DAT-230, a Novel Microtubule Inhibitor, Induced Aberrant Mitosis and Apoptosis in SGC-7901 Cells

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2-Methoxy-5-(2-(3,4,5-trimethoxyphenyl)thiophen-3-yl)aniline (DAT-230) is a novel synthesized compound of combretastatin-A-4 derivative with more stability. The present study is to investigate its anti-tumor activity and molecular mechanisms in human gastric adenocarcinoma SGC-7901 cells. DAT-230 inhibited SGC-7901 cells growth. The treatment of DAT-230 resulted in microtubule de-polymerization and G2/M phase arrest. Besides the accumulation and translocation of Cyclin B1, reduction of p-14/15-cdc2 and mitosis delay denoted the Cyclin B1-cdc2 complex active and M phase arrest in SGC-7901 cells treated with DAT-230. Mitochondria pathway participated in apoptosis after G2/M arrest in SGC-7901 cells treated with DAT-230, which was characterized by DNA fragmentation, cleavage of poly(ADP-ribose) polymerase (PARP), activation of caspase-3 and caspase-9, changes of Bel-2 and Bax expression, decrease of mitochondrial membrane potential and release of cytochrome c from mitochondria. In vivo, DAT-230 delayed tumor growth in BALB/c nude mice with human gastric adenocarcinoma xenografts. Besides apoptosis was detected with terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay in tumor tissue. In conclusion, DAT-230 is a promising microtubule inhibitor with great anti-tumor activity to SGC-7901, in vitro and in vivo. Its potential to be a candidate of anti-cancer agent is worth of being further investigated.

Key words microtubule inhibitor; cell cycle arrest; apoptosis; SGC-7901; Xenograft

Gastric cancer is the fourth most commonly diagnosed cancer with the second highest mortality rate worldwide.1) The highest incidence rate occur in Eastern Asia, where the rates are 46 per 100000 males and 21 per 100000 females.2) Chemotherapy plays a necessary role in the comprehensive treatment of gastric cancer. However, the use of chemotherapy drugs is limited, due to resistance and serious adverse reactions. Therefore, searching for new alternatives for prevention and treatment of gastric cancer is extremely important.3)

Microtubules are considered as a susceptible target by numerous therapeutic drugs, as they are important elements of cellular cytoskeleton and involved in cellular fundamental functions such as cell movement, organelle localization, intracellular material transportation and cell division regulation.4–6) Microtubules Damaging Agents (MDA) such as vinblastine, colchicines and taxanes have been used in cancer chemotherapy for a long time.7–9) However, they had high toxicity and clinical resistance in clinic for presented as P-glycoprotein (P-gp) substrate.10) In this respect, one of the most interesting compounds is Combretastatin A-4 (CA-4), an alkaido extracted from the South African Willow, Combretum caffrum, which shows anti-mitotic effects by interaction with the colchicine binding site of tubulin and has the peculiarity of having a low toxicity, not acting as a substrate of multidrug resistance pump.11–14) CA-4 is undergoing II phase clinical research, but its use is limited by its low aqueous solubility and low bioavailability, for isomerizing to the thermodynamically more stable but inactive trans-isomer.15) Moreover, CA-4 has dose-limited side effects such as cardiovascular toxicity and tumor pain.16–18) Therefore, more potent and selective active analogs of CA-4 are eagerly demanded. It’s more important that further studies are needed to assess the anti-tumor activity of newly synthesized analogs in vitro and in vivo to complete and better understand the structure–activity relationships of new analogs.19)

Considering that the cis-stilbenic double bonds can easily isomerize under the influence of heat, light and protic solvent, thiophene rings was introduced to replace the double bond to improve stability and activity of CA-4 by our research group. And a series of newly designed compounds of CA-4 analogues were synthesized. Among these, 3-hydroxy group replaced by amino-group named DAT-230 (Fig. 1) stood out by its significant anti-tumor activity against a wide variety of human cancer cell lines including human gastric adenocarcinoma SGC-7901 cells.20) In this study, the mechanisms of DAT-230 were identified from microtubule target, cell-cycle progression and associated regulators to mitochondrial related proteins and apoptotic cascades in SGC-7901 cells.

