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Structure−Activity Relationship and in Vitro and in Vivo Evaluation of the Potent Cytotoxic Anti-microtubule Agent *N*-(4-Methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium Chloride and Its Analogues As Antitumor Agents

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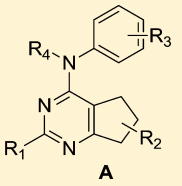
Supporting Information

ABSTRACT: A series of 21 substituted cyclopenta[*d*]pyrimidines were synthesized as an extension of our discovery of the parent compound (±)-**1**·HCl as an anti-microtubule agent. The structure−activity relationship indicates that the *N*-methyl and a 4*N*-methoxy groups appear important for potent activity. In addition, the 6-substituent in the parent analogue is not necessary for activity. The most potent compound **30**·HCl was a one to two digit nanomolar inhibitor of most tumor cell proliferations and was up to 7-fold more potent than the parent compound (±)-**1**·HCl. In addition, **30**·HCl inhibited cancer cell proliferation regardless of Pgp or βIII-tubulin status, both of which are known to cause clinical resistance to several anti-tubulin agents. In vivo efficacy of **30**·HCl was demonstrated against a triple negative breast cancer xenograft mouse model. Compound **30**·HCl is water-soluble and easily synthesized and serves as a lead compound for further preclinical evaluation as an antitumor agent.



A

INTRODUCTION

Microtubule targeting agents have proven to be some of the most valuable drugs used in the treatment of both solid tumors and hematological malignancies.[1−4] Microtubule targeting agents are classified into two groups: microtubule stabilizers, which promote tubulin polymerization, typified by the taxanes and the epothilones, and inhibitors of microtubule assembly, which include the vinca alkaloids and colchicine site agents. At low antiproliferative concentrations, both types of agents suppress microtubule dynamics without great effects on net microtubule mass, leading to mitotic arrest and subsequent cell death.[2,4]

Inhibitors of tubulin assembly are known to bind to a variety of sites on tubulin, two of which have been extensively studied. The first is the vinca site to which vincristine, vinblastine, and vinorelbine bind. These vinca alkaloids are important in the treatment of hematological malignancies, non-small-cell lung cancer, and testicular cancer.[1,5,6] The second site can be occupied by colchicine and a diverse collection of small molecules known as colchicine site agents. This binding site is found primarily on the β-tubulin subunit, at its interface with α-tubulin.[7] Colchicine itself is not used in the treatment of cancer due to its high toxicity. However, it is approved for use in the treatment of gout and familial Mediterranean fever. While there are no clinically approved anticancer agents that bind to the colchicine site on tubulin, a number of combretastatins or closely related analogues are currently being tested in clinical trials.[8] In preclinical studies combretastatin A-4 (CA-4) was shown to have properties superior to other colchicine binding agents and an impressive vascular disrupting activity leading to its clinical development.[9−11]

While the taxanes and the vinca alkaloids have had unprecedented success in cancer chemotherapy, innate and acquired resistance is a major limitation to their clinical use. Overexpression of the P-glycoprotein (Pgp) drug transporter has been reported following treatment with microtubule targeting agents, and Pgp overexpression correlates with poor prognosis.[12−16] The clinical failure of Pgp inhibitors highlights the need for the development of agents that are not substrates for Pgp. The taxanes, paclitaxel and docetaxel, and the vinca alkaloids are well-known substrates for Pgp, and a recent report

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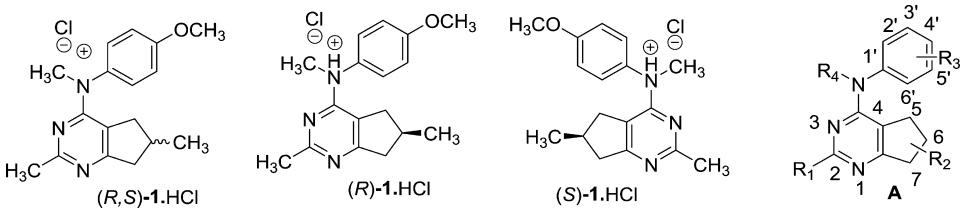

Figure 1. Structure of (±)-**1**·HCl and **A**.

noted that the newer microtubule stabilizer, ixabepilone, is also a substrate for Pgp.[17] A second mechanism of drug resistance involves the overexpression of specific subtypes of β-tubulin, particularly the overexpression of the βIII isotype, which has been shown to be associated with resistance to paclitaxel, docetaxel, and vinorelbine.[18−21] Colchicine site agents are hypothesized to have advantages over currently available microtubule targeting drugs because of their ability to circumvent βIII-mediated resistance. Thus, the clinical development of new colchicine site agents may provide new opportunities for patients whose tumors express this tubulin isotype.[22] Another major limitation of some microtubule targeting drugs is their poor aqueous solubility.[2] This property of the taxanes and ixabepilone require formulation in Cremophor, which is linked with hypersensitivity reactions.[6] Thus, the discovery and development of potent, water-soluble colchicine site agents may provide significant advances in cancer chemotherapy.

We[23,24] recently reported the discovery of a potent antitubulin compound (*R*,*S*)-*N*-(4-methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidine-4-aminium chloride, (±)-**1**·HCl (Figure 1), and identified the contribution of the two enantiomers [(*S*)-**1**·HCl and (*R*)-**1**·HCl] to the antitubulin and cytotoxic properties of the racemate (Figure 1). Compound (±)-**1**·HCl is a water-soluble colchicine site agent that inhibits microtubule assembly and the growth of cancer cells with a GI_{50} in the nanomolar range. In addition, compound (±)-**1**·HCl overcomes drug resistance associated with overexpression of Pgp and βIII-tubulin. Herein we report the synthesis of a variety of analogues of (±)-**1**·HCl with variations at R_1, R_2, R_3, and R_4 in structure A (Figure 1) to determine the structure−activity relationship (SAR) of the cyclopenta[*d*]pyrimidine scaffold for anti-microtubule and antitumor activity. These small molecule derivatives are readily synthesized, are potent, and show better efficacy against both sensitive and multidrug-resistant cancer cell lines.

RATIONALE AND SYNTHESIS

The modifications of (±)-**1**·HCl were focused on variations of R_1, R_2, R_3, and R_4, as shown in structure **A** (Figure 1). We[24] previously reported that the 4′-OCH_3 moiety is important for activity and that the preferable location of the methoxy group is the *para*-position rather than the *meta*- or *ortho*-positions. In addition, we established that an 4-*N*-methyl was preferred over an unsubstituted NH. It was now of interest to determine the biological activity of other variations on the aniline ring, as well as the cyclopenta[*d*]pyrimidine scaffold, in an attempt to optimize activity.

Compounds **3**−**5** (Table 1) were designed to isosterically replace the oxygen of (±)-**1**·HCl with a sulfur atom at the *para*-position of the phenyl ring (**3**), and **4**−**5** provide an electron withdrawing group at the *para*- and *meta*-positions, respectively, of the phenyl ring. In compound **10** (Table 1), the 2-CH_3 of (±)-**1**·HCl is replaced with a 2-NH_2 moiety to determine the importance of potential hydrogen bonding ability at this position. The N4−H isomer (**9**) was also synthesized to confirm that the N4-alkyl (methyl) group is necessary for activity as in (±)-**1**·HCl. Compound **30**·HCl (Table 1) lacks the 6-methyl moiety of (±)-**1**·HCl and was synthesized to determine the importance of the 6-CH_3 group for biological activity.

The first three compounds synthesized were **3**·HCl to **5**·HCl (Scheme 1), which replace the 4-OCH_3 with a 4-SCH_3 moiety (**3**·HCl) or introduce an electron-withdrawing group at the *para*- (**4**·HCl) or *meta*-position (**5**·HCl) in the aniline ring. The free bases of **3**−**5** were synthesized from **2**[14] using the appropriate *N*-methyl anilines in *i*-PrOH in the presence of 2−3 drops of conc. HCl. Compounds **3**·HCl to **5**·HCl precipitated out as white solids when anhydrous HCl gas was bubbled through a solution of the corresponding free base in anhydrous ether.

The next structural modification involved the replacement of the 2-methyl group with a 2-amino group. We have reported molecular modeling of our lead compounds (±)-**1**·HCl and the (*R*)- and (*S*)-enantiomers.[23,24] Using similar methodology, we determined that the 2-NH_2 could function as both a potential hydrogen donor and a potential hydrogen acceptor and could afford additional H-bonds with the amino acids in the colchicine binding pocket of tubulin, such as Lys254 and Asn258.[24] Thus, it was of interest to synthesize **9** and **10**. Compounds **9** and **10** (Scheme 2) were obtained starting from the commercially available 3-methyladipic acid **6** following a modified synthetic route reported for **1**.[24] Reaction of **6** with conc. H_2SO_4 at reflux in ethanol and a Dieckmann condensation in the presence of elemental sodium in toluene followed by treatment with guanidine carbonate afforded **7** (34%). Chlorination of **7** to **8** (34%) was accomplished by heating with $POCl_3$ for 3 h. Nucleophilic substitution of **8** with appropriate anilines in *i*-PrOH gave **9** (55%) and **10** (26%), respectively, as white solids. Compound **10**·HCl was obtained (65%) as a white precipitate when anhydrous HCl gas was bubbled through a solution of **10** in anhydrous ether.

A logical extension of the 6-CH_3 group of **1** was homologation of the methyl group to determine spatial requirements for biological activity at the 6-position. Target compounds **19** and **20** were synthesized as shown in Scheme 3. Ring-opening of 4-vinyl-1-cyclohexene 1,2-epoxide **11** in dilute HCl afforded the diol **12** (85%).[25,26] Compound **12** was treated with tris(cetylpyridinium) 12-tungstophosphate (CWP), which was prepared independently from 12-tungstophosphoric acid and 3 equiv of cetylpyridinium chloride as catalyst in *t*-BuOH in the presence of hydrogen peroxide to give dioic acid **13**.[27] Compound **13** was treated with thionyl chloride in methanol to afford the dimethyl ester **14** (20% yield over two steps). Condensation of **14** with guanidine carbonate and acetamidine hydrochloride afforded bicyclic **15** and **16**, respectively.

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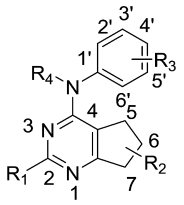
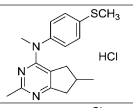
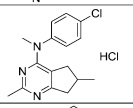
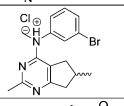
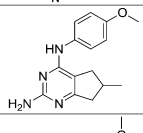
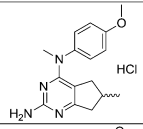
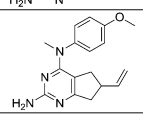
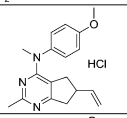
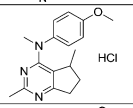
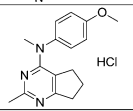
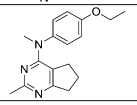
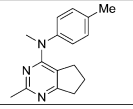
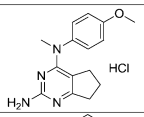
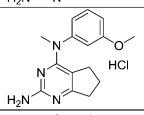
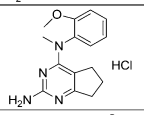
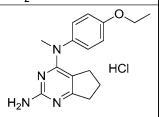
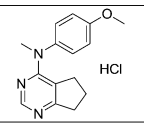
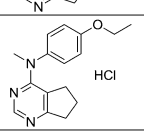
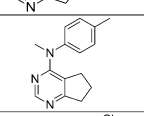
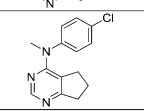
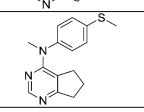
Table 1. IC_{50} Values for Growth Inhibition for Compounds Tested in MDA-MB-435 Cells and EC_{50} for Cellular Microtubule Depolymerization



general structure of targets

Compd # (Original ID)	Structure	IC_{50} ± SD in MDA-435 Cells	EC_{50} for Microtubule Depolymerization in A·10 Cells
(±)-**1**·HCl		17.0 ± 0.7 nM	56 nM
(±)-**3**·HCl		15.6 ± 6 nM	64 nM
(±)-**4**·HCl		2.3 ± 0.2 μM	No activity up to 40 μM
(±)-**5**·HCl		1.5 ± 0.2 μM	15 μM
(±)-**9**		>10 μM	No activity up to 40 μM
(±)-**10**·HCl		29.9 ± 1.2 nM	143 nM
(±)-**19**		205.2 ± 8.5 nM	899 nM
(±)-**20**·HCl		73.2 ± 2.4 nM	140 nM
(±)-**26**·HCl		18.8 ± 0.4 nM	47 nM
30·HCl		7.0 ± 0.7 nM	25.9 nM
31·HCl		15.1± 0.2 nM	64 nM
32		360 ± 8.7 nM	10.5 μM

Compd # (Original ID)	Structure	IC_{50} ± SD in MDA-435 Cells	EC_{50} for Microtubule Depolymerization in A·10 Cells
35·HCl		16.7 ± 0.8 nM	110 nM
36·HCl		>1 μM	No activity up to 10 μM
37·HCl		>1 μM	No activity up to 10 μM
38·HCl		20.3 ± 0.3 nM	148 nM
43·HCl		43.0 ± 1.1 nM	655 nM
44·HCl		48.7 ± 0.7 nM	730 nM
45		ND	No activity up to 10 μM
46		ND	No activity up to 10 μM
47		42.8 ± 0.9 nM	675.2 nM

Chlorination of **15,16** to **17,18** was performed at reflux in $POCl_3$ for 3 h. Nucleophilic substitution of **17,18** with 4-methoxy-*N*-methylaniline in *i*-PrOH afforded compounds, respectively. Compound **20** was a liquid and was converted to the corresponding HCl salt by bubbling anhydrous HCl gas through a solution of **20** in anhydrous ether.

