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Discovery of isopenicin A, a meroterpenoid as a novel inhibitor of tubulin polymerization

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ABSTRACT

Microtubules are involved in celluar processes of movement, intracellular trafficking and mitosis, thus microtubule-targeting agents have been widely used in cancer therapy. Herein, we report isopenicin A, a novel meroterpenoid isolated from the plant endophytic fungus of *Penicillium* sp. sh18, as a novel microtubule binding molecule that efficiently depolymerizes microtubule polymerization to evoke G2/M cell cycle arrest and subsequent cell apoptosis, contributing to proliferation inhibition of human tumor cell lines. The discovery of isopenicin A provides a new chemotype for discovery and development of promising microtubule inhibitors.

1. Introduction

Microtubules are highly dynamic cytoskeletal fibres that are formed by the assembly of tubulins in a head-to-tail structure [1]. The two types of non-equilibrium dynamics of microtubules are crucial to a wide range of cellular functions, such as the assembly of the mitotic spindle, the movement of organelles, vesicles and proteins, and the associated cell signaling, as well as in the development and maintenance of cellular shape [2]. Therefore, microtubule binding agents are widely used in clinical cancer therapy, such as paclitaxel and colchicine [3]. Tubulin interactors are classified into two categories containing tubulin stabilizing agents and tubulin destabilizing agents based on their interference action and the binding sites on the tubulin dimer have been identified [4]. Between them, compounds binding to paclitaxel [5] or laulimalide [6] sites promote tubulin polymerization while interactions with the other sites (vinblastine [7], colchicine [8], maytansine [5], laulimalide [9]) lead to inhibition of polymerization. These diverse mechanisms of tubulin inhibition provide several options for microtubule drug development.

Fungal metabolites have played a leading role in drug discovery and development resulting in the clinical application of useful agents [10], antibacterial penicillins, cholesterol-lowering lovastatin, antifungal echinocandin B, and immunosuppressive cyclosporin A, demonstrating potential of exploring fungal sources for new medicines. Of note, the *Taxus brevifolia* barks were the primary source of the approved tubulin binding agent paclitaxel until the discovery of paclitaxol in an endophytic fungus *Taxomyces andreanae* in 1993 [11], and more than 20 genera of taxol-producing endophytic fungus have been identified [12]. Fungal secondary metabolites have always been regarded as an important source for drug discovery due to their diverse chemical structures and bioactivities.

Fungal meroterpenoids, a unique type of secondary metabolites derived from a hybrid terpenoid-polyketide pathway, associated with a broad spectrum of biological properties for their complex and intriguing structures. Andrastin A, a meroterpenoid as a potential lead for anticancer drugs, serves as a node in the molecular networking of *Penicillium* sp. sh18 [13]. Subsequently, isopenicin A was found in the metabolites of *Penicillium* sp. sh18 as a novel antitumor meroterpene analog to andrastin A. Isopenicin A, a novel meroterpenoid possessing two types of unprecedented terpenoid-polyketide hybrid skeletons, showed antineoplastic activity and the Wnt/β-catenin signaling pathway inhibitory of isopenicin A in

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our previous research [13], while the anti-tumor target of isopenicin A has been little studied up to now. Herein, isopenicin A was identified as a novel natural microtubule inhibitor, which induced G2/M cell cycle arrest and subsequent apoptosis to attenuate the proliferation of cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

Isopenicin A was isolated from the cultures of *Penicillium* sp. sh18. The structure was determined through synergetic use of extensive spectroscopic analysis, quantum-chemical calculation with ANN-PRA analysis, and X-ray crystallographic analysis [13]. Isopenicin A was dissolved at 10 mM in dimethyl sulfoxide (DMSO) as a stock solution and diluted to desired concentrations according to the research requirement. Antibodies against PARP, Bcl-XL and XIAP were purchased from Santa Cruz Biotechnology. Antibodies against Cdc2 and Cyclin B1 were purchased from Cell Signaling Technology and antibody against tubulin was from Sigma. The Annexin V-FITC/PI Apoptosis kit and Tubulin Polymerization Assay Kit were purchased from BD Biosciences and Cytoskeleton, respectively.

