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4′-Alkoxyl substitution enhancing the anti-mitotic effect of 5-(3′,4′,5′-substituted)anilino-4-hydroxy-8-nitroquinazolines as a novel class of anti-microtubule agents

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Abstract—Mitosis inhibitors are powerful anticancer drugs. Based on a novel anti-microtubule agent of 5-(4′-methoxy)anilino-4-hydroxy-8-nitroquinazoline, a series of 5-(3′,4′,5′-substituted)anilino-4-hydroxy-8- nitroquinazolines were designed and synthesized to investigate the effect of the substitution on the inhibitory activity against mitotic progression of tumor cells. The large alkoxyl substitution on the 4′-position of 5-anilino ring is beneficial for the potency. The 5-(3′,4′,5′-trimethoxy)anilino-8-nitroquinazoline (**1h**) displays an overwhelming activity in arresting the cells at the G2/M phase, providing a promising new template for further development of potent microtubule-targeted anti-mitotic drugs.

Quinazolines are a class of fused pyrimidine derivatives which display diverse range of biological activities.[1] Among them, 4-anilinoquinazolines are the most promising small molecule EGFR tyrosine kinase inhibitors. For example, the best inhibitors of EGFR tyrosine kinase PD153035 (6,7-dimethoxy-4-(3′-bromophenyl)amino-quinazoline) and IRESSA™ (gefitinib, ZD1839),[2] developed from this compound family, are presently the only approved and granted drugs by the FDA for the treatment of advanced non-small-cell lung cancer (NSCLC). However, the activity of the quinazoline family could be greatly modulated by the substitution change on the quinazoline core structure.[3] Recently, we developed a new class of 5-substituted-4-hydroxy-8-nitroquinazolines which displayed potent antiproliferative effect against EGFR or ErbB-2 overexpressing tumor cell lines but not targeting the EGFR tyrosine kinase.[4] Further study on the molecular mechanism disclosed that the 5-(4-methoxy)anilino-4-hydroxy-8-nitroquinazoline (**LJK-11**) functions as an anti-microtubule agent by inhibiting the microtubule polymerization.[5]

Microtubules are critical elements in a variety of fundamental cell functions, including formation and maintenance of cell shape, transportation of vesicles and protein complexes, and regulation of motility and cell division.[6] The essential role of microtubules in mitosis and cell division makes them and their regulatory proteins important targets for anticancer drugs. Anti-microtubule agents, such as nocodazole, vinorelbine, colchicines, and paclitaxel (Fig. 1), have been successfully used in treating a variety of cancers.[7,8]

Despite the success of taxanes and vinca alkaloids to inhibit the progression of certain cancers in clinical use, resistance to anti-microtubule agents is encountered in many tumor types, particularly during multiple cycles of therapy.[9–11] Therefore, there has been great interest in identifying and developing novel anti-microtubule drugs. Our preliminary study has revealed that **LJK-11** is a specific cell mitosis blocker, which inhibits microtubule polymerization, arrests cells at early stage of mitosis, and induces apoptosis of tumor cells.[5]

Since **LJK-11** represents a novel anti-microtubule agent, we are intrigued to conduct SAR study to explore the

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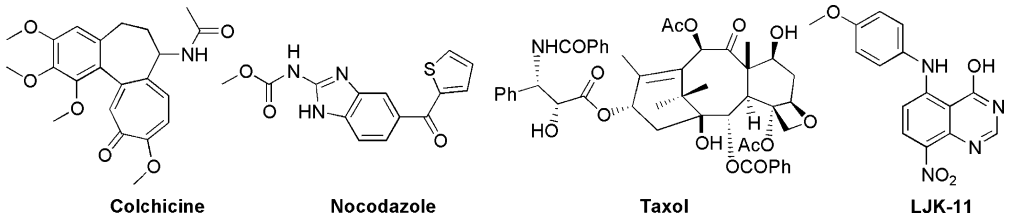


Figure 1. The representative structures of anti-microtubule agents.

