

Synthesis and Structure–Activity Relationship Study of Antimicrotubule Agents Phenylahistin Derivatives with a Didehydropiperazine-2,5-dione Structure

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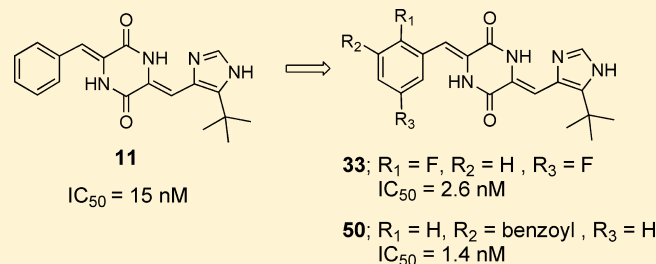
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Supporting Information

ABSTRACT: Plinabulin (**11**, NPI-2358) is a potent microtubule-targeting agent derived from the natural diketopiperazine “phenylahistin” (**1**) with a colchicine-like tubulin depolymerization activity. Compound **11** was recently developed as VDA and is now under phase II clinical trials as an anticancer drug. To develop more potent antimicrotubule and cytotoxic derivatives based on the didehydro-DKP skeleton, we performed further modification on the *tert*-butyl or phenyl groups of **11**, and evaluated their cytotoxic and tubulin-binding activities. In the SAR study, we developed more potent derivatives **33** with 2,5-difluorophenyl and **50** with a benzophenone in place of the phenyl group. The anti-HuVEC activity of **33** and **50** exhibited a lowest effective concentration of 2 and 1 nM for microtubule depolymerization, respectively. The values of **33** and **50** were 5 and 10 times more potent than that of CA-4, respectively. These derivatives could be a valuable second-generation derivative with both vascular disrupting and cytotoxic activities.



INTRODUCTION

Antimicrotubule agents have contributed to effective chemotherapy for a variety of cancers.¹ In particular, the microtubule-stabilizing taxanes² and vinca alkaloids,³ which recognize taxoid and vinca alkaloid sites on tubulin, respectively, are routinely used in the clinic. However, after long-term treatment, tumors typically become resistant to these compounds.⁴ Therefore, there is a significant need for the development of novel antimicrotubule agents for use in clinical oncology. Another chemotherapeutic target site on tubulin is the colchicine binding site, whose ligands generally inhibit tubulin polymerization. Although a number of natural products and their derivatives that recognize the colchicine site such as colchicine,⁵ podophyllotoxin,⁶ combretastatin A-4 (CA-4),⁷ curacin A,⁸ ZD6126,⁹ AVE8062A,¹⁰ ABT-751,¹¹ steganacine,¹² and noco-

dazole¹³ were reported, none are as yet approved except for colchicine as a drug for treating acute gouty arthritis.

Agents acting at the colchicine binding site induce a tumor-selective vascular collapse, resulting in the prevention of blood supply to tumor tissues, which leads to the regression of the tumor.¹⁴ We have been focusing on a low molecular weight natural product, phenylahistin (PLH, **1**, halimide), which has a novel diketopiperazine structure (Figure 1) and acts at the colchicine binding site on tubulin.¹⁵ This compound inhibits tumor cell growth in vitro with a submicromolar cytotoxic IC_{50} value against a variety of tumor cell lines and shows mild antitumor activity in the P388 leukemia and Lewis lung carcinoma models in mice.^{15c}

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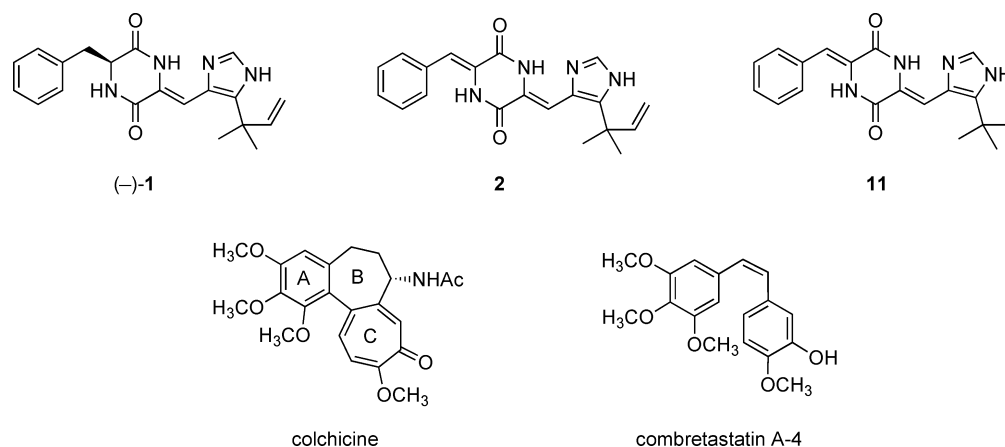


Figure 1. Structures of (–)-1, 2, 11, colchicine, and combretastatin A-4.

Compound **1** was discovered as a mixture of enantiomers from the agar-cultured medium of *Aspergillus ustus* NSC-F038 by Kanoh et al.^{15a} by the screening of new cell cycle inhibitors and independently as halimide from a marine microorganism by Fenical et al.¹⁶ The more potent enantiomer (–)-1 specifically inhibited the cell cycle at the G₂/M phase through the inhibition of tubulin polymerization, which is important for spindle formation in the mitotic process. In competitive binding assays using radiolabeled ligands, (–)-1 interacted at or near the colchicine binding site on tubulin, but not with the vinca alkaloid recognition site.^{15b}

The chemical structure of (–)-1 is that of a 2,5-diketopiperazine (DKP) derivative, consisting of L-phenylalanine and a unique (*Z*)-isoprenylated dehydrohistidine residue, as shown in Figure 1. This DKP provided a novel heterocyclic template for inducing antimicrotubule activity and a relatively hydrophilic template in comparison to that of the other naturally occurring antimicrotubule agents mentioned above. Therefore, the design and synthesis of highly potent compound **1** derivatives were thought to be a valuable strategy to develop a new anticancer drug with pharmacological, toxicological, and resistance profiles distinct from other antimicrotubule agents. Moreover, the simple chemical structure of compound **1** derivatives would be manufactured at a low price, and this would contribute to the medical economy.

In our previous studies of chemically modified or totally synthesized compound **1** derivatives,¹⁷ it was understood that important structural requirements that elicit potent cytotoxic activity were (1) the L-form of the Phe residue, (2) a rigid and planar pseudotricyclic structure formed by hydrogen bonding between DKP and imidazole rings, and (3) the *gem*-dimethyl structure at the 5-position of the imidazole ring (Figure 2). Kanzaki et al. reported that a biotransformed derivative of compound **1** with an additional α,β -unsaturation joining the DKP core with the benzyl substituent (dehydrophenylalhistin, **2**, Figure 1) showed a highly increased antiproliferative activity in sea urchin eggs.¹⁸ On the basis of this information, we focused on compound **2** to develop highly potent cytotoxic derivatives of compound **1**. Namely, modifications at the phenyl and dimethylallyl moieties were carried out to understand the precise structure–activity relationship (SAR) of the derivatives.

In this SAR study, we developed its *tert*-butyl derivative (**11**, KPU-2/NPI-2358, Figure 1),¹⁹ and its antimicrotubule and

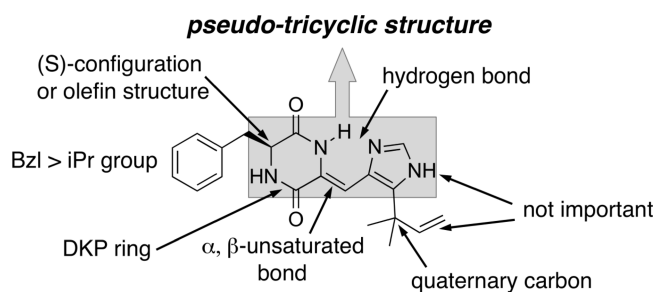
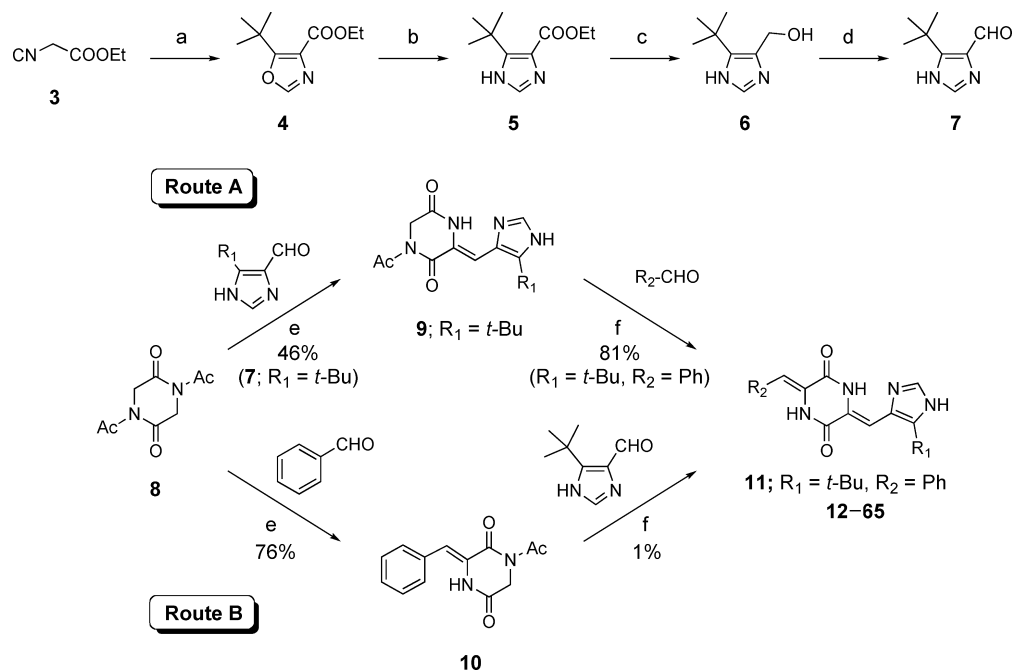


Figure 2. Structural entities required for potent biological activity of compound **1**.

cytotoxic activities were evaluated. Compound **11** showed a further 2.8-fold increase in cytotoxic potency against human colon adenocarcinoma cell lines (HT-29), as compared to compound **2** (Table S1 in the Supporting Information). The cytotoxic activity of **11** was similar to that of colchicine. Similar results were observed in the microtubule polymerization assay and for cytotoxic activity against HeLa cells, suggesting that the *tert*-butyl group at the 5-position of the imidazole ring is preferable for potency. In addition, compound **11** exhibited a similar biological effect to the other antimicrotubule agents (e.g., paclitaxel and nocodazole), as cell cycle progression of HeLa cells was inhibited at the G₂/M phase (Figure S1 in the Supporting Information). This result suggests that the major cytotoxic function of compound **11** is derived from the inhibition of microtubule polymerization. The potency of compound **11** was maintained in multidrug resistant (MDR) tumor cell lines and was able to rapidly induce tubulin depolymerization and monolayer permeability in normal human umbilical vein endothelial cells (HuVECs).^{19a} Furthermore, the activity of compound **11** against human prostate carcinoma cell line (DU 145) was further investigated in an *in vivo* tumor model. A dose-dependent reduction in tumor growth rate and tumor size was observed, with animals treated with 3.75 or 7.5 mg/kg compound **11** having significantly smaller tumors relative to vehicle control treated mice (Figure S2A,B in the Supporting Information). Compound **11** was well-tolerated, with all the mice gaining weight over the duration of the experiment (Figure S2C in the Supporting Information).

On the basis of these and other preclinical tumor models, tumor blood flow measurements, pharmacokinetics, distribution, metabolism, excretion, and safety testing (data not shown), compound **11** was selected as a clinical candidate

Scheme 1. Synthesis of Compounds 11–65^a

^aReagents and conditions: (a) pivalic anhydride, DBU, THF, rt, 16 h, 99%; (b) formamide, reflux (165 °C), 14 h, 59%; (c) LiAlH₄, THF, 0 °C to rt, 4 h, 69%; (d) MnO₂, acetone, rt, 79%; (e) Cs₂CO₃, DMF, rt, 12 h; (f) Cs₂CO₃, DMF, 80 °C, 4 h.

Table 1. Cytotoxic Activity of Compound 11 Derivatives with Substitutions of 5-Position at the Imidazole

compd	R ₁	K _d (μM)	IC ₅₀ (nM)/HT-29 ^a
11	<i>tert</i> -butyl	1.0 ± 0.5	14.9 ± 3.8
12	methyl	7.4 ± 2.3	339 ± 41.0
13	<i>n</i> -propyl	4.9 ± 1.4	153 ± 18.0
14	<i>n</i> -butyl	4.6 ± 1.1	112 ± 16.0
15	isopropyl	1.1 ± 0.3	15.5 ± 1.9
16	<i>sec</i> -butyl	2.0 ± 0.7	30.5 ± 4.5
colchicine		3.3 ± 0.3	16 ± 3.0

^aValues are the mean or mean ± SEM (for only potent compounds) from at least three independent dose–response curves.

with the USAN “plinabulin” and is currently in phase II clinical trials. In this report, we described the synthetic route of **11**, which was not disclosed, and report further derivatization from **11** to develop second-generation compounds. Then, the dissociation constant of binding (K_d) to tubulin and the effect on HuVECs were assessed in order to understand the vascular disrupting ability of the derivatives.

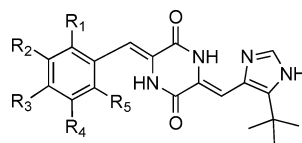
CHEMISTRY

To synthesize various derivatives of compound **2**, we examined the effective synthetic route of **11**. As shown in Scheme 1, the synthesis of the *tert*-butylimidazole aldehyde **7** was commenced with condensation of ethyl isocyanoacetate **3** with pivalic anhydride in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), following the report by Suzuki et al.,²⁰ to give oxazole ester **4**. After purification using silica gel column chromatography, oxazole ester **4** was converted to imidazole ester **5** by heating in formamide,²¹ which was reduced with LiAlH₄ in THF to give alcohol **6**. In the conversion of alcohol

to aldehyde, MnO₂ was employed for the oxidation to prepare aldehyde **7**. The total yield of imidazolealdehyde **7** was 32% from **3** in four steps.

In the construction of the bisdehydro-DKP template, we modified the reported sequential two-step condensation reaction between diacetyl-2,5-piperazinedione **8** and two different kinds of aldehydes in the presence of Cs₂CO₃ as a base in DMF.²² Namely, compound **8** was reacted with the first aldehyde at room temperature for 12 h, followed by the reaction with the second aldehyde at 80 °C for 4 h in degassed DMF under an Ar atmosphere. The degassed conditions were employed to avoid probable oxidation of activated α -carbon atoms at the DKP ring in the presence of Cs₂CO₃.²³ Comparison of the two synthetic routes differing in the order of condensation of the two aldehydes indicated that aldehyde **7** should be condensed first (Scheme 1, route A) to increase the yield of **11**. The low reactivity of aldehyde **7** is attributed to the high steric hindrance imposed by the bulky *tert*-butyl group at the 5-position of the imidazole ring. After reverse-phase

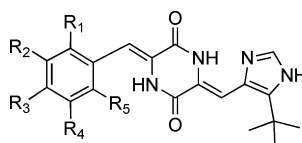
Table 2. Effect of the Methoxy Group Substitution on Cytotoxic Activity



compd	R ₁	R ₂	R ₃	R ₄	R ₅	IC ₅₀ (nM)/HT-29 ^a
11	H	H	H	H	H	14.9 ± 3.8
17	OMe	H	H	H	H	75.5 ± 25
18	H	OMe	H	H	H	26.1 ± 8.5
19	H	H	OMe	H	H	6570
20	OMe	OMe	H	H	H	868
21	OMe	H	OMe	H	H	5080
22	OMe	H	H	OMe	H	970
23	OMe	H	H	H	OMe	5170
24	H	OMe	OMe	H	H	2020
25	H	OMe	H	OMe	H	45.4 ± 12

^aValues are the mean or mean ± SEM (for only potent compounds) from at least three independent dose–response curves.

