Synthesis and biological evaluation of oxazinonaphthalene-3-one derivatives as potential anticancer agents and tubulin inhibitors

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ABSTRACT

Objectives: In the present study, a new series of oxazinonaphthalene-3-one analogs was designed and synthesized as novel tubulin inhibitors.

Materials and Methods: The cytotoxic activity of the synthesized compounds was evaluated against four human cancer cell lines including A2780 (human ovarian carcinoma), A2780/RG1 (cisplatin resistant human ovarian carcinoma), MCF-7 (human breast cancer cells), and MCF-7/MX (mitoxantrone resistant human breast cancer cells), those compounds which demonstrated the most antiproliferative activity in the MTT test were selected to investigate their tubulin inhibition activity and their effects on cell cycle arrest (at the G2/M phase). Moreover, molecular docking studies of the selected compounds in the catalytic site of tubulin (PDB ID: 4O2B) were carried out to describe the results of biological experiments.

Results: Most of our compounds exhibited significant to moderate cytotoxic activity against four human cancer cell lines. Among them, Compounds 4d, 5c, and 5g, possessing trimethoxy phenyl, showed the most antiproliferative activity with IC50 values ranging from 4.47 to 52.8 μM.

Conclusion: The flow cytometric analysis of A2780 cancer cell line treated with compounds 4d, 5c, and 5g showed that these compounds induced cell cycle arrest at the G2/M phase. Compound 5g, the most antiproliferative compound, inhibited tubulin polymerization in a dose-dependent manner. Molecular docking studies of 5g into the colchicine-binding site of tubulin displayed a possible mode of interaction between this compound and tubulin.

Keywords:
Anticancer activity
Molecular docking
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Resistant cancer cells
Tubulin polymerization

Introduction

Cancer is a public health problem in the United States and many other countries. It is nowadays the second reason of death in the United States and is predicted to exceed heart diseases as the leading cause of death in the future (1). Therefore, there is a crucial need to discover and develop novel and more effective drugs to combat cancer. Although chemotherapy is the usual method for treatment of many cancer types, it fails to cure most cancer patients with advanced disease; occurrence of drug resistance is the main cause of this failure (2, 3).

Therefore, increasing interest has been devoted to the design and discovery of more effective anticancer agents in current medicinal chemistry.

Microtubules play an essential role in mitosis and have long been considered an important target for the development of novel anticancer drugs. Colchicine (Figure 1) was the first drug known to bind tubulin, and it binds at a specific site called the colchicine domain (2, 3). There are a number of small molecules discovered, which bind at the colchicine site of tubulin, and hence by inhibiting microtubule polymerization cause cell cycle arrest and lead to cell death (4-8). Combretastatins, isolated from the South African tree, Combretum caffrum, are also a group of antimitotic compounds and combretastatin A-4 (CA-4, Figure 1) is one of the well-known natural anti-tubulin molecules, which exerts its antimitotic effect by binding to colchicine’s binding site of tubulin. The cis-double bond in CA-4 is readily converted to the more stable trans geometry during storage or use, unfortunately, this causes a dramatic decrease in both cytotoxicity and anti-tubulin activity (9-11). Many structural modifications of this compound, such as variation of substitutions on the A- and B-rings, have been reported (12-14). Numerous structure–activity relationship (SAR) studies suggested that the main pharmacophoric components are trimethoxyphenyl and p-methoxyphenyl units situated in a cis-configuration (12, 14, 15). A huge number of compounds with a modified central bridge have also been synthesized, and ultimately isomerization of the cis-double bond in CA-4 has led to extensive studies of rigid ring modifications (13-15). Maytansine (Figure 1), a benzansaamacrolide possessing 1,3-oxazinan-2-one, is a highly potent microtubule-targeted compound that induces mitotic arrest and kills tumor cells at subnanomolar concentrations(16).