pharmacophore and then improve the activity. A focused library containing 5,8-disubstituted quinazoline structure was designed. In the present work, we have focused on investigating the effect of the 3′,4′-substitution of the 5-anilino portion on the anti-mitosis activity (Fig. 2). Thus, a series of 5-(3′,4′-substituted) anilino-4-hydroxy-8-nitroquinazoline analogs were designed and synthesized. The activity to inhibit the growth and block the mitosis of the tumor cell lines was evaluated. The alkoxyl substitution on the 3′,4′-position of the 5-anilino moiety was found to potentiate the anti-mitotic potency of this novel class of microtubule polymerization inhibitors.

A series of 5-anilino-4-hydroxy-8-nitroquinazolines with various substitution on 3′,4′-position of 5-anilino moiety was designed and synthesized. The choice of the substituent structure was referred to the previous SAR study of the 5-substituted-4-hydroxy-8-nitroquinazolines as EGFR inhibitors.[4] The alkoxyl, alkyl, and aminoacyl groups containing α,β-unsaturated carboxyl ester were incorporated into the 4-position of the 5-anilino ring, in combination with additional substitution on 3- and 5-position with chloro and methoxy groups, respectively, in some cases. The general synthesis is similar to the procedure reported previously,[4] starting from the commercially available 3-chloro-2-methyl aniline and employing Neimentowski synthesis as the key step to construct the quinazoline core. As depicted in Scheme 1, the current effort was focused on the synthesis of the various 3′,4′-substituted anilines (**3a**–**h**) which react with the common intermediate **2** to furnish the desired 5-(3′,4′-substituted)anilino-4-hydroxy-8-nitroquinazolines **1a**–**h**.[12]

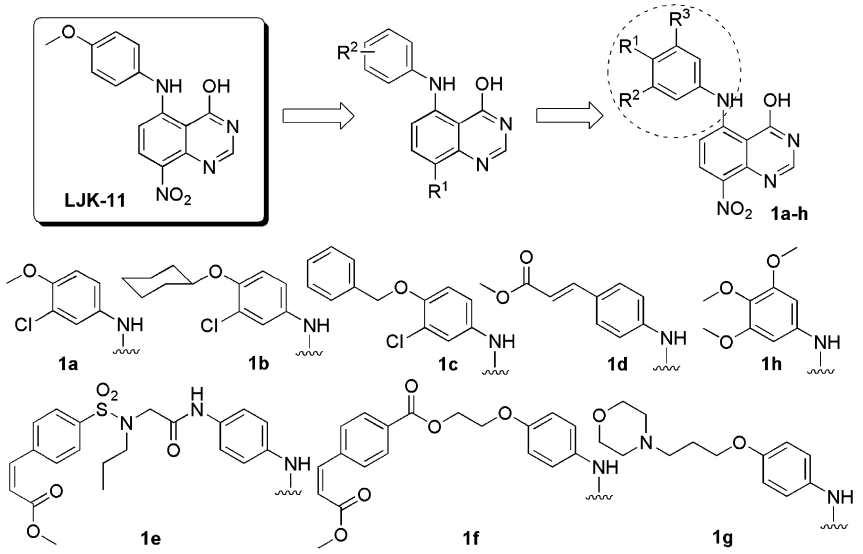
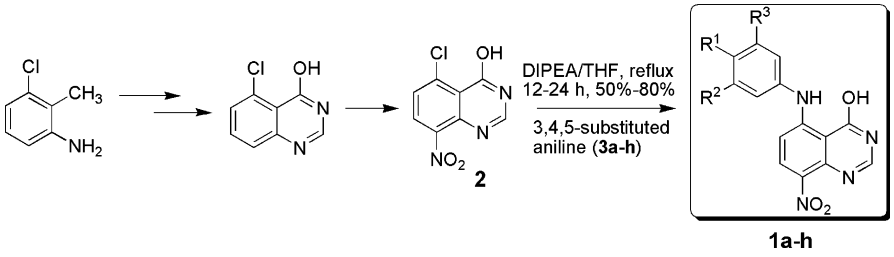