Table 3. Cytotoxic Activity of Compound 11 Derivatives with Substitutions on the Phenyl Ring



compd	R ₁	R ₂	R ₃	R ₄	R ₅	K _d (μM)	IC ₅₀ (nM)/HT-29 ^a
26	Me	H	H	H	H	2.6 ± 0.4	45.8 ± 11
27	H	Me	H	H	H	2.9 ± 0.1	46.7 ± 10
28	H	H	Me	H	H	8.9 ± 2.2	483
29	Me	H	H	H	Me	8.0 ± 0.7	430
30	F	H	H	H	H	nt ^b	30.0 ± 17
31	H	F	H	H	H	0.7 ± 0.1	13.1 ± 5.3
32	H	H	F	H	H	11.8 ± 2.4	501
33	F	H	H	F	H	0.20 ± 0.11 ^c	2.6 ± 0.5
34	Cl	H	H	H	H	2.5 ± 1.4	46.3 ± 13.5
35	H	Cl	H	H	H	1.6 ± 0.3	24.5 ± 8.7
36	H	H	Cl	H	H	nt ^b	709
37	Cl	Cl	H	H	H	nt ^b	29.9 ± 4.5
38	Br	H	H	H	H	nt ^b	40.5 ± 11
39	H	Br	H	H	H	1.9 ± 0.7	31.1 ± 7.3
40	H	H	Br	H	H	nt ^b	714
41	NO ₂	H	H	H	H	nt ^b	50.3 ± 12.4
42	H	NO ₂	H	H	H	nt ^b	44.7 ± 11.9
43	H	H	NO ₂	H	H	nt ^b	>20000
44	OH	H	H	H	H	nt ^b	6100
45	H	OH	H	H	H	nt ^b	364
46	H	OEt	H	H	H	nt ^b	45.9 ± 10.2
47	H	CN	H	H	H	nt ^b	35.6 ± 14
48	H	vinyl	H	H	H	nt ^b	17.0 ± 1.52
49	H	CF ₃	H	H	H	nt ^b	44.0 ± 7.49
50	H	benzoyl	H	H	H	0.62 ± 0.07 ^c	1.4 ± 0.4
51	F	H	H	H	Cl	nt ^b	24.9 ± 1.31

^aValues are the mean or mean ± SEM (for potent compounds) from at least three independent dose–response curves. ^bnt = not tested. ^cThe activity against porcine tubulin. The K_d value of compound 11 against porcine tubulin was similar (1.06 μM) to that against bovine tubulin (1.0 μM).

preparative high-performance liquid chromatography (HPLC) purification using an octadecylsilyl (ODS) column in a dark place, a yellow solid of **11** with >98% purity was obtained as a trifluoroacetic acid (TFA) salt. The total yield of compound **11** via route A was 37% in two steps while the yield via route B was only about 0.4%. Therefore, a variety of derivatives **12–65** for

the SAR study were synthesized according to route A. In the synthesis of compounds **12–17**, imidazolealdehydes with different alkyl chains at the 5-position were also prepared with corresponding acylanhydrides according to the same synthetic scheme starting from isonitrile **3** (Schemes S1 and S2 in the Supporting Information). The final HPLC purification of

every derivative removed the undesired *E*-forms (about 10%) at the benzylidene, which were generated as byproducts of the condensation reaction or upon exposure to ambient light during the workup process. The purity of each synthesized derivative prior to biological evaluation was over 95%. Derivatives with the *E*-configuration at the imidazolemethylene moiety were not observed, indicating that the reaction of **8** with aldehyde **7** was *Z*-form selective due to the hydrogen bond formation between imidazole and DKP nitrogen atoms.

RESULTS AND DISCUSSION

SAR Study from Compound 11. *1. Modification of the Alkyl Group on the Imidazole Ring.* To perform the optimization on the 5-position of the imidazole ring, the *tert*-butyl group of compound **11** was replaced to other alkyl groups and the synthesized derivatives were evaluated in both the cytotoxic and tubulin-binding assays (Table 1). The derivatives with normal alkyl groups, i.e., methyl (compound **12**), *n*-propyl (compound **13**), and *n*-butyl (compound **14**) groups, showed significantly lower potency than compound **11**. However, the derivatives with branched alkyl groups, i.e., isopropyl (compound **15**) and *sec*-butyl (compound **16**) groups maintained potency, particularly isopropyl derivative, which exhibited the same potency as compound **11**. This result suggests that the branched chain at 5-position of the imidazole ring is critical to form a tight binding with tubulin and to exhibit a potent cytotoxic activity and is consistent with earlier findings on (–)-**1**.^{17a}

2. Modification of the Phenyl Ring. To develop more potent antimicrotubule and cytotoxic derivatives based on compound **11**, a precise SAR study focused on its phenyl ring was performed. It is known that natural antimicrotubule agents such as colchicine, combretastatin, and derivatives, which recognize the same colchicine binding site on β -tubulin as compound **1**, are structurally characterized by multiple methoxy groups, which make marked contributions to their potent antimicrotubule activity.²⁴ Therefore, we evaluated analogs of compound **11** bearing methoxy substituents to the phenyl ring for cytotoxicity to HT-29 cells. As shown in Table 2, in the case of a single substitution, the ortho-substitution (compound **17**) resulted in a 5-fold decrease in cytotoxic potency, while meta-substitution (compound **18**) maintained potency similar to that of compound **11**. However, the para-substitution (compound **19**) resulted in a marked decrease in potency (>400-fold). Double substitution on the phenyl ring resulted in a marked decrease in potency for most derivatives, except for the 3,5-substitution (compound **25**), which showed a slightly weaker potency than compound **11**. These results indicate that both meta-positions have a potential for the modification of potency. Methoxy derivatives with triple substitutions similar to those occurring in colchicine demonstrated low potency (data not shown). These results suggest that tubulin recognizes the phenyl ring of compound **11** rigorously and the space for accepting the para-substituent is highly limited. In addition, in comparison to the relatively high potency of the single ortho-substitution (compound **17**), the observed reduction in potency of the double ortho-substitution (compound **23**) suggests that proper spatial orientation of the phenyl ring is critical for the activity. Specifically, rotation of the ring induced by steric hindrance may preclude the derivatives from adopting the most active conformation. Moreover, the collective SAR data for this series suggest that the recognition of the methoxy groups by the colchicine binding site on tubulin

is probably different from that of colchicine, suggesting that compound **11** recognizes tubulin in a different manner than colchicine and its biological homologues.

An expanded SAR study on the phenyl ring was performed by means of the introduction of a variety of substituents. As shown in Table 3, in the case of single substitutions with the methyl group, halogen atoms or a nitro group, ortho- or meta-substituted derivatives exhibited relatively high potency in both tubulin binding and cytotoxic assays, while all efforts at the para-position completely failed to exhibit even moderate potency. Among the ortho- or meta-substituted derivatives, compound **31** with the *m*-fluoro group showed potent cytotoxic activity ($IC_{50} = 13.1$ nM), which was similar to that of compound **11**. Introduction of a hydroxyl group at the ortho- or meta-position resulted in significant reduction of potency. The *m*-hydroxyl derivative **45** exhibited a 16-fold higher potency for cytotoxic activity than the *o*-hydroxyl derivative **44**, but was 24-fold less potent than compound **11**. The attenuated activity of the *m*-hydroxyl derivative suggests that a hydrogen bond donor at this position is undesirable; however, a hydrogen bond acceptor at this position may be tolerated, since the *m*-fluoro analog maintained good potency. The comparable activity of the *m*-fluoro analog with **11** may simply reflect the isosteric nature of the fluoro group. Overall, while single substitutions of the phenyl ring did not improve potency relative to **11**, substitutions at the meta-position were generally well-tolerated, suggesting an opportunity for further exploration.

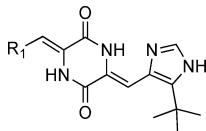
For the double modifications at the phenyl ring, 2,5-difluoro substitution (compound **33**) showed an increase in potency of 5.7-fold ($IC_{50} = 2.6$ nM) compared to compound **11**. This may reflect the unique properties of fluorinated organic compounds, including improved entropies of ligand binding associated with these agents.²⁵ On the other hand, 2,3-dichloro (compound **37**) and 2-fluoro-6-chloro (compound **51**) substitutions resulted in only slight decreases in potency, while the 2,6-dimethyl substitution (compound **29**) resulted in low potency. These results supported further modifications to the meta-position and indicated that the phenyl ring is specifically recognized by tubulin in a highly restricted manner.















Since our examination indicated that modification at the meta-position generally resulted in a greater potency than that of the ortho-position, further modifications to the meta-position were undertaken to increase potency. A vinyl group substitution at the meta-position (compound **48**) showed potent activity ($IC_{50} = 17$ nM), equivalent to that of compound **11**, while substitution with the *m*-ethoxy (compound **46**), *m*-cyano (compound **47**), or *m*-trifluoromethyl (compound **49**) group slightly decreased the potency; of note, the latter is both isosteric and comparable in activity to the *m*-methyl analog **27**. With the introduction of the carbonyl group, compound **50** with a *m*-benzoyl group provided the most potent activity in the present study, with 11-fold more potent cytotoxicity ($IC_{50} = 1.4$ nM) and 1.6-fold higher tubulin binding constant ($K_d = 0.6$ μ M) than compound **11**, although the derivative with a hydrophilic *m*-carbamoyl group showed very weak potency (data not shown). The enhanced potency of benzophenone analog **50** may reflect, in part, an entropic effect, with improved binding energetics of this more rigid ligand for its target. Spatially, these findings suggest the existence of a narrow, but deep, crevice in the binding site on tubulin that accepts the additional planar structure at the meta-position. Ideally, this crevice could effectively function as an adaptive space for

increasing the activity of compound **50**. Finally, the hydrophobic property of the benzophenone moiety of compound **50** might increase cell penetration, leading to highly potent cytotoxic activity in comparison to tubulin binding potency.

Encouraged by the results of replacing the phenyl ring with benzophenone, we expanded the SAR study to explore replacement with other ring systems; the results on tubulin binding and cytotoxic assays are shown in Table 4.

Table 4. Cytotoxic Activity of Compound 11 Derivatives with Other Ring Systems



compd	R ₁	K _d (μM)	IC ₅₀ (nM) / HT-29 ^a
52		nt ^b	14.9 ± 2.1
53		nt ^b	1730
54		4.5 ± 0.6	140 ± 31
55		1.2 ± 0.3	21 ± 2.1
56		nt ^b	>20000
57		nt ^b	103 ± 11.6
58		nt ^b	548
59		nt ^b	712
60		nt ^b	57.2 ± 20.0
61		nt ^b	38.2 ± 8.9
62		nt ^b	35.9 ± 10.3
63		nt ^b	27.4 ± 6.29
64		nt ^b	55.8 ± 6.2
65		nt ^b	78.9 ± 14.1

^aValues are the mean or mean ± SEM (for potent compounds) from at least three independent dose–response curves. ^bnt = not tested.

Replacement with the naphthalene ring, 1-naphthyl derivative **52**, showed almost the same potency as compound **11**, while the 2-naphthyl derivative **53** exhibited very weak potency. This observation was consistent with the result obtained with the benzophenone derivative **50**. An additional planar ring system connected at ortho- and meta-positions of the phenyl ring was well-tolerated for tubulin binding. The 5-isoquinoline derivative **55** showed similar potency to compound **11**, although the 8-quinoline derivative **54** was 7-fold less potent than compound **55**. An appropriate alignment of the electron-donating aromatic nitrogen is apparently important for maintaining activity. Importantly, this basic nitrogen atom may increase the water solubility of the derivatives as an acid salt; although the series of compound **1** may appear to be equipped with inherent ionization potential via the imidazole ring, conjugation of the imidazole with the DKP core renders this imidazole an extremely weak base (data not shown); thus, the incorporation

of a basic nitrogen into compound **1** derivatives provides added value.

The pyridine derivatives **56–58** showed decreased potency, although each position of the nitrogen atom on the pyridine ring afforded a different influence. As described above, the meta-position was relatively well tolerated. However, the 2-pyridyl derivative **56** was completely inactive. Since the 2-pyridyl nitrogen atom could form a hydrogen bond with the DKP amide NH via a pseudo-six-membered-ring formation, the conformation of the pyridine ring would be able to be fixed on the same plane as the DKP ring, resulting in a planar pseudopentacyclic conformation. This suggested that a certain degree of spatially distorted positioning between both rings was critical for the activity (Figure 4).

We also replaced the phenyl ring with smaller ring systems such as furan, thiophene, and pyrrole. Compound **59** with a 2-furyl group had a very weak potency; however, the introduction of a methyl group (compound **60**) or chlorine atom (compound **61**) at the 5-position of the furan ring restored the potency. The derivatives with 3-furyl (compound **62**), 2-thienyl (compound **63**), and 3-thienyl (compound **64**) groups also showed a slightly lower potency than compound **11**. The replacement of the phenyl ring by the cyclohexyl ring (compound **65**) resulted in a lower potency, suggesting that the more rigid, planar aromatic ring system was favorable to elicit the high potency in the cytotoxicity assay.

Inhibition of Microtubule Polymerization with the Potent Derivatives. Since 2,5-difluoro derivative **33** and benzophenone derivative **50** showed a higher potency in cytotoxic activity against HT-29 cell lines than compound **11**, we performed in vitro microtubule depolymerization assay using purified porcine tubulin to investigate whether these derivatives exhibit antimicrotubule activity (Table 5). The

Table 5. Inhibition of in Vitro Tubulin Polymerization and Tubulin Binding Activity

compd	inhibition of tubulin polymerization IC ₅₀ (μM) ^a	tubulin binding K _d (μM)
11	1.31 ± 0.10	1.0 ± 0.5
33	0.51 ± 0.02	0.20 ± 0.11
50	0.76 ± 0.14	0.62 ± 0.07
55	0.55 ± 0.04	1.2 ± 0.3
combretastatin A-4	0.55 ± 0.01	nt ^b
colchicine	4.12 ± 0.63	3.3 ± 0.3

^aValues are the mean or mean ± SEM (for potent compounds) from at least three independent dose–response curves. ^bnt = not tested.

activity of the 5-isoquinoline derivative **55** was also evaluated because it possessed favorable solubility in water (data not shown) as well as exhibiting a similar cytotoxic activity to compound **11**. In this assay, compounds **33**, **50**, and **55** exhibited more potent microtubule depolymerization activity than compound **11** and similar activity to combretastatin A-4.

Cell Cycle Progression Analysis. The effects of compounds **11**, **33**, **50**, and **55** on cell cycle progression were investigated using flow cytometry. After treatment with different concentrations of compound for 18 h, HeLa cells were fixed, stained with propidium iodide, and counted (Table 6 and Figures S3–S8 in the Supporting Information). All of these derivatives caused significant arrest of the cells at the G₂/M phase, compared to the untreated control. The ability of

Table 6. Effect of 11, 33, 50, and 55 on Cell Cycle Progression

compd	conc (nM)	cell population (%)		
		G ₀ /G ₁	S	G ₂ /M
control	0	43.9	39.9	16.2
11	10	44.6	37.5	17.9
	30	4.2	40.4	55.3
	100	0.0	2.4	97.6
33	3	41.5	42.1	16.4
	10	1.2	6.7	92.3
50	3	24.7	29.6	45.7
	10	0.5	6.5	93.0
55	30	48.2	37.6	14.2
	100	9.8	4.8	85.5
combretastatin A-4	3	34.6	28.1	37.4
	10	0.4	8.8	90.7
colchicine	10	47.5	31.5	21.0
	30	1.3	8.9	89.9

compound **33** to induce arrest of the cell cycle was more potent than that of **11**, although compound **55** showed less potency than **11**. Compound **50** caused maximum arrest of the cell at the G₂/M phase at the concentration of 3 nM, and its potency was similar to that of combretastatin A-4. The order of cell arrest ability at the G₂/M phase was **50** > **33** > **11** > **55**, consistent with their cytotoxic activities against HT-29 cells.

In Vitro Vascular Disrupting Activity. Compound **11** significantly increased endothelial cell permeability, and this activity was more potent than that of either colchicine or vincristine, while the profile of combretastatin A-4 was similar to that of compound **11**.^{19a} In addition, compound **11** rapidly induced morphological changes such as membrane blebbing and the rounding up of cells in HuVECs, which is probably a downstream effect of **11**-mediated microtubule disruption. To understand the vascular disrupting function of the present plinabulin derivatives, cytotoxic and antimicrotubule activities of compounds **33**, **50**, and **55** against HuVECs were examined. As shown in Table 7, all of the compound **2** derivatives

Table 7. Antimicrotubule and Cytotoxic Activities of Compound 11 Derivatives against HuVECs

compd	IC ₅₀ (nM) /HuVECs ^a	30 min HuVECs microtubule depolymerization	
		ED ₁₀₀ (nM) ^b	doses tested (nM)
11	11 ± 0.6	3	3, 5, 10
33	1.5 ± 0.3	2	1, 2, 5, 50
50	0.5 ± 0.1	1	0.1, 1, 5, 10
55	13 ± 4	20	10, 20, 50
combretastatin A-4	2.4 ± 0.8	10	2, 10, 20, 50

^aValues are the mean or mean ± SEM (for potent compounds) from at least three independent dose–response curves. ^bLowest concentration tested where complete depolymerization was observed.

demonstrated slightly greater potency against HuVECs than HT-29 cells. Particularly, compound **50** showed 22-times greater potency than compound **11**, with an IC₅₀ value of 0.5 nM, which was about 5-times more potent than combretastatin A-4. Moreover, the lowest effective dose of compound **50** for antimicrotubule activities was 1 nM, which was 3 and 10 times more potent than compound **11** (3 nM) and combretastatin A-

4 (10 nM), respectively. The results indicated that compound **50** was the most potent VDA in the present study.

