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Graphical abstract

Synthesis and biological evaluation of quinoline analogues of flavones as potential anticancer agents and tubulin polymerization inhibitors

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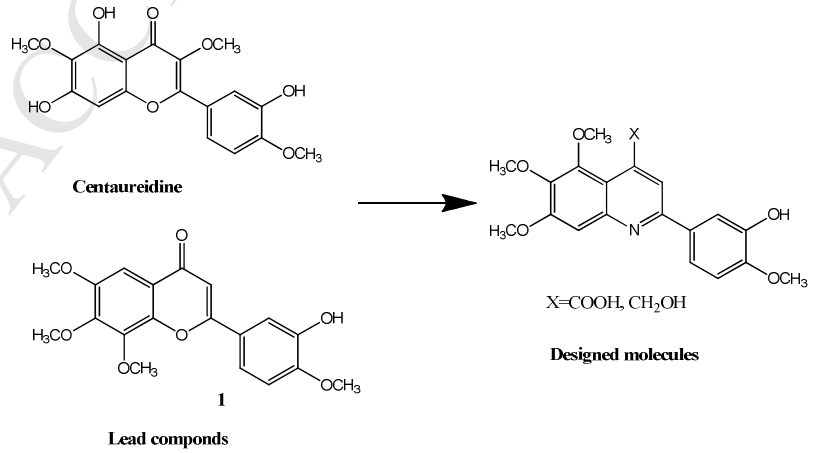
A new series of 2-aryl-trimethoxyquinoline analogues was designed and synthesized as tubulin inhibitors using methoxylated flavones as the lead compounds. The cytotoxic activity of the synthesized compounds was evaluated against four human cancer cell lines including MCF-7, MCF-7/MX, A-2780, and A-2780/RCIS. The effect of quinolines on tubulin polymerization was also evaluated. Compound **6e** was identified as the most potent inhibitor of tubulin polymerization. Molecular docking studies of **6e** into the colchicine-binding site of tubulin displayed possible mode of interaction between this compound and tubulin.



Synthesis and biological evaluation of quinoline analogues of flavones as potential anticancer agents and tubulin polymerization inhibitors

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Abstract

A new series of 2-aryl-trimethoxyquinoline analogues was designed and synthesized as tubulin inhibitors using methoxylated flavones as the lead compounds. The cytotoxic activity of the synthesized compounds was evaluated against four human cancer cell lines including MCF-7, MCF-7/MX, A-2780, and A-2780/RCIS. All the alcoholic derivatives (**6a**-**6e**) showed significant cytotoxic activity with IC_{50} in the range of 7.98-60 µM. The flow cytometry analysis of the four human cancer cell lines treated with **6e** and **5b** showed that **6e** induced cell cycle arrest at G2/M phase and apoptosis as well. The effect of quinolines on tubulin polymerization was also evaluated. Compound **6e** that demonstrated the best antiproliferative activity in the series was identified as the most potent inhibitor of tubulin polymerization as well. Molecular docking studies of **6e** into the colchicine-binding site of tubulin displayed possible mode of interaction between this compound and tubulin.

Keywords: Quinolines; Tubulin polymerization; Anticancer activity; Molecular docking; Resistant Cancer Cells

1. Introduction

Cancer is the cause of one-quarter of all deaths in developed countries. It is now the second leading cause of death in the United States, and is anticipated to surpass heart diseases as the leading cause of death in the futures. [1] Therefore, there is an urgent need to discover and develop novel and more effective drugs. Although, the chemotherapy is the usual method for treatment for different cancer types, it fails to cure most cancer patients with advanced disease due to the occurrence of drug resistance.[2, 3]Consequently; increasing interest has been devoted to the design and discovery of more effective anticancer agents in current medicinal chemistry.

Microtubules play essential role in mitosis and have long been considered as an important target for the development of novel anticancer drugs.[4]

In general, antitubulin agents exert their effects through binding to one of the three established drug domains on the tubulin heterodimer: the colchicine, the paclitaxel and the vinca alkaloid binding sites. Agents that target the colchicine's domain (e.g., colchicine and podophyllotoxin) or to the vinca alkaloid binding site (e.g., vincristine) are defined as inhibitors of tubulin assembly, that is, microtubule destabilizing agents. On the contrary, agents that bind to the paclitaxel binding site (e.g., paclitaxel) are known to act as tubulin promoters, that is, microtubule-stabilizing agents. [5]Combretastatins which are isolated from the South African tree *Combretum caffrum* are also a group of antimitotic compounds and combretastatin A-4 (CA-4, Fig. 1) is one of the well-known natural antitubulin molecule which exerts its antimitotic effect by binding to colchicine's binding site of tubulin.[6]

Centaureidin which is isolated from the tropical plant Potymtifruticosa is the first known example of a flavone with antimitotic activity.[7] Some other synthetic flavones such as compound **1** has been reported as cytotoxic agents and tubulin inhibitors.[8]In the present study some new quinoline derivatives have been designed and synthesized as tubulin inhibitors, as an attempt to check if the replacement of the benzopyrone moiety with the quinoline one is bioisosteric. These quinolines all are possessing trimethoxy phenyl fragment and some of our compounds (**5b** and **6b**) possess also 3-hydroxy-4-methoxy phenyl moiety which are present in some potent tubulin

inhibitors (Fig. 1). **5b** and **6b** were designed to have similarly substituted aryls positioned at C-2 to the aryl substituent at C-2 of flavones centaureidin and compound **1**(Fig. 1). The synthesized compounds were evaluated for their cytotoxic activity towards four different cancer cell lines including MCF-7 (Human Breast Cancer Cells), MCF-7/MX (Mitoxantrone resistant human Breast Cancer Cells), A-2780 (human ovarian carcinoma), and A2780/RCIS (Cisplatin resistant human ovarian carcinoma). The compounds were also investigated for their activity in a microtubular polymerization assay. Moreover, trying to explain the results of biological experiments docking studies were carried out.

2. Results and discussion

2.1. Synthesis

A one-step Doebner reaction was used to prepare the target 5,6,7-trimethoxy-2-arylquinoline-4-carboxylic acid derivatives **5a-5h**. As illustrated in Scheme 1, substituted benzaldehyde**2**, pyruvic acid **3** and 3,4,5-trimethoxyaniline **4** were refluxed in ethanol to afford 4-carboxy quinolines **5a-5h**[9, 10]and then reduction of carboxyl group to alcoholic substituent was carried out using $LiAlH_4$ in dry THF.[11]

However, the well-known Doebner-type synthesis of quinoline did not yield the expected 2-(3,4,5-trimethoxyphenyl)-quinoline-4-carboxylic (**5e**) in ethanol solvent. Therefore, the desire quinoline derivative was prepared in acetic acid. The compounds were characterized by nuclear magnetic resonance, infrared and mass spectrometry.

2.2. Biological evaluation

2.2.1. *In vitro* anticancer activity

The cytotoxic activity of the synthesized compounds were evaluated against four human cancer cell lines including MCF-7 (Human Breast Cancer Cells), MCF-7/MX (Mitoxantrone resistant human Breast Cancer Cells), A-2780 (human ovarian carcinoma) and A-2780/RCIS (Cisplatin resistant human ovarian carcinoma), employing the MTT assay. As depicted in Table 1, all the alcoholic derivatives showed significant cytotoxic activity with IC_{50} in the range of 7.98-60 µM. However, carboxylic derivatives except **5b** did not display cytotoxic activity at concentrations below 100 µM. This may be due to the poor ability of carboxylic derivatives to penetrate the cell membrane because of their high polarity. The only carboxyl derivative that showed cytotoxicity was **5b**. It caused moderate cytotoxicity in MCF-7 and A-2780 and had no cytotoxic effect on the resistant cell lines at concentrations below 100 µM. Generally the alcoholic derivatives (**6a-**

6e) showed more cytotoxicity in A-2780 cell line in comparison to other three cell lines. Interestingly, resistant human Breast Cancer Cells (MCF-7/MX) were more sensitive to all the alcoholic derivatives except **6a** in comparison to parental cells (MCF-7). In contrast, they caused more cytotoxicity in A-2780 cell line in comparison to resistant human ovarian carcinoma (A-2780/RCIS), indicating the possibility that our compounds exerted their cytotoxic activity through different mechanism in different tumor cell types. Overall, among the quinolines, compound **6e,** possessing trimethoxy phenyl group at position 2 of quinoline ring, demonstrated the strongest cytotoxicity against the cancer cell lines and had the same effectiveness against both parental and resistant cell lines.