Moving the 6-methyl group to the 5-position was anticipated to influence the conformation of the *N*-methylaniline moiety and to decrease its flexibility. Thus the regio 5-methyl isomer of **1** was expected to provide information on the importance of the methyl at the 6-position in addition to the conformational flexibility of the *N*-methylaniline. The 5-methyl analogue (±)-**26** was synthesized from commercially available methyl 3-oxoheptanoate **21** (Scheme 4). Compound **21** was treated with *p*-acetamidobenzene sulfonylazide in the presence of triethylamine via diazo-transfer to afford diazo **22** (63%).[28]

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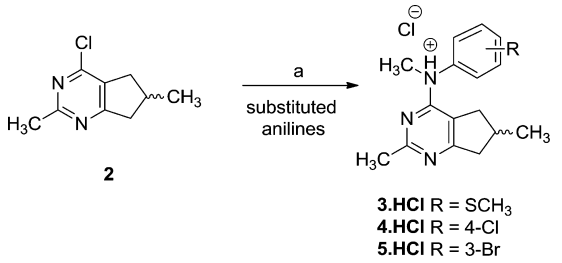
Scheme 1[a]



[a]Conditions: (a) (1) *i*-PrOH, 2−3 drops of HCl, substituted *N*-methyl-aniline; (2) anhydrous HCl gas, anhydrous ether.

Compound **22** was converted to cyclopentane (±)-**23** (43%) with a rhodium(II) acetate mediated intramolecular C−H insertion.[28,29] Condensation of **23** with acetamidine hydrochloride in the presence of *t*-BuOK afforded bicyclic (±)-**24** (79%). Chlorination of (±)-**24** to (±)-**25** was performed at reflux with $POCl_3$ for 3 h (32%). Nucleophilic aromatic substitution of (±)-**25** with 4-methoxy-*N*-methylaniline in *i*-PrOH afforded the free base (±)-**26**, which was converted to the HCl salt (±)-**26** as described above.

It was also of interest to ascertain the importance of the 6-CH_3 group of (±)-**1**·HCl to biological activity. In addition, the 4*N*-ethoxy analogue was synthesized as a simple homologue of (±)-**1**·HCl to determine size requirements at the 4*N*-position. The synthesis of the 2-methyl-6-desmethyl compounds **30**−**32** is shown in Scheme 5. Condensation of methyl-2-oxocyclopentane carboxylate **27** with acetamidine hydrochloride in the presence of *t*-BuOK afforded bicyclic **28** (81%). Chlorination of **28** with $POCl_3$ at reflux for 3 h afforded **29** (69%). Compound **29** was unstable and easily reverts to **28**. Thus, immediate reaction of **29** with the appropriate *N*-methylanilines in *i*-PrOH in the presence of 2−3 drops of concentrated HCl was necessary to afford **30**−**32**. The liquids **30** and **31** were converted to their HCl salts **30**·HCl and **31**·HCl following the method described above.

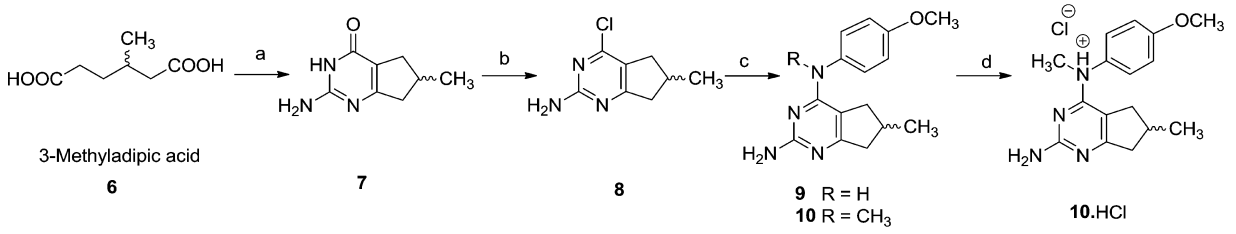
A preliminary evaluation of the antiproliferative effect of compound **30**·HCl (Table 1) showed it to be more potent than the lead (±)-**1**·HCl. Thus, the 2-amino analogues of **30**·HCl, with variations in the aniline ring substitutions, were also synthesized. Target compounds **35**−**38** were prepared as shown in Scheme 6. Condensation of **27** with guanidine carbonate in the presence of *t*-BuOK afforded bicyclic **33** (83%). Chlorination of **33** with $POCl_3$ at reflux for 3 h afforded **34** (56%). Compound **34** reacted with the appropriate *N*-methylanilines in *i*-PrOH in the presence of 2−3 drops of concentrated HCl to afford the free bases **35**−**38**. The HCl salts of **35**−**38** were obtained as solids when anhydrous HCl gas was bubbled through anhydrous ether solutions of the free bases.

Finally, the 2-desmethyl-6-desmethyl compounds **43**−**47** (Scheme 7) were also synthesized, to determine the importance to biological activity of the 2-substituent. Treatment of compound **27** with ammonium formate in methanol gave **39** (91%). Compound **39** reacted with formic acid in acetic anhydride to afford **40** (77%), which was condensed with ammonium formate in formamide to afford bicyclic **41** (83%). Chlorination of **41** with $POCl_3$ at reflux for 3 h gave **42** (60%). Nucleophilic aromatic substitution of **42** with appropriate *N*-methylanilines in *i*-PrOH afforded the free bases **43**−**47**. The liquid compounds **43** and **44** were converted to their HCl salts on treatment with anhydrous HCl gas as described above.

BIOLOGICAL EVALUATIONS AND SAR

Compounds **3**−**5**·HCl, **9**, **10**·HCl, **19**, **20**·HCl, **26**·HCl, **30**−**31**·HCl, **32**, **35**−**38**·HCl, **43**−**44**·HCl, **43**, and **45**−**47** were tested for antiproliferative activity against the drug-sensitive MDA-MB-435 cancer cell line using the sulforhodamine B assay (SRB assay), and IC_{50} values (concentration required to cause 50% inhibition of proliferation) were calculated.[30,31] The ability of the compounds to cause microtubule depolymerization in cells was also evaluated, and an EC_{50}, concentration required to cause approximately 50% microtubule loss, was determined. For substitution on the aniline ring, the data (Table 1) showed that an electron-withdrawing moiety was detrimental to both antiproliferative activity and cellular microtubule loss: the 4-chloro compound **4**·HCl and the 3-bromo compound **5**·HCl were, respectively, about 130- and 90-fold less potent in antiproliferative activity and >700- and 260-fold less potent against microtubule depolymerization than (±)-**1**·HCl. When the 2-methyl group was replaced with a 2-amino, compound **10**·HCl retained the potent antiproliferative effect as it was only about 1.7-fold less active than (±)-**1**·HCl. This indicated that a potential hydrogen bond donor or acceptor at the 2-position is not necessary for potent activity. For the 2-methyl series, an analogue with a one carbon extension at the 6-position, compound (±) **20**·HCl, with a 6-vinyl moiety, had a reduced antiproliferative effect, with an IC_{50} of 73 nM (about 4-fold less potent than (±)-**1**·HCl). When the 6-methyl group was moved to the 5-position, the resulting compound (±)-**26**·HCl had activity comparable (IC_{50} of 19 nM) to that of (±)-**1**·HCl, and the 6-desmethyl compound **30**·HCl had an IC_{50} of 7 nM (2.4-fold more potent than (±)-**1**·HCl). Thus the 6-methyl or vinyl group on the cyclopentane ring does not play a major role in the inhibitory activity and can be removed to afford a more potent analogue, one that has no

Scheme 2[a]



[a]Conditions: (a) (1) Ethanol, conc. sulfuric acid, reflux, 8 h; (2) Na, toluene, reflux, 3 h; (3) guanidine carbonate, *t*-BuOH, *t*-BuOK; (b) $POCl_3$, reflux, 3 h; (c) *N*-methyl-4-methoxyaniline, *i*-PrOH, 2−3 drops HCl; (d) anhydrous HCl gas, ether.

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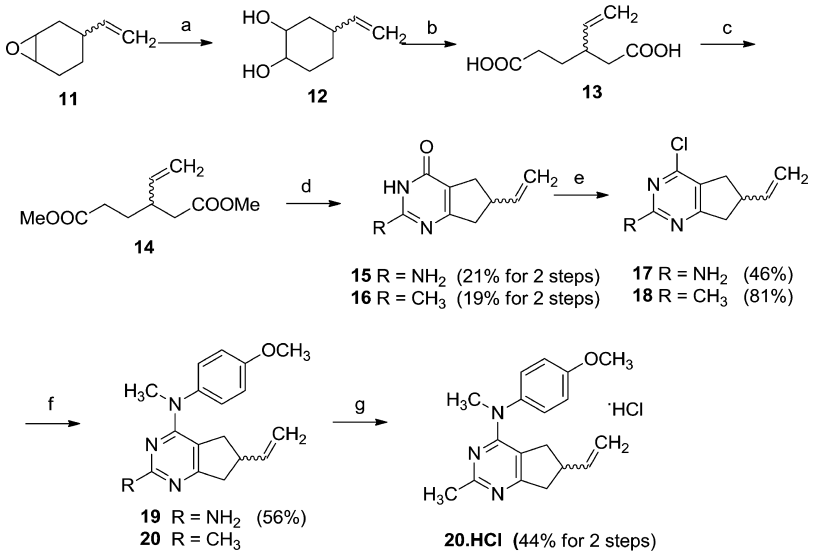
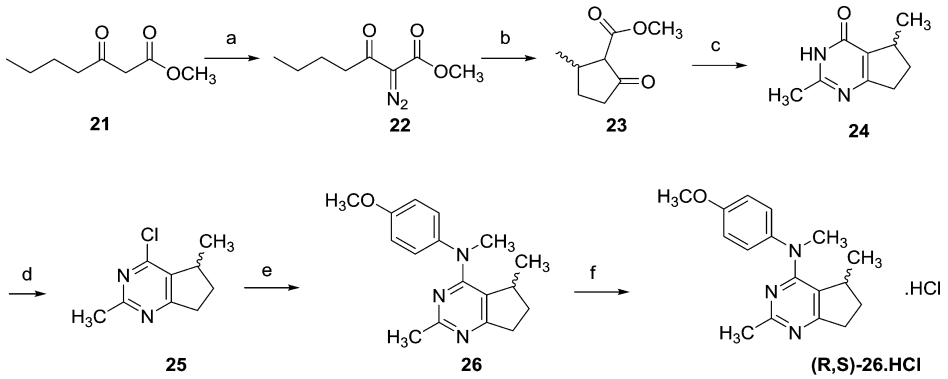
Scheme 3[a]



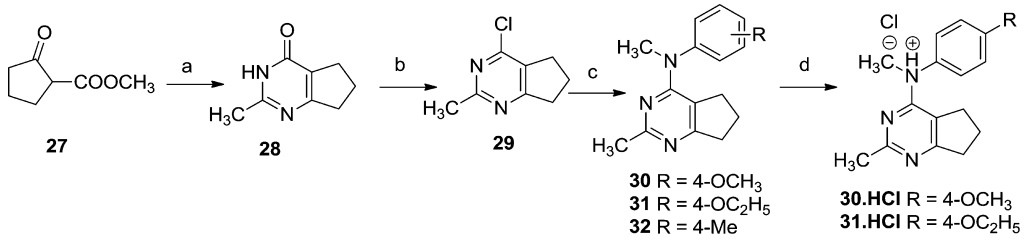
[a]Reagents and conditions: (a) H_2O, HCl; (b) *t*-BuOH, H_2O_2, CWP; (c) $SOCl_2$, CH_3OH; (d) (1) Na, toluene, reflux; (2) guanidine carbonate or acetamidine hydrochloride, *t*-BuOH, *t*-BuOK; (e) $POCl_3$, reflux; (f) *i*-PrOH, conc. HCl, 4-methoxy-*N*-methylaniline; (g) HCl gas, anhydrous ether.

Scheme 4[a]



[a]Reagents and conditions: (a) (1) *p*-acetamidobenzene sulfonylazide, CH_3CN, Et_3N, rt, 8 h; (b) $Rh_2(OAc)_4$, CH_2Cl_2; (c) acetamidine hydrochloride, *t*-BuOK, *t*-BuOH; (d) $POCl_3$, reflux, 3 h; (e) 4-methoxy-*N*-methylaniline, *i*-PrOH, 2 drops of conc. HCl, reflux, 8 h; (f) HCl gas, anhydrous ether.