Correlation between K_d and IC₅₀ of the Derivatives.

To understand whether the cytotoxic effect of the present DKP derivatives is dependent on their antimicrotubule activity, we examined the relationship between the K_d (1/K_d) in the tubulin binding assay and IC₅₀ values in the HT-29 assay based on the results from 19 derivatives for which both tubulin binding and cytotoxicity assays were performed.²⁶ As shown in Figure 3, the

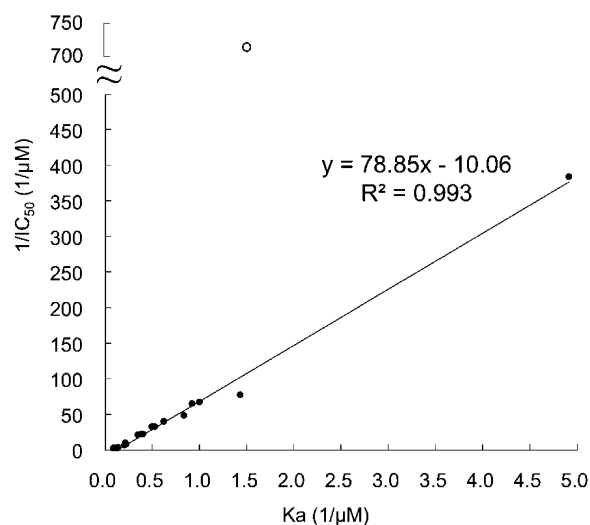


Figure 3. Correlation of the binding activity to purified porcine tubulin (K_d) vs cytotoxic activity (IC_{50}) among 19 compounds: (filled circle) compounds **11–16**, **26–29**, **31–35**, **39**, **54**, and **55**; (open circle) compound **50**.

double reciprocal plots of each value from 18 compounds, except for compound **50**, showed a high positive correlation ($R^2 = 0.993$), suggesting that derivatives of **11** have a high correlation between tubulin binding and cytotoxic potency. Therefore, it is revealed that the cytotoxic activity of the DKP derivatives is largely attributed to their antimicrotubule action. On the other hand, compound **50** with the benzophenone structure appeared to fall outside of this correlation, with high cytotoxic potency in comparison to its tubulin binding. Considering the result of tubulin depolymerization assay and cell cycle analysis, it is suggested that the benzophenone derivative **50** might have another mode of action, such as a higher cell penetration property, in addition to the tubulin depolymerization in order to induce the potent cytotoxicity.

Active Conformation of the Derivatives. Conformational analysis of the compound **1** derivatives may provide insights into the active conformation that supports ligand binding to tubulin. Clearly, the conformation of compound **1** derivatives is tightly restricted by the pseudotricyclic structure formed by the hydrogen bonding between the imidazole and DKP rings, as supported by the crystal structure of **11**. However, conformational analysis of the rotatable phenyl ring has not been previously explored. Thus, the X-ray crystal structures of potent compound **11** and inactive compound **56**, with the *o*-pyridyl group, were analyzed. As shown in Figure 4B, inactive compound **56** formed an additional hydrogen bond between the *o*-pyridine nitrogen atom and the NH group of the DKP ring, which was apparent in the ¹H NMR spectra, resulting in the planar structure. This result indicated that the uniplanar structure with a pseudotricyclic ring represented a

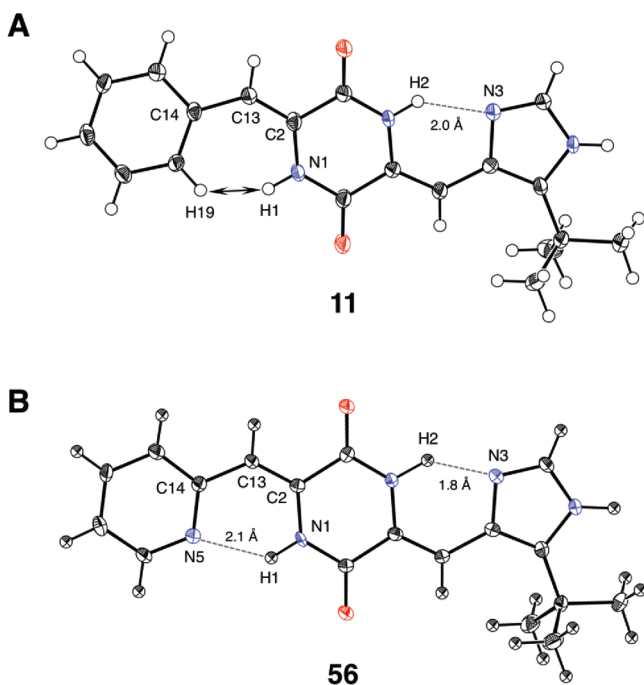


Figure 4. ORTEP drawing of compounds **11** and **56**. The arrow indicates the steric repulsion between H1 and H19.

biologically unfavorable conformation. On the other hand, the active compound **11** exhibited a twisted conformation between the phenyl and DKP rings induced by steric repulsion (Figure 4A (arrow)). The dihedral angle was about 30° . These findings suggest that the active conformation required a certain extent of dihedral angle around the C13–C14 bond. This is supported by evaluation of other analogs in the series. For example, the low potency of 2-furyl derivative **59** may be explained by the hydrogen bond formation between the oxygen atom on the 2-furyl ring and the NH group of the DKP ring, as observed in the *o*-pyridine derivative **56**. In addition, introduction of bulky substituents at the adjacent 5-position of the 2-furyl ring (compound **60** and **61**) might prevent this hydrogen bonding by steric hindrance, resulting in an increase in cytotoxic potency. The potent activity observed in the 2-thienyl derivative **63** might be due to the presence of the sulfur atom, which will not serve as a hydrogen bond acceptor for the DKP NH. It is known that the active conformation of colchicines requires the defined dihedral angles between A and C rings,²⁷ which is preserved among other colchicine site binding agents.²⁸ However, this conformational property appears not to apply to our derivatives, because the superimposition study between colchicine and compound **11** failed due to their low structural similarity, suggesting a different binding mode of our derivatives at or near to the colchicine site on tubulin.²⁹

Furthermore, 3D-QSAR was carried out using comparative molecular field analysis (CoMFA)³⁰ to create the pharmacophore model at the phenyl moiety in **11** derivatives, since the **11**-binding site on tubulin has not precisely been determined (Figure S9 in the Supporting Information). In this analysis, the meta-position of the phenyl ring was indicated as a favorable area, while the para-position was indicated as an unfavorable area for introducing the substituting group. These results are also consistent with the findings of the SAR study, especially concerning derivatives **31**, **33**, **35**, and **39**.

CONCLUSION

A series of highly potent and low molecular weight (<450) antimicrotubule agents with a DKP core structure was designed and synthesized on the basis of the modification of compound **11** derived from compound **1**. SAR studies were performed on the basis of substituents of both the benzyl and imidazole moieties, which are protruding from the biologically important pseudotricyclic structure of compound **1**. Several synthesized compounds inhibited tumor cell growth in vitro with nanomolar IC_{50} values against HT-29 human carcinoma cells and exhibited potent tubulin binding with a K_d value similar to or lower than that of colchicine. In addition, compounds **33** and **50**, which were more potent compounds than compound **11**, also exhibited highly potent antimicrotubule and cytotoxic activities against HuVECs, a model cell system for evaluating vascular disrupting activity in vitro. As second-generation compounds of **11**, potent compounds **33** and **50** could contribute toward tumor vascular disrupting therapy both as single agent and in combination with other chemotherapeutic agents or radiation for the treatment of solid tumors and toward gaining a better understanding for the development of new antimicrotubule agents for use in clinical oncology.

EXPERIMENTAL SECTION

General Methods. 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 used without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70–230 mesh). TLC was performed using Merck silica gel 60F₂₅₄ precoated plates. Melting points were measured on a Yanagimoto micromelting 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: linear gradient of CH₃CN in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, detected at UV 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 μ Bondasphere C18, 5 μ m, 100 Å) with a binary solvent system: linear gradient of CH₃CN in 0.1% aqueous TFA or methanol in water at a flow rate of 5–12 mL/min, detected at UV 230 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ¹H and ¹³C NMR spectra were obtained on JEOL 300 MHz spectrometer, a Varian Mercury 300 spectrometer (300 MHz), Bruker DPX-400 spectrometer (400 MHz) or a Bruker AV600 spectrometer (600 MHz) with TMS as an internal standard. IR spectra were recorded on JASCO FT/IR 4100 spectrometer. High-resolution mass spectra (ESI or EI) were recorded on a micramass Q-ToF Ultima API or a JEOL JMS-GCmate BU-20 spectrometer. X-ray crystallographic analysis was carried out on a Mac Science DIP-2020. The elemental analyses were performed on a PerkinElmer 2400 Series II analyzer (N241-0610) or an Elementar Vario EL elemental analyzer. The purity of all tested compounds was measured by a reverse-phase HPLC with the following conditions, and the purity of all compounds were >95%.

HPLC Conditions. Method 1: solvent A, 0.1% TFA–water; solvent B, 100% acetonitrile; flow rate of 0.9 mL/min, from 0% B to 100% B in 40 min. Method 2: solvent A, 0.1% TFA–water; solvent B, 100% acetonitrile; flow rate of 0.9 mL/min, from 0% B to 100% B in 35 min. Method 3: solvent A, 0.1% TFA–water; solvent B, 100% acetonitrile; flow rate of 0.9 mL/min, from 10% B to 50% B in 40 min.

Ethyl 5-(*tert*-butyl)oxazole-4-carboxylate (4**) [CAS No. 714273–89–9].** According to our previous report,²⁹ to a solution of ethyl isocyanacetate **3** (25 g, 221 mmol) in THF (200 mL) were added DBU (34.3 mL, 243 mmol) and pivalic anhydride (49.3 mL, 243 mmol), and the mixture was stirred overnight at room temperature. After the solvent was removed by evaporation in vacuo, the residue action was extracted with AcOEt (200 mL); washed with 10% Na₂CO₃, 10% citric acid, and saturated NaCl three

times; and dried over anhydrous Na_2SO_4 , and the solvent was concentrated in vacuo. The residual oil was purified by silica gel column chromatography using hexane–AcOEt (20:1 to 4:1) to give an oil of **4** (66.4 g, 99%): ^1H NMR (300 MHz, CDCl_3) δ 7.70 (s, 1H), 4.39 (q, $J = 7.2$ Hz, 2H), 1.46 (s, 9H), 1.41 (t, $J = 7.2$ Hz, 3H); HRMS (EI) m/z 197.1050 (M^+) (calcd for $\text{C}_{10}\text{H}_{15}\text{NO}_3$ 197.1052).

Ethyl 5-(tert-butyl)imidazole-4-carboxylate (5). A mixture of **4** (11 g, 55.77 mmol) and formamide (66 mL, 1.66 mol) was heated at 165 °C for 14 h. After cooling, the reaction mixture was extracted with AcOEt (200 mL) and the organic layer was washed with 10% Na_2CO_3 and saturated NaCl three times, dried over anhydrous Na_2SO_4 , and concentrated in vacuo. The residual dark brown oil was purified by column chromatography on silica gel using CHCl_3 –MeOH (100:1 to 50:1) as an eluant to give 6.44 g (59%) of a white solid **5**: ^1H NMR (300 MHz, CDCl_3) δ 7.51 (s, 1H), 4.36 (q, $J = 6.9$ Hz, 2H), 1.46 (s, 9H), 1.40 (t, $J = 7.2$ Hz, 3H); HRMS (EI) m/z 196.1213 (M^+) (calcd for $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_2$ 196.1212).

5-(tert-Butyl)imidazol-4-yl)methanol (6). To a solution of **5** (10 g, 50.95 mmol) in THF (200 mL) was added LiAlH_4 (1.93 g, 50.95 mmol) portionwise under argon atmosphere at 0 °C, and the mixture was stirred for 4 h at room temperature. To this solution was added saturated aqueous NH_4Cl slowly at 0 °C, and the resulting precipitate was removed by Celite filtration. The filtrate was extracted with AcOEt, washed with 10% Na_2CO_3 and saturated NaCl twice, dried over anhydrous Na_2SO_4 , and concentrated in vacuo. The residual oil was purified by silica gel chromatography using CHCl_3 –MeOH (200:1) to give an oil of **6** (5.4 g, 69%): ^1H NMR (300 MHz, CDCl_3) δ 7.55 (br s, 1H), 4.75 (s, 2H), 1.37 (s, 9H); HRMS (EI) m/z 154.1118 (M^+) (calcd for $\text{C}_8\text{H}_{14}\text{N}_2\text{O}$ 154.1103).

5-(tert-Butyl)imidazole-4-carboxaldehyde (7). To a solution of **6** (4.1 g, 26.6 mmol) in acetone (70 mL) was added MnO_2 (16.2 g, 186.2 mmol), and the mixture was stirred at room temperature for 2 h. After filtration to remove MnO_2 , the solvent was removed by evaporation, and the residual white powder was purified by silica gel column chromatography using CHCl_3 –MeOH (200:1) as an eluate to give 3.2 g (79%) of off-white solid **7**: ^1H NMR (300 MHz, CDCl_3) δ 10.09 (s, 1H), 7.78 (s, 1H), 1.50 (s, 9H); HRMS (EI) m/z 152.0950 for (M^+) (calcd 152.0947 for $\text{C}_8\text{H}_{12}\text{N}_2\text{O}$).

1-Acetyl-3-[(Z)-1-[5-(tert-butyl)-1H-4-imidazolyl]methylidene]-2,5-piperazinedione (9). To a solution of 5-(tert-butyl)imidazole-4-carboxaldehyde **7** (200 mg, 1.31 mmol) in DMF (2 mL) was added compound **8** (390.7 mg, 1.97 mmol) and the solution was repeatedly evacuated in a short time to remove oxygen and flushed with Ar, followed by the addition of Cs_2CO_3 (642.6 mg, 1.97 mmol), and the evacuation–flushing process was repeated again. The resultant mixture was stirred for 12 h at room temperature. After the solvent was removed by evaporation, the residue was dissolved in the mixture of AcOEt and water, and the organic phase was washed with saturated NaCl twice, dried over Na_2SO_4 , and concentrated in vacuo. The residual oil was purified by column chromatography on silica gel using CHCl_3 –MeOH (200:1 to 50:1) as an eluant to give 380 mg (46%) of a pale yellow solid **9**: ^1H NMR (300 MHz, CDCl_3) δ 12.14 (br s, 1H), 9.22 (br s, 1H), 7.57 (s, 1H), 7.18 (s, 1H), 4.47 (s, 2H), 2.65 (s, 3H), 1.47 (s, 9H); HRMS (EI) m/z 290.1382 (M^+) (calcd for $\text{C}_{14}\text{H}_{18}\text{N}_4\text{O}_3$ 290.1379).

1-Acetyl-3-(Z)-phenylmethylidene-2,5-piperazinedione (10) [CAS No. 30166–30–4]. This known compound^{22b} was synthesized using a procedure similar to that of compound **9**. Namely, benzaldehyde (2.7 mL, 26.5 mmol) and compound **8** (5 g, 25.2 mmol) were reacted in DMF (70 mL) in the presence of Cs_2CO_3 (8.6 g, 26.5 mmol) under Ar atmosphere for 2 h at room temperature: yield 76%; ^1H NMR (300 MHz, CDCl_3) δ 7.92 (br s, 1H), 7.37–7.50 (m, 5H), 7.26 (s, 1H), 7.19 (s, 1H), 4.52 (s, 2H), 2.66 (s, 3H); HRMS (ESI) m/z 245.0921 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{13}\text{H}_{13}\text{N}_2\text{O}_3$ 245.0926).

3-[(Z)-1-[5-(tert-butyl)-1H-4-imidazolyl]methylidene]-6-[(Z)-1-phenylmethylidene]-2,5-piperazinedione (11). To a solution of **9** (100 mg, 0.34 mmol) in DMF (4 mL) was added benzaldehyde (52.25 μL , 0.51 mmol) and the solution was repeatedly evacuated in a short time to remove oxygen and flushed with Ar, followed by the addition of Cs_2CO_3 (224.6 mg, 0.68 mmol), and the evacuation–

flushing process was repeated again. The resultant mixture was heated for 4 h at 80 °C. After the solvent was removed by evaporation, the residue was dissolved in EtOAc, washed with water two times and saturated NaCl three times, dried over Na_2SO_4 , and concentrated in vacuo. The resulting residue was dissolved in 70% aqueous MeCN, applied to a reverse-phase HPLC column ($\mu\text{Bondasphere}$ 5C₁₈ 100 Å, 19 × 150 mm), and eluted using a linear gradient from 20 to 80% CH_3CN in 0.1% aqueous TFA over 30 min at a flow rate of 12 mL/min, and the desired fraction was collected, concentrated by evaporation, and then lyophilized to give 94.0 mg of the title compound **11** as a yellow solid: yield 81%; mp 160–162 °C (dec); IR (KBr, cm^{-1}) 3500, 3459, 3390, 3117, 3078, 2963, 2904, 1673, 1636, 1601, 1413, 1371, 1345; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.26 (s, 2H), 10.16 (br s, 1H), 7.86 (s, 1H), 7.53 (d, $J = 7.4$ Hz, 2H), 7.42 (t, $J = 7.5$ Hz, 2H), 7.32 (t, $J = 7.4$ Hz, 1H), 6.86 (s, 1H), 6.75 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.2, 156.4, 145.3, 137.4, 134.5, 133.1, 129.1, 128.6, 127.9, 126.4, 113.9, 112.0, 104.5, 37.4, 27.7; HRMS (EI) m/z 336.1591 (M^+) (calcd for $\text{C}_{19}\text{H}_{20}\text{N}_4\text{O}_2$ 336.1586). Anal. ($\text{C}_{19}\text{H}_{20}\text{N}_4\text{O}_2 \cdot 0.25\text{H}_2\text{O} \cdot \text{CF}_3\text{COOH}$) C, H, N. HPLC (method 1) 99.4% ($t_{\text{R}} = 18.87$ min).