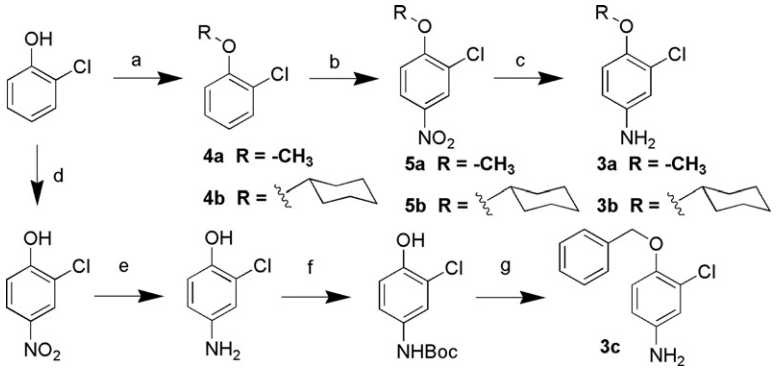
Figure 2. The designed series of 5-(3′,4′-substituted)anilino-4-hydroxy-8-nitroquinazolines.



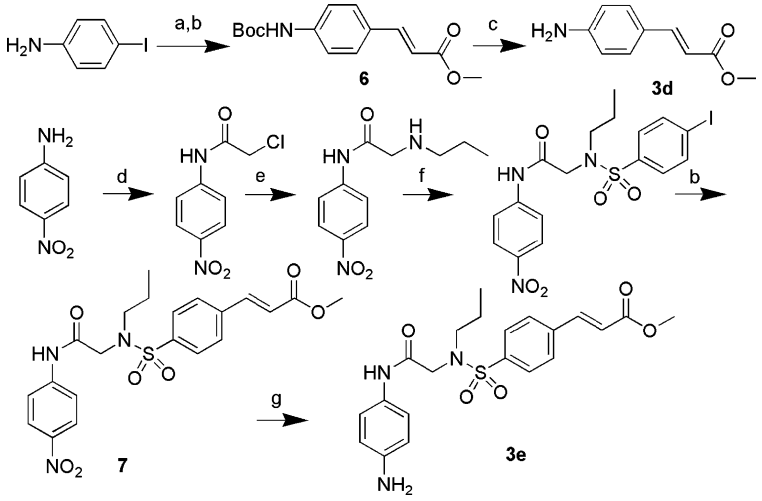
Scheme 1.

According to the synthetic approaches, there are three types of 3,4-substituted anilines: one is 3-chloro-4-alkoxy-aniline (**3a**–**c**), the second is the 4-alkyl or aminoacyl-aniline (**3d**–**e**), and the third is the alkoxyl linked functional group as 4-substituent (**3f**–**g**). 3,4,5-trimethoxyl-aniline (**3h**) is a special case. The synthesis is described in Schemes 2–5, respectively.

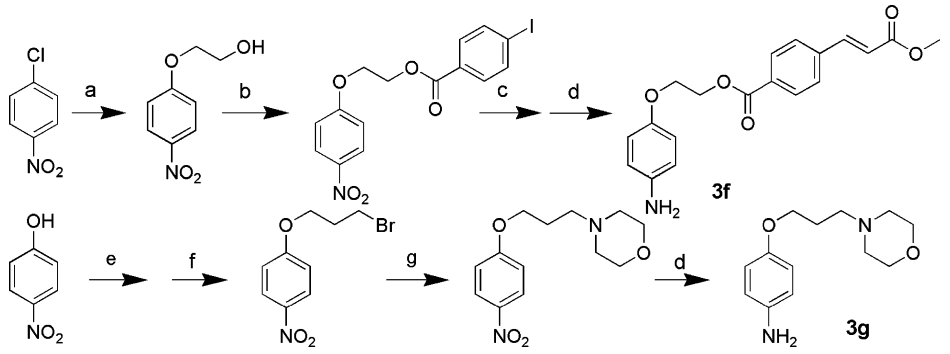
Generally speaking, the 3-chloro-4-alkoxy-aniline (**3a**–**c**) was prepared from *o*-chlorophenol via a selective nitra-



