Inhibition of FOXM1 by thiostrepton sensitzes medulloblastoma to the effects of chemotherapy

JIAPING LIN*, YIPENG ZHENG*, KUN CHEN, ZHENGSONG HUANG, XINJIAN WU and NU ZHANG

Department of Neurosurgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510080, P.R. China

Received June 13, 2013; Accepted July 22, 2013

DOI: 10.3892/or.2013.2654

Abstract. Medulloblastoma (MB) is the most common malignant brain tumor in children and is highly invasive and metastatic. Despite recent advances, most MB patients suffer significant therapy-related morbidity, and the survival rate for patients with metastatic MB remains unsatisfactory. Altered expression of FOXM1 has been detected in many types of cancers, and the inhibition of FOXM1 has been studied as a cancer therapy. In the present study, we evaluated the impact of the inhibition of FOXM1 by thiostrepton in Daoy MB cells. Cells were treated with different concentrations of thiostrepton alone or in combination with cisplatin. Cell viability was measured with CCK-8 assays, and cell cycle distribution and apoptosis were assessed by flow cytometric analysis. Changes in protein expression were examined by western blotting. RNAi experiments were performed using siRNA oligonucleotides. The invasion and migration studies were performed using 8-µm Transwell plates. Inhibition of FOXM1 by thiostrepton significantly decreased MB cell proliferation. Cell arrest at the G2/M phase and apoptosis were significantly increased in MB cell lines that were treated with thiostrepton or transfected with siRNA. Thiostrepton decreased the IC50 value of cisplatin for MB treatment by enhancing cisplatin-induced apoptosis. Thiostrepton also decreased cell invasion and migration, which are crucial steps for tumor progression. Our data suggest that targeting FOXM1 with small-molecule inhibitors results in potent antitumor activity and chemosensitizing effects in human medulloblastoma cells.

Introduction

Medulloblastoma (MB) is the most common pediatric malignant brain tumor, and satisfactory treatment results are notoriously difficult to achieve. MB has a high propensity to metastasize, as ~30% of patients have evidence of leptomeningeal dissemination at initial diagnosis (1). Despite recent advances in surgery, radiotherapy and chemotherapy, only 60 to 75% of affected children are cured, and the majority of them suffer from considerable long-term morbidity after aggressive multimodal therapy (2). Attempts to further improve outcomes and decrease the morbidity associated with MB have been restricted by the conventional cytotoxic approaches used and the infiltrative nature of the disease (2-4). Therefore, increased understanding of the mechanisms underlying MB is necessary to develop novel therapeutic approaches.

The oncogenic transcription factor FOXM1 is known to stimulate proliferation by promoting cell cycle transition and is involved in the proper execution of mitosis (5,6). Elevated expression of FOXM1 has been detected in a wide range of human tumors and has been implicated in cellular transformation, tumor initiation and progression (7). Accumulating evidence demonstrates that increased expression of FOXM1 is associated with poor prognosis in various types of cancers (8-11), including MB (12). Furthermore, previous studies have shown that FOXM1 mediates resistance to a diverse spectrum of anticancer drugs in breast cancer (13-15). A recent study confirmed that suppression of FOXM1 enhanced the chemosensitivity of various types of cancer cells to the DNA-damaging reagent doxorubicin (16). Therefore, the inhibition of FOXM1 activity has emerged as an attractive goal for cancer therapy. However, since FOXM1 displays a proliferation-specific expression pattern and is essential for embryonic development (5), the impact of inhibiting FOXM1 activity in non-malignant cells must be considered.