Scheme 5[a]



[a]Reagents and conditions: (a) acetamidine hydrochloride, *t*-BuOH, *t*-BuOK; (b) $POCl_3$, reflux; (c) *i*-PrOH, conc. HCl, substituted *N*-methylaniline; (d) HCl gas, anhydrous ether.

chiral carbons. Deletion of the 2-methyl group from the heterocyclic ring (**43**·HCl) afforded a compound with lower activity (IC_{50} of 43 nM), 6-fold less potent than **30**·HCl. However, compound **44** with a 4′-ethoxy moiety at the 4′-position on the aniline ring, has comparable potency (IC_{50} of 49 nM) with the 4′-methoxy analogue **43**·HCl. This is consistent with the trend shown in both the 2-methyl and the 2-amino series when comparing **31**·HCl with **30**·HCl and **38**·HCl with **35**·HCl. When the 4′-methoxy group was replaced with a 4′-methylthio group, the resulting analogue **47** showed similar potency (IC_{50} of 43 nM) as **43**·HCl, as is also the case in comparing **3**·HCl with (±)-**1**·HCl. However, when a methyl group was introduced at the 4′-position on the aniline ring (compound **45**), the cellular microtubule depolymerization

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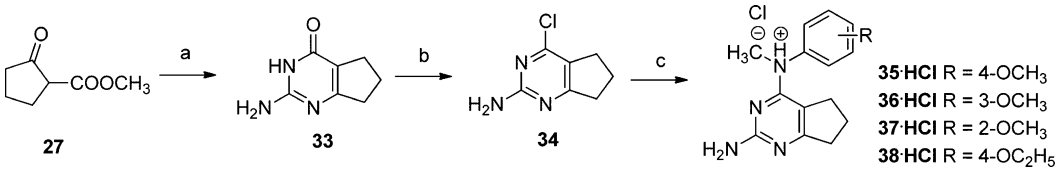
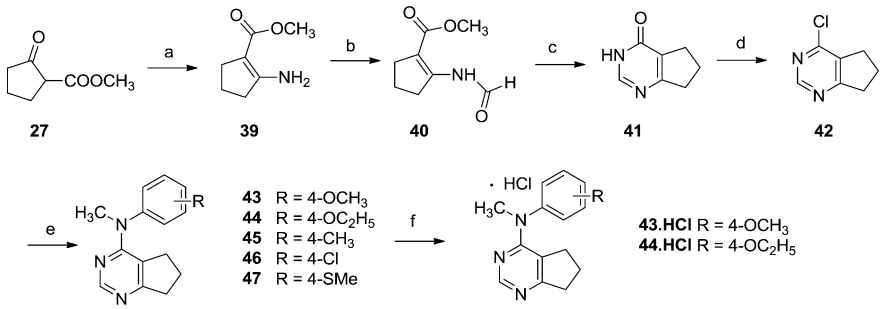
Scheme 6[a]



[a]Reagents and conditions: (a) guanidine carbonate, *t*-BuOH, *t*-BuOK; (b) $POCl_3$, reflux; (c) (1) *i*-PrOH, conc. HCl, substituted-*N*-methylaniline; (2) HCl gas, anhydrous ether.

Scheme 7[a]



[a]Reagents and conditions: (a) ammonium formate, CH_3OH; (b) formic acid and acetic anhydride; (c) formamide, ammonium formate; (d) $POCl_3$, reflux; (e) *i*-PrOH, conc. HCl, 4-methoxy-*N*-methylaniline; (f) HCl gas, anhydrous ether.

activity was lost (compare with compound **44**·HCl). Analogue **46** with an electron-withdrawing group at the 4′-position on the aniline ring had no activity. These results indicate that the substituent at the 4′-position on the aniline ring is crucial for activity, and thus far a substituent with an electron-donating group along with a hydrogen bond acceptor atom, such as OCH_3 or SCH_3, is advantageous.

For analogues with a 2-amino scaffold, the potency trend is similar to the 2-methyl scaffold: the aniline 4′-methoxy moiety is important for potent activity, and the optimum location for the methoxy group is at the 4′-position, since both **36**·HCl, which has a 3′-methoxy, and **37**·HCl, which has a 2′-methoxy, were inactive. With a vinyl group at the 6-position of the cyclopentane ring, compound **19** was about 7-fold less potent than **10**·HCl. When the 6-methyl group was removed from the scaffold, compound **35**·HCl was more potent than **10**·HCl, but **35**·HCl was less potent than the 2-methyl **30**·HCl. Comparing **30**·HCl with **35**·HCl and **43**·HCl, the greatest antiproliferative activity occurred with a 2-methyl moiety. When the methyl moiety on the aniline N was removed to yield compound **9**, antiproliferative activity was lost (IC_{50} >10 μM). This confirmed that the methylation of the aniline N is crucial for potency as observed in the 2-methyl series analogues and is in agreement with our previous reports.[23,24]

Further Mechanistic Studies of 30·HCl. Of the target compounds listed in Table 1, compound **30**·HCl was the most potent analogue, over twice as active as (±)-**1**·HCl, and it was therefore selected for further mechanistic studies.

Evaluation of Anti-microtubule and Antiproliferative Effects. The effects of (±)-**1**·HCl and **30**·HCl on interphase microtubules (Figure 2) were evaluated in a cell-based phenotypic screen. Compounds (±)-**1**·HCl and **30**·HCl led to drastic microtubule depolymerization, similar to the effects of colchicine and CA-4. The EC_{50} was determined to be 7 nM for CA-4, 56 nM for (±)-**1**, and 26 nM for **30**·HCl. Like other microtubule targeting agents, (±)-**1**·HCl and **30**·HCl cause the formation of aberrant mitotic spindles with concurrent mitotic

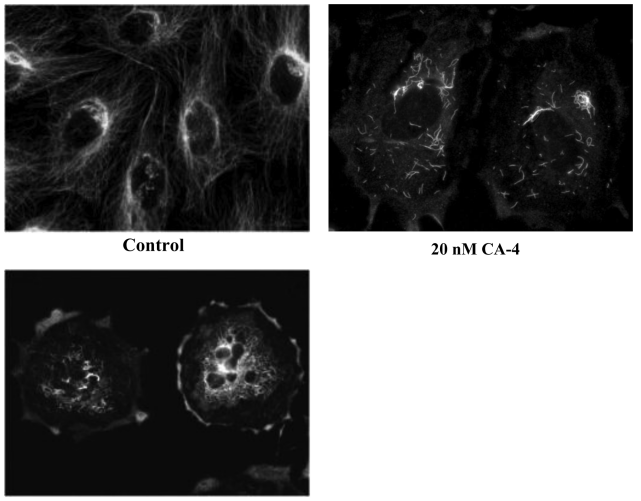


Figure 2. The effects of CA-4 and **30**·HCl on interphase microtubules. A-10 cells were treated with vehicle or compounds as indicated for 18 h and fixed, and microtubules were visualized by indirect immunofluorescence techniques.

accumulation when measured by flow cytometry (Figure 3). These data show that compound **30**·HCl is a potent antimicrotubule agent.

The ability to circumvent Pgp-mediated drug resistance was evaluated using an SK-OV-3 isogenic cell line pair (Table 2). Similar to (±)-**1**·HCl, **30**·HCl was 1.5-times more resistant to cells overexpressing the Pgp drug transporter. On the other hand, paclitaxel, a known Pgp substrate, was 868-times more resistant. These data suggest that **30**·HCl is a poor substrate for Pgp and therefore might have advantages over some clinically useful tubulin-targeting drugs.

A second clinically relevant mechanism of drug resistance to these microtubule agents is the expression of the βIII isotype of tubulin. An isogenic HeLa cell line pair was used to test the

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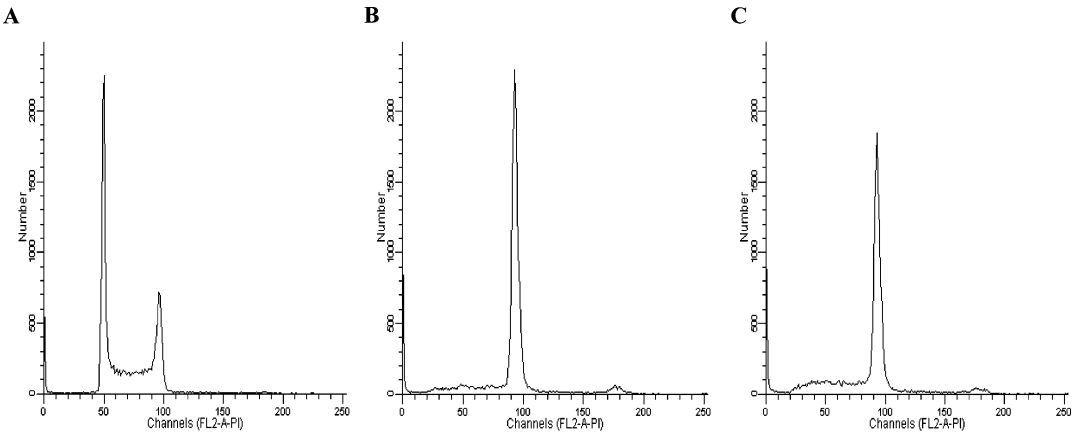

Figure 3. Cell cycle distribution. MDA-MD-435 cells were treated with vehicle (A), 12.5 nM paclitaxel (B), or 25 nM **30**·HCl (C) for 24 h, the cells were harvested, stained, and evaluated by flow cytometry.

Table 2. (±)-1 and 30·HCl Circumvent Clinically Relevant Models of Drug Resistance

	effect of Pgp on drug sensitivity[a]			parental and βIII-tubulin transfected HeLa cells[b]		
	IC_{50} (nM)			IC_{50} (nM)		
drug	SK-OV-3	SK-OV-3 MDR-M6-6	Rr[c]	HeLa	WT βIII	Rr[c]
paclitaxel	3.0 ± 0.06	2,600 ± 270	864	1.6 ± 0.2	7.7 ± 0.2	4.7
CA-4	4.5 ± 0.2	6.6 ± 1.3	1.5	4.7 ± 0.2	5.7 ± 0.4	1.2
(±)-**1**·HCl	38.6 ± 3.1	44.4 ± 3.2	1.2	37.3 ± 4.1	23.9 ± 1.7	0.6
30·HCl	11.3 ± 0.2	16.4 ± 0.4	1.5	9.3 ± 0.3	8.2 ± 0.8	0.8

[a]Antiproliferative effects of (±)-**1**·HCl and **30** HCl in parental and PgP overexpressing cell lines in comparison with other microtubule disrupting agents ($n = 3 \pm$ SD). [b]Effects of the expression of βIII-tubulin on the sensitivity of cell lines to microtubule-targeting agents. The IC_{50} values were determined using SRB assay ($n = 3 \pm$ SD). [c]Relative resistance. The Rr was calculated by dividing the IC_{50} of the transduced cell line by the IC_{50} of the parental cell line.

Table 3. Comparison of Cancer Cell Growth Inhibitory Activity (NCI) GI_{50} (nM) of 30·HCl with (±)-1·HCl

	GI_{50} (nM)			GI_{50} (nM)			GI_{50} (nM)			GI_{50} (nM)	
panel/cell line	(±)-1·HCl	30·HCl	panel/cell line	(±)-1·HCl	30·HCl	panel/cell line	(±)-1·HCl	30·HCl	panel/cell line	(±)-1·HCl	30·HCl
leukemia			colon cancer			melanoma			renal cancer		
CCRF-CEM	16.3	<10	COLO 205	18.3	<10	LOX IMVI	22.7	18.4	786 - 0	34.8	17.8
HL-60(TB)	<10	<10	HCC-2998	24.0	16.4	M14	10.2	<10	A498	<10	<10
K-562	<10	<10	HCT-116	15.1	<10	MDA-MB-435	<10	<10	ACHN	14.6	<10
MOLT-4	31.8	27.8	HCT-15	<10	<10	SK-MEL-2	33.1	11.9	CAKI-1		<10
RPMI-8226	15.7	14.3	HT29	11.8	<10	SK-MEL-28	<10	<10	RXF 393	<10	<10
SR	<10	<10	KM12	<10	<10	SK-MEL-5	<10	<10	SN12C	31.8	33.0
NSCLC			SW-620	<10	<10	UACC-62	<10	<10	TK10	292	42.7
A549/ATCC	24.4	15.0	CNS cancer			ovarian cancer			UO-31	13.4	46.1
EKVX	19.6	34.8	SF-268	15.2	13.9	IGROVI	11.0	12.8	prostate cancer		
HOP-62	19.4	<10	SF-295	<10	<10	OVCAR-3	<10	<10	PC-3	14.6	<10
HOP-92	55.4	16.0	SF-539	11.4	<10	OVCAR-4	26.8	22.9	DU-145	21.4	19.6
NCI-H226	31.5	34.7	SNB-19	36.9	17.0	OVCAR-5	38.5	36.4	breast cancer		
NCI-H23	16.4	22.3	SNB-75	<10	<10	OVCAR-8	32.8	21.8	MCF7	<10	<10
NCI-H322M	59.0	48.8	U251	12.5	<10	NCI/ADR-RES	<10	<10	MDA-MB-231/ATCC	24.6	25.9
NCI-H460	23.4	10.3				SK-OV-3	27.5	14.6	HS 578T	<10	<10
NCI-H522	<10	<10							BT-549	21.9	21.8
									MDA-MB-468	<10	<10

ability of these compounds to overcome the overexpression of βIII. Compound **30**·HCl has a Rr value of 0.8 (Table 2) in this cell line pair suggesting that it can overcome drug resistance mediated by βIII tubulin compared with paclitaxel with a Rr of 4.7.