2.2.2. Tubulin polymerization assay

To investigate whether the cytotoxic activity of alcoholic derivatives were related to the binding to tubulin, compounds **6a**, **6b**, **6d** and **6e** and reference compounds CA4 (polymerization suppressor) and a polymerization promoter (paclitaxel) were evaluated for tubulin polymerization inhibitory effect. As shown in Figure 2, these compounds proved to be inhibitor of tubulin polymerization in a mode similar to that of CA4. The order of tubulin inhibitory activity by alcoholic derivatives was **6e**>>**6d**>**6a**>**6c**. These data showed that the increase of lipophilic properties of substituents on the C-2 quinoline ring increased their affinity to bind to tubulin. Furthermore, compound **6e** that demonstrated the most anti proliferative agent was identified as the most potent tubulin inhibitor as well. The tubulin polymerization activity of carboxyl derivatives compounds **5a-5h** at concentration of 100 µM is also shown in Figure 2. Compounds **5c** and **5h** which are possessing hydroxyl group at *para* position revealed most inhibition activity of tubulin assembly. Surprisingly, **5b** which was most cytotoxic carboxyl derivative did not show any significant influence on tubulin assembly. This may be explained by other mechanisms involved in cytotoxicity other than tubulin inhibition. Although, the carboxyl derivatives **5a** and **5h** were not cytotoxic at the concentration of 100 µM, they showed tubulin assembly inhibitory activity at this concentration equal to combretastatin A4 at 10 µM concentration, this disparity might be due to their poor ability to penetrate the cell membrane of cancer cells. SAR (structure-activity relationship) data acquired from inhibition of tubulin by

carboxyl and alcoholic derivatives indicated that the interaction of carboxyl derivatives and alcoholic analogues with tubulin seems to be different.

2.2.3. Cell Cycle analysis using flow cytometry

The anti-mitotic drugs arrest cell cycle at G2/M phase in cancer cells due to destruction of the microtubular cytoskeleton. [12] To get further insight into the mechanism of action, the effect of compound **5b** at 100 µM concentration and **6e** at 50 µM concentration on the cell cycle were analyzed by flow cytometry against four human cancer cell lines including MCF-7, MCF-7/MX, A-2780 and A-2780/RCIS. As depicted in Figure 3, when the cells were treated with **5b** and **6e** for 24 h, the percentage of cells in the G2/M phase were 62.15% and 79.59% in A-2780 and 43.12 and 62.31 in A-2780/RCIS, respectively. 79.59% of MCF-7cells and 44.79% of MCF-7/MX cells were arrested at G2/M after treatment with **6e** at 50 µM concentration while **5b** did not arrest MCF-7 and MCF-7/MX cells at 100 µM concentration. These findings were consistent with the inhibitory effect of **6e** on tubulin polymerization and also on the proliferation of resistant cells and their parents.

2.2.4. Apoptosis assay

DNA fragmentation occurs due to cell-cycle arrest in the G2/M phase. To investigate whether compound **5b** and **6e** exhibiting high cytotoxic activity induce apoptosis, the percentage of sub-G1 apoptotic cells in **5b** and **6e** treatments was determined by flow cytometry.

Cells were treated with **5b** at 100 µM concentration and **6e** at 50 µM concentration for 72 h. As shown in Figure 4, the results proved that **6e**, in addition to its anti-proliferative activity, also induced apoptosis in the four tested cancer cell lines, especially in A2780/RCIS, which is in good agreement to its cytotoxic activity as well.

3. Molecular modeling (docking) studies

In order to investigate the mode of interactions between the tubulin and its inhibitors in a 3D fashion, the compounds were docked into the colchicine binding site of tubulin. The quality and validity of docking procedure was assessed by docking of co-crystalized ligand into the binding site of tubulin. The top binding position from docking studies showed a similar alignment in the binding pocket to the co-crystallized ligand found in crystal structure (PDB ID of1SA0). The root mean square deviation (RMSD) between co crystallized ligand into the binding site and

ligand docked in the crystal structure for tubulin was 1.4 Å indicating good capability to reproduce the ligand binding mode detected in the experimental data (Fig. 5).

As mentioned above, compound **6e** possessing alcoholic substituent at position 4 and trimethoxy phenyl at position 2 of quinoline ring demonstrated the most potent inhibitory effects. Studying ligand interaction mode of **6e** by LigX module of MOE software revealed that hydroxyl group has made suitable site for hydrogen binding (Fig. 6) hydrogen bonds were observed between residues Asn 249 and hydrogen atom and Ala 250 and oxygen atom in hydroxyl group. In addition, trimethoxy phenyl at position 2 of quinoline ring is surrounded by hydrophobic residues like Ala 316 and Ala 354. These hydrophobic interactions can describe potent inhibitory effects of this compound in comparison to the others. Green area in figure 5 and hydrophobic amino acids colored as green in figure 6, demonstrated that the existence of hydrophobic residues in colchicine binding site of tubulin is significant, and can be considered as a point to develop potent tubulin inhibitors.

4. Conclusions

A new series of 2-aryl-trimethoxyquinoline analogues was synthesized and evaluated for their cytotoxic activity against four human cancer cell lines including MCF-7, MCF-7/MX, A-2780 and A-2780/RCIS. All the alcoholic derivatives (**6a**-**6e**) showed significant cytotoxic activity, both in resistant cells and their parents. Compound **6e** possessing trimethoxyphenyl group at position 2 of quinoline ring demonstrated the highest cytotoxicity among quinolines against the tumor cell lines and showed similar cytotoxic efficacy on both parental cells and resistant cell lines. It was also identified as the most potent tubulin inhibitor and induced cell cycle arrest at G2/M phase and apoptosis as well. Finally, molecular docking studies of **6e** into the colchicine-binding site of tubulin demonstrated the possible interaction of this compound in the active site of tubulin.

5. Experimental section

All chemicals, reagents and solvents used in this study were purchased from Merck AG and Aldrich Chemical. Melting points were determined with a Thomas–Hoover capillary apparatus. Infrared spectra were acquired using a Perkin Elmer Model 1420 spectrometer. Bruker FT-

500and 400 MHz instruments (Brucker Biosciences, USA) was used to acquire ^{1}HNMR spectra and A Bruker FT-300 MHz instrument was used to acquire ^{13}CNMR spectra with TMS as internal standard. Chloroform-D and DMSO-D6 were used as solvents. Coupling constant (J) values are assessed in hertz (Hz) and spin multiples are given as s (singlet), d (double), t (triplet), q (quartet), m (multiplet). The mass spectral measurements were performed on a 6410 Agilent LCMS triple quadrupole mass spectrometer (LCMS) with an electrospray ionization (ESI) interface.

5.1. General procedure for preparation of 5,6,7-trimethoxy-2-arylquinoline-4-carboxylic acid (Doebner reaction)

A solution of appropriate benzaldehyde (9.45 mmol) and pyruvic acid (1.26 g, 14.3 mmol) in ethanol or acetic acid (5 ml) was heated for 30 min then 3,4,5-trimethoxyaniline (9.45 mmol) was added to the solution and refluxed overnight. After cooling, the produced precipitate was filtered and washed with ethanol and hexane and recrystallized in ethanol.

5.1.1. 5,6,7-trimethoxy-2-(4-methoxyphenyl)7quinoline-4-carboxylic acid(5a)

Yield: 16%; yellow crystalline powder; mp = 203-205°C; IR (KBr): ν (cm^{-1}) 3378 (OH), 1652 (C=O), ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.79 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 7.02-7.04 (d, 2H, 4-methoxy phenyl H_3& H_5, J = 8.64 Hz), 7.29 (s, 1H, quinoline H_8), 7.81 (s, 1H, quinoline H_3), 8.19-8.21 (d, 2H, 4-methoxy phenyl H_2& H_6, J = 8.64 Hz), 13.2 (s, 1H, COOH), ^{13}C NMR (DMSO, 75 MHz): δ 55.76, 56.57, 61.17, 61.65, 105.13, 113.32, 114.18, 114.67, 128.97, 130.89, 140.10, 141.27, 146.16, 146.90, 155.23, 156.64, 161.19, 170.47, LC-MS (ESI): 370.1 $(M+1)^{+}$.

5.1.2. 2-(3-hydroxy-4-methoxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5b)

Yield: 52%; yellow crystalline powder; mp =282-284 °C; IR (KBr): ν (cm^{-1}) 3416 (OH), 1618 (C=O), ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.80 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 6.98–7.00 (d, 1H, 3-hydroxy-4-methoxy phenyl H_5, J = 8.53 Hz), 7.25 (s, 1H, quinoline H_8), 7.62-7.65 (dd, 1H, 3-hydroxy-4-methoxy phenyl H_6, J = 8.53 Hz, J = 2.19 Hz), 7.72 (s, 1H, quinoline H_3), 7.73-7.74 (d, 1H, 3-hydroxy-4-methoxy phenyl H_2, J = 2.18 Hz), 9.14 (s, 1H, OH), 13.29 (s, 1H, COOH), ^{13}C NMR (DMSO, 75 MHz): δ 56.10, 56.55,

61.16, 61.65, 105.06, 112.48, 112.98, 113.38, 114.42, 119.03, 131.27, 139.96, 141.24, 146.13, 146.92, 197.25, 149.92, 155.42, 156.61, 170.48, LC-MS (ESI): 386.1 $(M+1)^+$.

