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Graphic Abstract

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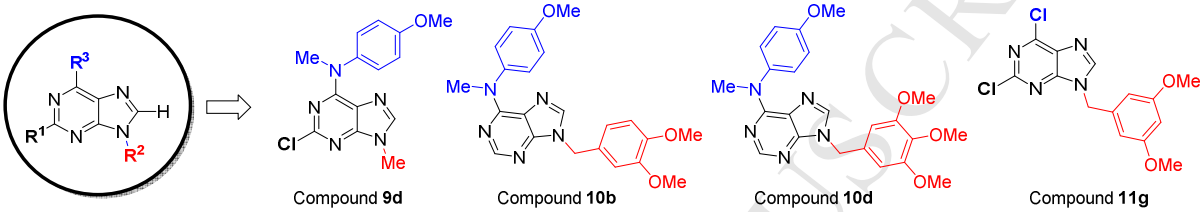

	Compound **9d**	Compound **10b**	Compound **10d**	Compound **11g**
K562: GI_{50} (μM) =	2.3±0.7	>100	>100	4.6±1.1
SH-SY5Y: GI_{50} (μM) =	3.4±0.6	0.43±0.05	0.26±0.07	1.7±0.7
AGS: GI_{50} (μM) =	0.26±0.05	>100	>100	3.8±0.5

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Abstract Two series of N-(4-methoxyphenyl)-N-methyl-9*H*-purin-6-amines (**9a-d** and **10a-h**) and 9-substituted benzyl-6-chloro-9*H*-purines (**11a-h**) were designed and synthesized. Their antiproliferative activities against human myelogenous leukemia (K562), human neuroblastoma (SH-SY5Y) and gastric cancer (AGS) cell lines were evaluated using the MTT assay. The preliminary results indicated that compounds **9d** and **11e-h** displayed low-micromole GI_{50} values against all tested cell lines. In addition, compounds **10b** and **10d** showed wonderful antiproliferative activities towards SH-SY5Y cells with selectivity of >230-fold over K562 and AGS cells. Among them, compounds **9d, 10b, 10d** and **11g** with good antitumor activities exhibited high selectivity for tumor cell lines over immortalized mouse hippocampal (HT22) cell line. Moreover, compound **9d** with sub-micromole GI_{50} values toward AGS cells exhibited moderate tubulin polymerization inhibitory activity**,** and induced apoptosis at G2/M phase arrest with a dose-dependent manner in the human AGS cells.

Key words: 9*H*-purins, antiproliferative activity, tubulin-polymerization inhibitors, apoptosis, cell cycle arrest.

1. Introduction

Cancer with the uncontrolled cell proliferation is a constant threat for human health [1]. Therefore, a continued effort of discovering new anticancer agents remains critically important. It is well known that nitrogen-containing heterocycles are widely distributed in nature and essential for the metabolism of all living cells. Among these heterocycles, the pyrimidine nucleus is an important drug-like scaffold. It is present in many pharmacologically active compounds, particularly in antitumor agents. Undoubtedly, tubulin is a good target for its essential in a diverse array of eukaryotic cell functions, and many small molecules have

been found[2-6]. Among tubulin-polymerization inhibitors, many are fused pyrimidine derivatives, such as quinazolines (**1a-b** [7]), cyclopenta[d]pyrimidin-4-amine **2** [8, 9] and 7*H*-pyrrolo[2,3-d]pyrimidine **3** [8]. 5-methyl-furo[2,3□d]pyrimidine **4** [10] with wonderful antitumor activity was reported as a potent microtubule depolymerizing agent. Pyrrolo[2,3-d]pyrimidine **5** [11] bearing the 7-benzyl group displayed good antiproliferative and antitubulin activities. Pyrazolo[4,3-d]pyrimidine **6** [12] bearing two 4-methoxybenzyl groups was identified as multi-target compound that targets both tubulin polymerization and cyclin-dependent kinases 1, 2 and 7.

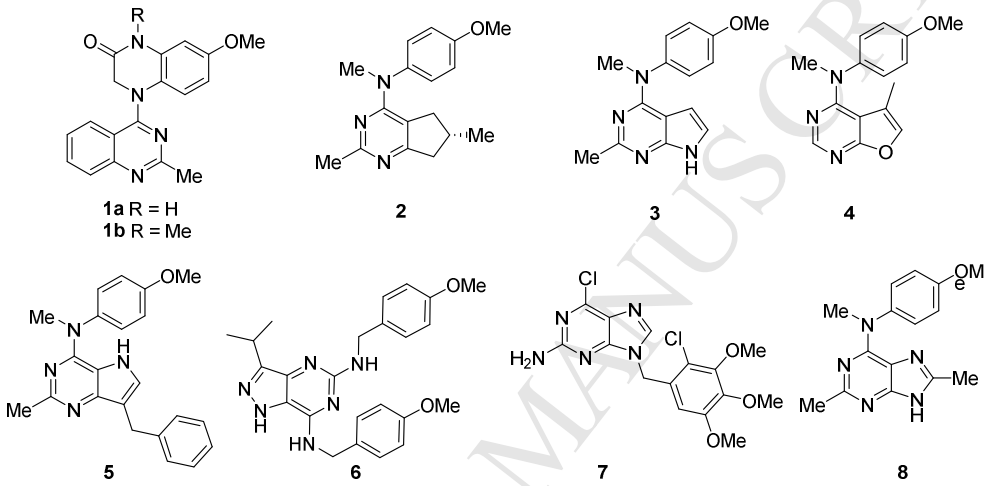


Fig. 1. Fused pyrimidine derivatives as anticancer agents.

