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Synthesis and biological evaluation of 3,4-diaryl-5-aminoisoxazole derivatives

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ABSTRACT

A series of *cis*-restricted 3,4-diaryl-5-aminoisoxazoles have been synthesized and evaluated for their biological activities. Among them, compound **11a** and **13a** displayed potent cytotoxic activities in vitro against five human cancer cell lines with IC_{50} values in the low micromolar range and two compounds inhibited tubulin polymerization with IC_{50} value of 1.8, and 2.1 μM, respectively, similar to that of CA-4. Compound **13a** could arrest at the G2/M phase of the cell cycle at the concentration of 0.1 and 1.0 μM and induce apoptosis at 0.1–1.0 μM.

1. Introduction

Microtubules represent an attractive target for antitumor drug discovery because they play an essential role in cellular functions including shape maintenance, division and intracellular transport.[1,2] Drugs interfering with the microtubule dynamics have attracted much attention. Among them, combretastatin A-4 (CA-4, **1**, Fig. 1), a natural *cis*-stilbene product, strongly inhibits the tubulin assembly by binding to the colchicine site and prevents tubulin polymerization. It also displayed potent cytotoxic activities against a variety of human cancer cell lines, including multidrug resistant cell lines.[3,4] The corresponding water-soluble disodium phosphate prodrug of CA-4 (CA-4P, **2**, Fig. 1) has been demonstrated to cause reversible vascular shutdown in established tumors in vivo, consistent with an anti-vascular mechanism of action.[5] These biological effects and the structural simplicity of CA-4 make this natural product an attractive lead compound in the development of new antitumor agents.

Extensive studies have been conducted to examine the structure–activity relationships of variously modified CA-4 analogues. The 3,4,5-trimethoxyphenyl ring (ring A) and the *cis*-orientation between the two aryl rings are essential requirements for the activity of CA-4.[6] However, the *cis*-stilbenes tend to isomerize to the more stable *trans*-forms which show dramatic reduction in both anti-tubulin and cytotoxic activities. Therefore, many studies have focused on design of analogs with a locked *cis*-type bridge, which was achieved by the introduction of a heterocyclic moiety between rings A and B.[7–9]

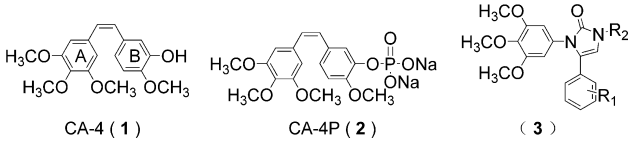
In our previously study, we reported the synthesis and evaluation of the cytotoxic activities of vicinal diaryl-substituted imidazol-2-one derivatives (**3**, Fig. 1), which can be considered as *cis*-restricted analogues of CA-4. Some of them showed potent in vitro/in vivo antitumor activity, strongly inhibited tumor cell growth, and caused arrest in the G2/M phase of the cell cycle.[10]

During our continuing studies on the synthesis of new CA-4 analogs, our attention was still focused on the central heterocyclic ring. Introducing 5-aminoisoxazole ring as the locked *cis*-type bridge may improve the solubility of CA-4 analogs and enhance the cytotoxic activity. We were also interested to investigate the biological evaluation of *N*-acetyl-5-aminoisoxazole derivatives. In this paper, the design, synthesis, molecular modeling, and in vitro cytotoxic activities of 3,4-diaryl-5-aminoisoxazole derivatives, as well as the inhibition data of tubulin polymerization and the effects on the cell cycle are reported.

2. Results and discussion

2.1. Chemistry

The synthetic routs to the target isoxazoles **11a–h**, **12a–h** and **13a–c** are outlined in Scheme 1. The phenylacetonitriles **9a–d** were synthesized following the method described in the literature with



CA-4 (**1**) CA-4P (**2**) (**3**)

Figure 1. Structure of CA-4 (**1**), CA-4P (**2**), and imidazol-2-one derivatives (**3**).

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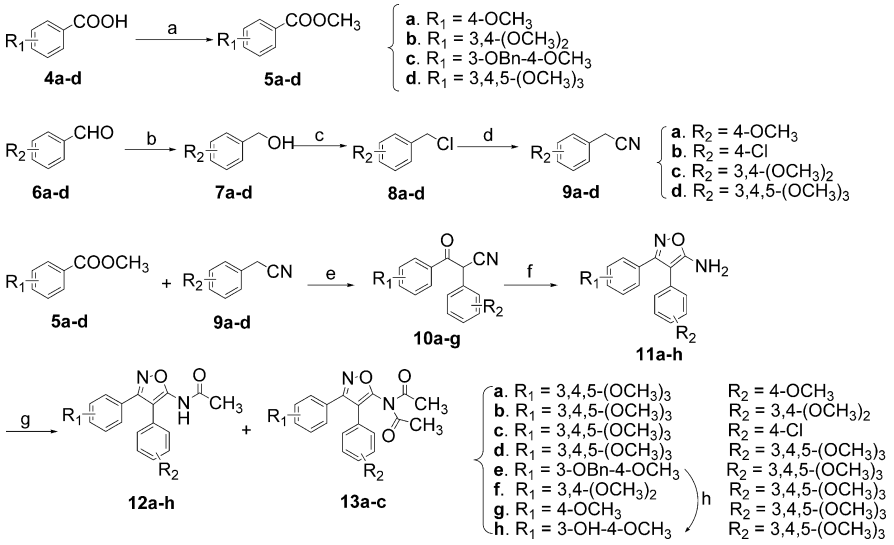

Scheme 1. Synthesis of target isoxazoles **11a**–**h**, **12a**–**h**, and **13a**–**c**. Reagents and conditions: (a) CH_3OH, H_2SO_4, reflux, 6 h; (b) KBH_4, CH_3OH, 0 °C, then rt, 3 h; (c) $SOCl_2$, DCM, Et_3N, 0–5 °C, then rt, 2 h; (d) NaCN, CH_3CN, reflux, 4 h; (e) NaH, THF, reflux, 5 h; (f) $NH_2OH{\cdot}HCl$, CH_3CH_2OH, reflux, 2 h; (g) $(CH_3CO)_2O$, pyridine, rt, 24 h; (h) 10% Pd-C, THF, rt, 12 h.

minor modifications.[11,12] Reduction of benzaldehydes **6a**–**d** with KBH_4 gave benzyl alcohols **7a**–**d** in high yields. Benzyl alcohols **7a**–**d** were chlorinated by $SOCl_2$, followed by treating with NaCN to afford phenylacetonitriles **9a**–**d**.

