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A small molecule MTBT prevents cancer cell growth by activating p38 MAPK

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Abstract

Cancer is a disease of unscheduled cell division and many anticancer drugs target the cell cycle to inhibit the proliferation of cancer cells. We conducted a screen for new anticancer drugs that induce cell cycle arrest using a small compound library. From this screen, we identified 2-(3-Methyl-thiophen-2-yl)-4-(3,4-dioxybenzene) thiazole (MTBT) that causes accumulation of cancer cells with 4N DNA content and inhibits colony formation for several cancer cell lines. We further showed that the treatment of cancer cells with this compound for a longer time leads to apoptosis, as indicated by the presence of cells with a sub-G1 peak and the appearance apoptotic markers. The increased phosphorylation of serine 10 on histone H3 in MTBT-treated cancer cells suggests cell cycle arrest in M-phase. Strikingly, MTBT-induced cell cycle arrest and enhanced H3 (Ser10) phosphorylation are abrogated by the pretreatment with SB203580, a specific inhibitor for mitogen-activated protein kinase p38. Moreover, treatment of cancer cells with MTBT induces the phosphorylation of p38, indicative of p38 activation. Together, we have identified a new compound that inhibits cancer cell proliferation, which is likely a consequence of p38 activation.

Keywords

Anticancer drug screen; small molecule; p38 MAPK; cell cycle; apoptosis

Introduction

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that transmit various extracellular stimuli to the nucleus.^[1,2] p38, a member of the MAPK family, is activated in response to a wide range of extracellular stress stimuli, such as hyperosmolarity, heat shock, genotoxic compounds, UV light, γ -irradiation, metabolic stress, and protein synthesis inhibition.^[3-6] In human cells, four genes encode p38 MAPK family members, p38 α , p38 β , p38 γ and p38 δ . Although both p38 α and p38 β are expressed

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ubiquitously, p38 β is expressed at very low levels. Previous work shows that p38 γ and p38 δ are only expressed in certain type of tissues, indicating their specialized functions.^[7] Accumulating evidence supports the role of p38 α as a tumor suppressor based on its negative role in cell cycle progression and the induction of apoptosis.

p38 α negatively regulates cell cycle progression at both G₁ to S and G₂ to M transitions. The downstream targets include cyclins, cyclin-dependent kinase (CDK) regulators, and tumor suppressor p53.^[8] Recent evidence shows that the activation of p38 MAPK pathway delays the G₂ to M transition by destabilizing Cdc25B, a phosphatase required to activate CDK.^[9, 10] Previous data also show that the introduction of activated recombinant p38 is able to induce mitotic arrest, but p38-specific inhibitors can abrogate cell cycle arrest when the integrity of the mitotic spindle is compromised.^[11] On the other hand, p38 activation is involved in the induction of apoptosis by different cellular stresses and the apoptotic effect of p38 might be mediated by activating p53 and caspase.^[12] Thus, activation of p38 could play a critical role in cancer treatment.

Recent studies indicate that p38 MAPK activation is necessary for the killing of cancer cells by a variety of anticancer agents. HeLa cells treated with microtubule-depolymerizing reagents (nocodazole, vincristine, and vinblastine) or a microtubule-stabilizing compound (taxol) show M-phase arrest and cell death. p38 activation is observed in these treated cells and this activation is necessary for the cell death induced by chemotherapeutic drugs.^[13] Cisplatin and doxorubicin are widely used in cancer chemotherapy and the treatment with these drugs leads to increased p38 activation as indicated by its phosphorylation.^[14] However, recent studies show that nocodazole inhibits p38 activation by UV treatment.^[15] The discrepancy in the previous reports could be a result of the different methods or cell lines used for the assessment of p38 activity. Therefore, the role of p38 in cancer treatment needs to be reevaluated.