MATERIALS AND METHODS

Materials DAT-230 was synthesized by our research group (Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang, China). The purification of DAT-230

Fig. 1. Chemical Structure of DAT-230

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was through column chromatography and its purity was 98.77%. Doxorubicin (DOX) was obtained from Pfizer Italia (Nerviano, Italy). RPMI-1640 medium was obtained from Gibco (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS) was obtained from TBD Biotechnology Development (Tianjin, China). Propidium iodide (PI), rhodamine-123 (Rh123), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and acridine orange (AO) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Primary antibodies against Cyclin B1, cdc25c, CDK7, cdc2, p-14/15-cdc2, Bax, Bel-2, poly(ADP-ribose) polymerase (PARP), caspase-9, caspase-3, cytochrome c, α-tubulin, β-actin, horseradish peroxidase (HRP)-conjugated secondary antibodies, and fluorescein isothiocyanate (FITC)/tetramethyl rhodamin isothiocyanate (TRITC)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enhanced chemiluminescence was purchased from Pierce Biotechnology (Rockford, IL, U.S.A.).

**Cell Culture** Human gastric adenocarcinoma cell line SGC-7901 was obtained from Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). Cells were cultured in RPMI-1640 containing 10% FBS, 100 μU/mL streptomycin and 100 μU/mL penicillin at 37°C in humidified atmosphere with 5% CO2 incubator.

**Growth Inhibition Assay** MTT assay was used to measure the cell viability after treatment. Briefly, SGC-7901 cells (7×10^5 cells/well) were seeded in 96-well plate (Corning, NY, U.S.A.) and cultured for 24 h, then treated with various concentrations of DAT-230 for indicated time. Then, MTT solution (5 mg/mL in phosphate buffered saline (PBS)) was added (20 μL/well) and incubated for another 4 h at 37°C. The purple formazan crystals were dissolved in 100 μL dimethyl sulfoxide. Then the plates were read on a plate reader (MK3, Thermo, Germany) at 490 nm. The percentage of cell growth inhibition was calculated as follow: inhibition ratio %=[A_{490 (control)}−A_{490 (DAT-230)}]/A_{490 (control)}×100%.

**Cell Cycle Analysis** SGC-7901 cells (1×10^6 cells) were incubated with various concentrations of DAT-230 or 0.1% dimethyl sulfoxide (DMSO) for indicated time. The cells were harvested by trypsinization, washed with PBS and fixed in ice-cold 70% ethanol. The fixed cells were harvested by centrifugation and re-suspended in 500 μL PBS containing 50 mg/mL RNase. After 30 min incubation at 37°C, the cells were stained with 50 mg/mL PI at 4°C in dark for 30 min. Then the samples were analyzed by FACSscan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

**Confocal Immuno-Fluorescence Microscopic Examination** SGC-7901 cells (1×10^5 cells) were seeded in 24-well plates with cover slips in each well and incubated with DAT-230 (100 nm) or 0.1% DMSO for 24 h or 36 h. After treatment, the cells were fixed with 4% paraformaldehyde for 15 min, washed twice with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min. Then the cells were blocked with 5% bovine serum albumin (BSA) in PBS for 10 min at room temperature. Microtubule or Cyclin B1 were detected with anti-α-tubulin primary antibody or anti-cyclin B1 primary antibody diluted 1:100 in PBS overnight at 4°C and FITC or TRITC conjugated secondary antibody diluted 1:100 in PBS for 1 h at 37°C respectively. After nuclei staining with 4-6-diamidino-2-phenylindole (DAPI) (1 mg/mL), the cells were imaged with a confocal laser microscopic system (TCS-SP2, Leica, German).

**Apoptosis Analysis** Apoptosis was detected by Annexin V-FITC and PI double staining. SGC-7901 cells were treated with various concentrations of DAT-230 or 0.1% DMSO for 60 h, then collected and stained using Annexin V-FITC/PI staining kit (Nanjing KeyGen Biotech, Nanjing, China) according to manufacturer’s instruction. Briefly, cells were harvested, washed with PBS and re-suspended in 500 μL PBS plus Annexin V-FITC and PI. For each sample at least 1×10^4 cells should be analyzed using FACSscan flow cytometry.

