Identification of novel and potent small-molecule inhibitors of tubulin with antitumor activities by virtual screening and biological evaluations

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Abstract

Microtubules (made up of α and β-tubulin subunits) play an essential role in the process of mitosis and cell proliferation, thus making them an ideal target for anticancer drugs discovery. Microtubule-targeted drugs, including taxanes and vinca alkaloids, can suppress microtubule dynamics, cause mitotic block and apoptosis, which have been widely used in the treatment of various cancers. There are three unique binding sites (taxanes, vinca alkaloids, and colchicine) in tubulin can be targeted to develop tubulin inhibitors. In this study, we selected the colchicine binding site in tubulin as our target. By performing molecular docking-based virtual screening combined with in vitro tubulin polymerization inhibition assay, we identified two novel and potent tubulin inhibitors (**9** and **19**). These two compounds arrested cell cycle progression at the G2/M phase and induced apoptosis at sub μM concentrations. In addition, they displayed potent antiproliferative activity with IC_{50} values in the nM range. Finally, the probable binding modes of **9** and **19** were probed by molecular docking. These two compounds with novel scaffold will shed new light on the lead molecules discovery and the design of new microtubule-targeting agents (MTAs).

Introduction

Uncontrolled cell division and fast proliferation are fundamental hallmarks of cancer cells. In mammalian, cancer cells divide into two daughter cells were typically by means of mitosis, which is closely related to resembling and disassembling of microtubules [1, 2]. Microtubules, which are key components of the cytoskeleton, play crucial roles in various cellular processes, including the development and maintenance of cell shape, cell signaling, cell division and mitosis [3, 4]. A lot of efforts have led to the identification of several compounds that specifically block the key factors of mitosis [5]. The mechanism of these inhibitors can be largely divided into two categories, the selective inhibitors of mitotic kinases and kinesins, as well as the inhibitors targeting microtubules. Comparing with the inhibitors of kinases, compounds directly targeting microtubules, such as paclitaxel, always showed more potent selectivity and efficiency [6].

Paclitaxel and the vinca alkaloids are the two most successful microtubule-targeted chemotherapeutic drugs, and their most potent actions are mainly due to the suppression of microtubule dynamics, rather than increasing or decreasing microtubule-polymer mass [6]. MTAs can be grouped into two major types (microtubule stabilizers and microtubule destabilizers) based on their mechanism of action (MOA), for example, taxanes belonged to microtubule stabilizers while vinca alkaloids and colchicine belonged to microtubule destabilizers. Both types of MTAs inhibit the necessary dynamics required for microtubule function [7].

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There are three main classes of sites on tubulin for microtubule-active drugs: the paclitaxel site, the vinca site, and the colchicine site. Although there are no FDA approved tubulin inhibitors drugs targeting the colchicine binding site currently, this binding site is more amenable to molecules with more favorable physiochemical properties that improve oral bioavailability over taxanes and vinca alkaloids binding sites, for example, have less drug–drug interaction inclination, and are less prone to develop multi-drug resistance [8]. Thus, discovery and design of colchicine site tubulin inhibitors have received extraordinary attention in anticancer drug development.

Virtual screening has been widely and successfully used in identifying modulators of critical targets [9–17]. In this study, colchicine binding site in tubulin was selected as the target, and we performed molecular docking-based virtual screening in order to find candidates with novel scaffolds and potent binding affinity of microtubules. After in vitro tubulin polymerization inhibition assay, two novel and potent tubulin inhibitors (9 and 19) were identified. As expected, these two compounds displayed potent anti-proliferative activity on tumor cells (CEM, human lymphocytic leukemia cells; HeLa, human cervical carcinoma cells) with IC50 values in the nM range, arrested cell cycle progression at the G2/M phase and induced apoptosis at sub μM concentrations. Finally, the probable binding modes of 9 and 19 were probed by molecular docking simulations.