In the present study, we report the design and synthesis of new compounds possessing pharmacophoric elements of antitubulins with a 1,3-oxazinan-2-one central bridge (Figure 1). The synthesized compounds were evaluated for their cytotoxic activity towards four different cancer cell lines including MCF-7, MCF-7/MX,

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A-2780, and A2780/RCIS. The effect of the most cytotoxic compounds to induce G2/M arrest was evaluated by flow cytometry analysis. The most cytotoxic compounds were also investigated for their activity in a microtubular polymerization assay. In addition, in order to explain the obtained biological results, docking studies have been carried out (Figure 1).

**Materials and Methods**

**General chemistry**

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. A Bruker FT-300 MHz instrument was used to acquire NMR spectra. 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 (doublet), t (triplet), q (quartet), m (multiplet). The mass spectral measurements were performed on a 3200 QTRAP LC/MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) interface.

### General procedure for preparation of 1-(substituted phenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (4a-f)

A mixture of β-naphthol or 6-quinolinol 1 (1 mmol), urea 2 (1.5 mmol) and substituted benzaldehydes 3 (1 mmol), and HOAc (0.3 mmol) were mixed. The reaction mixture was placed in a Pyrex test tube and irradiated for 4-20 min with a power of 700 W. After cooling, the reaction mixture was washed with water and then recrystallized from EtOAc/Hexane (25/75) to afford the pure products 4a-4f (Scheme 1).

#### 1-(p-tolyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (4a)

Yield: 30.9%; yellow crystalline powder; mp: 173-175°C; IR(KBr): v (cm⁻¹) 3525 (NH) 1714 (CO); 1H NMR (400 MHz-DMSO): δ (ppm) 2.31 (s, 3H, CH₃), 6.06-6.07 (d, 1H, CH, J=1.8 Hz), 6.16 (s, 1H, NH), 7.12-7.13 (d, 2H, ArH, J=3.6 Hz), 7.15-7.18 (d, 2H, ArH, J=9 Hz), 7.34-7.36 (d, 1H, ArH, J=6.4Hz), 7.42-7.44 (m, 2H, ArH), 7.58-7.60 (m, 1H, ArH), 7.85-7.89 (m, 2H, ArH).

#### 1-(4-methoxyphenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (4b)

Yield: 36.21%; yellow crystalline powder; mp: 193-195°C; IR(KBr): v (cm⁻¹) 3407 (NH), 1726 (CO); 1H NMR (400 MHz-DMSO): δ (ppm) 3.77 (s, 3H, OCH₃), 6.064 (d, 1H, CH, J=1.8 Hz), 6.17 (s, 1H, NH), 6.84-6.86 (d, 2H, ArH, J=6 Hz), 7.21-7.23(d, 1H, ArH, J=6 Hz), 7.32-7.34 (d, 1H, ArH, J=8.4 Hz), 7.43-7.45 (m, 3H, ArH), 7.57-7.60(m, 1H, ArH), 7.86-7.89 (m, 2H, ArH).

### Scheme 1

Reagents and conditions: (a) AcOH, MW (b) DMF, NaH, CH₃I or benzyl bromide, rt
1-(3,4-dimethoxyphenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (4c)
Yield: 36.84%; yellow crystalline powder; mp: 216-219°C; IR(KBr): δ (ppm) 3490 (NH), 1748 (CO); 1H NMR (500 MHz-DMSO): δ (ppm) 3.69 (s, 3H, OCH3), 3.73 (s, 3H, OCH3), 5.65 (s, 2H, ArH), 7.33-7.34 (d, 1H, ArH, J=9 Hz), 7.40-7.43 (t, 1H, ArH, J=6 Hz), 7.46-7.49 (t, 1H, ArH, J=6 Hz), 7.82-7.84 (d, 1H, ArH, J=9 Hz), 7.90-7.92 (d, 1H, ArH, J=9 Hz), 7.94-7.96 (d, 1H, ArH, J=6 Hz), 8.73-8.74 (d, 1H, NH, J=3 Hz).