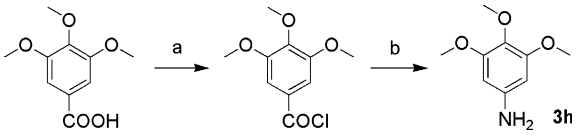
Scheme 2. Reagents and conditions: (a) CH_3I, CH_2Cl_2, Na_2CO_3, rt, yield 95% for **4a** or cyclohexene, $BF_3{\cdot}Et_2O$, toluene, rt, yield 49% for **4b**; (b) $Bi_5O(OH)_9(NO_3)_4$/ $SOCl_2$, rt, yield 36% for **5a**, 58% for **5b**; (c) Fe, NH_4Cl, $MeOH/H_2O$, refluxed, yield 98% for **3a**, 97% for **3b**; (d) $NaNO_2$, NaAc, $AcOH/H_2O$, rt, 70%; (e) Zn/NH_4Cl, $MeOH/H_2O$, refluxed, 98%; (f) $(Boc)_2O$, $NaHCO_3$, THF, rt, 100%; (g) i—BnBr, $NaHCO_3$, THF, rt, 55%; ii—CF_3COOH, CH_2Cl_2, rt, 100%.



Scheme 3. Reagents and conditions: (a) $(Boc)_2O$, $NaHCO_3$, THF, rt, 100%; (b) ZnBr, DIPEA, 0.05 equiv $Pd(PPh_3)_2Cl_2$, methyl acrylate, THF, rt, yield 88% for **6**, 51% for **7**; (c) CF_3COOH, CH_2Cl_2, rt, 100%; (d) TEA, $ClCH_2COCl$, CH_2Cl_2, rt, 97%; (e) propan-1-amine, THF, rt, 98%; (f) DIPEA, 4-iodo-benzenesulfonyl chloride THF, rt, 94%; (g) Fe/NH_4Cl, $MeOH/H_2O$, refluxed, 80%.



Scheme 4. Reagents and conditions: (a) Na_2CO_3, $OHCH_2CH_2OH$, dioxane, refluxed, 69%; (b) 4-iodobenzoic acid, cat. TsOH, toluene, refluxed, 21%; (c) ZnBr, DIPEA, 0.05 equiv $Pd(PPh_3)_2Cl_2$, methyl acrylate, THF, rt, 66%; (d) Zn/NH_4Cl, $MeOH/H_2O$, refluxed, 30%; (e) Na_2CO_3, KI, $BrCH_2CH_2CH_2OH$, acetone, refluxed, 41%; (f) NBS, PPh_3, CH_2Cl_2, refluxed, 60%; (g) morpholine, cat. DMAP, THF, rt, 62%.



Scheme 5. Reagents and conditions: (a) $SOCl_2$, CH_2Cl_2, refluxed, 100%; (b) i—NaN_3, H_2O/THF, 0 °C; ii—toluene, refluxed; iii—5 N NaOH, then 6 N HCl, overall yield for three steps 70%.

tion and *O*-alkylation, followed by the reduction of the nitro into amino group, as depicted in Scheme 2. The selective 4-nitration of aniline was achieved with bismuth subnitrate and thionyl chloride,[13] and the reduction proceeded smoothly with zinc or iron powder in ammonium chloride solution. But for the synthesis of 4-benzyloxyl substituted-3-chloro-aniline (**3c**), the reduction of nitro group should take place prior to the benzylation of the phenol group.

For the 4-alkyl or 4-aminoacyl-aniline (**3d**–**e**), both contain an α,β-unsaturated carboxyl ester on the 4-substituent, which was introduced by a Heck coupling reaction, as depicted in Scheme 3.

