MCL-1 Degradation Mediated by JNK Activation via MEKK1/TAK1-MKK4

Contributes to Anticancer Activity of New Tubulin Inhibitor MT189

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Running title: The JNK-MCL-1 axis contributes to the anticancer activity of MT189.

Key words: MT189; tubulin inhibitor; JNK; MCL-1; MEKK1/TAK1

Abbreviations list: MCL-1, myeloid cell leukemia sequence 1; JNK, c-Jun N-terminal kinase; MEKK1, mitogen-activated protein kinase kinase kinase 1; MKK4, mitogen-activated protein kinase kinase 4; MKK7, mitogen-activated protein kinase kinase 7; MDR, multidrug resistance; HIF-1α, hypoxia inducible factor 1α; BCL-2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra large; BAX, bcl-2-associated X protein; BAK, bcl-2 homologous antagonist killer; CA4, combretastatin A4; ADR, adriamycin; VCR, vincristine; SRB, sulforhodamine B; IC₅₀, 50%-proliferation-inhibition concentration; CCK-8, Cell count kit 8; PBS,
phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RTV, relative tumor volume; MMP, mitochondrial membrane potential; PARP, poly (ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; CDK1, cyclin-dependent kinase 1.

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Conflicts of interest

The authors declare no conflicts of interest related to this work.

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Abstract

Colchicine-site-targeted tubulin inhibitors are a promising type of anticancer drugs. MT189 is a new derivative of MT119, a previously reported colchicine-site-binding antitubulin agent. In this study, MT189 was demonstrated to retain the property of MT119 in disrupting microtubulin via binding to the colchicine site, causing mitotic arrest and inducing apoptosis, and to display 8.7-fold enhanced proliferative inhibition in a panel of cancer cells. MT189 was shown to elicit in vivo anticancer effects on MDA-MB-231 xenografts in nude mice, and the tumor growth was suppressed by 35.9% over 14 days. MT189 led to degradation of MCL-1, a member of the antiapoptotic BCL-2 protein family. Its overexpression reduced but its silenced expression increased the apoptotic induction followed by the treatment with MT189. Moreover, the treatment with MT189 caused activation of the MEKK1/TAK1-MKK4-JNK signaling pathway. The activated JNK resulted in phosphorylation of MCL-1, which facilitated its ubiquittination-mediated degradation. Our results show that MT189 inhibits microtubulin polymerization by binding to the colchicine site. Relief of apoptotic suppression by MCL-1 degradation together with mitotic arrest contributes to the anticancer activity of MT189. (177 words)
Introduction

In recent years, colchicine-binding-site-targeted tubulin inhibitors have become a promising type of anticancer drugs with several of them entering into clinical trials (1). We previously constructed a combinatorial library of 6H-Pyrido[2’,1’:2,3]imidazo[4,5-c]isoquinolin-5(6H)-ones (2) from which two new tubulin inhibitors, MT7 and its structurally-modified antiproliferation-activity-improved derivative MT119, were obtained. Both cause microtubulin depolymerization, persistent M phase arrest and apoptosis. MT119 was confirmed to bind to the colchicine-site on tubulin (3-5). Although both produce potent in vitro antiproliferative effects and overcome tumor multidrug resistance (MDR), they did not show obvious in vivo anticancer activity. In considering their structural novelty and potential new modes of action, we put persistent efforts into further structural optimization on MT119. Finally, we acquired a new colchicine-site binder 2-(6-fluoro-3-((4-methoxybenzyl)amino)imidazo[1,2-α] pyridin-2-yl) phenol (designated as MT189) (Suppl. Fig. 1).

Tubulin inhibitors, including those targeting the colchicine site, can disrupt cellular microtubulin assembly, followed by persistent M arrest and subsequent apoptosis. Both M arrest and apoptosis contribute to their anticancer effects (3, 4, 6). Besides, other mechanisms of action and/or signaling pathways might be involved. We previously found that the naturally occurring colchicine-site binder pseudolaric acid B (7) could activate the JNK-c-Jun-hypoxia inducible factor 1α (HIF-1α) signaling axis to inhibit angiogenesis and overcome tumor MDR (8, 9). JNK can be activated by its upstream MAP kinase kinase kinases (MAP3Ks; at least 14 kinases,
including MEKK1 and TAK1) through MAP kinase kinases (MAP2Ks; MKK4 and MKK7). Both MKK4 and MKK7 phosphorylate JNK but MKK4 also activates p38 (10). However, whether the new colchicine-site binder MT189 could also activate JNK and if so, which upstream kinases are involved, remain to be clarified. In addition, the relationship between microtubulin disruption, mitotic arrest and JNK activation triggered by MT189 needs investigating.

Besides c-Jun, JNK has many other substrates, one of which is the antiapoptotic protein MCL-1 (11). MCL-1 is a member of the BCL-2 protein family. It promotes survival via inhibiting apoptosis by suppressing proapoptotic proteins BAX and BAK (11, 12). Antitubulin agents paclitaxel and vincristine that bind to the taxane site and the vinca site on tubulin, respectively, have been shown to cause phosphorylation of MCL-1 via JNK, which subsequently leads to ubiquitination-mediated MCL-1 degradation (11). So it is intriguing whether the colchicine-site binder MT189 also affects MCL-1 via JNK and if so, what effects will be caused on its anticancer activity.

In this study, we first examined the effects of MT189 on microtubulin dynamics, cell cycle and apoptosis to determine whether it retains the characteristics of its parental compound MT119. Next, its in vitro and in vivo activities against tumor cells were measured. After that, we further tested the impacts of MT189 on MCL-1 and the JNK signaling pathway. Finally, whether MT189-induced microtubule disruption is directly related to its activation of the JNK signaling axis was explored.