2.2. Cell lines and cell culture

Human colorectal cancer cells SW480 were obtained from American Type Culture Collection (ATCC), and human mammary cancer cells MDA-MB-231 and human chronic myelogenous leukemia cells K562 were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SW480 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. K562 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Tubulin polymerization assay

The Tubulin Polymerization Assay was conducted following the manufacture's protocols. The condition was 2 mg/mL tubulin in 80 mM PIPES pH 6.9, 2.0 mM $MgCl_2$, 0.5 mM EGTA, 1.0 mM GTP and 15% glycerol. In brief, Tubulin Reaction Mix that acquired by mixing the assay components were utilized to react with compounds. The reaction was detected at 410–460 nm in kinetic mode with excitation filters of 340–360 nm using the EnVision Multimode Plate Reader.

2.4. Immunofluorescence staining

The MDA-MB-231 cells grown on cover slips were fixed in 4% paraformaldehyde for 20 min at 37 °C and permeabilized using 0.1% Triton X-100 for 10 min at room temperature. The cells were blocked with 3% BSA serum for 1 h at room temperature. To observe microtubules polymerization, cells were incubated with anti-tubulin antibody and corresponding FITC conjugated secondary antibody before staining the nuclei with DAPI. Images were later observed using a Laser confocal microscope.

2.5. Cytotoxicity assay and IC_{50} determination

Cell viability was assessed by the MTS assay. Cells were dealt with the indicated compounds at concentrations of 2.5, 5, 10, 20 and 40 μM in 96-well plates. After 48 h, MTS was added to each well, for a final concentration of 20%. The samples were incubated at 37 °C for 1–4 h and the optical density (OD) was measured at 490 nm using a microplate reader (PerkinElmer). The IC_{50} values were determined with GraphPad Prism software by non-linear regression analysis.

2.6. Cell cycle analysis

SW480 cells were incubated with isopenicin A for 24 h, and cells were collected and fixed in 70% ethanol overnight. Fixed cells were resuspended in PBS the next day, and then incubated with RNase A (50 μg/mL) for 30 min and stained with propidium iodide (PI) solution (50 μg/mL final concentration) in the dark for 20 min. FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) was used to analyze the fluorescence intensity, and the cell cyle distribution was determined using FlowJo 7.6.1.

2.7. Cell apoptosis assay

The Annexin V-FITC/PI Apoptosis Kit was used to analyze cell apoptosis according to the manufacturer's protocols. Cells were seeded in 6-well plates at a density of 2×10^5 cells/well and incubated with certain concentrations of isopenicin A for 48 h. The cells were collected and washed twice with cold PBS, and then resuspended in 1 × binding buffer containing Annexin V-FITC and propidium iodine (PI). After incubation for 15 min at room temperature in the dark, the fluorescent intensity was measured using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ).

2.8. Western blotting

Total cell lysates were prepared by direct lysis in 2×Laemmli buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.004% bromophenol blue). Samples were