We are interested in the isolation of new anticancer drugs that target cell cycle progression. From a small molecule library, we identified a new compound MTBT that causes cell cycle arrest and apoptosis as evidenced by the accumulation of cancer cells with 4N DNA content and the increase of cells positive for apoptotic markers. Enhanced H3 (Ser10) phosphorylation in MTBT-treated cells indicates cell cycle arrest in M-phase. The results of colony-formation assay demonstrate the anticancer activity of MTBT. Strikingly, MTBT-induced cell cycle arrest and H3 (Ser10) phosphorylation were abrogated when the cells were pretreated with a specific inhibitor for p38. Consistently, the phosphorylation of p38 is increased shortly after cells are treated with MTBT. Therefore, we have identified a new anticancer compound that inhibits cancer cell growth, and this inhibition is like a consequence of p38 activation.

Results

MTBT inhibits proliferation of cancer cells

We are interested in the isolation of new anticancer drugs that target cell cycle progression. For this purpose, we used MTT assay to measure the viability loss of cancer cells after treatment with compounds from a small molecule library (10,000 compounds). Among

10,000 compounds from this library, about 80 of them show anticancer activity at 2 $\mu\text{g/ml}$ and they were selected for further analysis. We found one compound MTBT that caused more than 50% viability loss of A549, HeLa and HepG2 cancer cells at 2 $\mu\text{g/ml}$. MTBT is a new compound without any reported bioactivity, and the structure of this compound is shown in Figure 1A. We further used a colony formation assay to evaluate the inhibition of MTBT on cancer cell proliferation. Cells were inoculated in 6-well plates at the density of 1000 cells per well, cultured for 24 hr and then treated with various concentrations of MTBT for 24 hr. After MTBT washout, we incubated the cells in fresh medium without MTBT for 14 days to assess colony formation. Compared to the cells treated with DMSO, the colony formation of A549, HeLa and HepG2 cells was inhibited significantly after treatment with 4.3 μM MTBT. Stronger inhibition of colony formation was observed in the cells treated with higher concentrations of MTBT (Fig. 1B). Therefore, we isolated a new compound MTBT that inhibits cancer cell proliferation and prevents colony formation of several cancer cell lines.

MTBT triggers cell cycle arrest in A549 cells

We next asked whether MTBT inhibits cancer cell growth by targeting cell cycle progression. For this purpose, A549 cells were first incubated for different amount of time in the presence of MTBT at various concentrations and followed by FACS analysis. After treatment with MTBT, we observed accumulation of A549 cells with 4N content of DNA, indicative of a G2/M arrest (Table 1; Fig. 2). For example, after 24 hr exposure to different concentrations of MTBT (1.08, 2.16 and 4.32 μM), the proportion of cells with 4N DNA content reached 30.22%, 63.02% and 66.57%, respectively, compared with 8.11% for the control. After treatment with 4.32 μM MTBT for 12, 24 and 36 hr, the percentage of A549 cells with 4N content of DNA increased to 25.11%, 68.12% and 80.40%, respectively. These results suggest that MTBT induces G2/M phase arrest in a dose and time-dependent manner.

MTBT induces apoptosis in A549 cells

The induction of apoptosis is a common mechanism for many anti-cancer drugs, thus we examined the apoptotic process in MTBT-treated cells. A549 cells were first incubated with MTBT at various concentrations for different amount of time and then subjected to AnnexinV-FITC labeling and PI staining as described in materials and methods. We used FACS analysis to determine the proportion of A549 cells positive for AnnexinV-FITC (FITC), PI, or both. The A549 cells positive for FITC or for both FITC and PI indicate that they are in early or late apoptotic stages, respectively. After treatment with different concentrations of MTBT for 24 hr, we noticed a clearly increased population of apoptotic cells. For example, only 7.03% (3.66+3.37) of control cells showed apoptotic signals, but the percentage increased to 15.70% (3.91+11.79) for cells treated with 4.32 μM . It was obvious that high concentrations of MTBT induced more dramatic apoptosis (Table 2, Fig. 3A). Moreover, we examined the apoptosis in cells treated with 8.64 μM MTBT for different times. The proportion of apoptotic cells increased from 11.65% (5.51+6.14) at 24 hr to 55.31% (53.42+1.89) at 72 hr (Table 2, Fig. 3B). In addition, the accumulation of cells positive for both PI and FITC became more significant after longer treatment with MTBT,

which indicates the increase of cells in late apoptotic stages. All these results suggest that MTBT also induces apoptosis in a dose and time-dependent manner.