Esterification of benzoic acids **4a**–**d** in methanol catalyzed by H_2SO_4 yielded methyl benzoates **5a**–**d**. Reaction of methyl benzoates **5a**–**d** with phenylacetonitriles **9a**–**d** resulted in α-cyanoketones **10a**–**g**, then followed condensation with hydroxylamine hydrochloride in refluxing alcohol to afford 3,4-diaryl-5-aminoisoxazoles **11a**–**g** in moderate yields.[13] Debenzyl-protecting group of **11e** by hydrogenation afforded phenol **11h**. When the 5-aminoisoxazoles **11a**–**c** were acetylated with acetic anhydride, it yielded a mixture of *N*-acetyl-5-aminoisoxazoles **12a**–**c** and *N*,*N*-diacetyl-5-aminoisoxazoles **13a**–**c** in approximate ratio of 2:1, which could be separated by flash chromatography. Otherwise, acetylation of 5-aminoisoxazoles **11d**–**g** at the same condition, the main products were *N*-acetyl-5-aminoisoxazoles **12d**–**g** and only a trace amount of **13d**–**g** were obtained. This is mainly attributed to compound **11d**–**g** possessing a sterically hindering group, 3,4,5-trimethoxy phenyl ring. Compound **12h** was got by removal of benzyl protecting group of **12e** under catalytic hydrogenation condition.

2.2. Biological evaluation

All the prepared compounds were evaluated for their cytotoxic activities in vitro against five human cancer cell lines (human myeloid leukemia cells K562, human esophageal carcinoma cells ECA-109, human non-small lung cancer cells A549, human hepatocellular carcinoma cells SMMC-7721, and human prostate carcinoma cells PC-3). CA-4 was used as a positive control. The results are summarized in Table 1.

As shown in Table 1, the tested compounds displayed more potent cytotoxic activities against leukemia cells K562 than other cell lines, with IC_{50} values in low micromolar range (IC_{50} 0.022–8.75 μM), some of them even in nanomolar range (e.g., **11a**, **11g**, and **13a**). On the other hand, compounds **11a** and **13a** presented significant cytotoxicities against five human cancer cell lines.

Obviously, the cytotoxic potency in 5-aminoisoxazoles series (**11a**–**h**) was highly dependent on the structures of 3- or 4-position substituents of isoxazole. Compound **11a**, 3-(3,4,5-trimethoxyphenyl)-4-(4-methoxyphenyl)-5-aminoisoxazole, revealed the highest cytotoxic activities with IC_{50} values in the range of 0.04–12.00 μM against all tested cancer cell lines. Transferring the trimethoxyphenyl group from 3-position of isoxazole ring to 4-position (**11a** vs **11g**), led to a loss of cytotoxic activity against most of tested cancer cell lines, except to leukemia cells K562. Compound **11a** with 4-methoxy group on B ring showed more potent activity than that of **11b** and **11d** with 3,4-dimethoxy or 3,4,5-trimethoxy groups on B ring. Replacement the 4-methoxy group of B ring with chlorine atom (**11c**), displayed a decrease in the cytotoxic activity by one order of magnitude against K562. This result implies that the methoxy group at the C-4 position of B ring is important for the cytotoxic activity profile in the tested compounds.

In the *N*-acetyl-5-aminoisoxazoles series, compounds **12a** and **12b** showed higher cytotoxic activities than those of their

Table 1
Cytotoxicity of compounds **11a**–**h**, **12a**–**h** and **13a**–**c** against five human cancer cell lines

Compd	Cytotoxicity (IC_{50} μM)[a]				
	K562	ECA-109	A549	SMMC-7721	PC-3
11a	0.04	5.84	4.49	12.00	3.03
11b	1.27	20.55	>50	>50	21.12
11c	0.64	>50	>50	>50	>50
11d	3.65	>50	>50	>50	>50
11e	ND[b]	>50	38.25	ND[b]	>50
11f	1.40	1.66	>50	>50	>50
11g	0.022	9.82	21.75	39.76	15.01
11h	ND[b]	>50	>50	ND[b]	>50
12a	0.38	10.84	10.69	>50	>50
12b	3.71	17.65	19.84	46.31	>50
12c	3.62	9.53	24.95	>50	1.91
12d	3.97	13.94	21.14	24.63	14.88
12e	ND[b]	>50	>50	ND[b]	>50
12f	8.75	>50	>50	>50	>50
12g	1.00	>50	>50	>50	>50
12h	ND[b]	>50	>50	ND[b]	>50
13a	0.068	0.39	0.43	1.36	0.36
13b	4.29	>50	>50	>50	>50
13c	3.96	39.83	>50	>50	2.63
CA-4	0.046	4.50	0.42	3.22	5.40

[a] IC_{50} values are means of three experiments.
[b] ND: Not Determined.

regioisomers **12g** and **12f** against tested cancer cell lines. It is also proved that 3,4,5-trimethoxyphenylgroup at the 3-position was essential for cytotoxicity.