2-(benzyl)-1-(3,4-dimethoxyphenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (5b)
Yield: 51.86%; yellow crystalline powder; mp: 103-105°C; 1H NMR (400 MHz-DMSO): δ (ppm) 2.98 (3H, NHCH3), 3.68 (3H, OCH3), 3.73 (3H, OCH3), 5.63 (s, 1H, CH), 6.67-6.73 (2H, ArH), 6.88-6.91 (dd, 1H, ArH, J=8.4Hz, J=2Hz), 7.18-7.24 (m, 1H, ArH), 7.30-7.35 (m, 2H, ArH), 7.58-7.61 (d, 1H, ArH, J=9 Hz), 7.71-7.74 (d, 2H, ArH, J=9 Hz); 13C NMR (75 MHz-DMSO): δ (ppm) 34.73, 55.88, 55.93, 62.45, 110.13, 111.12, 114.90, 116.82, 120.48, 122.33, 125.08, 127.42, 128.88, 129.96, 130.39, 130.89, 131.74, 146.89, 149.42, 149.72, 150.32.

2-methyl-1-(3,4,5-trimethoxyphenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (5c)
Yield: 55.10%; yellow crystalline powder; mp: 137-140°C; 1H NMR (300 MHz-DMSO): δ (ppm) 3.00 (3H, OCH3), 3.70 (s, 9H, OCH3), 5.62 (s, 1H, CH), 6.47 (s, 2H, ArH), 7.18-7.24 (m, 1H, ArH), 7.29-7.40 (m, 2H, ArH), 7.61-7.63 (d, 1H, ArH, J=8.46Hz), 7.72-7.75 (d, 2H, ArH, J=8.46 Hz); 13C NMR (75 MHz-DMSO): δ (ppm) 34.90, 56.10, 56.24, 60.79, 62.83, 104.75, 113.97, 116.81, 122.32, 125.16, 127.48, 128.93, 128.98, 130.52, 130.90, 134.85, 138.36, 146.97, 150.29, 153.75.

2-benzyl-1-(p-tolyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (5d)
Yield: 77.74%; white crystalline powder; mp: 159-162°C; 1HNMR: δ (37.1 (3H, CH3 ) , 4.16-4.22 (d, 1H, J=16.4 Hz, CH2 , 4.91-4.96 (d, 1H, J=16.4 Hz, CH2 ), 6.14 (s, 1H, CH ), 6.91-6.94 (2H, J=9 Hz, Ar-H), 7.26-7.34 (m, 5H, Ar-H), 7.43-7.51 (m, 5H, Ar-H), 7.85-7.87 (d, 1H, J=6 Hz, Ar-H ), 7.94-7.96 (d, 1H, J=6 Hz, Ar-H), 7.99-8.02 (d, 1H, J=9 Hz, Ar-H ), 13CNMR:50.03, 55.57, 58.71, 114.97, 115.64, 117.13, 123.17, 125.77, 127.85, 128.01, 128.82, 129.06, 129.20, 129.30, 130.80, 130.95, 132.52, 136.88, 145.98, 146.84, 150.34, 159.69.

2-benzyl-1-(p-tolyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (5e)
Yield: 69.53%; white crystalline powder; mp: 163-166°C; 1HNMR: δ (37.1 (3H, CH3 ) , 4.17-4.22 (d, 1H, J=15 Hz, CH2 ), 4.91-4.96 (d, 1H, J=15 Hz, CH2 ), 6.14 (s, 1H, CH ), 6.92-6.94 (2H, J=6 Hz, Ar-H ), 7.26-7.35 (m, 5H, Ar-H), 7.43-7.54 (m, 5H, Ar-H), 7.85-7.87 (d, 1H, J=6 Hz, Ar-H ), 7.95-7.97 (d, 1H, J=6 Hz, Ar-H), 8.00-8.03 (d, 1H, J=9 Hz, Ar-H ), 13CNMR:50.04, 55.88, 55.93, 56.10, 56.24, 60.79, 62.83, 104.75, 113.97, 116.81, 122.32, 125.16, 127.48, 128.93, 128.98, 130.52, 130.90, 134.85, 138.36, 146.97, 150.29, 153.75.

