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Development of a New Benzophenone−Diketopiperazine-Type Potent Antimicrotubule Agent Possessing a 2-Pyridine Structure

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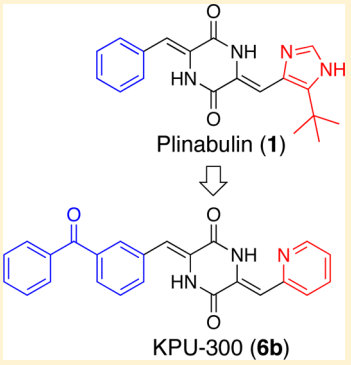
Supporting Information

ABSTRACT: A new benzophenone−diketopiperazine-type potent antimicrotubule agent was developed by modifying the structure of the clinical candidate plinabulin (**1**). Although the right-hand imidazole ring with a branched alkyl chain at the 5-position in **1** was critical for the potency of the antimicrotubule activity, we successfully substituted this moiety with a simpler 2-pyridyl structure by converting the left-hand ring from a phenyl to a benzophenone structure without decreasing the potency. The resultant compound **6b** (KPU-300) exhibited a potent cytotoxicity, with an IC_{50} value of 7.0 nM against HT-29 cells, by strongly binding to tubulin (K_d = 1.3 μM) and inducing microtubule depolymerization.



KEYWORDS: *Cyclic dipeptide, diketopiperazine, antimicrotubule agent, anticancer*

Microtubules are tubular polymers formed by the polymerization of α- and β-tubulin heterodimers and are major cytoskeletal components in eukaryotic cells.[1] The dynamic and mutual interconversion of tubulins and microtubules is responsible for a variety of cellular processes involved in maintaining the cellular structure, providing an intracellular rail-like transport platform for the motor proteins kinesin and dynein, and enabling cell division through spindle formation during mitosis, which pulls apart the eukaryotic chromosomes.[1] Because of these important roles, microtubules are attractive molecular targets in cancer chemotherapy.[2,3] Several antimicrotubule agents that interfere with the dynamic functions of microtubules have been developed as clinical drugs for the treatment of various cancers.[4−6] These are taxanes (paclitaxel and dosetaxel), which can stabilize the polymerized states of microtubules,[4,5] and the vinca alkaloids (vincristine, vinblastine, and vindesine), which can depolymerize microtubules to form α/β-tubulin heterodimers.[6]

Antimicrotubule agents generally recognize one of three specific major drug binding sites on β-tubulin.[1−3] The taxanes and vinca alkaloids bind specifically to taxane and vinca sites, respectively. The third binding site is a colchicine site, to which colchicine and many other natural products, such as podophyllotoxin,[7,8] combretastatin A-4 (CA-4),[9,10] steganacine,[11] and phenylahistin[12−16] can bind. No anticancer drugs have yet been developed using colchicine site-binding compounds, probably due to the strong in vivo toxicity of these compounds or the difficulties associated with demonstrating superiority over existing drugs that bind to other sites.[17−19]

Colchicine site binders (Figure 1) were recently recognized as vascular disrupting agents (VDAs)[20] that damage tumor-induced immature vascular endothelial cells, resulting in the selective disruption of the angiogenic blood vessels that are formed in and around solid tumor tissues. Clinical trials have been conducted in which these vascular disrupting compounds were tested as anticancer drugs.[21] Because vascular disruption prevents oxygen and nutrition supplies from reaching solid tumor tissues, VDA can induce tumor regression.