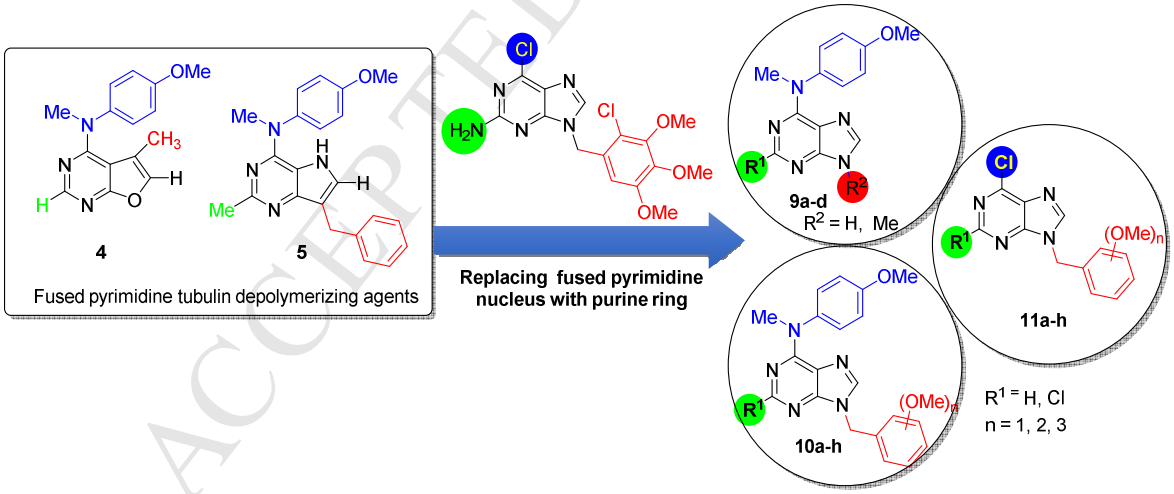


Fig. 2. The structures of target compounds

The replacement of fused pyrimidine nucleus with purine is a promising strategy for the discovery of novel therapeutic agents, and propitious to increase antitumor molecular diversity. In addition, purine derivatives have attracted considerable attention for their important chemo-preventive and chemo-therapeutic effects on cancer [13-19]. For example, 6-chloro-9*H*-purin-2-amine bearing substituted benzyl moieties (**7**) [20] was reported to

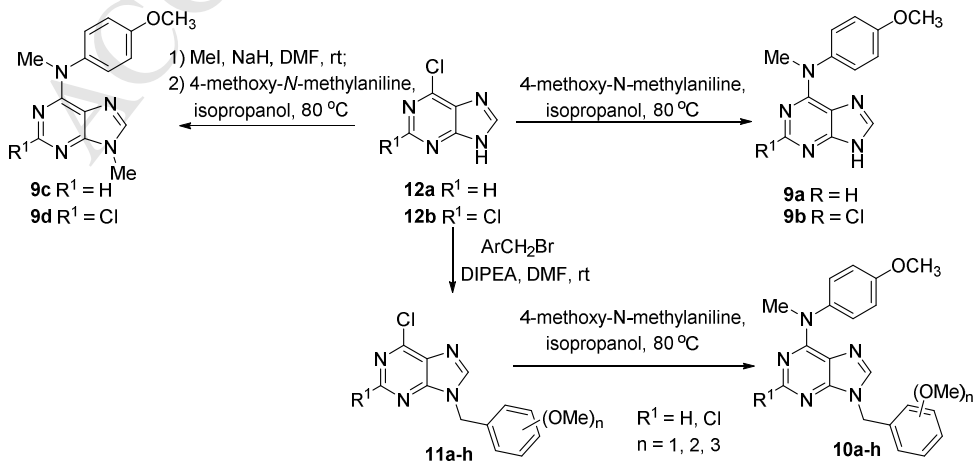
exhibit excellent antiproliferative activities against various tumor cell lines. Replacing the skeleton of compounds **2** and **3** with purine ring gave phenylaminopurine **8** [13], which displayed good antiproliferative activities and induced G2/M phase arrest as reported tubulin depolymerizing agents.

Inspired by the above considerations, N-(4-methoxyphenyl)-N-methyl-9*H*-purin-6-amines (**9a-d** and **10a-h**) and 9-substituted benzyl-6-chloro-9*H*-purines (**11a-h)** were designed and synthesized by replacing 7*H*-pyrrolo[2,3-d]pyrimidine and furo[2,3□d]pyrimidine ring in compounds **4** and **5** with purine ring (Fig. 2 and Scheme 1)**.** Their antiproliferative activities towards three human cancer cell lines (K562, SH-SY5Y and AGS) and one non-tumor cell line (immortalized mouse hippocampal cell line, HT22) were performed. The apoptosis assay, cell-cycle analyses and tubulin polymerization assay *in vitro* of selected compounds have also been further performed.

2. Results and discussion

2.1 Chemistry

Target compounds **9a-d**, **10a-h** and **11a-h** were synthesized as described in Scheme 1 using 6-chloro-9*H*-purines **12a-b** as starting materials. Compounds **9a-b** were obtained directly by the substitution reaction of 4-methoxy-N-methylaniline with 6-chloro-9*H*-purines **12a-b.** Compounds **9c-d** were synthesized from 9-methyl-6-chloro-9*H*-purines**,** which were the methylation products of compounds **12a-b**. The substitution reaction of 6-chloro-9*H*-purines **12a-b** with substituted benzyl bromine gave compounds **11a-h,** which could be converted into compounds **10a-h** by C-N cross coupling reaction.



Scheme 1. The synthesis route of target compounds **9a-d**, **10a-h** and **11a-h**.

2.2 Pharmacology

2.2.1 In vitro antiproliferative activities

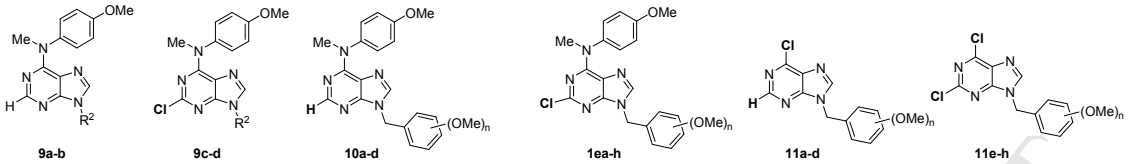
Antiproliferative activities of target compounds **9a-d**, **10a-h** and **11a-h** were examined in three human cancer cell lines, including K562, SH-SY5Y and AGS cell lines, using MTT method [21]. Fluorouracil (5-Fu) and colchicine were used as a reference cytotoxic compound in the experiments. The results were expressed as growth inhibitory concentration (GI_{50}) values which represent the compound concentrations required to produce a 50% growth inhibition of cell growth after 48 h of incubation compared to untreated controls (Table 1).

