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Synthesis and structure–activity relationships of benzophenone-bearing diketopiperazine-type anti-microtubule agents

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ABSTRACT

KPU-105 (**4**), a potent anti-microtubule agent that contains a benzophenone was derived from the diketopiperazine-type vascular disrupting agent (VDA) plinabulin **3**, which displays colchicine-like tubulin depolymerization activity. To develop derivatives with more potent anti-microtubule and cytotoxic activities, we further modified the benzophenone moiety of **4**. Accordingly, we obtained a 4-fluorobenzophenone derivative **16j** that inhibited tumor cell growth in vitro with a subnanomolar IC_{50} value against HT-29 cells (IC_{50} = 0.5 nM). Next, the effect of **16j** on mitotic spindles was evaluated in HeLa cells. Treatment with 3 nM of **16j** partially disrupted the interphase microtubule network. By contrast, treatment with the same concentration of CA-4 barely affected the microtubule network, indicating that **16j** exhibited more potent anti-mitotic effects than did CA-4.

1. Introduction

Microtubules, formed by α- and β-tubulin heterodimers, are major cytoskeletal components with important roles in a variety of cellular functions, for example, maintenance of cell shape, intracellular transport, and mitosis.[1] These crucial functions make microtubules attractive targets for anti-cancer chemotherapeutics. To date, numerous anti-microtubule agents have been developed with anti-mitotic properties and anticancer potential. With respect to their mode of action, there are three well established drug binding sites on β-tubulin: the vinca domain, the taxane site and the colchicine site.[1–3] Anti-microtubule agents such as the microtubule stabilizing taxanes[4] (e.g., paxlitaxel, docetaxel) and the microtubule depolymerizing vinca alkaloids[5] (e.g., vinblastine, vincristine) have been used as effective chemotherapies for a variety of cancers. By contrast, the microtubule depolymerizing agent colchicine (**1**, Fig. 1) has not been approved for clinical use due to its extreme toxicity.[6] However, over the past ten years, various agents possessing colchicine-like tubulin depolymerizing activities (e.g., combretastatin A-4 [**2**, CA-4 (Fig. 1)],[7] ZD6126,[8] AVE8062[9] and ABT-751[10]) have been recognized to act as both cytotoxic and vascular disrupting agents (VDAs).[11] The latter class of molecules induce the collapse of established tumor vasculature via rapid microtubule depolymerization. This leads to a loss of blood supply and eventual contraction of the tumor. Hence, the microtubule-depolymerizing agents represent a promising new class of anti-cancer drugs.

Plinabulin (**3**, NPI-2358/KPU-2, Fig. 1), which we developed, is a potent anti-microtubule agent derived from the natural diketopiperazine (DKP) 'phenylahistin'[12] with a colchicine-like tubulin depolymerization activity. Compound **3** was developed as a VDA in 2006,[13] and is now in clinical trials as an anticancer drug in four countries including the United States. In our previous study, we modified the phenyl group and 5-position of the imidazole ring on compound **3**, and evaluated their cytotoxic and tubulin-binding activities. We performed a structure–activity relationship (SAR) study using compound **3** as a starting point. One of the derivatives (compound **4**, KPU-105, Fig. 1), which possess a benzophenone, *m*-benzoyl derivative, was more potent than the original structure.[14] The vascular disrupting activity of compound **4** as evaluated with human vascular endothelial cells (HuVECs) was 10- and 3-fold more potent than that of compounds **2** and **3**, respectively. This result indicated that compound **4** was a valuable anti-microtubule and vascular disrupting agent. At the same time, we knew that several benzophenone-type CA-4 analogues, including

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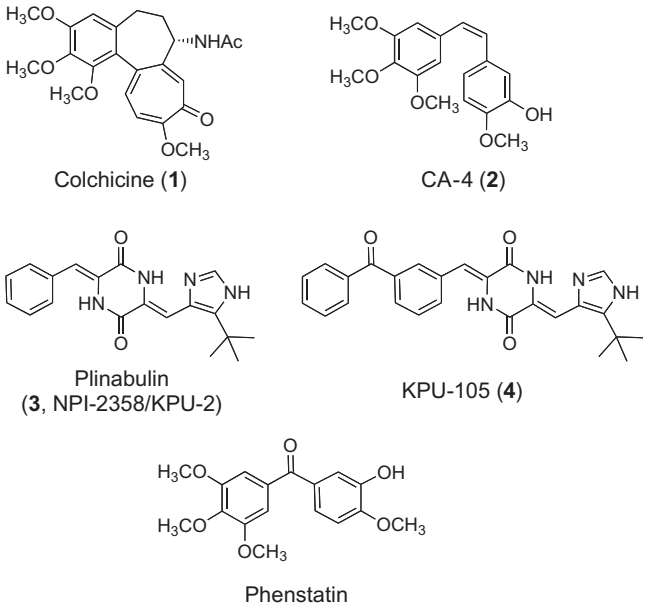


Figure 1. Structure of anti-microtubule agents.

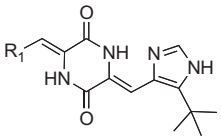
phenstatin[15] (Fig. 1), that exhibited relatively potent anti-microtubule activities, developed enhanced anti-microtubule potency through substitutions on the benzophenone moiety.[16] Therefore, we thought it worthwhile to pursue further modification and optimization on the benzophenone part of compound **4** in order to develop more potent derivatives, and to better understand the pharmacophore of diketopiperazine-type vascular disrupting agents. Here, we present the design and synthesis of a series of benzophenone-containing anti-microtubule agents, and the outcome of an SAR study on these molecules.

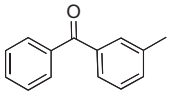
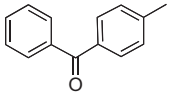
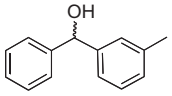
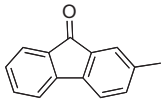
2. Results and discussion

2.1. Effect of benzophenone on anti-microtubule potency

Previously, we developed the potent *m*-benzoyl derivative **4** containing a benzophenone structure, suggesting that the benzophenone could play a crucial role in enhancing anti-microtubule activity. However, we did not previously optimize the position for introducing the benzoyl group to compound **3**. To address that issue, we designed and synthesized the *p*-benzoyl derivative (**5**). The *o*-benzoyl derivative of compound **3** was not examined because the synthesis was thought to be complicated due to steric hindrance. Furthermore, to understand the effects of the benzophenone carbonyl group and skeletal backbone, we designed and synthesized benzhydrol (**6**) and fluorenone derivatives (**7**). Compounds **5–7** were synthesized by an aldol condensation between 1-acetyl-3-{(*Z*)-1-[5-(*tert*-butyl)-1*H*-4-imidazolyl]methylidene}]-2, 5-piperazinedione (**15**) and the corresponding aldehyde in the presence of Cs_2CO_3 according to the conventional procedure (Supplementary data, Scheme S1). [14] Then, their cytotoxic activities were evaluated using HT-29 cells. As shown in Table 1, the *p*-benzoyl derivative **5** was very weakly active (IC_{50} = 1.2 μM). This result indicated that the *m*-benzoyl substitution on the phenyl ring of compound **3** was better able to induce potent cytotoxic activity than the *p*-substitution. Similarly, the benzhydrol derivative **6** was 29-fold less potent than the parent compound **4**, suggesting that the carbonyl group of the benzophenone was important for potent cytotoxicity. The conformationally restricted fluorenone derivative **7** was also significantly less potent (2000-fold) as compared to

Table 1
Cytotoxic activity of plinabulin (**3**) derivatives against HT-29 cells



Compound	R_1	IC_{50}[a] (nM)
1 (Colchicine)		16 ± 3.0
2 (CA-4)		12,000[b]
3 (Plinabulin)		15 ± 3.8
4		1.4 ± 0.40
5		1200[c]
6		41 ± 19[c]
7		2800

[a] Values represent the mean or mean ± SEM (for potent compounds) from at least three independent dose response curves.
[b] HT-29 cells were particularly unresponsive toward CA-4.[17]
[c] Activity as determined by the XTT assay. The IC_{50} value of colchicine determined by this assay was similar (9.3 nM) to the value obtained using the Resazurin assay (16 nM).