In our efforts to develop a potent microtubule depolymerization agent based on an original skeleton that can provide a pharmacological profile distinct from that of the known colchicine site binders, we focused on a natural cyclic dipeptide (diketopiperazine, DKP), phenylahistin,[12−16] which was isolated from *Aspergillus ustus* in 1997. This compound has a

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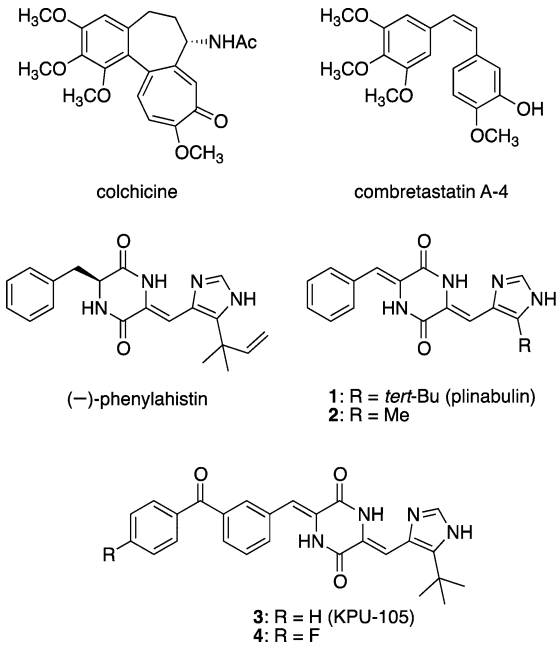


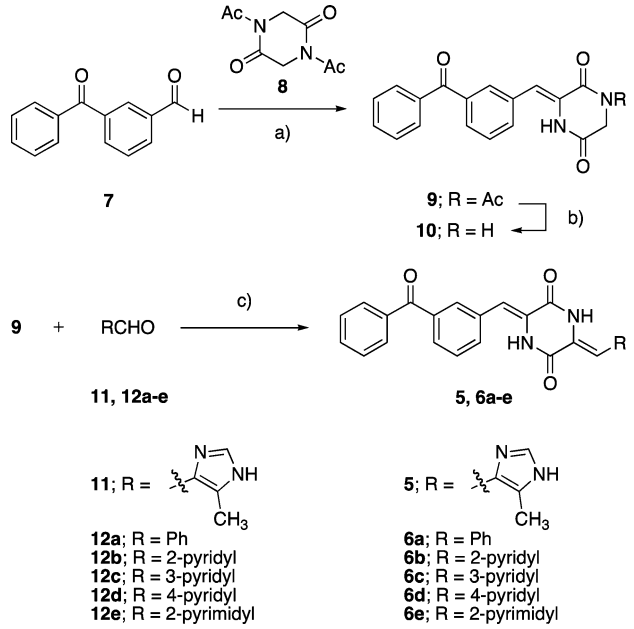
Figure 1. Structures of natural colchicine-site binders, plinabulin, and its representative derivatives.

distinctive hydrophilic chemical structure, displays moderate colchicine-like microtubule depolymerization activity, and has been used to develop a DKP-type antimicrotubule clinical candidate (phase II), plinabulin (**1**, Figure 1).[22] Agent **1** exhibited a potent cytotoxicity, with an IC_{50} value of 10 nM level against a variety of human cancer cell lines in addition to vascular disrupting activity. A further structure−activity relationship (SAR) study of agent **1** was used to identify a more potent derivative **3** (KPU-105) having a benzoyl group at the C-3 position of the phenyl ring in agent **1**.[22] The cytotoxicity against HT-29 (human colon adenocarcinoma) cells and the binding dissociation constant of this benzophenone derivative **3** to porcine tubulin were 1.4 and 65 nM, respectively. Its derivative **4**, bearing a fluorine atom substitution at the 4-position of the additional benzoyl group, showed a more potent cytotoxicity (IC_{50} = 0.5 nM) and a similar binding dissociation constant (K_d = 72 nM).[23]

The potent activities of the derivatives **3** and **4** suggested that the benzophenone moiety (the left-hand unit of the structure) was important for tubulin recognition. Further structural modifications of these derivatives were, therefore, attractive for the development of a potent DKP-derivative composed of a new pharmacophore. In the present study, we focused on the imidazole moiety (right-hand unit) at the opposite site of the benzophenone moiety in the derivative **3** to synthesize a series of new derivatives. The cytotoxicities of the compounds in this series were evaluated to discover a new DKP pharmacophore that displayed good antimicrotubule activity.