Compounds **12–16** were prepared from compound **8** over two steps according to the procedure (route A) described for the synthesis of **11**.

(3Z,6Z)-3-Benzylidene-6-((5-methyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (12). Yield of 3% from **8** (two steps); mp >280 °C; IR (KBr, cm^{-1}) 3185, 3107, 3032, 2872, 2776, 2681, 1682, 1645, 1393; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.72 (br s, 1H), 11.85 (br s, 1H), 10.08 (s, 1H), 8.00 (s, 1H), 7.54 (d, $J = 7.7$ Hz, 2H), 7.42 (t, $J = 7.5$ Hz, 2H), 7.33 (t, $J = 7.0$ Hz, 1H), 6.76 (s, 1H), 6.58 (s, 1H), 2.31 (s, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.8, 157.0, 135.4, 133.6, 129.7, 129.5, 129.2, 128.5, 127.0, 114.5, 103.8, 9.6; HRMS (ESI) m/z 295.1201 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{16}\text{H}_{15}\text{N}_4\text{O}_2$ 295.1195); HPLC (method 1) 97.7% ($t_{\text{R}} = 12.10$ min). Anal. ($\text{C}_{16}\text{H}_{15}\text{N}_4\text{O}_2 \cdot \text{CF}_3\text{COOH} \cdot 0.67\text{H}_2\text{O}$) C, H, N.

(3Z,6Z)-3-Benzylidene-6-((5-propyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (13). Yield of 9% from **8** (two steps); mp >280 °C (dec); IR (KBr, cm^{-1}) 3219, 3176, 3088, 3036, 2966, 2930, 2871, 1684, 1638, 1394; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.70 (br s, 1H), 11.91 (s, 1H), 10.09 (s, 1H), 8.00 (s, 1H), 7.54 (d, $J = 7.5$ Hz, 2H), 7.42 (t, $J = 7.4$ Hz, 2H), 7.32 (t, $J = 7.2$ Hz, 1H), 6.76 (s, 1H), 6.58 (s, 1H), 2.68 (t, $J = 7.3$ Hz, 2H), 1.63–1.55 (m, 2H), 0.88 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.8, 156.9, 135.6, 133.7, 133.6, 129.7, 129.2, 128.5, 127.0, 114.5, 103.7, 25.8, 23.1, 13.9; HRMS (ESI) m/z 323.1519 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{18}\text{H}_{19}\text{N}_4\text{O}_2$ 323.1508); HPLC (method 1) 98.1% ($t_{\text{R}} = 14.76$ min). Anal. ($\text{C}_{18}\text{H}_{19}\text{N}_4\text{O}_2 \cdot 0.65\text{CF}_3\text{COOH} \cdot 0.33\text{H}_2\text{O}$) C, H, N.

(3Z,6Z)-3-Benzylidene-6-((5-butyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (14). Yield of 13% from **8** (two steps); mp >270 °C (dec); IR (KBr, cm^{-1}) 3180, 3120, 3069, 2955, 2928, 2870, 1675, 1635, 1418; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.56 (s, 1H), 12.01 (s, 1H), 10.04 (s, 1H), 7.90 (s, 1H), 7.54 (d, $J = 7.5$ Hz, 2H), 7.42 (t, $J = 7.4$ Hz, 2H), 7.32 (t, $J = 7.2$ Hz, 1H), 6.75 (s, 1H), 6.58 (s, 1H), 2.71 (t, $J = 7.4$ Hz, 2H), 1.60–1.50 (m, 2H), 1.35–1.23 (m, 2H), 0.89 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.9, 156.7, 135.7, 134.0, 133.7, 132.8, 129.7, 129.2, 128.5, 127.1, 124.2, 114.3, 104.4, 32.0, 23.6, 22.1, 14.1; HRMS (ESI) m/z 337.1659 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{19}\text{H}_{21}\text{N}_4\text{O}_2$ 337.1665); HPLC (method 1) 97.3% ($t_{\text{R}} = 16.39$ min). Anal. ($\text{C}_{19}\text{H}_{21}\text{N}_4\text{O}_2 \cdot 0.25\text{H}_2\text{O}$) C, H, N.

(3Z,6Z)-3-Benzylidene-6-((5-isopropyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (15). Yield of 6% from **8** (two steps); mp >270 °C (dec); IR (KBr, cm^{-1}) 3137, 3056, 2972, 2937, 2874, 1675, 1649, 1389; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.72 (br s, 1H), 11.92 (s, 1H), 10.08 (s, 1H), 8.00 (s, 1H), 7.54 (d, $J = 7.5$ Hz, 2H), 7.42 (t, $J = 7.4$ Hz, 2H), 7.32 (t, $J = 7.5$ Hz, 1H), 6.76 (s, 1H), 6.61 (s, 1H), 3.29–3.20 (m, 1H), 1.24 (d, $J = 6.9$ Hz, 6H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.8, 156.8, 139.4, 135.8, 133.7, 129.7, 129.2, 128.5, 127.0, 114.4, 110.0, 104.1, 24.5, 23.0; HRMS (ESI) m/z 323.1502 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{18}\text{H}_{19}\text{N}_4\text{O}_2$ 323.1508); HPLC (method 1) 99.2% ($t_{\text{R}} = 14.31$ min). Anal. ($\text{C}_{18}\text{H}_{18}\text{N}_4\text{O}_2 \cdot \text{CF}_3\text{COOH} \cdot 0.75\text{H}_2\text{O}$) C, H, N.

(3Z,6Z)-3-Benzylidene-6-((5-sec-butyl-1H-imidazol-4-yl)-methylene)piperazine-2,5-dione (16). Yield of 20% from **8** (two steps); mp 226–228 °C; IR (KBr, cm^{-1}) 3433, 3185, 3161, 2961, 2876, 2679, 1692, 1670, 1399; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.67 (br s, 1H), 11.93 (s, 1H), 10.08 (s, 1H), 8.02 (s, 1H), 7.54 (d, $J = 7.5$ Hz, 2H), 7.42 (t, $J = 7.4$ Hz, 2H), 7.32 (t, $J = 7.2$ Hz, 1H), 6.78 (s, 1H), 6.59 (s, 1H), 3.00–2.93 (m, 1H), 1.67–1.50 (m, 2H), 1.23 (d, $J = 7.0$ Hz, 3H), 0.75 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.2, 156.3, 137.4, 135.3, 133.1, 129.2, 128.6, 127.9, 126.4, 113.9, 103.2, 30.9, 29.2, 20.2, 11.9; HRMS (ESI) m/z 337.1668 ($\text{M} + \text{H}^+$) (calcd for $\text{C}_{19}\text{H}_{21}\text{N}_4\text{O}_2$ 337.1665); HPLC (method 1) 95.9% ($t_{\text{R}} = 16.00$ min). Anal. ($\text{C}_{19}\text{H}_{20}\text{N}_4\text{O}_2 \cdot \text{CF}_3\text{COOH} \cdot \text{H}_2\text{O}$) C, H, N.

Compounds **17–65** were prepared from compound **9** according to the procedure described for the synthesis of **11**.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-methoxyphenyl)methylidene]-2,5-piperazinedione (17). Yield of 29% from **9**; mp 260–262 °C (dec); IR (KBr, cm^{-1}) 3196, 2973, 1671, 1637, 1417; ^1H NMR (300 MHz, CDCl_3) δ 12.32 (s, 1H), 9.45 (s, 1H), 8.59 (s, 1H), 7.59 (s, 1H), 7.32–7.38 (m, 2H), 7.01 (s, 1H), 7.00 (s, 1H), 6.97–7.06 (m, 2H), 3.94 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.5, 157.2, 156.9, 140.6, 134.7, 130.5, 130.3, 126.8, 122.3, 121.1, 111.9, 110.7, 104.9, 56.0, 32.3, 31.0; HRMS (EI) m/z 366.1696 (M^+) (calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3$ 366.1692); HPLC (method 1) 97.2% ($t_{\text{R}} = 16.11$ min). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3 \cdot 0.25\text{H}_2\text{O}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3-methoxyphenyl)methylidene]-2,5-piperazinedione (18). Yield of 41% from **9**; mp 251–253 °C (dec); IR (KBr, cm^{-1}) 3175, 2968, 1672, 1634, 1416; ^1H NMR (300 MHz, CDCl_3) δ 12.37 (s, 1H), 9.33 (br s, 1H), 8.11 (s, 1H), 7.58 (s, 1H), 7.26–7.39 (m, 1H), 7.02 (s, 1H), 7.00 (s, 1H), 6.97 (s, 1H), 6.86–6.92 (m, 2H), 3.84 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 159.3, 157.5, 156.3, 140.4, 134.5, 134.4, 130.7, 129.8, 126.8, 123.8, 121.5, 114.5, 113.9, 113.8, 105.1, 55.1, 31.9, 30.7; HRMS (EI) m/z 366.1691 (M^+) (calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3$ 366.1692); HPLC (method 3) 95.4% ($t_{\text{R}} = 18.82$ min). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3 \cdot \text{CF}_3\text{COOH} \cdot 0.5\text{H}_2\text{O}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(4-methoxyphenyl)methylidene]-2,5-piperazinedione (19). Yield of 54% from **9**; mp 268–270 °C (dec); IR (KBr, cm^{-1}) 3409, 3248, 2975, 1668, 1622, 1608, 1406, 1344; ^1H NMR (300 MHz, CDCl_3) δ 12.32 (s, 1H), 9.30 (br s, 1H), 8.06 (s, 1H), 7.58 (s, 1H), 7.35 (d, $J = 8.4$ Hz, 2H), 7.00 (s, 1H), 6.95 (d, $J = 8.4$ Hz, 2H), 6.96 (s, 1H), 3.85 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 159.6, 158.0, 157.1, 140.6, 134.8, 131.4, 131.2, 126.1, 125.5, 124.4, 114.7, 114.6, 105.1, 55.7, 32.4, 31.1; HRMS (EI) m/z 366.1693 (M^+) (calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3$ 366.1692); HPLC (method 1) 99.1% ($t_{\text{R}} = 19.20$ min). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3 \cdot 0.5\text{MeOH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2,3-dimethoxyphenyl)methylidene]-2,5-piperazinedione (20). Yield of 15% from **9**; mp 141–143 °C (dec); IR (KBr, cm^{-1}) 3212, 2966, 1670, 1632, 1413; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.37 (br s, 1H), 12.22 (s, 1H), 9.73 (s, 1H), 7.88 (s, 1H), 7.02–7.17 (m, 3H), 6.85 (s, 1H), 6.76 (s, 1H), 3.84 (s, 3H), 3.71 (s, 3H), 1.35 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 157.1, 156.1, 152.8, 146.2, 140.5, 134.4, 130.7, 127.3, 127.2, 124.6, 123.7, 122.1, 113.1, 109.8, 105.3, 60.7, 55.8, 32.0, 30.7; HRMS (EI) m/z 396.1790 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 95.1% ($t_{\text{R}} = 14.98$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot \text{CF}_3\text{COOH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2,4-dimethoxyphenyl)methylidene]-2,5-piperazinedione (21). Yield of 27% from **9**; mp 266–268 °C (dec); IR (KBr, cm^{-1}) 3397, 3259, 2969, 1663, 1637, 1417; ^1H NMR (300 MHz, CDCl_3) δ 12.26 (s, 1H), 9.41 (br s, 1H), 8.68 (s, 1H), 7.57 (s, 1H), 7.28 (s, 1H), 6.99 (s, 1H), 6.95 (s, 1H), 6.53–6.60 (m, 2H), 3.92 (s, 3H), 3.85 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 161.5, 158.5, 157.5, 157.0, 140.5, 134.7, 131.5, 131.2, 125.3, 124.4, 115.0, 110.8, 106.1, 105.0, 99.1, 56.2, 55.9, 32.4, 31.1; HRMS (EI) m/z 396.1795 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 97.9% ($t_{\text{R}} = 16.74$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 0.75\text{H}_2\text{O} \cdot \text{CH}_3\text{OH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2,5-dimethoxyphenyl)methylidene]-2,5-piperazinedione (22). Yield of 5% from **9**; mp 257–259 °C (dec); IR (KBr, cm^{-1}) 3196, 3140, 2968, 1657, 1395; ^1H NMR (300 MHz, CDCl_3) δ 12.36 (s, 1H), 9.57 (br s, 1H), 8.81 (s, 1H), 7.57 (s, 1H), 7.01 (s, 1H), 6.92 (s, 1H), 6.82–6.97 (m, 3H), 3.89 (s, 3H), 3.79 (s, 3H), 1.42 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 157.1, 156.9, 153.1, 151.3, 140.2, 134.4, 126.8, 122.6, 115.8, 115.0, 112.7, 110.8, 104.0, 56.2, 55.6, 32.0, 30.5; HRMS (EI) m/z 396.1799 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 98.1% ($t_{\text{R}} = 20.04$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot \text{CF}_3\text{COOH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2,6-dimethoxyphenyl)methylidene]-2,5-piperazinedione (23). Yield of 50% from **9**; mp 161–163 °C (dec); IR (KBr, cm^{-1}) 3196, 3048, 2979, 1688, 1652, 1406; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.52 (br s, 1H), 12.00 (br s, 1H), 9.20 (s, 1H), 8.01 (s, 1H), 7.33 (t, $J = 8.4$ Hz, 1H), 6.80 (s, 1H), 6.75 (s, 1H), 6.71 (d, $J = 8.4$ Hz, 2H), 3.83 (s, 6H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 158.1, 158.0, 156.8, 156.7, 140.5, 134.7, 130.8, 127.1, 110.6, 107.6, 104.6, 104.0, 56.3, 32.3, 31.0; HRMS (EI) m/z 396.1789 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 99.2% ($t_{\text{R}} = 16.22$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 1.5\text{CF}_3\text{COOH} \cdot \text{H}_2\text{O}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3,4-dimethoxyphenyl)methylidene]-2,5-piperazinedione (24). Yield of 30% from **9**; mp 264–266 °C (dec); IR (KBr, cm^{-1}) 3393, 3253, 3096, 2972, 1670, 1638, 1412; ^1H NMR (300 MHz, CDCl_3) δ 12.32 (s, 1H), 9.23 (s, 1H), 8.12 (s, 1H), 7.58 (s, 1H), 7.01 (s, 2H), 6.95 (s, 2H), 6.85 (s, 1H), 3.93 (s, 3H), 3.90 (s, 3H), 1.46 (s, 9H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 158.0, 157.0, 149.3, 149.0, 140.6, 134.8, 131.2, 126.2, 125.5, 124.4, 122.8, 115.0, 113.4, 112.3, 105.2, 56.0, 55.9, 32.4, 31.1; HRMS (EI) m/z 396.1806 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 96.4% ($t_{\text{R}} = 15.14$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 1.5\text{H}_2\text{O} \cdot 0.5\text{MeOH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3,5-dimethoxyphenyl)methylidene]-2,5-piperazinedione (25). Yield of 22% from **9**; mp 266–268 °C (dec); IR (KBr, cm^{-1}) 3197, 2969, 1670, 1647, 1601, 1419; ^1H NMR (300 MHz, CDCl_3) δ 12.36 (s, 1H), 9.29 (s, 1H), 8.13 (s, 1H), 7.58 (s, 1H), 7.02 (s, 1H), 6.93 (s, 1H), 6.51 (s, 2H), 6.44 (s, 1H), 3.82 (s, 6H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 160.5, 157.5, 156.3, 140.4, 135.0, 134.4, 130.7, 127.0, 123.8, 114.0, 107.1, 105.2, 100.3, 55.2, 32.0, 30.7; HRMS (EI) m/z 396.1790 (M^+) (calcd for $\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4$ 396.1797); HPLC (method 1) 96.4% ($t_{\text{R}} = 16.67$ min). Anal. ($\text{C}_{21}\text{H}_{24}\text{N}_4\text{O}_4 \cdot 0.25\text{CF}_3\text{COOH}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-methylphenyl)methylidene]-2,5-piperazinedione (26). Yield of 70% from **9**; mp 148–150 °C (dec); IR (KBr, cm^{-1}) 3167, 3066, 3033, 2974, 2877, 1686, 1668, 1652, 1401; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.57 (br s, 1H), 12.03 (s, 1H), 9.85 (s, 1H), 8.04 (s, 1H), 7.28–7.41 (m, 1H), 7.21–7.25 (m, 3H), 6.80 (s, 1H), 6.79 (s, 1H), 2.26 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 157.6, 157.0, 140.5, 137.3, 134.7, 132.7, 130.7, 129.0, 128.5, 127.6, 126.5, 113.6, 104.7, 32.3, 30.9, 20.2; HRMS (EI) m/z 350.1734 (M^+) (calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_2$ 350.1743); HPLC (method 2) 95.8% ($t_{\text{R}} = 15.59$ min). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_2 \cdot 0.9\text{CF}_3\text{COOH} \cdot 0.5\text{H}_2\text{O}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3-methylphenyl)methylidene]-2,5-piperazinedione (27). Yield of 62% from **9**; mp 156–158 °C (dec); IR (KBr, cm^{-1}) 3194, 3118, 3040, 2975, 2875, 2652, 1689, 1651, 1399; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.66 (br s, 1H), 12.00 (br s, 1H), 10.10 (s, 1H), 8.06 (s, 1H), 7.28–7.38 (m, 3H), 7.12–7.20 (m, 1H), 6.81 (s, 1H), 6.73 (s, 1H), 2.34 (s, 3H), 1.38 (s, 9H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) 157.6, 156.4, 140.4, 137.8, 134.4, 133.1, 130.7, 129.7, 128.8, 128.6, 126.5, 126.4, 123.8, 114.1, 105.1, 32.0, 30.7, 21.1; HRMS (EI) m/z 350.1743 (M^+) (calcd for $\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_2$ 350.1743); HPLC (method 2) 97.5% ($t_{\text{R}} = 16.78$ min). Anal. ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_2 \cdot \text{CF}_3\text{COOH} \cdot 0.5\text{H}_2\text{O}$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(4-methylphenyl)methylidene]-2,5-piperazinedione (28). Yield of 50% from **9**; mp 168–170 °C (dec); IR (KBr, cm^{-1}) 3369, 3168, 3040, 2975, 2878, 1688, 1654, 1400; ^1H NMR (300 MHz,