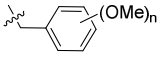
As shown in Table 1, the antiproliferative activities (GI_{50}) of target compounds **9a-d**, **10a-h** and **11a-h** against these cancer cell lines ranged from 0.26 μM to >100 μM. Among these compounds, compounds **9a-b** and **11a-d** were found to be inactive toward all tested cells. However, compounds **9d** and **11e-h** demonstrated wonderful antiproliferative activities with low-micromole GI_{50} values against all tested cancer cell lines. Particularly, compound **9d** showed best antiproliferative activity (GI_{50} = 0.26 μM) against gastric cancer (AGS) cell lines. In addition, compounds **10a**, **10b**, **10d**, **10e**, **10f** and **10h** exhibited selective antiproliferative activities against SH-SY5Y cells. Moreover, compounds **10b** and **10d** showed wonderful antiproliferative activities against SH-SY5Y cells with selectivity of >230-fold over K562 and AGS cells. The GI_{50} values of compounds **10b** and **10d** (GI_{50} = 0.43 μM and 0.26 μM respectively) were about 28- and 46-fold over that of 5-FU (GI_{50} = 11.9 μM), respectively. In addition, compounds **9d**, **10b**, **10d** and **11g** with good antitumor activities displayed lower cytotoxicities toward non-tumor HT22 cell line. More interesting, compound **9d** exhibited at least 23-, 43- and 430-fold higher selectivity for tumor cells over HT22. Compound **10b** and **10d** showed at least 230- and 88-flod higher selectivity for SH-SY5Y cell over HT22 cell, respectively.

Careful analysis on SAR of these compounds revealed that the substituents have primary influences on their antiproliferative activities and selectivity. Firstly, 2-Cl group is one of primary influences on the antiproliferative activities. For example, N-(4-methoxyphenyl)-N-methyl-9*H*-purin-6-amines bearing a Cl atom at 2-position (**9c-d** and **10e-h**) displayed higher activities against K562 and AGS cells than their corresponding purine derivatives (**9a-b, 10a-d**). 9-Substituted benzyl-6-chloro-9*H*-purines bearing a 2-H group (**11a-d)** displayed

inactivities for all tested cells, while 9-substituted benzyl-2,6-dichloro-9*H*-purines bearing a 2-Cl group (**11e-h)** shown wonderful antiproliferative activities against all tested cells.

Table 1 In vitro evaluation of compounds **9a-d**, **10a-h** and **11a-h**.



Compounds	R^2 or 	antiproliferative activity (GI_{50}, μM) [a]			CC_{50} (uM)[b]	tubulin polymerization inhibition (%)[c]
		K562	SH-SY5Y	AGS	HT22	
9a	H	>100	>100	>100	NT	NT[d]
9b	methyl	>100	>100	>100	NT	NT
9c	H	> 100	>100	30±1.5	NT	NT
9d	methyl	2.3±0.7	3.4±0.6	0.26±0.05	>100	41±1. 5
10a	4-methoxybenzyl	>100	27±2. 3	>100	NT	NT
10b	3,4-dimethoxybenzyl	>100	0.43±0.05	> 100	>100	18±0. 6
10c	3,5-dimethoxybenzyl	42±1. 2	>100	>100	NT	NT
10d	3,4,5-trimethoxybenzyl	>100	0.26±0.07	>100	23±3	20±0. 5
10e	4-methoxybenzyl	>100	31±1.9	>100	NT	NT
10f	3,4-dimethoxybenzyl	27±2.1	7.3±1.0	56±2.5	NT	25±0. 8
10g	3,5-dimethoxybenzyl	42±2.7	>100	69±3.9	NT	NT
10h	3,4,5-trimethoxybenzyl	41±2.3	3.0±0.5	85±4.1	NT	12±0. 6
11a[e]	4-methoxybenzyl	> 50	> 50	29±1.3	NT	NT
11b	3,4-dimethoxybenzyl	> 50	>100	>100	NT	NT
11c	3,5-dimethoxybenzyl	>100	>100	>100	NT	NT
11d	3,4,5-trimethoxybenzyl	> 50	>100	> 50	NT	NT
11e[f]	4-methoxybenzyl	36±2.1	7.7±1.6	19±1.5	NT	25±1. 1
11f	3,4-dimethoxybenzyl	7.0±1.3	5.5±1.1	11±1.2	NT	22±0. 6
11g	3,5-dimethoxybenzyl	4.6±1.1	1.7±0.7	3.8±0.5	>100	13±0. 8
11h	3,4,5-trimethoxybenzyl	4.3±0.6	9.6±0.9	9.4±0.8	NT	13±1. 1
5-Fu		19±1.1	12±1.2	25±1.0	NT	NT
Colchicine		0.02±0. 1	0.04±0. 1	0.04±0. 1	NT	77±3

[a] 50% Growth inhibition and data are expressed as means ± SDs (standard deviations) from at least two independent experiments.

[b] CC_{50} values represent the 50% cytotoxic concentration after 48h, the mean values of three repeated experiments.

[c] The tested concentration of compounds is 10 μM, and tubulin concentration is 5 mg/mL. Data are expressed as means ± SDs (standard deviations) from three independent experiments.

[d] NT stands for "Not Tested".

[e] The structure of compound **9a** has been reported[22]

[f] The structure of compound **9e** has been reported [23, 24]

Secondly, the substituents exert significant influences on their antiproliferative activities against SH-SY5Y cells. For example, the replacement of 9-H or 9-Me group of compound

9a-d with substituted benzyl group lead to compounds **10a**, **10b**, **10d**, **10e**, **10f** and **10h** with selective antiproliferative activities towards SH-SY5Y cells. However, compounds **10c** and **10g** bearing 3,5-dimethoxyphenyl group are inactive for SH-SY5Y cells. In addition, compounds **10a-d** bearing a 2-H group exhibited higher activities against SH-SY5Y cells than corresponding purine derivatives **10e-h** bearing a 2-Cl group**.** The aforementioned phenomena illustrate the position of methoxy moiety on the benzyl group and 2-substituents (R^1) have impacts on their activities against SH-SY5Y cells.

Base on the obtained antiproliferative activities, *in vitro* tubulin polymerization inhibition test was performed on the nine selected compounds with good activities at 10 μM, and colchicine was used as the reference (Table 1). Interestingly, their tubulin polymerization inhibitory activities are related to their antiproliferative activities. For example, compound **9d** also exhibited the most potent antitubulin polymerization inhibition activity (41% at 10 μM, Figure S1 and Table 1).