It is interesting that compound **13a**, a *N*,*N*-diacetyl derivative of compound **11a**, showed similar or more potent cytotoxic activities compared with that of **11a** as well as CA-4.

Further study on the tubulin polymerization inhibitory activity was performed with selected compounds **11a** and **13a**. Compounds **11a** and **13a**, with IC_{50} = 1.8 and 2.1 μM, showed similar tubulin polymerization inhibitory activity in comparison with CA-4 (IC_{50} = 1.2 μM) (Table 2).

To gain further insight into the mechanisms of action of these new compounds, the most cytotoxic compound **13a** was assayed for its effect on cell cycle (by flow cytometry). SMMC-7721 cells were treated with **13a** at different concentrations for 24 h.

In 0.1 μM **13a** treatment group, 41.5% cells were arrested in G2/M phase and 50.4% cells were arrested in S phase, in 1.0 μM **13a** treatment group, 70.0% cells were arrested in G2/M phase and 24.3% cells were arrested in S phase, while in control group, 25.0% and 38.0% cells were observed in G2/M phase and S phase (Fig. 2). Further research showed that compound **13a**, at graded concentrations of 0.1 μM, 0.5 μM, and 1.0 μM, induced apoptosis in SMMC-7721 cells for 48 h, the percentages of apoptotic cells were 37%, 70%, and 72%, respectively (Fig. 3).[14]

2.3. Molecular modeling

Molecular modeling studies were performed to investigate the binding ability of the isoxazoles to the colchicines binding site of α,β-tubulin. The proposed mechanism of action was supported by docking studies of compound **13a** in the colchicine site of tubulin by using the reported high-resolution crystal structure of tubulin-DAMAcolchicine complex. Figure 4 showed the docking conformation of **13a** well overlaps with CA-4 in the crystallized protein complex. The trimethoxyphenyl moiety (ring A) and 4-methoxyphenyl moiety (ring B) of isoxazole **13a** was positioned in the hydrophobic pocket between Alaβ250∼Alaβ316 and Valα181∼Metβ259, respectively. The oxygen atom of 3-methoxy group of ring A formed a hydrogen bond with the thiol group of Cysβ241, which was also found in CA-4. Besides, the oxygen atom of isoxazole ring formed one hydrogen bond with the NH of Alaβ250 and the oxygen atom of an acetyl group of 5-NH_2 formed one hydrogen bond with the NH of Asnα101. Although **12a** had a similar binding mode as **13a**, but it showed weak cytotoxicity, we think maybe **13a** has a more restrictive configuration than **12a** because of the diacetyl substitution.

3. Conclusions

A novel series of 3,4-diaryl-5-aminoisoxazoles bearing structural similarity to CA-4 have been synthesized and evaluated for their biological activities. Some of these compounds exhibited potent cytotoxic activities against five human cancer cell lines in vitro. The most potent compounds **11a** and **13a** displayed comparable cytotoxic activities with that of CA-4 against the tested cancer cell lines and they are potent inhibitors of tubulin polymerization. Cell cycle distribution analysis showed that cells exposed to **13a** at the concentration of 0.1 and 1.0 μM arrested at the G2/M phase of the cell cycle and induced cell apoptosis at 0.1–1.0 μM. Future progress on related series will be reported in due course.

Table 2
Inhibition of tublin polymerization by compound **11a**, **13a** and CA-4

Compd	IC_{50}[a] (μM)
11a	1.8
13a	2.1
CA-4	1.2

[a] IC_{50} values are means of three experiments.

4. Experimental

Melting points were obtained on a B-540 Buchi melting point apparatus and are uncorrected. ^{1}H NMR spectra was recorded on a Bruker AM 400 instrument at 400 MHz (chemical shifts are expressed as δ values relative to TMS as internal standard). Mass spectra (MS) and ESI (positive) were recorded on an Esquire-LC-00075 spectrometer. Element analysis was obtained on an Eager 300 instrument.

4.1. Synthesis

4.1.1. Synthesis of α-cyanoketones (10): general procedure

To a solution of corresponding phenylacetonitrile (1.0 mmol) in anhydrous THF (10 mL), NaH (2.4 mmol) was added at room temperature. The suspension was stirred for a few minutes, a solution of methyl benzoate (1.2 mmol) in anhydrous THF (20 mL) was added dropwise and the mixture was refluxed for 5 h. The reaction mixture was quenched with water and THF was removed under reduced pressure. The residue was acidic with 3 M HCl solution and extracted with CH_2Cl_2 (20 mL×3). The organic layers were combined, washed with brine (20 mL×2), dried over anhydrous sodium sulfate, and concentrated in vacuo to obtain the crude product. Purification by crystallization from EtOH afforded the corresponding α-cyanoketone.

4.1.1.1. 2-(4-Methoxyphenyl)-3-oxo-3-(3,4,5-trimethoxyphenyl)propanenitrile (10a). White solid (78%); mp 90–92 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (s, 3H, OCH_3), 3.85 (s, 6H, OCH_3×2), 3.90 (s, 3H, OCH_3), 5.56 (s, 1H, *HC*–CN), 6.91 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.18 (s, 2H, Ar–H), 7.35 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 342 $[M+1]^+$; Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.77; H, 5.69; N, 4.31.

4.1.1.2. 2-(3,4-Dimethoxyphenyl)-3-oxo-3-(3,4,5-trimethoxyphenyl)propanenitrile (10b). White solid (71%); mp 159–160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.88 (s, 6H, OCH_3×2), 3.89 (s, 6H, OCH_3×2), 3.92 (s, 3H, OCH_3), 5.48 (s, 1H, *HC*–CN), 6.88 (d, 1H, *J* = 8.4 Hz, Ar–H), 6.92 (s, 1H, Ar–H), 6.99 (d, 1H, *J* = 8.4 Hz, Ar–H), 7.21 (s, 2H, Ar–H); MS (ESI) *m*/*z* = 372 $[M+1]^+$; Anal. Calcd for $C_{20}H_{21}NO_6$: C, 64.68; H, 5.70; N, 3.77. Found: C, 64.53; H, 5.87; N, 3.63.

