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Structural Optimization of Indole Derivatives Acting at Colchicine Binding Site as Potential Anticancer Agents

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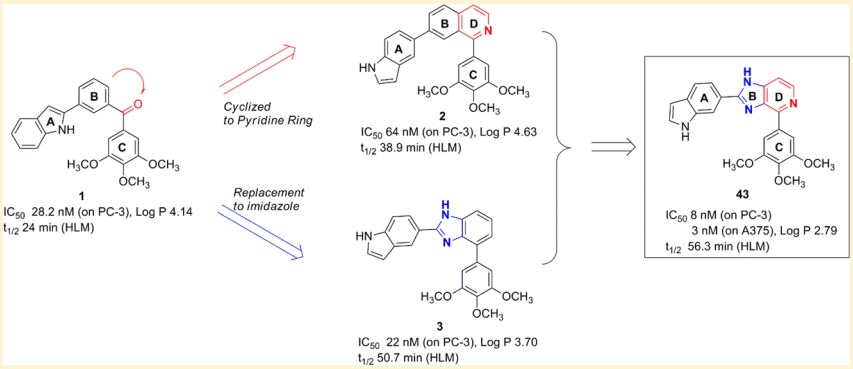
Supporting Information



ABSTRACT: A new series of indole analogues based on our earlier lead compound, 2-(1*H*-indol-5-yl)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazo[4,5-*c*]pyridine (**42**), was prepared as tubulin inhibitors in an effort to find a molecule with improved cytotoxic potency and metabolic stability. A series of indolyl-imidazopyridines (IIP) were synthesized and exhibited potent tubulin polymerization inhibitory activity with potent IC_{50} values ranging from 3 to 175 nM against a panel of human melanoma and prostate cancer cell lines. Among these compounds, the 6-indolyl compound **43** showed improved cytotoxic potency (average IC_{50} of 9.75 nM vs 55.75 nM) and metabolic stability in human liver microsomes (half-life time was 56.3 min vs. 45.4 min) as compared to previously reported **42**. It was also shown to be effective against P-glycoprotein (P-gp) mediated multiple drug resistance (MDR) and taxol resistance.

KEYWORDS: *Colchicine-binding site, tubulin polymerization inhibitor, prostate cancer, melanoma, antiproliferative activity, structure−activity relationship (SAR), multiple drug resistance, liver microsomal stability*

Microtubules are tubular polymers of tubulin that have been crucial chemotherapeutic targets in a variety of cancers.[1] Interfering with tubulin dynamics in tumor cells is a validated approach in developing antimitotic drugs to treat cancers.[2] Using potent cytotoxic drugs that inhibit microtubules has been useful in inducing cancer cells to undergo apoptosis through various mechanisms.[3] Most of the small molecule antitubulin agents bind to one of the three best characterized binding sites on α,β-tubulin subunits. For example, the taxane, vinca alkaloids, and colchicine binding sites bind to paclitaxel, vinblastine, or colchicine, respectively. Antitubulin agents are also classified according to whether they prevent microtubule degradation (e.g., taxanes, epothilones) or induce microtubule-destabilization (e.g., vinca alkaloids, colchicine).[4]

While a number of tubulin inhibitors binding to the taxane or vinca alkaloid sites exhibit high potency and have been approved by the FDA for the treatment of various cancers, the emergence of resistant phenotypes still leads to diminished efficacy in tumors expressing drug efflux transporters.[1,5] Compared to the taxane or vinca alkaloid binding sites, targeting the colchicine binding site may provide a better opportunity for structural optimization to overcome such ABC-transporter mediated drug resistance while complying with Lipinski's Rule of Five.[6] Recently, colchicine scaffolds in *in vitro* studies showed strong cancer inhibition and also overcame resistant phenotypes of carcinoma.[7,8] A number of potent tubulin inhibitors targeting the colchicine binding site have been reported, including combretastatin A-4 (CA-4),[9] BRP0L075,[10] phenstatin,[11] ARAP,[12] and SD400[13] as shown in Figure S1 of the Supporting Information. Following our discovery of (3-(1*H*-indol-2-yl)phenyl)(1*H*-indol-2-yl)-methanone[14] as an antitubulin agent, our group showed several diaryl-ketone chemotypes as tubulin inhibitors that bind to the colchicine domain, including phenyl ring as linker (I-387),[15] phenylaminothiazoles (PAT),[4] arylbenzoylimidazoles (ABI),[16] reverse ABIs (RABI),[17] and 4-substituted methoxybenozyl aryl thiazoles (SMART).[18] Unsubstituted examples of these chemotypes are shown in Figure S1. Among them, the SMART, ABI, and PAT analogues have shown very strong *in vivo* efficacy in human melanoma and prostate cancer xenograft models.[18]