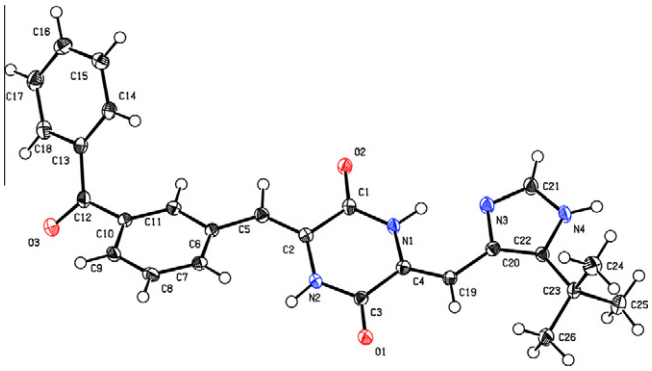
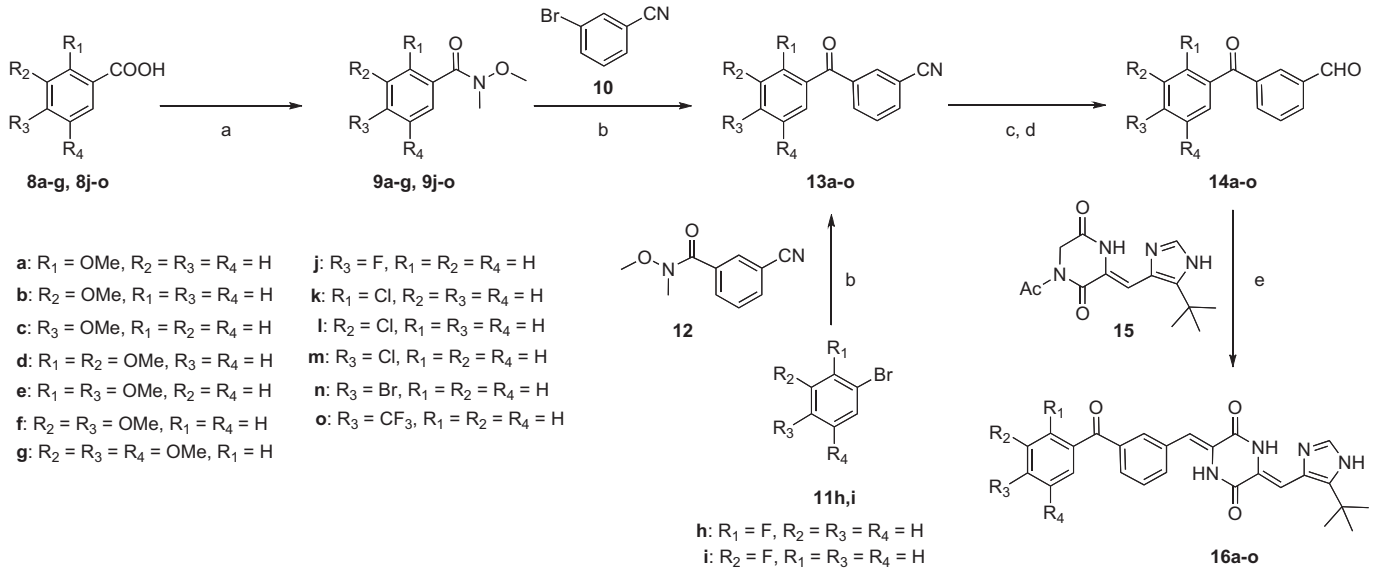


Figure 2. ORTEP drawing of compound **4**.

compound **4**. This suggested that the replacement of benzophenone with a planar structure was not an appropriate strategy to retain activity. The molecular structure of compound **4** was determined by single-crystal X-ray crystallography. The ORTEP drawing of compound **4** is presented in Figure 2. It revealed that compound **4** existed in a twisted conformation with the two aromatic rings of benzophenone at a torsion angle of 58° relative to each other. This hydrophobic topology could be crucial for optimal tubulin binding and potent cytotoxicity.

2.2. Synthesis of substituted benzophenone-type plinabulin derivatives

Encouraged by the results obtained in Section 2.1, we expanded the SAR study to include modifications on the benzophenone



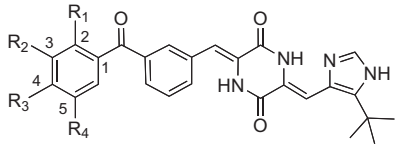
Scheme 1. Synthesis of benzophenone derivatives **16a–o**. Reagents and conditions: (a) MeONHMe·HCl, EDC·HCl, Et_3N, DMF, rt; (b) *n*-BuLi, THF, −78 °C; (c) DIBAL-H, THF, −78 °C; (d) PDC, molecular sieves 4 Å, CH_2Cl_2, rt; (e) Cs_2CO_3, DMF, 70–80 °C followed by HPLC purification.

moiety of compound **4**. Natural anti-microtubule agents such as colchicine, CA-4, and phenstatin, which recognize the same colchicine binding site on β-tubulin, are structurally characterized by multiple methoxy groups, which markedly contribute to their potent anti-microtubule activity.[18] In addition, it is reported that the methoxy group of CA-4 can be replaced by halogen substituents.[19] Based on this information, we designed the benzophenone-type plinabulin derivatives **16a–o** bearing methoxy, halogen or trifluoromethyl substituents, and synthesized them according to Scheme 1. First, we synthesized the 3-benzoylbenzonitrile derivatives **13a–o** via two routes. In the first, the benzoic acids **8a–g** and **8j–o** were converted to the corresponding Weinreb amides, **9a–g** and **9j–o**, respectively, and these Weinreb amides were condensed with 3-bromobenzonitrile (**10**) via a bromo-lithium exchange reaction[20] to give the corresponding benzoylbenzonitriles **13a–g** and **13j–o**. In the second, compounds **13h** and **i** were obtained by a bromo-lithium exchange reaction between the bromo-fluorobenzenes **11h** and **i** and 3-cyano-*N*-methoxy-*N*-methylbenzamide[21] (**12**) in one step. The low yields observed for this bromo-lithium exchange reaction were probably due to side reactions occurring on the amide nitrogen of the Weinreb amide (**9**) and the steric hinderance between substituent groups. Next, the nitrile and carbonyl groups of the benzophenone derivatives **13a–o** were reduced with diisobutylaluminium hydride (DIBAL-H), and, without further purification, the resulting benzhydryl alcohols were oxidized to the benzophenones with pyridinium dichromate (PDC)[22] to obtain the substituted benzophenone aldehydes **14a–o**. Finally, the aldehydes **14a–o** were condensed with monodehydroDKP (**15**) by an aldol reaction in the presence of Cs_2CO_3 to afford the desired didehydroDKP derivatives **16a–o**.[23] The crude **16a–o** products were purified by preparative reversed-phase HPLC, and the eluant was lyophilized to obtain purified **16a–o** as yellow powders. The purity of all tested compounds was >92%.

2.3. In vitro cytotoxic activity

To perform an SAR study, the cytotoxic activities of the synthesized derivatives **16a–o** were evaluated using the human colon adenocarcinoma cell line HT-29. First, the effect of the methoxy substitutions was examined. As shown in Table 2, of the derivatives bearing a single methoxy substitution at the 2, 3 or 4-position (**16a**, **b,** and **c** respectively), **16a** and **b** showed markedly reduced cytotoxic activity (257- and 28-fold, respectively) as compared to the parent compound **4**. By contrast, the 4-substituted derivative **16c** was only slightly less potent (2.7-fold, IC_{50} = 4 nM) than compound **4**. Thus, a single methoxy group at the 4-position was tolerated. This result suggested that it might be possible to further modify the 4-position. On the other hand, double substitutions with methoxy groups at the 2, 3-, 2, 4- or 3, 4-positions (compounds **16d–f**) drastically decreased potency. The triply modified, 3,4,5-methoxy derivative also was not significantly effective, although anti-microtubule agents such as colchicine, combretastatin, and phenstatin possess multiple methoxy groups that contribute to their activity. Other triply substituted trimethoxy derivatives (2,3,4-, 2,3,5- or 2,4,5-substitutions) exhibited no

Table 2
Cytotoxic activity of derivatives **16a–o** against HT-29 cells



Compound	R_1	R_2	R_3	R_4	IC_{50} (nM)[a]
4	H	H	H	H	1.4 ± 0.4
16a	OMe	H	H	H	360 ± 66
16b	H	OMe	H	H	39 ± 12
16c	H	H	OMe	H	3.8 ± 0.4
16d	OMe	OMe	H	H	955
16e	OMe	H	OMe	H	1800
16f	H	OMe	OMe	H	7300
16g	H	OMe	OMe	OMe	2500
16h	F	H	H	H	3.0 ± 2.0
16i	H	F	H	H	0.6 ± 0.1
16j	H	H	F	H	0.5 ± 0.1
16k	Cl	H	H	H	6.0 ± 0.5
16l	H	Cl	H	H	2.0 ± 0.8
16m	H	H	Cl	H	1.1 ± 0.0
16n	H	H	Br	H	4.0 ± 0.7
16o	H	H	CF_3	H	147 ± 15

[a] Values represent the mean or mean ± SEM (for potent compounds) from at least three independent dose response curves.

Table 3
Biological activities of compounds **1**, **2**, **4** and **16j**

Compound	Tubulin binding K_d (μM)	Inhibition of MT polymerization IC_{50}[a] (μM)	Cytotoxicity IC_{50} (nM)	
			HeLa	A549
1	3.3 ± 0.3	4.12 ± 0.63	20.2 ± 2.1	50.6 ± 3.8
2	0.28 ± 0.12	0.55 ± 0.01	2.2 ± 0.3	5.3 ± 1.5
4	0.065 ± 0.015	0.76 ± 0.14	2.3 ± 0.2	1.9 ± 0.6
16j	0.072 ± 0.010	0.90 ± 0.03	1.7 ± 0.1	0.8 ± 0.2

[a] Values represent the mean or mean ± SEM (for potent compounds) from at least three independent dose response curves.