MTBT induces M-phase arrest

The Ser10 of histone H3 becomes highly phosphorylated by Aurora B kinase on condensed chromosomes during mitosis, thus H3 (Ser10) phosphorylation has been used as a sensitive marker for mitotic cells.^[16,17] To determine if the accumulation of cells with 4N DNA content is a consequence of mitotic arrest, we analyzed H3 (Ser10) phosphorylation in MTBT-treated cells. A549 cells were first cultured with 4.32 and 8.64 μM MTBT for 24 hr and then probed with a phospho-antibody specific for H3 (Ser10). After incubation with FITC-conjugated secondary antibody and DNA dye (PI), the cells were subjected to FACS analysis to determine H3 (Ser10) phosphorylation and DNA content. Compared to untreated cells, the treatment with 4.32 or 8.64 μM MTBT caused a 2.12 and 2.18 fold increase of the phosphorylation of H3(Ser10), respectively (Fig. 4A). We found that more cells with 4N DNA content became positive for H3 (Ser10) phosphorylation. Only 4.62% of untreated cells that showed 4N DNA content were positive for H3 (Ser10) phosphorylation, but the treatment with 4.32 or 8.64 μM MTBT increased the number to 37.9% and 28.7%, respectively (Fig. 4B). It is surprise that we did not see the dose-dependent accumulation of cells with 4N content of DNA. We speculate that some arrested cells underwent apoptosis, and this speculation is supported by the appearance cells with sub-G1 peak after treatment with higher concentrations of MTBT (Fig. 2). Therefore, the treatment with MTBT causes cell cycle arrest in M-phase, which may contribute to the anticancer activity of this compound.

Functional p38 MAPK is required for MTBT-induced cell cycle arrest

Previous studies have confirmed that mitogen-activated protein kinases (MAPKs) elicit the rapid phosphorylation of Ser10 on H3.^[18] The use of specific MAP kinase inhibitors enables the dissection of the role of different MAPK pathways in H3 phosphorylation. SB203850, a specific inhibitor of p38 MAPK, has been shown to block H3 (Ser10) phosphorylation induced by a protein synthesis inhibitor.^[19] Therefore, we tested if MTBT-induced cell cycle arrest requires the activity of p38. For this purpose, we examined MTBT-induced cell cycle arrest in cells pretreated with p38 inhibitor SB203580. A549 cells were first incubated with 50 μM or 100 μM SB203580 for 2 hr followed by the treatment with 4.32 μM MTBT. After further incubation for 18 hr, the cells were collected and analyzed for cell cycle distribution using FACS. In the absence of pretreatment with p38 inhibitor, MTBT induced significant accumulation of cells with 4N DNA content. In clear contrast, pretreatment with 50 or 100 μM SB203580 blocked the cell cycle arrest induced by MTBT, as evidenced by the decrease of a 4N DNA peak (Fig. 5A). We found that 66.63% of A549 cells showed 4N DNA content after exposure to 4.32 μM MTBT, but pretreatment with 50 or 100 μM p38 inhibitor lowered the number to 25.36% and 17.96%, respectively. Cells treated with p38 inhibitor alone did not show an increased 4N DNA peak (Table 3).