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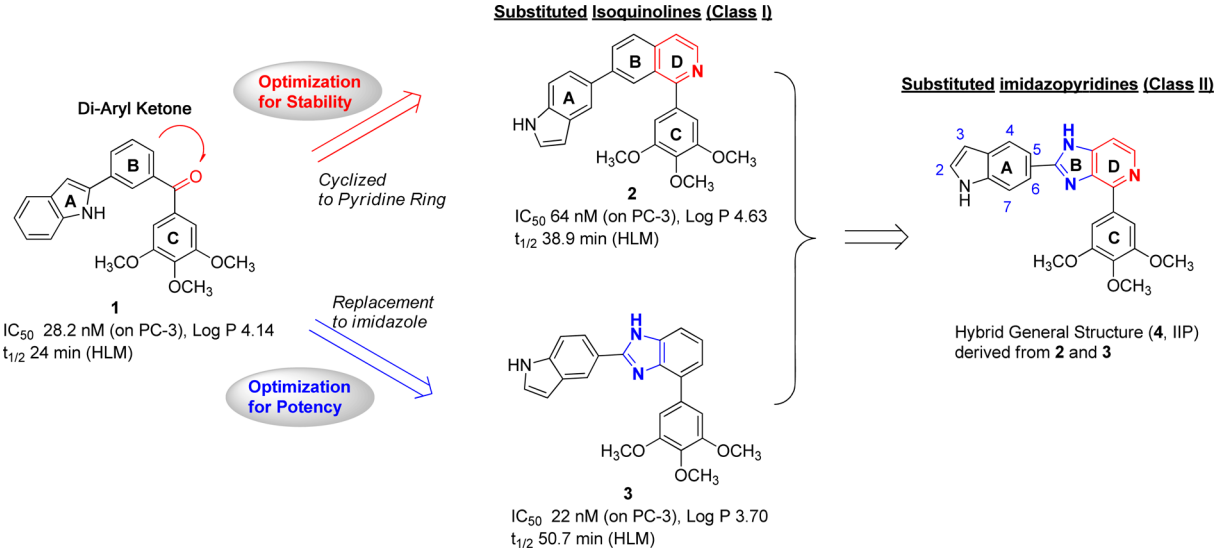
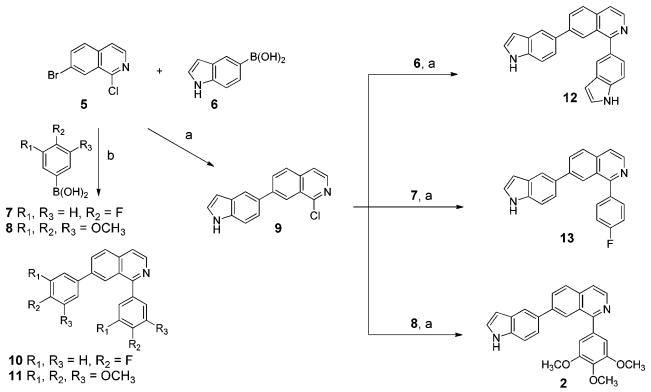


Figure 1. Hybrid indolyl-imidazopyridine general structure **4** (IIP) derived from a combination of isoquinoline (**2**) and imidazo-benzene (**3**) templates.