5.1.3. 2-(4-hydroxy-3-methoxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5c)

Yield: 25%; yellow crystalline powder; mp =266-268 °C; IR (KBr): ν (cm^{-1}) 3430 (OH), 1614 (C=O), ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.83 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 6.85-6.87 (d, 1H, 3-methoxy-4- hydroxy phenyl H_6, J = 8.28 Hz), 7.27 (s, 1H, quinoline H_8), 7.70-7.72 (dd, 1H, 3-methoxy-4- hydroxyphenyl H_6, J = 8.28 Hz, J = 2.01 Hz), 7.81-7.82 (d, 1H, 3-methoxy-4- hydroxyphenyl H_2, J = 2.01 Hz), 7.82 (s, 1H, quinoline H_3) 9.40 (s, 1H, OH), 13.28 (s, 1H, COOH), ^{13}C NMR (DMSO, 75 MHz): δ 56.25, 56.57, 61.16, 61.64, 105.12, 111.18, 112.87, 113.38, 116.11, 120.83, 129.79, 139.97, 141.12, 146.08, 146.91, 148.41, 149.09, 155.54, 156.54, 170.53, LC-MS (ESI): 386.1 $(M+1)^+$.

5.1.4. 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5d)

Yield: 30%; yellow crystalline powder; mp =143-145 °C; IR (KBr): ν (cm^{-1}) 3428 (OH), 1643 (C=O), ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.79 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.96 (s, 3H, OCH3), 7.03-7.05 (d, 1H, 3,4-dimethoxy phenyl H_5, J = 9.07 Hz), 7.29 (s, 1H, quinoline H_8), 7.82-7.83 (m, 2H, 3,4-dimethoxy phenyl H_2& H_6) 7.88 (s, 1H, quinoline H_3), 13.30 (s, 1H, COOH),^{13}C NMR (DMSO, 75 MHz): δ 56.07, 56.14, 56.61, 61.18, 61.66, 105.18, 110.61, 112.17, 113.04, 113.50, 120.51, 131.07, 140.06, 141.27, 146.07, 146.88, 149.49, 150.95, 155.27, 156.61, 170.47, LC-MS (ESI): 400.1 $(M+1)^+$.

5.1.5. 5,6,7-trimethoxy-2-(3,4,5-trimethoxyphenyl)8quinoline-4-carboxylic acid (5e)

Yield: 52%; yellow crystalline powder; mp =178-179 °C; IR (KBr): ν (cm^{-1}) 3366 (OH), 1652 (C=O),^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.70 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.88 (s, 6H, $2OCH_3$), 3.97 (s, 3H, OCH3), 7.31 (s, 1H, quinoline H_8) 7.55 (s, 2H, 3,4,5-trimethoxy phenyl H_2 & H_6), 7.98 (s, 1H, quinoline H_3), 13.20 (s, 1H, COOH), ^{13}C NMR (DMSO, 75 MHz): δ 56.56, 56.63, 60.59, 61.19, 61.68, 104.87, 105.25, 113.31, 113.90, 133.89, 139.59, 140.03, 141.49, 145.96, 146.81, 153.70, 155.13, 156.67, 170.36, LC-MS (ESI): 430.1 $(M+1)^+$.

5.1.6. 2-(4-hydroxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5f)

Yield: 53%; yellow crystalline powder; mp =263-265 °C; IR (KBr): ν (cm^{-1}) 3389 (OH), 1649 (C=O, ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.82 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3), 6.84-6.86 (d, 2H, 4-hydroxyphenyl H_3 & H_5 , J = 8.72 Hz), 7.26 (s, 1H, quinoline H_8), 7.73 (s, 1H, quinoline H_3), 8.08-8.10 (d, 2H, 4-hydroxyphenyl H_2 & H_6, J = 8.72 Hz), 9.82 (s, 1H, OH), 13.1 (s, 1H, COOH),^{13}C NMR (DMSO, 75 MHz): δ 56.54, 61.15, 61.63, 105.06, 112.82, 113.18, 116.05, 129.05, 139.97, 141.11, 146.16, 146.91, 153.70, 155.56, 156.57, 159.61, 170.47,), LC-MS (ESI): 356.1 $(M+1)^+$.

5.1.7. 2-(3-hydroxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5g)

Yield: 54%; yellow crystalline powder; mp =270-272 °C; IR (KBr): ν (cm^{-1}) 3400 (OH), 1615 (C=O), ^{1}H NMR (500MHz-DMSO-d_6): δ (ppm) 3.84 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.83–6.86 (dd, 1H, 3-hydroxy phenyl H_6, J = 8.01 Hz, J = 2.3 Hz), 7.26-7.29 (m, 2H, 3-hydroxy phenyl H_5 &quinoline H_8), 7.60-7.61 (d, 1H, 3-hydroxy phenyl H_4, J = 7.88 Hz), 7.65-7.66 (d, 1H, 3-hydroxy phenyl H_2, J = 1.78 Hz), 7.74 (s, 1H, quinoline H_3), 9.54 (s, 1H, OH), 13.32 (s, 1H, COOH), ^{13}C NMR (DMSO, 75 MHz): δ 56.60, 61.19, 61.68, 105.20, 113.44, 113.87, 114.11, 117.27, 118.36, 130.35, 139.84, 140.12, 141.59, 146.11, 146.85, 155.57, 156.72, 158.26, 170.33, LC-MS (ESI): 356.1 $(M+1)^+$.

5.1.8. 2-(2,4-dihydroxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5h)

Yield: 15%; yellow crystalline powder; mp =250-252 °C; IR (KBr): ν (cm^{-1}) 3330 (OH), 1608 (C=O), ^{1}H NMR (300MHz-DMSO-d_6): δ (ppm) 3.88 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 6.34-6.36 (d, 1H, 2,4-dihydroxy phenyl H_3, J = 10 Hz), 6.39–7.42 (d, 1H, 2,4-dihydroxy phenyl H_6, J = 15 Hz, J = 5 Hz), 7.33 (s, 1H, quinoline H_8), 7.96 (s, 1H, quinoline H_3), 8.03–8.06 (d, 1H, 2,4-dihydroxy phenyl H_5, J = 15 Hz), 9.93 (s, 1H, OH), 9.99 (s, 1H, OH), 14.72 (s, 1H, COOH),^{13}C NMR (DMSO, 75 MHz): δ 56.80, 61.20, 61.73, 103.37, 103.92, 108, 111.12, 112.15, 112.60, 129.73, 140.77, 141.20, 142.94, 147.13, 157.30, 157.42, 161.55, 169.99, 191.56.

5.2. General procedure for preparation of 2-aryl- 5,6,7-trimethoxyquinoline-4-yl)methanol

$LiAlH_4$ (0.45 g, 12 mmol) was addedto dry THF (20 ml) under a nitrogen atmosphere. Under vigorous stirring a solution of appropriate acid (5.67 mmol) in dry THF was added drop wise to keep the reaction mixture somewhat boiling. After stirring for 5 h at room temperature, the result suspension was carefully hydrolyzed with NaOH solution (10%) till no more hydrogen was formed. The solid was filtered off and washed thoroughly with chloroform. The filtrate was dried over Na_2SO_4 and the solvent was removed under reduced pressure to yield brown oil which was purified with column chromatography.

5.2.1. (5,6,7-trimethoxy-2-(4-methoxyphenyl)quinolin-4-yl)methanol (6a)

Yield: 25%; yellow crystalline powder; mp= 172-174;IR (KBr): ν (cm^{-1}) 3427 (OH), ^{1}H NMR (500MHz-$CDCl_3$-d_6): δ (ppm) 3.83-4.12 (m, 13H, 4OCH_3, OH), 5.07 (s, 2H, CH_2), 6.94-7.04 (m, 3H, 4-methoxy phenyl H_3 & H_5, quinoline H_8), 7.77 (s, 1H, quinoline H_3), 8.06-8.07 (d, 2H, 4-methoxy phenyl H_2 & H_6, J = 8.7 Hz), ^{13}C NMR (DMSO, 75 MHz): δ 50.62, 51.27, 51.34, 57.83, 96.26, 104, 109.38, 109.77, 115.17, 123.78, 123.95, 127.66, 139.77, 140.58, 144.58, 147.46, 150.21, 155.75, LC-MS (ESI): 356.1 $(M+1)^+$.