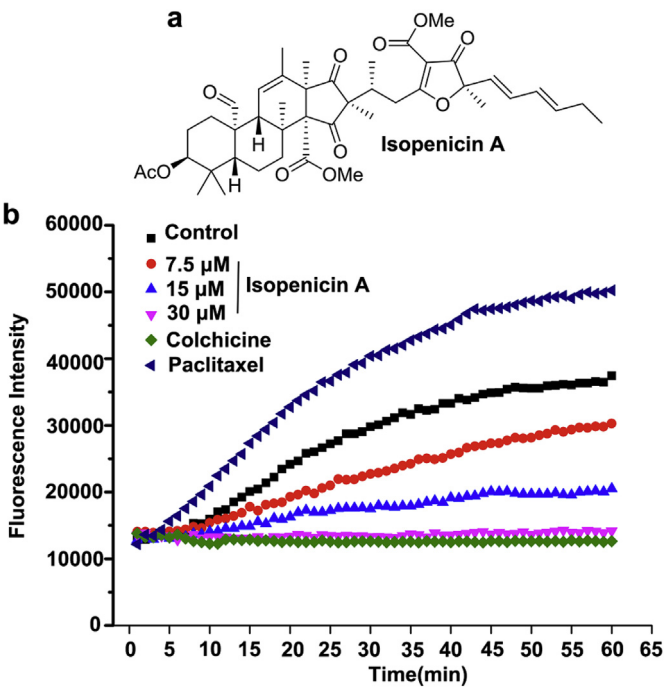

Fig. 1. Isopenicin A inhibited tubulin polymerization in vitro. (a) Structure of isopenicin A. (b) After reacting with Tubulin Reaction Mix, the effect of compounds on the tubulin polymerization was detected using the EnVision Multimode Plate Reader. Isopenicin A dose-dependent inhibited the tubulin polymerization.

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fractionated in 12% acrylamide gel, transferred to a PVDF membrane (Bio-Rad), and then incubated with specific primary antibodies followed by the corresponding peroxidase-conjugated secondary antibodies. Proteins of interest were visualized by chemiluminescent detection on an ImageQuant LAS mini 4000 (GE Healthcare).

3. Results

3.1. Isopenicin A inhibited tubulin polymerization in vitro

Isopenicin A (structure designated in Fig. 1a), a meroterpenoid isolated from the cultures of *Penicillium* sp. sh18, was found to suppress the proliferation of various cancer cells in our previous study, in which the obvious G2/M cell cycle arrest was observed [13]. It is generally known that tubulin-binding agents induce the G2/M phase arrest due to disruption of microtubule dynamics. Thus, we tested the effect of isopenicin A on tubulin polymerization in a cell-free system. Purified tubulin alone was polymerized, and Paclitaxel promoted microtubule polymerization while colchicine accelerated depolymerization. Meanwhile, isopenicin A inhibited the tubulin polymerization in a concentration-dependent manner (Fig. 1b).

3.2. Isopenicin A inhibited tubulin polymerization in vivo

Tubulin-targeted agents disrupt the microtubule network, then immunofluorescence staining was undertaken to evaluate the effect of isopenicin A on the cytoskeleton network in MDA-MB-231 cells. As shown in Fig. 2a, compared to the typical cytoskeleton structures of untreated cells which had long and dense microtubules extending throughout the cytoplasm, paclitaxel-induced microtubule polymerization with cellular microtubule gathered closely together, while colchicine-induced microtubule depolymerization caused