Thiostrepton, a natural product originally isolated from Streptomyces azureus, has captured a great deal of attention because of its potent anticancer activity as a FOXM1 inhibitor (17-19). The mechanism by which thiostrepton affects FOXM1 remains unknown. Hegde et al (18) reported that thiostrepton interacts directly with FOXM1 protein to inhibit the transcriptional activity of FOXM1, whereas Bhat et al (19) suggested that thiostrepton functions as a proteasomal
inhibitor. However, in a wide variety of tumor cell types, thiostrepton-induced apoptosis is dependent on the expression of FOXM1 (10,17,20). Importantly, thiostrepton appears to exert minimal toxicity against non-malignant cells (17,21). Thiostrepton has shown anticancer activity in rodent xenograft models without observable toxicity (10,22,23). Collectively, these data suggest that thiostrepton is an ideal treatment for MB, particularly in children. In this study, we examined the antitumor effects of thiostrepton in Daoy MB cells. More importantly, we assessed the ability of thiostrepton to sensitize MB cell lines to cisplatin, which is commonly used for the treatment of MB.

Materials and methods

Chemicals and reagents. Thiostrepton purchased from Tocris Cookson Inc. (Ellisville, MO, USA) was freshly dissolved in dimethyl sulfoxide (DMSO) to make a 10 mmol/l stock solution. Cisplatin (Qilu Pharmaceutical Co., Ltd., Shandong, China) was dissolved at a stock concentration of 2 mmol/l and divided into aliquots. An antibody specific for FOXM1 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) Antibodies specific for Bel-2, Bax, caspase-3, PARA and β-actin were obtained from Cell Signaling Technology, Inc.

Cell culture. The Daoy human MB cell line was obtained from ATCC (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco-BRL) and incubated at 37˚C in a humidified incubator in the presence of 5% CO₂.

siRNA transfection. RNA interference was performed by transfecting Daoy cells with 21-nucleotide RNA duplexes. FOXM1 siRNA and mock siRNA were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA-NC did not target any known mammalian gene and was synthesized by Shanghai GenePharma Co., Ltd. siRNA transfection was carried out with Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the procedure recommended by the manufacturer. Six hours after transfection with siRNA NC at various concentrations, the cells were analyzed on a FACSCalibur flow cytometer equipped with Cell Quest software (Becton-Dickinson, San Jose, CA, USA).

Western blot analysis. The cells were collected and lysed in lysis buffer on ice. The cell lysates were centrifuged at 10,000 x g for 10 min at 4˚C, and the protein content in the supernatants was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., USA). Equal amounts of protein lysate were electrophoretically separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 1% bovine serum albumin (BSA) for 2 h at room temperature and then incubated with anti-FOXM1, anti-Bel-2, anti-Bax, anti-caspase 3, anti-PARA or anti-β-actin primary antibody overnight at 4˚C. Horseradish peroxidase (HRP)-conjugated secondary antibody was added for 2 h at room temperature. Detection was performed by enhanced chemiluminescence (ECL; Pierce Biotechnology, Inc.).

Cell proliferation analysis. Cell proliferation assays were performed using the CCK-8 kit (Cell Counting Kit-8; Dojindo Laboratories), according to the manufacturer’s instructions. In brief, Daoy cells were seeded in a 96-well plate at a density of 5x10^3 cells/ml. The following day, the medium was replaced with DMEM containing 10% FBS with or without agents. The cells were incubated for 24, 48 or 72 h, and CCK-8 was used according to the manufacturer’s instructions. Extinction was measured at 450 nM, and the reference extinction was subtracted. Each experiment was performed in triplicate and repeated 3 times.

Cell cycle analysis. Daoy cells were seeded in 6-well plates in DMEM containing 10% FBS. The following day, the medium was replaced with DMEM containing 10% FBS with or without agents. The cells were detached after 24 h and fixed with 500 µl of 70% ethanol at -20˚C for 2 h. Subsequently, the cells were washed twice with PBS and then stained with propidium iodide (PI) (50 µg/ml propidium iodide and 100 µg/ml RNase A in PBS) at 37˚C for 30 min. Cell cycle analysis was performed on a FACScan flow cytometer (Becton-Dickinson).

Apoptosis analysis. Daoy cells were seeded in 6-well plates in DMEM containing 10% FBS. The following day, the medium was replaced with DMEM containing 10% FBS without or with agents. The cells were collected after 24 h, and cell apoptosis was detected by Annexin V-FITC/PI staining. The experiments were performed in triplicate for each sample, and analyses were performed using a FACScan flow cytometer in accordance with the manufacturer’s guidelines.