DMSO-*d*₆) δ 12.60 (br s, 1H), 11.97 (s, 1H), 10.02 (s, 1H), 8.06 (s, 1H), 7.44 (d, *J* = 7.8 Hz, 2H), 7.24 (d, *J* = 7.8 Hz, 2H), 6.80 (s, 1H), 6.73 (s, 1H), 2.33 (s, 3H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 157.5, 156.9, 140.3, 138.0, 134.5, 130.6, 130.5, 129.5, 129.5, 128.3, 126.0, 114.7, 32.1, 30.6, 21.2; HRMS (EI) *m/z* 350.1734 (*M*⁺) (calcd for C₂₀H₂₂N₄O₂ 350.1743); HPLC (method 1) >99.9% (*t*_R = 16.91 min). Anal. (C₂₀H₂₂N₄O₂·1.2CF₃COOH) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(2,6-dimethylphenyl)methylidene]-2,5-piperazinedione (29). Yield of 51% from **9**; mp 158–160 °C (dec); IR (KBr, cm⁻¹) 3434, 3174, 3063, 3034, 2973, 2876, 1692, 1670, 1649, 1405; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.63 (br s, 1H), 11.98 (br s, 1H), 9.67 (s, 1H), 8.10 (s, 1H), 7.04–7.29 (m, 3H), 6.78 (s, 1H), 6.73 (s, 1H), 2.16 (s, 6H), 1.37 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 157.5, 156.6, 140.5, 136.6, 134.7, 132.1, 128.6, 128.0, 127.8, 114.4, 32.3, 30.8, 20.5; HRMS (EI) *m/z* 364.1894 (*M*⁺) (calcd for C₂₁H₂₄N₄O₂ 364.1899); HPLC (method 1) >99.9% (*t*_R = 16.71 min). Anal. (C₂₁H₂₄N₄O₂·0.25CF₃COOH) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(2-fluorophenyl)methylidene]-2,5-piperazinedione (30). Yield of 68% from **9**; mp 142–145 °C (dec); IR (KBr, cm⁻¹) 3174, 3047, 2676, 2877, 1691, 1653, 1399; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.58 (br s, 1H), 12.09 (br s, 1H), 10.28 (s, 1H), 8.04 (s, 1H), 7.57 (t, *J* = 7.8 Hz, 1H), 7.34–7.43 (m, 1H), 7.21–7.29 (m, 2H), 6.83 (s, 1H), 6.70 (s, 1H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 161.5, 159.0, 157.7, 156.6, 140.7, 134.8, 131.1, 130.6, 130.5, 128.8, 125.0, 125.0, 121.6, 121.4, 116.3, 116.1, 107.0, 105.2, 32.3, 30.9; HRMS (EI) *m/z* 354.1501 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂F 354.1492); HPLC (method 1) 99.8% (*t*_R = 15.52 min). Anal. (C₁₉H₁₉N₄O₂F·CF₃COOH·0.5H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(3-fluorophenyl)methylidene]-2,5-piperazinedione (31). Yield of 63% from **9**; mp 169–171 °C (dec); IR (KBr, cm⁻¹) 3419, 3184, 3124, 3048, 1692, 1661, 1624; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.59 (br s, 1H), 12.05 (br s, 1H), 10.32 (s, 1H), 8.05 (s, 1H), 7.30–7.49 (m, 3H), 7.10–7.19 (m, 1H), 6.82 (s, 1H), 6.74 (s, 1H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 163.4, 161.0, 157.7, 156.2, 140.5, 135.7, 135.6, 134.4, 130.7, 130.6, 130.5, 127.6, 125.6, 125.5, 123.7, 115.9, 115.7, 114.8, 114.6, 112.4, 105.3, 32.0, 30.7; HRMS (EI) *m/z* 354.1493 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂F 354.1492); HPLC (method 3) 99.3% (*t*_R = 18.59 min). Anal. (C₁₉H₁₉N₄O₂F·CF₃COOH·H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(4-fluorophenyl)methylidene]-2,5-piperazinedione (32). Yield of 83% from **9**; mp 168–170 °C (dec); IR (KBr, cm⁻¹) 3179, 3045, 2973, 2878, 2669, 1689, 1655, 1623, 1402; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.60 (br s, 1H), 12.00 (br s, 1H), 10.22 (s, 1H), 8.06 (s, 1H), 7.52–7.61 (dd, *J* = 5.7, 8.4 Hz, 2H), 7.25 (t, *J* = 8.7 Hz, 2H), 6.81 (s, 1H), 6.75 (s, 1H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 163.3, 160.8, 157.9, 157.1, 140.5, 134.7, 132.0, 132.0, 130.1, 130.1, 126.9, 116.2, 116.0, 113.8, 104.6, 32.3, 30.9; HRMS (EI) *m/z* 354.1488 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂F 354.1492); HPLC (method 1) 95.4% (*t*_R = 15.59 min). Anal. (C₁₉H₁₉N₄O₂F·CF₃COOH·0.75H₂O) C, H, N.

(3*Z*,6*Z*)-3-((5-*tert*-Butyl-1*H*-imidazol-4-yl)methylene)-6-(2,5-difluorobenzylidene)piperazine-2,5-dione (33). Yield of 75% from **9**; mp >290 °C; IR (KBr, cm⁻¹) 3406, 3192, 3117, 2979, 2875, 1693, 1655, 1398; ¹H NMR (300 MHz, CDCl₃) δ 12.62 (br s, 1H), 12.09 (br s, 1H), 10.51 (s, 1H), 8.07 (s, 1H), 7.21–7.41 (m, 3H), 6.84 (s, 1H), 6.63 (s, 1H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 159.0, 158.3, 158.0, 157.3, 157.2, 156.6, 155.8, 154.7, 140.2, 134.2, 129.2, 122.5, 122.4, 122.3, 122.2, 117.0, 116.7, 116.3, 116.2, 116.1, 116.0, 105.1, 104.8, 31.8, 30.3; HRMS (EI) *m/z* 372.1934 (*M*⁺) (calcd for C₁₉H₁₈N₄O₂F₂ 372.1397); HPLC (method 1) >99.9% (*t*_R = 20.14 min). Anal. (C₁₉H₁₈F₂N₄O₂·CF₃COOH) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(2-chlorophenyl)methylidene]-2,5-piperazinedione (34). Yield of 42% from **9**; mp 160–162 °C (dec); IR (KBr, cm⁻¹) 3166, 3041, 2973, 2874, 1689, 1668, 1399; ¹H NMR (270 MHz, CDCl₃) δ 12.52 (br s, 1H), 11.60 (br s, 1H), 10.48 (s, 1H), 8.35 (s, 1H), 7.43–

7.57 (m, 2H), 7.30–7.38 (m, 1H), 7.15–7.19 (m, 1H), 7.04 (s, 1H), 6.96 (s, 1H), 1.43 (s, 9H); ¹³C NMR (150 MHz, DMSO-*d*₆) 157.8, 156.6, 140.8, 134.8, 133.5, 132.2, 131.1, 130.0, 129.9, 128.9, 127.8, 110.6, 105.4, 32.4, 31.0; HRMS (EI) *m/z* 370.1191 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂Cl 370.1196); HPLC (method 1) >99.9% (*t*_R = 16.56 min). Anal. (C₁₉H₁₉N₄O₂Cl·CF₃COOH·H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(3-chlorophenyl)methylidene]-2,5-piperazinedione (35). Yield of 76% from **9**; mp 276–278 °C (dec); IR (KBr, cm⁻¹) 3185, 3114, 3072, 3039, 2978, 2876, 2656, 1690, 1655, 1402; ¹H NMR (270 MHz, CDCl₃) δ 12.53 (s, 1H), 11.61 (br s, 1H), 8.48 (s, 1H), 7.55 (s, 1H), 7.18–7.43 (m, 4H), 7.06 (s, 1H), 6.86 (s, 1H), 1.44 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 157.7, 156.6, 140.4, 135.6, 134.5, 133.4, 130.5, 129.0, 128.3, 127.9, 127.8, 112.8, 104.6, 32.1, 30.6; HRMS (EI) *m/z* 370.1188 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂Cl 370.1196); HPLC (method 2) 98.2% (*t*_R = 17.46 min). Anal. (C₁₉H₁₉N₄O₂Cl·CF₃COOH·H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(4-chlorophenyl)methylidene]-2,5-piperazinedione (36). Yield of 55% from **9**; mp 166–168 °C (dec); IR (KBr, cm⁻¹) 3166, 3041, 2974, 2876, 2673, 1688, 1656, 1400; ¹H NMR (270 MHz, CDCl₃) δ 12.51 (br s, 1H), 11.62 (br s, 1H), 8.41 (s, 1H), 7.55 (s, 1H), 7.33–7.47 (m, 4H), 7.05 (s, 1H), 6.87 (s, 1H), 1.44 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 157.7, 156.2, 140.5, 134.4, 132.4, 132.3, 131.1, 130.7, 128.6, 127.3, 123.8, 112.6, 105.3, 32.0, 30.7; HRMS (EI) *m/z* 370.1201 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂Cl 370.1196); HPLC (method 1) 95.8% (*t*_R = 17.24 min). Anal. (C₁₉H₁₉ClN₄O₂·0.5CF₃COOH) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(2,3-dichlorophenyl)methylidene]-2,5-piperazinedione (37). Yield of 27% from **9**; mp 165–168 °C (dec); IR (KBr, cm⁻¹) 3181, 3048, 2975, 2874, 1692, 1656, 1404; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.54 (br s, 1H), 12.17 (br s, 1H), 10.40 (s, 1H), 8.01 (s, 1H), 7.37–7.60 (m, 3H), 6.84 (s, 1H), 6.72 (s, 1H), 1.38 (s, 9H); ¹³C NMR (150 MHz, DMSO-*d*₆) 157.9, 156.3, 140.9, 135.0, 134.9, 132.4, 131.3, 130.1, 129.9, 129.7, 128.8, 110.4, 110.0, 105.8, 32.4, 31.0; HRMS (EI) *m/z* 404.0801 (*M*⁺) (calcd for C₁₉H₁₈N₄O₂Cl₂ 404.0806); HPLC (method 1) 98.8% (*t*_R = 18.52 min). Anal. (C₁₉H₁₈Cl₂N₄O₂·CF₃COOH·H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(2-bromophenyl)methylidene]-2,5-piperazinedione (38). Yield of 52% from **9**; mp 146–148 °C (dec); IR (KBr, cm⁻¹) 3182, 3041, 2973, 2873, 1692, 1656, 1399; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.58 (br s, 1H), 12.10 (br s, 1H), 10.33 (s, 1H), 8.04 (s, 1H), 7.69 (d, *J* = 7.5 Hz, 1H), 7.57 (d, *J* = 7.2 Hz, 1H), 7.44 (t, *J* = 7.2 Hz, 1H), 7.27 (t, *J* = 7.5 Hz, 1H), 6.82 (s, 1H), 6.72 (s, 1H), 1.38 (s, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) 157.5, 156.4, 140.5, 134.5, 133.7, 132.8, 131.0, 130.0, 128.4, 128.1, 124.1, 112.9, 32.1, 104.8, 32.1, 30.6; HRMS (EI) *m/z* 414.0694 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂Br 414.0691); HPLC (method 1) 95.4% (*t*_R = 16.80 min). Anal. (C₁₉H₁₉BrN₄O₂·CF₃COOH·0.5H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(3-bromophenyl)methylidene]-2,5-piperazinedione (39). Yield of 55% from **9**; mp 173–175 °C (dec); IR (KBr, cm⁻¹) 3383, 3113, 3038, 2978, 2876, 1689, 1656, 1404; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.58 (br s, 1H), 12.05 (br s, 1H), 10.46 (s, 1H), 8.04 (s, 1H), 7.70 (s, 1H), 7.44–7.55 (m, 2H), 7.35 (t, *J* = 7.8 Hz, 1H), 6.82 (s, 1H), 6.71 (s, 1H), 1.38 (s, 9H); ¹³C NMR (150 MHz, DMSO-*d*₆) 158.1, 156.7, 140.8, 136.2, 134.8, 132.0, 131.0, 128.9, 128.1, 122.4, 112.7, 105.4, 32.4, 31.0; HRMS (EI) *m/z* 414.0687 (*M*⁺) (calcd for C₁₉H₁₉N₄O₂Br 414.0691); HPLC (method 1) 99.4% (*t*_R = 17.79 min). Anal. (C₁₉H₁₉N₄O₂Br·CF₃COOH·0.25H₂O) C, H, N.