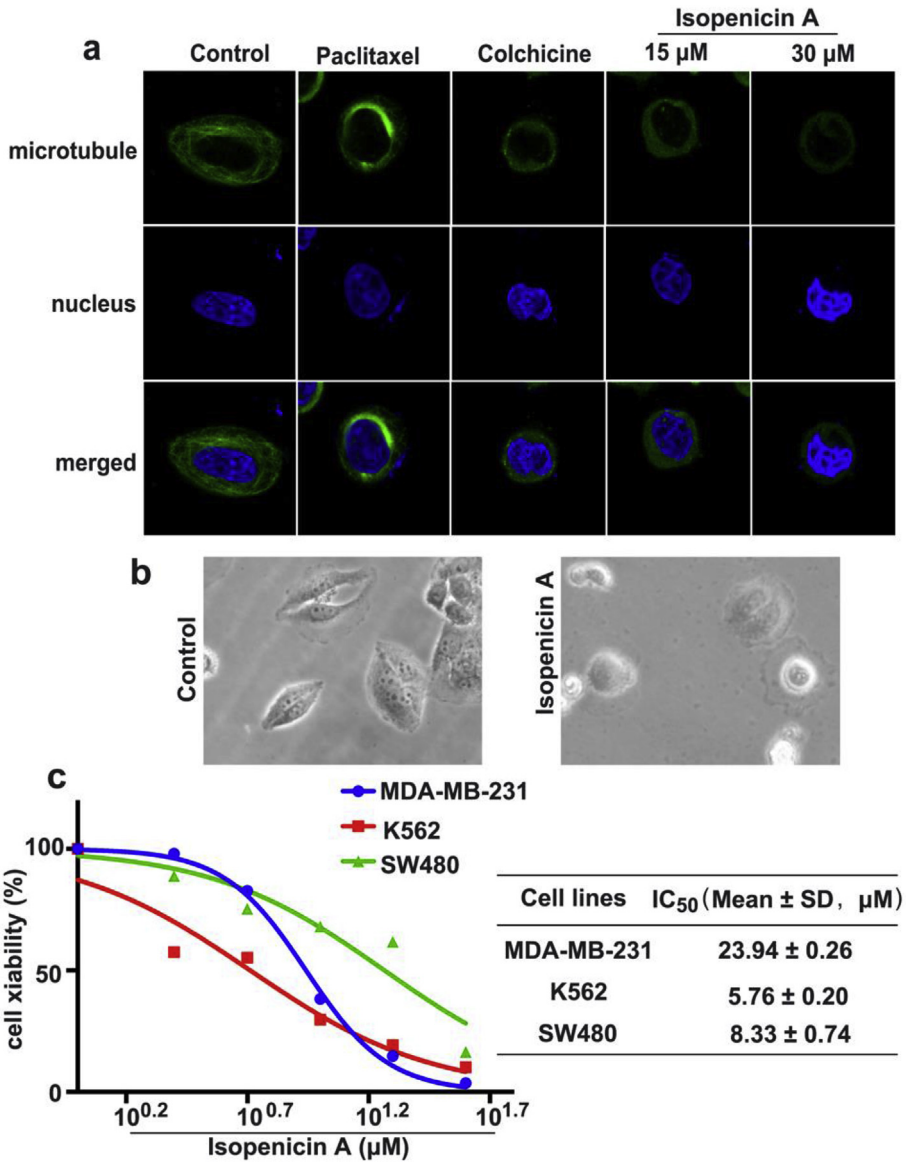

Fig. 2. Isopenicin A inhibited tubulin polymerization in vivo. (a) Cells pretreated with isopenicin A and processed for immunostaining with anti-tubulin antibody. Nuclei of cells were stained with DAPI (blue) and tubulin was visualized by green fluorescence. (b) Morphological changes of SW480 cells after treated with isopenicin A were observed and recorded with a microscope. (c) Effects of isopenicin A on the growth of various cell lines (48 h). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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cellular microtubule incompact. After exposure to isopenicin A at different concentrations (15 μM, 30 μM) for 2 h, the microtubule networks in cytosol were disrupted, indicating that isopenicin A induced a dose-dependent collapse of the microtubule networks. Microtubules are involved in celluar processes of cell shape maintenance and mitosis, the morphological change and cytotoxicity were detected in isopenicin A treated cancer cells. As shown in Fig. 2b, long time treatment of isopenicin A caused the celluar morphology change. The IC_{50} values of isopenicin A for 48 h treatment were 23.94 ± 0.26 μmol/L, 5.76 ± 0.20 μmol/L and 8.33 ± 0.74 μmol/L for MBA-MD-231, K562 and SW480 cells, respectively (Fig. 2c).

3.3. Isopenicin A induced G2/M phase arrest and regulated the expression of G2/M-related proteins in SW480 cells

In the M phase of cell cycle, microtubules are remodeled and assembled into mitotic spindles to direct and drive the chromosomes separation, thus disruption of tubulin dynamics leads to G2/M cell cycle arrest [14]. To further confirm the microtubule depolymerization effect of isopenicin A, we examined the G2/M cell cycle arrest activity of isopenicin A with propidiumiodide (PI) staining by flow cytometry analysis in SW480 cells. Cells were treated with 7.5 μM, 15 μM, and 30 μM isopenicin A for 24 h, then cell cycle distribution of isopenicin A-treated cells was analyzed. As shown in Fig. 3a and b, exposure to isopenicin A increased the number of G2/M phase cells, while the number of cells in the S and G1 phases was contemporaneously reduced. The percentages of cells arrested at the G2/M phase were 29.79%, 32.44%, and 34.83% at the concentrations of 7.5 μM, 15 μM, and 30 μM respectively, while the percentage of G2/M in control group was 12.06%. Cyclin B1 [15] and Cdc2 [16] play a key role in the control of cell cycle progression from G2 to M phase, which have been implicated in the tumorigenesis and the development of malignancy. The expression of these proteins then be analyzed by western blot. The western blot analysis demonstrated that the expression of Cyclin B1 and Cdc2 were decreased after treated with isopenicin A (Fig. 3c).