4.1.1.3. 2-(4-Chlorophenyl)-3-oxo-3-(3,4,5-trimethoxyphenyl)propanenitrile (10c). White solid (80%); mp 148–150 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.65 (s, 6H, OCH_3×2), 3.83 (s, 3H, OCH_3), 5.29 (s, 1H, *HC*–CN), 6.52 (s, 2H, Ar–H), 7.23 (d, 2H, *J* = 8.4 Hz, Ar–H), 7.33 (d, 2H, *J* = 8.4 Hz, Ar–H); MS (ESI) *m*/*z* = 346 $[M+1]^+$; Anal. Calcd for $C_{18}H_{16}ClNO_4$: C, 62.52; H, 4.66; N, 4.05. Found: C, 62.61; H, 4.54; N, 4.13.

4.1.1.4. 3-Oxo-2,3-bis(3,4,5-trimethoxyphenyl)propanenitrile (10d). White solid (68%); mp 174–176 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (s, 6H, OCH_3×2), 3.83 (s, 6H, OCH_3×2), 3.90 (s, 3H, OCH_3), 5.44 (s, 1H, *HC*–CN), 6.62 (s, 2H, Ar–H), 7.19 (s, 2H, Ar–H); MS (ESI) *m*/*z* = 402 $[M+1]^+$; Anal. Calcd for $C_{21}H_{23}NO_7$: C, 62.83; H, 5.78; N, 3.49. Found: C, 62.91; H, 5.62; N, 3.56.

4.1.1.5. 3-(3-Benzyloxy-4-methoxyphenyl)-3-oxo-2-(3,4,5-trimethoxyphenyl) propanenitrile (10e). White solid (54%); mp 135–136 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.83 (s, 9H, OCH_3×3),

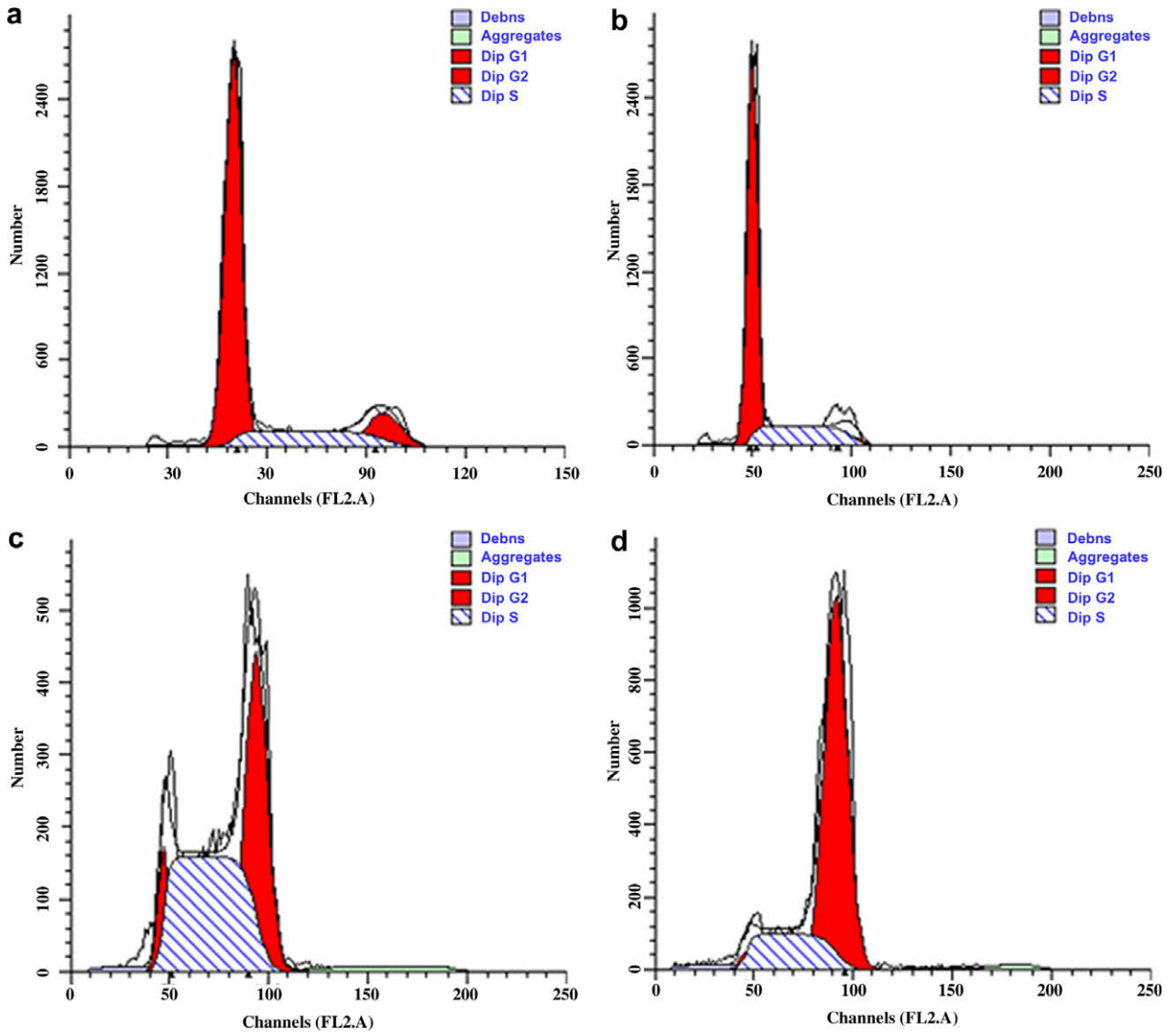

Figure 2. Effect of **13a** on the cell cycle as determined by flow cytometry. SMMC-7721 cells were treated with **13a** at the concentrations of 0 μM (control, a), 0.01 μM (b), 0.1 μM (c), and 1.0 μM (d) for 24 h.