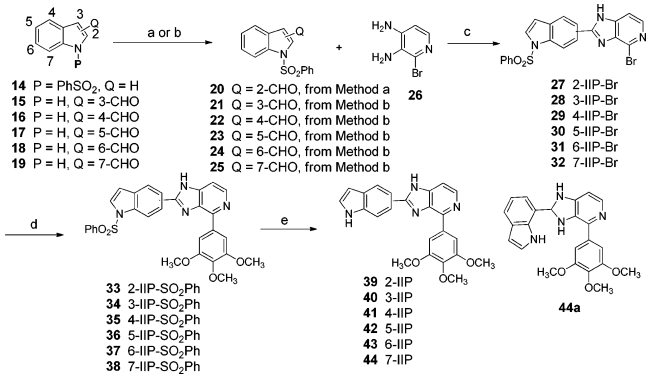
However, the ketone represented a metabolically labile site in human liver microsomes (HLM) studies and thus new approaches were sought to improve antimitotic effects and reduce untoward effects.[19] In an effort to improve potency and metabolic stability, we explored general structure **4**. As shown in Figure 1, **4** was designed as a fusion of metabolically stable nonketone templates **2**[15] and **3**.[19] The building-blocks for analogues of **4** are indoles and pyrido-imidazoles connected with (3,4,5-trimethoxyphenyl)-(TMP-) template. The imidazole ring is a widely used template in many drugs.[20] Also the indole group has been investigated in many antitubulin agents,[21] such as 3-formyl-2-phenylindoles, heterocombretastatins, diarylindoles, 2-aroylindoles, D-24851, 2-aryl-3-aroylindoles, 3-aroyl- and 1-aroylindoles, and arylthioindoles.[21] We herein report the evaluation of two classes of nonketone antitubulin agents that contain indole and imidazole moieties. Isoquinolines (class I) and imidazopyridines (class II), as shown in Schemes 1 and 2, are bioisosteric chemotypes explored herein as new classes of tubulin inhibitors binding to the colchicine site.

Scheme 1. Synthesis of Class I (1,7-Disubstituted Isoquinolines) Antitubulin Agents[a]



[a]Reagents and conditions: (a) 1 equiv of each boric acid **6**–**8**, $Pd(PPh_3)_4$, K_2CO_3, DMF, H_2O, reflux; (b) 2 equiv of boric acid **7** or **8**, $Pd(PPh_3)_4$, K_2CO_3, DMF, H_2O, reflux.

Scheme 2. Synthesis of Class II (2,4-Disubstituted 1*H*-Imidazo[4,5-*c*]pyridines) Antitubulin Agents[a]



[a]Reagents and conditions: (a) *n*-BuLi, DMF, −78 °C; (b) $PhSO_2Cl$, KOH, TBAHS, DCM, 16 h, room temperature; (c) *p*-toluenesulfonic acid, 1,4-dioxane, reflux; (d) **8**, $Pd(PPh_3)_4$, aqueous Na_2CO_3, THF/MeOH/H_2O, 100 °C, MW; (e) aqueous NaOH, EtOH, reflux.

The structure–activity relationship (SAR) was optimized with regard to site of indole attachment to the core motif in an effort to develop new therapeutic candidates for future *in vivo* studies. 6-Indolyl (**43**) demonstrated high potency and metabolic stability *in vitro*. In class I of Scheme 1, five isoquinolines analogues (**2**, **10**–**13**) with TMP-, 5-indolyl-, or 4-fluorophenyl-groups in A- and/or C-rings were prepared and tested for their cytotoxicity against prostate cancer and human melanoma cell lines,[22] and their metabolic stability is discussed. The majority of the isoquinolines derivatives and intermediates were prepared by Suzuki-cross coupling of commercially available aryl halides (**5**, **9**) with boric acids (**6**–**8**) using $Pd(PPh_3)_4$ as a catalyst. Compounds **7** and **8** were synthesized using 2 equiv of boric acid (**7** and **8**, respectively).

Scheme 2 shows the synthesis of class II agents, indole-based 2,4-disubstituted 1*H*-imidazo[4,5-*c*]pyridines **39**–**44**. Each of the six substitutable positions of the indolyl moiety was synthesized using different indolylaldehydes **14**–**19** as shown in Scheme 2. The protected aldehydes (**20**–**25**) were prepared by known procedures.[23] Condensation of the *N*-protected

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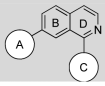
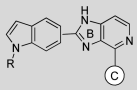
Table 1. Antiproliferative Activities of Isoquinoline Analogues (Class I) and 1*H*-Imidazo[4,5-*c*]pyridine Analogues (Class II) in Parental and MDR (LLC6R1) or Paclitaxel Resistant (TxR) Cancer Cell Lines