Previous studies suggest that the disruption of microtubules impairs the activation of p38 induced by DNA damage.^[20,21] However, several groups showed that microtubule-interfering agents stimulate the activation of MAPK superfamily, including p38 in certain

cell contexts. [22, 23-26] Since we showed that MTBT-induced G₂/M arrest depends on functional p38, it is possible that mitotic arrest induced by other drugs is also p38 dependent. Thus, we tested if p38 is required for the cell cycle arrest induced by microtubule disruption under our experimental conditions. Nocodazole is a microtubule-depolymerizing agent and it has been widely used to disrupt the spindle structure and induce G₂/M arrest. After exposure to 0.13 μ M nocodazole for 18 hr, 83.25% of A549 cells showed 4N DNA content, suggesting mitotic arrest. The pretreatment with 50 and 100 μ M p38 inhibitor SB203580 lowered the number to 73.76% and 58.21%, respectively (Table 3, Fig. 5A), which is much less significant compared to the suppression of MTBT-induced G₂/M arrest by p38 inhibitor. Vincristine is another microtubule-depolymerizing agent that causes G₂/M arrest. We found that the suppression of vincristine-induced cell cycle arrest in A549 cells by SB203580 was not significant either (data not show).

In contrast to nocodazole and vincristine, an anti-cancer drug taxol stabilizes microtubules, which also leads to G₂/M arrest. After exposure to 0.03 μ M taxol for 18 hr, 72.56% of A549 cells showed 4N DNA content, indicating G₂/M arrest. The pretreatment with 50 and 100 μ M p38 inhibitor SB203580 lowered the number to 67.61% and 57.77%, respectively (Table 4, Fig. 5A). Thus, compared with MTBT-treated A549 cells, the suppression of nocodazole or taxol-induced G₂/M arrest by p38 inhibitor is much less efficient. This discrepancy suggests that MTBT likely induces cell cycle arrest through a mechanism different from microtubule-interfering agents.

Since the phosphorylation of H3 (Ser10) occurs in mitotic cells, we also examined whether pretreatment with p38 inhibitor suppresses the MTBT-induced H3 (Ser10) phosphorylation. As described above, treatment of A549 cells with 4.32 μ M MTBT induced a clear phosphorylation of Ser10 on H3, but this modification was abolished completely by the pretreatment with 50 μ M SB203580 for 2 hr. Compared to untreated cells, the treatment of A549 cells with 4.32 μ M MTBT caused a 2.32 fold increase in H3 (Ser10) phosphorylation (Fig. 5B), but the increase was barely detected in cells pretreated with p38 inhibitor (1.01 fold). After treatment with 4.32 μ M MTBT, 32.9% of cells showed 4N DNA content and these cells were also positive for H3 (Ser10) phosphorylation, but pretreatment with p38 inhibitor lowered the number to 3.67% (Fig. 5C). Therefore, pretreatment of A549 cells with p38 inhibitor blocks MTBT-induced cell cycle arrest at M-phase.

MTBT induces the activation of p38 MAPK

The family of p38 MAPK is activated by dual phosphorylation in the TGY motif in the activation loop.^[27] To further determine if MTBT treatment triggers the phosphorylation of p38, we examined the phosphorylation status of p38 MAPK in A549 cells treated with MTBT. A549 cells treated with 4.32 μ M MTBT were collected at 0, 1, 5 and 12 hr. The cells were probed with phospho-p38 MAPK (Thr180/Tyr182) antibody and FITC-conjugated secondary antibody for FACS analysis. Compared to untreated cells, the treatment with 4.32 μ M MTBT for 1, 5, and 12 hr caused 1.98, 1.76 and 1.86 fold increase in p38 phosphorylation, respectively. A substantial increase in p38 phosphorylation was detected in cells treated with MTBT only for 1 hr, but further incubation with MTBT did not enhance this phosphorylation (Fig. 6A). The western-blotting result further confirms the induction of

p38 phosphorylation by MTBT (Fig. 6B). Therefore, the treatment of MTBT likely triggers immediate p38 phosphorylation and activation, supporting the possibility that the mitotic arrest induced by MTBT treatment is secondary to p38 activation.