Materials and methods

Molecular docking-based virtual screening

Molecular docking-based virtual screening was performed using the Autodock 4.2 program [18, 19]. The crystal structure of the tubulin-colchicine complex (PDB ID: 4O2B [20]) was used to construct the docking model. Prior to docking studies, one α and one β tubulin of microtubules as well as crystallographic waters that have interactions with colchicine were retained. The missing hydrogen atoms were added, Gasteiger charges were assigned, and the protein was parameterized with AD4 type by Autodock Tools 1.5.6. Finally, the resulting enzyme structure was used as an input for the Autogrid program. Autogrid performed a pre-calculated atomic affinity grid maps for each atom type in the ligand plus an electrostatics map and a separate de-solvation map presented in the substrate molecule. Grid map with $60 \times 60 \times 60$ points was made according to the conformation of co-crystal ligand, and the grid spacing was set to 0.375 Å. All the SPECS database compounds were prepared using the program LigPrep from the Schrödinger (Schrödinger Release 2015-2: LigPrep, Schrödinger, LLC, New York, NY, 2015) to generate stereoisomers and different protonation states conformers. The OPLS3 force field was used, and the possible ionization states were predicted at pH (7.0 ± 2.0) using Epik. Docking calculations were carried out using the Lamarckian genetic algorithm (LGA). Colchicine was first redocked to tubulin to validate the docking methods, then the prepared SPECS database compounds were docked to tubulin for virtual screening. Pipeline Pilot 8.0 (Pipeline Pilot; Accelrys Software Inc., San Diego, CA) software was used to finish the cluster analysis. The clustering algorithm is a partitioning method in which the original data set is partitioned into ever-smaller regions that define the clusters, and a number of representative objects are chosen from the data set. The corresponding clusters are found by assigning each remaining object to each representative object, selecting the object that is the closest. The representative objects are called the cluster centers, while the other objects are the cluster members.

All the obtained compounds were purchased from commercial supplier (SPECS database). The purity of these compounds was > 95% as declared by the chemical vendor (Table S1).

Tubulin polymerization inhibition assay

Bovine brain tubulin (3 mg/mL, purity > 99%) was purchased from Cytoskeleton, Inc., USA. Tubulin protein was incubated with DMSO (control) or the tested compounds or colchicine (positive control) in general tubulin buffer (100 mM PIPES, 1.0 mM MgCl2, 1 mM EGTA, 1 mM GTP and 5% glycerol) at 0 °C. The reaction was started by warming the samples to 37 °C. Then the absorbance of wavelength at 340 nm (indicated the mass of polymer formation) was detected every 1 min for 20 min by Spectra Max 190 spectrophotometer (Molecular Device).

Tumor cell growth inhibition assay

Cytotoxic effects were examined in the CEM human lymphocytic leukemia cells and HeLa human cervical carcinoma cells by MTT assay. CEM and Hela cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% FBS and 1 × Pen/Strep. To determine the cytotoxic effects of compound 9 and 19, the cells $(2 \times 10^5$ cells per well) were placed in 100 μL of serum containing media with compounds in 96-well flat-bottomed microtiter plates in triplicate cultures. After three days incubation, the absorbance at 570 nm was recorded on a fully automatic enzyme-labeled meter (Labsystems Inc. Wellscan MK-2, Finland). Compounds were tested in triplicate in at least three independent assays, and the average median inhibitory concentration (IC50) values were reported.

Cell cycle and apoptosis analysis

CEM cells were seeded overnight at 2×10^5 cells/well. Next, DMSO or compounds were added for 24 h. For cell cycle analysis, cells were harvested and washed three times with PBS and at last fixed with 75% ethanol at -20 °C overnight. The cells were then stained with propidium iodide dye (BD Biosciences) for 20 min in dark conditions at room temperature, followed by subjected to flow cytometry (Cytomics FC 500MPL, Beckman Coulter) detection. For cell apoptosis analysis, harvested cells were measured using AnnexinV-FITC Apoptosis Detection Kit (Vazyme Biotech) according to the manufacturer's protocol. These results were analyzed by FlowJo V7.6.1.