2.2.2. Effects on cell cycle distribution and apoptosis assay

Many studies have confirmed that the tubulin polymerization inhibitors can arrest cells in the G2/M phase of the cell cycle, and induce their apoptosis[6, 25-27]. The excellent inhibitory activity of **9d** promoted us to further investigate its cellular mechanisms by flow cytometry analysis. As shown in Fig. 3, AGS cells treated with compound **9d** were arrested at the G2/M phase in a dose-dependent manner, and induced an increase in the percentage of cells at G2/M. The percentages of cells at G2/M phase were increased to 64.39% at high concentration from 9.18% for the blank control group, which is accompanied by a concurrent reduction in the percentage of cells at G0/G1 and S phase.

To investigate whether compound **9d** induces apoptosis, the apoptotic effect of compound **9d** was also evaluated by an annexin V FITC/PI (AV/PI) dual staining assay using DMSO as the control. The AGS cells were treated with compound **9d** at different concentration to examine the apoptotic effect. Annexin V-FITC/propidium iodide analysis revealed that compound **9d** induced apoptosis of AGS cells in a concentration-dependent manner. As shown in Fig. 4, compound **9d** induced 33.9% apoptosis in the cancer cells at higher concentration.

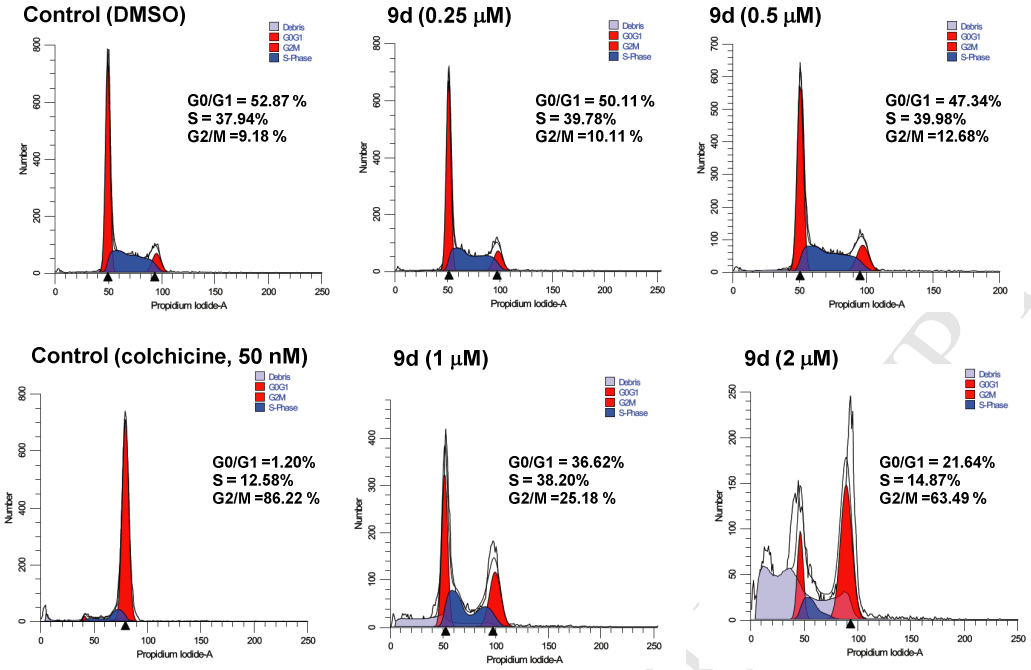


Fig. 3. Cell cycle distribution of AGS cells cell lines after treatment with compound **9d** at different concentration. It determined by flow cytometry analysis after 24 h co-culture using DNA intercalating dye, propidium iodide (PI). DMSO was used as blank control, and colchicine was used as positive control.

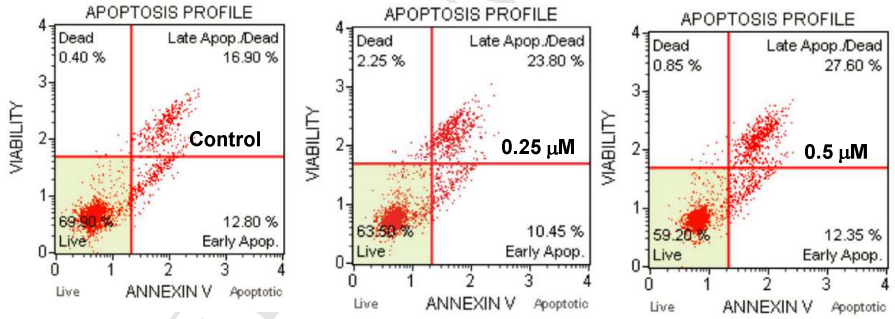


Fig. 4. Annexin V-FITC/propidium iodide analyses on apoptosis of AGS cells 24 h after co-culture with compound **9d.** The four quadrants identified as: live, early apoptotic; late apoptotic; Dead. DMSO was used as control.

2.2.3. Molecular docking

To support the discussion of the previous biological results, compound **9d** was chosen to dock in the tubulin at the colchicine site (PDB: 4O2B, 2.30 Å[28], Fig. 5). As shown in Fig. 5, the docked conformation of compound **9d** (magenta) exhibits similar conformation with colchicine (white) with some apparent variances in the colchicine site. The 9*H*-purin scaffold of compound **9d** overlaps the trimethoxybenzene ring of colchicine, hydrophobically interacts

with *β*/Ala250, *β*/Asp251, *β*/Lys254 and *β*/Lys255. 9-Methyl group of compound **9d** hydrophobically interacts hydrophobic interactions with *β*/Cys241and *β*/Leu242, while the 2-chloro group interacts with *β*/Ala317, *β*/Thr353 and *β*/Ala354. The N-methyl group of compound **9d** simulates bridging role of the cycloheptane ring of colchicine, and forms hydrophobic interactions with *β*/Lys254, *β*/Leu255 and *β*/Asn258 of tubulin. The 4-meothoxyphenyl ring of compound **9d** occupies the place of the cyclohepta-2,4,6-trien-1-one of colchicine with some variances, and forms hydrophobic interactions with *β*/Asn258, *β*/Met259, *β*/Val351, *β*/Lys352 and *α*/Val181 of tubulin.