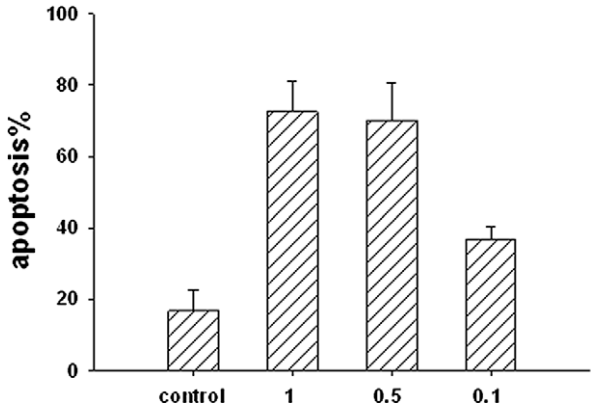

Figure 3. Compound **13a** induced apoptosis in SMMC-7721 cell. Cells were treated without (Control), 1.0 μM, 0.5 μM (c), and 0.1 μM **13a** for 48 h, respectively.

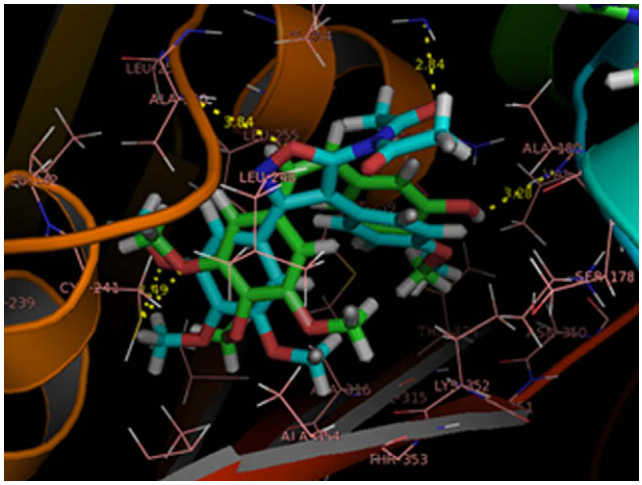

Figure 4. Proposed binding mode for compound **13a** (in blue), and CA-4 (in green) in the colchicine site of tubulin.

3.93 (s, 3H, OCH_3), 5.15 (s, 2H, CH_2), 5.48 (s, 1H, *HC*–CN), 6.60 (s, 2H, Ar–H), 6.89 (d, 1H, *J* = 8.4 Hz, Ar–H), 7.32–7.45 (m, 5H, Ar–H), 7.56–7.61 (m, 2H, Ar–H); MS (ESI) *m*/*z* = 448 $[M+1]^+$; Anal. Calcd for $C_{26}H_{25}NO_6$: C, 69.79; H, 5.63; N, 3.13. Found: C, 69.65; H, 5.71; N, 3.33.

4.1.1.6. 3-(3,4-Dimethoxyphenyl)-3-oxo-2-(3,4,5-trimethoxyphenyl)propanenitrile (10f). White solid (72%); mp 123–124 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.78 (s, 3H, OCH_3), 3.80 (s, 6H, $OCH_3\times2$), 3.86 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 5.53 (s, 1H, *HC*–CN), 6.60 (s, 2H, Ar–H), 6.84 (d, 1H, *J* = 8.8 Hz, Ar–H), 7.47 (s, 1H, Ar–H), 7.56 (d, 1H, J = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 372 $[M+1]^+$; Anal. Calcd for $C_{20}H_{21}NO_6$: C, 64.48; H, 5.70; N, 3.77. Found: C, 64.59; H, 5.59; N, 3.84.

4.1.1.7. 3-(4-Methoxyphenyl)-3-oxo-2-(3,4,5-trimethoxyphenyl)propanenitrile (10g). White solid (72%); mp 94–96 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.83 (s, 3H, OCH_3), 3.85 (s, 6H, $OCH_3\times2$), 3.87 (s, 3H, OCH_3), 5.48 (s, 1H, *HC*–CN), 6.62 (s, 2H, Ar–H), 6.94 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.95 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 342 $[M+1]^+$; Anal. Calcd for $C_{19}H_{19}NO_5$: C, 66.85; H, 5.61; N, 4.10. Found: C, 66.98; H, 5.54; N, 4.24.

4.1.2. Synthesis of 3,4-diaryl-5-aminoisoxazole derivative (11): general procedure

$NH_2OH.HCl$ (1.2 mmol) was added to a solution of the corresponding α-cyanoketone (1.0 mmol) in EtOH (5 mL), and the reaction was refluxed for 2–3 h. The reaction mixture was cooled, the solvent was evaporated off, and the residue was recrystallized from EtOH/H_2O to give the corresponding 3,4-diaryl-5-aminoisoxazole.

4.1.2.1. 4-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11a). White solid (65%); mp 157–159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68 (s, 6H, $OCH_3\times2$), 3.83 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.51 (br s, 2H, NH_2), 6.72 (s, 2H, Ar–H), 6.94 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.19 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 357 $[M+1]^+$; Anal. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. Found: C, 64.23; H, 5.42; N, 7.98.