Results and discussion

Virtual screening and tubulin polymerization inhibition assay identified 2 tubulin inhibitors

Molecular docking-based virtual screening was used to screen small-molecule tubulin inhibitor candidates from SPECS database (http://www.specs.net/) which contains 212,449 compounds. In order to validate the molecular modeling methodology, the inhibitor colchicine that was co-crystalized with tubulin (PDB code: 4O2B) was first redocked to tubulin protein. As shown in Figure S1, the docking result could well reproduce the crystal structure, which indicated that the method was suitable for docking study of tubulin. The workflow of virtual screening was shown in Fig. 1a. After docking, the top-246 compounds ranked based on the docking score were retained for visual inspection to remove the compounds with unfavored geometry, unfavored chemical fitness with the binding sites or binding outside

the defined pocket. In addition, compounds with similar chemical scaffolds were clustered by chemical fingerprints, and characteristic compounds were selected in each cluster according to the following considerations: (1) At least one molecule was retained in each cluster to guarantee the diversity of chemical space. (2) Molecules with simple structure and small molecular weight were preferred. (3) Molecules that could form hydrogen bonds with V181 of tubulin and the water around colchicine were preferred. Finally, 36 compounds were selected for experimental validation. Among them, compounds 9 and 19 displayed potent tubulin polymerization inhibition activity with IC_{50} values of 1.68 μM and 2.33 μM, respectively (Fig. 1b). As a reference compound, combretastatin A-4 (CA-4) was included.

Growth-inhibitory activity of compounds 9 and 19 in three cancer cell lines

The anti-proliferative activity of the identified two compounds **9** and **19** were evaluated for their growth-inhibitory activity in three human cancer cell lines [lymphoblastic leukemia (CEM) cells, cervical carcinoma (HeLa) cells and colon cancer (HT-29) cells]. As can be seen from Fig. 2, both of this two compounds displayed potent anti-proliferative effects on all the three human cancer cells (CEM, Hela and HT-29), with IC_{50} values in the nM range.

Inhibition of cell cycle progression and inducement of cell apoptosis in HT-29 human colon cancer cell line

Since MTAs usually arrest cancer cells in G2/M phase and induce cancer cells apoptosis [21]. Meanwhile, given the fact that compound **9** was found to have growth inhibitory effect on human cancer cells, we continued to investigate its
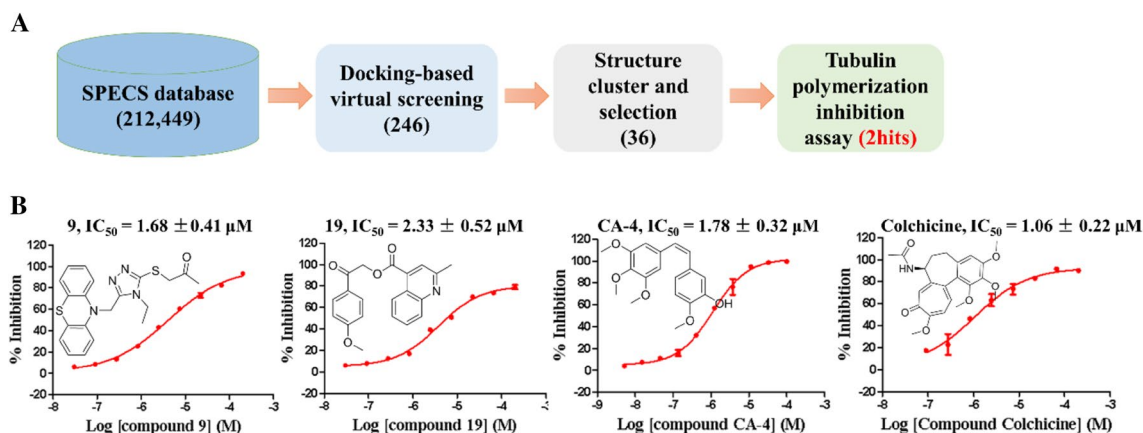