Class I			IC_{50} (nM)						
	A	C	Melanoma cells			Prostate Cancer cells[22]			
			A375	WM164	M14	LNCaP	PC-3	DU 145	PPC-1
2	5-indolyl	TMP	929	227	74	81	64	92	61
10	4-F-phenyl	4-F-phenyl	> 30000	> 30000	> 30000	> 30000	> 30000	> 30000	> 30000
11	TMP	TMP	10749	6673	9740	ND [a]	ND [a]	ND [a]	ND [a]
12	5-indolyl	5-indolyl	93	147	111	98	72	113	48
13	5-indolyl	4-F-phenyl	66	33	49	38	26	47	28
Class II			IC_{50} (nM)						
	Indolyl position	R	C	Melanoma cells			Prostate Cancer cells			
				A375	M14	M14/LCC6R1	PC-3	PC-3/TxR	DU145	DU145/TxR
29	4-	SO_2Ph	Br	> 30000	> 30000	> 30000	> 30000	> 30000	> 30000	> 30000
35	4-	SO_2Ph	TMP	263	670	730	956	570	406	325
38	7-	SO_2Ph	TMP	551	471	545	623	525	1029	> 30000
39	2-	H	TMP	175	164	191	170	120	1704	2035
40	3-	H	TMP	16	26	88	27	7	222	74
41	4-	H	TMP	3	14	25	7	12	14	17
42	5-	H	TMP	22	53	116	27	34	121	164
43	6-	H	TMP	3	16	53	8	13	12	12
44	7-	H	TMP	24	28	53	43	65	177	> 30000
44a [b]	7-	H	TMP	984	1063	975	1243	689	2539	3661

[a]ND: Not determined. [b]2-(1*H*-Indol-7-yl)-4-(3,4,5-trimethoxyphenyl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]pyridine, which is dihydro-imidazole on B-ring.

indolylaldehydes (**20**[23] and **21**–**25**[24]) with pyridodiamine **26** was accomplished using TsOH in 1,4-dioxane, or catalytic amount of c-H_2SO_4 in toluene/DMF solution using a Dean–Stark trap, to give the desired imidazopyridines **27**–**32**. The Suzuki cross-coupling reaction was performed between this series of protected bromides **27**–**32** and 3,4,5-trimethoxyphenylboric acid (**8**) to obtain compounds **33**–**38**.

The deprotection of the phenysulfonyl group (SO_2Ph) was performed using aqueous NaOH to generate the desired IIP products **39**–**44**. Interestingly, we also found and purified 2-(1*H*-indol-7-yl)-4-(3,4,5-trimethoxyphenyl)-2,3-dihydro-1*H*-imidazo[4,5-*c*]pyridine (**44a**) when modifying the conditions by heating ethanol to reflux in conditions c (Scheme 2), rather than using 1,4-dioxane. Compound **44a** could only be separated at the final step and was tested in our assay system in order to compare it to compound **44**. The *in vitro* cytotoxic/antiproliferative activity of the synthesized class I and II compounds against a panel of human melanoma and prostate cancer cell lines was characterized using the MTS assay (Tables 1). Compounds in class II were further tested in P-gp overexpressing multidrug resistant (MDR) and taxol resistant cancer cell lines. The results for class I showed that compounds with 5-indolyl A-rings (**2**, **12**, and **13**, 26–929 nM) had higher growth inhibition potency than compounds with TMP or 4-fluorophenyl A-rings (**10** and **11**, 10 to >30 μM ranges). Compound **13** was the most potent class I compound bearing a 5-indolyl A-ring with a 4-fluorophenyl C-ring. Compound **13** had potent activity against prostate cell lines in the range of 26 to 47 nM, which was better than that observed with the TMP analogue **2**. Class I, with the six-membered B-ring, has increased intramolecular repulsion more than class II. This may be due to the increased steric overlap between the A- and C-rings. Therefore, 4-fluorophenyl of **13** (class I) may have less internal strain as compared to the relatively bulky TMP C-ring of **2**. Perhaps rotation of bond connecting the C- and D-rings in class I compounds is not tolerated within the colchicine binding pocket.