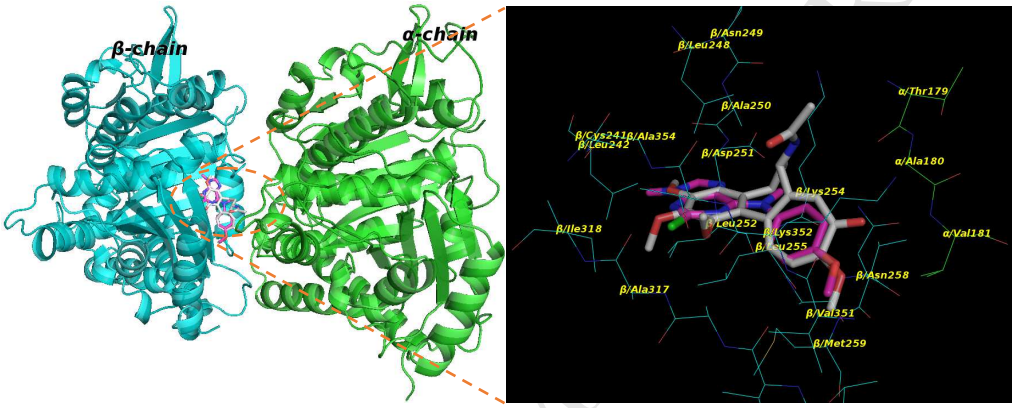


Fig. 5. Potential configuration of compound **9d** in the colchicine site of tubulin (PDB: 4O2B[28]). The C atoms in compound **9d** are colored in magenta, while the C atoms in colchicine is colored in white. The total docking score of compound **9d** is 6.689, while the colchicine is 8.722.

3. Conclusion

In summary, seventeen 9*H*-purins were designed and synthesized in this paper. Their preliminary antiproliferative activities against K562, SH-SY5Y and AGS cell lines indicated that their activities varied along with the substituents. Among them, compounds **9d** and **11e-h** showed higher antiproliferative activities against all tested cells than 5-Fu. Moreover, 2-chloro-9-methyl-N-(4-methoxyphenyl)-N-methyl-9*H*-purin-6-amine (**9d**) exhibited good tubulin polymerization inhibitory activity, and induce apoptosis at G2/M phase arrest in a dose-dependent manner in the human AGS cells. Meanwhile, 9-substituted benzyl-N-(4-methoxyphenyl)-N-methyl-9*H*-purin-6-amines **10a-b, 10d**, **10e-f** and **10h** displayed selective antiproliferative activities against SH-SY5Y cells. Compounds **10b** and**10d** showed the wonderful antiproliferative activities against SH-SY5Y cells with sub-micromole GI_{50} values. In addition, compounds **9d**, **10b**, **10d** and **11g** showed very lower

cytotoxicities toward non-tumor HT22 cell line. Considering their attractive antitumor activities and selectivity, further efforts for novel 9*H*-purins analogues with higher anticancer activities are presently going on in our laboratories.

4. Experimental Section

4.1 Chemistry

ESI spectra were measured on a Waters UPLC/Quattro Premier XE mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 using a Varian Mercury 400 spectrometer and TMS as an internal reference. Element analyses were carried out on a Vario ELIII CHNSO elemental analyzer. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. All the chemicals were of analytical grade and used without further purification.

*4.1.2 General procedures of compounds **9a-b***

6-chloro-9-*H*-purines (0.5 mmol), isopropanol (3 mL) and 4-methoxy-N-methylaniline (82 mg, 0.6 mmol) was added into a round flask (5 mL) with a stir bar. The resulting mixture was stirred at 80 ^{o}C until the starting material disappeared on TLC. Then the solvent was removed under reduced pressure to afford crude product, which was purified by chromatography on silica gel (petroleum ether/acetic ether = 1: 1, v/v) to get target compound.

*4.1.2.1 N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine **9a***: White solid. Yield: 22%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H), 3.79 (s, 3H), 6.95 (d, J = 8.6 Hz, 2H), 7.24 (d, J = 8.6 Hz, 2H), 8.04 (s, 1H), 8.18 (s, 1H), 12.98 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.6, 55.6, 114.5, 119.6, 128.5, 138.9, 139.1, 151.9, 152.0, 154.5, 157.7. ESI-MS (m/z): 271.14 ($[M+Na]^+$). And Anal. Calcd. for $C_{13}H_{13}N_5O$: C, 61.17; H, 5.13; N, 27.43; Found: C, 61.54; H, 5.36; N, 27.09.

*4.1.2.2 2-chloro-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine **9b***: White solid. Yield: 36%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.73 (s, 3H), 3.80 (s, 3H), 6.97 (d, J = 8 Hz, 2H), 7.27(d, J = 8 Hz, 2H), 8.03 (s, 1H), 13.12 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.8, 55.6, 114.6, 118.7, 128.4, 138.2, 139.5, 152.5, 153.152, 154.8, 158.1. ESI-MS (*m/z*): 290.70 ($[M+H]^+$). And Anal. Calcd. for $C_{13}H_{12}ClN_5O$: C, 53.89; H, 4.17; N, 24.17; Found: C, 53.71; H, 4.52; N, 24.39.

*4.1.3 General procedures of compounds **9c-d***

To a solution of 9*H*-purines (1.5 mmol) and sodium hydride (0.12 g, 60%) in anhydrous DMF (3 mL) was added iodomethane (0.42g, 3 mmol). The resulting mixture was stirred at room temperature

until the starting material disappeared on TLC. Then the reaction solution was poured into water (20 mL), and extracted three times with EtOAc (15 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield crude 9-methyl-9*H*-purine, which was used in next step without further purification.

And then the synthesis of compounds ***9c-d*** was performed as previous procedures of compounds ***9a-b*** using obtained 9-methyl-9*H*-purine as reactant**.**

4.1.3.1 N-(4-methoxyphenyl)-N,9-dimethyl-9H-purin-6-amine **9c**: White solid. Yield: 23%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.80 (s, 3H), 3.85 (s, 6H), 6.97 (d, J = 8 Hz, 2H), 7.25 (d, J = 8 Hz, 2H), 7.66 (s, 1H), 8.41 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.6, 40.7, 55.3, 114.5, 120.3, 127.9, 138.7, 139.6, 151.4, 152.4, 154.8, 158.0. ESI-MS (m/z): 270.50 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{15}N_5O$: C, 62.44; H, 5.61; N, 26.01; Found: C, 62.32; H, 5.28; N, 25.98.

4.1.3.2 2-chloro-N-(4-methoxyphenyl)-N,9-dimethyl-9H-purin-6-amine ***9d***: White solid. Yield: 53%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.72 (s, 3H), 3.73 (s, 3H), 3.85 (s, 3H), 6.95 (d, J = 8 Hz, 2H), 7.22 (d, J = 8 Hz, 2H), 7.54 (s, 1H). ^{13}C NMR (100 MHz, CDCl3) δ 29.9, 40.7, 55.4, 114.4, 119.3, 127.9, 137.9, 140.0, 152.6, 153.7, 155.0, 158.4. ESI-MS (m/z): 305.02 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{14}ClN_5O_2$: C, 55.36; H, 4.65; N, 23.06; Found: C, 55.49; H, 4.34; N, 22.85.