5.2.2. 5-(4-(hydroxymethyl)-5,6,7-trimethoxyquinolin-2-yl)-2-methoxyphenol (6b)

Yield: 30%; yellow crystalline powder; mp = 100-102°C; IR (KBr): ν (cm^{-1}) 3406 (OH), ^{1}H NMR (500MHz-$CDCl_3$-d_6): δ (ppm) 3.90 (s, 3H, OCH_3), 3.96 (m, 4H, OCH_3, OH), 4.03 (s, 3H, OCH_3), 4.09 (s, 3H, OCH_3), 5.10 (s, 2H, CH_2), 6.88–6.90 (d, 1H, 3-hydroxy-4-methoxy phenyl H_5, J = 8.21 Hz), 7.25 (s, 1H, quinoline H_8), 7.49 (s, 1H, OH), 7.60-7.62 (d, 1H, 3-hydroxy-4-methoxy phenyl H_6, J = 8.21 Hz), 7.70 (s, 1H, 3-hydroxy-4-methoxy phenyl H_2), 7.75 (s, 1H, quinoline H_3), ^{13}C NMR ($CDCl_3$, 75 MHz): δ 56.01, 56.09, 61.09, 61.93, 65.06, 106.10, 110.78, 113.68, 115.56, 116.63, 119.52, 126.55, 128.47, 132.86, 141.83, 145.91, 147.86, 147.92, 155.28, 156.30, LC-MS (ESI): 372.1 $(M+1)^+$.

5.2.3. 4-(4-(hydroxymethyl)-5,6,7-trimethoxyquinolin-2-yl)-2-methoxyphenol (6c)

Yield: 32%; yellow crystalline powder; mp = 168-170°C; IR (KBr): ν (cm^{-1}) 3382 (OH), ^{1}H NMR (500MHz-$CDCl_3$-d_6): δ (ppm) 3.93-4.07 (m, 13H, 4OCH_3, OH), 5.09 (s, 2H, CH_2), 6.95-6.97 (d, 1H, 3-methoxy-4-hydroxy phenyl H_5, J = 8.21 Hz), 7.25 (s, 1H, quinoline H_8), 7.54-7.55 (d, 1H, 3-methoxy-4-hydroxy phenyl H_6, J = 8.24 Hz),7.70 (s, 1H, OH), 7.79 (m, 2H, 3-methoxy-4-hydroxy phenyl H_2&quinoline H_3), LC-MS (ESI): 372.1 $(M+1)^+$.

5.2.4. (2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxyquinolin-4-yl)methanol (6d)

Yield: 23%; yellow crystalline powder; mp= 152-154; IR (KBr): ν (cm^{-1}) 3425 (OH), ^{1}H NMR (500MHz-$CDCl_3$-d_6): δ (ppm) 3.78-4.07 (m, 16H, 5OCH_3, OH), 5.09 (s, 2H, CH_2), 6.83–6.97 (m, 4H, 3,4-dimethoxy phenyl H_2 & H_5 & H_6&quinoline H_8), 7.80 (s, 1H, quinoline H_3). LC-MS (ESI): 386.2 $(M+1)^+$.

5.2.5. (5,6,7-trimethoxy-2-(3,4,5-trimethoxyphenyl)quinolin-4-yl)methanol (6e)

Yield: 35%; yellow crystalline powder; mp= 145-147°C; IR (KBr): ν (cm^{-1}) 3434 (OH), ^{1}H NMR (500MHz-$CDCl_3$-d_6): δ (ppm) 3.89-4.07 (m, 19H, 6OCH_3, OH), 5.09 (s, 2H, CH_2), 7.31 (s, 2H, 3,4,5-trimethoxy phenyl H_2 & H_6), 7.46 (s, 1H, quinoline H_8), 7.71 (s, 1H, quinoline H_3), ^{13}C NMR (DMSO, 75 MHz): δ 56.09, 56.30, 60.95, 61.10, 61.94, 65, 104.55, 104.69, 106.07, 115.75, 116.73, 134.97, 139.40, 142.02, 146.30, 146.65, 147.85, 153.50, 155.43, 156.40, LC-MS (ESI): 416.2 $(M+1)^+$.

5.3. Cytotoxicity assay

5.3.1. General procedure

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) based assay was carried out by seeding 5×10^3 cancer cells per 180 µL RPMI complete culture medium in each well of 96-well culture plates. The day after seeding, culture medium was replaced with medium containing standard anti-tumor agent CA-4 as well as different concentrations of newly synthesized quinolines and RPMI control (no drug). Cells were then incubated at 37 ºC in 5% O_2 incubator for 48h. Then 25 µL of MTT solution (4mg ml^{-1}) was added to each well and further incubated at 37 ºC for 3h. At the end of incubation, formazan crystals were dissolved in 100 µL of DMSO and plates were read in a plate reader (Synergy H4, USA) at 540 nm. This experiment was performed in triplicate determination each time.

5.4. Tubulin polymerization assay

Tubulin polymerization assay were performed by employing a commercial kit (Tubulin Polymerization Assay Kit (Porcine tubulin and Fluorescence based Kit (Cat. No. BK011P,Cytoskeleton, USA)), according to the manufacturer's protocol.[13-15]Briefly, tubulin protein was suspended in tubulin buffer (80 mM PIPES,2 mM MgCl2, 0.5 mM EGTA, 1 mM GTP, 60% (v/v) glycerol, PH 6.9) then added to wells on a 96-well plate containing the cytotoxic

compounds or vehicle and mixed well. The effects of compound **5a-5h** at100 µM concentration and **6a**, **6b**, **6d** and **6e** at 50 µM concentration on tubulin polymerization were evaluated.

Tubulin polymerization was followed by monitoring the fluorescence enhancement due to the integration of a fluorescence reporter into microtubules as polymerization happens. Polymerization was measured by excitation at 360 nm and emission at 420 nm for 1 h at 1 min intervals in a plate reader (Synergy H4, USA).Paclitaxel at 3µM concentration and CA4 at 10µM concentration were used as positive stabilizing and destabilizing controls, respectively.

5.5. Cell cycle and apoptosis analysis using flow cytometry

Tumor cells (2.5×10^5 cells) were seeded in 6-well cell culture plates for 24 h, then treated with **5b** at concentration of 100 M, **6e** at concentration of 50 µM and CA4 at concentration of 3 µM or vehicle alone (0.05% DMSO) . The treated and untreated cells were incubated for 72 h, washed with PBS and fixed with 70% ethanol, then washed twice with PBS, and then incubated for 0.5 h at 37 °C in a PBS solution containing 1 mg/mL RNase A and propidium iodide (PI).

The influence of the compounds on the cells Cycle was analyzed by flow cytometer using FL2 channel (FACSculibur flow cytometer, Germany). And the percentage of cells in different phases of cell cycle and sub-G1 area were calculated employing Modfit 2.8 software.

5.6. Molecular modeling

Mode of interaction between synthesized ligands and tubulin was investigated by docking. 2D structure of chemicals was prepared in Chem Draw ultera 8.0 software and 3D structures were prepared by Hyperchem 7 software using molecular mechanic force filed pre-optimization followed by AM1 semi empirical calculation. The X-ray crystal structure of tubulin (PDB ID: 1SA0) was downloaded from the Protein Data Bank (www.rcsb.org). Further modification such as polar hydrogen addition and water molecules removal was performed by MOE software. Synthesized chemicals were docked into the binding site of tubulin by MOE software. All atoms within a 5 Å around the co-crystallized ligand in crystal coordinates of tubulin was chosen as binding site. The docking simulations were done employing triangle matcher placement algorithm in combination with London dG scoring function and force field as refinement method. For each compound, the top-score docking poses were chosen for final ligand–target interaction analysis employing LigX module in MOE Software. Validation of docking procedure was first evaluated by docking of co-crystalized ligand into the tubulin binding site.