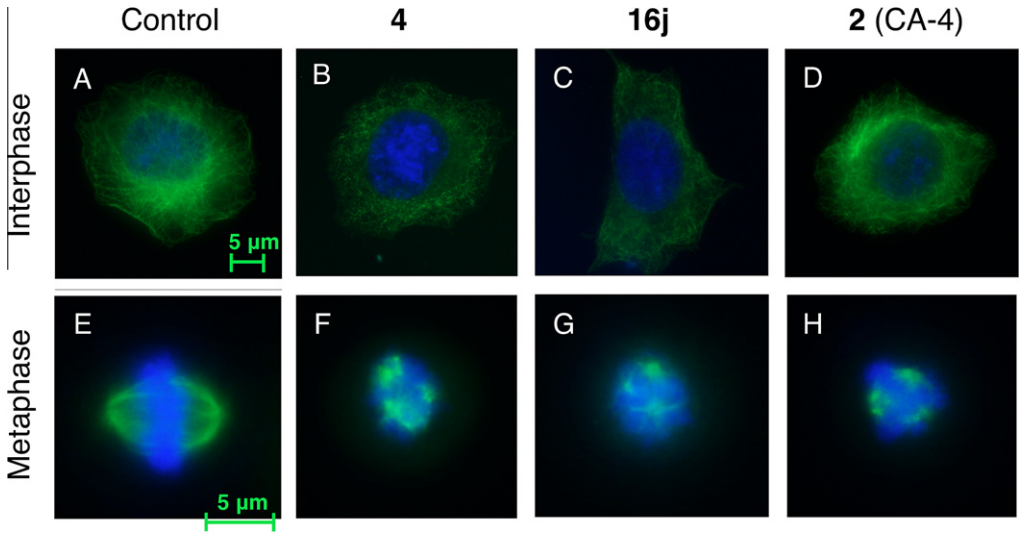


Figure 3. Effect of compounds **2**, **4** and **16j** on spindle structures. HeLa cells were treated with vehicle (A and E), 3 nM compound **4** (B and F), 3 nM compound **16j** (C and G), or 3 nM compound **2** (D and H). α-Tubulin antibody was used to visualize microtubules, and the DNA was visualized by DAPI staining. Microtubules are depicted in green and DNA in blue.

cytotoxicity (data not shown). These results suggested that tubulin rigorously recognizes the benzophenone of compound **4** and the space for accepting substituents is highly limited. Moreover, the data indicated that compound **4** might bind to tubulin in a different manner than colchicine and its biological homologues. This finding is consistent with previously reported data from the SAR study of compound **3**.[14]

Next, in an effort to obtain more potent compounds, we evaluated the effects of substitution with halogen atoms (F, Cl, Br) or the trifluoromethyl group. As shown in Table 2, with respect to single substitutions with a fluorine atom, the 3- and 4-substituted derivatives (**16i** and **j**) exhibited increases in potency of 2.3- and 2.8-fold, respectively, as compared to compound **4**. By contrast, the 2-substituted derivative **16h** had slightly decreased potency. With respect to chlorine substitutions, the 3- and 4-substituted derivatives (**16l** and **m**) were similar in potency to compound **4**, but weaker than the fluoro derivatives **16i** and **16j**. The 2-chloro substituted derivative **16k** was less potent than compound **16h**. Since these results showed that modifications at the 4-position generally improved the potency, we examined the effects of additional modifications at this position. Substituting with a bromine atom at the 4-position (compound **16n**) did not substantially improve the activity relative to compound **4**, and adding a trifluoromethyl group (compound **16o**) decreased the potency 105-fold. These results indicated that modifications with F or Cl at the 4-position improved cytotoxic activity, but modifications with bulky electron-withdrawing groups, such as Br and CF_3, had the opposite effect.

2.4. Tubulin binding and inhibition of microtubule depolymerization by highly potent derivatives

Since the 4-fluorobenzophenone derivative **16j** exhibited the highest cytotoxic activity against HT-29 cells of all the compounds tested, we performed a tubulin binding assay and an in vitro microtubule depolymerization assay with **16j** to understand its mechanism of action (Table 3). The dissociation constant (K_d) from tubulin was measured at 37 °C using purified porcine tubulin as described previously.[21,24] The K_d of compounds **4** and **16j** were calculated to be 0.065 and 0.072 μM, respectively. The binding affinity of compound **16j** was also similar to that of compound **4**, suggesting that the introduction of a fluorine atom to the benzophenone moiety did not result in an increased binding affinity to tubulin. With respect to the microtubule depolymerization assay, the IC_{50} value of compound **16j** (0.90 μM) was only slightly higher than that of compound **4** (0.76 μM). Thus, the introduction of a fluorine atom to compound **4** had little effect on the microtubule depolymerizing activity.

To confirm the cytotoxic potency of compound **16j**, we repeated the cytotoxicity assays using other two target cell lines, HeLa and human lung carcinoma A549 cells, and assessed their response to treatment with compounds **1**, **2**, **4** and **16j** (Table 3). Consistent with the results obtained using HT-29 cells, compound **16j** showed the most potent cytotoxic activity of all the compounds tested (e.g., IC_{50} = 0.6 nM against A549 cells). The potent cytotoxic activity displayed by compound **16j** might be due to improved cell membrane permeability by the introduction of a hydrophobic fluorine,

or to poor recognition by multidrug efflux pumps such as *P*-glycoprotein.

2.5. Antimitoic effects induced by potent derivatives

Anti-microtubule agents such as colchicine and CA-4 analogues generally initiate the formation of highly abnormal mitotic spindles. The effects of compounds **2**, **4** and **16j** on mitotic spindles were evaluated in HeLa cells. HeLa cells were treated with each compound at 1, 3 and 10 nM for 6 h, and then the microtubules and chromosomes were visualized with an anti-α-tubulin antibody and 4,6-diamidino-2-phenylindole (DAPI), respectively (Fig. 3 and Supplementary data, Fig. S1). As shown in Figure 3A, untreated cells possessed well-defined microtubule networks at interphase. At metaphase, untreated cells contained well-organized dipolar spindles, and all of the chromosomes were organized at the metaphase plate (Fig. 3E). No notable damage was observed in cells treated with 1 nM of any of the compounds (Supplementary data, Fig. S1). By contrast, treatment with 3 nM of compounds **4** and **16j**, but not compound **2**, partially disrupted the interphase microtubule network (Fig. 3B–D). These results suggested that compounds **4** and **16j** exhibited more potent anti-mitotic activity than compound **2**. Treatment of metaphase cells with 3 nM of any of the three compounds led to multipolar mitotic spindles and defects in chromosome congression (Fig. 3F–G), both of which are characteristic of cells with depolymerized microtubules. In addition, treatments with all tested compounds at 10 nM completely disrupted the interphase microtubule network (Supplementary data, Fig. S1). Together, these results indicated that benzophenone-type DKP anti-microtubule agents, such as compound **16j**, could strongly interfere with the normal mitotic progression of cancer cells.

3. Conclusion

A series of potent diketopiperazine-type anti-microtubule agents with a benzophenone structure was designed and synthesized based on modifications of compound **4**, derived from plinabulin (**3**). Then, a SAR study was performed, which indicated that we had developed a potent 4-fluorobenzophenone derivative (**16j**) possessing subnanomolar IC_{50} values against HT-29 cells. In general, modifications at the 4-position of the benzophenone structure were well tolerated with respect to activity. Notably, modifications with F or Cl atom at the 4-position of benzophenone increased the cytotoxicity, while modifications with bulky electron-withdrawing groups, such as Br and CF_3, did not. Moreover, our data suggested that the non-planar conformation of the benzophenone was crucial to effective tubulin binding and potent cytotoxicity. Next, to understand the mechanism of the potent cytotoxic effects of compound **16j**, we performed tubulin binding and microtubule depolymerization assays. Surprisingly, both the tubulin binding affinity and the microtubule depolymerizing ability of the 4-fluorobenzophenone derivative **16j** were slightly reduced as compared to those of compound **4.** Thus, to confirm the initial observations of cytotoxic potency on HT-29 cells, cytotoxicity assays were repeated using HeLa cells and human lung carcinoma A549 cells. The results were the same as the initial studies in HT-29 cells, indicating that compound **16j** was more potent than compound **4** in cell-based assays. Next, the effects of compounds **4** and **16j** on mitotic spindles were evaluated in HeLa cells. Treatment with 3 nM of compound **16j** partially disrupted the interphase microtubule network, whereas treatment with CA-4 at the same concentration had almost no effect, indicating that compound **16j** had more potent anti-mitotic activity than CA-4. Although we have not yet determined the mechanism underlying the improved activity of compound **16j** relative to compound **4**, the highly potent compound could be used as a next-generation derivative of compound **3**, which is now in clinical trials as a tumor vascular disrupting therapy in combination with other chemotherapeutic agents or radiation for the treatment of solid tumors.

4. Experimental

4.1. General

Reagents and solvents were obtained from Wako Pure Chemical Ind., Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co., Inc. (Milwaukee, WI), and were used without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70–230 mesh). TLC was performed using Merck Silica gel $60F_{254}$ precoated plates. Melting points were measured on a Yanagimoto micro-melting apparatus without correction. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system and a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min. UV detection was at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS-AM or 19 × 150 mm; Waters mBondasphere C18, 5 mm, 100Å) with a binary solvent system and a linear gradient of CH_3CN in 0.1% aqueous TFA or methanol in water at a flow rate of 5–12 mL/min. UV detection was at 230 and 360 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^1H and ^{13}C NMR spectra were obtained on a JEOL 300 MHz spectrometer, a Varian Mercury 300 spectrometer (300 MHz), or a BRUKER AV600 spectrometer (600 MHz) with tetramethylsilane as an internal standard. High-resolution mass spectra (ESI or EI) were recorded on a micromass Q-Tof Ulitima API or a JEOL JMS-GCmate BU-20 spectrometer. Mass spectra (ESI) were recorded on LCMS-2010EV (SHIMADZU).