**Materials and Methods**
**Drugs, chemicals and reagents**

MT119 was prepared as reported previously (4). MT189 was obtained through a de-construction approach to break the tetracyclic ring of MT-119 (Suppl. Fig.1). Their purity ($\geq 99\%$) was determined by RP-HPLC at two wavelengths of 214 nm and 254 nm. Paclitaxel, combretastatin A4 (CA4), adriamycin (ADR), SP600125 and PD169316 were purchased from Sigma-Aldrich (St. Louis, MO). RO3306 was obtained from Tocris Bioscience (Bristol, UK). JNK-IN-8 was purchased from Merck Millipore (Billerica, MA). Z-VAD-FMK was obtained from Beyotime (Shanghai, China). Colchicine was obtained from Sangon (Shanghai, China). Vincristine (VCR) was obtained from J&K (Shanghai, China). BODIPY FL-vinblastine was purchased from Invitrogen (Carlsbad, CA). All the antibodies were commercially available, including those respectively against histone H3, p-histone H3, JNK, MCL-1, MEKK1 and p-MEKK1 from Santa Cruz Biotechnology (Santa Cruz, CA); p38, p-p38, p-MCL-1, MKK4, p-MKK4, MKK7, p-MKK7, TAK1 and p-TAK1 from Cell Signaling Technology (Danvers, MA); p-JNK from Abcam (Cambridge, UK); $\beta$-Actin from Abgent (San Diego, CA); MPM-2 from Merck Millipore (Billerica, MA); and $\alpha$-Tubulin from Invitrogen (Carlsbad, CA).

**Cell culture**

BEL-7402 (2002), SPC-A4 (2003), SMMC-7721 (2006) and MDA-MB-231 (2009) cell lines were kept in the Shanghai institute of Materia Medica of the Chinese Academy of Sciences (Shanghai, China). MCF-7 (2002), MKN-45 (2003) and SKOV-3 (2007) were obtained from the Japanese Foundation of Cancer Research (Tokyo, Japan). The adriamycin-selected resistant MCF7/ADR cell line was purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China) in 2003 (4, 13, 14). The vincristine-selected resistant KB/VCR cell line was purchased from the Sun Yat-Sen University of Medical Sciences (Guangzhou, China) in 2003 (4, 13, 14). All the other cell lines except WI-38 (obtained in Dec., 2013) were authenticated by short tandem repeat (STR) profiling in Beijing Microread Gene Technology Co, Ltd. (Beijing, China; HeLa and DU145, Jul., 2012) or in Shanghai Genesky Bio-Tech CO., LTD.(Shanghai, China; MDA-MB-231, MCF-7 and MCF7/ADR, Mar., 2013; HT-29, KB, MDA-MB-468 and KB/VCR, May, 2013; A549 and SGC-7901, Jun., 2013; HCT116, SPC-A4, SMMC-7721 and BEL-7402, Jul., 2013; PC-3 and SKOV-3, Aug., 2013; and MKN-45, Nov., 2013). The cells were also periodically authenticated by morphologic inspection and tested for Mycoplasma contamination. The cell lines were cultured according to the suppliers’ instructions.

**Proliferation inhibition assays**

Sulforhodamine B (SRB) assays were done as reported previously (4) to measure the 50%-proliferation-inhibition concentration (IC₅₀) values of different agents in the cells exposed to their gradient concentrations for 72 h. Cell counting kit-8 (CCK-8)
assays were performed to determine the viability of the cells treated with MT189 for shorter than 72 h. For this purpose, cells were seeded into 96-well plates, cultured overnight and treated with MT189 for the indicated time. Then, 10-µl CCK-8 solution (Dojindo Laboratories, Japan) was added into 100-µl culture medium each well according to the manufacturer’s instructions, and the cells were incubated for 4 h at 37°C. The absorbance at 450 nm was measured with spectra-MAX190 (Molecular Devices, Sunnyvale, CA). The percentage of cell proliferation inhibition was calculated as: proliferation inhibition (%) = [1-(A450treated/A450control)] × 100%. The averaged IC₅₀ values were determined with the Logit method from three independent tests.

**Western blotting**

Western blotting was performed as previously described (8).

**Immunofluorescence-based laser confocal microscopy**

Cells were seeded on glass coverslips, cultured for 24 h and treated with appropriate agents for the indicated time. Then, the cells were fixed for 15 min with 4% paraformaldehyde, permeabilized for 5 min with 0.2% TritonX-100, blocked with 3% bovine serum albumin for 15 min, incubated with the primary antibody for 1 h, and stained with fluorescence-conjugated secondary antibody for 1 h. Finally, after being counterstained with DAPI, the cells were imaged under an Olympus confocal microscope (Olympus, Tokyo, Japan).

**In vitro tubulin polymerization assays**

Tubulin was purchased from Cytoskeleton (Denver, CO). In *vitro* tubulin
polymerization was assessed by the turbidity assay as previously described (4).

**Cellular microtubulin polymerization assays**

The cells were treated with the given agent and harvested in the lysis buffer (100 mM PIPES pH6.9, 1 mM EGTA, 1 mM MgCl₂, 30% glycerol, 5% DMSO, 1% NP-40, 5 mM GTP and protease inhibitors). The polymerized tubulin fraction (precipitate) and the soluble tubulin fraction (supernatant) were separated by centrifugation at 180,000 g at 37°C for 1 h. Finally, α-tubulin from equal aliquots of the polymerized tubulin fraction and the soluble tubulin fraction was determined by Western blotting (4).