3.4. Isopenicin A induced apoptosis of SW480 cells

Microtubule inhibitors induced disruption of mitosis and cell cycle arrest lead to cell apoptosis activation and an obvious typical apoptosis sub-G1 accumulation was observed in isopenicin A treated cells (Fig. 3a and b), we deduced that isopenicin A caused cell apoptosis. Annexin V-FITC/PI apoptosis detection kit assay was performed to assess the apoptosis induction effect of isopenicin A. As Fig. 4 illustrated, the percentage of apoptotic cells in the control group was only 10.38%, while the percentages of early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) apoptosis induced by isopenicin A increased to 15.56%, 59.435%, and 69.68% at 7.5, 15 and 30 μM for 48 h respectively. Western blot analysis (Fig. 4c) also demonstrated that isopenicin A significantly induced the cleavage of PARP-1 [17] in the cancer cells detected. The protein level of Bcl-xL, a key regulator of anti-apoptotic pathway [18], was down-regulated by isopenicin A treatment. Moreover, the level of XIAP (X-linked inhibitor of apoptosis), a member of the inhibitor of apoptosis (IAP) [19], was dramatically decreased in isopenicin A treated cells. These

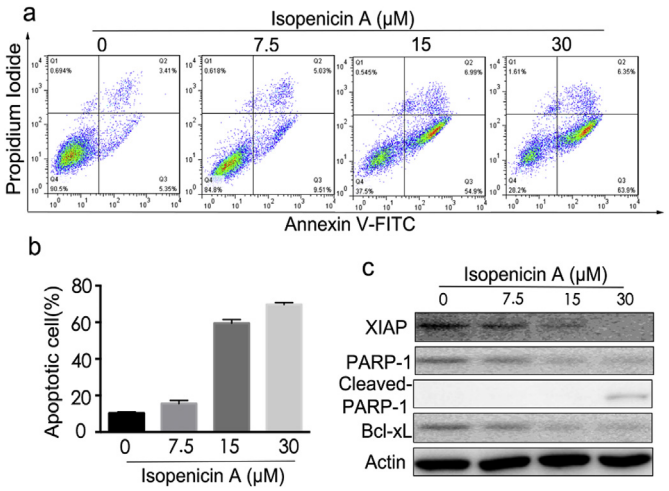

Fig. 4. Isopenicin A induced apoptosis of SW480 cells. (a, b) SW480 cells were incubated with Isopenicin A at concentrations of 7.5, 15, and 30 μM for 48 h. Apoptosis was analyzed by Annexin V-FITC/PI staining. Isopenicin A induced dramatic apoptosis in SW480 cells. (c) Cells were treated with isopenicin A for 24 h and cell lysates were subjected to western blot analysis with antibodies indicated. β-actin were used as loading controls.