4.1.2.2. 4-(3,4-Dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11b). Yellow solid (59%); mp 159–160 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.68 (s, 6H, $OCH_3\times2$), 3.76 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.57 (br s, 2H, NH_2), 6.73 (d, 1H, *J* = 2.0 Hz, Ar–H), 6.75 (s, 2H, Ar–H), 6.84 (dd, 1H, *J* = 2.0, 8.0 Hz, Ar–H), 6.91 (d, 1H, *J* = 8.0 Hz, Ar–H); MS (ESI) *m*/*z* = 387 $[M+1]^+$; Anal. Calcd for $C_{20}H_{22}N_2O_6$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.28; H, 5.62; N, 7.35.

4.1.2.3. 4-(4-Chlorophenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11c). Yellow solid (70%); mp 170–172 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.84 (br s, 2H, NH_2), 3.68 (s, 6H, $OCH_3\times2$), 3.84 (s, 3H, OCH_3), 6.64 (s, 2H, Ar–H), 7.18 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.34 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 387 $[M+1]^+$; Anal. Calcd for $C_{20}H_{22}N_2O_6$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.28; H, 5.62; N, 7.35.

4.1.2.4. 3,4-Bis(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11d). Yellow solid (47%); mp 160–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70 (s, 6H, $OCH_3\times2$), 3.78 (s, 6H, $OCH_3\times2$), 3.86 (s, 6H, $OCH_3\times2$), 4.60 (br s, 2H, NH_2), 6.47 (s, 2H, Ar–H), 6.76 (s, 2H, Ar–H); MS (ESI) *m*/*z* = 417 $[M+1]^+$; Anal. Calcd for $C_{21}H_{24}N_2O_7$: C, 60.57; H, 5.81; N, 6.73. Found: C, 60.25; H, 5.93; N, 6.38.

4.1.2.5. 3-(3-Benzyloxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11e). Yellow solid (48%); mp 156–158 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74 (s, 6H, $OCH_3\times2$), 3.87 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.57 (br s, 2H, NH_2), 4.98 (s, 2H, CH_2), 6.47 (s, 2H, Ar–H), 6.83 (d, 1H, *J* = 8.4 Hz, Ar–H), 7.07 (d, 1H, *J* = 8.4 Hz, Ar–H), 7.11 (s, 1H, Ar–H), 7.28–7.33 (m, 5H, Ar–H); MS (ESI) *m*/*z* = 463 $[M+1]^+$; Anal. Calcd for $C_{26}H_{26}N_2O_6$: C, 67.52; H, 5.67; N, 6.06. Found: C, 67.27; H, 5.55; N, 6.31.

4.1.2.6. 3-(3,4-Dimethoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11f). Yellow solid (58%); mp 132–134 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.75 (s, 9H, $OCH_3\times3$), 3.86 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.61 (br s, 2H, NH_2), 6.46 (s, 2H, Ar–H), 6.80 (d, 1H, *J* = 8.0 Hz, Ar–H), 7.04 (dd, 1H, *J* = 1.6, 8.0 Hz, Ar–H), 7.10 (d, 1H, *J* = 1.6 Hz, Ar–H); MS (ESI) *m*/*z* = 387 $[M+1]^+$; Anal. Calcd for $C_{20}H_{22}N_2O_6$: C, 62.17; H, 5.74; N, 7.25. Found: C, 62.31; H, 5.54; N, 7.29.

4.1.2.7. 3-(3-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11g). Yellow solid (63%); mp 146–148 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 (s, 6H, $OCH_3\times2$), 3.82 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.60 (br s, 2H, NH_2), 6.41 (s, 2H, Ar–H), 6.87 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.44 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 357 $[M+1]^+$; Anal. Calcd for $C_{19}H_{20}N_2O_5$: C, 64.04; H, 5.66; N, 7.86. Found: C, 64.22; H, 5.52; N, 7.75.

4.1.2.8. 3-(3-Hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-amine (11h). To a solution of **11e** (463 mg, 1.0 mmol) in THF (25 mL), 10% Pd/C (106 mg, 0.1 mmol) was added and the reaction mixture was stirred at room temperature under hydrogen for 12 h. The suspension was filtered on a Celite layer and concentrated in vacuo. The residue was purified by flash chromatography using pertoleum/EtOAc (1:2) to afford **11h**. Yellow solid (36%); mp 131–133 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.86 (s, 6H, $OCH_3\times2$), 3.92 (s, 6H, $OCH_3\times2$), 6.49 (s, 2H, Ar–H), 6.76–6.77 (m, 2H, Ar–H), 7.39–7.40 (m, 1H, Ar–H); MS (ESI) *m*/*z* = 373 $[M+1]^+$; Anal. Calcd for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41; N, 7.52. Found: C, 61.43; H, 5.61; N, 7.37.

4.1.3. Synthesis of mono- and di-acetylisoxazoles (12, 13): general procedure

Acetic anhydride (5 mmol) was added to a solution of the 5-amineisoxazole derivative (2 mmol) in pyridine (0.3 mL). After stirred for 24 h at room temperature, the reaction was poured into EtOAc, washed with 2 M HCl, 5% $NaHCO_3$ and brine, and the organic layers were dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo. The crude product was purified by chromatography (petroleum ether/EtOAc 4:1–1:2) to give mono- and diacetylisoxazole.

4.1.3.1. *N*-(4-(4-Methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-isoxazol-5-yl)-acetamide (12a) and *N*-acetyl-*N*-(4-(4-methoxyphenyl)-3-(3,4,5-trimethoxy-phenyl)isoxazol-5-yl)acetamide (13a). Compound **12a**, yellow solid (62%); mp 129–131 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.19 (s, 3H, O═C–*CH*$_3$), 3.64 (s, 6H, $OCH_3\times2$), 3.80 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.68 (s, 2H, Ar–H), 6.92 (d, 2H, *J* = 8.4 Hz, Ar–H), 7.16 (d, 2H, *J* = 8.4 Hz, Ar–H), 7.49 (br s, 1H, O═C–*NH*); MS (ESI) *m*/*z* = 399 $[M+1]^+$; Anal. Calcd for $C_{21}H_{22}N_2O_6$: C, 63.31; H, 5.57; N, 7.03. Found: C, 63.05; H, 5.75; N, 7.23.