Procedure for synthesis of 2-methyl-1-phenyl-1,2-dihydro-3H-naphtho[1,2-e][1,3]oxazin-3-one and 2-benzyl-1-phenyl-1,2-dihydro-3H-naphtho[1,2-e][1,3]oxazin-3-one (5a-g)
To a solution of 4a-4d (1mmol) in DMF (at 0°C) was added sodium hydride (60% oil suspension, 3mmol), then methyl iodide or benzyl bromide (3 mmol) was then added. The mixture was stirred at 0°C for 1hr, then diluted with water and the crude product was filtrated and recrystallized in ethanol.
7.91-7.96 (t, 2H, J=9 Hz, Ar-H ), 7.99-8.02 (d, 1H, J=9 Hz, Ar-H ). 13CNR: 50.15, 55.92, 56.02, 59.14, 111.88, 112.28, 112.75, 115.59, 117.11, 120.25, 123.31, 125.69, 127.87, 129.99, 129.92, 129.02, 129.16, 130.80, 130.95, 132.88, 136.98, 146.86, 149.27, 150.37.

2- benzyl-1-(3,4,5-trimethoxyphenyl)-1H-naphtho[1,2-e][1,3]oxazin-3(2H)-one (5g)

Yield: 79.32%; white crystalline powder; mp: 171-174 °C. 1HNMR: 3.60 (s, 3H, OCH3 ), 3.72 (s, 6H, OCH3 ), 4.36-4.41 (d, 1H, J=15 Hz, CH2 ), 4.89-4.95 (d, 1H, J=15 Hz, CH2 ). 6.17 (s, 1H, CH), 6.81 (s, 2H, Ar-H), 7.23-7.36 (m, 5H, Ar-H), 7.44-7.48 (m, 2H, Ar-H), 7.52-7.57 (t, 1H, J=6 Hz, Ar-H ), 7.94-7.97 (d, 1H, J=9 Hz, Ar-H ), 7.99-8.03 (m, 2H, Ar-H). 13CNR: 31.13, 50.53, 56.38, 59.77, 60.35, 105.39, 115.31, 117.13, 123.37, 125.75, 127.78, 127.88, 128.08, 128.91, 128.97, 129.18, 130.97, 136.16, 136.42, 137.06, 137.91, 146.97, 150.37, 153.66.

Biological evaluation

Antiproliferative activity assay

The levels of in vitro proliferative responses of cancer cells and normal cells were estimated using MTT dye assay. Briefly, all stock cell lines were grown in T-75 flasks at 37 °C with 5% CO2 in air. Cells were seeded in a 96-well plate at a density of 5000 cells per well, with 200 µl RPMI1640 or DMEM medium for 24 hr at 37 °C in a 5% CO2 incubator. Then they were exposed to different concentrations of the newly synthesized compounds (compounds were prepared in concentration 0.5% DMSO and diluted with culture medium), standard antitumor agent CA-4, and RPMI control (no drug) for 48 hr. Thereafter, 20 µl of MTT solution (5 mg/ml) was added to each well and incubation continued at 37 °C for 3 hr. Then, the suspension was removed, and formazan crystals were solubilized in 200 µl of dimethyl sulfoxide (DMSO). Plates were shaken vigorously (400 rpm) for 5 min. The absorbance of the solution at 545 nm and emission at 420 nm for 1 hr at 1 min intervals in a plate reader (Synergy H4, USA).