Colony formation assays. Daoy cells growing in log phase were seeded at a density of 1,000 cells/well in a 6-well plate in complete growth medium containing 10% FBS. The cells were allowed to adhere for 24 h, and the medium was replaced with fresh complete growth medium containing the indicated concentrations of thiostrepton. The cells were cultured at 37˚C for 10 days with medium changes every third or fourth day. Colony formation was detected by crystal violet staining.

Cell invasion and migration assay. Daoy cells were treated with the indicated concentrations of thiostrepton for 48 h, and equal numbers of cells were suspended in serum-free medium and seeded into either uncoated Transwell inserts (for migration assays) or growth factor-reduced Matrigel-coated Transwell inserts (for invasion assays) (BD Biosciences, Bedford, MA, USA). The bottom wells were filled with complete medium, and after 12 h, the cells were stained with crystal violet and photographed under a fluorescence microscope. The number of cells that penetrated the membrane was determined by counting the mean cell number of 5 randomly selected high-power fields.

Statistical analysis. All data were analyzed using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). t-tests were used for pairwise comparisons. The synergistic or antagonistic effects of 2 drugs were evaluated according to the formula \( Q = E_a + b/E_b - E_a \times E_b \). In this equation, \( E_a + b \) represents the inhibition rate of the combined drug therapy on tumor cell proliferation, and \( E_a \) represents...
and Eb represent the inhibition rates of drug A and drug B individually. Q-values ranging from 0.85 to 1.15 indicate that the effects of the 2 drugs are simply additive, Q-values >1.15 indicate a synergistic effect, and Q-values <0.85 indicate an antagonistic effect for the combined drug therapy.

Results

Thiostrepton inhibits cell proliferation and decreases the colony formation capacity of Daoy cells. We first investigated the effect of thiostrepton on Daoy cell proliferation. Daoy cells were treated with various concentrations of thiostrepton for 24, 48 or 72 h. Cell viability was assessed by CCK-8 assay. (B) Following treatment with the indicated concentrations of thiostrepton, the clonogenicity of Daoy cells was determined. (C) The graph displays the means ± SD of 3 independent clonogenicity formation experiments. *P<0.05, statistically significant (Student’s t-test).

Table I. IC<sub>50</sub> values for thiostrepton in Daoy cells were calculated at 24, 48 or 72 h.

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM) for thiostrepton</td>
<td>2.14</td>
<td>1.69</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Thiostrepton-mediated inhibition of FOXM1 induces cell cycle arrest and leads to apoptosis of Daoy cells. (A-C) Thiostrepton inhibits FOXM1 expression and induces G2-M arrest and apoptosis in a dose-dependent manner. (D) Daoy cells were transfected with either negative control (NC) siRNA or 3 FOXM1-specific siRNAs, which targeted different sites of the FOXM1 gene. FOXM1 protein levels were determined by western blotting. All 3 FOXM1 siRNAs induced (E) G2-M arrest and (F) apoptosis in Daoy cells.
of thiostrepton on Daoy cells, we investigated the expression of FOXM1 following thiostrepton treatment and examined whether thiostrepton affects cell cycle distribution and apoptosis. The results of these experiments showed that thiostrepton inhibited FOXM1 expression in a dose-dependent manner and induced a gradual dose-dependent G2/M arrest and apoptosis in Daoy cells (Fig. 2A-C). To validate the target specificity of thiostrepton, we downregulated FOXM1 expression using RNAi. Three different FOXM1 siRNA oligonucleotides, but not the negative control (NC) oligonucleotides, efficiently knocked down FOXM1 protein expression (Fig. 2D) and similarly resulted in a significant G2/M cell cycle phases arrest and apoptosis in Daoy cells (Fig. 2E and F). These results indicate that thiostrepton induces G2/M cell cycle phase arrest and leads to apoptosis in Daoy cells by targeting FOXM1.