**Tubulin-site competitive binding assays**

Tubulin (3 μM) was incubated with the tested agents at 37°C for 1 h. Then colchicine or BODIPY FL-vinblastine was added to the final concentration of 3 μM. The fluorescence was determined after 30 min at 37°C with a PerkinElmer fluorometer (4, 15).

**Cell cycle assays**

Cells were seeded into 6-well plates, cultured overnight and treated with MT189 for the indicated time. In order to examine the reversibility of cell cycle arrest, the cells treated with 0.2 μM MT189 for 12 h were washed with medium 3 times and then incubated in drug-free full medium for the indicated time. Cells were then harvested and washed with PBS, fixed with pre-cooled 70% ethanol at 4°C. Staining went along in PBS containing 40 μg/ml RNase A and 10 μg/ml propidium iodide in the dark for 30 min. For each sample, at least 1×10⁴ cells were collected with FACS Calibur (BD
Biosciences, Franklin Lakes, NJ) and analyzed using the CELLQUEST software (BD Biosciences, Franklin Lakes, NJ).

**TUNEL assays**

Cells were seeded into 6-well plates, cultured overnight and treated with MT189. Then, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100 and washed with phosphate-buffered saline (PBS). TUNEL assays were performed with an *in situ* cell death detection kit (Roche Applied Science, PA) according to the manufacturer’s instructions.

**Annexin V-FITC apoptosis detection**

Cells were seeded into 6-well plates, cultured overnight and treated with different agents. Then, cells were harvested, washed and stained by using the AnnexinV-FITC apoptosis detection kit (Beyotime, Shanghai, China). Fluorescence of the cells was determined immediately by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

**Mitochondrial membrane potential detection**

Cells were seeded into 6-well plates, cultured overnight and treated with MT189. Then, cells were harvested, washed and stained by using JC-1 kit (Beyotime, Shanghai, China). Fluorescence of the cells was determined immediately by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

**Plasmid transfection**

The plasmid expressing wild-type MCL-1 (#25392) was obtained from Addgene, Cambridge, MA. The transfection was conducted as reported previously (8).
RNA interference

MCL-1 expression was knocked down with specific small interfering RNA (siRNA) duplexes from RiboBio (Cat. No. Q000004170-1-A). The transfection of siRNAs was carried out with the RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Quantitative real-time PCR

Total RNA prepared with the TRIzol reagent (Invitrogen, Carlsbad, CA) was reverse-transcribed into cDNA with the PrimeScript RT reagent kit (TaKaRa, Dalian, China). cDNA was amplified with the SYBR Premix EX TaqII Kit (TaKaRa, Dalian, China) in a 7500 fast real-time PCR system (Applied Biosystems, Carlsbad, CA). The PCR program was as follows: 95°C, 30 s; 40 cycles (for each cycle 95°C, 5 s; 64°C, 20 s; 72°C, 15 s); 72°C, 10 min. All primers were synthesized by Sangon (Shanghai, China) as follows: (5'-3') GGGCAGGATTGTGACTCTCATT (forward) and GATGCAGCTTTCTTGGTTTATGG (reverse) for mcl-1; TCTACAATGAGCTGCGTGTG (forward) and GGTGAGGATCTTCATGAGGT (reverse) for β-actin.

Immunoprecipitation assays

Cells treated with MT189 for 24 h were lysed with the NP40 lysis buffer (Beyotime, Shanghai, China). Then, followed by centrifugation at 12000 g at 4°C for 5 min, the supernatant was precleared with 20 μl protein A/G agarose beads at 4°C for 2 h. After that, centrifugation was done at 1000 g at 4°C for 3 min and the resulting supernatant was exposed to the indicated antibodies for 4 h followed by the addition of 20 μl protein A/G agarose for overnight at 4°C. When harvested, the beads were
washed thrice with 1 ml NP40 lysis buffer or PBS for 10 min each. The precipitates were dissolved with the SDS loading buffer for Western blotting.

**In vivo anticancer activity**

A subcutaneous MDA-MB-231 xenograft model in nude mice was used to evaluate the anticancer activity of the tested compounds. The model was established by the transplantation of 2×10^6 MDA-MB-231 cells subcutaneously on the back of each 6-week-old female Balb/c nude mouse. When the average tumor volume reached around 150 mm³, 24 nude mice were selected based on the tumor volume and randomly assigned into 3 treatment groups (8 per group). For 2 weeks, these animals were administered intraperitoneally with MT189 (100 mg/kg/d) or with vehicle. During the treatment period, the implanted tumors were measured by caliper twice a week in a blind fashion. The tumors were measured for the maximum width (X) and length (Y) and the tumor volumes (V) were calculated using the formula: $V = \frac{X^2Y}{2}$. Then relative tumor volume (RTV) was calculated as follows: $RTV = \frac{V_t}{V_0}$. $V_0$ was the tumor volume before treatments, and $V_t$ was the tumor volume after treatments. The *in vivo* anticancer activity was judged by their T/C (%) values. T/C (%) was calculated as $(T_{RTV} / C_{RTV}) \times 100\%$, where $T_{RTV}$ represented the RTV of the treatment group and $C_{RTV}$ represented the RTV of the vehicle group. The animal body weights were also measured at the same time. The animal use and experimental protocol were reviewed and approved by the Animal Care and Use Committee of the Medicilon Company, Shanghai, China.