In vitro tubulin polymerization assay

A tubulin polymerization assay was performed by monitoring the fluorescence enhancement due to the integration of a fluorescence reporter into microtubules as polymerization happens. In this study, we have employed a commercial kit (cytoskeleton, cat. #BK011P) for the tubulin polymerization (21). The final buffer used for tubulin polymerization contained 80.0 mM piperazine-N,N' -bis(2-ethanesulfonic acid) sequisodium salt (pH 6.9), 2.0 mM MgCl2, 0.5 mM EGTA, 1.0 mM GTP, and 10.2% glycerol. First, 5 µl of the test compound at different concentrations, Paclitaxel (a polymerization promoter) at 3 µM concentration and CA4 (a polymerization suppressor) at 5 µM concentration, were poured into wells of a 96-well plate, and the mixture was warmed to 37 °C for 1 min; then, the reaction was initiated by the addition of 55 µl of the tubulin solution. Polymerization was measured by excitation at 360 nm and emission at 420 nm for 1 hr at 1 min intervals in a plate reader (Synergy H4, USA).

Cell cycle analysis using flow cytometry

Flow cytometric analysis (FACS) was performed to calculate the distribution of the cell population with propidium iodide (PI) through the cell cycle phases. Tumor cells (2.5 x 105 cells) were seeded in 6-well cell culture plates for 24 hr, then treated with different concentrations of the compounds 5g, 5c, and 4d, and vehicle alone (0.05% DMSO). Treated and untreated cells were incubated for 48 hr, washed with PBS and fixed with 70% ethanol, then washed twice with PBS, and incubated for 0.5 hr at 37 °C in a PBS solution containing 0.1 mg/mL RNase A and propidium iodide. The data regarding the number of cells in different phases of the cell cycle were analyzed using the Flowjo software package.

Molecular docking

The molecular docking process was performed using AutoDock4.2 (http://autodock.scripps.edu/). The x-ray crystal structure of tubulin (PDB ID:4O2B) with resolution 2.5 Å was downloaded from the Protein Data Bank (PDB). Water molecules, co-crystalized ligand, and all ions were removed and polar hydrogen atoms were added to the receptor then the protein was saved in the pdbqt format using the graphical user interface Autodock tools (ADT, 1.5.6). The 2D structure of the target compounds was prepared using Chem Draw Ultra 8.0 software (http://www.cambridgesoft.com/) and converted to 3D format by HyperChem7 (Hyper cube Inc, USA) using AM1 semi-empirical method, and the pdbqt format of the target compounds was prepared using ADT. The target compounds were docked into the active site of tubulin. A grid box size of 50 x 50 x 50 points with 0.375 Å spacing between the grid points was used. Grid box center was located at center of co-crystalized ligand (x =15.761, y =13.765, z =−17.608). AutoGrid 4.2 was used to generate the grid map files for the docking calculations. Then AutoDock4.2 was run. Lamarckian Genetic Algorithm (GA) parameters were set to 100 GA runs with a population size of 150; a maximum number of 2.5 x 105 energy evaluation and 2.7 x 104 generation were used. The other parameters were set as default. The output DLG files were converted to pdb format and Molecular Operating Environment (MOE) (www.chemcomp.com) was employed to view the docking results.

Results

Synthesis

As illustrated in scheme 1, β-naphthol, substituted benzaldehydes and urea were combined in the presence of a catalytic amount of acetic acid under microwave irradiation to obtain the target compounds 4a-4e, then benzyl or methyl derivatives 5a-5g were prepared using benzyl bromide or methyl iodide in the presence of sodium hydride. The compounds were characterized by nuclear magnetic resonance and infrared spectrometry.