Discussion

Most of the anticancer drugs target the cell cycle machinery to block cell division, and we are interested in the isolation of new anticancer drugs that arrest the cell cycle. From the small molecule library,^[28] we identified MTBT, a new compound that causes cell cycle arrest with 4N DNA content. The increased phosphorylation of Ser10 on histone H3 indicates mitotic arrest. Moreover, the treatment of cancer cells with MTBT for a longer time (24 hr) induces apoptosis. Interestingly, MTBT-induced M-phase arrest and the increased H3 (Ser10) phosphorylation can be alleviated by pretreatment with p38 inhibitor. Furthermore, we showed that the phosphorylation of p38 increased significantly after MTBT treatment. Although the microtubule-depolymerizing agent nocodazole also induces cell cycle arrest at M phase, we found that p38 inhibitor is unable to alleviate nocodazole-induced arrest. Therefore, we identified a new anticancer drug that arrests the cell cycle in M-phase. This arrest is likely a consequence of p38 activation, but it remains to be determined if MTBT activates p38 directly.

MTBT is a derivative of 2, 4-two-aryl-thiazolidine without any anticancer activity reported. Thiazol has a heterocyclic ring and is widely used in anticancer drug development. Many natural chemotherapeutic agents containing thiazol moiety have been discovered and studied, such as tiazofurin, distamycin and netropsin.^[29-31] Some thiazol derivatives inhibit the growth of cancer cells by distinct mechanisms, such as the inhibition of an essential cytoplasmic purine metabolic enzyme IMPDH, induction of DNA damage, and inhibition of the activity of topoisomerase II and tyrosine kinase.^[29-34] Here we showed that a derivative of Thiazol, MTBT, blocks the cell cycle in M-phase likely by activating p38 MAPK. In addition to the further investigation of the anticancer mechanism of MTBT, we will also synthesize new compounds based on the structure of MTBT in order to find more efficient anticancer derivatives.

In conclusion, we found a new compound MTBT that inhibits the proliferation of several cancer cell lines by inducing M-phase arrest and apoptosis. The activation of p38 MAPK is likely responsible for the cell cycle arrest and apoptosis. Our data also show that MTBT can inhibit colony formation of several cancer cell lines, indicating the potential for cancer therapy. Our future interest is to define the direct target of this compound, synthesize more efficient anticancer derivatives using MTBT as a lead compound, and evaluate the *in vivo* anticancer activity of this compound by using animal models.

Materials and methods

Cells and growth

The cell culture media and fetal bovine serum (FBS) were purchased from Hyclone (HyClone Laboratories Inc., USA). The cell lines were obtained from the Cell Culture

Centre, Institute of Basic Medical Science at the Chinese Academy of Medical Sciences (China).

A549 cells (Human non-small cell lung cancer) were maintained in Ham's F-12 medium supplemented with 10% FBS. HepG2 cells (human hepatoma cells) were grown in minimum essential medium (MEM) containing 10% FBS. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂.

Chemicals

2-(3-Methyl-thiophen-2-yl)-4-(3,4-dioxybenzene) thiazole (MTBT) was purchased from Enamine Ltd (Kiev, Ukraine). The compounds used for the screen were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml as stock solution. p38 MAPK inhibitor SB203580 and DMSO were purchased from Sigma (Shanghai, China).

Colony-formation assay

Cells were plated out in 6-well plates at 1000 cells per well and incubated for 24 hr. After treatment with MTBT for 24 hr, MTBT was washed off with fresh medium and the cells were further incubated for 14 days. Then the cells were washed with cold phosphate-buffered saline (PBS), fixed with ice-cold methanol for 10 min and stained with 10% Giemsa for 2 min. The cells were examined with a microscope and the colony formation efficiency was calculated by the following formula: colony formation rate (%) = colony number/1000 × 100%.