**Fluorescence Microscopy Examination** The apoptotic nuclear morpholgy was assessed with the fluorescent DNA-binding dye AO. After treatment with DAT-230 (50, 100, 200 nm) for 72 h, the cells were stained with AO (20 μg/mL) for 15 min, and then the nuclear morphology was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

**Measurement of Mitochondrial Membrane Potential** The mitochondrial membrane potential was measured using fluorescent probe Rh123 as described elsewhere. After incubation with DAT-230 (100 nm) for 0, 48, 60 and 72 h, SGC-7901 cells were collected and suspended in 1 mL PBS containing 1 μg/mL Rh123 and incubated at 37°C for 15 min, then washed with PBS. The fluorescent intensity of the cells was analyzed by FACSscan flow cytometry with excitation and emission wavelengths of 480 and 525 nm, respectively.

**Nuclear Protein Extraction** The method used for the extraction of nuclear proteins is described elsewhere. SGC-7901 cells were harvested and washed in 1 mL of cold PBS after various time incubated with 100 μM DAT-230. The cell pellets were re-suspended in 100–400 μL of cold hypotonic buffer [10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES)/KOH, 2 mM MgCl2, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.9)] on ice for 10 min, then vortexed and centrifuged at 15000×g for 10 min. The pellets of nuclei were gently re-suspended in cold saline buffer [50 mM Hepes/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (w/v) glycerol, 1 mM DTT, 0.5 mM PMSF (pH 7.9)] on ice for 20 min. After centrifugation (15000×g for 10 min at 4°C) the supernatant was stored at −70°C as the nuclear lysate.

**Western-blot Analysis** After treatment, cells were collected and lysed in radio immunoprecipitation assay (RIPA) lysis buffer at 4°C for 40 min. The sub-cellular fractions (mitochondria and cytosol) were obtained using a Mitochondria Isolation Kit (Nanjing KeyGen Biotech, Nanjing, China). After centrifugation at 12000×g for 15 min at 4°C, the protein content of the supernatant was determined using a protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). The protein lysates were separated by electrophoresis on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Millipore Corporation, Billerica, MA, U.S.A.). The membranes were soaked in blocking buffer (5% skimmed milk), probed with primary antibodies and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence.

**Subcutaneous Tumor Model** Female BALB/c nude mice aged 7 weeks (HFK Bio-Technology, Peking, China) were injected subcutaneously in right flank with SGC-7901 cells (2×10^6 cells/0.2 mL of saline). All animals were housed 12
hourly cycles of light and dark in an air-conditioned room, had un-restricted access to water and food in SPF center of Shenyang Pharmaceutical University. Tumor growth was measured every five days using digital caliper in two directions. Tumor volumes were calculated based on the following formula: tumor volume = (length × width²)/2. Once tumors reached about 200 mm³ in average, animals were divided randomly into three groups: Matrix (5% ethanol with 20% tween-20 in saline) group, DOX (4 mg/kg in saline) group and DAT-230 (80 mg/kg in saline with 5% ethanol and 20% tween-20) group. DAT-230 or Matrix were intraperitoneally (i.p.) administered three times weekly from 13th day to 35th day after tumor implantation; DOX was i.p. administered once daily for 4 d as positive control. All experiments were carried out in

Fig. 2. DAT-230 Inhibited Proliferation of SGC-7901
(A) The cells were treated with different concentrations of DAT-230 for the indicated time periods, and the inhibitory ratio was measured. The values shown are mean±S.D. (n=3 of individual experiments). (B) The morphologic alterations of SGC-7901 cells treated with DAT-230 (100 nM) for 12, 24, and 48 h. Scale bar: 100 µm.