As shown in Scheme 4, 4-alkoxyl substituted aniline (**3f**–**g**) was prepared from 1-chloro-4-nitrobenzene via an S_NAr reaction followed by an acylation then a Heck coupling (**3f**), or from 1-hydroxy-4-nitrobenzene via an *O*-alkylation followed by an S_N2 reaction (**3g**). In this series, a linker was used to join the anilino group and another functional group, such as morpholine or 3-phenyl-acrylic acid methyl ester.

The special 3,4,5-trimethoxyaniline (**3h**) was converted from 3,4,5-trimethoxybenzoic acid via a Curtius rearrangement, as indicated in Scheme 5.

Anti-proliferative effect of 5-(3′,4′,5′-substituted)anilino-4-hydroxy-8-nitro-quinazolines on the tumor cells. We examined the growth inhibitory activity of the new series of quinazolines using human tumor cell lines of different tissue origin, including lung adenocarcinoma cell line A549 and gastrointestinal cancer cell line HGC-27.[14] The effect of the compounds **1a**–**h** on the cell proliferation was evaluated using MTT assay.[15] As shown in Figure 3, compounds **1c** and **1f**–**h**, bearing an alkoxyl substituent on the 5-anilino portion, exhibited potent inhibition on the growth of HGC-27 cells at the concentration of 50 μM, while compounds **1a**, **1b**, **1d** and **1e** displayed poor potency. Furthermore, this series was less effective in inhibiting the proliferation of A549 cells, though compound **1c** was still the best inhibitor (Fig. 3).

Previous studies have found that the lipophilic electron-donating groups such as methoxy or benzyloxy at the 4′-position were beneficial for the 5-(4′-substituted)-anilino-4-hydroxy-8-nitroquinazoline in inhibiting the growth of breast cancer cells MDA-MB-468, and 3′-chloro substituent on the aniline ring improved the inhibitory activity against the SK-BR-3 cell line.[4] But in our case, the simultaneous substitution of 3′-chloro-4′-methoxy (compound **1a**) resulted in a big loss of the potency compared to **LJK-11** (IC_{50} = 20 μM for A549 and HGC-27 cells). And the cyclohexyloxy group (compound **1b**) is disfavored in 4′-position either. However, the large alkoxy group at 4′-position (compounds **1c, f** and **g**) enhanced the antiproliferative effect against both HGC-27 and A549 cells. On the contrary, the large alkyl or amide substituent (compounds **1d** and **e**) is detrimental to the activity. As expected, the 3,4,5-trimethoxyl substitution confers an effective inhibitor (compound **1h**) for the two tumor cell lines.

Several lines of evidence have indicated that this 5-(4′-substituted)anilino-8-nitroquinazoline family inhibits proliferation and induces apoptosis by blocking mitosis of tumor cells. It functions by inhibiting microtubule polymerization, similar to that of colchicines.[5] So we would like to examine the effect of these analogs on the mitosis of tumor cells.[16]

Arresting effect of 5-(3′,4′,5′-substituted)anilino-8-nitro-quinazolines on the mitosis of tumor cells. We analyzed

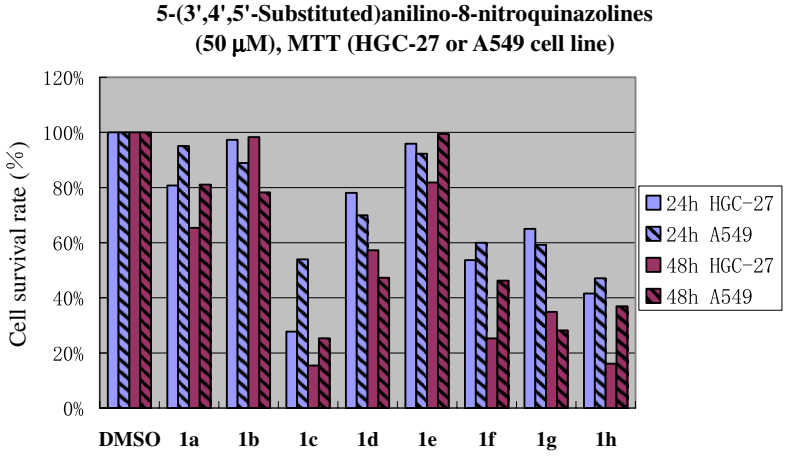


Figure 3. Effect of compounds **1a**–**h** on the proliferation of HGC-27 or A549 cells after the incubation of 24 and 48 h, respectively. The cell viability was measured by MTT assay and is expressed as a percentage of the compound-treated viable cells divided by the viable cells of the untreated control. The data are means of triplicates ±SD.