N-Protected class II compounds with TMP as the C-ring (**35** or **38**) showed moderate activity (average ~400 nM), but compound **29**, which had a bromide substituent instead of the C-ring, was inactive (>30 μM). This indicated again that TMP on the C-ring position was critical for antiproliferative activity. Generally, all the tested compounds with A-ring indole substituents (**39**–**44**) showed strong antiproliferative activity against melanoma and prostate cancer cell lines, including taxol resistant PC-3/TxR and DU145/TxR cells, and overcame the P-gp mediated MDR in M14/LCC6R1 cells. In this series, 2-indolyl **39** was less cytotoxic with IC_{50} values at around a few hundred nanomolar. 3- and 7-Indolyl compounds **40** and **44** presented comparable potency (average IC_{50} values in the low nanomolar range) to the previously reported 5-indolyl compound **42** (average 55.75 nM). The dihydro-imidazole **44a**, which was made as a side product, showed 10–20-fold lower potency in melanoma and prostate cell lines than imidazole analogue **44**, indicating that the imidazole ring is an important moiety for inducing apoptosis. This suggests that the planar central ring of **44** is more complementary to the colchicine binding site, which apparently does not tolerate the nonplanar arrangement of the A- and C-rings caused by the dihydro-imidazole of **44a**. The most active A-ring indolyl compounds were the 4- and 6-indolyl **41** and **43**, which demonstrated IC_{50} values as low as 3 nM in human melanoma A375 cells. Mechanistic and molecular modeling studies suggested that selected compounds **41**, **42**, and **43** maintained their mode of action as tubulin polymerization inhibitors as shown in Figure S2 of the Supporting Information. To

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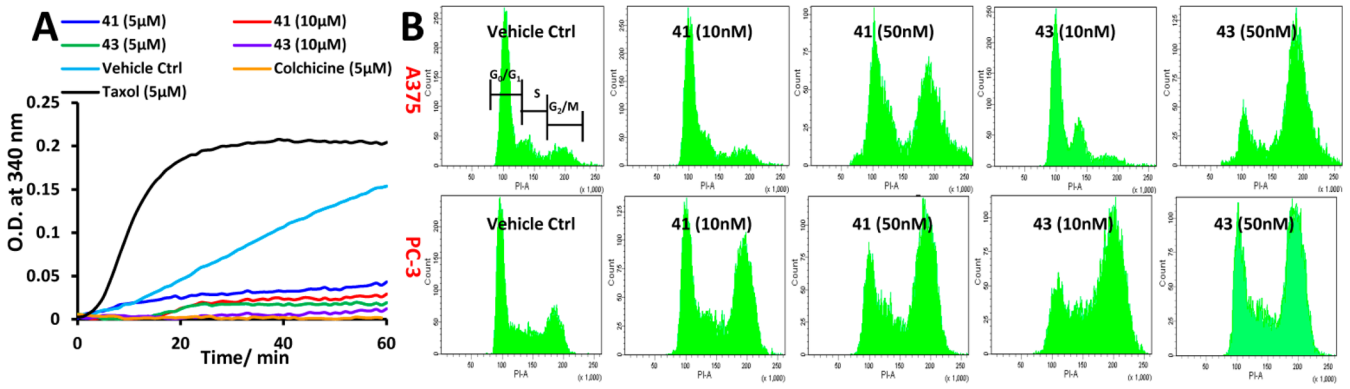

Figure 2. (A) Compounds **41** and **43** effectively inhibited the tubulin polymerization *in vitro*. The microtubule polymerization was monitored by measuring the absorbance at 340 nm in the absence or presence of drugs ($N = 3$). A representative experiment is shown. Vehicle control (sky blue); paclitaxel (5 μM) (black); colchicine (5 μM) (brown); **41** (5 μM) (blue), **41** (10 μM) (red); **43** (5 μM) (green), **43** (10 μM) (purple). (B) Compounds **41** and **43** arrested human melanoma A375 and human prostate cancer PC-3 cells in G2/M phase *in vitro* ($N = 3$).