Fig. 3. DAT-230 Disrupted Microtubule Network and Induced Aberrant Mitosis
(A, B, C and A', B', C') SGC-7901 cells were incubated for 24 and 36 h; (D, E, F and D', E', F') SGC-7901 cells were treated with DAT-230 (100 nM) for 24 and 36 h. After treatment, the cells were fixed and stained with primary antibody to α-tubulin. Then FITC-labeled secondary antibodies were used (green) and nuclei were counter-stained with DAPI (blue). Cells were detected by a confocal laser microscopic system. Thick arrows indicated cells went through mitosis smoothly; thin arrows indicated abnormal mitosis cells with deformed nuclei; scale bar=50 µm. (Color images were converted into gray scale.)
accordance with the guidelines of Shenyang Pharmaceutical Animal Experimental Ethics Committee.

Apoptosis in Tumor Tissues Was Analysed by Terminal Deoxynucleotidyl Transferase Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay To explore the apoptosis-inducing effect of DAT-230 in tumor tissues, TUNEL assay was performed. Tumors were removed and fixed with 4% paraformaldehyde in PBS. Samples were dehydrated in alcohol and embedded with paraffin. About 5 μm sections were prepared for TUNEL assay. De-waxed sections were washed three times (5 min each) in PBS, and permeabilized in proteinase K for 10 min. Endogenous peroxidase was deactivated by 0.3% hydrogen peroxide. These sections were washed three times again. Then, they were incubated with the mixture of terminal deoxynucleotidyl transferase and biotin-dUTP at 37°C for 1 h, and incubated with HRP-streptavidin solution at 37°C for 1 h. Then these sections were stained with 3,3′-diaminobenzidine (DAB), after hematoxylin post-staining, observed under light microscope.

Statistical Analysis Statistical analysis was performed using SPSS 11.0 soft-ware (Chicago, IL, U.S.A.). All results were expressed as mean±S.D./S.E., an analysis of variance (ANOVA) and Dunnett t test were used to evaluate statistical significance. In all statistical analysis, p values less than 0.05 were considered statistically significant.

RESULTS

DAT-230 Inhibited Proliferation of SGC-7901 Cells DAT-230 inhibited SGC-7901 cell growth with an IC₅₀ (at 72 h) of 98±2.4 nm (Fig. 2A). Cell morphological changes were detected in the groups treated with DAT-230. Compared with the control group, cell morphological changes, such as membrane bleb, rounded up and detached from the substratum, were observed in the groups treated with 100 nm DAT-230 from 12 to 48 h (Fig. 2B).

DAT-230 Disrupted Microtubule Network and Induced Aberrant Mitosis Microtubules are the target of CA-4, which are critical elements of the cellular cytoskeleton and relate to cell division closely. So immuno-fluorescence staining was used to investigate possible alterations of the cellular microtubule network in SGC-7901 cells treated with DAT-230. The microtubule network of SGC-7901 cells exhibited normal arrangement and organization in the control group (Figs. 3B, C). However, after DAT-230 (100 nm) treatment for 24 h, we observed a great loss of microtubules throughout the...
cytoplasm (Figs. 3E, F). As time went by, the SGC-7901 cells without drug exposure went through mitosis smoothly (Fig. 3C′). While, the SGC-7901 cells treated with DAT-230 didn’t, as showed in Figs. 3D′, E′ and F′: microtubules arranged irregularly into spindle-like status shape with predominant aster array and mono-polarity, besides nuclei also deformed.

**DAT-230 Induced G2/M Phase Arrest Preceding Apoptosis**

As demonstrated in Figs. 4A and C, 25–100 nM DAT-230 caused a dramatic increase in G2/M phase cell counts, with the percentages rising from 25.4% ± 2.1% to 70.8% ± 6.0%, in a concentration-dependent manner. On the other hand, treatment of SGC-7901 cells with DAT-230 (100 nM) for 48 h caused a significant increase in G2/M phase cells by 78.8% ± 3.5% compared to the control (25.4% ± 2.1%).