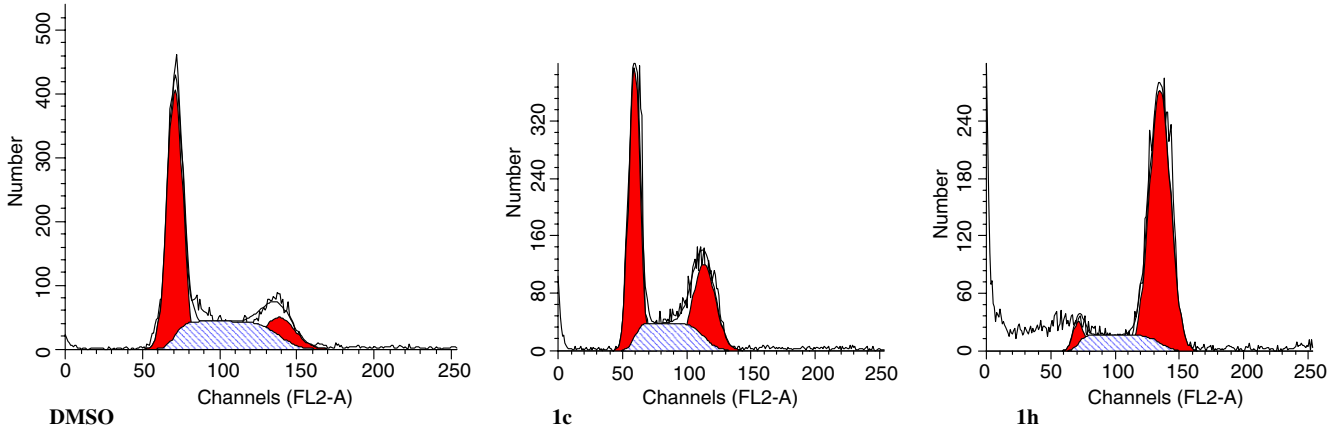


Figure 4. Flow cytometry analysis of 5-(3′,4′,5′-substituted)anilino-8-nitroquinazoline-treated tumor cells. HGC cells were incubated with 50 μM concentration of **1c** or **1h** for 24 h. The cells were then fixed and stained with PI, and analyzed by flow cytometry.

the effect of compounds **1a**–**h** on cell cycle.[17] It is clear that the treatment with potent inhibitors bearing 4′-alkoxyl substituent, that is, **1c**, **1f**, **1g**, and **1h** induced G2/M arrest of HGC-27 cells after 24 h of drug exposure (Figs. 4, 5). Especially for 3,4,5-trimethoxylaniline containing analog **1h**, 81% of the cell population was blocked in G2/M phase when they were exposed to 50 μM of **1h** for 24 h (Fig. 5). The potency to block the mitosis of the cells is correlated with the activity to inhibit the growth of the tumor cells. Therefore, this series exhibited an inferior effect on the mitosis of A549 cells (Fig. 6). However, the 5-(3′,4′,5′-trimethoxy)anilino-8-nitroquinazoline (**1h**) displays an overwhelming potency in arresting the tumor cells at G2/M phase, for both HGC-27 and A549 cells. Considering the structures of well known anti-microtubule agents such as colchicine and combretastatin A4, the 3,4,5-trimethoxyphenyl moiety in common plays an important role in the binding to the tubuline. The observation could be rationalized by molecular modeling.