As shown in Scheme 1, the new benzophenone−DKP derivatives **5** and **6a**−**6e** were synthesized via a tandem Aldol condensation with two aldehydes onto the DKP ring.[22] First, the benzophenone−DKP intermediate **9** was synthesized by the first Aldol condensation between 3-benzoylbenzaldehyde **7** and *N*,*N*′-diacetylpiperazine-2,5-dione **8** in the presence of *t*-BuOK and *t*-BuOH in DMF under an Ar atmosphere. The intermediate **9** was hydrolyzed in the presence of an excess amount of 28% NH_4OH to afford a deacetylated reference

Scheme 1. Synthesis of Benzophenone−DKP Derivatives[a]



[a]Reagents and conditions: (a) *t*-BuOK, *t*-BuOH, DMF, −15 °C, 2.5 h, 64%; (b) 28% NH_4OH, DMF, rt, 1 h, 53%; (c) Cs_2CO_3, degassed DMF, 110 °C, 2 h, 14−86%.

compound **10** for biological evaluation. Next, the intermediate **9** was used for the second Aldol condensation with a variety of aromatic aldehydes **11** and **12a**−**12e** in the presence of Cs_2CO_3 in degassed DMF under an Ar atmosphere. These aldehydes were purchased commercially, except for **12e**, which was prepared by the reduction of the 2-cyanopyrimidine with DIBAL-H and was used directly without purification. All derivatives used for the biological evaluation were purified using silica-gel column chromatography to obtain with the desired purity (>95%), as characterized by a reversed-phase HPLC analysis monitored at 230 nm. The chemical structures of all derivatives were characterized by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) and IR and NMR spectroscopy (see the Supporting Information).

Cytotoxicities of the synthesized derivatives were evaluated against human HT-29 colorectal cancer cells. In a previous study, we observed that branched alkyl chains, such as the *tert*-butyl group at the 5-position of the imidazole ring, were critical for generating potent activity in agent **1**.[22] By contrast, the methyl derivative **2** (Figure 1), possessing a plinabulin skeleton, showed a dramatically reduced activity (IC_{50} = 339 nM, Table 1).[22] The imidazole moiety bearing an appropriate blanched alkyl group was, therefore, indispensable in the right-hand unit for tubulin recognition by the DKP-type antimicrotubule agents derived from natural DKP, (−)-phenylahistin. The introduction of a methyl group into the imidazole ring of the benzophenone derivative **3** yielded the corresponding derivative **5**, which, however, retained its potent cytotoxicity (IC_{50} = 15 nM). Although this cytotoxicity was a factor of 10.7 less than that of the corresponding benzophenone derivative **3** bearing a *tert*-butyl group, the activity was nearly equal to the value obtained from agent **1**. This result suggested that the quaternary carbon (i.e., *tert*-butyl group) was not essential for the potent activity of the benzophenone−DKP derivatives, probably due to the strong interactions between the tubulin molecules and the benzophenone moiety. Complete removal of the imidazole

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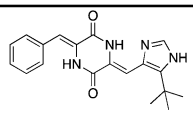
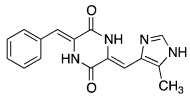
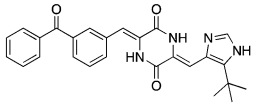
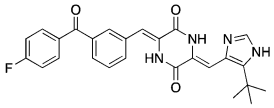
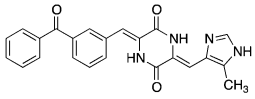
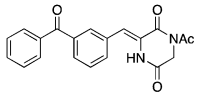
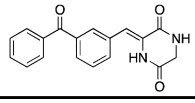
Table 1. Cytotoxicities of the Plinabulin (1) Derivatives against HT-29 Cells