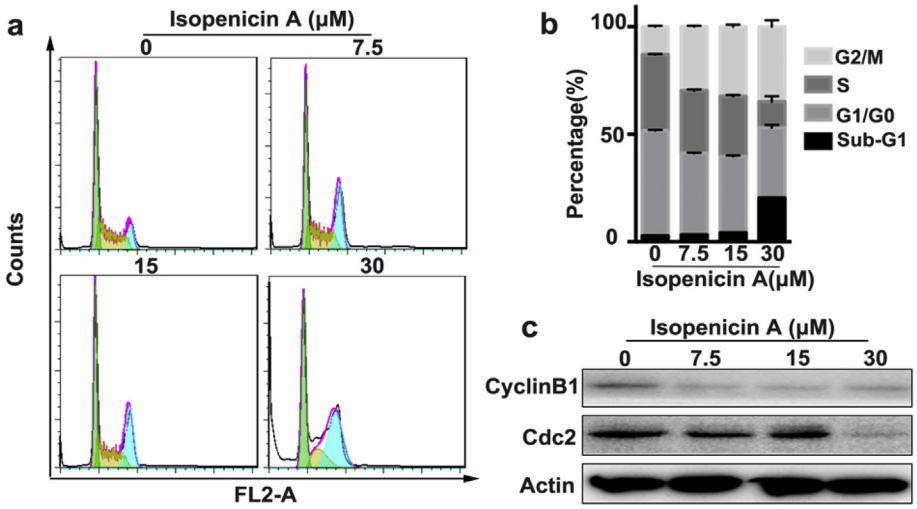

Fig. 3. Isopenicin A induced G2/M phase arrest and regulated the expression of G2/M-related proteins in SW480 cells. (a, b) SW480 cells were treated respectively with 7.5 μM, 15 μM, 30 μM isopenicin A for 24 h. Cells were harvested and subjected to cell cycle analysis. The percentage of cells of different phases of cell cycle was analyzed by FlowJo. (c) Lysates from cells treated with Isopenicin A for 24 h were subjected to western blot analysis with Cdc2 and Cyclin B antibodies.

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results confirmed that isopenicin A effectively induced cell apoptosis in SW480 cells in a dose-dependent manner.

4. Discussion

Microtubules, polymerized by two subunits (α-tubulin and β-tubulin) in a dynamic status, polymerization and depolymerization, are involved in celluar processes of movement, cell shape maintenance, intracellular trafficking and mitosis [20]. As tubulin is among the most established and clinically validated targets and tubulin-targeted chemotherapeutic drugs are widely used in clinical, microtubules have been an attractive target for the development of highly efficient anticancer drugs. Natural microtubule-interactive products are important sources of microtubule binding agents in cancer therapy, the discovery of potent tubulin-targeted inhibitors and structures are the important content of drug research and development.

Isopenicin A is an effective anti-cancer compound with unprecedented terpenoid-polyketide hybrid skeletons that could induce potent G2/M phase arrest of SW620 cells in our previous study [14], a typical characteristic of tubulin inhibitors, indicating the compound may serve as an effective anti-microtubule agent. In the present study, isopenicin A was discovered to be a tubulin-targeted agent that could depolymerize microtubules in a cell-free system and disrupt the in-vivo microtubule networks in cancer cells, as well as the cellular morphology change and proliferation inhibition, suggesting that isopenicin A is a novel tubulin depolymerization agent. Furthermore, the microtubule disruptive effect was further confirmed by the G2/M cell cycle arrest modulated by isopenicin A in cell cycle analysis, a typical characteristic due to the disruption of microtubule dynamics. Besides, since an obvious typical apoptosis sub-G1 was observed in cell cycle analysis, the cell apoptosis was investigated with Annexin V-FITC/PI binding assay. The apoptosis analysis confirmed that isopenicin A effectively induced cell apoptosis in SW480 cells in a dose-dependent manner, with upregulation of proapoptotic modulator cleaved-PARP-1 and downregulation of anti-apoptotic proteins Bcl-xL and XIAP. The proliferation inhibitory activity of isopenicin A on cancer cells was detected, and the growth of SW480, K562 and MDA-MB-231 cells was effectively suppressed by Isopenicin A.

In conclusion, our study identified isopenicin A as a novel natural microtubule depolymerization agent with tumor proliferation inhibitory activity by inducing G2/M cell cycle arrest and cell apoptosis. Moreover, the discovery of isopenicin A provides a new chemotype for discovery and development of promising microtubule inhibitors.

Declaration of competing interest

The authors declare no conflict of interest.