Based on the recently reported X-ray crystal structure of α,β-tubulin complexed with *N*-deacetyl-*N*-(2-mercaptoacetyl) colchicine (DAMA-colchicine) (PDB code 1SA0),[18] the automated molecular docking can produce

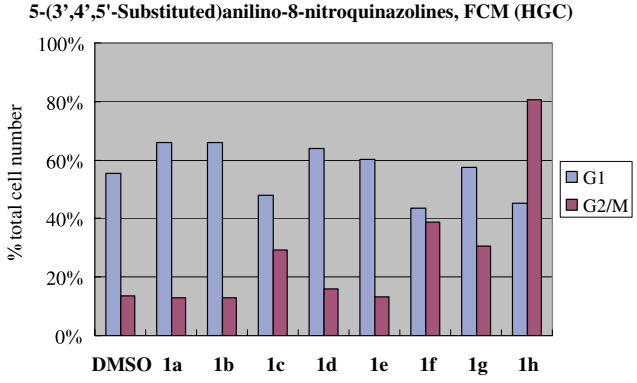


Figure 5. Percentage of HGC cells in G2/M phase after 24-h treatment with fixed concentration of various 5-anilino-8-nitroquinazolines, examined by the flow cytometry analysis. The data are means of triplicates ±SD.

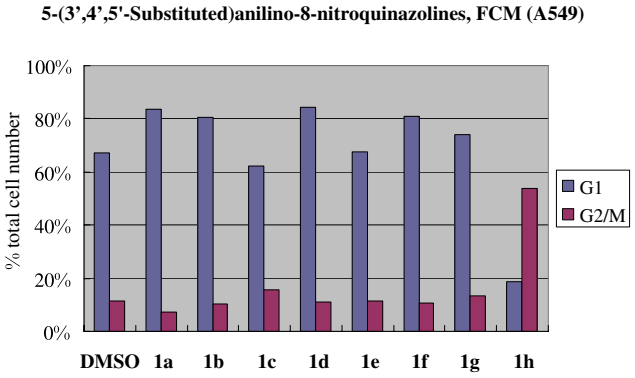


Figure 6. Percentage of A549 cells in G2/M phase after 24-h treatment with 50 μM concentration of various 5-anilino-8-nitroquinazolines, evaluated by the flow cytometry analysis. The data are means of triplicates ±SD.

several optional conformations of our inhibitors.[19,20] Among them, the lowest binding energy one was selected as the possible binding conformation, as shown in Figure 7.

In the final model, the hydrogen bonds between **1h** and tubulin are as follows: (1) the trimethoxyphenyl (TMP) is hydrogen bonded to the thiol group of Cys β241; (2) the 4-OH of quinazolinone is hydrogen bonded to the carbonyl oxygen atom of Asn β258; (3) of particular significance to **1h**, the 8-nitro group is involved in the interaction with the colchicine site via its oxygen atom forming hydrogen bond with the terminal NH of Lys β254. For the diaryl system of **1h**, the phenyl plane and quinazolinone plane have a tilt of 57°, and conform to the shape of the colchicine site. The TMP moieties of DAMA-colchicine and **1h** occupy similar cartesian space and are buried in a hydrophobic pocket containing Leu255, Val238, Cys241, Val318, Ala354, and Ala317 of the β-tubulin structure. That is, why the 3,4,5-trimethoxy substitution endows a significant anti-microtubule activity.

In conclusion, we have synthesized and assessed a series of 5-(3′,4′,5′-substituted)anilino-8-nitroquinazolines as a