**Results**
MT189 inhibits microtubule polymerization by binding to the colchicine site, which leads to mitotic arrest and spindle assembly disruption.

We previously reported that MT7 (3) and its efficacy-improved derivative MT119 (4) were new colchicine-site-binding tubulin inhibitors. Both are easy to be synthesized and MT119 is also active even in multidrug resistant tumor cells. Disappointingly, however, both of them lack in vivo anticancer activities, possibly due to their relative weak bioactivity. So based on MT119, continuous structural optimization has been carried out. Among other efforts, one de-construction approach was taken to break the tetracyclic ring of MT119 to a new intra-molecular hydrogen bonding scaffold (iMHB scaffold). A phenolic group was introduced to the alpha position of phenyl ring to form a pseudo-ring through the intra-molecular hydrogen bonding between the imidazole nitrogen and the phenolic hydrogen atom to lock the planar conformation and to mimic the original tetracyclic ring space in MT119 (Suppl. Fig.1). This strategy produced a new series of compounds, in which a compound designated as MT189 (Suppl. Fig.1) showed an averaged 8.7-fold enhancement in the in vitro proliferation inhibition against a panel of 6 different tissue-derived cancer cells when compared with its parental compound MT119 (Suppl. Fig.2).

To characterize MT189, we tested whether it retained the property of MT119 in impairing microtubule assembly and arresting cell cycle. The classical microtubule stabilizer paclitaxel promoted microtubulin polymerization as expected. In contrast, MT189 inhibited tubulin polymerization in a concentration-dependent manner, similar to the microtubule destabilizer colchicine (Fig.1A). In addition, MT189 competitively
reduced the binding of colchicine to tubulin as the positive control CA4 (a known colchicine-site binder) did. However, MT189 did not affect the fluorescent intensity of BODIPY FL-vinblastine-tubulin complexes that was apparently lowered by VCR (Fig.1B). The data indicate that MT189 binds to tubulin at the colchicine site rather than the vinblastine site.

In interphase cells, microtubules radiate from the microtubule-organizing center located at the centrosome in the cytoplasm, involved in the maintenance of cell shape (16). The treatment of SMMC-7721 cells with MT189 resulted in scattered tubulin staining and long microtubule fibers could rarely be observed in the cell (Fig.1C). Moreover, MT189-triggered microtubule depolymerization occurred very early (within 0.5 h) and then persistently progressed (Suppl. Fig.3A). In addition, MT189 caused an increase in the free microtubulin fraction (supernatant) and simultaneously a decrease in the polymerized microtubulin fraction (precipitate) in the cell although the levels of total tubulin kept unchanged (Fig.1D). The data indicate that MT189 can disrupt the microtubule assembly by inhibiting tubulin polymerization in the cell.

MT189 was further shown to lead to a typical, reversible G2/M arrest in SMMC-7721 cells in a time- and concentration-dependent manner (Fig.1E and Suppl. Fig.3B). The enhanced levels of the two mitotic markers MPM-2 and Ser10-phosphorylated histone 3 (17) (Fig.1F) in MT189-treated SMMC-7721 cells indicated that MT189 caused cell cycle arrest in M rather than G2 phase. During mitosis, cellular microtubules are normally assembled into bipolar mitotic spindles to guarantee equal segregation of the chromosomes (18, 19) (Fig.1G). However, MT189
resulted in multipolar spindle damage and scattered chromosome misalignments in SMMC-7721 cells (Fig.1G). The data indicate that MT189 interferes with mitosis progression by disrupting mitotic spindle assembly.

All the above results show that MT189 retains the property of its parental compound MT119 in impairing microtubule assembly (4).

**MT189 induces intrinsic apoptosis and exerts enhanced *in vitro* and *in vivo* activities against tumor cells.**

Persistent mitotic arrest caused by microtubule depolymerization has been demonstrated to trigger apoptosis (20, 21). So we investigated whether and how MT189 induced apoptosis. The treatment with MT189 led to a progressive increase in apoptosis in a time-dependent manner in SMMC-7721 cells (Fig.2A). MT189 also caused apparent loss of mitochondrial membrane potential (MMP) in these cells in a concentration-dependent manner (Figs. 2B and 2C). Apoptosis can roughly be classified into two types: caspase-8-mediated extrinsic apoptosis and caspase-9-initiated intrinsic apoptosis (22, 23). MT189 caused the cleavage of PARP, caspase-3 and caspase-9 rather than caspase-8 in a time- and concentration-dependent manner (Fig.2D), indicating that it mediates mitochondria-involved caspase-9-initiated intrinsic apoptosis.

MT189 displayed potent proliferation inhibition in 14 cancer cell lines derived from different tissues of origin with an averaged IC$_{50}$ value of 0.1 μM ranging from 0.01 μM (HeLa) to 0.3 μM (DU145) (Fig.2E) in contrast to its relatively weak activity in the normal WI-38 cell line (Proliferation was inhibited by 35.09%±7.30%
at 10 μM). The data indicate that MT189 possesses a broad in vitro antiproliferative spectrum and potential selectivity over normal cells. Moreover, the in vitro antiproliferative activity of MT189 was time- and concentration-dependent as shown in HeLa and SMMC-7721 cells (Suppl. Fig.4). As expected, MT189 could also overcome multidrug resistance (Table 1), indicating that MT189, in this regard, also retains the excellent feature of its parental compound MT119 (4). With its enhanced in vitro antiproliferative activity as compared to MT119, MT189 elicited in vivo anticancer effects by inhibiting the growth of MDA-MB-231 xenografts in nude mice by 35.9% over 14 days but did not obviously affect the body weight of the tested animals during the same period (Fig.2F).