NCI Cytotoxicity Studies. The improved potency of compound **30**·HCl prompted its selection and evaluation against the NCI 60 tumor cell line panel, and the results (Table 3) are compared with those for (±)-**1**·HCl. The data (Table 3) showed that **30**·HCl was a potent inhibitor (GI_{50} of one to two digit nanomolar) of almost all of the cancer cell lines and in

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most instances was more potent than (±)-**1**·HCl, displaying up to 7-fold greater potency than (±)-**1**·HCl, further establishing **30**·HCl as the lead analogue for further preclinical development.

In Vivo Activity of 30·HCl. Compound **30**·HCl was further evaluated for potential antitumor effects in the MDA-MB-231 triple negative breast cancer murine xenograft model. Total doses ranging from 50.8 to 75.0 mg/kg were well tolerated, resulting in no greater than 9% weight loss, even when dosing was done twice a day for a total of 12 doses of 50 mg/kg. At these concentrations, **30**·HCl was efficacious at preventing tumor growth, causing a 27−44% tumor growth delay (T-C/C) with net log kills of 1.1−1.5 (Table 4 and Figure 4). There were no drug related deaths, and the maximum tolerated dose was never reached. A total dose of 75 mg/kg seemed to be the most efficacious, as it resulted in the smallest tumors 17% and 19% (T/C) at day 21. These initial studies suggest **30**·HCl has antitumor efficacy in this murine model of triple negative breast cancer and should be evaluated further in additional preclinical models.

Table 4. In Vivo Activity of 30·HCl in the MDA-MB-231 Breast Cancer Murine Xenograft Model[a]

	tumor free on day 41	max % relative weight loss	optimal % T/C (day)	growth delay % T-C/C	net log cell kill
vehicle control, 10% DMSO in saline/ Tween 80	0	6.7			
30·HCL, 50 mg/kg, QD × 5, day 14(27)	0	7.7	20 (31)	27	−1.40
30·HCL, 75 mg/kg, QD × 5, day 14(27)	0	7.9	17 (31)	40	−1.10
30·HCL, 75 mg/kg, Q2D × 5, day 14(27)	0	9.0	19 (31)	44	−1.50
30·HCL, 50 mg/kg, BID × 6, day 14(16,18,27,29,31)	0	7.8	25 (31)	32	−1.30

[a]T/C is the median tumor mass of a specific treatment group (T) divided by the median tumor mass of the control group (C), expressed as percent. T-C/C is the median number of days between the time the treatment groups (T) and control group (C) take to reach a predetermined size. This number is normalized to the control group (C) and expressed as a percent. QD refers to daily administration of drug dose, Q2D refers to administration of dose every other day, and BID refers to twice daily dosing. Animals were dosed according to their schedule for 5 or 6 days starting on day 14 and allowed to recover, and dosing started again on day 27. Study consisted of 8 animals per experimental treatment group and 16 animals in the control group.

■ BIOCHEMICAL EVALUATIONS

The effects of (±)-**1**·HCl and **30**·HCl on the polymerization of purified tubulin were evaluated. This allows for the study of the direct interaction of the compounds with their intracellular target. The data (Figure 5) show that this compound is an effective and potent inhibitor of tubulin assembly. Further quantitative studies were conducted and confirmed that **30**·HCl inhibits tubulin assembly with somewhat greater potency than (±)-**1**·HCl and somewhat less potency than CA-4 (Table 5). The ability of **30**·HCl to bind to the colchicine site on tubulin was evaluated by measuring inhibition of [^{3}H]colchicine binding to tubulin. From Table 5, it is clear that at 1 and 5 μM concentrations, **30**·HCl inhibited the binding of [^{3}H]-colchicine, again somewhat more potently than (±)-**1**·HCl and somewhat less potently than CA-4. It is therefore likely that **30**·HCl binds in the colchicine site.

Molecular Modeling and Computational Studies. In an attempt to determine the structural basis of the inhibition of tubulin assembly at the colchicine site, it was of interest to determine the possible conformation(s) and binding poses of the most potent analogue, **30** HCl, in the known colchicine binding site as defined by the crystal structure at 3.58 Å resolution (PDB ID 1SAO).[7] This crystal structure is composed of two αβ-tubulin dimers complexed with *N*-deacetyl-*N*-(2-mercaptoacetyl) colchicine (DAMA-colchicine), a close structural analogue of colchicine (Figure 6). The crystal structure indicates a rather large binding site. A variety of small molecules have been docked into this binding site, and pharmacophores of the various small molecule colchicine site compounds have been proposed.[32−34]

Docking of DAMA-Colchicine. To validate the utility of MOE 2007.09[35] for docking ligands into the binding site, DAMA-colchicine, the ligand in the crystal structure (PDB 1SA0) was built using the molecule builder, energy minimized, and redocked into the binding site. The best-docked pose of DAMA-colchicine displayed an rmsd of 0.95 Å compared with the crystal structure pose of DAMA-colchicine. MOE 2007.09 was thus validated for our docking studies.

Binding of DAMA-Colchicine to Tubulin. A description of the binding of colchicine to the tubulin dimer has been published.[33,36,37] The colchicine binding site is located primarily in β-tubulin at its interface with α-tubulin. The large colchicine binding site (~10 Å × ~10 Å × ~4−5 Å) is composed of the T7 loop, S8 and S9 strands, and H7 and H8 helices, where the β-subunit joins loop T5 of the α-subunit.[33,36] Thrα179 and Valα181 from α-tubulin have been shown to form crucial interactions with DAMA-colchicine and other colchicine site inhibitors.[33,37] Additionally, the oxygen atom of the 3-methoxy in the A-ring of colchicine forms hydrogen bonds with Cysβ241. Side chain atoms of Valα181 and Metβ259 provide additional stabilization for DAMA-colchicine binding.[33,36] The C-ring carbonyl group of DAMA-colchicine hydrogen bonds with Lysβ352 (residue numbers follow the convention in ref 7).

A variety of low-energy conformations (within 1 kcal/mol of the best pose) were obtained on docking the most potent analogue **30**·HCl, as well as other analogues. Figure 7 shows one of the docked conformations of **30**·HCl. This pose was ranked fourth in the results and had a score (−6.9850 kcal/mol) within 1 kcal/mol of the best-scored pose. In this pose, the 4-methoxy of **30**·HCl approximates the 3-methoxy group in the A-ring of DAMA-colchicine. A hydrogen bond between Cysβ241 and the oxygen atom of the 4′-methoxy of **30**·HCl, as seen with the 3-methoxy group of DAMA-colchicine, is formed with this conformation of **30**·HCl. The phenyl ring of **30**·HCl mimics the A-ring of colchicine and could form hydrophobic interactions with Leuβ248, Alaβ250, Leuβ255, and Alaβ316 from the β-tubulin subunit. The 4′-methoxy methyl moiety of **30**·HCl could interact with the side chain of Valβ318 or with the side chain of Ileβ378. The *N*-CH_3 group of **30**·HCl is oriented toward the C5- and C6-bridging carbon atoms of the B-ring of colchicine and interacts with the side chain carbon atoms of Lysβ254 and Alaβ250. Additionally, the *N*-CH_3 moiety of **30**·HCl could form hydrophobic interactions with the side chain carbon atom of Leuβ248 aided by the flexible nature of the protein. These interactions could help stabilize

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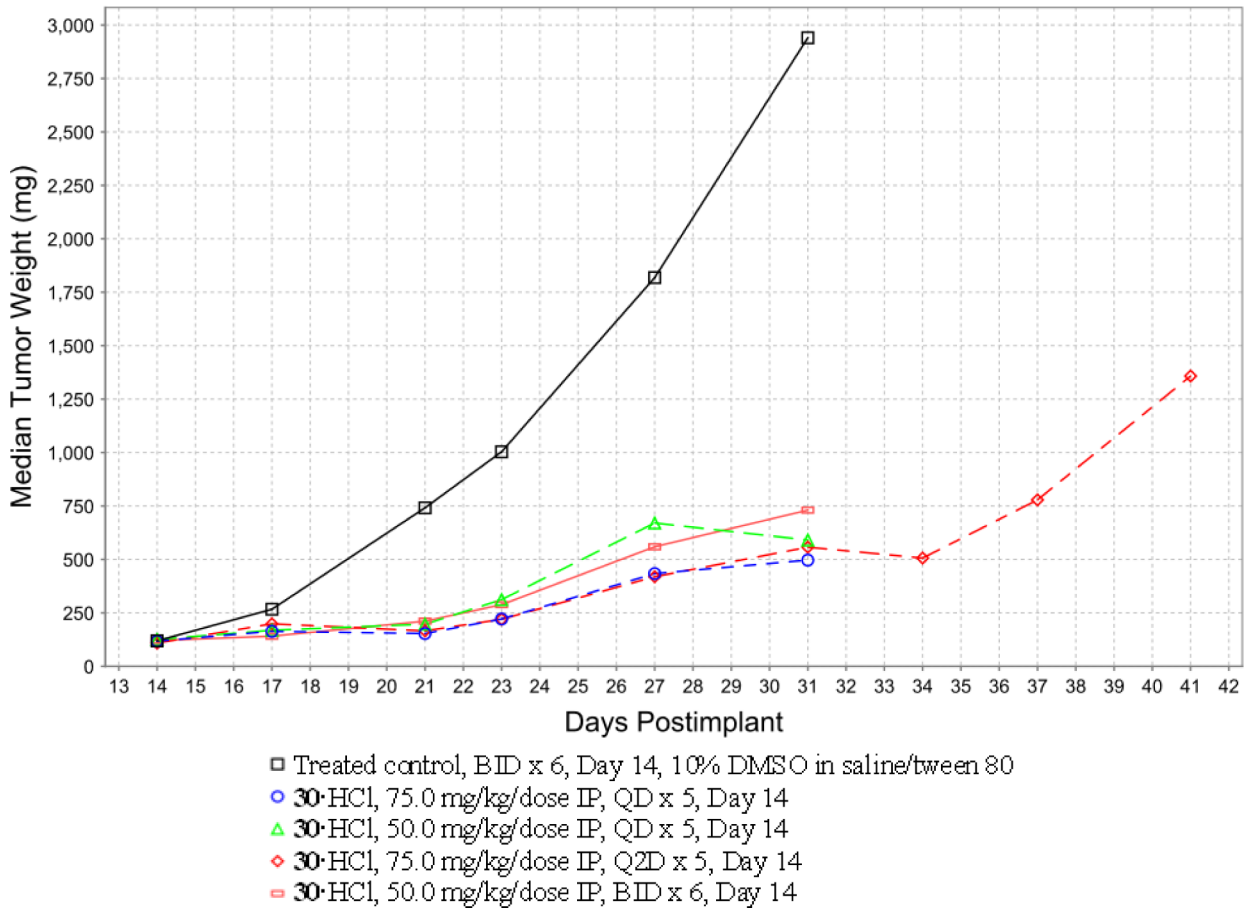

Figure 4. In vivo evaluation of **30**·HCl.

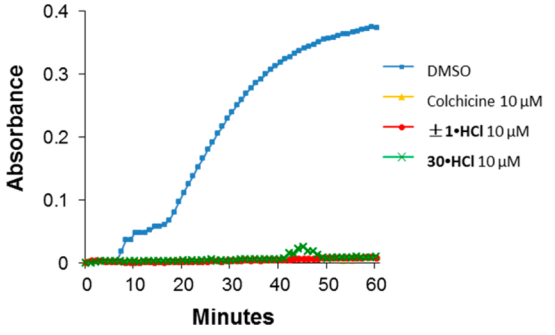

Figure 5. Effects of select compounds on purified tubulin polymerization. Colchicine was used as a positive control. The assays were conducted using porcine tubulin in the presence of 10% glycerol, 2 mg/mL purified tubulin, and GTP.

Table 5. Inhibition of Tubulin Assembly and Colchicine Binding by CA-4, (±)-1·HCl and 30·HCl

compound	inhibition of tubulin assembly, IC_{50} (μM) ± SD	inhibition of colchicine binding, % inhibition ± SD	
		1 μM	5 μM
CA-4	1.0 ± 0.09	88 ± 2	99 ± 0.2
(±)-**1**·HCl	1.9 ± 0.01	60 ± 2	84 ± 3
30·HCl	1.6 ± 0.1	70 ± 2	92 ± 0.7

the docked conformation of **30**·HCl. The *N*-CH_3 moiety was similarly oriented in the docked conformations of other

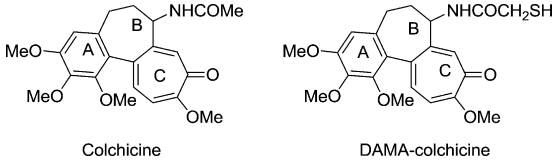

Figure 6. Colchicine and DAMA-colchicine.

analogues (not shown) in this series and explains, in part, the loss of activity of the NH analogue **9**, which would lack these additional *N*-CH_3 interactions with tubulin. Docked poses of compounds with the *N*-CH_3 group consistently scored higher (by ~1 kcal/mol) than those of compounds that lack the *N*-CH_3 group.