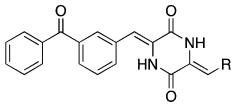
Compd	Structure	IC_{50} (nM)[a]
1 Plinabulin		15 ± 3.8 [b]
2		339 ± 41 [b]
3 KPU-105		1.4 ± 0.4 [c]
4		0.5 ± 0.1 [c]
5		15 ± 3.0
9		8800
10		>20000

[a]The values represent the mean ± SD from at least three independent dose response curves, except for compounds **9** and **10**, the IC_{50} values of which were only measured once because their activities were quite low. [b]The IC_{50} values are reported in ref 22. [c]The IC_{50} values are reported in ref 23.

moiety from the benzophenone derivative **3** yielded the derivative **10**, which showed almost no cytotoxicity and its N-acetylated derivative **9** showed a dramatically reduced activity with an IC_{50} value on the micromolar level. Therefore, some side-chain structure at the right-hand part would be required to exhibit the activity. This knowledge suggests a new route to converting the right-hand imidazole moiety into other units while retaining the potent activity.

Encouraged by these findings, we attempted to modify the critical imidazole moiety of derivative **3** to form other heteroaromatic structures. A pseudoplanar tricyclic structure formed by hydrogen bonding between the DKP-amide NH group and the imidazole nitrogen is thought to be critical for the potent activity;[12−16,22] therefore, the position of the nitrogen atom in the aromatic ring was considered in the substitutions of the right-hand moiety. The introduction of a simple 2-pyridyl ring in place of the alkylated imidazole ring yielded the derivative **6b** (KPU-300), which intriguingly showed a sufficiently high potent cytotoxicity (IC_{50} = 7.0 nM), twice the activity of agent **1** and the benzophenone−methyl derivative **5**. This result successfully identified a new pharmacophore comprising a DKP-type microtubule depolymerization agent. The cytotoxicities of the other pyridine derivatives **6c** and **6d** bearing 3- and 4-pyridyl rings, respectively, were lost, whereas the phenyl and 2-pyrimidyl derivatives **6a** and **6e** showed reduced but significant activities (IC_{50} = 94.3 and 75.3 nM, respectively) (Table 2). These results suggested that the nitrogen atom at the 2-position of the

Table 2. Cytotoxicities of the Benzophenone−DKP Derivatives against HT-29 Cells



Compd	R	IC_{50} (nM)
6a		94.3[a]
6b KPU-300		7.0 ± 1.1[b]
6c		>1000[b]
6d		>1000[b]
6e		75.3 ± 8.1[b]

[a]The values represent the average from two independent dose response curves. [b]The values represent the mean ± SD from at least three independent dose response curves.

pyridine ring in derivative **6b** could form a hydrogen bond to the DKP ring, thereby retaining the pseudoplanar tricyclic structure critical for eliciting potent antimicrotubule activity among the DKP-type compounds. The observed lower activity of the phenyl derivative **6a** was attributed to a lack of planarity due to repulsion between the DKP and phenyl rings. This conformation would have precluded hydrogen bond formation with the DKP ring. However, the introduction of a nitrogen atom at the other sites on the pyridine ring reduced the activity. The complete loss of activity in the 3- and 4-pyridyl derivatives **6c** and **6d** was most likely due to the unfavorable effects of the nitrogen atom, in addition to preventing hydrogen bond formation. The significant activity of the 2-pyrimidyl derivative **6e** (IC_{50} = 75.3 nM) suggested that hydrogen bond formation between the DKP and pyrimidine rings somewhat restored the negative effects of the other unfavorable nitrogen atom on the pyrimidine ring. These results suggested that the hydrogen bond was necessary for maintaining the pseudoplanar tricyclic structure, which was critical to the antimicrotubule activity of the DKP-type compounds.