Notably, MT189 caused M arrest much earlier than apoptosis. Treatments with 0.2 μM MT189 took only 3 h to arrest cell cycle in M phase (Figs.1E and 1F), but about 36 h to result in apoptosis (Figs.2A and 2D). The results suggest that apoptosis might result from persistent mitotic arrest in these cells. Apparently, growth inhibition in mitosis followed by apoptosis elicits the anticancer effect of MT189.

**Degradation of the antiapoptotic protein MCL-1 via the ubiquitin-proteosome pathway potentiates the apoptotic induction of MT189.**

The BCL-2 family proteins are key regulators of apoptosis by regulating mitochondrial membrane permeabilization. This family consists of two types of members: antiapoptotic ones such as BCL-2, BCL-XL and MCL-1 and proapoptotic ones such as BAK and BAX. The change in the dynamic balance of those members may result in either inhibition or promotion of apoptosis (24). MT189 was revealed to
reduce the levels of MCL-1 protein in a time-dependent manner, but did not obviously change the protein levels of the other tested members (Fig.3A). Moreover, the caspase inhibitor Z-VAD-FMK did not prevent MCL-1 reduction but did prevent PARP cleavage induced by MT189 (Suppl. Fig.5A). The results suggest that MCL-1 might be associated with MT189-induced apoptosis.

To clarify whether MT189-induced MCL-1 reduction contributes to its in vitro proliferation-inhibitory activity, we compared its effect on the levels of MCL-1 protein in 3 relative sensitive cells (SMMC-7721, HT-29 and HeLa) with those in 2 relative insensitive cells (DU145 and SKOV-3). MT189 was 11.8 times more potent in SMMC-7721, HT-29 and HeLa cells (with an averaged IC₅₀ of 0.025 μM) than in DU145 and SKOV-3 (with an averaged IC₅₀ of 0.295 μM) (Fig.3B). The treatment with MT189 for 24 h led to reduction of MCL-1 protein levels in a concentration-dependent manner in all three relative sensitive cell lines, but did not obviously changed the levels of MCL-1 protein in the insensitive cell lines (Fig.3B). Nevertheless, MCL-1 protein levels in DU145 cells also decreased in a concentration-dependent manner when the treatment time with MT189 was prolonged to 48 h (Suppl. Fig.5B). However, MT189 induced apparently less apoptosis in DU145 cells than in HeLa cells at the same conditions (Suppl. Fig.5C). Moreover, the ectopic overexpression of MCL-1 attenuated (but did not abolish) the cleavage of caspase-9, caspase-3 and PARP (Fig.3C) and the loss of viable cells (Fig.3D) caused by MT189 in the pWT-MCL-1-transfected SMMC-7721 cells. In contrast, downregulation of MCL-1 protein potentiated these effects in the mcl-1-siRNA
(siMCL-1)-transfected cells (Figs.3E and 3F). These results indicate that MT189-induced apoptosis is potentiated by MCL-1 reduction in the cells, which contributes to its in vitro proliferation-inhibitory activity.

However, MT189 did not lower the mRNA level of MCL-1 (Suppl. Fig.6). But the proteosome inhibitor MG132 could rescue MCL-1 protein reduction by MT189 (Fig.3G). Moreover, MT189 apparently increased the ubiquitination of MCL-1 protein (Fig.3H). The data reveal that MT189 promotes the degradation of MCL-1 protein via the ubiquitin-proteosome pathway.

**MT189-mediated JNK activation causes degradation of MCL-1 protein.**

The MAPK kinases JNK and p38 have been shown to participate in the regulation of the MCL-1 protein stability (11, 25, 26). MT189 was revealed indeed to increase their activated (phosphorylated) forms but not to change their total proteins levels (Fig.4A). However, it was the JNK inhibitor SP600125 but not the p38 inhibitor PD169316 that prevented the MT189-mediated Ser159/Thr163 phosphorylation and degradation of MCL-1 protein in SMMC-7721 cells (Fig.4B, upper). Consistently, SP600125 rather than PD169316 partially reversed the cleavage of caspase-9, caspase-3 and PARP (Fig.4B, lower), apoptosis (Figs.4C and 4D) and the loss of viable cells (Fig.4E) induced by the treatment with MT189 in SMMC-7721 cells. Moreover, p-JNK was shown to be colocalized with both MCL-1 and p-MCL-1 in the MT189-treated SMMC-7721 cells (Fig.4F). The results indicate that MT189 mediates JNK activation, which leads to MCL-1 phosphorylation that facilitates its ubiquitination-driven degradation.
MT189 mediates JNK activation via the MEKK1/TAK1-MKK4 pathway.

The JNK inhibitor SP600125 also inhibits CDK1 (27). So we used another JNK inhibitor JNK-IN-8 (28) and a CDK1 inhibitor RO3306 (29-31). The result showed that the MT189-mediated MCL-1 degradation could be reversed only by the JNK inhibitors but not by the CDK1 inhibitor (Fig.5A). Additionally, both JNK and CDK1 were reported to mediate phosphorylation of the antiapoptotic protein BCL-2 (32-34). However, the CDK1 inhibitor RO3306 did not affect the MT189-mediated increase in the phosphorylation level of BCL-2 (Fig.5B). Moreover, either overexpression or silence of MCL-1 did not change the cell cycle arrest induced by MT189 (Fig.5C). These data strengthen the evidence supporting the conclusion that the MT189-mediated JNK activation rather than the mitotic arrest-related CDK1 activation causes the increased phosphorylation levels of MCL-1 followed by its ubiquitination-driven degradation.