The C-ring of DAMA-colchicine partly overlaps the cyclopenta[2,3-*d*]pyrimidine ring of **30**·HCl and interacts with side chain carbon atoms of Lysβ252, Leuβ255, and Asnβ258. The C7 of **30**·HCl overlaps the C9-carbonyl carbon atom of DAMA-colchicine. The C2-methyl group of **30**·HCl could be involved in a potential hydrophobic interaction with Alaα180.

In summary, 21 analogues of the lead compound (±)-**1**·HCl were synthesized and evaluated, and SAR for substitutions in the 2-, 4-, 4*N*-anilinyl, and 6-positions were studied. Compound **30**·HCl, the 6-desmethyl analogue of the lead compound (±)-**1**·HCl, was 2.4-fold more potent than the parent compound as an antiproliferative agent, with single digit nanomolar potency. This analogue also showed promising

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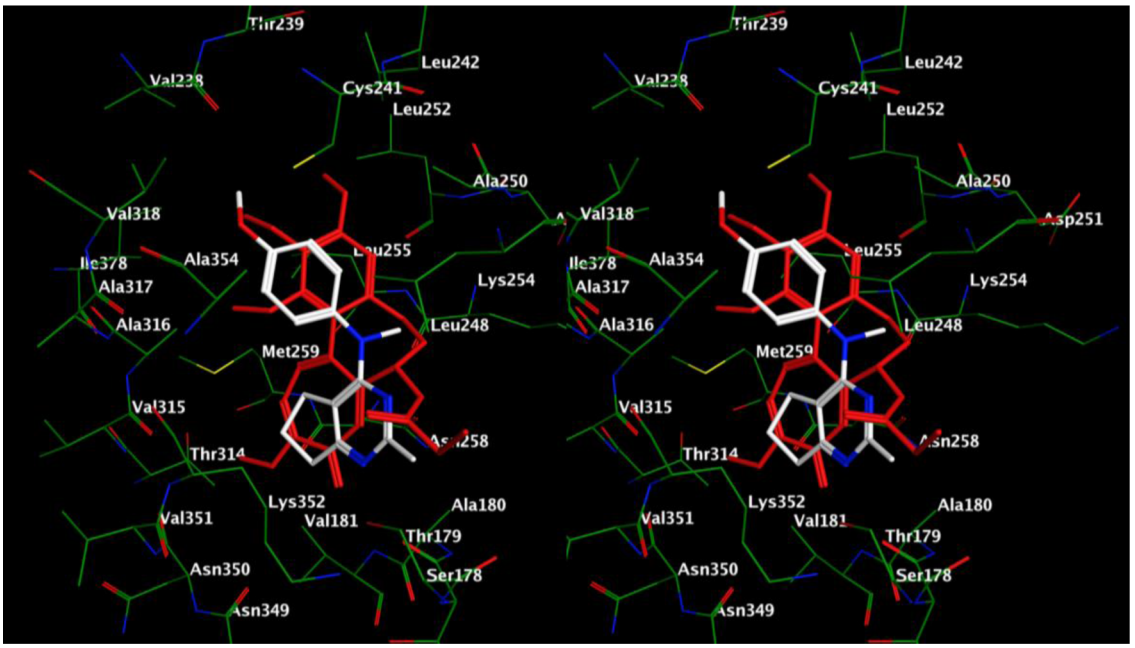

Figure 7. Docking mode of **30**·HCl (white) overlaid with DAMA-colchicine (red).

antitumor activity in vivo and serves as the analogue for further preclinical development.

EXPERIMENTAL SECTION

Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P_2O_5 at 50 °C. Melting points were determined on a digital MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for protons (^{1}H NMR) were recorded on a Bruker Avance II 400 (400 MHz) or on a 500 (500 MHz) NMR systems. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230−400 mesh silica gel (Fisher Scientific) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Elemental compositions are within ±0.4% of the calculated values and indicate >95% purity of the compounds. Fractional moles of water or organic solvents found in some analytical samples could not be prevented despite 24−48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. All solvents and chemicals were purchased from Sigma-Aldrich Co. or Fisher Scientific Inc. and were used as received.

General Procedure for Chlorination. A mixture of the appropriate 4-oxo compound and $POCl_3$ (10 mL) was heated at reflux for 3 h. The reaction mixture was cooled and evaporated at reduced pressure. The residue was diluted with chloroform (50 mL) and neutralized slowly in an ice bath with NH_4OH. The organic portion was washed with water (3 × 30 mL) and dried with anhydrous Na_2SO_4. Concentration of the organic solvent with 1 g of silica gel afforded a dry plug. This plug was placed on the top of a silica gel column and eluted with chloroform. Fractions containing the product were pooled and evaporated to afford the chlorinated compound.

General Procedure for Nucleophilic Displacement from Chlorinated Compound. Chlorinated compound and the appropriate substituted *N*-methyl anilines or aniline were dissolved in isopropyl alcohol (5 mL). To this solution was added 37% HCl (2−3 drops). The mixture was heated at reflux for 3−6 h. Then the reaction mixture was cooled and evaporated at reduced pressure. The residue was diluted with chloroform, neutralized with NH_4OH, and washed with water (2 × 30 mL). After drying with anhydrous Na_2SO_4, the organic solvent with 1 g silica gel was evaporated under reduced pressure to give a dry plug. This plug was placed on the top of a silica gel column and eluted with chloroform. Fractions containing the product were pooled and evaporated to afford pure compound.

General Method To Make HCl Salt from Base. HCl gas was bubbled through the solution of free base in anhydrous ether until no more solid was precipitated. The solid was collected by filtration and dried to give the HCl salt.

*N,2,6-Trimethyl-N-(4-(methylthio)phenyl)-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-aminium Chloride (**3**·HCl).* Compound **3**·HCl was synthesized from **2** (0.06 g, 0.39 mmol) and *N*-methyl-4-methylmercaptoaniline (0.049 g, 0.46 mmol) following the general procedure for nucleophilic displacement from the chlorinated compound and the general method to make a HCl salt from the base to afford, after purification 0.052 g (40% over two steps) as a gray solid: TLC R_f 0.33 (chloroform/methanol, 10:1); mp 214.4−215.5 °C; ^{1}H NMR (DMSO-d_6) δ 0.89 (d, J = 6.8 Hz, 3H), 1.44−1.53 (m, 1H), 1.97−2.06 (m, 1H), 2.30−2.39 (m, 1H), 2.53 (s, 3H), 2.63 (s, 3H), 3.03−3.08 (m, 2H), 3.54 (s, 3H), 7.37 (s, 4H), 15.02 (bs, 1H); Anal. calcd for ($C_{17}H_{22}ClN_3S$·0.1H_2O) C, H, N, Cl, S.

*N-(4-Chlorophenyl)-N,2,6-trimethyl-6,7-dihydro-5H-cyclopenta-[d]pyrimidin-4-aminium Chloride (**4**·HCl).* Compound **4**·HCl was synthesized from **2** (0.25 g, 1.37 mmol) and 4-chloro-*N*-methylaniline (0.19 g, 1.37 mmol) following the general procedure for nucleophilic displacement from the chlorinated compound and the general method to make a HCl salt from the base to afford after purification 0.228 g (58% over two steps) as a white solid: TLC R_f 0.50 ($CHCl_3$/CH_3OH, 10:1); mp 114.4−116.0 °C; ^{1}H NMR (DMSO-d_6) δ ^{1}H NMR (400 MHz, DMSO) δ 0.89 (d, J = 6.8 Hz, 3H), 1.48 (dd, J = 16.3, 6.1 Hz, 1H), 1.99 (dd, J = 16.9, 5.9 Hz, 1H), 2.27−2.41 (m, 1H), 2.62 (s, 3H), 3.05 (dd, J = 17.6, 7.9 Hz, 1H), 3.54 (s, 3H), 7.48 (d, J = 8.7 Hz, 2H), 7.59 (d, J = 8.7 Hz, 2H)., 15.04 (bs, 1H); Anal. calcd. for ($C_{16}H_{18}ClN_3$) C, H, N, Cl.

*N-(3-Bromophenyl)-N,2,6-trimethyl-6,7-dihydro-5H-cyclopenta-[d]pyrimidin-4-aminium Chloride (**5**·HCl).* Compound **5**·HCl was synthesized from **2** (0.085 g, 0.55 mmol) and 3-bromo-*N*-methyl aniline (0.06 mL) following the general procedure for nucleophilic displacement from chlorinated compound and the general method to make a HCl salt from base to afford after purification 0.090 g (52% over two steps) as a white solid: TLC R_f 0.40 ($CHCl_3$/CH_3OH, 10:1); mp 200.5−202.0 °C; ^{1}H NMR (DMSO-d_6) δ 0.86 (d, J = 6.8 Hz, 3H),

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1.37−1.47, 1.89−1.99, 2.43−2.44, 2.98−3.05 (m, 5H), 2.60 (s, 3H), 3.51 (s, 3H), 3.80 (s, 3H), 7.01−7.03, 7.33−7.35 (m, 4H), 14.88 (br, 1H); Anal. calcd. for ($C_{16}H_{19}BrN_3Cl·0.2H_2O$) C, H, N, Cl, Br.

*2-Amino-6-methyl-3,5,6,7-tetrahydro-4H-cyclopenta[d]-pyrimidin-4-one (**7**).* 3-Methyladipic acid (1.60 g, 10 mmol) was heated under reflux in an ethanol/conc. H_2SO_4 solution (35 mL, v/v = 2.5/1) for 8 h. The solution was neutralized with NH_4OH to pH = 7, then diluted with ethyl acetate (100 mL), and washed with water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated to afford a light yellow liquid used in the next step. The liquid was diluted with anhydrous toluene (100 mL), and sodium (0.23 g) was added to the solution. The mixture was heated under reflux for 3 h and cooled, neutralized with a 1 N HCl solution, and washed with water. After drying with anhydrous Na_2SO_4, the organic phase was evaporated to afford a light brown liquid used in the next step. The liquid was diluted with *t*-BuOH. Guanidine carbonate (2.70 g, 15 mmol) and potassium *tert*-butoxide (1.68 g, 15 mmol) were added, and the mixture was heated under reflux overnight. The reaction mixture was cooled, and a precipitate was removed by filtration. The precipitate was washed twice with warm methanol (30 mL × 1, 15 mL × 1). The filtrate and washings were combined and evaporated under reduced pressure, and the residue was purified by column chromatography using chloroform/methanol (100/1) as eluent to afford **7** as a white solid (0.23 g, 19% yield for 3 steps): TLC R_f 0.36 ($CHCl_3$/CH_3OH, 10:1); mp 319−321 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (d, J = 6.8 Hz, 3H), δ 1.35−1.46, 1.99−2.20, 2.38−2.72, 2.92−2.98 (m, 5H), 6.32 (bs, 2H), 10.47 (bs, 1H); Anal. calcd. for ($C_8H_{11}N_3O·0.1CH_3OH$) C, H, N.

*4-Chloro-6-methyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-2-amine (**8**).* Compound **8** was synthesized from compound **7** (0.297 g, 1.8 mmol) following the general procedure for chlorination described above to afford after purification 0.112 g (34%) as an off-white solid: TLC R_f 0.48 ($CHCl_3$/CH_3OH, 10:1); mp 181.5−182.9 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (d, J = 6.8 Hz, 3H), 1.55−1.65, 2.15−2.38, 2.49−2.62, 2.83−2.92 (m, 5H), 6.83 (bs, 2H); Anal. calcd. for ($C_8H_{10}ClN_{33}OH$) C, H, N, Cl.

*N^4-(4-Methoxyphenyl)-6-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine-2,4-diamine (**9**).* Compound **9** was synthesized from **8** (0.1 g, 0.54 mmol) and 4-methoxylaniline (0.074 g, 0.65 mmol) following the general procedure for nucleophilic displacement from chlorinated compound described above to afford after purification 0.045 g (55%) as a light pink solid: TLC R_f 0.28 ($CHCl_3$/CH_3OH, 10:1); mp 187.4−189.5 °C; ^{1}H NMR (DMSO-d_6) δ 1.09 (d, J = 6.8 Hz, 3H), 2.16−2.25 (m, 2H), 2.38−2.47 (m, 1H), 2.74 (dd, J = 16.3, 8.3 Hz, 1H), 2.83 (dd, J = 15.3, 8.2 Hz, 1H), 3.71 (s, 3H), 5.84(bs, 2H), 6.83 (d, J = 9.0 Hz, 2H), 7.64 (d, J = 9.0 Hz, 2H), 8.05 (bs, 1H); Anal. calcd. for ($C_{15}H_{18}N_4O·0.1CHCl_3$) C, H, N.