4.2. Synthesis

4.2.1. (3*Z*,6*Z*)-3-(4-Benzoylbenzylidene)-6-((5-*tert*-butyl-1*H*-imidazol-4-yl)methylene)piperazine-2,5-dione (5)

To a solution of (*Z*)-1-acetyl-3-((5-*tert*-butyl-1*H*-imidazol-4-yl)methylene)piperazine-2,5-dione[14] **15** (20 mg, 0.069 mmol) in DMF (10 mL) was added 4-benzoylbenzaldehyde (Supplementary data, compound **S8**, 29 mg, 0.138 mmol) and the solution was repeatedly evacuated over a short time period to remove oxygen and flushed with Ar. Then, Cs_2CO_3 was added (45 mg, 0.138 mmol) and the evacuation-flushing process was repeated again. The resultant mixture was heated overnight at 70 °C. After the solvent was removed by evaporation, the residue was dissolved in EtOAc, washed with water, 10% citric acid, 5% $NaHCO_3$, and saturated NaCl, dried over Na_2SO_4 and concentrated in vacuo. The resulting residue was dissolved in DMSO and purified by preparative HPLC with a linear gradient of 35–55% CH_3CN in 0.1% aq TFA over 40 min to give a yellow powder of the desired compound **5** (5.0 mg, 17%); ^1H NMR (600 MHz, DMSO-d_6) δ 12.48 (br s, 1H), 12.22 (br s, 1H), 10.38 (s, 1H), 7.96 (br s, 1H), 7.76–7.75 (m, 4H), 7.72–7.68 (m, 3H), 7.59 (dd, J = 7.7, 7.7 Hz), 6.87 (s, 1H), 6.82 (s, 1H), 1.39 (s, 1H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 195.1, 157.5, 156.0, 140.4, 137.6, 136.9, 135.7, 134.3, 132.6, 129.8, 129.5, 129.2, 128.5, 128.2, 112.4, 105.0, 31.8, 30.4; HRMS (ESI): *m/z* 441.1912 $[M+H]^+$ (Calcd for $C_{26}H_{25}N_4O_3$: 441.1927).

4.2.2. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(hydroxy(phenyl)methyl)benzylidene)piperazine-2,5-dione (6)

Compound **6** was prepared from compound **15** and 3-(hydroxy(phenyl)methyl)benzaldehyde[24] according to the procedure described for the synthesis of **5**.

11% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.40 (br s, 1H), 12.18 (br s, 1H), 9.93 (s, 1H), 7.92 (br s, 1H), 7.52 (s, 1H), 7.41–7.38 (m, 3H), 7.35 (dd, J = 7.6, 7.6 Hz), 7.32–7.28 (m, 3H), 7.21 (dddd, J = 7.3, 7.3, 1.3, 1.3 Hz, 1H), 6.84 (s, 1H), 6.73 (s, 1H), 5.74 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 157.3, 156.2, 146.0, 145.3, 140.2, 134.2, 132.8, 128.5, 128.0, 127.5, 126.9, 126.7, 126.4, 126.2, 126.0, 113.8, 104.8, 73.9, 31.8, 30.5; HRMS (ESI): m/z 443.2093 $[M+H]^+$ (Calcd for $C_{26}H_{27}N_4O_3$: 443.2083).

4.2.3. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-((9-oxo-9*H*-fluoren-2-yl)methylene)piperazine-2,5-dione (7)

Compound **7** was prepared from compound **15** and 9-oxo-9*H*-fluorene-2-carbaldehyde (**S4**, Supplementary data) according to the procedure described for the synthesis of **5**.

5% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.43 (br s, 1H), 12.20 (br s, 1H), 10.55 (s, 1H), 7.94 (br s, 1H), 7.85–7.83 (m, 2H), 7.75 (s, 1H), 7.71 (dd, J = 8.1, 1.3 Hz, 1H), 7.64 (ddd, J = 7.4, 1.5, 1.5 Hz, 2H), 7.40 (ddd, J = 8.2, 3.8, 3.8 Hz, 1H), 6.86 (s, 1H), 6.78 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 192.8, 157.6, 156.2, 143.6, 142.8, 140.2, 136.4, 135.5, 134.5, 134.3, 133.5, 133.3, 129.5, 127.4, 124.4, 123.9, 121.3, 112.8, 104.8, 31.8, 30.5; HRMS (EI): m/z 438.1700 $[M]^+$ (Calcd for $C_{26}H_{22}N_4O_3$: 438.1692).

4.2.4. *N*,2-Dimethoxy-*N*-methylbenzamide (9a)

To a solution of 3-cyanobenzoic acid **8a** (3.0 g, 19.7 mmol) in DMF was added *N*,*O*-dimethylhydroxylamine hydrochloride (2.0 g, 20.7 mmol), Et_3N (2.88 mL, d = 0.73, 20.7 mmol) and EDC·HCl (4.0 g, 20.7 mmol). After the mixture was stirred for 3 h at room temperature, the solvent was removed in vacuo and the residue was dissolved in EtOAc, washed with 10% citric acid, 10% $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. Then, the solvent was removed to give a colorless oil of compound **9a** (3.0 g, 79%); ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.42–7.36 (m, 1H), 7.21 (dd, J = 7.4, 1.7 Hz, 1H), 7.07 (d, J = 8.3 Hz, 1H), 6.98 (dd, J = 7.4, 7.4 Hz, 1H), 3.78 (s, 3H), 3.45 (br s, 1H), 3.07 (s, 3H), 3.19 (br s, 2H); HRMS (ESI): m/z 218.0795 $[M+Na]^+$ (Calcd for $C_{10}H_{13}NO_3Na$: 218.0793).

Compounds **9b–g** and **9j–o** were prepared from compounds **8b–g** or **8j–o** according to the procedure described for the synthesis of **9a**.

4.2.5. *N*,3-Dimethoxy-*N*-methylbenzamide (9b)

86% Yield from **8b**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.37–7.31 (m, 1H), 7.21–7.16 (m, 2H), 7.06–7.01 (m, 1H), 3.82 (s, 3H), 3.58 (s, 3H), 3.28 (s, 3H); MS (ESI): m/z 217.95 $[M+Na]^+$ (Calcd for $C_{10}H_{13}NO_3Na$: 218.08).

4.2.6. *N*,4-Dimethoxy-*N*-methylbenzamide (9c)

81% Yield from **8c**; ^{1}H NMR1H NMR (300 MHz, $CDCl_3$) δ 7.60 (d, J = 9.0 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 3.58 (s, 3H), 3.37 (s, 3H), 2.41 (s, 3H); MS (ESI): m/z 217.95 $[M+Na]^+$ (Calcd for $C_{10}H_{13}NO_3Na$: 218.08).

4.2.7. *N*,2,3-Trimethoxy-*N*-methylbenzamide (9d)

92% Yield from **8d**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.09 (d, J = 3.0 Hz, 1H), 6.83 (d, J = 3.0 Hz, 1H), 6.81 (d, J = 3.0 Hz, 1H), 3.87 (s, 6H), 3.80 (s, 6H); MS (ESI): m/z 248.00 $[M+Na]^+$ (Calcd for $C_{11}H_{15}NO_4Na$: 248.09).

4.2.8. *N*,2,4-Trimethoxy-*N*-methylbenzamide (9e)

93% Yield from **8e**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.15 (d, J = 9.0 Hz, 1H), 6.59 (d, J = 3.0 Hz, 1H), 6.55 (dd, J = 9.0, 3.0 Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H), 3.54 (s, 3H), 3.18 (s, 3H); MS (ESI): m/z 247.95 $[M+Na]^+$ (Calcd for $C_{11}H_{15}NO_4Na$: 248.09).

4.2.9. *N*,3,4-Trimethoxy-*N*-methylbenzamide (9f)

87% Yield from **8f**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.36–7.29 (m, 2H), 6.99 (d, J = 9.0 Hz, 1H), 3.86 (s, 3H), 3.84 (s, 3H), 3.61 (s, 3H), 3.27 (s, 3H); MS (ESI): m/z 248.05 $[M+Na]^+$ (Calcd for $C_{11}H_{15}NO_4Na$: 248.09).

4.2.10. *N*,3,4,5-Tetramethoxy-*N*-methylbenzamide (9g)

82% Yield from **8g**; ^{1}H NMR (300 MHz, acetone-d_6) δ 6.97 (s, 2H), 3.86 (s, 6H), 3.77 (s, 3H), 3.64 (s, 3H), 3.28 (s, 3H); MS (ESI): m/z 277.95 $[M+Na]^+$ (Calcd for $C_{12}H_{17}NO_5Na$: 278.10).

4.2.11. 4-Fluoro-*N*-methoxy-*N*-methylbenzamide (9j)

96% Yield from **8j**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.78–7.72 (m, 2H), 7.24–7.17 (m, 2H), 3.58 (s, 3H), 3.30 (s, 3H); MS (ESI): m/z 205.95 $[M+Na]^+$ (Calcd for $C_9H_{10}FNO_2Na$: 206.06).

4.2.12. 2-Chloro-*N*-methoxy-*N*-methylbenzamide (9k)

93% Yield from **8k**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.54–7.38 (m, 4H), 3.46 (s, 3H), 3.29 (s, 3H); MS (ESI): m/z 221.85 $[M+Na]^+$ (Calcd for $C_9H_{10}ClNO_2Na$: 222.03).

4.2.13. 3-Chloro-*N*-methoxy-*N*-methylbenzamide (9l)

61% Yield from **8l**; ^{1}H NMR (300 MHz, $CDCl_3$) δ d 767dd, J = 1.8, 1.8 Hz, 1H), 7.57 (ddd, J = 7.6, 1.4, 1.4 Hz, 1H), 7.46–7.42 (m, 1H), 7.35 (dd, J = 7.8, 7.8 Hz, 1H), 3.56 (s, 3H), 3.37 (s, 3H); HRMS (ESI): m/z 222.0303 $[M+Na]^+$ (Calcd for $C_9H_{10}ClNO_2Na$: 222.0298).

4.2.14. 4-Chloro-*N*-methoxy-*N*-methylbenzamide (9m)

87% Yield from **8m**; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (d, J = 9.0 Hz, 2H), 7.38 (d, J = 9.0 Hz, 2H), 3.53 (s, 3H), 3.36 (s, 3H); MS (ESI): m/z 221.90 $[M+Na]^+$ (Calcd for $C_9H_{10}ClNO_2Na$: 222.03).