The next question is how MT189 mediates JNK activation. Either JNK or p-JNK did not appear in the immunoprecipitated α-tubulin complexes in the control or MT189-treated SMMC-7721 cells (Fig.5D). In the control cells, both JNK and p-JNK were observed mainly in nuclei, but they were not revealed to be colocalized with tubulin. The treatment with MT189 did not change this feature of either JNK or p-JNK, although it increased the levels of p-JNK (Suppl. Fig.7). The data suggest that there is no direct interaction between tubulin and JNK, and JNK phosphorylation followed by the treatment with MT189 must be caused by some kinase(s).

JNK has been shown to be activated via MAP kinase kinase kinases (MEKK1
and TAK1) through MAP kinase kinases (MKK4 and MKK7) (10). Indeed, the treatment with MT189 apparently enhanced the phosphorylation levels of JNK, p38, MEKK1, TAK1 and MKK4, but just marginally changed the level of p-MKK7 in SMMC-7721 cells. At the same time, the levels of their corresponding total proteins basically kept unchanged (Fig.5E). However, either intact or disrupted microtubules were not colocalized with p-MEKK1 or p-TAK1 or their non-phosphorylated forms in SMMC-7721 cells (Data not shown). The results indicate that JNK is activated via the MEKK1/TAK1-MKK4 pathway responding to the MT189-caused microtubulin disruption, but which upstream kinase(s) are involved in the activation of TAK1 and/or MKK4 remains to be clarified.

Discussion

Colchicine binding site inhibitors are promising anticancer agents, several of which, including CA4 derivatives, are at different phases of clinical trials (1). These inhibitors display differential characteristics in anticancer spectrum, drug resistance and antivascular effects when compared with the classical tubulin inhibitors such as paclitaxel and vincristine (1, 33). We previously reported MT7 and MT119 derived from a combinatorial library of 6H-Pyrido[2′,1′:2,3]imidazo[4,5-c]isoquinolin-5(6H)-ones as colchicine site-targeted tubulin inhibitors (2-4). They are new chemical entities with a distinct mode of tubulin polymerization inhibition from colchicines, but disappointingly, both do not show in vivo anticancer activity (3, 4). MT189 is a newest analog of this series, obtained by taking a de-construction approach to break the tetracyclic ring of MT119.
In this study, we found that MT189, a new optimized derivative of MT119 (4) retained its property in impairing microtubule assembly by depolymerizing microtubules via binding to the colchicine site, leading to M arrest and inducing apoptosis. MT189 showed significantly improved in vitro proliferation-inhibitory activity and direct killing of MDR tumor cells. However, its in vivo anticancer activity is still limited. MT189 has very low solubility in water, and thus it was administered by intraperitoneal injection rather than by intravenous injection (as taxol) in this study. When administered at 100 mg/kg/d once a day to nude mice, MT189 caused tumor growth inhibition by 35.9% on day 14. At this time-point, all tested animals were alive and showed no obvious loss of the body weight. However, on day 17, the animals began to die, and till day 21, among 8 tested animals in the MT189 group, only one was still alive. Therefore, demonstrating what are the primary toxicity organs and the related toxicity mechanisms of MT189 is critical for our future work.

MT189 binds to cellular microtubules via the colchicine site, leading to rapid microtubule depolymerization (within 0.5 h) followed by persistent M arrest and subsequent apoptotic induction. In addition, MT189 also relieves the apoptosis suppression of the antiapoptotic protein MCL-1 by promoting its degradation via the ubiquitination-proteosome pathway, which potentiates its apoptotic induction and thus its anticancer effects. Consequently, persistent mitotic arrest, apoptosis and MCL-1 degradation collectively contributes to the anticancer activity of MT189 (Fig.5F).

MCL-1 is an antiapoptotic member of the BCL-2 protein family. It can block proapoptotic proteins BAX and BAK to repress apoptosis. Both taxol and vincristine
can lead to degradation of MCL-1 *via* different pathways, such as JNK (11), CDK1 (12, 34) and NOXA (35, 36). However, whether colchicine-binding-site tubulin inhibitors elicit similar effects remains to be clarified. In this study, we demonstrated that the colchicine-site-targeted tubulin inhibitor MT189 sequentially activated MEKK1/TAK1, MKK4 and JNK (Fig. 5E). Activation of JNK caused phosphorylation of MCL-1 that facilitated its ubiquitination and subsequent degradation. However, we did not observe that microtubulin (either intact or disrupted) was colocalized with JNK, MEKK1 or TAK1 or their respective phosphorylated equivalents. The result suggests that MT189 could mediate the activation of the MEKK1/TAK1-MKK4-JNK pathway via unknown intermediary kinase(s) in a microtubulin depolymerization-dependent fashion (Fig. 5F).