An X-ray crystal analysis of the potent derivative **6b**, as shown in Figure 2, revealed that the 2-pyridyl and DKP rings retained their planarity, with a dihedral angle of only 0.99°. The DKP−amide NH and 2-pyridyl nitrogen atoms were proximally positioned to permit hydrogen bonding. The formation of hydrogen bonds was also supported by the NMR analysis.[15] A

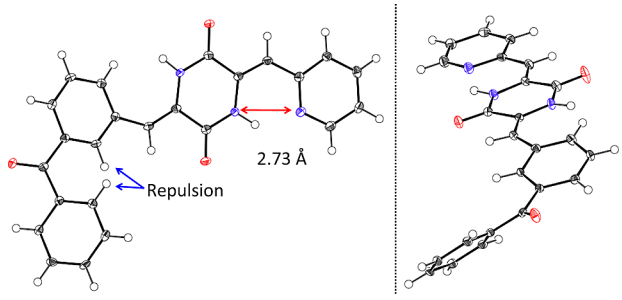


Figure 2. ORTEP drawing of compound **6b**.[24]

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downfield shift in the DKP amide NH peak was observed in compounds **6b** and **6e** (δ 12.60 and 12.02 ppm, respectively) in the presence of the *o*-nitrogen atom, although the downfield shift was not observed in **6a**, **6c**, and **6d** (around δ 10.63 ppm), which did not include the relevant nitrogen atom (see the Supporting Information). Two phenyl rings in the benzophenone moiety, which provided new interaction sites to the tubulin molecule, were positioned in a different plane with a dihedral angle of 48.2° resulting from the repulsive forces between the two hydrogen atoms positioned proximally on their respective rings. The bending geometry was important for the potent activity. We previously reported that a derivative of agent **1** with a planar fluorenone moiety, in which two phenyl rings in the benzophenone were covalently connected, displayed a dramatically reduced cytotoxicity.[23]

The effects of the potent 2-pyridyl derivative **6b** on microtubule function were investigated by evaluating this derivative for both tubulin binding and tubulin depolymerization activities. As shown in Table 3, compound **6b** had a tubulin binding dissociation constant (K_d =1.3 μM) similar to that of agent **1** (1.0 μM). The IC_{50} value of **6b** (1.2 μM) for tubulin polymerization was slightly lower than that of **1** (1.8 μM). These results suggested that the new derivative **6b** interacted with tubulin in a fashion similar to that of other DKP antimicrotubule derivatives. The cytotoxicity of the compounds toward other cancer cell lines was tested using HeLa and A549 cells. The derivative **6b** showed a higher cytotoxicity compared to agent **1** against both cell lines (the cytotoxicities of **6b** were factors of 3.8 and 2.5 greater than the respective values obtained from agent **1**). These results suggested that the cytotoxicity of **6b** arose mainly from the antimicrotubule activity. Meanwhile, the cytotoxic IC_{50} values of derivative **6b** toward primary human cell lines (human primary fibroblasts NHSF46 and human peripheral blood mononuclear cells (HMNC)) were 77 and 481 ± 73 nM, respectively, resulting in being at least 10-fold less potent than that against HT-29 cells. This result suggested that the cancer cells were more sensitive to derivative **6b** than the normal cells.

Table 3. Biological Activities of Plinabulin and 6b

			cytotoxicity IC_{50} (nM)[a]	
compd	tubulin binding K_d (μM)[a]	inhibition of tubulin polymerization IC_{50} (μM)[a]	HeLa	A549
1 Plinabulin	1.0 ± 0.5	1.8 ± 0.3	20.6 ± 3.2	35.7 ± 25.1
6b	1.3 ± 0.3	1.2 ± 0.1	5.4 ± 0.4	14.2 ± 1.2

[a]The values represent the mean ± SD from at least three independent dose response curves.