4.2.15. 4-Bromo-*N*-methoxy-*N*-methylbenzamide (9n)

68% Yield from **8n**; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60–7.52 (m, 4H), 3.53 (s, 3H), 3.36 (s, 3H); MS (ESI): m/z 265.90 $[M+Na]^+$ (Calcd for $C_9H_{10}BrNO_2Na$: 265.98).

4.2.16. *N*-Methoxy-*N*-methyl-4-(trifluoromethyl)benzamide (9o)

82% Yield from **8o**; ^{1}H NMR (300 MHz, acetone-d_6) δ 7.86–7.78 (m, 4H), 3.59 (s, 3H), 3.33 (s, 3H); MS (ESI): m/z 255.95 $[M+Na]^+$ (Calcd for $C_{10}H_{10}FNO_2Na$: 256.06).

4.2.17. 3-(2-Methoxybenzoyl)benzonitrile (13a)

To a solution of compound **9a** (2.0 g, 10.2 mmol) and 3-bromobenzonitrile **10** (1.86 g, 10.2 mmol) in anhydrous THF was added dropwise *n*-BuLi (2.0 M solution in *n*-hexane, 10.2 mL, 20.5 mmol) at −78 °C under Ar. After the addition of *n*-BuLi, the solution was poured into ice-cold 1 M HCl, and the organic phase was extracted with AcOEt, washed with brine twice, and dried over Na_2SO_4. Then, the solvent was removed under reduced pressure and the resulting brown oil was purified by silica-gel column chromatography (*n*-hexane:EtOAc = 5:1) to yield the benzophenone derivative **13a** as a white solid (457 mg, 19%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.06–8.02 (m, 2H), 7.82 (ddd, J = 7.7, 1.1, 1.1 Hz, 1H), 7.61–7.51 (m, 2H), 7.42 (dd, J = 7.5, 1.7 Hz, 1H), 7.09 (ddd, J = 7.5, 7.5, 0.9 Hz, 1H), 7.02 (d, J = 8.4 Hz, 1H); HRMS (ESI): m/z 238.0864 $[M+H]^+$ (Calcd for $C_{15}H_{12}NO_2$: 238.0863).

Compounds **13b–g** and **13j–o** were prepared from compound **9b–g** or **9j–o** according to the procedure described for the synthesis of **13a**.

4.2.18. 3-(3-Methoxybenzoyl)benzonitrile (13b)

25% Yield from **9b**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.14 (ddd, J = 1.8, 1.8, 0.9 Hz, 1H), 8.11–8.05 (m, 2H), 7.81 (ddd, J = 7.8, 7.8, 0.6 Hz, 1H), 7.52–7.47 (m, 1H), 7.37–7.33 (m, 2H), 7.29–7.25 (m,

1H), 3.88 (s, 3H); MS (EI): *m/z* 237.10 [M^+] (Calcd for $C_{15}H_{11}NO_2$: 237.08).

4.2.19. 3-(4-Methoxybenzoyl)benzonitrile (13c)

25% Yield from **9c**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.23–8.16 (m, 2H), 8.11–8.09 (m, 1H), 7.85–7.75 (m, 3H), 7.12 (d, J = 9.0 Hz, 2H), 3.89 (s, 3H); MS (EI): *m/z* 237.10 [M^+] (Calcd for $C_{15}H_{11}NO_2$: 237.08).

4.2.20. 3-(2,3-Dimethoxybenzoyl)benzonitrile (13d)

12% Yield from **9d**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.10–8.03 (m, 2H), 7.88–7.74 (m, 2H), 7.30–7.19 (m, 2H), 6.98 (dd, J = 9.0 Hz, 1H), 3.93 (s, 3H), 3.64 (s, 3H); MS (EI): *m/z* 267.10 [M^+] (Calcd for $C_{16}H_{13}NO_3$: 267.09).

4.2.21. 3-(2,4-Dimethoxybenzoyl)benzonitrile (13e)

24% Yield from **9e**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.02–7.97 (m, 3H), 7.72 (ddd, J = 9.0, 9.0, 3.0 Hz, 1H), 7.49 (d, J = 12.0 Hz, 1H), 6.72–6.68 (m, 2H), 3.92 (s, 3H), 3.70 (s, 3H); MS (EI): *m/z* 267.10 [M^+] (Calcd for $C_{16}H_{13}NO_3$: 267.09).

4.2.22. 3-(3,4-Dimethoxybenzoyl)benzonitrile (13f)

12% Yield from **9f**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.12–8.09 (m, 2H), 8.01–7.97 (m, 1H), 7.78–7.73 (m, 1H), 7.40 (d, J = 3.0 Hz, 1H), 7.32 (dd, J = 9.0, 3.0 Hz, 1H), 7.11 (d, J = 9.0 Hz, 1H), 3.87 (s, 3H), 3.83 (s, 3H); HRMS (EI): *m/z* 267.0900 [M^+] (Calcd for $C_{16}H_{13}NO_3$: 267.0895).

4.2.23. 3-(3,4,5-Trimethoxybenzoyl)benzonitrile (13g)

11% Yield from **9g**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.15 (ddd, J = 1.8, 1.8, 0.6 Hz, 1H), 8.12–8.04 (m, 2H), 7.82–7.77 (ddd, J = 7.8, 7.8, 0.6 Hz, 1H), 7.12 (s, 2H), 3.87 (s, 6H), 3.85 (s, 3H); MS (EI): *m/z* 297.10 [M^+] (Calcd for $C_{17}H_{15}NO_4$: 297.10).

4.2.24. 3-(4-Fluorobenzoyl)benzonitrile (13j)

17% Yield from **9j**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.15–8.14 (m, 1H), 8.11–8.06 (m, 2H), 7.94 (dd, J = 14.7, 5.4 Hz, 2H), 7.84–7.79 (m, 1H); MS (EI): *m/z* 225.05 [M^+] (Calcd for $C_{14}H_8FNO$: 225.06).

4.2.25. 3-(2-Chlorobenzoyl)benzonitrile (13k)

9% Yield from **9k**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.15–8.13 (m, 1H), 8.12–8.06 (m, 2H), 7.81 (ddd, J = 7.8, 7.8, 0.9 Hz, 1H), 7.63–7.54 (m, 4H); MS (EI): *m/z* 241.05 [M^+] (Calcd for $C_{14}H_8ClNO$: 241.03).

4.2.26. 3-(3-Chlorobenzoyl)benzonitrile (13l)

46% Yield from **9l**; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (dd, J = 1.3, 1.3 Hz, 1H), 8.03 (ddd, J = 7.8, 1.5, 1.5 Hz, 1H), 7.90 (ddd, J = 7.8, 1.4, 1.4 Hz, 1H), 7.78 (dd, J = 1.8, 1.8 Hz, 1H), 7.69–7.61 (m, 3H), 7.48 (dd, J = 7.8, 7.8 Hz, 1H); HRMS (ESI): *m/z* 242.0378 [M+H]$^+$ (Calcd for $C_{14}H_9ClNO$: 242.0373).

4.2.27. 3-(4-Chlorobenzoyl)benzonitrile (13m)

26% Yield from **9m**; ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.89 (d, J = 8.7 Hz, 2H), 7.53 (d, J = 8.7 Hz, 2H), 7.37–7.29 (m, 4H); MS (EI): *m/z* 241.05 [M^+] (Calcd for $C_{14}H_8ClNO$: 241.03).

4.2.28. 3-(4-Bromobenzoyl)benzonitrile (13n)

53% Yield from **9n**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.17–8.16 (m, 1H), 8.12–8.07 (m, 2H), 7.84–7.79 (m, 5H); MS (EI): *m/z* 285.05 [M^+] (Calcd for $C_{14}H_8BrNO$: 284.98).

4.2.29. 3-(4-(Trifluoromethyl)benzoyl)benzonitrile (13o)

48% Yield from **9o**; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.21–8.20 (m, 1H), 8.17–8.09 (m, 2H), 8.06–7.93 (m, 4H), 7.87–7.81 (m, 1H); MS (EI): *m/z* 275.10 [M^+] (Calcd for $C_{15}H_8F_3NO$: 275.06).

4.2.30. 3-(2-Fluorobenzoyl)benzonitrile (13h)

To a solution of 1-bromo-2-fluorobenzene **11h** (3.0 g, 17.1 mmol) and 3-cyano-*N*-methoxy-*N*-methylbenzamide[21] **12** (3.3 g, 17.1 mmol) in anhydrous THF was added dropwise *n*-BuLi (1.66 M solution in *n*-hexane, 20.1 mL, 34.3 mmol) at −78 °C under Ar. After the addition of *n*-BuLi, the solution was poured into ice-cold 1 M HCl, and the organic phase was extracted with AcOEt, washed with brine twice, and dried over Na_2SO_4. Then, the solvent was removed under reduced pressure and the resulting brown oil was purified by silica-gel column chromatography (*n*-hexane:EtOAc = 2:1) to yield the benzophenone derivative **13h** as a white solid (544 mg, 19%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09–8.05 (m, 2H), 7.88 (ddd, J = 7.7, 1.4, 1.4 Hz, 1H), 7.66–7.57 (m, 3H), 7.36–7.30 (m, 1H), 7.23–7.17 (m, 1H); MS (ESI): *m/z* 226.0665 [M+H$^+$] (Calcd for $C_{14}H_9FNO$: 226.0668).

4.2.31. 3-(3-Fluorobenzoyl)benzonitrile (13i)

Compounds **13i** were prepared from compound **11i** according to the procedure described for the synthesis of **13h**. 39% Yield from **11i**. ^{1}H NMR (300 MHz, acetone-d_6) δ 8.17 (ddd, J = 1.7, 1.7, 0.6 Hz, 1H), 8.14–8.07 (m, 2H), 7.82 (ddd, J = 7.8, 7.8, 0.6 Hz, 1H), 7.67–7.64 (m, 2H), 7.60–7.46 (m, 2H); MS (EI): *m/z* 225.05 [M^+] (Calcd for $C_{14}H_8FNO$: 225.06).