Fig. 1 The workflow of tubulin inhibitor discovery and the inhibitory activity of the two hit compounds against tubulin polymerization. **a** The workflow of tubulin inhibitor discovery. Numerals denote the number of molecules in each stage. **b** Chemical structures and IC_{50} curves of compound **9**, **19**, CA-4 and Colchicine (positive control). Data shown are mean ± SD of three replicates

Compounds	9	19	Colchicine
IC_{50} (nM)	14.1	23.4	10.2

Compounds	9	19	Colchicine
IC_{50} (nM)	18.6	21.0	5.34

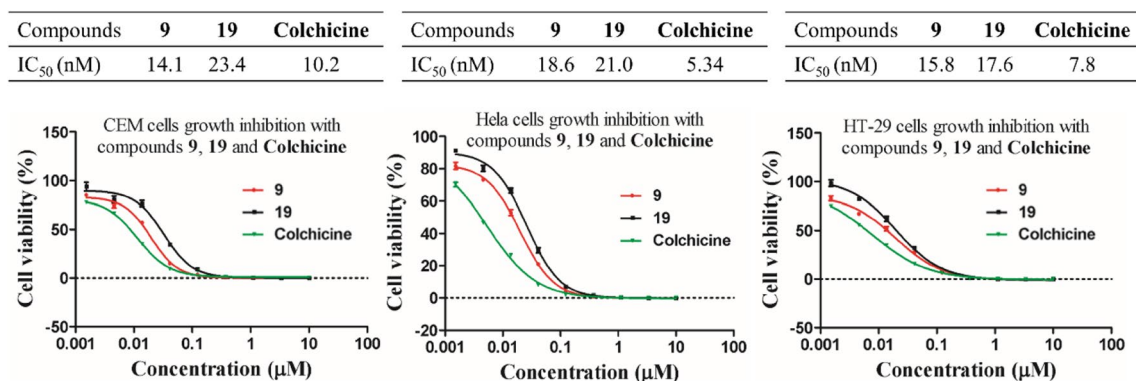| Compounds	9	19	Colchicine
IC_{50} (nM)	15.8	17.6	7.8

Fig. 2 The effects of compounds **9**, **19** and Colchicine (positive control) on the proliferation of three human cancer cell lines. IC_{50} is presented as the mean ± SD from at least three independent experiments. The top panel depicted the IC50 values, and the low panel showed the IC_{50} curves

actual cellular mechanism of action in human cells. Since MTAs usually induce G2/M cell cycle arrest, the effect of **9** on the cell cycle was studied in HT-29 colon cancer cells for 24 h by flow cytometry. As shown in Fig. 3, an accumulation of cells in G2/M phase was observed after 24 h treatment with compound **9** at the concentrations of 5 nM and 10 nM, respectively. This data indicated an antimitotic mode of action of compound **9**.

The effect of compound **9** on cell apoptosis was also examined. Results showed a concentration-dependent increased apoptosis (Fig. 4) upon treatment with compound **9** for 48 h on HT-29 cells. These data together with cell cycle arrest results supported the idea that compound 9 were not only cell-cycle arrestors but also inducers of apoptosis.