4.1.2 General procedures of compounds ***10a-h*** *and* ***11a-h.***

To a solution of 9*H*-purines (1.5 mmol) and N,N-diisopropylethylamine (DIPEA, 3 mmol) in DMF (4 mL) was added substituted benzyl bromide (1.5 mmol). The resulting mixture was stirred at room temperature until the starting material disappeared on TLC. Then the reaction solution was poured into water (40 mL), and extracted three times with EtOAc (15 mL × 3). The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield crude product. Purification was accomplished by chromatography on a silica gel column, eluted with petroleum ether-acetic ether (1/l, v/v), to give compounds **11**. And then the synthesis of compounds ***10a-h*** was performed as previous procedures of compounds ***9a-b*** using obtained compounds **11a-h** as reactants.

4.1.3.1 9-(4-methoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10a***: White solid. Yield: 59%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H), 4.22 (s, 3H), 5.32 (s, 2H), 6.42 (s, 1H), 6.46 (s, 2H), 8.10 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 41.2, 46.8, 55.3, 55.4, 114.4, 114.6, 120.2, 127.5, 127.9, 138.2, 139.3, 150.7, 151.6, 158.4, 159.7. ESI-MS (m/z): 376.50 ($[M+H]^+$), 398.50 ($[M+Na]^+$). And Anal. Calcd. For $C_{21}H_{21}N_5O_2$: C, 67.18; H, 5.64; N, 18.65; Found: C, 66.92; H, 5.71; N,18.42.

4.1.3.2 9-(3,4-dimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10b***: White solid.

Yield: 30%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (s, 3H), 3.84 (s, 6H), 3.85 (s, 3H), 5.26 (s, 2H), 6.79-6.87 (m, 3H), 6.96 (d, J = 8 Hz, 2H), 7.24 (d, J = 8 Hz, 2H), 7.64 (s, 1H), 8.42 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.7, 47.0, 55.4, 56.0, 111.3, 114.5, 120.5, 128.0, 128.2, 138.7, 138.9, 149.1, 149.4, 151.1, 152.5, 154.9, 158.1. ESI-MS (*m/z*): 406.85 ($[M+H]^+$), 429.13 ($[M+Na]^+$). And Anal. Calcd. for $C_{22}H_{23}N_5O_3$: C, 65.17; H, 5.72; N, 17.27; Found: C, 64.98; H, 5.464; N,17.13.

4.1.3.3 9-(3,5-dimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10c***: White solid. Yield: 14%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.74 (s, 6H), 3.84 (s, 6H), 5.26 (s, 2H), 6.38 (s, 1H), 6.40 (s, 2H), 6.96 (d, J = 8 Hz, 2H), 7.25 (d, J = 8 Hz, 2H), 7.65 (s, 1H), 8.42 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.8, 47.1, 55.4, 55.4, 99.9, 105.8, 114.5, 120.3, 128.0, 138.0, 138.6, 139.1, 151.1, 152.5, 154.9, 158.1, 161.3. ESI-MS (*m/z*): 406.87 ($[M+H]^+$), 428.62 ($[M+Na]^+$). And Anal. Calcd. for $C_{22}H_{23}N_5O_3$: C, 65.17; H, 5.72; N, 17.27; Found: C, 65.34; H, 5.41; N,17.57.

4.1.3.4 9-(3,4,5-trimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10d***: White solid. Yield: 29%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.81 (s, 9H), 3.84 (s, 6H), 5.25 (s, 2H), 6.54 (s, 2H), 6.97 (d, J = 8 Hz, 2H), 7.25 (d, J = 8 Hz, 2H), 7.66 (s, 1H), 8.43 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.8, 47.4, 55.4, 56.2, 60.8, 105.2, 114.5, 120.3, 128.0, 131.3, 138.0, 138.6, 139.0, 151.0, 152.5, 153.6, 154.9, 158.2. ESI-MS (*m/z*): 436.54 ($[M+H]^+$), 458.56 ($[M+Na]^+$). And Anal. Calcd. for $C_{23}H_{25}N_5O_4$: C, 64.44; H, 5.79; N, 16.08; Found: C, 64.71; H, 5.46; N,15.98.

4.1.3.5 2-chloro-9-(4-methoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10e***: White solid. Yield: 82%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70 (s, 3H), 3.78 (s, 3H), 3.83 (s, 3H), 5.19 (s, 2H), 6.84 (d, J = 8 Hz, 2H), 6.94 (d, J = 8 Hz, 2H), 7.20-7.22 (m, 4H), 7.47 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.7, 40.6, 46.7, 55.3, 55.4, 114.37, 114.41, 119.3, 127.3, 128.0, 129.6, 137.9, 139.2, 152.2, 153.8, 155.0, 158.4, 159.7. ESI-MS (*m/z*): 410.30 ($[M+H]^+$). And Anal. Calcd. for $C_{21}H_{20}ClN_5O_2$: C, 61.54; H, 4.92; N, 17.09; Found: C, 61.35; H, 4.73; N, 16.89.

4.1.3.6 2-chloro-9-(3,4-dimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10f***: White solid. Yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70 (s, 3H), 3.83 (s, 6H), 3.85 (s, 3H), 5.18 (s, 2H), 6.81 (q, J = 8 Hz, 2H), 6.90 (s, 1H), 6.94 (d, J = 8 Hz, 2H), 7.22 (d, J = 8 Hz, 2H), 7.50 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.6, 47.2, 55.4, 55.9, 111.2, 111.6, 114.4, 119.3, 120.7, 127.6, 127.9, 137.8, 139.2, 149.2, 149.3, 152.2, 153.7, 155.0, 158.4. ESI-MS (*m/z*): 440.4 ($[M+H]^+$), 462.4 ($[M+Na]^+$). And Anal. Calcd. for $C_{22}H_{22}ClN_5O_3$: C, 60.07; H, 5.04; N, 15.92; Found: C, 59.70; H, 4.86; N,15.67.