Acknowledgment

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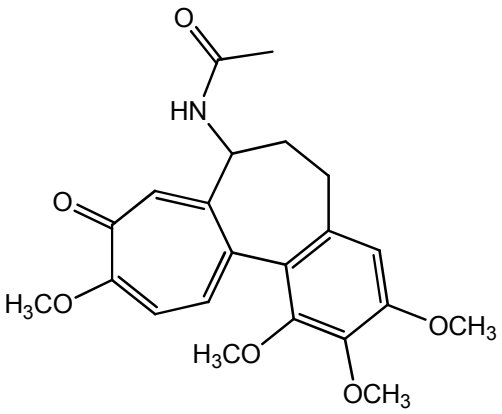
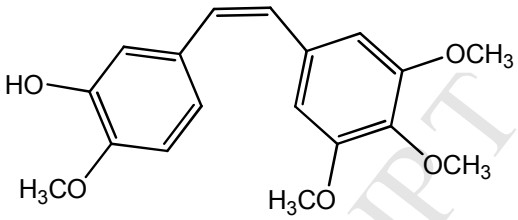
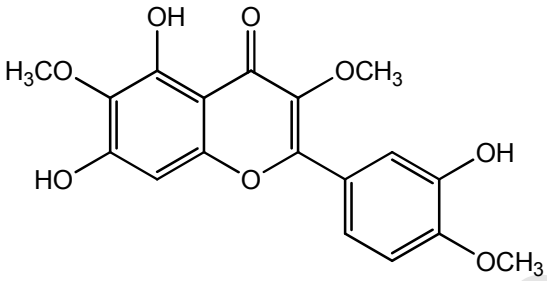


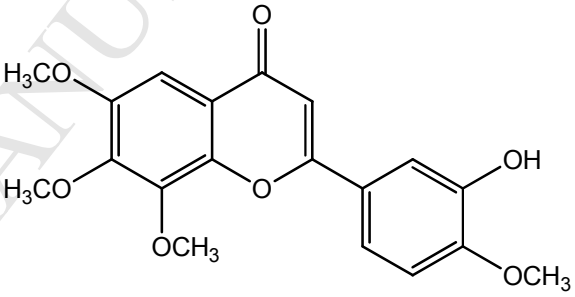
Colchicine



Combretastatin A4



Centaureidine



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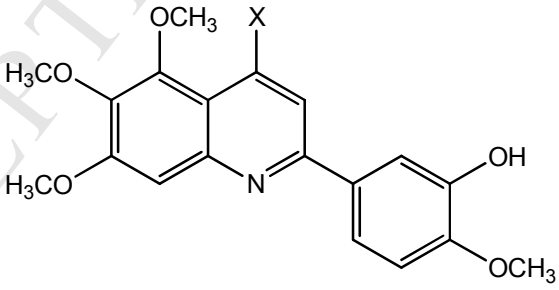
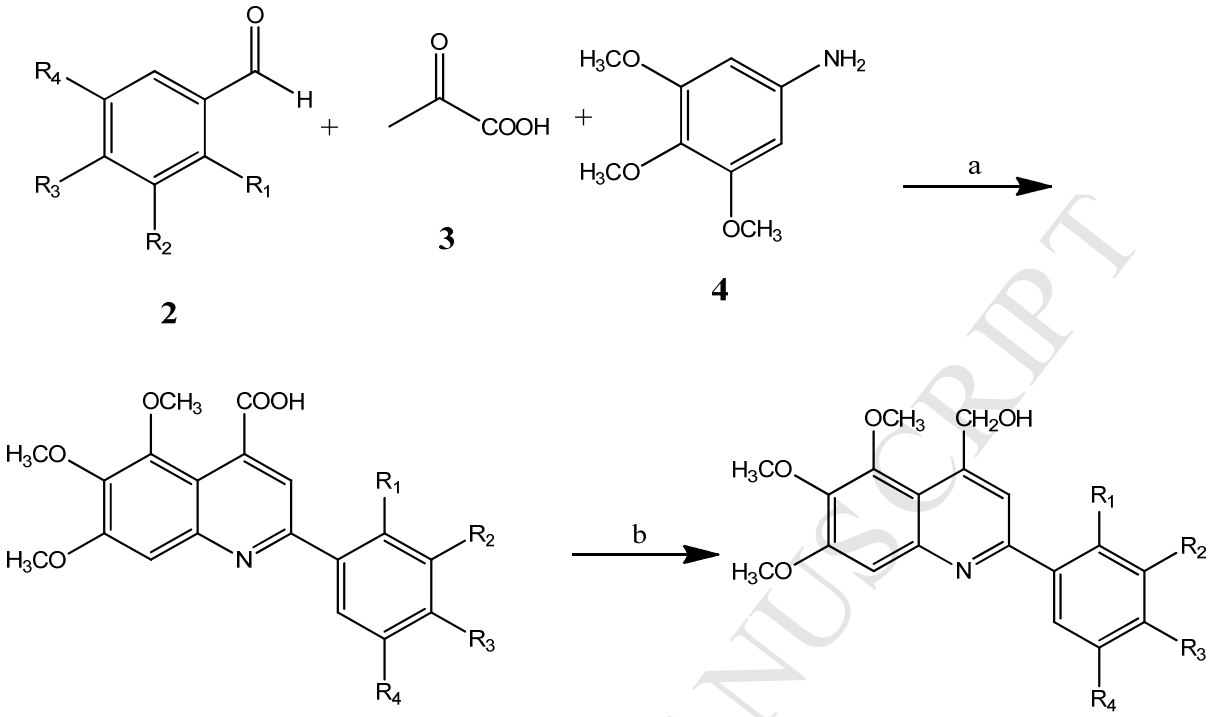


X=COOH, CH_2OH

Designed molecules

Figure 1. Chemical structures of known tubulin inhibitors and designed compounds



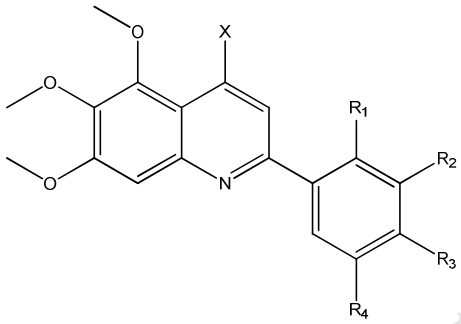
5a, R_1=R_2=R_4=H, R_3=OCH_3
5b, R_1=R_4=H, R_2=OH, R_3=OCH_3
5c, R_1=R_4=H, R_2=OCH_3, R_3=OH
5d, R_1=R_4=H, R_2=R_3=OCH_3
5e, R_1=H, R_2=R_3=R_4=OCH_3
5f, R_1=R_2=R_4=H, R_3=OH
5g, R_1=R_3=R_4=H, R_2=OH
5h, R_2=R_4=H, R_1=R_3=OH

6a, R_1=R_2=R_4=H, R_3=OCH_3
6b, R_1=R_4=H, R_2=OH, R_3=OCH_3
6c, R_1=R_4=H, R_2=OCH_3, R_3=OH
6d, R_1=R_4=H, R_2=R_3=OCH_3
6e, R_1=H, R_2=R_3=R_4=OCH_3

Scheme 1. Reagents and conditions: (a) Ethanol, reflux, (b) LiAlH4, dry THF

Table 1

The in vitro antiproliferative activities of quinoline derivatives and CA4 against human cancer cell lines



Compound	X	R_1	R_2	R_3	R_4	MCF7 (IC_{50} μM)	MCF-7/MX (IC_{50} μM)	A2780 (IC_{50} μM)	A2780RCIS (IC_{50} μM)
6a	CH_2OH	H	H	OCH_3	H	16.42	39.83	8.04	28.40
6b	CH_2OH	H	OCH_3	OH	H	59.47	21.48	29.60	43.31
6c	CH_2OH	H	OH	OCH_3	H	49.71	53.86	9.19	52.16
6d	CH_2OH	H	OCH_3	OCH_3	H	60.67	37.15	7.98	23.09
6e	CH_2OH	H	OCH_3	OCH_3	OCH_3	16.28	14.06	11.44	8.15
5a	COOH	H	H	OCH3	H	>100	>100	>100	>100
5b	COOH	H	OCH_3	OH	H	56.30	98.55	38.73	>100
5c	COOH	H	OH	OCH_3	H	>100	>100	>100	>100
5d	COOH	H	OCH_3	OCH_3	H	>100	>100	>100	>100
5e	COOH	OCH_3	OCH_3	OCH_3	H	>100	>100	>100	>100
5f	COOH	H	H	OH	H	>100	>100	>100	>100
5g	COOH	H	OH	H	H	>100	>100	>100	>100
5h	COOH	OH	H	H	OH	>100	>100	>100	>100
CA-4	-	-	-	-	-	1.23	2.97	2.84	2.95