Compound **13a**, yellow solid (27%); mp 119–121 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.34 (s, 6H, O═C–$CH_3\times2$), 3.68 (s, 6H, $OCH_3\times2$), 3.85 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.75 (s, 2H, Ar–H), 6.94 (d, 2H, *J* = 8.8 Hz, Ar–H), 7.12 (d, 2H, *J* = 8.8 Hz, Ar–H); MS (ESI) *m*/*z* = 441 $[M+1]^+$; Anal. Calcd for $C_{23}H_{24}N_2O_7$: C, 62.72; H, 5.49; N, 6.36. Found: C, 62.54; H, 5.56; N, 6.24.

4.1.3.2. *N*-(4-(3,4-Dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)-isoxazol-5-yl)-acetamide (12b) and *N*-acetyl-*N*-(4-(3,4-dimethoxyphenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)acetamide (13b). Compound **12b**, white solid (63%); mp 137–139 °C; ^{1}H

NMR (400 MHz, $CDCl_3$) δ 2.20 (s, 3H, O=C–CH_3), 3.66 (s, 6H, $OCH_3\times2$), 3.75 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.72 (s, 2H, Ar–H), 6.76 (d, 1H, J = 2.0 Hz, Ar–H), 6.82 (dd, 1H, J = 2.0, 8.4 Hz, Ar–H), 6.89 (d, 1H, J = 8.4 Hz, Ar–H), 7.69 (br s, 1H, O=C–*NH*); MS (ESI) m/z = 429 $[M+1]^+$; Anal. Calcd for $C_{22}H_{24}N_2O_7$: C, 61.67; H, 5.65; N, 6.54. Found: C, 61.88; H, 5.42; N, 6.76.

Compound **13b**, white solid (25%); mp 139–140 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (s, 6H, O=C–$CH_3\times2$), 3.67 (s, 6H, $OCH_3\times2$), 3.76 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 6.74 (s, 2H, Ar–H), 6.78 (d, 1H, J = 2.0 Hz, Ar–H), 6.85 (dd, 1H, J = 2.0, 8.4 Hz, Ar–H), 6.91 (d, 1H, J = 8.4 Hz, Ar–H); MS (ESI) m/z = 471 $[M+1]^+$; Anal. Calcd for $C_{24}H_{26}N_2O_8$: C, 61.27; H, 5.57; N, 5.95. Found: C, 61.54; H, 5.32; N, 5.69.

4.1.3.3. *N*-(4-(4-Chlorophenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)acetamide (12c) and *N*-acetyl-*N*-(4-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)acetamide (13c).

Compound **12c**, white solid (57%); mp 162–164 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.18 (s, 3H, O=C–CH_3), 3.66 (s, 6H, $OCH_3\times2$), 3.86 (s, 3H, OCH_3), 6.63 (s, 2H, Ar–H), 7.20 (d, 2H, J = 8.8 Hz, Ar–H), 7.37 (d, 2H, J = 8.8 Hz, Ar–H), 7.90 (s, 1H, O=C–*NH*); MS (ESI) m/z = 403 $[M+1]^+$; Anal. Calcd for $C_{20}H_{19}ClN_2O_5$: C, 59.63; H, 4.75; N, 6.95. Found: C, 59.43; H, 4.63; N, 6.86.

Compound **13c**, white solid (21%); mp 165–166 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (s, 6H, O=C–$CH_3\times2$), 3.67 (s, 6H, $OCH_3\times2$), 3.86 (s, 3H, OCH_3), 6.63 (s, 2H, Ar–H), 7.20 (d, 2H, J = 8.8 Hz, Ar–H), 7.39 (d, 2H, J = 8.8 Hz, Ar–H); MS (ESI) m/z = 445 $[M+1]^+$; Anal. Calcd for $C_{22}H_{21}ClN_2O_6$: C, 59.40; H, 4.76; N, 6.30. Found: C, 59.12; H, 4.62; N, 6.56.

4.1.3.4. *N*-(3,4-Bis(3,4,5-trimethoxyphenyl)isoxazol-5-yl)acetamide (12d).

Yellow solid (55%); mp 160–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.24 (s, 3H, O=C–CH_3), 3.67 (s, 6H, $OCH_3\times2$), 3.75 (s, 6H, $OCH_3\times2$), 3.85 (s, 6H, $OCH_3\times2$), 6.48 (s, 2H, Ar–H), 6.73 (s, 2H, Ar–H), 7.81 (br s, 1H, O=C–*NH*); MS (ESI) m/z = 459 $[M+1]^+$; Anal. Calcd for $C_{23}H_{26}N_2O_3$: C, 60.26; H, 5.72; N, 6.11. Found: C, 60.43; H, 5.51; N, 6.47.

4.1.3.5. *N*-(3-(3-(Benzyloxy)-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-isoxazol-5-yl)acetamide (12e).

White solid (50%); mp 178–180 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (s, 3H, OCH_3), 3.72 (s, 6H, $OCH_3\times2$), 3.87 (s, 6H, $OCH_3\times2$), 4.96 (s, 2H, CH_2), 6.41 (s, 2H, Ar–H), 6.82 (d, 1H, J = 8.4 Hz, Ar–H), 7.04 (d, 1H, J = 8.4 Hz, Ar–H), 7.08 (s, 1H, Ar–H), 7.26–7.32 (m, 5H, Ar–H), 7.83 (s, 1H, O=C–NH); MS (ESI) m/z = 505 [M+1]+; Anal. Calcd for $C_{28}H_{28}N_2O_7$: C, 66.66; H, 5.59; N, 5.55. Found: C, 66.45; H, 5.76; N, 5.33.