In fact, various extracellular stimuli such as cytokines and protein synthesis inhibitors can stimulate phosphorylation of JNK (37). Many other anticancer agents with different molecular targets or mechanisms of action can also lead to the activation of this pathway (10, 37, 38). Notably, detailed time-effect analyses showed that taxol, a microtubulin-polymerization-promoting anticancer agent, activated JNK at early stages (30 min) (37, 38), just as MT189 did (0.5 h; Fig. 5E). All the lines of evidence suggest that the depolymerization of microtubules is an essential factor for JNK activation. JNK has multiple substrates and is involved in extensive physiological and pathophysiological processes and therapeutic responses (8, 10, 39-41). Apparently, it is deserved to put continuous efforts into demonstrating which unknown factor(s) and how they mediate JNK activation.
We have already shown that in MT189-treated cancer cells, microtubule depolymerization causes both M arrest (Figs. 1E, 1F and 5F) and JNK activation following with MCL-1 degradation (Figs. 4, 5E and 5F). Notably, however, both microtubule depolymerization and JNK activation took place at very early stages (0.5 h; Fig. 5E and Suppl. Fig. 3), but M arrest occurred at relatively late stages (3 h or later; Figs. 1E and 1F) in those cells. Moreover, both tested JNK inhibitors, but not the CDK1 inhibitor, could reverse MCL-1 degradation (Fig. 5A); the CDK1 inhibitor did not prevent the increase in the MT189-induced BCL-2 phosphorylation (Fig. 5B) although CDK1 was reported to mediate phosphorylation of BCL-2 (34); and the changes in MCL-1 protein expression did not affect the MT189-induced M arrest (Fig.5C). Additionally, MT189 was shown to mediate JNK activation via the MEKK1/TAK1-MKK4 pathway. Therefore, JNK activation is independent of M arrest in MT189-treated cancer cells (Fig.5F).

Together, this study presents a new tubulin inhibitor MT189 with anticancer activity and retaining the colchicine-site-targeted characteristics of its parental compound MT119. Besides its mitosis arrest and apoptosis induction, we show that relief of apoptosis suppression due to MCL-1 degradation subsequent to the activation of the MEKK1/TAK1-MKK4-JNK pathway potentiates apoptotic induction of MT189. We believe that demonstrating how to activate this pathway is helpful to further understand the mechanisms of action of MT189 and thus to promote the optimization and development of its new derivatives.

References


and Orai3 channels more important than calcium influx for cell proliferation?


Table 1. Effects of MT189 on multidrug resistant cancer cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (μM)</th>
<th>RF</th>
<th>IC$_{50}$ (μM)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KB</td>
<td>KB/VCR</td>
<td>MCF-7</td>
<td>MCF-7/ADR</td>
</tr>
<tr>
<td>MT189</td>
<td>0.003±0.002</td>
<td>0.008±0.005</td>
<td>2.7</td>
<td>0.588±0.319</td>
</tr>
<tr>
<td>vincristine</td>
<td>0.008±0.003</td>
<td>0.596±0.139</td>
<td>74.5</td>
<td>-</td>
</tr>
<tr>
<td>adriamycin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.033±0.010</td>
</tr>
</tbody>
</table>

IC$_{50}$ from three separate experiments was expressed as mean ± SD. Resistance factor (RF) was calculated as the ratio of the IC$_{50}$ value of the drug-resistant cells to that of the corresponding parental cells.
Figure Legends

Figure 1

MT189 inhibits microtubule polymerization by binding to the colchicine site, induces mitotic arrest, and disrupts spindle assembly. A. MT189 inhibited in vitro polymerization of microtubulin in a cell-free system. Colchicine and paclitaxel were used as references. B. The binding site of MT189 on tubulin was determined. The competitive binding assay showed that MT189 bound to the colchicine site rather than the vinblastine site on microtubule. Vincristine (VCR) and CA4 were used as references. Data from 3 independent experiments were expressed as mean ± SD. C. SMMC-7721 cells were treated with 0.2 μM colchicine, 0.2 μM paclitaxel or 0.2 μM MT189 for 6 h, and then interphase microtubulin was imaged by immunofluorescence microscopy. Scale bar: 10 μm. D. SMMC-7721 cells were treated with 0.2 μM colchicine, 0.2 μM paclitaxel, 0.1 μM or 0.2 μM MT189 for 6 h. Then, the polymerized fraction and the free fraction of tubulin were separated by ultracentrifugation and processed for Western blotting. E. SMMC-7721 cells were treated with MT189 at indicated concentrations for 12 h or with 0.2 μM MT189 for the indicated time. Cell cycle was analyzed by flow cytometry and representative histograms were presented. F. SMMC-7721 cells were treated with 0.2 μM MT189 for the indicated time and then, were lysed and immunoblotted for mitotic molecular markers MPM2 and p-histone 3 (Ser10). G. SMMC-7721 cells were treated with 0.2 μM MT189 for 12 h, and then the mitotic spindle assembly was shown by immunofluorescence staining. Scale bar: 10 μm.
**Figure 2**

MT189 induces intrinsic apoptosis and elicits tumor growth delay. **A.** Apoptosis of SMMC-7721 cells was detected by TUNEL assays after the treatment with 0.2 μM MT189 for the indicated time. Scale bar: 10 μm. **B** and **C.** MT189 caused loss of MMP in SMMC-7721 cells treated with MT189 for 36 h analyzed by flow cytometry. Representative images were presented in **B** and the data from 3 independent experiments were expressed as mean ± SD in **C.** **D.** Effects of MT189 on PARP and caspases in SMMC-7721 cells were examined by Western blotting. **E.** MT189 exerted potent antiproliferative activity against a panel of cancer cells revealed by the 72-h IC_{50} values from 3 independent experiments determined by SRB assays. **F.** MT189 displayed tumor growth inhibition in human breast cancer MDA-MB-231 xenograft models in nude mice, but marginally affected the body weight of the tested animals.