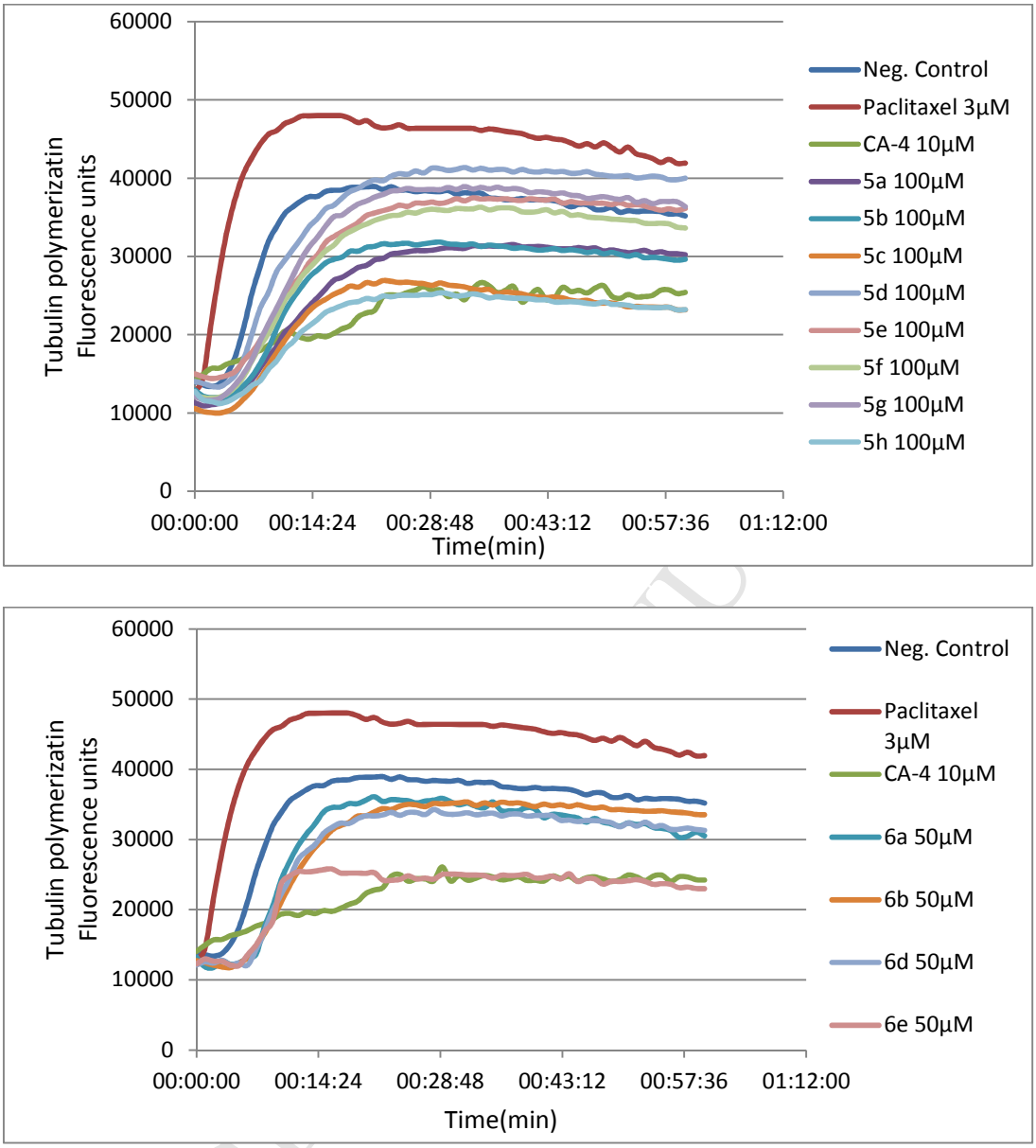
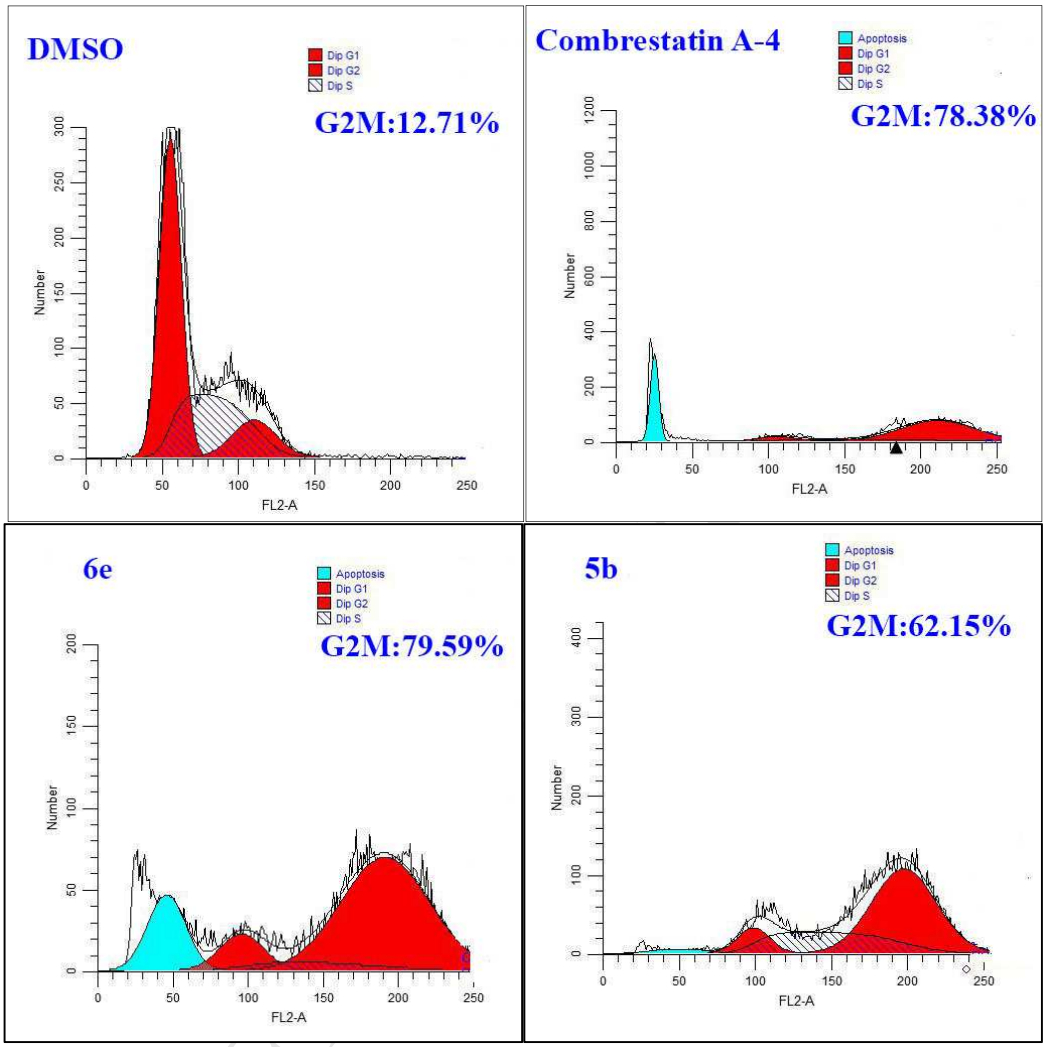
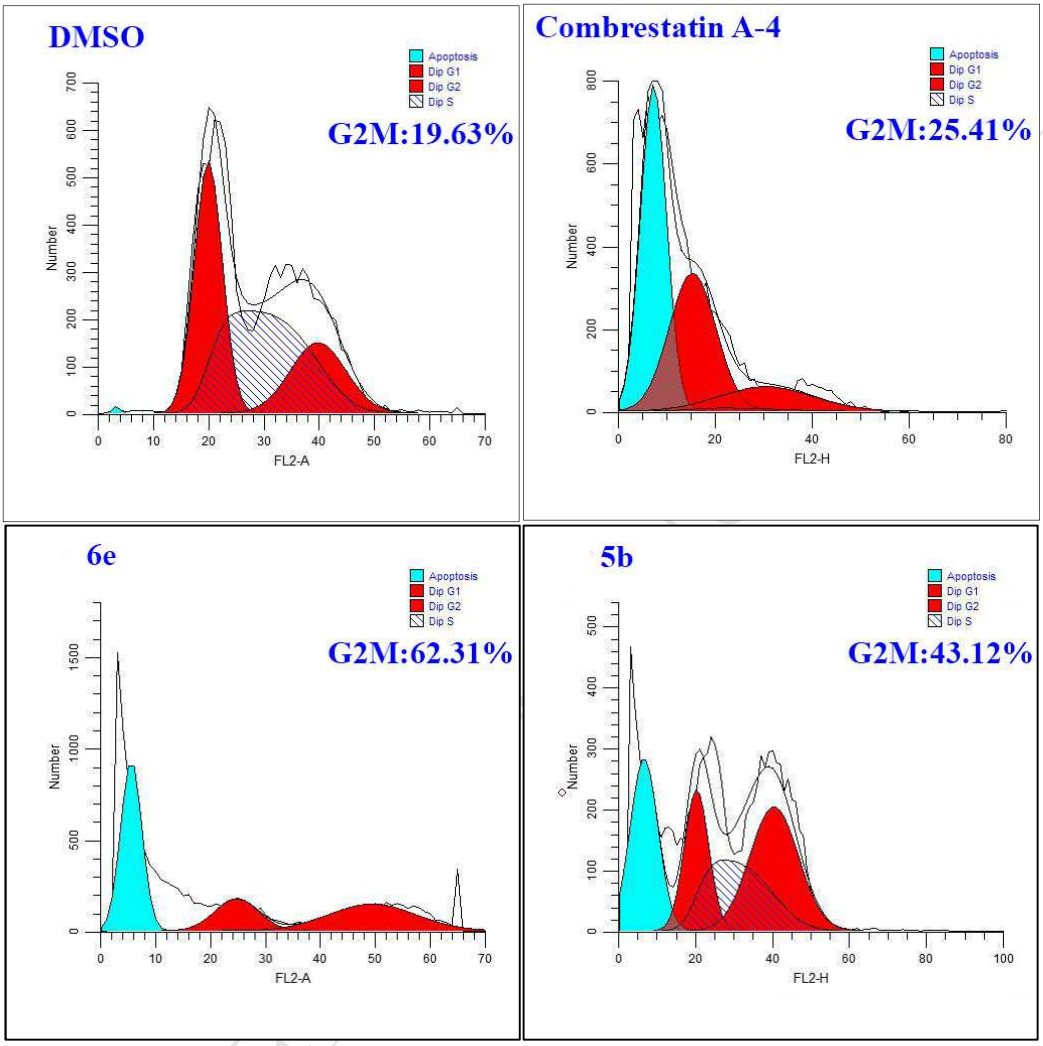