4.1.3.7 2-chloro-9-(3,5-dimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10g***:

White solid. Yield: 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.71 (s, 3H), 3.75 (s, 6H), 3.85(s, 3H), 5.20 (s, 2H), 6.38 (s, 1H), 6.41 (s, 2H), 6.96 (d, J = 8 Hz, 2H), 7.22 (d, J = 8 Hz, 2H) 7.70 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.7, 47.8, 55.4, 55.5, 100.2, 106.2, 114.7, 127.9, 137.0, 139.5, 161.3. ESI-MS (*m/z*): 440.6 ($[M+H]^+$), 462.5 ($[M+Na]^+$). And Anal. Calcd. for $C_{22}H_{22}ClN_5O_3$: C, 60.07; H, 5.04; N, 15.92; Found: C, 60.34; H, 4.89; N,15.87.

4.1.3.8 2-chloro-9-(3,4,5-trimethoxybenzyl)-N-(4-methoxyphenyl)-N-methyl-9H-purin-6-amine ***10h***: White solid. Yield: 55%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.71 (s, 3H), 3.82 (s, 9H), 3.84(s, 3H), 5.19 (s, 2H), 6.58 (s, 2H), 6.96 (d, J = 8 Hz, 2H), 7.22 (d, J = 8 Hz, 2H), 7.69 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.70, 47.87, 55.4, 56.2, 60.8, 105.7, 114.6, 127.9, 130.5, 137.5, 138.2, 139.3, 152.0, 153.6, 154.0, 154.8, 158.7. ESI-MS(*m/z*): 470.5 ($[M+H]^+$), 492.5 ($[M+Na]^+$). And Anal. Calcd. for $C_{23}H_{24}ClN_5O_4$: C, 58.79; H, 5.15; N, 14.90; Found: C, 58.64; H, 5.01; N,14.78.

4.1.3.9 6-chloro-9-(4-methoxybenzyl)-9H-purine ***11a***[22]: White solid. Yield: 33%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.79 (s, 3H), 5.38 (s, 2H), 6.89 (d, J = 8 Hz, 2H), 7.28 (d, J = 8 Hz, 2H), 8.08 (s, 1H), 8.77 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 47.4, 55.3, 114.5, 126.4, 128.5, 129.5, 144.9, 150.9, 151.7, 152.0, 159.9. ESI-MS (*m/z*): 275.16 ($[M+H]^+$). ESI-MS (*m/z*): 275.16 ($[M+H]^+$). And Anal. Calcd. for $C_{13}H_{11}ClN_4O$: C, 56.84; H, 4.04; N, 20.40; Found: C, 56.51; H, 3.90; N, 20.35.

4.1.3.10 6-chloro-9-(3,4-dimethoxybenzyl)-9H-purine ***11b****:* White solid. Yield: 36%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (s, 3H), 3.88 (s, 3H), 5.39 (s, 2H), 6.84-6.92 (m, 3H), 8.08 (s, 1H), 8.79 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 47.9, 56.0, 56.0, 111.3, 111.5, 120.8, 126.8, 131.6, 144.9, 149.6, 151.2, 151.8, 152.1. ESI-MS (*m/z*): 305.92 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{13}ClN_4O_2$: C, 55.18; H, 4.30; N, 18.39; Found: C, 55.40; H, 4.11; N, 18.50;

4.1.3.11 6-chloro-9-(3,5,-dimethoxybenzyl)-9H-purine ***11c****:* White solid. Yield: 34%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.76 (s, 6H), 5.37 (s, 2H), 6.43 (s, 3H), 8.11 (s, 1H), 8.79 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 48.0, 55.4, 100.25, 106.1, 131.4, 136.6, 145.0, 151.2, 151.8, 152.3, 161.5. ESI-MS (*m/z*): 305.6 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{13}ClN_4O_2$: C, 55.18; H, 4.30; N, 18.39; Found: C, 55.42; H, 4.29; N, 18.17.

4.1.3.12 6-chloro-9-(3,4,5-trimethoxybenzyl)-9H-purine ***11d***: White solid. Yield: 30%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.82 (s, 6H), 3.83 (s, 3H), 5.37 (s, 2H), 6.52 (s, 2H), 8.12 (s, 1H), 8.81 (s, 1H). ^{13}C

NMR (100 MHz, $CDCl_3$) δ 48.3, 56.2, 60.8, 105.4, 129.7, 131.1, 138.5, 144.9, 151.1, 151.7, 152.2, 153.8. ESI-MS (*m/z*): 335.76 ($[M+H]^+$). And Anal. Calcd. for $C_{15}H_{15}ClN_4O_3$: C, 53.82; H, 4.52; N, 16.74; Found: C, 53.51; H, 4.42; N, 16.89.

4.1.3.13 2,6-dichloro-9-(4-methoxybenzyl)-9H-purine ***11e*** [23, 24]: White solid. Yield: 41%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.81 (s, 3H), 5.34 (s, 2H), 6.91 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 10.4 Hz, 2H), 8.02 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 47.71, 55.4, 114.7, 125.9, 129.8, 130.3, 130.7, 145.5, 151.8, 153.1, 160.2. ESI-MS (*m/z*): 309.91 ($[M+H]^+$). And Anal. Calcd. for $C_{13}H_{10}Cl_2N_4O$: C, 50.51; H, 3.26; N, 18.12; Found: C, 50.21; H, 3.20; N, 18.20;

4.1.3.14 2,6-dichloro-9-(3,4-dimethoxybenzyl)-9H-purine ***11f***: White solid. Yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.86 (s, 3H), 3.88 (s, 3H), 5.33 (s, 2H), 6.86 (d, J = 8 Hz, 1H), 6.90-6.92 (m, 2H), 8.04 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 48.2, 56.0, 56.0, 111.5, 121.0, 126.2, 130.8, 130.9, 145.9, 149.7, 151.9, 153.1. ESI-MS (*m/z*): 340.14 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{12}Cl_2N_4O_2$: C, 49.58; H, 3.57; N, 16.52; Found: C, 49.61; H, 3.55; N, 16.45;

4.1.3.15 2,6-dichloro-9-(3,5-dimethoxybenzyl)-9H-purine ***11g***: White solid. Yield: 35%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.77 (s, 6H), 5.32 (s, 2H), 6.43 (s, 3H), 8.06 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 48.1, 55.5, 100.4, 106.3, 130.7, 136.0, 145.6, 151.9, 153.2, 153.2, 161.5. ESI-MS (*m/z*): 339.88 ($[M+H]^+$). And Anal. Calcd. for $C_{14}H_{12}Cl_2N_4O_2$: C, 49.58; H, 3.57; N, 16.52; Found: C, 49.31; H, 3.49; N, 16.61;