*N^4-(4-Methoxyphenyl)-N^4,6-dimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidine-2,4-diamine (**10**).* Compound **10** was synthesized from **8** (0.094 g, 0.51 mmol) and *N*-methyl-4-methoxylaniline (0.084 g, 0.61 mmol) following the general procedure for nucleophilic displacement from chlorinated compound described above to afford after purification 0.08 g (55%) as a white solid: TLC R_f 0.26 ($CHCl_3$/CH_3OH, 10:1); mp 146.2−147.5 °C; ^{1}H NMR (DMSO-d_6) δ ^{1}H NMR (400 MHz, DMSO) δ 0.81 (d, J = 6.1 Hz, 3H), 1.26−1.40 (m, 1H), 1.82 (dd, J = 15.2, 7.3 Hz, 2H), 2.00−2.14 (m, 1H), 2.54−2.66 (m, 1H), 3.25 (s, 3H), 3.75 (s, 3H), 5.90 (bs, 2H, NH_2, exch), 6.92 (d, J = 8.4 Hz, 5H), 7.10 (d, J = 8.6 Hz, 5H); Anal. calcd. for ($C_{16}H_{20}N_4O$) C, H, N.

*2-Amino-N-(4-methoxyphenyl)-N,6-dimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-aminium Chloride (**10**·HCl).* The **10**·HCl was prepared from **10** (0.15 g) following the general method to make a HCl salt from base described above to yield 0.11 g (65%) as a white solid: mp 232.8−233.4 °C; ^{1}H NMR (DMSO-d_6) δ 0.84 (d, J = 6.8 Hz, 3H), 1.24−1.36 (m, 1H), 1.74−1.88 (m, 1H), 2.17−2.37 (m, 2H), 2.87 (dd, J = 17.4, 8.2 Hz, 1H), 3.41 (s, 3H), 3.80 (s, 3H), 7.01 (d, J = 8.7 Hz, 2H), 7.31 (d, J = 8.8 Hz, 2H), 7.70 (bs, 2H), 12.93 (bs, 1H); Anal. calcd. for ($C_{16}H_{21}N_4OCl·0.6CH_3OH$) C, H, N, Cl.

*4-Vinylcyclohexane-1,2-diol (**12**).* The suspension of 4-vinyl-1-cyclohexene 1,2-epoxide **11** (3 mL) and conc. H_2SO_4 (3 mL) in distilled water (10 mL) in a 50 mL round-bottom flask was stirred at room temperature overnight. The reaction mixture was diluted with distilled water (30 mL) and extracted with ethyl acetate (20 mL × 3). The organic layer was dried over Na_2SO_4. Concentration of the organic solvent with 6 g of silica gel afforded a dry plug. This plug was placed on the top of a silica gel column and eluted with chloroform/*n*-hexane (1:1). Fractions containing the product were pooled and evaporated to afford **12** as a colorless liquid (2.81 g, 85%): TLC R_f 0.17 (chloroform/methanol, 10:1); ^{1}H NMR (400 MHz, CDCl3) δ 1.31−1.72 (m, 6 H), 2.27−2.35 (m,1 H), 3.41 (p, J = 4.4, 3.6; 1 H), 3.51 (p, J = 4.4, 4.0; 1 H), 4.47 (d, J = 3.6; 1 H, exch), 4.53 (d, J = 4.0; 1 H, exch), 4.87−4.93 (m, 2H), 5.71−5.79 (m, 1 H).

*3-Vinylhexanedioic Acid (**13**).* A mixture of **12** (2.0 g, 14 mmol), 35% H_2O_2 (4.2 mL), and CWP (1.069 g, 5.86 mmol) in *t*-BuOH (50 mL) was heated at reflux for 1 d. Then 10% sodium bisulfate was added to decompose the excess H_2O_2. The mixture was extracted with ethyl acetate (40 mL × 3) and dried over Na_2SO_4. Concentration of the organic solvent with 5 g of silica gel afforded a dry plug. This plug was placed on the top of a silica gel column and eluted with chloroform/methanol (50:1). Fractions containing the product were pooled and evaporated to afford **13** as a colorless liquid (0.68 g, 28%): TLC R_f 0.11 (chloroform/methanol, 10:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.42−1.68 (m, 2 H), 2.13−2.31 (m, 4 H), 2.34−2.45 (m, 1 H), 4.97−5.01 (m, 2 H,), 5.56−5.65 (m, 1 H), 12.05 (bs, 2 H).

*Dimethyl 3-Vinylhexanedioate (**14**).* Thionyl chloride (1.43 mL, 19.6 mmol) was added dropwise to a solution of compound **13** (0.68 g, 3.95 mmol) in methanol (6 mL) in an ice-bath. Then the mixture was stirred at room temperature overnight. Concentration of the organic solvent with 6 g of silica gel afforded a dry plug. This plug was placed on the top of a silica gel column and eluted with hexane/ethyl acetate (50:1). Fractions containing the product were pooled and evaporated to afford **14** as a colorless liquid (0.38 g, 48%): TLC R_f 0.43 (hexane/ethyl acetate, 3:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.47−1.70 (m, 2 H), 2.23−2.43 (m, 5 H), 3.55 (s, 3 H), 3.56 (s, 3 H), 4.96−5.00 (m, 2 H), 5.54−5.64 (m, 1 H).

*2-Amino-6-vinyl-6,7-dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**15**).* To a solution of **14** (1.10 g, 5.5 mmol) in anhydrous toluene, elemental sodium (0.19 g, 8.2 mmol) was added in portions. The mixture was heated at reflux for 3 h. The reaction mixture was neutralized with 1 N HCl and extracted with ethyl acetate (20 mL × 3). The organic layer was dried over Na_2SO_4 and concentrated. The resulting yellow liquid was diluted with *tert*-butanol (15 mL). To this solution were added potassium *tert*-butoxide (1.85 g, 16.4 mmol) and guanidine carbonate (1.49 g, 8.2 mmol). The mixture was heated at reflux overnight. The solid was filtered and washed with hot methanol. Silica gel (3 g) was added to the filtrate, and the solvent was evaporated. The resulting silica gel plug was loaded onto a silica gel column and eluted with chloroform/methanol (50:1). The fractions containing the product (TLC) were pooled, and the solvent was evaporated to give a sticky solid. The solid was washed with anhydrous ether and filtered to afford pure **15** as a white solid (0.21 g, 21% for two steps): TLC R_f 0.17 (chloroform/methanol, 10:1); mp >260 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.62−2.97 (m, 5 H), 4.85−5.06 (m, 2 H), 5.82−5.92 (m, 1 H), 6.36 (bs, 2 H), 10.13 (bs, 1 H); Anal. calcd. for ($C_9H_{11}N_3O·0.15C_4H_{10}O$) C, H, N.

*2-Methyl-6-vinyl-6,7-dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**16**).* To a solution of **14** (1.00 g, 5 mmol) in anhydrous toluene, sodium metal (0.23 g, 10 mmol) was added in small portions. The mixture was heated at reflux for 3 h. The reaction mixture was neutralized with 1 N HCl and extracted with ethyl acetate (20 mL × 3). The organic layer was dried over Na_2SO_4 and concentrated. The resulting yellow liquid was diluted with *t*-BuOH (15 mL). To this solution were added potassium *tert*-butoxide (1.69 g, 15 mmol) and acetamidine hydrochloride (7.5 mmol). The mixture was heated at reflux overnight. The solid obtained on cooling was filtered, and the filtered material was washed with hot methanol. To the filtrate was added silica gel (3 g), and the solvent was removed by evaporation. The resulting silica gel plug was loaded onto a silica gel column and eluted with chloroform/methanol (50:1). The fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford **16** as an off-white solid (0.17 g, 19% over two steps): TLC R_f 0.19

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(chloroform/methanol, 10:1); mp 189.0−190.9 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.62−2.97 (m, 5 H), 2.24 (s, 3 H), 4.90−5.08 (m, 2 H), 5.83−5.98 (m, 1 H), 12.20 (bs, 1 H); Anal. calcd. for ($C_{10}H_{12}N_2O$) C, H, N.

*4-Chloro-6-vinyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-2-amine (**17**).* Compound **17** was synthesized from **15** (0.3 g, 1.69 mmol) following the general chlorination procedure described above to afford after purification 0.152 g (46%) as an off white solid: TLC R_f 0.36 (chloroform/methanol, 10:1); mp 142.8−144.6 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.54−3.74 (m, 5 H), 4.94−5.11 (m, 2 H), 5.79−5.90 (m, 1 H), 6.85 (bs, 2 H); Anal. calcd. for ($C_9H_{10}ClN_3$) C, H, N, Cl.

*4-Chloro-2-methyl-6-vinyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine (**18**).* Compound **18** was synthesized from **16** (0.460 g, 2.61 mmol) following the general procedure for chlorination described above to afford after purification 0.414 g (81%) as a colorless liquid: TLC R_f 0.45 (chloroform/methanol, 10:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.17−3.93 (m, 5 H), 2.56 (s, 3 H), 5.00−5.14 (m, 2 H), 5.84−6.01 (m, 1 H).

*N^4-(4-Methoxyphenyl)-N^4-methyl-6-vinyl-6,7-dihydro-5H-cyclopenta[d]pyrimidine-2,4-diamine (**19**).* Compound **19** was synthesized from **17** (0.1 g, 0.51 mmol) and *N*-methyl-4-methoxyaniline (0.077 g, 0.56 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.084 g (56%) as a white solid: TLC R_f 0.30 (chloroform/methanol, 10:1); mp 144.8−147.0 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.58 (m, 1H), 1.81 (m, 1H), 2.30−2.40 (m, 1H), 2.54−2.70 (m, 2H), 3.27 (s, 3H), 3.76 (s, 3H), 4.84 (dd, J = 20.1, 15.0 Hz, 2H), 5.63−5.77 (m, 1H), 5.92 (bs, 2H), 6.93 (d, J = 8.9 Hz, 2H), 7.13 (d, J = 8.8 Hz, 2H); Anal. calcd. for ($C_{17}H_{20}N_4O$) C, H, N.

*N-(4-Methoxyphenyl)-N,2-dimethyl-6-vinyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine Hydrochloride (**20**·HCl).* Compound **20**·HCl was synthesized from **18** (0.4 g, 2.05 mmol) and *N*-methyl-4-methoxyaniline (0.310 g, 2.26 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification a light yellow liquid: TLC R_f 0.28 (chloroform/methanol, 10:1). Using the general procedure to make a HCl salt described above, the yellow liquid was converted to **20**·HCl, 0.3 g (44% over two steps) as a white solid: mp 191−193.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.64−1.75 (m, 1H), 1.88−1.97 (m, 1H), 2.62 (s, 3H), 2.72−2.76 (m, 1H), 2.88−2.92 (m, 1H), 3.04−3.10 (m, 1H), 3.53 (s, 3H), 3.82 (s, 3H), 4.90−4.95 (m, 2H), 5.70−5.77 (m, 1H), 7.04 (d, J = 9.1 Hz, 1H), 7.36 (d, J = 8.6 Hz, 1H), 15. 06 (bs, 1H); Anal. calcd. for ($C_{18}H_{22}ClN_3O$) C, H, N, Cl.

*Methyl 2-Diazo-3-oxoheptanoate (**22**).* A mixture of methyl 3-oxoheptanoate **21** (0.2 mL, 1.26 mmol), 4-acetamidobenzene sulfonylazide (0.319 g, 1.33 mmol), and trimethylamine (0.35 mL, 2.51 mmol) in acetonitrile (3 mL) was stirred at room temperature for 8 h. The reaction mixture was diluted with 1 N NaOH and extracted with ethyl acetate. After drying with anhydrous Na_2SO_4, the organic solvent with 1 g of silica gel was evaporated under reduced pressure to give a dry plug. This plug was placed on the top of a silica gel column and eluted with 1% ethyl acetate in hexane. Fractions containing the product were pooled and evaporated to afford **22** as a light yellow oil (0.145 g, 63%): TLC R_f 0.52 (hexane/ethyl acetate, 5:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.87 (t, J = 7.4 Hz, 3H), 1.29 (dq, J = 14.7, 7.4 Hz, 2H), 1.51 (dt, J = 15.1, 7.5 Hz, 2H), 2.77 (t, J = 7.4 Hz, 2H), 3.76 (s, 3H).

*2,5-Dimethyl-6,7-dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**24**).* $Rh_2(OAc)_4$ (0.036 g, 0.08 mmol) was suspended in dry dichloromethane (10 mL). A solution of **22** (0.3 g, 1.63 mmol) in dichloromethane was then added dropwise to a stirred slurry of $Rh_2(OAc)_4$ over 3 h. The reaction mixture was then filtered through a fritted funnel to recover the catalyst (20 mg). The solvent was evaporated to give **23** (0.110 g, 43%) as a light green oil. A mixture of **23** (0.1 g, 0.64 mmol), *t*-BuOK (0.215 g, 1.92 mmol), and acetamidine hydrochloride (0.091 g, 0.96 mmol) in *t*-BuOH (20 mL) was heated at reflux overnight. The reaction mixture was then filtered and washed with methanol. To the filtration was added silica gel (300 mg), and the solvent was evaporated under reduced pressure to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with chloroform/methanol (100:1). Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford **24** as a white solid (0.083 g, 79%): TLC R_f 0.28 (chloroform/methanol, 10:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.16 (d, J = 6.9 Hz, 3H), 1.44−1.52 (m, 1H), 1.75 (s, 1H), 2.12−2.20 (m, 1H), 2.24 (s, 3H), 2.54−2.62 (m, 1H), 2.68−2.76 (m, 1H), 3.03−3.08 (m, 1H), 12.14 (bs, 1H); Anal. calcd. for ($C_9H_{12}N_2O$·0.2H_2O) C, H, N.