**Fig. 5. DAT-230 Induced Apoptosis in SGC-7901 Cells**

(A) SGC-7901 cells were incubated with DAT-230 (50, 100, 200 nM) for 60 h and apoptosis was analyzed by staining phosphatidylserine translocation with Annexin V-FITC. (B) SGC-7901 cells were treated with DAT-230 (50, 100, 200 nM) for 72 h and analyzed by flow cytometry through PI-stained DNA content. (C) SGC-7901 cells were treated with DAT-230 (50, 100, 200 nM) for 72 h then the morphological changes were examined by AO staining. Arrow indicates nuclear condensation and fragments. The results shown here are representative of three independent experiments. Scale bar: 100 µm.

**Fig. 6. Changes of the Expression of Cell Cycle Related Proteins and Localization of Cyclin B1 in SGC-7901 Cells with DAT-230 Treatment**

(A) DAT-230 regulated the cell cycle related proteins. SGC-7901 cells were treated with DAT-230 (100 nM) for indicated time (6, 12, 24 and 48 h), then were harvested and lysed for detection of Cyclin B1, cdc25c, CDK7, cdc2, p-14/15-cdc2 and the internal control β-actin. The nuclear fraction protein was extracted as described in Materials and Methods. (B, C, D and E) DAT-230 induced Cyclin B1 translocation into nuclei. SGC-7901 cells were incubated with vehicle (0.1% DMSO, control) or DAT-230 (100 nM) for 48 h. The cells were fixed, permeabilized, incubated with primary antibodies to Cyclin B1 and stained with TRITC-labeled secondary antibodies. Nuclei were counter stained with DAPI. Then cells were detected by a confocal laser microscopic system. B and C represented the fluorescence pictures of Cyclin B1 (red); D and E showed the merged pictures of Cyclin B1 and nuclei (blue). Scale bar: 50 µm. (Color images were converted into gray scale.)
48 h induced a dramatic increase in the counts of cells in G2/M phase, with the percentages rising from 35.2% ± 2.6% to 70.8% ± 6.0% (Figs. 4B, D), in a time-dependent manner. Data in Fig. 4 demonstrated that DAT-230 induced SGC-7901 cells G2/M arrest in both concentration and time dependent manners.

Previous studies have established that microtubule-inhibiting agents caused G2/M arrest and then triggered apoptosis. Thus, Annexin V-PI double staining method was employed to detect the apoptosis following cell cycle arrest. Figure 5A showed that the percentage of Annexin V-positive cells increased from 18.4% ± 1.1% to 48.6% ± 4.3% in SGC-7901 cells treated with DAT-230 (50–100 nM). Figure 5B also revealed that apoptosis occurred in SGC-7901 cells after 72 h exposure to DAT-230 (50, 100, 200 nM). The percentages of SubG1 cells increased from 24.7% ± 2.3% to 40.2% ± 4.1% using PI staining method. Further, apoptosis was confirmed by examining the nuclear morphology with AO staining. As shown in Fig. 5C, the nuclei in control group were stained homogeneously with AO, whereas exposure to DAT-230 resulted in marked chromatin condensation and nuclear fragmentation in SGC-7901 cells, a hallmark of apoptosis. Therefore, DAT-230 caused G2/M cell-cycle arrest and subsequent apoptosis in SGC-7901 cells.

**DAT-230 Changed the Expression and Localization of the Cell Cycle Regulatory Proteins**

To better understand the mechanisms of DAT-230-induced G2/M arrest, we examined G2/M phase related regulatory proteins. As shown in Fig. 6A, DAT-230 up-regulated the expression of Cyclin B1 and down-regulated cdc2 phosphorylated form at Thr14/Tyr15 residues, while the expression of cdc2 was sustained, from 12 to 48 h. On this condition, cdc25c was down-regulated. However, the expression of CDK7, a CDK-activating kinase, was nearly no change (Fig. 6A). Additionally, the translocation of Cyclin B1 into the nuclei promotes the natural progression of cell cycle before the nuclear envelope breakdown during prophase.24) Immuno-fluorescence staining of Cyclin B1 showed that the fluorescence intensity was increased in SGC-7901 cells treated with DAT-230 (100 nM) for 48 h compared to control group, it indicated the expression of Cyclin B1 was up-regulated (Figs. 6B, C); besides the red fluorescence labeled Cyclin B1 was overlapped with the blue fluorescence indicated nuclei, it suggested that DAT-230 notably triggered the translocation of Cyclin B1 from cytoplasm into nuclei in SGC-7901 cells (Figs. 6C, D). Furthermore the expression of Cyclin B1 was up-regulated in nuclei fraction (Fig. 6A).