4.2.32. 3-(2-Methoxybenzoyl)benzaldehyde (14a)

To a solution of compound **13a** (150 mg, 0.632 mmol) in anhydrous THF (10 mL) was added dropwise DIBAL-H (1.00 M solution in toluene 2.53 mL, 2.53 mmol) at −78 °C under Ar. Then, the cooling bath was removed and the reaction mixture was stirred for 2 h at 0 °C. The reaction mixture was quenched by the addition of MeOH–AcOH (2:1), diluted with AcOEt, and filtered over a Celite pad to remove the produced $Al(OH)_3$. After the resultant organic phase was washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4, the solvent was removed under reduced pressure to obtain a colorless oil (171 mg). To a solution of this oil (153 mg, 0.632 mmol) in CH_2Cl_2 (10 mL) was added molecular sieves 4 Å (1.2 g) and pyridinium dichromate (571 mg, 1.52 mmol), and the mixture was stirred for 2 h at room temperature. Then, the suspension was removed by Celite filtration, and the solvent was removed in vacuo. The residue was purified by silica gel column chromatography using $CHCl_3$ as an eluent to give a colorless oil of compound **14a** (53 mg, 35% over two steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.06 (s, 1H), 8.26 (dd, J = 1.7, 1.7 Hz, 1H), 8.08 (dd, J = 7.7, 1.7 Hz, 2H), 7.62 (dd, J = 7.7, 7.7 Hz, 1H), 7.56–7.50 (m, 1H), 7.43 (dd, J = 7.4, 7.4, 0.8 Hz, 1H), 7.02 (d, J = 8.4 Hz, 1H), 3.71 (s, 3H); HRMS (ESI): *m/z* 241.0865 [M+H]$^+$ (Calcd for $C_{15}H_{13}O_3$: 241.0865).

Compounds **14b–o** were prepared from compounds **13b–o** according to the procedure described for the synthesis of **14a**.

4.2.33. 3-(3-Methoxybenzoyl)benzaldehyde (14b)

84% Yield from **13b** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.16 (s, 1H), 8.30 (ddd, J = 1.7, 1.7, 0.3 Hz, 1H), 8.23–8.09 (m, 2H), 7.83–7.78 (m, 1H), 7.52–7.47 (m, 1H), 7.37–7.34 (m, 2H), 7.28–7.24 (m, 1H), 3.88 (s, 3H); MS (EI): *m/z* 240.10 [M^+] (Calcd for $C_{15}H_{12}O_3$: 240.08).

4.2.34. 3-(4-Methoxybenzoyl)benzaldehyde (14c)

70% Yield from **13c** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.16 (s, 1H), 8.25–8.24 (m, 1H), 8.20–8.16 (m, 1H), 8.07–8.03 (m, 1H), 7.87–7.76 (m, 3H), 7.10 (d, J = 9.0 Hz, 2H), 3.93 (s, 3H); MS (EI): *m/z* 240.10 [M^+] (Calcd for $C_{15}H_{12}O_3$: 240.08).

4.2.35. 3-(2,3-Dimethoxybenzoyl)benzaldehyde (14d)

39% Yield from **13d** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.13 (s, 1H), 8.27 (ddd, J = 1.7, 1.7, 0.3 Hz, 1H), 8.21–8.17 (m, 1H),

8.09–8.06 (m, 1H), 7.80–7.75 (m, 1H), 7.30–7.20 (m, 2H), 6.97 (dd, J = 7.5, 1.8 Hz, 1H), 3.94 (s, 3H), 3.63 (s, 3H); MS (EI): m/z 270.10 [M^+] (Calcd for $C_{16}H_{14}O_4$: 270.09).

4.2.36. 3-(2,4-Dimethoxybenzoyl)benzaldehyde (14e)

11% Yield from **13e** (2 steps); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.08 (s, 1H), 8.14–8.11 (m, 2H), 7.98–7.94 (m, 1H), 7.75–7.70 (m, 1H), 7.42 (d, J = 8.4 Hz, 1H), 6.72 (dd, J = 5.1, 2.1 Hz, 1H), 6.68 (d, J = 2.4 Hz, 1H), 3.87 (s, 3H), 3.63 (s, 3H); MS (EI): m/z 270.10 [M^+] (Calcd for $C_{16}H_{14}O_4$: 270.09).

4.2.37. 3-(3,4-Dimethoxybenzoyl)benzaldehyde (14f)

42% Yield from **13f** (2 steps); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.11 (s, 1H), 8.20–8.01 (m, 3H), 7.81–7.76 (m, 1H), 7.41 (d, J = 1.8 Hz, 1H), 7.33 (dd, J = 8.4, 2.1 Hz, 1H), 7.12 (d, J = 8.4 Hz, 1H), 3.88 (s, 3H), 3.83 (s, 3H); MS (EI): m/z 270.15 [M^+] (Calcd for $C_{16}H_{14}O_4$: 270.09).

4.2.38. 3-(3,4,5-Trimethoxybenzoyl)benzaldehyde (14g)

56% Yield from **13g** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.17 (s, 1H), 8.20–8.18 (m, 1H), 8.13–8.11 (m, 1H), 7.83–7.77 (m, 1H), 7.72–7.65 (m, 1H), 7.13 (s, 2H), 3.87 (s, 6H), 3.85 (s, 3H); MS (EI): m/z 300.10 [M^+] (Calcd for $C_{17}H_{16}O_5$: 300.10).

4.2.39. 3-(2-Fluorobenzoyl)benzaldehyde (14h)

57% Yield from **13h** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.15 (s, 1H), 8.33–8.32 (m, 1H), 8.25–8.22 (m, 1H), 8.15–8.11 (m, 1H), 7.84–7.79 (m, 1H), 7.76–7.64 (m, 2H), 7.43 (ddd, J = 7.5, 7.5, 0.9 Hz, 1H), 7.37–7.31 (m, 1H); MS (EI): m/z 228.05 [M^+] (Calcd for $C_{14}H_9FO_2$: 228.06).

4.2.40. 3-(3-Fluorobenzoyl)benzaldehyde (14i)

44% Yield from **13i** (2 steps); ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.12 (s, 1H), 8.26–8.20 (m, 1H), 8.10–8.06 (m, 1H), 7.84–7.75 (m, 1H), 7.68–7.56 (m, 4H); MS (EI): m/z 228.10 [M^+] (Calcd for $C_{14}H_9FO_2$: 228.06).

4.2.41. 3-(4-Fluorobenzoyl)benzaldehyde (14j)

82% Yield from **13j** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.16 (s, 1H), 8.30–8.29 (m, 1H), 8.23–8.20 (m, 1H), 8.12–8.08 (m, 1H), 7.97–7.92 (m, 2H), 7.84–7.79 (m, 1H); MS (EI): m/z 228.05 [M^+] (Calcd for $C_{14}H_9FO_2$: 228.06).

4.2.42. 3-(2-Chlorobenzoyl)benzaldehyde (14k)

34% Yield from **13k** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.14 (s, 1H), 8.30–8.29 (m, 1H), 8.26–8.22 (m, 1H), 8.11–8.08 (m, 1H), 7.85–7.79 (m, 1H), 7.64–7.60 (m, 2H), 7.57–7.55 (m, 2H); MS (EI): m/z 244.05 [M^+] (Calcd for $C_{14}H_9ClO_2$: 244.03).

4.2.43. 3-(3-Chlorobenzoyl)benzaldehyde (14l)

87% Yield from **13l** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.17 (s, 1H), 8.32 (ddd, J = 1.7, 1.7, 0.5 Hz, 1H), 8.25–8.08 (m, 2H), 7.86–7.73 (m, 4H), 7.65–7.60 (m, 1H); HRMS (ESI): m/z 245.0365 [M+H]$^+$ (Calcd for $C_{14}H_{10}ClO_2$: 245.0369).

4.2.44. 3-(4-Chlorobenzoyl)benzaldehyde (14m)

61% Yield from **13m** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.16 (s, 1H), 8.30 (ddd, J = 1.8, 1.8, 0.6 Hz, 1H), 8.24–8.09 (m, 2H), 7.88–7.82 (m, 3H), 7.66–7.61 (m, 2H); MS (EI): m/z 244.10 [M^+] (Calcd for $C_{14}H_9ClO_2$: 244.03).

4.2.45. 3-(4-Bromobenzoyl)benzonitrile (14n)

68% Yield from **13n** (2 steps); ^{1}H NMR (300 MHz, acetone-d_6) δ 10.16 (s, 1H), 8.31–8.30 (m, 1H), 8.24–8.21 (m, 1H), 8.13–8.10 (m, 1H), 7.85–7.74 (m, 5H); HRMS (EI): m/z 287.9778 [M^+] (Calcd for $C_{14}H_9BrO_2$: 287.0785).

4.2.46. 3-(4-(Trifluoromethyl)benzoyl)benzonitrile (14o)

59% Yield from **13o** (2 steps); ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.10 (s, 1H), 8.28–8.27 (m, 1H), 8.17–8.08 (m, 3H), 7.92–7.88 (m, 4H); HRMS (EI): m/z 278.0558 [M^+] (Calcd for $C_{15}H_9F_3O_2$: 278.0554).

Compounds **16a–o** were prepared from compound **15** and aldehydes **14a–o** according to the procedure described for the synthesis of **5**.