Biological evaluation

In vitro anticancer activity

The cytotoxic activity of the synthesized compounds was evaluated against MCF-7, A2780, MCF-7/MX, A2780/RCIS, employing MTT assay using combretastatin A-4 as the positive control. As illustrated in Table 1, in general, the benzyl derivative 5g showed the most cytotoxic effect.
in cancer cells in comparison with other compounds. The simplest compound, 4a, did not show significant cytotoxic effect in cancer cells. Inserting three methoxy groups in ring A of 4a, increased the cytotoxicity on MCF-7/MX, A2780, and A2780RCIS cancer cells (compare the cytotoxic activity of compounds 4a and 4d). Compound 4e possessing 3-hydroxy-4-methoxyphenyl moiety showed significant cytotoxic effects in all cancer cell lines. In general, N-methyl and N-benzyl derivatives of 4a-4f demonstrated more cytotoxicity against all cancer cells in comparison with their parents 4a-4f, this may be due to better ability of 5a-5g to penetrate the cell membrane because of their high lipophilicity or better interaction of these compounds with tubulin. Compounds 5a, 5b, and 5c, N- methyl derivatives of 4a, 4c, 4d, and compounds 5d and 5g, N-benzyl derivatives of 4a and 4d showed also more cytotoxicity in comparison with their parents. Replacing the methyl group of 5d with the methoxy group, increased the cytotoxicity, significantly (compare the cytotoxic activity of compounds 5d and 5e). Also, in compounds 5d-5g with increasing the number of methoxy groups on the A ring, the cytotoxic activity of the compounds increased (5g>5f>5e>5d).

\textbf{Tubulin polymerization assay}

To examine whether the antiproliferative activity of compounds was due to their binding to tubulin, compound 5g, which demonstrated the most antiproliferative activity, was selected to test the inhibitory effect on microtubule assembly \textit{in vitro}, CA-4 as the polymerization suppressor and paclitaxel as the polymerization promoter were used (22). For characterization of tubulin polymerization, the fluorescence intensity was examined every minute and the results were shown in Figure 2. Compound 5g at 25-100 µM concentrations inhibited tubulin polymerization in a dose-dependent manner and in a manner similar to that of CA-4 (5 µM).

\textbf{Cell cycle analysis using flow cytometry}

The anti-mitotic drugs arrest cell cycle at the G2/M phase in cancer cells due to damage of the microtubular cytoskeleton(23). We examined the effect of the compounds 5g, 5c, and 4d on the cell cycle using the

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**Table 1.** \textit{In vitro} antiproliferative activities (IC50 (µM) a) of synthesized compounds and CA-4 against human cancer cell lines

| Compound | R1 | R2 | R3 | R4 | X | MCF-7 | MCF7-MX | A2780 | A2780-RCIS |
|----------|----|----|----|----|---|-------|---------|--------|------------|
| 4a       | H  | CH3| C  | N  | H | >100  | 100     | >100   | 58.7±4.33  |
| 4b       | CH3| CH3| C  | N  | H | ND    | ND      | ND     | ND         |
| 4c       | OCH3| H  | C  | N  | H | >100  | >100    | 31.5±2.35 | 46.7±3.89  |
| 4d       | OCH3| OCH3| C  | N  | H | ND    | 47.6±3.94 | 24.3±2.78 | 52.8±3.98  |
| 4e       | OH | OCH3| C  | N  | H | 26.87±3.67 | 15.31±2.91 | 23.47±1.84 | 17.43±2.34 |
| 4f       | OCH3| OCH3| OCH3| N | H | ND    | ND      | ND     | ND         |
| 5a       | H  | CH3| CH3| CH3| C | 30.74±3.04 | 40.9±4.24 | 24.3±2.63 | 32.6±3.04  |
| 5b       | OCH3| OCH3| CH3| C  | C | 33.43±3.71 | >100    | 26.9±2.72 | 31.4±2.56  |
| 5c       | OCH3| OCH3| OCH3| CH3| C | 32.5±3.21 | 9.0±1.03 | 12.2±1.45 | 18.3±2.32  |
| 5d       | H  | CH3| H  | benzyl| C | 100±4.91 | ND      | 31.5±3.25 | 28.3±2.45  |
| 5e       | H  | OCH3| H  | benzyl| C | 98.11±4.56 | 70.9±4.76 | 20.7±2.11 | 25.6±2.17  |
| 5f       | OCH3| OCH3| H  | benzyl| C | 30.3±2.44 | 71.10±5.36 | 18.7±1.84 | 24.2±2.16  |
| 5g       | OCH3| OCH3| OCH3| benzyl| C | 11.17±1.57 | 20.8±1.25 | 4.47±1.14 | 19.8±2.19  |
| CA-4     |    |    |    |    |   | 1.23±0.42 | 2.97±0.41 | 2.84±0.27 | 2.95±0.61  |