Fluorescence-Activated Cell Sorter (FACS) analysis

A549 cells in exponential growth phase were treated with MTBT or 0.1% DMSO (control). Cells were harvested by trypsin digestion followed by centrifugation. After washed with cold 1×PBS, the cells were resuspended in ice-cold 70% ethanol at 4°C for at least 30 min. The fixed cells were then collected by brief centrifugation and resuspended in PBS containing RNase A (Sigma, USA) and propidium iodide (PI) (Sigma, USA). After incubation for 30 min at room temperature, the samples were subjected to cell cycle analysis using FACS.

To analyze the phosphorylation of Ser10 of histone H3 and p38 MAPK, cells were first washed with cold 1×PBS and then fixed in 4% paraformaldehyde at room temperature for 40 min. After washed with PBS, cells were blocked in 1×PBS plus 0.5% FBS and 0.2% Tween X-100 for 10 min at 4°C. The cells were then incubated with monoclonal phospho-H3 (Ser10) antibody or phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology, 1:40 dilution) for 2 hr at room temperature, followed by FITC-conjugated secondary antibody (1:100 dilution) for 1 hr at room temperature. The cells incubated only with FITC-conjugated secondary antibody were used as a negative control. The fluorescence signals were detected by FACS. The relative fluorescence intensity (FI) after MTBT treatment was calculated by the following formula $(FI_{\text{MTBT}} - FI_{\text{negative control}})/(FI_{\text{control}} - FI_{\text{negative control}})$

To analyze the cell cycle distribution of A549 cells, the cells stained with monoclonal phospho-H3 (Ser10) antibody and FITC-conjugated secondary antibody (1:100 dilution) were incubated with PI for 30 min at room temperature and then subjected to FACS analysis.

FITC-Annexin V/PI Apoptosis Assay

A549 cells were either treated with 0, 2.16, 4.32 and 8.64 μ M of MTBT for 24 hr, or treated with 8.64 μ M MTBT for 24, 48 and 72 hr. The treated cells were harvested, washed three times with 1 $\times$ PBS, and resuspended in 500 ml binding buffer (10 mM Hepes/sodium hydroxide (pH7.4), 140 mM sodium chloride, and 2.5 mM CaCl₂). Then 5 μ l FITC-labeled Annexin V (Zhongshan JinQiao Biotechnology Ltd., Beijing) was added and the cells were incubated in the dark for 10 min. We added 5 μ l of propidium iodide (PI) (10 mg/ml in binding buffer) to each sample before FACS analysis.

Western-Blotting Analysis

A549 cells were incubated with or without 4.32 μ M MTBT for 1, 3, 5 and 12 hr. The cells were washed twice with cold 1 $\times$ PBS and lysed in RAPI buffer (Applygen technologies Inc) for 30 min and centrifuged at 14,000 rpm for 20 min at 4 °C. We separated 5 μ l of total protein (5 μ g/ μ l) with 10% SDS-PAGE gels and transferred the proteins to PVDF membrane. After blocking with 5% (W/V) nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20 (TBST) for 1 hr, the membrane was incubated with monoclonal phospho-p38 MAPK (Thr180/Tyr182) antibody or p38 MAPK antibody (Cell Signaling Technology, 1:1000 dilution) for 2 hr and then with peroxidase-conjugated secondary antibodies for 1 hr. The protein bands were detected using ECL System (Piscataway, New Jersey, USA). The level of β -actin was used as a loading control.

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Abbreviations

MTBT	2-(3-Methyl-thiophen-2-yl)-4-(3,4-dioxybenzene) thiazole
DMSO	dimethyl sulfoxide
MAPK	Mitogen activated protein kinase
PI	propidium iodide

Figure 1.

MTBT inhibits cancer cell growth. **A.** The chemical structure of MTBT. **B.** MTBT inhibits colony formation of A549, HeLa, and HepG2 cancer cells. Cells were inoculated in 6-well plates at the density of 1000 cells/well and cultured for 24 hr. The cells were treated with the indicated concentrations of MTBT for 24 hr. After MTBT washout, the cells were incubated with fresh medium lacking MTBT for 14 days. The cells were washed with cold 1xPBS, fixed with ice-cold methanol for 10 min, and stained with 10% Giemsa for 2 min. The stained samples were counted for the numbers of colonies by microscopy. The average from three repeats is shown.