4.2.47. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(2-methoxybenzoyl)benzylidene)piperazine-2,5-dione (16a)

33% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.56 (br s, 1H), 12.07 (br s, 1H), 10.33 (s, 1H), 8.03 (br s, 1H), 7.80 (s, 1H), 7.76 (d, J = 7.4 Hz, 1H), 7.58–7.53 (m, 3H), 7.35 (dd, J = 7.4, 1.8 Hz, 1H), 7.20 (d, J = 8.4 Hz, 1H), 7.10 (ddd, J = 7.3, 7.3, 0.8 Hz, 1H), 6.82 (s, 1H), 6.77 (s, 1H), 3.71 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 195.2, 157.4, 156.6, 156.2, 140.2, 137.2, 134.2, 133.8, 133.5, 132.1, 129.5, 128.9, 128.8, 128.0, 127.4, 120.4, 112.9, 112.0, 104.5, 55.5, 31.8, 30.4; HRMS (EI): m/z 470.1946 [M^+] (Calcd for $C_{27}H_{26}N_4O_4$: 470.1954).

4.2.48. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(3-methoxybenzoyl)benzylidene)piperazine-2,5-dione (16b)

43% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.64 (br s, 1H), 12.02 (br s, 1H), 10.42 (s, 1H), 8.09 (br s, 1H), 7.83 (s, 1H), 7.76 (d, J = 7.7 Hz, 1H), 7.64 (d, J = 7.8 Hz, 1H), 7.58 (dd, J = 7.6, 7.6 Hz, 1H), 7.50 (dd, J = 7.9, 7.9 Hz, 1H), 7.36 (d, J = 7.6 Hz, 1H), 7.30 (dd, J = 2.5, 1.5 Hz, 1H), 7.28–7.26 (m, 1H), 6.82 (s, 1H), 6.80 (s, 1H), 3.83 (s, 3H), 1.37 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 195.2, 159.2, 157.4, 156.3, 140.1, 138.2, 137.3, 134.2, 133.4, 133.3, 130.0, 129.7, 128.8, 128.7, 127.5, 122.4, 118.7, 114.0, 113.0, 104.3, 55.3, 31.8, 30.3; HRMS (EI): m/z 470.1947 [M^+] (Calcd for $C_{27}H_{26}N_4O_4$: 470.1954).

4.2.49. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(4-methoxybenzoyl)benzylidene)piperazine-2,5-dione (16c)

25% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.52 (br s, 1H), 12.10 (br s, 1H), 10.40 (s, 1H), 8.01 (br s, 1H), 7.83 (ddd, J = 8.9, 2.0, 2.0 Hz, 2H), 7.78 (s, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.60 (ddd, J = 7.6, 1.4, 1.4 Hz, 1H), 7.57 (dd, J = 7.6, 7.6 Hz, 1H), 7.12–7.10 (m, 2H), 6.81 (s, 2H), 3.87 (s, 3H), 1.37 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.1, 162.9, 157.4, 156.2, 140.1, 138.0, 134.2, 133.3, 132.7, 132.2, 129.8, 129.2, 128.6, 128.5, 127.4, 113.9, 113.0, 104.6, 55.4, 31.8, 30.4; HRMS (EI): m/z 470.1947 [M^+] (Calcd for $C_{27}H_{26}N_4O_4$: 470.1954).

4.2.50. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(2,3-dimethoxybenzoyl)benzylidene)piperazine-2,5-dione (16d)

31% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.59 (br s, 1H), 12.06 (br s, 1H), 10.43 (s, 1H), 8.05 (br s, 1H), 7.82 (s, 1H), 7.78 (d, J = 7.4 Hz, 1H), 7.58 (ddd, J = 7.7, 1.5, 1.5 Hz, 1H), 7.55 (dd, J = 7.4, 7.4 Hz, 1H), 7.26 (dd, J = 8.3, 1.5 Hz, 1H), 7.20 (dd, J = 8.0, 8.0 Hz, 1H), 6.92 (dd, J = 7.6, 1.4 Hz, 1H), 6.82 (s, 1H), 6.78 (s, 1H), 3.87 (s, 3H), 3.61 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 195.0, 157.4, 156.2, 152.3, 146.0, 140.1, 137.1, 134.2, 134.0, 133.6, 133.3, 129.6, 128.9, 128.9, 127.5, 124.1, 119.7, 115.0, 112.8, 104.4, 60.9, 55.8, 31.8, 30.3; HRMS (EI): m/z 500.2050 [M^+] (Calcd for $C_{28}H_{28}N_4O_5$: 500.2059).

4.2.51. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(2,4-dimethoxybenzoyl)benzylidene)piperazine-2,5-dione (16e)

12% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.44 (br s, 1H), 12.18 (br s, 1H), 10.26 (s, 1H), 7.94 (br s, 1H), 7.74 (s, 1H), 7.72 (d, J = 7.7 Hz, 1H), 7.56 (ddd, J = 7.8, 1.3, 1.3 Hz, 1H), 7.52 (dd, J = 7.4, 7.4 Hz, 1H), 7.36 (d, J = 8.5 Hz, 1H), 6.83 (s, 1H), 6.76 (s,

1H), 6.71 (d, J = 2.3 Hz, 1H), 6.66 (dd, J = 8.3, 2.3 Hz, 1H), 3.86 (s, 3H), 3.69 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.1, 163.0, 159.0, 157.4, 156.1, 140.2, 138.4, 134.2, 133.3, 133.2, 131.5, 129.5, 128.6, 127.3, 120.4, 112.9, 105.4, 98.8, 55.6, 55.4, 31.8, 30.4; HRMS (EI): m/z 500.2051 [M$^+$] (Calcd for $C_{28}H_{28}N_4O_5$: 500.2059).

4.2.52. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(3,4-dimethoxybenzoyl)benzylidene)piperazine-2,5-dione (16f)

32% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.60 (br s, 1H), 12.05 (br s, 1H), 10.41 (s, 1H), 8.05 (br s, 1H), 7.79 (s, 1H), 7.73 (d, J = 7.7 Hz, 1H), 7.61 (d, J = 7.6 Hz, 1H), 7.57 (dd, J = 7.6, 7.6 Hz, 1H), 7.42 (d, J = 2.0 Hz, 1H), 7.39 (dd, J = 8.3, 2.1 Hz, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.82 (s, 1H), 6.80 (s, 1H), 3.87 (s, 3H), 3.83 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.1, 157.4, 156.3, 152.9, 148.6, 140.1, 138.1, 134.2, 133.3, 132.7, 129.8, 129.1, 128.6, 128.5, 127.4, 125.2, 113.1, 111.6, 110.6, 104.4, 55.7, 55.5, 31.8, 30.3; HRMS (EI): m/z 500.2066 [M$^+$] (Calcd for $C_{28}H_{28}N_4O_5$: 500.2059).

4.2.53. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(3,4,5-trimethoxybenzoyl)benzylidene)piperazine-2,5-dione (16g)

32% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.50 (br s, 1H), 12.13 (br s, 1H), 10.37 (s, 1H), 7.99 (br s, 1H), 7.83 (s, 1H), 7.75 (d, J = 7.7 Hz, 1H), 7.67 (d, J = 7.6 Hz, 1H), 7.58 (dd, J = 7.7, 7.7 Hz, 1H), 7.08 (s, 2H), 6.82 (s, 2H), 3.82 (s, 6H), 3.78 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.5, 157.5, 156.2, 152.6, 141.5, 140.2, 137.5, 134.2, 133.5, 133.1, 131.9, 129.9, 128.8, 128.6, 127.5, 112.9, 107.3, 104.6, 60.1, 56.0, 31.8, 30.4; HRMS (EI): m/z 530.2158 [M$^+$] (Calcd for $C_{29}H_{30}N_4O_6$: 530.2165).

4.2.54. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(2-fluorobenzoyl)benzylidene)piperazine-2,5-dione (16h)

20% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.55 (br s, 1H), 12.10 (br s, 1H), 10.43 (s, 1H), 8.02 (br s, 1H), 7.87 (s, 1H), 7.80 (d, J = 7.6 Hz, 1H), 7.71–7.67 (m, 1H), 7.65 (d, J = 7.6 Hz, 1H), 7.63 (ddd, J = 7.6, 7.6, 1.7 Hz, 1H), 7.59 (dd, J = 7.7, 7.7 Hz, 1H), 7.42–7.39 (m, 2H), 6.82 (s, 1H), 6.79 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 192.3, 160.1, 158.5, 157.4, 156.2, 140.2, 136.9, 134.3, 134.2, 133.7, 133.7, 130.6, 130.6, 129.8, 129.0, 128.9, 127.6, 126.2, 126.1, 124.7, 124.7, 116.4, 116.3, 112.6, 104.6, 31.8, 30.4; HRMS (EI): m/z 458.1759 [M$^+$] (Calcd for $C_{26}H_{23}FN_4O_3$: 458.1754).

4.2.55. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(3-fluorobenzoyl)benzylidene)piperazine-2,5-dione (16i)

20% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.52 (br s, 1H), 12.12 (br s, 1H), 10.39 (s, 1H), 8.00 (br s, 1H), 7.84 (s, 1H), 7.77 (d, J = 7.7 Hz, 1H), 7.67–7.63 (m, 3H), 7.60 (dd, J = 7.8, 7.8 Hz, 1H), 7.58–7.55 (m, 2H), 6.82 (s, 2H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.2, 162.6, 161.0, 157.5, 156.1, 140.3, 139.1, 139.0, 136.8, 134.2, 133.6, 133.5, 130.8, 130.7, 130.1, 128.8, 127.6, 126.1, 126.0, 119.7, 119.6, 116.0, 115.8, 112.6, 104.9, 31.8, 30.5; HRMS (EI): m/z 458.1747 [M$^+$] (Calcd for $C_{26}H_{23}FN_4O_3$: 458.1754).