Molecular modelling

To gain detailed insights into the binding of this two compounds with tubulin, molecular docking simulation was performed. As shown in Fig. 5a, b, one hydrogen bond that the colchicine carbonyl oxygen makes with the backbone NH of Val181 were established. In addition, there are three water mediated hydrogen bonds that are involved in the interactions of colchicine with tubulin. Although the hydrogen bond established between colchicine and V181 residue does not exist in the interactions of **9** and **19** with tubulin, the water mediated hydrogen bond were maintained. And there are both two water-mediated hydrogen bonds (rendered as red dash lines) established between 9 and tubulin as well as
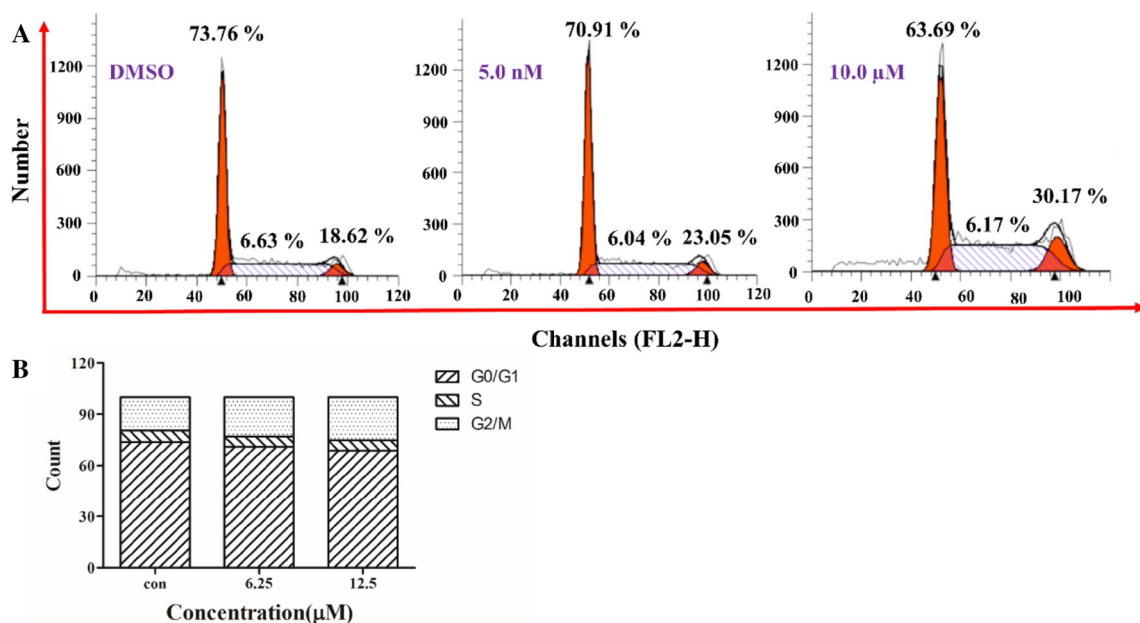

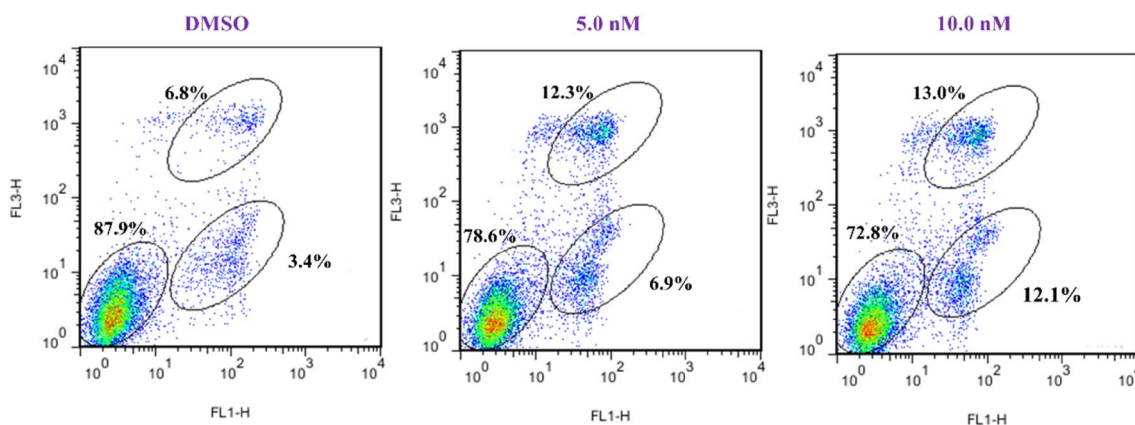**Fig. 3** Treatment with compound **9** led to cell cycle arrest at G2/M phase