Figure 2.

MTBT induces G2/M phase arrest in A549 cells. A549 cells were suspended in 5% fetal bovine serum (FBS) F-12 medium in 6-well plates. **A.** After cells reached 70% confluence, they were treated with 1.08, 2.16 and 4.32 μM MTBT for 24 hr. The cells were collected and stained with PI for FACS analysis. **B.** The cells treated with 4.32 μM MTBT for 12, 24 and 36 hr. The cells were collected and stained with PI for FACS analysis. The experiment was repeated three times.

Figure 3.

MTBT induces apoptosis in A549 cells. A549 cells were suspended in 5% fetal bovine serum (FBS) F-12 medium in 6-well plates. **A.** After cells reached 70% confluence, they were treated with 0, 4.32, 8.64 and 17.28 μ M MTBT for 24 hr. The cells were collected and stained with PI and FITC-labeled Annexin V for FACS analysis. The four quadrants in this figure represent: 1, dead cells (PI positive, FITC negative); 2, later apoptotic cells (PI and FITC positive); 3, live cells (PI and FITC negative); 4, early apoptotic cells (PI negative, FITC positive). **B.** The cells were treated with 8.64 μ M MTBT for 24, 48 and 72 hr and then collected and stained with PI and FITC-labeled Annexin V for FACS analysis. The experiment was repeated three times.

Figure 4.

MTBT induces increased phosphorylation of Serine 10 on Histone H3 and accumulation of cells with 4N DNA content. **A.** MTBT treatment induces the phosphorylation of Ser10 on histone H3. A549 cells were treated with 4.32 or 8.64 μM MTBT in 6-well plates for 24 hr and collected for staining. The treated cells were stained with anti-phospho-H3 (Ser10) primary antibody and followed by FITC-conjugated secondary antibody. The cells were analyzed with FACS to detect H3 (Ser10) phosphorylation. The blue line represents negative control, in which the cells were only treated with FITC-conjugated secondary antibody. The brown line indicates the level of phospho-H3 (Ser10) in control cells treated with DMSO. The green and orange lines represent the cells treated with 4.32 or 8.64 μM MTBT, respectively. Increased phosphorylation of H3 (Ser10) indicates accumulation of cells in M-phase. **B.** MTBT treatment leads to the increase of cell population with 4N DNA content and are positive for H3 (Ser10) phosphorylation. A549 cells were treated with 4.32 or 8.6 μM MTBT in 6-well plates for 24 hr. The collected cells were stained for both DNA (PI) and phosphorylated H3 (Ser10). The DNA content and the level of H3 (Ser10) phosphorylation are shown. The cells within the top right section have 4N DNA content and are also positive for H3 (Ser10) phosphorylation, indicating that these cells are in M-phase.

Figure 5.

The p38 inhibitor alleviates MTBT-induced cell cycle arrest. A549 cells were first treated with 50 or 100 μM p38-specific inhibitor SB203580 for 2 hr. Untreated and SB203580-treated cells were further incubated with 4.32 μM MTBT, 0.13 μM nocodazole or 0.03 μM Taxol for 18 hr and then collected for the following analysis. **A.** SB203580 treatment blocks MTBT-induced cell cycle arrest but not the arrest induced by microtubule-interfering agents. A portion of the above cells were stained with PI followed by FACS analysis to determine the DNA content. **B.** SB203580 treatment inhibits the increase of H3 (Ser10) phosphorylation induced by MTBT. The treated cells were probed with anti-phospho-H3 (Ser10) primary antibody and FITC-conjugated secondary antibody followed by FACS analysis. The blue line represents negative control, in which the cells were only treated with FITC-conjugated secondary antibody. The sky blue line represents the level of phospho-H3 (Ser10) in cells treated with DMSO. The brown and red lines represent the cells treated with 50 μM SB203580 or 4.32 μM MTBT, respectively. The green line represents the cells treated with 50 μM SB203580 and 4.32 μM MTBT. Increased phosphorylation of H3 (Ser10) indicates accumulation of cells in M-phase. **C.** MTBT-induced M-phase arrest is suppressed by pretreatment with SB203580. A portion of treated cells were subjected to staining for H3 (Ser10) phosphorylation and DNA (PI) and followed by FCAS analysis. The