\(a\) compound concentration required to inhibit tumor cell proliferation by 50%. Data are presented as the mean±SD from the dose−response curves of three independent experiments.
propidium iodide staining method (24). A2780 cells were treated with increasing concentrations of compounds 5g, 5c, and 4d for 48 hr. They were stained with propidium iodide and then analyzed by flow cytometry. A2780 cells treatment with compound 5g at 5 and 10 µM, displayed enhancement in the G2/M population from 9% (control) to 11.34% and 25.65%, respectively in a dose-dependent manner (Figure 3).

4d caused G2/M arrest in a concentration-dependent manner. Only 9% of the A2780S cells were arrested in G2/M phase in the control group after 48 hr treatment, the percentage of G2/M phase increased to 20.35% and 28.08% when cells were exposed to 20 µM and 40 µM of 4d. Similarly, the cell accumulation percentage at the G2/M phase increased from 9% (control) to 18.44% and 24.12 % with a concentration rising from 15 to 25 µM of 5c. The results suggest that 5g, 4d, and 5c induced cell cycle arrest in the G2/M phase in A2780S cells.

Molecular modeling studies
In order to investigate the binding mode of the target compounds in the catalytic site of the tubulin molecular docking studies were carried out on the synthesized oxazinonaphthalene-3-one analogs and co-crystallized ligand against the colchicine binding site of the tubulin crystal structure (PDB ID: 4O2B) using Auto Dock 4.2 (25).

The docking study showed that the top-ranked conformation of all the selected compounds was well accommodated inside the colchicine binding site of the tubulin. Compound 5g has shown one hydrogen bond interaction between the carbonyl group with residues Lys 254β (Figure 4 B). In addition, compound 5g is surrounded by hydrophobic residues like Met 259β, Ile 378β, Ala 180, Cys 241β, Leu 242β, Lys 352β, Thr 353β, and Leu 248β. These hydrogen bonds and hydrophobic interactions can describe tubulin inhibitory effects of this compound.

Discussion
In the current study, a new series of oxazinonaphthalene-3-one analogs were synthesized and evaluated for their
in vitro antiproliferative activities by MTT assay against four human cancer cell lines including MCF-7, MCF-7/MX, A-2780, and A-2780/RCIS. In general, 5a-5g, N-methyl and N-benzyl derivative of 4a-4f demonstrated more cytotoxicity against all cancer cells in comparison with their parent 4a-4f, this may be due to the better ability of 5a-5g to penetrate the cell membrane because of their high lipophilicity or better interaction of these compounds with tubulin. Among them, 4d, 5c, and 5g possessing trimethoxy groups in the A ring showed significant cytotoxic activity with IC50 values ranging from 4.4-52.8 µM stronger than the other compounds. The cell cycle analysis indicated that 4d, 5c, and 5g disrupted the microtubule network and arrested cells in the G2/M phase of the cell cycle in the A2780 cancer cell line.

Conclusion

Among the synthesized compounds, 5g possessing trimethoxy groups and N-benzyl group were found to be the most potent cytotoxic agents against four human cancer cell lines with an IC50 value of 4.47-20.81 µM. Also, compound 5g inhibited tubulin polymerization in a dose-dependent manner and arrested cells in the G2/M phase of the cell cycle in the A2780 cancer cell line. Finally, molecular docking studies of 5g into the colchicine-binding site of tubulin represented the possible interaction of this compound in the active site of tubulin. These results suggested that oxazinonaphthalene-3-one derivatives could be promising lead compounds for further development of anticancer agents through inhibition of tubulin.

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Conflicts of Interest

The authors declare no conflicts of interest.

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