4.2.56. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(4-fluorobenzoyl)benzylidene)piperazine-2,5-dione (16j)

14% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.52 (br s, 1H), 12.12 (br s, 1H), 10.39 (s, 1H), 8.00 (br s, 1H), 7.93–7.90 (m, 2H), 7.82 (s, 1H), 7.76 (d, J = 7.7 Hz, 1H), 7.64 (ddd, J = 7.7, 1.3, 1.3 Hz, 1H), 7.59 (dd, J = 7.6, 7.6 Hz, 1H), 7.42–7.39 (m, 2H), 6.82 (s, 1H), 6.81 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.1, 165.5, 163.8, 157.5, 156.2, 140.2, 137.2, 134.2, 133.4, 133.4, 133.3, 133.3, 132.7, 132.7, 130.0, 128.8, 128.7, 127.5, 115.7, 115.6, 112.8, 104.6, 31.8, 30.4; HRMS (EI): m/z 458.1762 [M$^+$] (Calcd for $C_{26}H_{23}FN_4O_3$: 458.1754).

4.2.57. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(2-chlorobenzoyl)benzylidene)piperazine-2,5-dione (16k)

7% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.50 (br s, 1H), 12.13 (br s, 1H), 10.42 (s, 1H), 7.99 (br s, 1H), 7.85 (s, 1H), 7.83–7.81 (m, 1H), 7.63–7.60 (m, 2H), 7.59–7.58 (m, 2H), 7.53–7.52 (m, 2H), 6.83 (s, 1H), 6.77 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.1, 157.4, 156.1, 140.2, 137.7, 135.9, 134.6, 134.2, 134.0, 131.8, 129.9, 129.8, 129.8, 129.2, 129.2, 129.1, 127.7, 127.3, 112.5, 104.7, 31.8, 30.4; HRMS (EI): m/z 474.1453 [M$^+$] (Calcd for $C_{26}H_{23}ClN_4O_3$: 474.1458).

4.2.58. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(3-chlorobenzoyl)benzylidene)piperazine-2,5-dione (16l)

49% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.55 (br s, 1H), 12.10 (br s, 1H), 10.40 (s, 1H), 8.02 (br s, 1H), 7.84 (s, 1H), 7.79–7.76 (m, 4H), 7.66–7.63 (m, 1H), 7.62–7.59 (m, 2H), 6.82 (s, 2H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.2, 157.4, 156.2, 140.1, 138.8, 136.7, 134.2, 133.6, 133.5, 133.4, 132.5, 130.5, 130.1, 128.9, 128.8, 128.8, 128.4, 127.6, 112.8, 104.6, 31.8, 30.4; HRMS (EI): m/z 474.1455 [M$^+$] (Calcd for $C_{26}H_{23}ClN_4O_3$: 474.1458).

4.2.59. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(4-chlorobenzoyl)benzylidene)piperazine-2,5-dione (16m)

13% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.54 (br s, 1H), 12.11 (br s, 1H), 10.40 (s, 1H), 8.01 (br s, 1H), 7.84 (ddd, J = 8.5, 2.0, 2.0 Hz, 2H), 7.83 (s, 1H), 7.76 (d, J = 7.6 Hz, 1H), 7.65 (d, J = 8.5 Hz, 3H), 7.59 (dd, J = 7.7, 7.7 Hz, 1H), 6.82 (s, 1H), 6.82 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.4, 157.5, 156.2, 140.2, 137.7, 136.9, 135.4, 134.2, 133.5, 133.4, 131.6, 130.1, 128.8, 128.7, 128.7, 127.6, 112.8, 104.7, 31.8, 30.4; HRMS (EI): m/z 474.1450 [M$^+$] (Calcd for $C_{26}H_{23}ClN_4O_3$: 474.1458).

4.2.60. (3*Z*,6*Z*)-3-(3-(4-Bromobenzoyl)benzylidene)-6-((5-*tert*-butyl-1*H*-imidazol-4-yl)methylene)piperazine-2,5-dione (16n)

44% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.51 (br s, 1H), 12.13 (br s, 1H), 10.39 (s, 1H), 7.99 (br s, 1H), 7.83 (s, 1H), 7.80 (ddd, J = 8.6, 2.0, 2.0 Hz, 2H), 7.76 (ddd, J = 8.6, 1.9, 1.9 Hz, 3H), 7.65 (ddd, J = 7.7, 1.4, 1.4 Hz, 1H), 7.59 (dd, J = 7.6, 7.6 Hz, 1H), 6.83 (s, 1H), 6.81 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.5, 157.5, 156.2, 140.2, 136.9, 135.8, 134.2, 133.5, 133.4, 131.7, 131.6, 130.1, 128.8, 128.7, 127.6, 126.8, 112.8, 104.6, 31.8, 30.4; HRMS (EI): m/z 518.0945 [M$^+$] (Calcd for $C_{26}H_{23}Br\ N_4O_3$: 518.0953).

4.2.61. (3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(3-(4-(trifluoromethyl)benzoyl)benzylidene)piperazine-2,5-dione (16o)

25% Yield from **15**; ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.51 (br s, 1H), 12.12 (br s, 1H), 10.40 (s, 1H), 8.00 (d, J = 8.0 Hz, 2H), 7.95 (s, 1H), 7.95 (d, J = 8.2 Hz, 2H), 7.87 (s, 1H), 7.79 (d, J = 7.9 Hz, 1H), 7.68 (d, J = 7.7 Hz, 1H), 7.61 (dd, J = 7.7, 7.7 Hz, 1H), 6.83 (s, 1H), 6.82 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 194.6, 157.5, 156.1, 140.4, 140.2, 136.4, 134.2, 133.8, 133.6, 132.1, 131.9, 130.4, 130.3, 128.9, 127.7, 125.5, 125.5, 124.6, 122.8, 122.7, 104.7, 31.8, 30.4; HRMS (EI): m/z 508.1727 [M$^+$] (Calcd for $C_{27}H_{23}F\ _3N_4O_3$: 508.1722).

4.3. Biological evaluation

4.3.1. Tubulin binding assay

Fluorescence spectra were measured at 37 °C as described previously.[21,24] Porcine tubulin (0.5 μM) in MES buffer (0.1 M MES,

0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8) was incubated with different concentrations of the test compounds (0–20 μM, 1% DMSO) at 37 °C for 1 h. After incubation, the fluorescence of each solution was measured (excitation at 295 nm, emission at 300–450 nm) using an FP-750 Spectrofluorometer (JASCO, JAPAN).

4.3.2. HT-29. cell culture conditions and Resazurin-based cytotoxicity assay

The cytotoxic activity assay was performed as described previously.[14] HT-29 cells were purchased from ATCC and maintained in McCoy's 5A medium containing 10% fetal bovine serum supplemented with 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. For the growth inhibition assays, cells were plated in 96-well plates at an appropriate density the day before compound addition. Stock solutions of compounds were prepared in DMSO. Serially diluted compounds were added to the cells, resulting in a final concentration range of 2 pM to 20 μM. Forty-eight hours later, 10 μL of a 0.2 mg/mL Resazurin solution in PBS buffer was added to each well and the cells were incubated for an additional 3–6 h. The fluorescence of the Resazurin reduction product was measured using a Fusion microplate fluorometer (Packard Bioscience) with λex = 535 nm and λem = 590 nm filters.

4.3.3. XTT/PMS-based cytotoxicity assay

The XTT/PMS assay was performed as described previously.[24b] Briefly, HT-29 cells were plated in 96-well plates at 5000 cells/well the day before compound addition. Stock solutions of compounds were prepared in DMSO. Serially diluted compounds were added to the cells, resulting in a final concentration range of 2 pM to 20 μM. Seventy-two hours later, 0.1 mg/mL XTT solution in PBS buffer containing 25 μM phenazine methosulfate was added to each well and the cells were incubated for an additional 40 min. The absorbance of the formazan product was measured at 492 nm on a plate reader (TECAN SAFIRE). To compensate for non-specific absorption, the absorbance at 690 nm was also measured.

4.3.4. HeLa and A549 cell culture conditions and WST-8-based proliferation assay

The human cervix epidermoid carcinoma cell line, HeLa, and the human lung carcinoma cell line, A549, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (Nichirei Bioscience Inc., Tokyo, Japan). Cells were grown at 37 °C in a 5% CO_2 atmosphere. For calculation of IC_{50} values, cells (3×10^4 cells/mL) were treated with various concentrations of each compound for 48 h. Cell growth was measured using the WST-8 cell counting kit (Dojindo, Kumamoto, Japan).

4.3.5. In vitro tubulin polymerization assay

Tubulin was purified from bovine brain using the two polymerization-depolymerization cycle method described previously.[25] Turbidity assays were performed by incubating 1 mg/mL tubulin in RB buffer (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.8) with 1 mM GTP and 1 M glutamate. An increase in the absorbance at 350 nm was monitored at 37 °C using a thermostatic spectrophotometer (Beckman Coulter Inc., Brea, CA).

4.3.6. Immunofluorescence

HeLa cells (3×10^4 cells /mL) were placed on sterile coverslips and treated with various concentrations of each compound for 6 hours. Coverslips were fixed with −20 °C MeOH for 5 min, and washed in PBS-B (PBS containing 0.5% w/v BSA). Next, coverslips were overlain with an anti-α-tubulin antibody (sc-32293, Santa Cruz Biotechnology Inc., Santa Cruz, CA) in PBS-B, and placed in a humidified container at 37 °C for 1 h. Then, coverslips were washed twice with PBS-B, overlain with a solution of Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen) in PBS-B, and incubated for 30 min. Finally, coverslips were washed with PBS and mounted with 0.1 μg/mL DAPI solution (Dojindo, Kumamoto, Japan). The morphology of chromosomes and microtubules was observed under a Leica LAS AF 6000 fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.059.

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