4.1.3.6. *N*-(3-(3,4-Dimethoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)-acetamide (12f).

White solid (49%); mp 168–170 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.25 (s, 3H, O=C–CH_3), 3.73 (s, 9H, $OCH_3\times3$), 3.86 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 6.44 (s, 2H, Ar–H), 6.79 (d, 1H, J = 8.0 Hz, Ar–H), 7.01 (dd, 1H, J = 1.6, 8.0 Hz, Ar–H), 7.07 (d, 1H, J = 1.6 Hz, Ar–H), 7.45 (br s, 1H, O=C–*NH*); MS (ESI) m/z = 429 $[M+1]^+$; Anal. Calcd for $C_{22}H_{24}N_2O_7$: C, 61.67; H, 5.65; N, 6.54. Found: C, 61.43; H, 5.78; N, 6.65.

4.1.3.7. *N*-(3-(4-Methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-isoxazol-5-yl)-acetamide (12g).

Yellow solid (65%); mp 175–177 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.21 (s, 3H, O=C–CH_3), 3.70 (s, 6H, $OCH_3\times2$), 3.80 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.41 (s, 2H, Ar–H), 6.85 (d, 2H, J = 8.4 Hz, Ar–H), 7.39 (d, 2H, J = 8.4 Hz, Ar–H), 7.88 (br s, 1H, O=C–*NH*); MS (ESI) m/z = 399 $[M+1]^+$; Anal. Calcd for $C_{21}H_{22}N_2O_6$: C, 63.31; H, 5.57; N, 7.03. Found: C, 63.23; H, 5.68; N, 7.05.

4.1.3.8. *N*-(3-(3-Hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)isoxazol-5-yl)acetamide (12h).

Light yellow solid (46%); mp 238–240 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.45 (s, 3H, O=C–CH_3), 3.62 (s, 6H, $OCH_3\times2$), 3.77 (s, 6H, $OCH_3\times2$), 6.20 (s, 2H, Ar–H), 6.51–6.53 (m, 2H, Ar–H), 6.69 (s, 1H, Ar–H), 7.86 (s, 1H, O=C–*NH*); MS (ESI) m/z = 415 $[M+1]^+$; Anal. Calcd For $C_{21}H_{22}N_2O_7$: C, 60.86; H, 5.35; N, 6.76. Found: C, 60.76; H, 5.21; N, 6.89.

4.2. Biology

4.2.1. Tubulin polymerization assay[15]

Tubulin polymerization assays were conducted with reagents as described by the manufacturer (Cytoskeleton, Inc.). In brief, compound **11a** and **13a** with variable concentrations (3 μM, 0.75 μM, 0.1875 μM) were incubated with purified bovine tubulin and buffer containing 20% glycerol, 1 mM GTP, 80 mM PIPES (pH 6.9), 2.0 mM $MgCl_2$, and 0.5 mM EGTA at 37 °C and the effect of compound **11a** and **13a** on tubulin polymerization was monitored kinetically using a fluorescent plate reader. The IC_{50} value is defined as the concentration of product which inhibits the rate of polymerization by 50%.

4.2.2. Cytotoxicity assay[16]

The cytotoxic activity in vitro against several cancer cell lines was measured by MTT assay. These cancer cell lines included human leukemia (K562), human esophageal carcinoma (ECA-109), human lung carcinoma (A-549), human liver carcinoma (SMMC-7721), and human prostate carcinoma (PC-3). MTT solution (10.0 μL/well) in RPMI-1640 (Sigma, St. Louis, MO) was added after cells were treated with drug for 48 h, and cells were incubated for a further 4 h at 37 °C. The purple formazan crystals were dissolved in 100.0 μl DMSO. After 5 min, the plates were read on an automated microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT) at 570 nm. Assays were performed in triplicate in three independent experiments. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated using the software 'Dose-Effect Analysis with Microcomputers'. In all of these experiments, three replicate wells were used to determine each point.

4.2.3. Flow cytometry analysis[17]

For flow cytometry analysis, SMMC-7721 cells (5×10^4/mL) were treated with DMSO, CA-4 and graded concentrations of **13a** (0.01–1.0 μM) for 24 h. Cells were washed twice with PBS and fixed in 75% ethanol at −20 °C. The cell pellet was resuspended in 100.0 μL of PBS containing 200.0 mg/mL RNase (Amersco, Solon, OH), then incubated at 37 °C for 0.5 h. After incubation, the cells were stained with 20.0 mL/L propidium iodide (PI, Sigma, St. Louis, MO) at 4 °C for 15 min. The fluorescence of cell was measured with FACSCalibur (Becton–Dickinson, Lincoln Park, NJ).

4.3. Molecular modeling[18]

Tubulin-DAMAcolchicine crystal structure (PDB ID: 1SA0) was chosen as a template. The functional A, B chains of were kept, polar hydrogens were added, and CHARMm force field was employed. Binding sphere (45.4407, 41.1246, 34.6219, 9) was selected from the active site using the binding site tools. For all tested compounds, hydrogens were added and CHARMm force fields were employed. Each of the compounds was minimized by Dreiding Minimize tool. CDOCKER (Discovery Studio 2.1) was used for the docking simulation. For each compound, the docking parameters were as follows: Top Hits: 25; Random Conformations: 10; Random Conformations Dynamics Steps: 1000; Grid Extension: 8.0; Random Dynamics Time Step: 0.002. Final docked conformations were scored by Calculated Cdocker-Energy and Cdocker interaction Energy.

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