3-((Z)-1-[5-(*tert*-Butyl)-1*H*-4-imidazolyl]methylidene)-6-[(Z)-1-(4-bromophenyl)methylidene]-2,5-piperazinedione (40). Yield of 50% from **9**; mp 176–178 °C (dec); IR (KBr, cm⁻¹) 3383, 3206, 3039, 2971, 2873, 2660, 1687, 1656, 1625, 1397; ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.60 (br s, 1H), 12.03 (br s, 1H), 10.28 (s, 1H), 8.06 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J* = 8.1 Hz, 2H), 6.81 (s, 1H), 6.70 (s, 1H), 1.38 (s, 9H); ¹³C NMR (150 MHz, DMSO-*d*₆) 157.9, 156.9, 140.6, 134.7, 133.0, 132.0, 131.8, 127.6, 121.5, 113.4,

104.8, 32.3, 30.9; HRMS (EI) m/z 414.0698 (M^+) (calcd for $C_{19}H_{19}N_4O_2Br$ 414.0691); HPLC (method 1) 99.1% ($t_R = 17.87$ min). Anal. ($C_{19}H_{19}BrN_4O_2 \cdot CF_3COOH \cdot 0.5H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(2-nitrophenyl)methylidene]-2,5-piperazinedione (41). Yield of 22% from **9**; mp 168–170 °C (dec); IR (KBr, cm^{-1}) 3384, 3189, 3053, 2973, 2872, 2664, 1691, 1657, 1407; 1H NMR (300 MHz, DMSO- d_6) δ 12.34 (br s, 1H), 12.31 (s, 1H), 10.35 (s, 1H), 8.14 (d, $J = 8.1$ Hz, 1H), 7.87 (s, 1H), 7.78 (t, $J = 7.5$ Hz, 1H), 7.64 (d, $J = 7.8$ Hz, 1H), 7.59 (t, $J = 8.1$ Hz, 1H), 6.93 (s, 1H), 6.86 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.8, 156.5, 148.2, 140.7, 134.8, 134.6, 132.1, 129.7, 129.0, 125.3, 111.2, 105.2, 32.3, 30.9; HRMS (EI) m/z 381.1429 (M^+) (calcd for $C_{19}H_{19}N_5O_4$ 381.1437); HPLC (method 1) 95.1% ($t_R = 14.40$ min). Anal. ($C_{19}H_{19}N_5O_4 \cdot CF_3COOH \cdot 0.25H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(3-nitrophenyl)methylidene]-2,5-piperazinedione (42). Yield of 50% from **9**; mp 273–275 °C (dec); IR (KBr, cm^{-1}) 3214, 3086, 2967, 1675, 1633, 1414, 1348; 1H NMR (300 MHz, DMSO- d_6) δ 12.50 (br s, 1H), 12.17 (br s, 1H), 10.64 (s, 1H), 8.29 (s, 1H), 7.82–8.16 (m, 3H), 7.67 (t, $J = 7.8$ Hz, 1H), 6.86 (s, 1H), 6.82 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (100 MHz, DMSO- d_6) 179.9, 157.9, 155.9, 147.9, 140.6, 135.9, 135.0, 134.5, 130.7, 129.9, 128.8, 123.9, 123.7, 122.3, 111.1, 105.5, 32.0, 30.7; HRMS (EI) m/z 381.1430 (M^+) (calcd for $C_{19}H_{19}N_5O_4$ 381.1437); HPLC (method 1) 99.2% ($t_R = 15.70$ min). Anal. ($C_{19}H_{19}N_5O_4 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(4-nitrophenyl)methylidene]-2,5-piperazinedione (43). Yield of 18% from **9**; mp 153–155 °C (dec); IR (KBr, cm^{-1}) 3411, 3300, 2957, 1672, 1630, 1417, 1336, 1108; 1H NMR (300 MHz, DMSO- d_6) δ 12.43 (br s, 1H), 12.29 (s, 1H), 10.54 (s, 1H), 8.23 (d, $J = 9$ Hz, 2H), 7.92 (s, 1H), 7.76 (d, $J = 8.7$ Hz, 2H), 6.88 (s, 1H), 6.80 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.2, 156.2, 146.3, 141.2, 141.0, 134.8, 131.1, 130.7, 124.1, 124.0, 111.2, 106.0, 32.3, 31.0; HRMS (EI) m/z 381.1442 (M^+) (calcd for $C_{19}H_{19}N_5O_4$ 381.1437); HPLC (method 2) 96.4% ($t_R = 15.32$ min). Anal. ($C_{19}H_{19}N_5O_4 \cdot 0.4CF_3COOH \cdot CH_3COOCH_2CH_3$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(2-hydroxyphenyl)methylidene]-2,5-piperazinedione (44). Yield of 67% from **9**; mp 220–222 °C (dec); IR (KBr, cm^{-1}) 3134, 2981, 2613, 1646, 1434, 1401, 1362; 1H NMR (300 MHz, DMSO- d_6) δ 12.61 (br s, 1H), 11.95 (br s, 1H), 10.85 (s, 1H), 10.16 (s, 1H), 8.07 (s, 1H), 7.36 (d, $J = 7.8$ Hz, 1H), 7.21 (t, $J = 7.8$ Hz, 1H), 6.94 (d, $J = 7.8$ Hz, 1H), 6.88 (t, $J = 7.8$ Hz, 1H), 6.79 (s, 1H), 6.75 (s, 1H), 1.37 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.1, 156.9, 154.5, 140.4, 134.7, 132.5, 130.6, 125.8, 121.0, 120.4, 116.7, 112.7, 104.3, 32.3, 30.8; HRMS (EI) m/z 352.1539 (M^+) (calcd for $C_{19}H_{20}N_4O_3$ 352.1535); HPLC (method 1) >99.9% ($t_R = 14.46$ min). Anal. ($C_{19}H_{20}N_4O_3 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(3-hydroxyphenyl)methylidene]-2,5-piperazinedione (45). Yield of 32% from **9**; mp 171–173 °C (dec); IR (KBr, cm^{-1}) 3167, 3094, 2965, 2879, 1683, 1611, 1423, 1403; 1H NMR (300 MHz, DMSO- d_6) δ 12.59 (br s, 1H), 12.01 (br s, 1H), 9.92 (s, 1H), 8.04 (s, 1H), 7.22 (t, $J = 7.8$ Hz, 1H), 6.93 (d, $J = 7.8$ Hz, 1H), 6.90 (s, 1H), 6.81 (s, 1H), 6.74 (d, $J = 7.8$ Hz, 1H), 6.67 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (100 MHz, DMSO- d_6) 157.5, 157.4, 156.3, 140.4, 134.4, 130.7, 129.8, 126.5, 123.8, 120.1, 115.6, 115.4, 114.1, 105.1, 32.0, 30.7; HRMS (EI) m/z 352.1534 (M^+) (calcd for $C_{19}H_{20}N_4O_3$ 352.1535); HPLC (method 3) 97.9% ($t_R = 13.54$ min). Anal. ($C_{19}H_{20}N_4O_3 \cdot CF_3COOH \cdot 0.5H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(3-ethoxyphenyl)methylidene]-2,5-piperazinedione (46). Yield of 28% from **9**; mp 158–160 °C (dec); IR (KBr, cm^{-1}) 2975, 2874, 1687, 1649, 1396; 1H NMR (300 MHz, DMSO- d_6) δ 12.58 (br s, 1H), 12.01 (br s, 1H), 10.10 (s, 1H), 8.04 (s, 1H), 7.32 (t, $J = 7.8$ Hz, 1H), 7.03–7.10 (m, 2H), 6.89 (d, $J = 8.4$ Hz, 1H), 6.81 (s, 1H), 6.73 (s, 1H), 4.06 (q, $J = 6.9$ Hz, 2H), 1.38 (s, 9H), 1.35 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (100 MHz, DMSO- d_6) 158.6, 157.5, 156.3, 140.4, 134.5, 134.4, 130.7, 129.8, 126.8, 123.8, 121.5, 115.0, 114.3, 113.9,

105.1, 63.0, 40.1, 39.9, 39.7, 39.5, 39.3, 39.1, 38.9, 31.9, 30.7, 14.7; HRMS (EI) m/z 380.1843 (M^+) (calcd for $C_{21}H_{24}N_4O_3$ 380.1848); HPLC (method 1) 98.8% ($t_R = 17.46$ min). Anal. ($C_{21}H_{24}N_4O_3 \cdot 0.25H_2O \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(3-cyanophenyl)methylidene]-2,5-piperazinedione (47). Yield of 41% from **9**; mp 163–165 °C (dec); IR (KBr, cm^{-1}) 3190, 3114, 3073, 3040, 2978, 2876, 1691, 1656, 1629, 1409; 1H NMR (300 MHz, DMSO- d_6) δ 12.58 (br s, 1H), 12.10 (br s, 1H), 10.58 (s, 1H), 8.04 (s, 1H), 7.98 (s, 1H), 7.76 (t, $J = 8.6$ Hz, 2H), 7.58 (t, $J = 7.8$ Hz, 1H), 6.84 (s, 1H), 6.74 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.2, 156.6, 140.9, 134.9, 134.8, 134.6, 133.2, 131.6, 130.1, 128.7, 119.3, 112.2, 111.9, 105.4, 32.4, 31.0; HRMS (EI) m/z 361.1536 (M^+) (calcd for $C_{20}H_{19}N_5O_2$ 361.1538); HPLC (method 1) 95.0% ($t_R = 14.83$ min). Anal. ($C_{20}H_{19}N_5O_2 \cdot CF_3COOH \cdot 0.5CH_3COOCH_2CH_3$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(3-phenylphenyl)methylidene]-2,5-piperazinedione (48). Yield of 67% from **9**; mp 183–185 °C (dec); IR (KBr, cm^{-1}) 3434, 3172, 3036, 2971, 2873, 1689, 1655, 1619, 1402; 1H NMR (300 MHz, DMSO- d_6) δ 12.64 (br s, 1H), 11.98 (br s, 1H), 10.24 (s, 1H), 8.09 (s, 1H), 7.59 (s, 1H), 7.36–7.48 (m, 3H), 6.81 (s, 1H), 6.77 (s, 1H), 6.77 (dd, $J = 11, 18$ Hz, 1H), 5.89 (d, $J = 18$ Hz, 1H), 5.31 (d, $J = 11$ Hz, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.9, 157.0, 140.6, 137.9, 136.9, 134.7, 134.0, 129.4, 129.2, 127.7, 127.2, 126.1, 115.3, 114.5, 104.9, 32.3, 30.9; HRMS (EI) m/z 362.1740 (M^+) (calcd for $C_{21}H_{22}N_4O_2$ 362.1743); HPLC (method 1) 96.7% ($t_R = 17.70$ min). Anal. ($C_{21}H_{22}N_4O_2 \cdot CF_3COOH \cdot 0.5H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-[3-(trifluoromethyl)phenyl]methylidene]-2,5-piperazinedione (49). Yield of 56% from **9**; mp 271–273 °C (dec); IR (KBr, cm^{-1}) 3180, 3115, 3073, 3038, 2977, 2879, 1690, 1652, 1407; 1H NMR (300 MHz, DMSO- d_6) δ 12.59 (br s, 1H), 12.08 (br s, 1H), 10.57 (s, 1H), 8.05 (s, 1H), 7.75–7.85 (m, 2H), 7.59–7.68 (m, 2H), 6.83 (s, 1H), 6.80 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.1, 156.8, 140.7, 134.8, 133.7, 130.0, 129.8, 129.6, 128.4, 126.2, 126.2, 125.5, 124.8, 124.8, 123.7, 112.8, 105.1, 32.3, 30.9; HRMS (EI) m/z 404.1466 (M^+) (calcd for $C_{20}H_{19}N_4O_2F_3$ 404.1460); HPLC (method 1) 99.6% ($t_R = 18.32$ min). Anal. ($C_{20}H_{19}F_3N_4O_2 \cdot CF_3COOH$) C, H, N.

(3Z,6Z)-3-(3-Benzoylbenzylidene)-6-((5-tert-butyl-1H-imidazol-4-yl)methylene)piperazine-2,5-dione (50). 3-Benzoylbenzaldehyde was synthesized as previously reported.³¹ Yield of 26% from **9**; mp 158–161 °C; IR (KBr, cm^{-1}) 3161, 3065, 2972, 2874, 1685, 1654, 1397; 1H NMR (300 MHz, DMSO- d_6) δ 12.47 (br s, 1H), 12.15 (br s, 1H), 10.38 (s, 1H), 7.96 (br s, 1H), 7.54–7.83 (m, 9H), 6.80 (s, 1H), 6.83 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 196.0, 158.0, 156.7, 140.8, 137.8, 137.3, 134.8, 134.0, 133.8, 133.3, 130.6, 130.3, 129.4, 129.3, 129.1, 128.1, 113.4, 105.3, 32.4, 31.0; HRMS (EI) m/z 440.1845 (M^+) (calcd for $C_{26}H_{24}N_4O_3$ 440.1848); HPLC (method 1) 96.1% ($t_R = 18.67$ min). Anal. ($C_{26}H_{24}N_4O_3 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(2-chloro-6-fluorophenyl)methylidene]-2,5-piperazinedione (51). Yield of 40% from **9**; mp 225–227 °C (dec); IR (KBr, cm^{-1}) 3189, 3049, 2974, 2875, 1693, 1654, 1406; 1H NMR (300 MHz, DMSO- d_6) δ 12.62 (br s, 1H), 12.13 (br s, 1H), 10.51 (s, 1H), 8.07 (s, 1H), 7.35–7.46 (m, 2H), 7.23–7.31 (m, 1H), 6.84 (s, 1H), 6.53 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 161.4, 159.8, 157.6, 156.0, 140.9, 134.8, 134.5, 134.5, 130.9, 130.8, 130.8, 125.7, 121.1, 121.0, 115.5, 115.3, 105.8, 104.6, 32.4, 30.9; HRMS (EI) m/z 388.1096 (M^+) (calcd for $C_{19}H_{18}N_4O_2ClF$ 388.1102); HPLC (method 1) 96.4% ($t_R = 20.71$ min). Anal. ($C_{19}H_{18}ClFN_4O_2 \cdot CF_3COOH \cdot 0.25H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[[Z]-1-(1-naphthyl)methylidene]-2,5-piperazinedione (52). Yield of 36% from **9**; mp 188–190 °C (dec); IR (KBr, cm^{-1}) 3434, 3178, 3044, 2972, 2873, 1686, 1650, 1401; 1H NMR (300 MHz, DMSO- d_6) δ 12.60 (br s, 1H), 12.11 (br s, 1H), 10.08 (s, 1H), 8.07 (s, 1H), 7.88–8.01 (m, 3H), 7.53–7.65 (m, 4H), 7.21 (s, 1H), 6.81 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.8, 156.7, 140.7, 134.8,

133.8, 131.7, 130.6, 129.0, 129.0, 128.8, 127.6, 127.0, 126.6, 126.3, 124.8, 112.0, 110.0, 105.3, 32.4, 31.0; HRMS (EI) m/z 386.1751 (M^+) (calcd for $C_{23}H_{22}N_4O_2$ 386.1743); HPLC (method 1) 95.4% (t_R = 18.12 min). Anal. ($C_{23}H_{22}N_4O_2 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-naphthyl)methylidene]-2,5-piperazinedione (53). Yield of 53% from **9**; mp 158–160 °C (dec); IR (KBr, cm^{-1}) 3394, 3178, 3042, 2974, 2873, 1686, 1651, 1401; 1H NMR (300 MHz, DMSO- d_6) δ 12.64 (br s, 1H), 12.01 (br s, 1H), 10.39 (s, 1H), 8.11 (s, 1H), 8.04–8.15 (m, 1H), 7.98–7.98 (m, 3H), 7.50–7.64 (m, 3H), 6.92 (s, 1H), 6.83 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (100 MHz, DMSO- d_6) 157.7, 156.4, 140.4, 134.4, 133.0, 132.4, 130.9, 130.8, 128.5, 128.3, 128.0, 127.5, 127.2, 127.0, 126.6, 126.4, 123.9, 114.0, 105.1, 32.0, 30.7; HRMS (EI) m/z 386.1737 (M^+) (calcd for $C_{23}H_{22}N_4O_2$ 386.1743); HPLC (method 1) 99.2% (t_R = 18.98 min). Anal. ($C_{23}H_{22}N_4O_2 \cdot CF_3COOH \cdot H_2O$) C, H, N.

(3Z,6Z)-3-((5-tert-Butyl-1H-imidazol-4-yl)methylene)-6-(quinolin-8-ylmethylene)piperazine-2,5-dione (54). Yield of 32% from **9**; mp >250 °C; IR (KBr, cm^{-1}) 3435, 3241, 2962, 1656, 1633, 1415; 1H NMR (300 MHz, DMSO- d_6) δ 12.31 (s, 2H), 8.99 (dd, J = 1.0, 4.6 Hz, 1H), 8.57 (dd, J = 1.0, 7.8 Hz, 1H), 8.04 (d, J = 8.0 Hz, 1H), 8.00 (d, J = 6.8 Hz, 1H), 7.88 (s, 1H), 7.75–7.66 (m, 2H), 7.15 (s, 1H), 6.88 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 156.7, 156.4, 148.4, 143.9, 140.2, 138.4, 134.9, 134.3, 132.8, 130.7, 129.2, 129.0, 127.2, 126.9, 124.1, 122.0, 112.0, 109.4, 104.7, 31.8, 30.5; HRMS (EI) m/z 387.1697 (M^+) (calcd for $C_{22}H_{21}N_5O_2$ 387.1695); HPLC (method 1) >99.9% (t_R = 16.00 min). Anal. ($C_{22}H_{21}N_5O_2 \cdot 2H_2O$) C, H, N.