**Thiostrepton enhances the anti-proliferative effects of cisplatin on Daoy cells.** The upregulation of FOXM1 has recently been reported to be closely related to chemotherapy resistance, and the inhibition of FOXM1 has been shown to increase the sensitivity of tumor cells to chemotherapeutic agents (13,15,24-26). We next aimed to ascertain whether combining FOXM1 inhibition with standard chemotherapeutic agents would enhance the effect. Daoy cells were cultured in the presence of cisplatin (0-40 µM) with or without thiostrepton (1 µM) for 24 h. Cytotoxicity was assessed using CCK-8 proliferation assays. The results demonstrated that combined treatment reduced the IC_{50} value of cisplatin in Daoy cells from 5.622 to 3.116 µmol/l (Fig. 3). Thiostrepton and cisplatin were shown to synergistically inhibit cell growth (Q>1.15) when the cells were treated with suboptimal concentrations of cisplatin (Table II).

<table>
<thead>
<tr>
<th>Cisplatin (µM)</th>
<th>Thiostrepton (1 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.35</td>
</tr>
<tr>
<td>2</td>
<td>1.31</td>
</tr>
<tr>
<td>3</td>
<td>1.78</td>
</tr>
<tr>
<td>4</td>
<td>1.66</td>
</tr>
<tr>
<td>5</td>
<td>1.29</td>
</tr>
<tr>
<td>10</td>
<td>1.04</td>
</tr>
<tr>
<td>20</td>
<td>1.03</td>
</tr>
<tr>
<td>40</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Q >1.15 corresponds to a synergistic interaction; *For all the various concentrations of cisplatin, 1 µm thiostrepton was used.

**Thiostrepton enhances cisplatin-induced caspase-mediated apoptosis in Daoy cells.** To characterize thiostrepton-mediated apoptotic enhancement, Daoy cells were treated with a low concentration of either thiostrepton (1 µM) or cisplatin (3 µM) or treated with both drugs for 24 h. Flow cytometric analysis showed that thiostrepton increased cisplatin-induced apoptosis from 14.3±2.46 to 30.2±3.16% (P<0.01) (Fig. 4A). To gain a better understanding of the mechanism leading to cell death, we measured the combined effect of thiostrepton and cisplatin on the expression of Bcl-2, Bax, caspase-3 and PARP proteins. As shown in Fig. 4B, the decreases in Bcl-2 expression were significantly greater in samples treated with both thiostrepton (1 µM) and cisplatin (3 µM) than those in...
the single treatment group. Moreover, combination treatment resulted in greater increases in Bax, caspase-3 and PARP protein expression than either drug alone (Fig. 4B).

**Thiostrepton impairs the migration and invasion of Daoy cells.** FOXM1 play a critical role in tumor cell metastasis (27-29). Since tumor cell migration and invasion are essential steps in tumor metastasis, we further examined the effects of thiostrepton on cell migration and invasion in Daoy cells. Our data clearly showed that thiostrepton inhibited the migration and invasion capacity of Daoy cells in a dose-dependent manner with minimal involvement of cell inhibition (Fig. 5).

**Discussion**

As the most common malignant brain tumor in children, medulloblastoma is characterized by aggressive invasion and early metastasis. Current standard therapies have unsatisfactory effects on survival, and therapy-associated side-effects have led to a concentrated search for novel therapeutic approaches for MB. Here, we report for the first time that thiostrepton inhibits FOXM1 in Daoy MB cells. We found that thiostrepton inhibited Daoy cell proliferation and triggered apoptosis in a dose-dependent manner. We further demonstrated that thiostrepton chemosensitized Daoy cells to cisplatin by enhancing cisplatin-induced apoptosis. Thiostrepton also showed important antiprogressive effects such as inhibition of migration and invasion in Daoy cells.