**Figure 3**

MT189-induced apoptosis is closely correlated with degradation of MCL-1. **A.** Effects of MT189 on the levels of BCL-2 family proteins in SMMC-7721 cells were determined by Western blotting. β-Actin was used as the loading control. **B.** Differential effects of MT189 on the levels of MCL-1 protein were detected by Western blotting in 3 relatively sensitive cell lines (SMMC-7721, HT-29 and HeLa) and in 2 relatively insensitive cell lines (DU145 and SKOV-3) treated with MT189 for 24 h. **C** and **D.** Overexpression of wild-type MCL-1 protein in SMMC-7721 cells partially rescued apoptosis (C) and reduction of viable cells (D) induced by the
treatment with MT189 for 48 h. E and F. Downregulation of MCL-1 protein with siRNA in SMMC-7721 cells increased apoptosis (E) and reduction of viable cells (F) induced by the treatment with MT189 for 24 h. Protein levels of MCL-1, PARP and caspases were evaluated by Western blotting (C and E). And reduction of viable cells was detected by CCK-8 assays (D and F). *, $P < 0.05$; **, $P < 0.01$. G. The proteosome inhibitor MG-132 reversed the reduction of MCL-1 protein induced by the treatment with MT189 for 24 h in SMMC-7721 cells. The cells were exposed to MT189 in the presence or absence of MG-132 for 24 h, and then the whole-cell lysates were collected for Western blotting. H. The treatment with MT189 for 24 h led to the increased ubiquitination of MCL-1 protein in SMMC-7721 cells. The cells were treated with MT189 and then collected for immunoprecipitation with an anti-MCL-1 antibody. Western blotting was conducted to detect the ubiquitinated MCL-1 with an anti-ubiquitination antibody in the immunoprecipitated complexes or the levels of MCL-1 protein in the corresponding inputs.

**Figure 4**

**Activation of JNK rather than p38 contributes to reduction of MCL-1 protein. A.**

The treatment with MT189 activated JNK and p38 detected by Western blotting in SMMC-7721 cells. B. The JNK inhibitor SP600125 rather than the p38 inhibitor PD169316 prevented the Ser159/Thr163 phosphorylation and reduction of MCL-1 protein (upper; 12-h treatments with MT189) or reversed the changes in the levels of other apoptosis-related proteins (lower; 48-h treatments with MT189) in SMMC-7721
cells. C-E. Effects of SP600125 and PD169316 on apoptosis induction detected by Annexin V-FITC assays (representative images in C and mean ± SD from 3 independent experiments in D) and reduction of viable cells determined by CCK-8 assays (mean ± SD from 3 independent experiments; E) elicited by the 48-h treatments with MT189 in SMMC-7721 cells. *, P < 0.05; **, P < 0.01. F. SMMC-7721 cells were treated with 0.2 μM MT189 for 12 h. Then, the locations of p-JNK, MCL-1 and p-MCL-1 proteins were detected by immunofluorescence-based laser confocal microscopy. Scale bar: 10 μm.

Figure 5

MT189 leads to activation of the MAPK pathway. A. The JNK inhibitors SP600125 and JNK-IN-8 rather than the CDK1 inhibitor RO3066 prevented the degradation of MCL-1 protein induced by 12-h treatments of MT189 in HeLa cells. B. The CDK1 inhibitor RO3306 did not reverse phosphorylation of JNK and BCL-2 in HeLa cells exposed to MT189 for 12 h. C. MCL-1 did not affect the cell cycle progression. Downregulation (siMCL-1) or overexpression (pWT-MCL-1) of MCL-1 did not change cell cycle progression in SMMC-7721 cells treated with or without 0.2 μM MT189 for 12 h analyzed by flow cytometry. D. JNK or p-JNK was not present in the immunoprecipitated complexes of α-tubulin. SMMC-7721 cells were treated with 0.2 μM MT189 for 12 h for α-tubulin immunoprecipitation and subsequent Western blotting. E. MT189 caused phosphorylation of the components in the MAPK pathway, including MEKK1, TAK1, MKK4, MKK7, JNK and p38 detected by Western blotting.
in SMMC-7721 cells. F. Schematic presentation of possible anticancer mechanisms of MT189.
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Fig. 4. Dose-dependent effect of MT189 on the activity of JNK and p38.

(A) Western blot analysis of the expression of JNK, p-JNK, p38, p-p38, MCL-1, p-MCL-1, and β-Actin in MDA-MB-231 cells treated with MT189 (0.2 µM) for 0, 6, 12, 16, and 20 hours.

(B) Western blot analysis of the expression of p-MCL-1, MCL-1, and β-Actin in MDA-MB-231 cells treated with MT189 (0.2 µM), SP600125 (10 µM), or PD169316 (10 µM) for 0, 6, 12, 16, and 20 hours.

(C) Flow cytometry analysis of apoptosis in MDA-MB-231 cells treated with MT189 (0.2 µM) for 0, 6, 12, 16, and 20 hours, and stained with Annexin V and PI.

(D) Bar graph showing the percentage of apoptosis in MDA-MB-231 cells treated with MT189 (0.2 µM) for 0, 6, 12, 16, and 20 hours.

(E) Bar graph showing the reduction rate of viable cells in MDA-MB-231 cells treated with MT189 (0.2 µM) for 0, 0.05, 0.1, and 0.2 µM, and stained with Annexin V and PI.

(F) Immunofluorescence images showing the expression of DAPI, p-JNK, MCL-1, and their merged images in control and MT189 (0.2 µM, 12 h) treated MDA-MB-231 cells.
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Molecular Cancer Therapeutics

MCL-1 Degradation Mediated by JNK Activation via MEKK1/TAK1-MKK4 Contributes to Anticancer Activity of New Tubulin Inhibitor MT189

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