4.1.3.16 2,6-dichloro-9-(3,4,5-trimethoxybenzyl)-9H-purine ***11h****:* White solid. Yield: 34%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (s, 9H), 5.32 (s, 2H), 6.59 (s, 2H), 8.09 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 48.6, 56.3, 60.9, 105.7, 129.3, 130.8, 138.7, 145.5, 152.0, 153.1, 153.1, 153.9. ESI-MS(*m/z*): 361.90 ($[M+H]^+$). And Anal. Calcd. for $C_{15}H_{14}C_{l2}N_4O_3$: C, 48.80; H, 3.82; N, 15.18; Found: C, 48.57; H, 3.75; N, 15.07;

4.2 Pharmacology

4.2.1. Compounds

Target compounds were dissolved in DMSO and stored as stock solutions (100 mM) at -20°C. For experimental use, all the compounds were prepared from stock solutions, diluted with growth medium

and used immediately.

4.2.2. Statistical Analysis.

Statistical analysis was performed and figures were produced using Prism 5 for Windows (Graph-Pad Software, Inc., San Diego, CA, USA). Data were expressed as mean ± standard Deviation (SD). All data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison tests for post hoc analyses between the groups, results were considered statistically significant when $p < 0.05$.

4.2.3 Cell culture

SH-SY5Y human neuroblastoma, K562 human myelogenous leukemia and AGS gastric cancer cell lines were cultured in RPMI 1640, supplemented with 4% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

4.2.4. In vitro anticancer activity

The *in vitro* cytotoxic activities of tested compounds was determined by MTT assay [21, 29, 30]. Briefly, cells in the log-phase of growth were harvested by trypsinization, seeded in 96 well plates (Nunc, Denmark) for 24 h. Then, the cells were treated with various concentrations of the compounds for 48 h. Fluorouracil (5-Fu) and DMSO were used as positive and negative controls, respectively. The final concentration of DMSO was less than 1%. After 48 h, the culture medium was removed, and cells were incubated with 200 mL of MTT solution (0.5 mg/mL) for 4 h. Then, the supernatant was removed and the formazan crystals were dissolved using DMSO. The stained formazan product was determined spectrophotometrically at 570 nm in GENios Pro microplate reader (TECAN).

4.2.5. Annexin V-FITC apoptosis assay

Further evaluation of the pro-apoptotic effect of compound **9d** on AGS cells was analyzed with the Annexin V/7-AAD assays using Muse® Annexin V and Dead Cell Assay Kit from Millipore. Annexin V, which is membrane permeable, labels all cells containing a nucleus. The second component of the kit is 7-AAD, which stains the membrane of the cells that have been compromised and are dying or dead. This synergistic functioning of the two components differentiates between cell populations based

on their health. The assay was performed according to manufacturer's protocol. Briefly, AGS cells were grown in 6-well culture plates (3×10^5 cells/well) and treated with compounds for 24 h. Following the treatment, cells were pelleted and then resuspended in 100 mL of complete growing media, followed by adding 100 mL of Muse Annexin V and Dead Cell Reagent to each tube. The tubes were mixed thoroughly and then incubated at room temperature for 20 min without light exposure. After incubation, the tubes were read individually using Muse TM Cell Analyzer. Default gate settings were used to distinguish the cells in the live, early-apoptotic, late-apoptotic and dead categories.

4.2.6. Cell cycle analysis

The SH-SY5Y cells were treated with different concentrations of compound **9d** for 24 h. After treatment, the cells were washed twice with ice-cold PBS, collected by centrifugation, and fixed in ice-cold 70% (v/v) ethanol, washed with PBS, re-suspended with 0.1 mg/mL RNase, stained with 40 mg/mL PI, and analyzed by flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions were analyzed using Cell- Quest software (Becton Dickinson).

4.2.7. In vitro tubulin polymerization assay

According to method described by Shelanski et al. [31], pig brain microtubule protein was isolated by employing three cycles of temperature-dependent assembly/disassembly. Homogeneous tubulin was prepared from microtubule protein by phosphocellulose (P11) chromatography as has been described previously [32]. The purified proteins were stored in aliquots at -70^{o}C. Microtubule polymerization of tubulin protein was monitored at 37^{o}C in solutions containing compounds (10 μM) in PEM buffer (100 mM PIPES, 1 mM $MgCl_2$, and 1 mM EGTA), 1 mM GTP, and 5% glycerol by using light scattering at 340 nm with a SPECTRA MAX 190 (MD) spectrophotometer. And colchicine was chosen as standard inhibitor of tubulin polymerization, while DMSO was used as negative control, and tubulin concentration is 5 mg/mL. The percent inhibition values for selected compounds were compared to the value of colchicine and measured the same day under the same conditions.

4.3. Molecular docking

Surflex-Dock program in Sybyl 7.3 was employed to dock compound **9d** in colchicine binding site of αβ-tubulin (PDB: 4O2B, 2.30 Å [28]). And an empirical scoring function and a patented search engine [33, 34] was adopted. Docking was initiated by removing all ligands and water followed by addition of random hydrogen atoms. The following procedures are performed according to our reported procedures [35-37]. Ligand was docked into the corresponding protein-binding site guided by protomol, which is an idealized representation of a ligand that makes every potential interaction with the binding site. To validate docking reliability, the co-crystalized ligand (colchicine) was removed from the active site and docked back into the known binding pocket. The root means square deviation (RMSD) between the predicted conformation and the actual conformation from the crystal structure of ligand was 1.96 Å, which was smaller than the resolution of X-ray crystallography 2.30 Å [28]. Thus, the parameters set for this Surflex-dock simulation successfully reproduced the ligand-binding motif in the X-ray structure, and were extrapolated to predict the binding conformations of the synthesized inhibitors.

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Research Highlights

- Two series of 9*H*-purin-6-amines and 6-dichloro-9*H*-purines were designed and synthesized.
- Compounds **9d** and **11e-h** exhibited low-micromole GI_{50} values against all test cell lines.
- Compound **9d** induced apoptosis at G2/M phase arrest in the human AGS cells.
- Compounds **10b** and **10d** showed selective antiproliferative activities towards SH-SY5Y cells.
- Compounds **10b** and **10d** displayed sub-micromole GI_{50} values against SH-SY5Y cells.