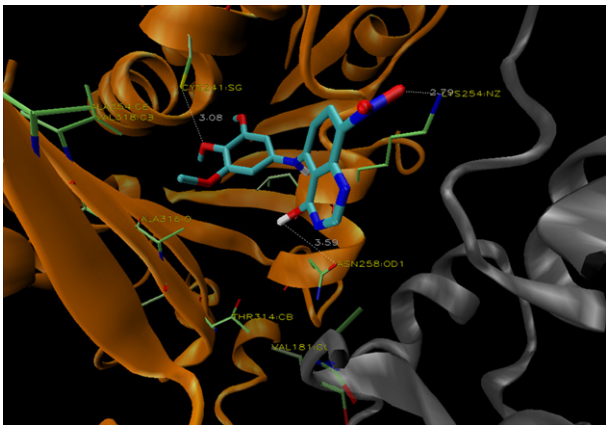


Figure 7. Binding model of **1h** with the α,β-tubulin polypeptide whose backbones are rendered as brown (β chain) and gray (α chain) ribbons. Residue side chains at the colchicine site (Thr α179, Val α181, Cys β241, Lys β254 Leu β255, Asn β258, Thr β314, Ala β316, Val β318, and Ala β354), **1h** is shown in stick with the carbon atoms of tubulin colored green and the carbon atoms of the **1h** cyan, nitrogen atoms are colored blue, oxygen atoms red, and sulfur atoms yellow. White dashed lines indicate potential intermolecular hydrogen bonds.

new class of anti-microtubule agents. The effect of the 3′,4′,5′-substitution on the 5-anilino ring was investigated. The large alkoxyl substitution on the 4′-position of 5-anilino portion is beneficial for the inhibitory activity against the growth and mitosis of the tumor cells. However, the alkyl or amide substituent at this position is disfavored. With respect to the antiproliferative effect and anti-mitosis activity, this series is more potent against HGC-27 tumor cells than A549 tumor cells. Significantly, the 5-(3′,4′,5′-trimethoxyl)anilino-8-nitroquinazoline (**1h**) displays an overwhelming potency in arresting the tumor cells at the G2/M phase of the cell cycle, providing new templates for further development of potent mitosis inhibitors with therapeutic potential in treating cancer.

Acknowledgment

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12. The physicochemical data of selected compounds: **1c**, mp: 152–154 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ ppm 8.30 (d, 1H), 8.27 (d, J = 9.0, 1H), 7.79 (d, J = 9.0, 1H), 7.47 (m, 5H), 7.19 (s, 1H), 7.09 (d, J = 8.3, 1H), 6.90 (d, J = 8.3, 1H), 5.16 (s, 2H). MS (EI) m/z 422 (M^+); Anal. Calcd for ($C_{21}H_{15}ClN_4O_4$) C, H, N: 59.65, 3.58, 13.25. Found: 59.83, 3.42, 13.47. **1h**, mp: 125–127 °C. ^{1}HNMR (DMSO-d_6, 300 MHz): δ ppm 8.22 (s, 1H), 8.14 (d, J = 8.3, 1h), 7.03 (d, J = 8.3, 1H), 6.28 (s, 2H), 3.65 (s, 6H), 3.58 (s, 3H). MS (EI) m/z 372 (M^+); Anal. Calcd for ($C_{17}H_{16}N_4O_6$). C, H, N: 54.84, 4.33, 15.05. Found: 54.76, 4.42, 15.14.
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14. Cell Growth Inhibition Assay. Two human carcinoma cell lines, A549 (lung adenocarcinoma) and HGC-27 (gastrointestinal carcinoma), were used for the cell proliferation assay. A549 cells were cultured in RPMI-1640 medium supplemented with 10% calf serum; HGC-27 cells were cultured in DMEM supplemented with 10% calf serum. The growth inhibition was examined by MTT assay.
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16. Cell mitosis assay by flow cytometry analysis. Tumor cells were incubated with indicated concentration of synthetic inhibitor for 24 h. The harvested cells were washed with PBS, resuspended in 1 mL of iced 75% ethanol at −20 °C. After being left to stand overnight, cell pellets were collected by centrifugation, resuspended in 500 μL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37 °C for 30 min. Then 25 μL of propidium iodide solution (50 μg /mL) was added, and the mixture was allowed to stand on ice for 1 h. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescent dye by FAC-Scan cytometry. The histogram of DNA distribution was modeled as a sum of G1, G2-M, S phase, and an aneuploid population close to the G1 peak, by using ModFitLT software.
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