Fig. 4 Cell apoptosis induction after treatment with **9**. Treatment with **9** induced apoptosis of HT-**29** cells measured after 48 h
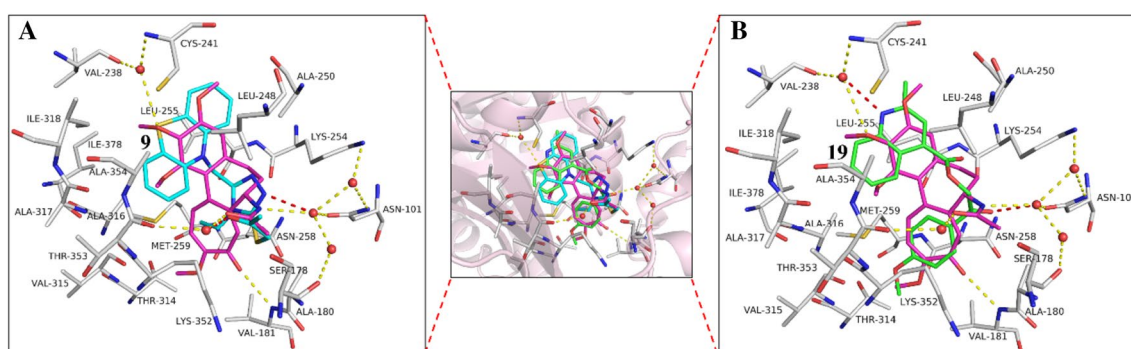

Fig. 5 The putative binding modes of **9** and **19** with tubulin. **a** The binding mode **9**. **b** The binding mode **19**. Tubulin is shown as light pink cartoon diagram, and **9**, **19** and colchicine are represented as cyan, green and magenta sticks, respectively. The interactive residues are labeled with carbon atoms colored in white. The water mediated hydrogen bonds between colchicine and tubulin are shown as yellow dash lines, while that between 9 and 19 and tubulin are shown as red dash lines. Colchicine binding mode was obtained by the original crystal structure (PDB code: 4O2B)

19 and tubulin, respectively. Besides, hydrophobic interaction was also the main interactions that contributed to the binding affinity between this two compounds and tubulin by further analysis (Fig. S2). In particular, both compounds **9** and **19** were located in the hydrophobic pocket which is composed of residues A180, C241, L242, L248, A250, L255, N258, M259, I318 and K352, except residue A180, the other residues belong to β tubulin. By comparing the binding modes of the two compounds with that of colchicine, we concluded that this two compounds could efficiently occupy the colchicine binding site of tubulin and showed similar binding modes with that of colchicine.

Conclusion

Owing to the key role in mitosis and other cellular processes, microtubules are regarded as an excellent target for anticancer drug development and have drawn much attention in recent years. Tubulin inhibitors that bind to the colchicine binding site have many advantages over taxanes and vinca alkaloids site binding inhibitors, such as the capacity to overcome multidrug resistance (MDR), favorable pharmacokinetics and well water solubility as well as the high selectivity in killing cancer cells over normal cells. However, up to date, there are no colchicine site binding inhibitors approved by FDA to treat cancer. Thus, it is an urgent need to discovery more colchicine site binding tubulin inhibitors. In this study, by performing molecular docking-base virtual screening, we identified two novel colchicine binding site tubulin inhibitors. Then their anti-proliferative effects on three different human cancer cells were confirmed, and the anti-proliferation mechanism was validated to the cell cycle arrest and cell apoptosis induce. Finally, the binding modes of this two compounds with tubulin were well studied by molecular docking simulation.

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