cells in the upper right section have 4N DNA content and are positive for H3 (Ser10) phosphorylation as well.

Figure 6.

MTBT treatment enhances p38 phosphorylation. A549 cells were treated with DMSO or 4.32 μ M MTBT and then collected at 0, 1, 5, and 12 hr. A. FCAS analysis for MTBT-induced p38 phosphorylation. After staining with phospho-p38 (Thr180/Tyr182) antibody and FITC-conjugated secondary antibody, the cells were subjected to FCAS analysis. The black line represents negative control, in which the cells were only treated with FITC-conjugated secondary antibody. The pink, green and blue lines represent the level of phospho-p38 (Thr180/Tyr182) in cells treated with 4.32 μ M MTBT for 0, 1, 5 and 12 hr, respectively. The right-shift of the curve indicates increased p38 phosphorylation. B. Western-blot analysis for MTBT-induced p38 phosphorylation. The level of p38 protein and phospho-p38 were detected after western blotting. β -actin is shown as a loading control

Table 1

Cell cycle distribution of A549 cells after MTBT treatment

Cell cycle stage	% cells after treatment for 24 h	% cells after treatment with different concentrations of MTBT (μ M)			% cells after MTBT (4.32 μ M) treatment for various amount of time (h)			% cells after DMSO treatment for various times (h)		
	0	1.08	2.16	4.32	12	24	36	12	24	36
G1	64.81	50.86	11.79	8.46	32.70	8.36	7.00	63.35	64.80	64.00
S	27.08	18.92	25.18	24.97	42.19	23.52	12.60	30.40	27.08	25.43
G2/M	8.11	30.22	63.03	66.57	25.11	68.12	80.40	6.25	8.12	10.57

Table 2

MTBT induces apoptosis at different concentrations.

% positive cells after 24 h treatment with different concentrations of MTBT (μM)				% positive cells after treatment with MTBT (8.64 μM) for different amount of times (h)				
	0	4.32	8.64	17.28	0	24	48	72
PI	1.08	1.41	3.01	1.95	0.82	0.76	12.07	33.29
PI/FITC	3.66	3.91	5.76	5.21	4.13	5.51	19.40	53.42
None	91.89	82.88	78.46	72.78	93.89	87.59	64.17	11.40
FITC	3.37	11.79	12.77	20.06	1.16	6.14	4.36	1.89

Table 3
The cell cycle profiles of A549 cells treated with MTBT alone or in combination with p38 inhibitor SB203850 (SB)

Cell cycle stage (%)	treatment					
	Control	50μM SB	100μM SB	MTBT	MTBT+50μM SB	MTBT+100μM SB
G1	56.41	62.55	66.81	8.89	31.78	32.64
S	38.23	30.67	23.80	24.48	42.86	49.40
G2/M	5.36	6.78	9.39	66.63	25.36	17.96

Table 4
The cell cycle profiles of A549 cells treated with nocodazole or taxol alone or in combination with p38 inhibitor SB203850 (SB)

Cell cycle stage (%)	treatment					
	Nocodazole	Nocodazole+ 50μM SB	Nocodazole+ 100μM SB	Taxol	Taxol+ 50μM SB	Taxol+ 100μM SB
G1	1.89	5.09	6.50	2.55	4.94	10.93
S	14.86	22.10	35.21	24.89	27.36	31.30
G2/M	83.25	72.81	58.29	72.56	67.61	57.77