We found that thiostrepton significantly inhibited the proliferation of Daoy cells. The IC$_{50}$ value of thiostrepton in Daoy cells after 48 h of treatment was 1.69 μmol/l, suggesting the sensitivity of Daoy cells to thiostrepton is comparable to that of other cancer cell types (20,22). We next performed colony formation assays, which are an excellent indication of the long-term survival of tumor cells. These assays showed that thiostrepton strongly suppressed the ability of Daoy cells to form colonies. Notably, 1.75 μM thiostrepton treatment had a more marked effect on colony formation than on cell proliferation (100 vs. 35.81% inhibition, respectively). These data suggest that thiostrepton may impact tumor cell self-renewal in addition to mitosis.

Furthermore, our studies demonstrated that thiostrepton treatment induced cell cycle arrest at the G2/M phase transition and triggered apoptosis. Similar results were obtained with RNAi-mediated inhibition of FOXM1 in Daoy cells. These results support the idea proposed by Priller et al (12) that FOXM1 inhibition results in failure of mitosis and leads to mitotic catastrophe in MB. Furthermore, our results demonstrated the efficient antitumor activity of thiostrepton in Daoy cells and suggest that the effects of thiostrepton may depend on the downregulation of FOXM1 target genes, which have been shown to be involved in the regulation of cell growth, apoptosis, cell cycle and progression (10).

Previous studies suggest that silencing of FOXM1 enhances sensitivity to chemotherapeutic agents in a wide range of human cancers (15,16,25). This prompted us to investigate whether thiostrepton-mediated FOXM1 inhibition sensitizes Daoy cells to chemotherapy. In this study, the IC$_{50}$ value of cisplatin in Daoy cells decreased from 5.622 to 3.116 μmol/l when it was combined with thiostrepton (1 μmol/l). Notably, all of the synergistic effects identified in this study occurred when thiostrepton was combined with suboptimal concentrations of chemotherapeutic drugs. Cisplatin is a standard chemotherapy agent used in MB treatment; however, its use results in drug-related morbidities including hearing loss and renal dysfunction. Therefore, a combination therapy approach using cisplatin and thiostrepton would not only improve the effect of cisplatin but also reduce the adverse side-effects of cisplatin by allowing it to be used at lower doses.

The ability to trigger tumor cell death is critical for successful treatment with chemotherapeutic agents. In the present study, flow cytometric analysis revealed a more marked increase in apoptosis in cells treated with a combination of thiostrepton and cisplatin than in those treated with either drug alone. To further explore the mechanism underlying the increased efficacy of combination treatment, we examined proteins known to regulate apoptosis. Bcl-2 is best known for its role in preventing apoptosis and conferring resistance to

![Image](image-url)
chemotherapeutic agents in various cell lines (30), whereas Bax accelerates cell death in response to certain apoptotic stimuli. Cells treated with both thiostrepton and cisplatin displayed a greater decrease in Bcl-2 protein expression than cells treated only with cisplatin, and thiostrepton also enhanced cisplatin-induced increases in Bax, PARP and caspase-3 protein expression. These results suggest that thiostrepton sensitizes Daoy cells to cisplatin partly through enhanced apoptosis, possibly via a caspase-3-dependent pathway.

FOXM1 has been reported to play a central role in tumor metastasis (27-29), and few studies have explored the antitumor activity of thiostrepton. In our study, thiostrepton significantly decreased the migration and invasion capacity of Daoy cells. As these processes are both necessary steps for metastasis, we believe that thiostrepton may be a promising agent with which to antagonize the highly metastatic behavior of MB.

Collectively, our findings showed that thiostrepton induces apoptosis and significantly decreases the proliferation and metastatic potential of Daoy cells. Thiostrepton also acts synergistically with cisplatin by enhancing cisplatin-induced apoptosis. These results demonstrate that thiostrepton may have wide therapeutic and/or adjuvant applications for MB chemotherapy. Thiostrepton may be particularly useful in very young children with metastatic disease who currently must receive more aggressive chemotherapeutics.

Acknowledgements

This study was supported by The National Natural Science Foundation of China (81001119), The Grant Awarded to New Teachers from the Chinese Ministry of Education (201101171120112) and The Fundamental Research Funds for the Central Universities (11ykzd06).

References