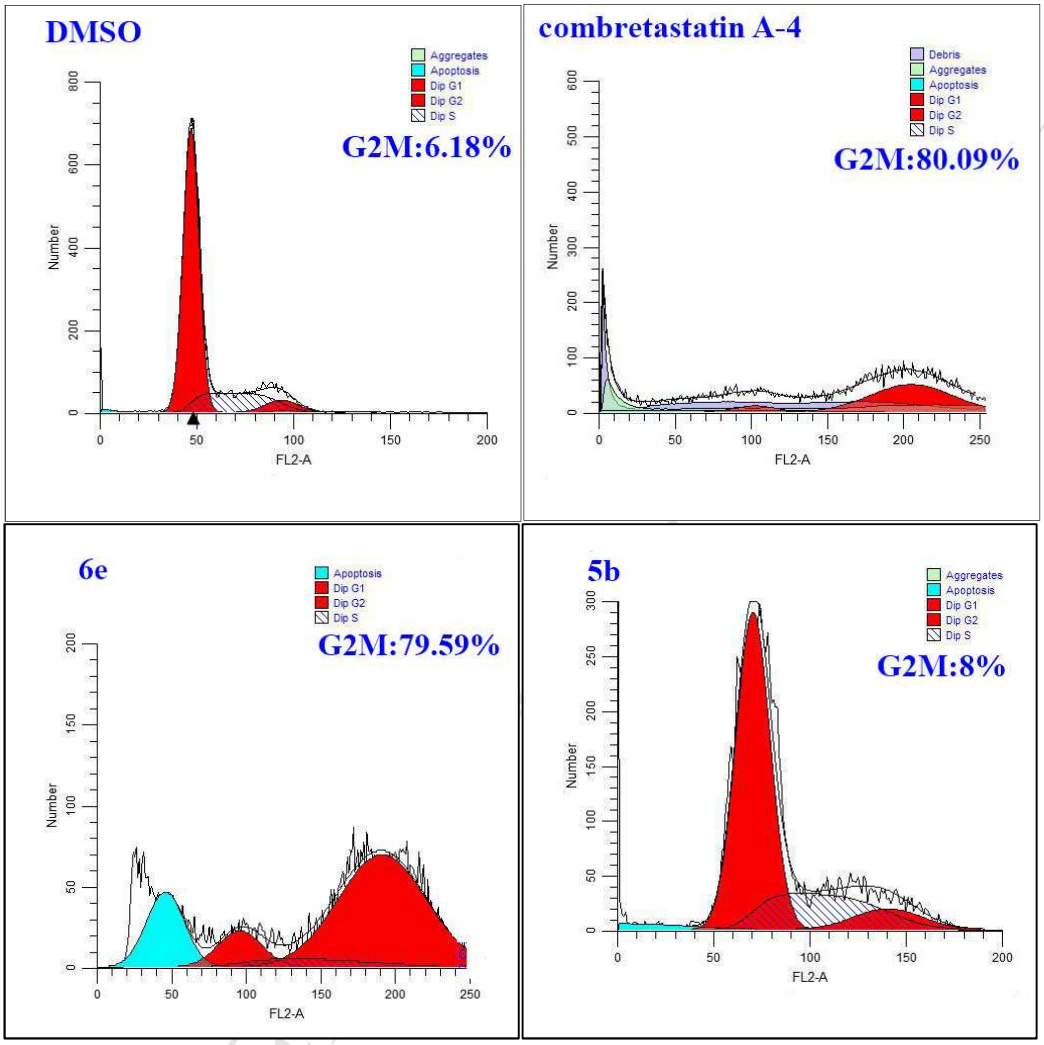
Figure 2.Effect of compounds on in vitro tubulin polymerization.


DMSO
Dip G1
Dip G2
Dip S
G2M:12.71%
Number
FL2-A
Combrestatin A-4
Apoptosis
Dip G1
Dip G2
Dip S
G2M:78.38%
Number
FL2-A
6e
Apoptosis
Dip G1
Dip G2
Dip S
G2M:79.59%
Number
FL2-A
5b
Apoptosis
Dip G1
Dip G2
Dip S
G2M:62.15%
Number
FL2-A

B


DMSO
Apoptosis
Dip G1
Dip G2
Dip S
G2M:19.63%
Number
FL2-A
Combrestatin A-4
Apoptosis
Dip G1
Dip G2
Dip S
G2M:25.41%
Number
FL2-H
6e
Apoptosis
Dip G1
Dip G2
Dip S
G2M:62.31%
Number
FL2-A
5b
Apoptosis
Dip G1
Dip G2
Dip S
G2M:43.12%
Number
FL2-H

C


DMSO
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
G2M:6.18%
Number
FL2-A
combretastatin A-4
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
G2M:80.09%
Number
FL2-A
6e
Apoptosis
Dip G1
Dip G2
Dip S
G2M:79.59%
Number
FL2-A
5b
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
G2M:8%
Number
FL2-A

D

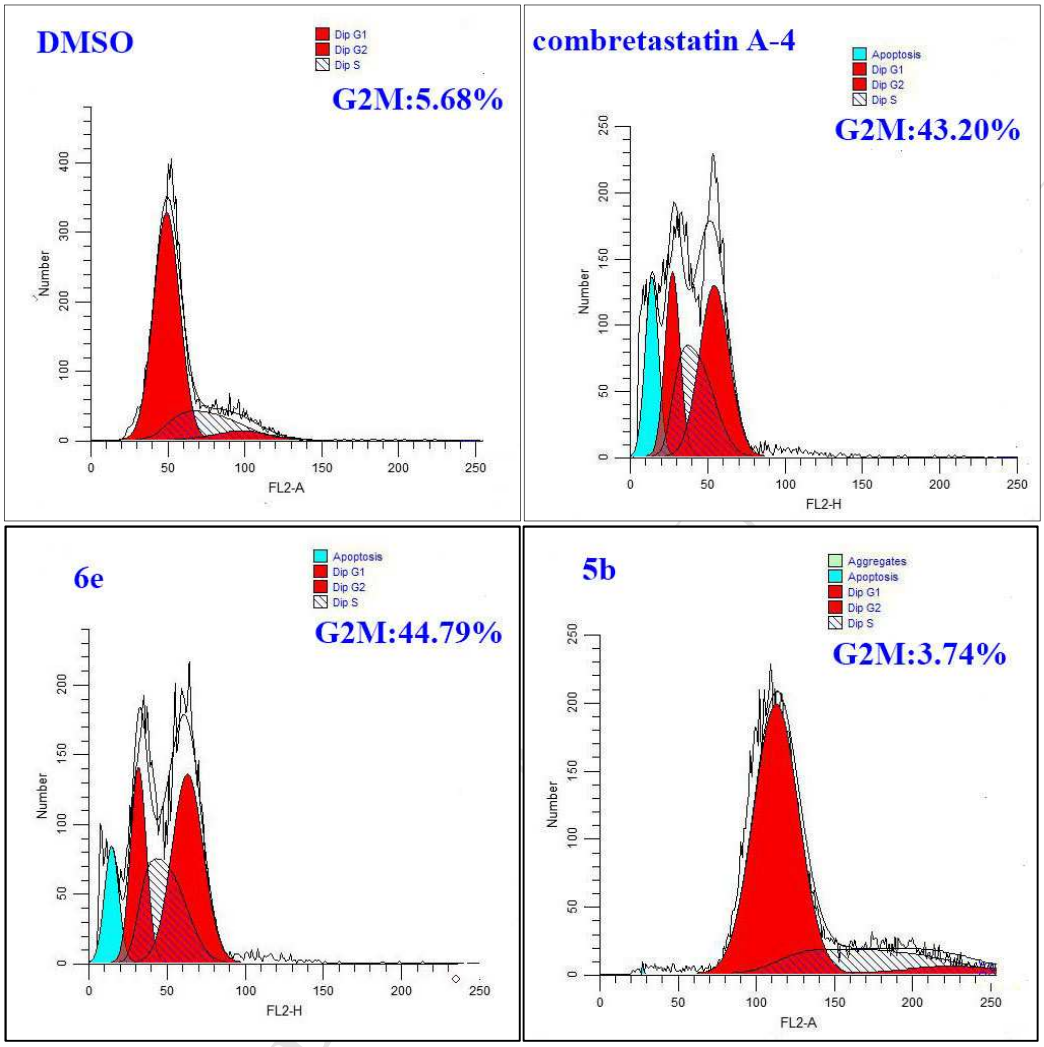


Figure 3. Flow cytometry analysis of compound **5b** and **6e**, (A) inA2780 cells. (B) in A2780/RCIS cells. (C) in MCF-7 (D) in MCF-7/MX.