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References

[1] Richard A. Stanton, et al., Drugs that target dynamic microtubules: a new molecular perspective, Med. Res. Rev. 31 (3) (2011) 443–481.
[2] Michael R. Harrison, Kyle D. Holen, Glenn Liu, Beyond taxanes: a review of novel agents that target mitotic tubulin and microtubules, kinases, and kinesins, Clin. Adv. Hematol. Oncol. 7 (1) (2009) 54–64.
[3] Charles Dumontet, Mary Ann Jordan, Microtubule-binding agents: a dynamic field of cancer therapeutics, Nat. Rev. Drug Discov. 9 (10) (2010) 790–803.
[4] Miguel A. Ojedalopez, et al., Transformation of taxol-stabilized microtubules into inverted tubulin tubules triggered by a tubulin conformation switch, Nat. Mater. 13 (2) (2014) 195–203.
[5] A.E. Prota, et al., Structural basis of microtubule stabilization by laulimalide and peloruside A, Angew. Chem. 53 (6) (2014) 1621–1625.
[6] Benoit Gigant, et al., Structural basis for the regulation of tubulin by vinblastine, Nature 435 (7041) (2005) 519–522.
[7] Ravelli, B.G. Raimond, et al., Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain, Nature 428 (6979) (2004) 198–202.
[8] A.E. Prota, et al., A new tubulin-binding site and pharmacophore for microtubule-destabilizing anticancer drugs, Proc. Natl. Acad. Sci. U.S.A. 111 (38) (2014) 13817–13821.
[9] S.K.G. Sharma, Manish, Ajit Kumar Saxena, Bedi Singh, Preet Mohinder, Thiazolidinone constraint combretastatin analogs as novel antitubulin agents: design, synthesis, biological evaluation and docking studies, Anti Canc. Agents Med. Chem. 11 (2017) 230–240.
[10] Antonio Evidente, et al., Fungal metabolites with anticancer activity, Nat. Prod. Rep. 31 (5) (2014) 617–627.
[11] Andrea A. Stierle, Gary A. Strobel, Donald B. Stierle, Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of Pacific yew, Science 260 (5105) (1993) 214–216.
[12] Weichuan Qiao, et al., Enhancing taxol production in a novel endophytic fungus, Aspergillus aculeatinus Tax-6, isolated from Taxus chinensis var. mairei, Fungal Biology 121 (12) (2017) 1037–1044.
[13] J. Tang, L. Kong, P. Puno, , et al.A.–C. Isopenicins, Two types of antitumor meroterpenoids from the plant endophytic fungus Penicillium sp. sh18, Org. Lett. 21 (3) (2019) 771–775.
[14] April L. Blajeski, et al., G1 and G2 cell-cycle arrest following microtubule depolymerization in human breast cancer cells, J. Clin. Invest. 110 (1) (2002) 91–99.
[15] Zhaoqing Wang, et al., Cyclin B1/cdk 1 coordinates mitochondrial respiration for cell-cycle G2/M progression, Dev. Cell 29 (2) (2014) 217–232.
[16] Min Ho Lee, et al., Parkin induces G2/M cell cycle arrest in TNF-α-treated HeLa cells, Biochem. Biophys. Res. Commun. 464 (1) (2015) 63–69.
[17] A. Hamid Boulares, et al., Role of poly (ADP-ribose) polymerase (PARP) cleavage in apoptosis CASPASE 3-RESISTANT PARP MUTANT INCREASES RATES OF APOPTOSIS IN TRANSFECTED CELLS, J. Biol. Chem. 274 (33) (1999) 22932–22940.
[18] Yite Hsu, Keith G. Wolter, Richard J. Youle, Cytosol-to-membrane redistribution of Bax and Bcl-XL during apoptosis, Proc. Natl. Acad. Sci. U.S.A. 94 (8) (1997) 3668–3672.
[19] Martin Holcik, Hilary Gibson, Robert G. Korneluk, XIAP: apoptotic brake and promising therapeutic target, Apoptosis 6 (4) (2001).
[20] S. Sharma, C. Kaur, A. Budhiraja, K. Nepali, M.K. Gupta, A.K. Saxena, P.M. Bedi, Chalcone based azacarboline analogues as novel antitubulin agents: design, synthesis, biological evaluation and molecular modelling studies, Eur. J. Med. Chem. 85 (2014) 648–660.

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