickinson, San Jose, CA, USA).

#### *4.2.11 Mitochondrial Membrane Potential Analysis*

After treatment with vehicle control 0.1% DMSO, and **20b** (7.5 nM, 15 nM, 30 nM) for 48 h, the cells were washed in PBS and resuspended in 500  $\mu$ L JC-1 incubation buffer at 37 °C for 15 min. The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis (Bectone-Dickinson, San Jose, CA, USA).

#### *4.2.12 Measurement of Intracellular ROS Generation*

Intracellular ROS production was detected by using the peroxide-sensitive fluorescent probe DCF-DA. In brief, after treatment with **20b** (7.5 nM, 15 nM, 30 nM) for 48 h, A431 cells were incubated with 10 mM DCF-DA at 37 °C for 15 min. The intracellular ROS mediated oxidation of DCF-DA to the fluorescent compound 2',7'-dichlorofluorescein (DCF). Then cells were harvested, and the pellets were suspended in 1 mL PBS. Samples were analyzed at an excitation wavelength of 480 nm and an emission wavelength of 525 nm by flow cytometry on a FC500 cytometer (Beckman Coulter).

#### *4.2.13 Solubility Evaluation*

Solubility was measured separately at pH 7.4 and pH 2.0 by using an HPLC–UV method. Compounds **20b** and **20d** were added to phosphate buffer (pH 7.4) or HCl aqueous (pH 2.0) at 20 °C. After shaking and centrifuging, the supernatant was taken to determine the concentration of compounds **20b** and **20d** in each solvent for calculation of the corresponding solubility. The mobile phase was menthol-water (80/20, v/v) at a flow rate of 1.0 mL/min with the detection wavelength at 254 nm. This experiment was repeated in triplicates.

#### *4.2.14 log P Measurement*

The DMSO stock solution (10 mg/mL, 20  $\mu$ L) of **20b**, **20d** and CA-4 were added into

the mixture solvents of *n*-octane (1 mL) and water (1 mL). The mixtures were stirred at room temperature for 24 h and then stood overnight. Each solution (0.5 mL) was transferred from two phases respectively into other vials for HPLC analysis. The instrument and conditions were the same as those for solubility determination. The log *P* was calculated by the peak area ratios in *n*-octane and in water.

#### *4.2.15 Plasma Stability Measurement*

The stability of compounds **20b** and **20d** in rat plasma was determined using HPLC method. Rat blood plasma was diluted with distilled water until the indicated volume, and then the solution was pre-incubated for 5 min at 37 °C. The stock solutions of the test compounds **20b**, **20d** and positive control (100 nM, 20 μL) were added to the rat plasma (480 μL), and the mixtures were incubated at 37 °C. The incubations were terminated at 0, 15, 30, 60, 120, and 180 min, by removing aliquots (80 μL) of the plasma samples and mixing them with an equal volume of acetonitrile. The mixture was stirred vigorously and centrifuged (5500 rpm, 5 min). The supernatant was filtered, and the filtrate was analyzed by HPLC. These tests were conducted three times for each compound. Remaining (%) was calculate by the ratio of peak area at each time point and peak area at 0 min.

#### *4.2.16 Determination of Acute Toxicity*

Five-week-old male Institute of Cancer Research (ICR) mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. An acute toxicity study by intravenous injection was conducted according to the guidelines of the Organization for Economic Co-operation and Development. The animals were weighed and divided at random into five groups of ten animals. Then, the mice were intravenously injected with **20b-P** (327.7, 409.6, 512.0, 640, and 800 mg/kg) in saline solutions. The animals were observed continuously for 14 days.

#### *4.2.17 Evaluation of In Vivo Anti-Tumor Effect*

A total of  $1 \times 10^6$  H22 cells were subcutaneously inoculated into the right flank of ICR mice according to protocols of tumor transplant research. After incubation for one day, mice were weighted and divided at random into eight groups with eight animals per groups. The groups treated with **20b**, IMB5046, and CA-4 were administered in a

vehicle of 10% DMF/2% Tween 80/88% saline, respectively. The groups treated with **20b-P** and CA-4P were administered 20 mg/kg in a vehicle of saline. The positive control group was treated with PTX (6 mg/kg) by intravenous injection. The negative control group received a vehicle of 10% DMF/2% Tween 80/88% saline through intravenous injection. Treatments of **20b**, **20b-P**, IMB5046, and CA-4 were conducted at a frequency of intravenous injection one dose per day for a total 21 consecutive days while the positive group was treated with PTX at a dose of 6 mg/kg every other day. Body weights and tumor volumes were measured every 2 days. After the treatments, all the mice were sacrificed and weighed. The following formula was used to determine tumor volumes: tumor volume =  $L \times W^2/2$ , where L is the length and W is the width. Ratio of inhibition of tumor (%) =  $(1 - \text{average tumor weight of treated group} / \text{average tumor weight of control group}) \times 100\%$ . All procedures were performed following institutional approval in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

#### *4.2.18 Wound Healing Assays*

HUVEC cells were grown in 6-well plates for 24 h. Scratches were made in confluent monolayers using 200  $\mu$ L pipette tips. Then, wounds were washed twice with PBS to remove non-adherent cell debris. Media containing different concentrations of **20b** (7.5 nM, 15 nM, 30 nM) were added to the petri dishes. Cells which migrated across the wound area were photographed using phase contrast microscopy at 0 h and 24 h.

#### *4.2.19 Tube Formation Assay*

EC Matrigel matrix was thawed at 4 °C overnight, and HUVECs suspended in F12K were seeded in 96-well culture plates at a cell density of 50,000 cells/well after polymerization of the Matrigel at 37 °C for 30 min. They were then treated with 20  $\mu$ L different concentrations of **20b** or vehicle for 6 h at 37 °C. Then, the morphological changes of the cells and tubes formed were observed and photographed under inverted microscope (OLYMPUS, Japan).

#### *4.2.20 Immunohistochemistry Assay*

Slides from mice tissues (control group and **20b**-treated group at 20 mg/kg in *in vivo* antitumor experiments) were embedded in paraffin and cut to sections of 4  $\mu$ m,

deparaffinized, and treated with citrate buffer. Then, they were blocked with avidin/biotin for 20 min. The slides were incubated with CD31 overnight at 4 °C. Next, the slides were treated with secondary antibody with horseradish peroxidase goat anti-rabbit for 1-3 h and developed with 3, 3-diaminobenzidine. Finally, the slides were counterstained with hematoxylin. The mean microvessel density (MVD) was measured by calculating the CD31-positive cells in randomly selected areas in each section using image analysis software and then analyzed with OriginPro 8.0 software.

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