**DAT-230 Induced Apoptosis through Caspase Activation**

Firstly, the effects of DAT-230 on PARP (a major substrate of caspases), procaspase-3 (an effector caspase) and procaspase-9 (an initiator caspase) were investigated. Figure 7 showed that DAT-230, at concentrations of 100 and 200 nM, induced activation of caspase-9 and caspase-3, which were accompanied with evident cleavage of PARP, denoting caspases involvement in DAT-230-triggered apoptosis. Recently, several lines of evidence suggest that MDAs are able to induce cdc2 activation and that triggers mitochondrial membrane permeable by targeting on Bcl-2 family proteins.25–27) As shown in Fig. 7, Bcl-2 was down-regulated and Bax was up-regulated after DAT-230 treatment in SGC-7901 cells.

**DAT-230 Induced Disruption of Mitochondria and**
Cytochrome c Release Mitochondrial membrane potential (MMP) regulates mitochondrial permeability, which plays an important role in triggering apoptosis. As shown in Figs. 8A and B, the retention of Rh123 decreased significantly after treatment with DAT-230 (100 nm) for 60h and 72h compared with control, indicating that DAT-230 induced mitochondria disruption. Depolarization of MMP is usually associated with cytochrome c release from mitochondria to cytosol. Therefore the level of cytochrome c was detected both in mitochondrial and cytosol. As shown in Fig. 8C, the level of cytochrome c decreased in mitochondrial but increased in cytosol.

DAT-230 Delayed Tumor Growth in Vivo As shown in Fig. 9A, tumors in the Matrix group grew remarkably fast, reaching 847.78 ± 192.33 mm³ in volume on 25th day after implantation. In contrast, the tumors of mice treated with 80 mg/kg of DAT-230 were significantly smaller than control group (p < 0.05), reaching only 465.88 ± 63.24 mm³ in volume. Moreover, DAT-230 treatment was well tolerated, as body weight didn’t significantly change during the treatment period (Fig. 9B), which indicated toxicity of DAT-230 was lower compared to DoX.

Histological Analysis by TUNEL Assay The tumors were removed and TUNEL assay was performed after DAT-230 treatment. Apoptosis was detected in tumor tissue of both DAT-230 and DOX group (Fig. 9C).

DISCUSSION

CA-4, a naturally stilbene, derived from South African tree Combretum caffrum, exhibits potent cytotoxicity against a broad spectrum of human cancer cell lines. The structural simplicity of CA-4 made it an attractive lead compound in development of new anti-tumor agents. In the present study, DAT-230, a novel synthetic CA-4 derivative, inhibited SGC-7901 cells growth associated with damaging microtubule.

The parental compound CA-4 has been reported as a microtubule-targeting agent. One of the key characteristics of anti-microtubule agents is induction of mitotic arrest. The mode of action of DAT-230 as a MDA was investigated using in situ immuno-fluorescence analysis. DAT-230 disrupted the microtubule network normal arrangement and organization through decreasing the contents of microtubule in cytoplasm (Figs. 3B, E), which indicated DAT-230 acted as a microtubule inhibitor. Besides DAT-230 delayed mitosis with characteristics that microtubules were arranged irregularly into a spindle-like status with star-shaped microtubules and monopolarity (Figs. 3E′, F′), while the SGC-7901 cells in the control group went to next cell cycle smoothly (Figs. 3B′, C′).