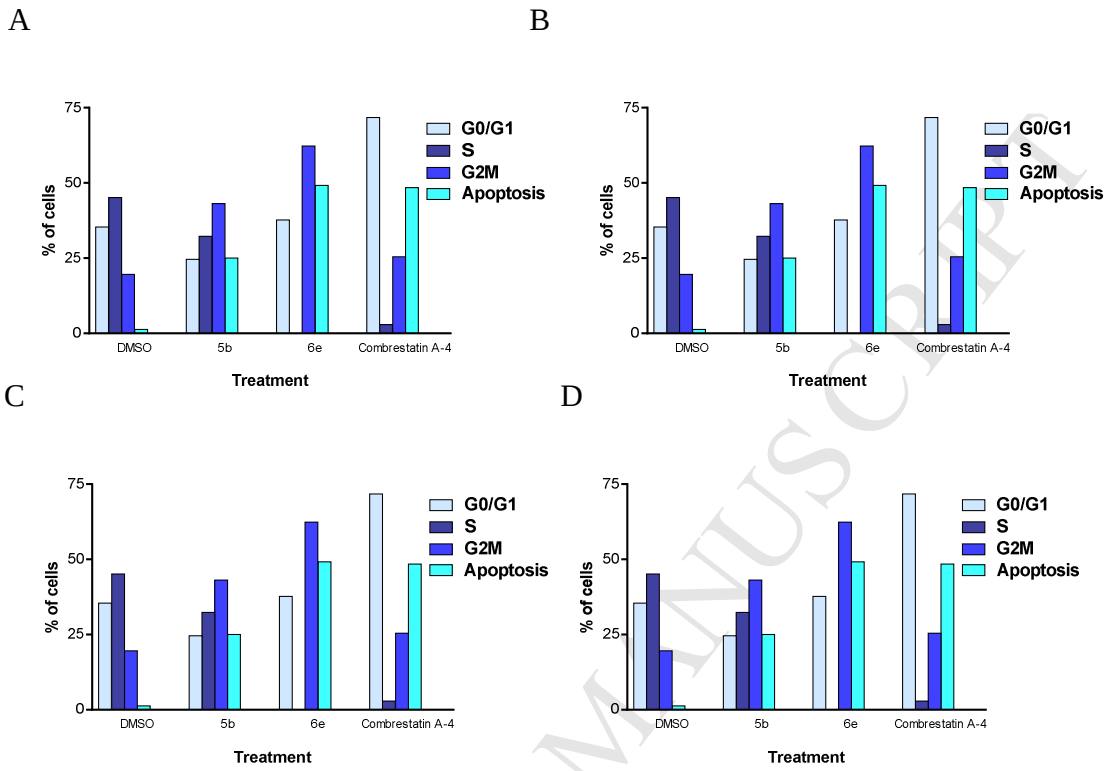


Figure 4. The statistical graph of cell cycle distribution of compound **5b** and **6e**, (A) in A2780/RCIS cells. (B) in A2780 cells. (C) in MCF-7/MX (D) in MCF-7.

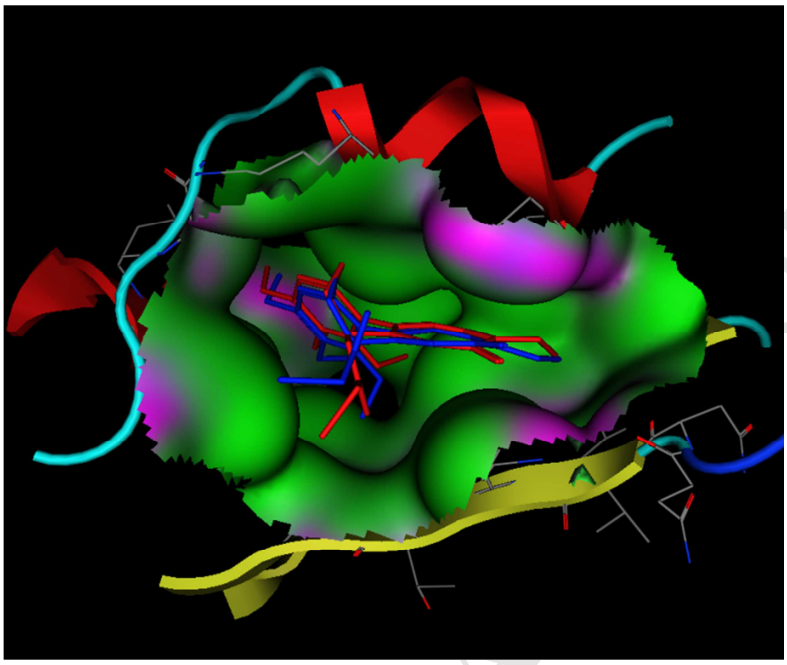


Figure 5: 3D representation of docked ligand (in red) into colchicine-binding site of tubulin using MOE program and co-crystallized ligand (in blue) in the crystal structure of tubulin (PDB ID: 1SA0).

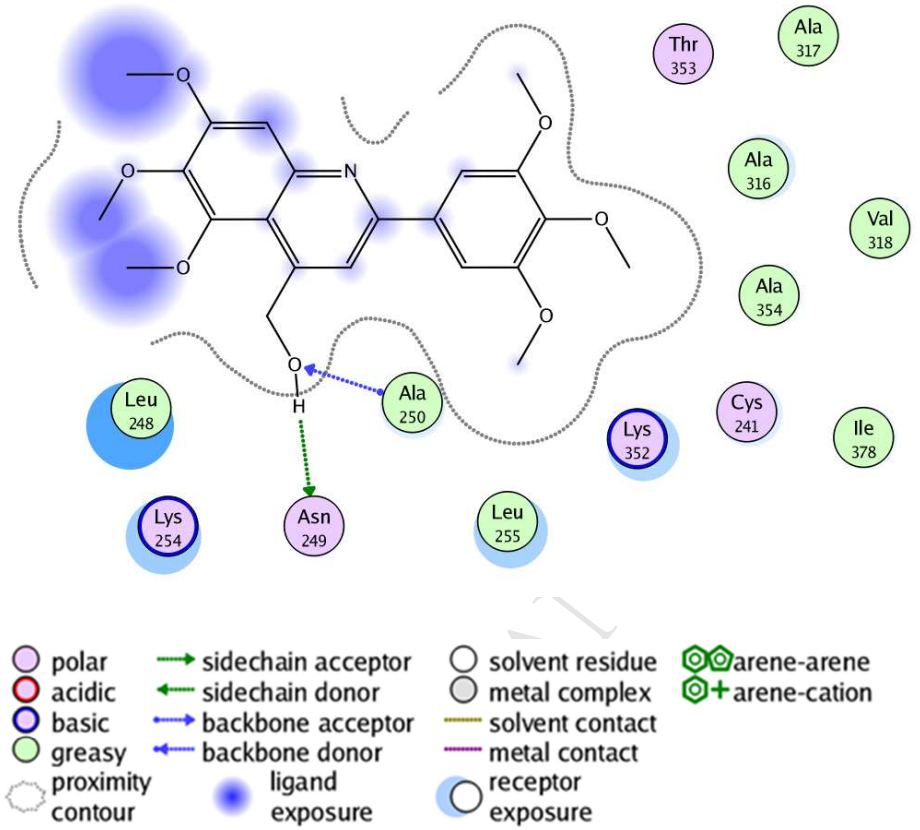


Figure 6: The 2D representation of the interaction between compound **6e** in the crystal structure of tubulin (PDB ID: 1SA0) using LigX in MOE.

Highlights

- Novel 2-aryl-trimethoxyquinoline analogues were synthesized
- Effects of quinolines on in vitro tubulin polymerization were investigated
- Some analogs displayed significant cytotoxic activity against resistant cancer cells
- Compound **6e** induced cell cycle arrest at G2/M phase and apoptosis as well