In general, antimicrotubule agents such as colchicine and CA-4 prevent the mitotic spindle formation. The effects of **6b** on the formation of the mitotic spindles were examined by treating HeLa cells with agent **1** (as a positive control) and derivative **6b** over 6 h in concentrations of 20, 30, and 70 nM (agent **1**) and 7 and 30 nM (derivative **6b**). After treatment, the microtubules and chromosomes were stained with an anti-α-tubulin antibody and 4,6-diamidino-2-phenylindole (DAPI), respectively. As shown in Figure 3, the untreated cells formed dipolar mitotic spindles with condensed chromosomes aligned at the metaphase plate during metaphase. By contrast, cells treated with agent **1** and derivative **6b** could not form a normal mitotic spindle in the presence of any concentration tested. At lower concentrations of the agent **1** (20 and 30 nM) and derivative **6b** (7 nM), multipolar spindle formation was observed, whereas at higher concentrations the cells completely lost the ability to form spindles. Abnormal mitosis is a well-known signature of microtubule-depolymerized cells. The results suggested that derivative **6b** mainly affected the tubulins and inhibited their polymerization in a manner similar to that of agent **1**.

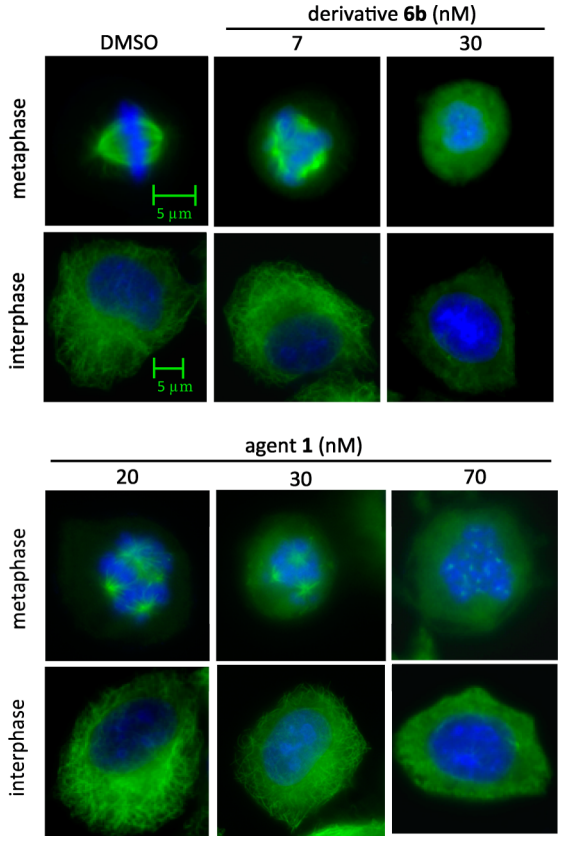

Figure 3. Effect of agent **1** and derivative **6b** on microtubules in HeLa cell. HeLa cells were treated with DMSO, derivative **6b** (7 and 30 nM), and agent **1** (20, 30, and 70 nM). α-Tubulin antibody was used to visualize microtubules, and the DNA was visualized by DAPI staining. Microtubules and DNA are depicted in green and blue, respectively.

In conclusion, we designed and synthesized a series of benzophenone−DKP antimicrotubule derivatives based on the structure of derivative **3** and developed a potent 2-pyridyl derivative **6b**. This derivative, which possessed a pseudotricyclic planar structure, as determined using X-ray crystal analysis, displayed a strong cytotoxicity with IC_{50} values on the nanomolar level against several human cancer cells. The derivative **6b** was found to bind strongly to tubulin and inhibit tubulin polymerization. This function induced abnormal mitotic spindle formation during metaphase in HeLa cells. These results suggested that derivative **6b** affected cancer cells by obstructing the tubulin function. The 2-pyridine and benzophenone moieties of the lead compound **6b** may potentially be modified toward the development of more potent derivatives. Studies along these lines are in progress.

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■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, characterization of new products, X-ray crystal data, biological assay protocols, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

VDA, vascular disrupting agent; DKP, diketopiperazine; SAR, structure−activity relationship; CA-4, combretastatin A4; DAPI, 4,6-diamidino-2-phenylindole

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(24) CCDC 998560 contains the supplementary crystallographic data for this letter. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.