On the other hand, we found that DAT-230 induced G2/M phase arrest (Fig. 4). The complex of cdc2 (Cdk1)/Cyclin B1 has been well known as the regulator governing the G2 to M progression. At the end of G2 phase, cdc2/Cyclin B1 complex is activated via de-phosphorylation at Thr14 and Tyr15 by cdc25c and translocates into nuclei, where it induces mitosis. Interestingly, we observed DAT-230 up-regulated the expression of Cyclin B1 and down-regulated cdc2 phosphorylated form at Thr14/Tyr 15 residues, while the expression of cdc2 was sustained (Fig. 6A). That indicated DAT-230-treatment induced activation of cdc2/Cyclin B1 complex. Thus, we speculated that DAT-230-treated cells were able to exit from G2 phase, and then entered into M phase, and were arrested at M phase. We all know that the expression of Cyclin B1 rises from prophase to metaphase and subsequently it is degraded during anaphase. Previous reports showed that accumulation of cyclinB1 in nuclei commonly indicated the mitotic entry. In Fig. 6, Cyclin B1 continuously increased from 12 to 48h and entered into nuclei at 48h, supporting the speculation that DAT-230 induced M phase arrest in SGC-7901 cells.
It has been suggested that mitotic arrest played a central role in apoptotic cell death induced by anti-microtubule agents. Moreover, cdc2 activation may be involved in some apoptotic signaling pathways. In this study, we found that DAT-230 induced mitotic arrest preceding apoptosis (Figs. 4, 5). Therefore, several cell-cycle regulators, in particular cdc2, were examined. Our data demonstrated that DAT-230 induced activation of cdc2 based on several observations, (i) the elevation of Cyclin B1 expression and (ii) its nuclei translocation, (iii) the de-phosphorylation of cdc2 on inhibitory Thr-14/Tyr-15 by cdc25c from 12 to 48h (Fig. 6). Furthermore, it is evident that the activity of cdc2 must be sustained from prophase to metaphase. Subsequently, the entry into anaphase is dependent on a rapid decline of the cdc2 activity. The sustained activation of cdc2 by DAT-230 may explain why the cells couldn’t go through mitosis smoothly, but went to apoptosis.

It is well accepted that microtubule inhibitors can induce apoptosis caused by cell cycle arrest. Here, our data also revealed that besides its induction of cell cycle arrest, DAT-230 promoted apoptosis (Fig. 5). The downstream apoptosis pathways following the cell-cycle arrest caused by a variety of pharmacological agents have been widely explored. The mitochondria-mediated signaling pathway is the most identified mechanism. Two major events have been noted in the apoptosis involving mitochondrial dysfunction. One event is the change in membrane permeability and subsequent loss of membrane potential. The other is the release of apoptotic proteins including cytochrome c from inter-membrane space of mitochondria into cytosol. Here, mitochondria membrane potential decreased, accompanying with the release of cytochrome c from mitochondria (Fig. 8). The Bcl-2 family proteins are critical regulators of mitochondria-mediated apoptosis by functioning as either promoters or inhibitors. Among these, Bcl-2 can stabilize the mitochondria permeability; in contrast, Bax increases the membrane permeability through interactions with pore proteins on the mitochondrial membrane, which leads to the release of cytochrome c from mitochondria, activation of caspase-9 and initiation of caspase activation for apoptosis. Here, we observed that DAT-230 induced down-regulation of Bcl-2 and up-regulation of Bax, activation of caspase-9 and caspase-3 (Fig. 7). It suggested that DAT-230 induced apoptosis through mitochondria pathway was due to, at least in part, the disruption of a balance between Bax and Bcl-2. It has been reported that Bcl-2 and/or Bax can be regulated by many upstream regulatory molecules, for example, abnormal cdc2 activation can make Bcl-2 lose its anti-apoptotic function through phosphorylation. The correlation between the change of Bcl-2 and Bax expression and upstream pathways needs further investigation.

In conclusion, the present study demonstrated DAT-230, a novel synthetic CA-4 analog, was a potent compound against human gastric adenocarcinoma both in vitro and in vivo. DAT-230 induced aberrant mitosis through disrupting intracellular microtubule and affecting key G2/M-phase regulatory proteins, and then caused apoptosis involving mitochondrial pathway. This study also provides a prototype structure that enables investigation of tubulin-targeting strategy in gastric cancer chemotherapy. To confirm the potency of DAT-230, further experiments concerning the anti-MDR property of DAT-230 are under way.

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