(3Z,6Z)-3-((5-tert-Butyl-1H-imidazol-4-yl)methylene)-6-(isoquinolin-5-ylmethylene)piperazine-2,5-dione (55). Yield of 11% from **9**; mp 204–206 °C; IR (KBr, cm^{-1}) 3179, 3042, 2975, 2875, 1692, 1671, 1415, 1405; 1H NMR (300 MHz, DMSO- d_6) δ 12.48 (br s, 1H), 12.31 (br s, 1H), 10.31 (s, 1H), 9.69 (s, 1H), 8.62 (d, J = 6.4 Hz, 1H), 8.34 (d, J = 8.1 Hz, 1H), 8.14 (d, J = 6.2 Hz, 1H), 8.07 (d, J = 7.1 Hz, 1H), 7.96 (s, 1H), 7.92 (t, J = 7.8 Hz, 1H), 7.12 (s, 1H), 6.85 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.0, 156.3, 150.5, 140.9, 137.9, 135.8, 134.9, 131.0, 130.6, 129.5, 129.5, 128.6, 124.7, 120.7, 109.3, 105.6, 32.4, 31.0; HRMS (EI) m/z 387.1696 (M^+) (calcd for $C_{22}H_{21}N_5O_2$ 387.1695); HPLC (method 1) >99.9% (t_R = 12.36 min). Anal. ($C_{22}H_{21}N_5O_2 \cdot 2CF_3COOH \cdot H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-pyridyl)methylidene]-2,5-piperazinedione (56). Yield of 51% from **9**; mp 270–272 °C (dec); IR (KBr, cm^{-1}) 3496, 3448, 3148, 3083, 2965, 1680, 1644, 1416; 1H NMR (300 MHz, DMSO- d_6) δ 12.66 (br s, 1H), 12.51 (s, 1H), 12.18 (s, 1H), 8.74 (d, J = 3.4 Hz, 1H), 8.06 (s, 1H), 7.91–8.00 (m, 1H), 7.66 (d, J = 8.1 Hz, 1H), 7.38–7.45 (m, 1H), 6.88 (s, 1H), 6.70 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.2, 155.9, 155.3, 149.0, 141.4, 138.2, 135.0, 131.8, 126.9, 122.8, 107.4, 106.3, 32.5, 31.0; HRMS (EI) m/z 337.1533 (M^+) (calcd for $C_{18}H_{19}N_5O_2$ 337.1538); HPLC (method 2) 99.2% (t_R = 15.15 min). Anal. ($C_{18}H_{19}N_5O_2 \cdot 0.75CF_3COOH \cdot H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3-pyridyl)methylidene]-2,5-piperazinedione (57). Yield of 29% from **9**; mp 198–200 °C; IR (KBr, cm^{-1}) 3422, 3189, 3124, 3093, 3045, 2886, 1697, 1677, 1661, 1420, 1396; 1H NMR (270 MHz, $CDCl_3$) δ 12.58 (s, 1H), 11.42 (br s, 1H), 8.78 (br s, 1H), 8.68–8.80 (m, 1H), 8.49–8.68 (m, 1H), 7.83 (d, J = 7.3 Hz, 1H), 7.54 (s, 1H), 7.41–7.52 (m, 1H), 7.08 (s, 1H), 6.88 (s, 1H), 1.44 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.2, 156.2, 146.7, 144.5, 141.4, 141.1, 134.9, 130.2, 125.7, 124.6, 108.7, 105.9, 32.4, 31.0; HRMS (EI) m/z 337.1534 (M^+) (calcd for $C_{18}H_{19}N_5O_2$ 337.1538); HPLC (method 2) 96.1% (t_R = 15.46 min). Anal. ($C_{18}H_{19}N_5O_2 \cdot 3CF_3COOH \cdot 1.25H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(4-pyridyl)methylidene]-2,5-piperazinedione (58). Yield of 50% from **9**; mp 195–197 °C; IR (KBr, cm^{-1}) 3463, 3182, 3055, 2886, 1692, 1676, 1633, 1399; 1H NMR (300 MHz, DMSO- d_6) δ 12.48 (br s, 1H), 12.42 (s, 1H), 10.87 (s, 1H), 8.78 (d, J = 5.7 Hz, 2H), 7.94 (d, J = 5.7 Hz, 2H), 7.93 (s, 1H), 6.93 (s, 1H), 6.77 (s, 1H), 1.39 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 158.3, 155.6, 143.4,

141.6, 141.4, 135.1, 133.4, 127.7, 126.5, 124.2, 108.1, 106.8, 32.5, 31.0; HRMS (EI) m/z 337.1531 (M^+) (calcd for $C_{18}H_{19}N_5O_2$ 337.1538); HPLC (method 2) 98.3% (t_R = 17.64 min). Anal. ($C_{18}H_{19}N_5O_2 \cdot 2CF_3COOH \cdot H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-furyl)methylidene]-2,5-piperazinedione (59). Yield of 45% from **9**; mp 245–247 °C; IR (KBr, cm^{-1}) 3123, 3034, 2973, 2875, 1686, 1648, 1416, 1394; 1H NMR (300 MHz, DMSO- d_6) δ 12.48 (br s, 1H), 12.20 (br s, 1H), 9.38 (s, 1H), 7.93 (s, 2H), 6.88 (s, 1H), 6.86 (s, 1H), 6.70–6.65 (m, 1H), 6.63 (s, 1H), 1.38 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.1, 156.3, 150.5, 145.3, 140.9, 134.8, 124.5, 114.7, 112.9, 105.3, 101.5, 32.4, 31.0; HRMS (EI) m/z 326.1373 (M^+) (calcd for $C_{17}H_{18}N_4O_3$ 326.1379); HPLC (method 1) >99.9% (t_R = 14.72 min). Anal. ($C_{17}H_{18}N_4O_3 \cdot CF_3COOH \cdot 0.75H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(5-methyl-2-furyl)methylidene]-2,5-piperazinedione (60). Yield of 19% from **9**; mp 271–273 °C (dec); IR (KBr, cm^{-1}) 3166, 2976, 2874, 1691, 1663, 1653, 1404; 1H NMR (300 MHz, $CDCl_3$) δ 12.44 (s, 1H), 11.70 (br s, 1H), 9.08 (s, 1H), 7.55 (s, 1H), 7.03 (s, 1H), 6.62 (s, 1H), 6.46 (d, J = 3.0 Hz, 1H), 6.14 (d, J = 2.2 Hz, 1H), 2.45 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.1, 156.4, 154.9, 149.2, 140.8, 134.8, 123.3, 116.2, 109.4, 105.3, 101.8, 32.4, 31.0, 14.2; HRMS (EI) m/z 340.1539 (M^+) (calcd for $C_{18}H_{20}N_4O_3$ 340.1535); HPLC (method 1) 99.1% (t_R = 15.96 min). Anal. ($C_{18}H_{20}N_4O_3 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(5-chloro-2-furyl)methylidene]-2,5-piperazinedione (61). Yield of 24% from **9**; mp 296–298 °C (dec); IR (KBr, cm^{-1}) 3165, 3032, 2974, 2874, 2658, 1693, 1661, 1627, 1404; 1H NMR (300 MHz, $CDCl_3$) δ 12.49 (s, 1H), 11.90 (br s, 1H), 8.89 (s, 1H), 7.58 (s, 1H), 7.04 (s, 1H), 6.59 (s, 1H), 6.60 (d, J = 3.2 Hz, 1H), 6.37 (d, J = 3.2 Hz, 1H), 1.45 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.3, 156.2, 150.4, 141.0, 136.7, 134.9, 125.0, 116.3, 110.2, 105.7, 100.7, 32.4, 31.0; HRMS (EI) m/z 360.0991 (M^+) (calcd for $C_{17}H_{17}ClN_4O_3$ 360.0989); HPLC (method 1) 96.8% (t_R = 16.35 min). Anal. ($C_{17}H_{17}ClN_4O_3 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3-furyl)methylidene]-2,5-piperazinedione (62). Yield of 43% from **9**; mp 250–252 °C (dec); IR (KBr, cm^{-1}) 3132, 3048, 2974, 1678, 1644, 1390; 1H NMR (270 MHz, $CDCl_3$) δ 12.44 (s, 1H), 11.54 (br s, 1H), 8.13 (s, 1H), 7.77 (d, J = 0.8 Hz, 1H), 7.53 (br s, 2H), 7.05 (s, 1H), 6.75 (s, 1H), 6.63 (d, J = 1.1 Hz, 1H), 1.44 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.8, 157.1, 144.5, 144.4, 140.5, 134.7, 125.7, 118.9, 111.1, 106.3, 104.6, 32.3, 30.8; HRMS (EI) m/z 326.1385 (M^+) (calcd for $C_{17}H_{18}N_4O_3$ 326.1379); HPLC (method 2) 95.0% (t_R = 10.44 min). Anal. ($C_{17}H_{18}N_4O_3 \cdot CF_3COOH$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(2-thienyl)methylidene]-2,5-piperazinedione (63). Yield of 42% from **9**; mp 274–276 °C; IR (KBr, cm^{-1}) 3177, 3114, 2973, 2875, 1686, 1673, 1653, 1396; 1H NMR (270 MHz, DMSO- d_6) δ 12.41 (s, 1H), 11.85 (br s), 8.67 (s, 1H), 7.62 (d, J = 5.4 Hz, 1H), 7.58 (br s, 1H), 7.47 (dd, J = 3.0, 5.1 Hz, 1H), 7.25 (dd, J = 1.1, 5.1 Hz, 1H), 7.02 (s, 1H), 6.89 (s, 1H), 1.44 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.8, 156.7, 140.9, 136.1, 134.8, 130.1, 128.9, 128.6, 125.2, 107.7, 105.6, 32.4, 31.0; HRMS (EI) m/z 342.1153 (M^+) (calcd for $C_{17}H_{18}N_4O_2S$ 342.1150); HPLC (method 1) 96.3% (t_R = 15.20 min). Anal. ($C_{17}H_{18}N_4O_2S \cdot 0.75CF_3COOH \cdot 0.5H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-(3-thienyl)methylidene]-2,5-piperazinedione (64). Yield of 39% from **9**; mp 271–273 °C; IR (KBr, cm^{-1}) 3166, 3099, 3030, 2974, 2874, 1686, 1646, 1390; 1H NMR (300 MHz, DMSO- d_6) δ 12.41 (s, 1H), 11.81 (br s, 1H), 8.59 (s, 1H), 7.57 (br s, 2H), 7.46 (dd, J = 2.4, 4.3 Hz, 1H), 7.25 (dd, J = 0.8, 4.6 Hz, 1H), 7.02 (s, 1H), 6.89 (s, 1H), 1.44 (s, 9H); ^{13}C NMR (150 MHz, DMSO- d_6) 157.8, 157.2, 140.6, 134.7, 134.3, 129.3, 127.1, 127.0, 125.6, 109.6, 104.8, 32.3, 30.9; HRMS (EI) m/z 342.1154 (M^+) (calcd for $C_{17}H_{18}N_4O_2S$ 342.1150); HPLC (method 1) 98.9% (t_R = 14.52 min). Anal. ($C_{17}H_{18}N_4O_2S \cdot CF_3COOH \cdot 0.5H_2O$) C, H, N.

3-((Z)-1-[5-(tert-Butyl)-1H-4-imidazolyl]methylidene)-6-[(Z)-1-cyclohexylmethylidene]-2,5-piperazinedione (65). Yield of 31% from **9**; mp 228–230 °C (dec); IR (KBr, cm^{-1}) 3201, 3133, 2928,

2855, 1682, 1647, 1393; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 12.59 (br s, 1H), 11.78 (br s, 1H), 10.30 (s, 1H), 8.05 (s, 1H), 6.74 (s, 1H), 5.69 (d, $J = 10.2$ Hz, 1H), 2.62–2.77 (m, 1H), 1.54–1.71 (m, 5H), 1.36 (s, 9H), 1.01–1.42 (m, 5H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) 157.8, 156.9, 140.1, 134.5, 126.1, 123.9, 79.6, 33.5, 32.3, 32.3, 30.9, 25.8, 25.4; HRMS (EI) m/z 342.2053 (M^+) (calcd for $\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_2$ 342.2056); HPLC (method 1) 99.4% ($t_R = 16.72$ min). Anal. ($\text{C}_{19}\text{H}_{26}\text{N}_4\text{O}_2 \cdot \text{CF}_3\text{COOH} \cdot \text{H}_2\text{O}$) C, H, N.

Tubulin Binding Assay. Fluorescence spectra were measured at 37 °C as described previously.^{29,31} Bovine or 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 (0–12 μM , 1% DMSO) at 37 °C for 1 h. After incubation, the fluorescence of each tubulin sample was measured (excitation at 290 nm, emission at 340 nm) using a spectrofluorometer (TECAN Spectra Fluor Plus).

Cytotoxic Activity Assay against HT-29 Cells and HuVECs.^{19a,32} HT-29 cells were purchased from ATCC and were 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. HuVECs were obtained from Cambrex Bio Science (Walkersville, MD), used between passages 2 and 6, and grown in EGM-2 medium 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 from 20 μM to 2 pM. Forty-eight hours later, 10 μL of 0.2 mg/mL resazulin solution in PBS buffer was added to each well, and the cells were incubated for an additional 3–6 h. The fluorescence of the reduction product of resazulin was measured using a Fusion microplate fluorometer (Packard Bioscience) with $\lambda_{\text{ex}} = 535$ nm and $\lambda_{\text{em}} = 590$ nm filters.

Preparation of Tubulin and Microtubule Polymerization Assay in Vitro. Tubulin except for MAPs was purified from bovine brain through the two cycles of the polymerization–depolymerization method described as previously.³³ Turbidity assays of tubulin 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. Increase of absorbance at 350 nm was monitored in cuvettes at 37 °C using a thermostatic spectrophotometer (Beckman Coulter Inc., Brea, CA).

Analysis of Cell Cycle Progression. HeLa cells (1×10^5 cells/mL) were treated with various concentrations of each compound for 18 h and then harvested and stained with propidium iodide solution. DNA contents were measured by flow cytometric analysis (PARTEC CyFlow PA; Partec GmbH, Munster, Germany). Cell populations of each cell cycle phase were calculated using Multi Cycle AV (PhoenixFlow Systems).

Microtubule Polymerization Assay on HuVECs. Microtubule depolymerization assay was performed as described previously.^{19a} HuVECs were plated onto sterile tissue culture treated coverslips (Fisher, Hampton, NH) in six-well plates. Following overnight incubation the proliferating HuVECs were treated with test compounds or vehicle [0.25% (v/v) DMSO]. The plates were returned to the incubator for 30 min. Following fixation in 10% (v/v) neutral buffered formalin at room temperature, the cells were permeabilized in 0.2% (v/v) Triton X-100/dPBS before transferring to a humidified chamber and blocking for 2 h in antibody buffer [2% (w/v) BSA/0.1% (v/v) Tween-20/dPBS]. The coverslips were incubated with 0.1 mg/mL mouse anti- α -tubulin in antibody buffer for 1 h before washing and incubation with 1 mg/mL goat antimouse-FITC for 1 h in the dark. Finally, to visualize the nuclei, the cells were washed and treated with 2 mg/mL DAPI before mounting with Vectashield mounting media. The cells were imaged using a 60 \times oil immersion objective on an upright microscope (Olympus BX51). The images were digitally captured using a CCD camera and Magnafire 2.0 software (Olympus, Melville, NY).

■ ASSOCIATED CONTENT

● Supporting Information

Synthesis of imidazole aldehydes, Table S1, Figures S1–S10, ^1H and ^{13}C NMR spectra, HPLC charts, crystallographic information files (Tables S2 and S3), and elemental analysis table. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

ATCC, American type culture collection; BSA, bovine serum albumin; CoMFA, comparative molecular field analysis; Cs_2CO_3 , cesium carbonate; DAPI, 4',6-diamidino-2-phenylindole; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DKP, dike-topiperazine; 3D-QSAR, three-dimensional quantitative structure–activity relationship; DU 145, human prostate carcinoma cell lines; EGTA, ethylene glycol tetraacetic acid; EI, electron ionization; ESI, electrospray ionization; FITC, fluorescein isothiocyanate; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HT-29, human colon adenocarcinoma cell lines; HuVECs, human umbilical vein endothelial cells; IC_{50} , 50% inhibitory concentration; K_a , association constant; K_d , dissociation constant; LiAlH_4 , lithium aluminum hydride; MAPs, microtubule associated proteins; MDR, multidrug resistance; MES, 2-(*N*-morpholino)ethanesulfonic acid; NSCLC, non-small-cell lung carcinoma; ODS, octadecylsilyl; ORTEP, Oak Ridge thermal ellipsoid plot; PBS, phosphate-buffered saline; SAR, structure–activity relationship; SEM, standard error of the mean; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TMS, tetramethylsilane; USAN, United States adopted name; VDA, vascular disrupting agent

■ REFERENCES

- (1) (a) Dumontet, C.; Jordan, M. A. Microtubule-binding agents: A dynamic field of cancer therapeutics. *Nat. Rev. Drug Discovery* **2010**, *9*, 790–803. (b) Shi, Q.; Chen, K.; Morris-Natschke, S. L.; Lee, K. H. Recent progress in the development of tubulin inhibitors as antimetabolic antitumor agents. *Curr. Pharm. Des.* **1998**, *4*, 219–248.
- (2) (a) Suffness, M.; Wall, M. E. *Taxol: Science and Applications*; Suffness, M., Ed.; CRC Press: Boca Raton, FL, 1995; pp 3–25. (b) Mekhail, T. M.; Markman, M. Paclitaxel in cancer therapy. *Expert Opin. Pharmacother.* **2002**, *3*, 755–766.