*4-Chloro-2,5-dimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidine (**25**).* Compound **25** was synthesized from **24** (0.3 g, 1.83 mmol) following the general procedure for chlorination described above to afford after purification 0.105 g (32%) as a colorless oil: TLC R_f 0.29 (hexane/ethyl acetate, 3:1); ^{1}H NMR ($CDCl_3$) δ 1.28 (d, J = 7.0 Hz, 3H), 1.76 (ddd, J = 17.2, 10.7, 6.4 Hz, 1H), 2.26−2.36 (m, 1H), 2.64 (s, 3H), 2.84−2.95 (m, 1H), 3.07 (dt, J = 17.5, 8.6 Hz, 1H), 3.31−3.41 (m, 1H).

*N-(4-Methoxyphenyl)-N,2,5-trimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-aminium Chloride (**26**·HCl).* Compound **26** was synthesized from **25** (0.1 g, 0.55 mmol) and 4-methoxy-*N*-methylaniline (0.083 g, 0.60 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification a colorless oil. Following the general method to make a HCl salt from a base, the oil was converted to **26**·HCl, 0.108 g (62% over two steps) as a white solid: TLC R_f 0.31 (chloroform/methanol, 10:1); mp 178.3−180.7 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.65 (d, J = 6.6 Hz, 3H), 1.48−1.57 (m, 1H), 1.88−2.02 (m, 2H), 2.62 (s, 3H), 2.84 (dd, J = 18.2, 9.4 Hz, 1H), 3.01 (dt, J = 18.1, 9.1 Hz, 1H), 3.54 (s, 3H), 3.81 (s, 3H), 7.04−7.05 (m, 2H), 7.40 (bs, 2H), 15.10 (s, 1H); Anal. calcd. for ($C_{17}H_{22}ClN_3O$) C, H, N, Cl.

*2-Methyl-6,7-dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**28**).* A mixture of compound **27** (1.25 mL, 9.9 mmol), acetamidine hydrochloride (1.42 g, 15.6 mmol), and *t*-BuOK (3.1 g, 27.7 mmol) in *t*-BuOH (50 mL) was heated at reflux overnight. The reaction mixture was cooled, and the precipitate was removed by filtration and washed with warm methanol twice (30 mL × 1, 15 mL × 1). The filtrate and washings were combined and evaporated under reduced pressure. The residue was purified by column chromatography using chloroform/methanol (100/1) as eluent to afford **28** as a white solid (1.2 g, 81%): TLC R_f 0.38 ($CHCl_3$/CH_3OH, 10:1); mp 234.4−236.6 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.86−2.00 (m, 2H), 2.24 (s, 3H), 2.58 (t, J = 7.4 Hz, 2H), 2.69 (t, J = 7.7 Hz, 2H), 12.17 (s, 1H); Anal. calcd. for ($C_8H_{10}N_2O$·0.2H_2O) C, H, N.

*4-Chloro-2-methyl-6,7-dihydro-5H-cyclopenta[d]pyrimidine (**29**).* Compound **29** was synthesized from **28** (0.55 g, 3.66 mmol) following the general procedure for chlorination described above to afford after purification 0.35 g (56%) as a yellow liquid: TLC R_f 0.67 ($CHCl_3$/CH_3OH, 10:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.03−2.12 (m, 2H), 2.55 (s, 3H), 2.90 (t, J = 7.5 Hz, 2H), 2.96 (t, J = 7.8 Hz, 2H).

*N-(4-Methoxyphenyl)-N,2-dimethyl-6,7-dihydro-5H-cyclopenta-[d]pyrimidin-4-aminium Chloride (**30**·HCl).* Compound **30** was synthesized from **29** (0.35 g, 2.1 mmol) and *N*-methyl-4-methoxylaniline (0.31 g, 2.3 mmol) using the general procedure for nucleophilic displacement from chlorinated compound described above to afford after purification a light yellow liquid. Using the general procedure to make a HCl salt from a base, the light yellow liquid was converted to **30**·HCl, 0.362 g (57% for two steps) as a white solid: TLC R_f 0.19 ($CHCl_3$/CH_3OH, 10:1); mp 233.7−234.4 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.71−1.87 (m, 4H), 2.62 (s, 3H), 2.81−2.92 (m, 2H), 3.53 (s, 3H), 3.81 (s, 3H), 7.03 (d, J = 8.9 Hz, 2H), 7.37 (d, J = 8.9 Hz, 2H), 15.18 (s, 1H); Anal. calcd. for ($C_{15}H_{18}N_4O$·0.7HCl·1.3H_2O) C, H, N, Cl.

*N-(4-Ethoxyphenyl)-N,2-dimethyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidin-4-aminium Chloride (**31**·HCl).* Compound **31** was synthesized from **29** (0.080 g, 0.48 mmol) and 4-ethoxy-*N*-methylaniline (0.079 g, 0.52 mmol) following the general procedure described for **30**·HCl to afford after purification 0.074 g (79%) as a light brown solid: TLC R_f 0.36 (chloroform/methanol, 10:1); mp 209.5−211.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.35 (t, J = 6.9 Hz, 3H), 1.79 (bs, 4H), 2.62 (s, 3H), 2.86 (bs, 2H), 3.52 (s, 3H), 4.07

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(q, J = 6.9 Hz, 2H), 7.01 (d, J = 8.8 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 15.11 (s, 1H); Anal. calcd. for ($C_{17}H_{22}ClN_3O{\cdot}0.3CH_3OH$) C, H, N, Cl.

*N,2-Dimethyl-N-p-tolyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine (**32**).* Compound **32** was synthesized from **29** (0.140 g, 0.83 mmol) and *N*-methyl-*p*-tulidine (0.101 g, 0.83 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.102 g (48%) as a light brown solid: TLC R_f 0.30 (chloroform/methanol, 10:1); mp 97.5−99.3 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.65 (p, J = 7.6 Hz, 2H), 1.78 (t, J = 7.3 Hz, 2H), 2.32 (s, 3H), 2.41 (s, 3H), 2.60 (t, J = 7.7 Hz, 2H), 3.36 (s, 3H), 7.10 (d, J = 8.2 Hz, 2H), 7.20 (d, J = 8.1 Hz, 2H); Anal. calcd. for ($C_{16}H_{19}N_3{\cdot}0.1H_2O$) C, H, N.

*2-Amino-6,7-dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**33**).* A mixture of **27** (1.25 mL), guanidine carbonate (2.71 g, 15 mmol), and *t*-BuOK (3.1 g, 27.7 mmol) in *t*-BuOH (50 mL) was heated at reflux overnight. The reaction mixture was cooled, and a precipitate was removed by filtration. The filtered material was washed twice with warm methanol (30 mL × 1, 15 mL × 1). The filtrate and washings were combined and evaporated under reduced pressure, and the residue was purified by column chromatography using chloroform/methanol (5/1) as eluent to afford **33** (1.53 g, 83%) as a white solid: TLC R_f 0.17 ($CHCl_3$/CH_3OH, 10:1); ^{1}H NMR (DMSO-d_6) δ 1.88−1.95 (m, 2H), 2.54−2.58 (m, 2H), 2.67 (t, J = 7.8 Hz, 2H), 12.16 (bs, 1H); Anal. calcd. for ($C_8H_{10}N_2O{\cdot}0.2H_2O$) C, H, N.

*4-Chloro-6,7-dihydro-5H-cyclopenta[d]pyrimidin-2-amine (**34**).* Compound **34** was synthesized from **33** (0.55 g, 3.66 mmol) following the general procedure for chlorination described above to afford after purification 0.35 g (56%) as a yellow liquid: TLC R_f 0.67 ($CHCl_3$/CH_3OH, 10:1); ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.96−2.06 (m, 2H), 2.69−2.77 (m, 2H), 2.82 (t, J = 7.8 Hz, 2H), 8.01 (bs, 2H).

*N^4-(4-Methoxyphenyl)-N^4-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine-2,4-diamine (**35**).* Compound **35** was synthesized from **34** (0.120 g, 0.71 mmol) and 4-methoxy-*N*-methylaniline (0.116 g, 0.85 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.136 g (71%) as a white solid: TLC R_f 0.41 ($CHCl_3$/CH_3OH, 10:1); mp 187.7−189.5 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.52−1.64 (m, 2H), 1.64−1.73 (t, J = 7.2 Hz, 2H), 2.44 (t, J = 7.7 Hz, 2H), 3.28 (s, 3H), 3.76 (s, 3H), 5.90 (bs, 2H), 6.93 (d, J = 8.8 Hz, 2H), 7.12 (d, J = 8.8 Hz, 2H); Anal. calcd. for ($C_{15}H_{18}N_4O{\cdot}0.3H_2O$) C, H, N.

*N^4-(3-Methoxyphenyl)-N^4-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine-2,4-diamine (**36**).* Compound **36** was synthesized from **34** (0.060 g, 0.35 mmol) and 3-methoxy-*N*-methylaniline (0.048 g, 0.35 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.054 g (56%) as a white solid: TLC R_f 0.36 ($CHCl_3$/CH_3OH, 10:1); mp 153.0−154.1 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.58−1.67 (m, 2H), 1.77 (t, J = 7.1 Hz, 2H), 2.47 (d, J = 7.7 Hz, 2H), 3.34 (s, 3H), 3.74 (s, 3H), 5.94 (bs, 2H), 6.69−6.77 (m, 2H), 6.80 (d, J = 8.9 Hz, 1H), 7.27 (t, J = 8.4 Hz, 1H); Anal. calcd. for ($C_{15}H_{18}N_4O{\cdot}0.1C_4H_{10}O$) C, H, N.

*N^4-(2-Methoxyphenyl)-N^4-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine-2,4-diamine (**37**).* Compound **37** was synthesized from **34** (0.085 g, 0.50 mmol) and 2-methoxy-*N*-methylaniline (0.067 g, 0.50 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.065 g (48%) as a light yellow solid: TLC R_f 0.21 ($CHCl_3$/CH_3OH, 10:1); mp 170.3−172.0 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.51−1.63 (m, 4H), 2.42 (t, J = 7.6 Hz, 2H), 3.21 (s, 3H), 3.76 (s, 3H), 5.84 (bs, 2H), 6.94 (t, J = 7.5 Hz, 1H), 7.08 (d, J = 8.3 Hz, 1H), 7.16 (d, J = 9.3 Hz, 1H), 7.28−7.34 (m, 1H); Anal. calcd. for ($C_{15}H_{18}N_4O{\cdot}0.3CH_3OH$) C, H, N.

*N^4-(4-Ethoxyphenyl)-N^4-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidine-2,4-diamine (**38**).* Compound **38** was synthesized from **34** (0.085 g, 0.51 mmol) and 4-ethoxy-*N*-methylaniline (0.076 g, 0.51 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.091 g (48%) as a light brown solid: TLC R_f 0.31 ($CHCl_3$/CH_3OH, 10:1); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.33 (t, J = 6.9 Hz, 3H), 1.58 (dt, J = 14.1, 7.2 Hz, 2H), 1.68 (t, J = 7.0 Hz, 2H), 2.44 (t, J = 7.7 Hz, 2H), 3.27 (s, 3H), 4.02 (q, J = 6.9 Hz, 2H), 5.89 (bs, 2H), 6.91 (d, J = 8.8 Hz, 2H), 7.11 (d, J = 8.8 Hz, 2H); Anal. calcd. for ($C_{16}H_{20}N_4O{\cdot}0.3H_2O$) C, H, N.

*Methyl 2-Aminocyclopent-1-enecarboxylate (**39**).* A mixture of methyl-2-oxocyclopentane carboxylate **27** (2 mL, 15.5 mmol), ammonium formate (5.4 g, 77.4 mmol), and methanol (30 mL) was heated at reflux for 24 h. To the solution was added silica gel (6 g), and the mixture was evaporated under reduced pressure to afford a plug. This plug was loaded on a dry silica gel column and flashed chromatographed with hexane/ethyl acetate (10:1). Fractions showing the major spot on TLC were pooled and evaporated to give **39**, 2 g (91%) as a white solid: TLC R_f 0.26 (hexane/ethyl acetate, 3:1); mp 94.3−95.8 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.67−1.73 (m, 2H), 2.34−2.44 (m, 4H), 3.53 (s, 3H), 6.74 (bs, 2H); Anal. calcd. for ($C_7H_{11}NO_2$) C, H, N.