experimentally determine whether the new analogues maintain their mode of action, we tested the two most potent compounds, **41** and **43**, in a microtubule polymerization assay *in vitro* (at 5 or 10 μM). Vehicle, taxol (5 μM), and colchicine (5 μM) were used as control groups and assayed under the same conditions (Figure 2A). Both **41** and **43** effectively inhibited the tubulin polymerization in a dose-dependent manner. Compound **43** at lower concentration (5 μM) was a more effective inhibitor than **41** at 10 μM. Human melanoma A375 cells and human prostate cancer PC-3 cells were treated with **41** or **43** for 24 h and analyzed through flow cytometry to determine their cell cycle distributions. As shown in the Figure 2B, while the general distributions of A375 cells were not significantly affected by either **41** or **43** at a low concentration of 10 nM, at a higher concentration of 50 nM, **41** and **43** effectively blocked A375 cells at the G2/M phase. The vehicle control group only had 4.0 ± 0.5% of A375 cells distributed in G2/M phase, but 42.0 ± 3.8% or 65.9 ± 2.2% of A375 cells were arrested in G2/M phase for **41** or **43** at 50 nM, respectively. In PC-3 cells, even at the low concentration of 10 nM, **41** or **43** efficiently arrested cells in G2/M phases as shown in Figure S3 of the Supporting Information. This dose-dependent G2/M phase block for **41** and **43** confirms the cytotoxic mechanism of action was conserved. The most potent compounds of each class were selected to be further evaluated for *in vitro* metabolic stability in human liver microsomes (HLM) and mouse liver microsomes (MLM). As shown in Table 2, Class I B-ring isoquinoline compounds (**2** and **13**) showed significantly better metabolic stability in HLM ($t_{1/2}$ = 38.9 and 30.7 min), than was observed in MLM ($t_{1/2}$ = 2.1 and 2.0 min). The replacement of TMP (for **2**) with 4-fluorophenyl group on C-ring (for **13**) increased the antiproliferative potency but decreased the metabolic stability slightly. Class II (IIP compounds) **41**, **42**, and **43** showed favorable metabolic stability in both HLM and MLM. Among them, **43** having an A-ring 6-indolyl substituent and showing the highest anticancer potency, also exhibited the best stability ($t_{1/2}$ = 56.3 min in HLM and 27.7 min in MLM). This suggests that the adjustment from 5-indolyl **42** to 6-indolyl **43** has improved both antitumor efficacy and metabolic stability.

Table 2. Half-Lives in Human (HLM) and Mouse (MLM) Liver Microsomes

compd ID		$t_{1/2}$ (min) HLM	$t_{1/2}$ (min) MLM
class I	**2**	38.9 ± 3.4	2.1 ± 1.1
	13	30.7 ± 2.6	2.0 ± 0.8
class II	**41**	33.1 ± 4.8	10.5 ± 1.8
	42	45.4 ± 3.0	16.1 ± 4.4
	43	56.3 ± 2.3	27.7 ± 6.1

In summary, our previous discovery of the 5-indolyl **42** demonstrated that the indolyl-imidazo[4,5-*c*]pyridine class II chemotype with TMP B-ring could potently bind the colchicine site and additionally provide a metabolic stability benefit. Herein we performed an "indole-walk" study to optimize the best configuration for biological activity on the 4-(3,4,5-trimethoxyphenyl)-1*H*-imidazo[4,5-*c*]pyridin-2-yl (IIP template). All six new indolyl derivatives were investigated for their *in vitro* cytotoxicities in cancer cell lines and stability to metabolism in liver microsomes. We also tested isoquinoline motifs (class I) to compare the structure−activity relationships of cytotoxicity and metabolic stability across nonketone classes I and II. The results showed the pyridine D-ring motif of IIP provided some benefits toward metabolic stability in HLM. Additionally, cytoxicity studies demonstrated compounds of general structure **4** (i.e., class II or IIP compounds) were very potent against the tested tumor cell lines. Among them, 6-indolyl derivative (6-IIP, **43**) showed the strongest inhibition activities (IC_{50} at 3 nM on A375 and 8 nM on PC-3) and best metabolic stability (56.3 min in HLM). We will evaluate the *in vivo* efficacy of the IIP series, which will be further characterized as potent novel anticancer agents. The IIP series can serve not only as a valuable tool for preclinical research of the modulation of tubulin polymerization dynamics but also as a promising candidate series for clinical development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00208.

Synthetic procedures, the structures of known colchicine inhibitors (Figure S1), molecular modeling (Figure S2), quantification data for cell cycle analysis (Figure S3), and the analytical spectra for **41**, **42**, and **43** and their derivatives (Figure S4) (PDF)

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Author Contributions

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Notes

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