- (3) Rowinsky, E. K.; Donehower, R. C. The clinical pharmacology and use of antimicrotubule agents in cancer chemotherapeutics. *Pharmacol. Ther.* **1991**, *52*, 35–84.
- (4) (a) Volm, M. Multidrug resistance and its reversal. *Anticancer Res.* **1998**, *18*, 2095–2917. (b) Ferlini, C.; Ojima, I.; Distefano, M.; Gallo, D.; Riva, A.; Morazzoni, P.; Bombardelli, E.; Mancuso, S.; Scambia, G. Second generation taxanes: From the natural framework to the challenge of drug resistance. *Curr. Med. Chem. Anti-Cancer Agents* **2003**, *3*, 133–138. (c) Kavallaris, M. Microtubules and resistance to tubulin-binding agents. *Nat. Rev. Cancer* **2010**, *10*, 194–204.
- (5) (a) Hastie, S. B. Interactions of colchicines with tubulin. *Pharmacol. Ther.* **1991**, *51*, 377–401. (b) Eigsti, O. J.; Dustin, P. Jr. *Colchicine in Agriculture, Medicine, Biology, and Chemistry*; Iowa State College Press: Ames, IA, 1955; Chapter 1.
- (6) (a) Kelly, M.; Hartwell, J. L. The biological effects and the chemical composition of podophyllin. *J. Natl. Cancer Inst.* **1954**, *14*, 967–1010. (b) Cortese, F.; Bhattacharyya, B.; Wolff, J. Podophyllo-toxin as a probe for the colchicines binding site of tubulin. *J. Biol. Chem.* **1977**, *521*, 1134–1140.
- (7) (a) Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E. Antimitotic natural products combretastatin A-4 and combretastatin A-2: Studies on the mechanism of their inhibition of the binding of colchicine to tubulin. *Biochemistry* **1989**, *28*, 6984–6991. (b) Pettit, G. R.; Singh, S. B.; Boyd, M. R.; Hamel, E.; Pettit, R. K.; Schmidt, J. M.; Hogan, F. Antineoplastic agents. 291. Isolation and synthesis of combretastatins A-4, A-5, and A-6(1a). *J. Med. Chem.* **1995**, *38*, 1666–1672.
- (8) (a) Verdier-Pinard, P.; Lai, J.-Y.; Yoo, H.-D.; Yu, J.; Marquez, B.; Nagle, D. G.; Nambu, M.; White, J. D.; Falck, J. R.; Gerwick, W. H.; Day, B. W.; Hamel, E. Structure–activity analysis of the interaction of curacin A, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells. *Mol. Pharmacol.* **1998**, *53*, 62–76. (b) Chang, Z.; Sitachitta, N.; Rossi, J. V.; Roberts, M. A.; Flatt, P. M.; Jia, J.; Sherman, D. H.; Gerwick, W. H. Biosynthetic pathway and gene cluster analysis of curacin A, an antitubulin natural product from the tropical marine cyanobacterium *Lyngbya majuscula*. *J. Nat. Prod.* **2004**, *67*, 1356–1367.
- (9) (a) Davis, P. D.; Dougherty, G. J.; Blakey, D. C.; Galbraith, S. M.; Tozer, G. M.; Holder, A. L.; Naylor, M. A.; Nolan, J.; Stratford, M. R. L.; Chaplin, D. J.; Hill, S. A. ZD6126: A novel vascular-targeting agent that causes selective destruction of tumor vasculature. *Cancer Res.* **2002**, *62*, 7247–7253. (b) Micheletti, G.; Poli, M.; Borsotti, P.; Martinelli, M.; Imberti, B.; Tarabozzi, G.; Giavazzi, R. Vascular-targeting activity of ZD6126, a novel tubulin-binding agent. *Cancer Res.* **2003**, *63*, 1534–1537.
- (10) (a) Ohsumi, K.; Hatanaka, T.; Nakagawa, R.; Fukuda, Y.; Morinaga, Y.; Suga, Y.; Nihei, Y.; Ohishi, K.; Akiyama, Y.; Tsuji, T. Synthesis and antitumor activities of amino acid prodrugs of amino-combretastatins. *Anti-Cancer Drug Des.* **1999**, *14*, 539–548. (b) Hori, K.; Saito, S. Microvascular mechanisms by which the combretastatin A-4 derivative AC7700 (AVE8062) induces tumour blood flow stasis. *Br. J. Cancer* **2003**, *89*, 1334–1344.
- (11) (a) Koyanagi, N.; Nagasu, T.; Fujita, F.; Watanabe, T.; Tsukahara, K.; Funahashi, Y.; Fujita, M.; Taguchi, T.; Yoshino, H.; Kitoh, K. In vivo tumor growth inhibition produced by a novel sulfonamide, E7010, against rodent and human tumors. *Cancer Res.* **1994**, *54*, 1702–1706. (b) Segreti, J. A.; Polakowski, J. S.; Koch, K. A.; Marsh, K. C.; Bauch, J. L.; Rosenberg, S. H.; Sham, H. L.; Cox, B. F.; Reinhart, G. A. Tumor selective antivasculature effects of the novel antimitotic compound ABT-751: An in vivo rat regional hemodynamic study. *Cancer Chemother. Pharmacol.* **2004**, *54*, 273–281.
- (12) Wang, R. W.-J.; Rebhun, L. I.; Kupchan, S. M. Antimitotic and antitubulin activity of the tumor inhibitor steganacin. *Cancer Res.* **1977**, *37*, 3071–3079.
- (13) (a) Hoebcke, J.; Nijen, G. V.; Brabander, M. D. Interaction of oncodazole (R 17934), a new anti-tumoral drug, with rat brain tubulin. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 319–324. (b) Samson, F.; Donoso, J. A.; Heller-Bettinger, I.; Watsonand, D.; Himes, R. H. Nocodazole action on tubulin assembly, axonal ultrastructure and fast axoplasmic transport. *J. Pharmacol. Exp. Ther.* **1979**, *208*, 411–417.
- (14) (a) Baguley, B. C.; Holdaway, K. M.; Thomsen, L. L.; Zhuang, L.; Zwi, L. J. Inhibition of growth of colon 38 adenocarcinoma by vinblastine and colchicine: Evidence for a vascular mechanism. *Eur. J. Cancer* **1991**, *27*, 482–487. (b) Hill, S. A.; Lonergan, S. J.; Denekamp, J.; Chaplin, D. J. Vinca alkaloids: Antivasculature effects in a murine tumor. *Eur. J. Cancer* **1993**, *29A*, 1320–1324. (c) Chaplin, D. J.; Pettit, G. R.; Parkins, C. S.; Hill, S. A. Antivasculature approaches to solid tumour therapy: Evaluation of tubulin binding agents. *Br. J. Cancer* **1996**, *74* (Suppl. XXVII), S86–S88. (d) Nihei, Y.; Suzuki, M.; Okano, A.; Tsuji, T.; Akiyama, Y.; Tsuruo, T.; Saito, S.; Hori, K.; Sato, Y. Evaluation of antivasculature and antimitotic effects of tubulin binding agents in solid tumor therapy. *Jpn. J. Cancer Res.* **1999**, *90*, 1387–1396. (e) Daenen, L. G. M.; Roodhart, J. M. L.; Shaked, Y.; Voest, E. E. Vascular disrupting agents (VDAs) in anticancer therapy. *Curr. Clin. Pharmacol.* **2010**, *5*, 178–185.
- (15) (a) Kanoh, K.; Kohno, S.; Asari, T.; Harada, T.; Katada, J.; Muramatsu, M.; Kawashima, H.; Sekiya, H.; Uno, I. (–)-Phenylahistin: A new mammalian cell cycle inhibitor produced by *Aspergillus ustus*. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2847–2852. (b) Kanoh, K.; Kohno, S.; Katada, J.; Takahashi, J.; Uno, I. (–)-Phenylahistin arrests cells in mitosis by inhibiting tubulin polymerization. *J. Antibiot.* **1999**, *52*, 134–141. (c) Kanoh, K.; Kohno, S.; Katada, J.; Hayashi, Y.; Muramatsu, M.; Uno, I. Antitumor activity of phenylahistin in vitro and in vivo. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 1130–1133.
- (16) (a) Fenical, W.; Jensen, P. R.; Cheng, X. C. Halimide, a cytotoxic marine natural product, derivatives thereof, and therapeutic use in inhibition of proliferation. PCT Int. Appl. WO 9948889, 1999. (b) Fenical, W.; Jensen, P. R.; Cheng, X. C. U.S. patent US6069146, 2000.
- (17) (a) Kanoh, K.; Kohno, S.; Katada, J.; Takahashi, J.; Uno, I.; Hayashi, Y. Synthesis and biological activities of phenylahistin derivatives. *Bioorg. Med. Chem.* **1999**, *7*, 1451–1457. (b) Hayashi, Y.; Orikasa, S.; Tanaka, K.; Kanoh, K.; Kiso, Y. Total synthesis of anti-microtubule diketopiperazine derivatives: Phenylahistin and auranti-amine. *J. Org. Chem.* **2000**, *65*, 8402–8405.
- (18) Kanzaki, H.; Yanagisawa, S.; Kanoh, K.; Nitoda, T. A novel potent cell cycle inhibitor dehydrophenylahistin: Enzymatic synthesis and inhibitory activity toward sea urchin embryo. *J. Antibiot.* **2002**, *55*, 1042–1047.
- (19) (a) Nicholson, B.; Lloyd, G. K.; Miller, B. R.; Palladino, M. A.; Kiso, Y.; Hayashi, Y.; Neulteboom, S. T. C. NPI-2358 is a tubulin-depolymerizing agent: In-vitro evidence for activity as a tumor vascular-disrupting agent. *Anti-Cancer Drugs* **2006**, *17*, 25–31. (b) Mita, M. M.; Spear, M. A.; Yee, L. K.; Mita, A. C.; Heath, E. I.; Papadopoulos, K. P.; Federico, K. C.; Reich, S. D.; Romero, O.; Malburg, L.; Pilat, M.; Lloyd, G. K.; Neulteboom, S. T. C.; Cropp, G.; Ashton, E.; LoRusso, P. M. Phase 1 first-in-human trial of the vascular disrupting agent plinabulin (NPI-2358) in patients with solid tumors or lymphomas. *Clin. Cancer Res.* **2010**, *16*, 5892–5899. (d) Ferrer, E.; Bolós, J.; Castañer, R. Plinabulin. *Drugs Future* **2010**, *35*, 11–15.
- (20) (a) Suzuki, M.; Iwasaki, T.; Miyoshi, M.; Okumura, K.; Matsumoto, K. Synthesis of amino acids and related compounds. 6. New convenient synthesis of α -C-acylamino acids and α -amino ketones. *J. Org. Chem.* **1973**, *38*, 3571–3575. (b) Makino, K.; Okamoto, N.; Hara, O.; Hamada, Y. Efficient enantioselective synthesis of (2R,3R)- and (2S,3S)-3-hydroxyleucines and their diastereomers through dynamic kinetic resolution. *Tetrahedron: Asymmetry* **2001**, *12*, 1757–1762.
- (21) (a) Weitman, M.; Lerman, L.; Cohen, S.; Nudelman, A.; Major, D. T.; Gottlieb, H. E. Facile structural elucidation of imidazoles and oxazoles based on NMR spectroscopy and quantum mechanical calculations. *Tetrahedron* **2010**, *66*, 1465–1471. (b) Bredereck, H.; Theilig, G. Untersuchungen in der oxazolreihe und umwandlungen von oxazolinen in imidazole mittels formamids (formamidreaktionen, II. mitteil.). *Chem. Ber.* **1952**, *86*, 88–96.
- (22) (a) Loughlin, W. A.; Marshall, R. L.; Carreiro, A.; Elson, K. E. Solution-phase combinatorial synthesis and evaluation of piperazine-2,5-dione derivatives. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 91–94. (b) Folkes, A.; Roe, M. B.; Sohal, S.; Golec, J.; Faint, R.; Brooks, T.;

Charlton, P. Synthesis and in vitro evaluation of a series of diketopiperazine inhibitors of plasminogen activator inhibitor-1. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2589–2592. (c) Gallina, C.; Liberatori, A. Condensation of 1,4-diacetylpiperazine-2,5-dione with aldehydes. *Tetrahedron* **1974**, *30*, 667–673.

(23) Watanabe, T.; Ishikawa, T. Mild air-oxidation of 1,3-dicarbonyl compounds with cesium salts: Novel α -hydroxylation accompanied by partial hydrolysis of malonate derivatives. *Tetrahedron Lett.* **1999**, *40*, 7795–7798.

(24) (a) Andreu, J. M.; Perez-Ramirez, B.; Gorbunoff, M. J.; Ayala, D.; Timasheff, S. N. Role of the colchicine ring A and its methoxy groups in the binding to tubulin and microtubule inhibition. *Biochemistry* **1998**, *37*, 8356–8368. (b) Nam, N.-H.; Kim, Y.; You, Y.-J.; Hong, D.-H.; Kim, H.-M.; Ahn, B.-Z. Synthesis and anti-tumor activity of novel combretastatins: Combretocyclopentenones and related analogues. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1955–1958.

(25) (a) Böhm, H.-J.; Banner, D.; Bendels, S.; Kansy, M.; Kuhn, B.; Müller, K.; Obst-Sander, U.; Stahl, M. Fluorine in medicinal chemistry. *ChemBioChem* **2004**, *5*, 637–643. (b) Olsen, J. A.; Banner, D. W.; Seiler, P.; Obst-Sander, U.; D'Arcy, A.; Stihle, M.; Müller, K.; Diederich, F. A fluorine scan of thrombin inhibitors to map the fluorophilicity/fluorophobicity of an enzyme active site: Evidence for C–F \cdots C=O interactions. *Angew. Chem., Int. Ed.* **2003**, *42*, 2507–2511. (c) Olsen, J. A.; Banner, D. W.; Seiler, P.; Wagner, B.; Tschoop, T.; Obst-Sander, U.; Kansy, M.; Müller, K.; Diederich, F. Fluorine interactions at the thrombin active site: Protein backbone fragments H–C α –C=O comprise a favorable C–F environment and interactions of CF with electrophiles. *ChemBioChem* **2004**, *5*, 666–675.

(26) To measure adequate intensity of intrinsic fluorescence derived from tubulin, highly concentrated tubulin solution (0.5 μ M) was used in tubulin binding assay. Therefore, K_d for tubulin binding was presented in the micromolar range. The K_d value of colchicine, which targets microtubule, is also presented in the micromolar range, although the IC₅₀ value of colchicine is shown in the nanomolar range.

(27) (a) Brossi, A.; Boye, O.; Muzaffar, A.; Yeh, H. J. C.; Toome, V.; Wegrzynski, B.; George, C. aS_{7S}-Absolute configuration of natural (–)-colchicine and allo-congeners. *FEBS Lett.* **1990**, *262*, 5–7. (b) Yeh, H. J. C.; Chrzanowska, M.; Brossi, A. The importance of the phenyl-tropolone 'aS' configuration in colchicine's binding to tubulin. *FEBS Lett.* **1988**, *229*, 82–86.

(28) (a) Zhang, S.-X.; Feng, J.; Kuo, S.-C.; Brossi, A.; Hamel, E.; Tropsha, A.; Lee, K.-H. Antitumor agents. 199. Three-dimensional quantitative structure–activity relationship study of the colchicine binding site ligands using comparative molecular field analysis. *J. Med. Chem.* **2000**, *43*, 167–76. (b) Kim, S.; Min, S. Y.; Lee, S. K.; Cho, W.-J. Comparative molecular field analysis study of stilbene derivatives active against A549 lung carcinoma. *Chem. Pharm. Bull.* **2003**, *51*, 516–521. (c) Combes, S.; Barbier, P.; Douillard, S.; McLeer-Florin, A.; Bourgarel-Rey, V.; Pierson, J.-T.; Fedorov, A. Y.; Finet, J.-P.; Boutonnat, J.; Peyrot, V. Synthesis and biological evaluation of 4-aryl coumarin analogues of combretastatins. Part 2. *J. Med. Chem.* **2011**, *54*, 3153–3162.

(29) Yamazaki, Y.; Kohno, K.; Yasui, H.; Kiso, Y.; Akamatsu, M.; Nicholson, B.; Deyanat-Yazdi, G.; Neuteboom, S.; Potts, B.; Lloyd, G. K.; Hayashi, Y. Tubulin photoaffinity labeling with biotin-tagged derivatives of potent diketopiperazine antimicrotubule agents. *ChemBioChem* **2008**, *9*, 3074–3081.

(30) Cramer, R. D. III; Patterson, D. E.; Bunce, J. D. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J. Am. Chem. Soc.* **1988**, *110*, 5959–5967.

(31) Yamazaki, Y.; Kido, Y.; Hidaka, K.; Yasui, H.; Kiso, Y.; Yakushiji, F.; Hayashi, Y. Tubulin photoaffinity labeling study with a plinabulin chemical probe possessing a biotin tag at the oxazole. *Bioorg. Med. Chem.* **2011**, *19*, 595–692.

(32) Mitchell, S. S.; Nicholson, B.; Teisan, S.; Lam, K. S.; Potts, B. C. M. Aureovercillactam, a novel 22-atom macrocyclic lactam from the marine actinomycete *Streptomyces aureovercillatus*. *J. Nat. Prod.* **2004**, *67*, 1400–1402.

(33) Castoldi, M.; Popov, A. V. Purification of brain tubulin through two cycles of polymerization–depolymerization in a high-molarity buffer. *Protein Expression Purif.* **2003**, *32*, 83–88.