*Methyl 2-Formamidocyclopent-1-enecarboxylate (**40**).* Formic acid (4.3 mL) was added to acetic anhydride (6.5 mL) while cooling in an ice bath. Compound **37** (1 g, 7.08 mmol) was added to the cold solution in small portions. The cooling bath was removed, and the resulting solution was stirred at room temperature for 4 h. The reaction mixture was extracted with ethyl acetate (30 mL × 3). The organic layer was dried over anhydrous Na_2SO_4 and then filtered. To the filtrate was added silica gel (5 g), and the mixture was evaporated under reduced pressure to afford a plug. This plug was loaded on a dry silica gel column and flashed chromatographed with hexane/ethyl acetate (10:1). Fractions showing the major spot on TLC were pooled and evaporated to give **40**, 0.927 g (77%) as a white solid: TLC R_f 0.26 (hexane/ethyl acetate, 3:1); mp 76.7−78.0 °C; ^{1}H NMR (DMSO-d_6) δ 1.75−1.95 (m, 2 H), 2.82−2.94 (m, 1H), 2.98−3.10 (m, 1H), 3.69 (s, 3H), 8.28 (bs, 0.5H), 8.57 (bs, 0.5H), 9.71 (bs, 0.5H), 10.28 (bs, 0.5H); Anal. calcd. for ($C_8H_{11}NO_3$) C, H, N.

*6,7-Dihydro-3H-cyclopenta[d]pyrimidin-4(5H)-one (**41**).* To a solution of ammonium formate (1 g, 16 mmol) in formamide (2 mL) at 150 °C was added compound **40** (0.5 mg, 2.96 mmol). The resulting solution was heated at 150 °C for 4 h and then allowed to stand at room temperature for 12 h. This mixture was loaded on a wet silica gel column and flashed chromatographed with chloroform/methanol (50:1). Fractions showing the major spot on TLC were pooled and evaporated to give **41**, 0.335 g (83%) as a white solid: TLC R_f 0.34 (chloroform/methanol, 10:1); mp 245.2−246.9 °C; ^{1}H NMR (DMSO-d_6) δ 1.90−1.99 (m, 2H), 2.62 (t, J = 7.4 Hz, 2H), 2.75 (t, J = 7.6 Hz, 2H), 8.01 (s, 1H), 12.26 (s, 1H); Anal. calcd. for ($C_7H_8N_2O$) C, H, N.

*4-Chloro-6,7-dihydro-5H-cyclopenta[d]pyrimidine (**42**).* Compound **42** was synthesized from **41** (0.330 g) following the general procedure for chlorination described above to afford after purification 0.223 g (60%) as a light yellow solid: TLC R_f 0.55 (chloroform/methanol, 10:1); mp 41.5−43.0 °C; ^{1}H NMR (DMSO-d_6) δ 2.05−2.14 (m, 2H), 2.96 (t, J = 7.6 Hz, 2H), 3.03 (t, J = 7.8 Hz, 2H), 8.78 (s, 1H); Anal. calcd. for ($C_7H_7ClN_2$) C, H, N, Cl.

*N-(4-Methoxyphenyl)-N-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidin-4-amine (**43**).* Compound **43** was synthesized from **42** (0.16 g, 1.04 mmol) and 4-methoxy-*N*-methylaniline (0.157 g, 1.14 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.231 g (87%) as a light yellow solid: TLC R_f 0.33 ($CHCl_3$/CH_3OH, 10:1); mp 95.7−96.5 °C; ^{1}H NMR (DMSO-d_6) δ 1.62−1.71 (m, 2H), 1.83 (t, J = 7.3 Hz, 2H), 2.65 (t, J = 7.8 Hz, 2H), 3.35 (s, 3H), 3.78 (s, 3H), 6.96 (d, J = 8.9 Hz, 2H), 7.19 (d, J = 8.8 Hz, 2H), 8.42 (s, 1H); Anal. calcd. for ($C_{15}H_{17}N_3O$) C, H, N.

*N-Methyl-N-p-tolyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-amine (**45**).* Compound **45** was synthesized from **42** (0.1 g, 0.65 mmol) and *N*-methyl-*p*-toluidine (0.087 g, 0.71 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.09 g (58%) as an off-white solid: TLC R_f 0.67 ($CHCl_3$/CH_3OH, 5:1); mp 79.1−80.5 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.67 (p, J = 7.6 Hz, 2H), 1.83

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(t, J = 7.3 Hz, 2H), 2.33 (s, 3H), 2.66 (t, J = 7.7 Hz, 2H), 3.37 (s, 3H), 7.13 (d, J = 8.2 Hz, 2H), 7.22 (d, J = 8.1 Hz, 2H), 8.45 (s, 1H); Anal. calcd. for ($C_{15}H_{17}N_3$) C, H, N.

*N-(4-Chlorophenyl)-N-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidin-4-amine (**46**).* Compound **46** was synthesized from **42** (0.080 g, 0.52 mmol) and 4-chloro-*N*-methylaniline (0.081 g, 0.57 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.115 g (85%) as an off white solid: TLC R_f 0.36 ($CHCl_3$/CH_3OH, 10:1); mp 106.8−108.1 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.67−1.79 (m, 2H), 1.90 (t, J = 7.2 Hz, 2H), 2.70 (t, J = 7.7 Hz, 2H), 3.41 (s, 3H), 7.26 (d, J = 8.6 Hz, 2H), 7.46 (d, J = 8.6 Hz, 2H), 8.49 (s, 1H); Anal. calcd. for ($C_{14}H_{14}ClN_3$) C, H, N, Cl.

*N-Methyl-N-(4-(methylthio)phenyl)-6,7-dihydro-5H-cyclopenta-[d]pyrimidin-4-amine (**47**).* Compound **47** was synthesized from **42** (0.1 g, 0.65 mmol) and *N*-methyl-4-(methylthio)aniline (0.109 g, 0.71 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound described above to afford after purification 0.135 g (77%) as a white solid: TLC R_f 0.68 ($CHCl_3$/CH_3OH, 5:1); mp 111.7−112.8 °C ; ^{1}H NMR (DMSO-d_6) δ 1.70 (p, J = 7.6 Hz, 2H), 1.89 (t, J = 7.3 Hz, 2H), 2.50 (s, 3H), 2.67 (t, J = 7.7 Hz, 2H), 3.38 (s, 3H), 7.19 (d, J = 8.5 Hz, 2H), 7.28 (d, J = 8.5 Hz, 2H), 8.46 (s, 1H); Anal. calcd. for ($C_{15}H_{17}N_3S$) C, H, N, S.

*N-(4-Methoxyphenyl)-N-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidin-4-aminium Chloride (**43**·HCl).* Compound **43**·HCl was synthesized from **43** (0.130 g, 0.51 mmol) using the general procedure to make a HCl salt from a base described above to afford 0.142 g (95%) as a light yellow solid: mp 224.1−225.9 °C; ^{1}H NMR (DMSO-d_6) δ 1.73−1.91 (m, 4H), 2.89 (t, J = 7.7 Hz, 2H), 3.54 (s, 3H), 3.81 (s, 3H), 7.04 (d, J = 8.9 Hz, 2H), 7.38 (d, J = 8.8 Hz, 2H), 8.86 (s, 1H), 15.19 (bs, 1H); Anal. calcd. for ($C_{15}H_{18}ClN_3O$) C, H, N, Cl.

*N-(4-Ethoxyphenyl)-N-methyl-6,7-dihydro-5H-cyclopenta[d]-pyrimidin-4-aminium Chloride (**44**·HCl).* Compound **44**·HCl was synthesized from **42** (0.092 g, 0.60 mmol) and 4-ethoxy-*N*-ethylaniline (0.090 g, 0.60 mmol) following the general procedure for nucleophilic displacement from a chlorinated compound and the general method to make a HCl salt described above to afford after purification 0.13 g (89%) as an off-white solid: TLC R_f 0.41 ($CHCl_3$/CH_3OH, 10:1); mp 228.0−229.2 °C ; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.35 (t, J = 7.0 Hz, 3H), 1.81 (ddd, J = 21.8, 15.7, 6.3 Hz, 4H), 2.89 (t, J = 7.7 Hz, 2H), 4.08 (q, J = 6.9 Hz, 2H), 7.02 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8.8 Hz, 2H), 8.86 (s, 1H),15.20 (bs, 1H); Anal. calcd. for ($C_{16}H_{20}N_3OCl$) C, H, N, Cl.

Cellular Studies. Effects on Cellular Microtubules. A-10 cells were used to evaluate the effects of the compounds on cellular microtubules using indirect immunofluorescence. EC_{50} values were calculated as previously described and represent a minimum of three independent experiments.[38]

SRB Assay. The antiproliferative and cytotoxic activity of all compounds was evaluated using the SRB assay as previously described.[39] The IC_{50}'s represent an average of at least three independent experiments plus or minus the standard deviation.

Cell Cycle Analysis. MDA-MB-435 cells were plated and allowed to adhere for 24 h. Drugs were then added, and cells were harvested 24 h later. Paclitaxel (12.5 nM) was used as a positive control. Once cells were harvested, they were stained with Krishan's reagent and analyzed for DNA content using a FacsCalibur flow cytometer.

In Vitro Tubulin Polymerization. The effects of the compounds on tubulin polymerization were initially measured using purified porcine brain tubulin (Cytoskeleton Inc.). The reactions contained 2 mg/mL of tubulin incubated with 1 mM GTP and 10% glycerol and drug as indicated. The polymerization of tubulin was monitored by measuring the absorbance at 340 nm at 37 °C in a SpectraMax 96-well plate spectrophotometer. Colchicine was used as a control.

Quantitative Tubulin Studies. Purified bovine brain tubulin[40] was used in these studies to determine IC_{50} values for tubulin polymerization and compound inhibition of colchicine binding. The techniques used in both assays have previously been described in detail[41,42] but are summarized below.

In the polymerization assay,[41] 10 μM (1.0 mg/mL) tubulin was preincubated for 15 min at 30 °C with varying compound concentrations in 0.8 M monosodium glutamate, taken from a 2.0 M stock solution adjusted to pH 6.6 with HCl. Reaction mixtures also contained 4% (v/v) dimethyl sulfoxide (compound solvent). Following the preincubation, reaction mixtures were placed on ice, and 10 μL of 10 mM GTP (0.4 mM final concentration) was added to each mixture. Reaction volume was 0.24 mL prior to GTP addition, and all concentrations are in terms of the final reaction volume of 0.25 mL. Reaction mixtures were transferred to 0 °C cuvettes in Beckman DU7400/7500 recording spectrophotometers equipped with electronic temperature controllers. Polymer formation was measured turbidimetrically at 350 nm. After baselines were established at 0 °C, temperature was rapidly jumped to 30 °C (less than 1 min), and the IC_{50} was defined as the compound concentration inhibiting the extent of assembly by 50% after 20 min at 30 °C.

In the colchicine binding assay,[42] 0.1 mL reaction volumes contained 1.0 μM tubulin, 5.0 μM [^{3}H]colchicine (from Perkin-Elmer), and potential inhibitors at 1.0 or 5.0 μM, together with reaction components that strongly stabilize the colchicine binding activity of tubulin.[42,43] Reaction mixtures also contained 5% dimethyl sulfoxide (compound solvent). Tubulin was the last component added to the reaction mixtures, which were prepared on ice. Binding of colchicine was initiated by transferring the reaction mixtures to a 37 °C water bath, and the mixtures were incubated for 10 min (a time when the reaction without inhibitor is 40−60% complete). Reactions were stopped with ice water, and the diluted reaction mixtures were filtered through a stack of two Whatman DEAE-cellulose filters obtained from GE Healthcare Life Sciences. Radiolabel bound to the filters was quantitated by scintillation counting.

In Vivo Evaluation. The purpose of this experiment was to determine the efficacy of **30**·HCl, when administered in different treatment regimens, in the MDA-MB-231T solid tumor xenograft model. A fragment of this tumor was implanted sc in female athymic nude mice, and tumors were staged to approximately 125 mg. Treatment to all groups commenced day 14 post-tumor implant. A second course of treatment was administered to all groups. This was a valid control.

Compound **30**·HCl was administered as a solution in 10% DMSO in saline/Tween 80. Groups 2 and 3 received 75 or 50 mg/kg per injection, respectively, ip for 5 consecutive days commencing days 14 and 27. Group 4 received 75 mg/kg per injection ip every other day for a total of five treatments commencing days 14 and 27. Group 5 received 50 mg/kg per injection ip twice a day, every other day, for a total of six treatments commencing on days 14 and 27.

A MTD was not administered to any group because there was no indication of toxicity as expressed by either excessive animal body weight loss or lethality. The following are the optimum T/C values for each group: group 2 (17), group 3 (20), group 4 (19) and group 5 (25). Efficacy was obtained in every treatment regimen with no marked differences between the regimens.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Elemental Analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

Pgp, P-glycoprotein; DAMA-colchicine, *N*-deacetyl-*N*-(2-mercaptoacetyl) colchicine; SAR, structure−activity relationship; CWP, tris(cetylpyridinium) 12-tungstophosphate; SRB, sulforhodamine B; CA-4, combretastatin A-4

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