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지도교수 이 은 식

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Antitumor effects of MutT homolog 1 inhibitors in human bladder cancer cells

by

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A thesis submitted to the Department of Urology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Urology) at Seoul National University College of Medicine

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Abstract

Introduction: We investigated the antitumor effects and the possible molecular mechanisms of MutT homolog 1 (MTH1) inhibitors, TH588 or TH287, in cisplatin-sensitive (T24) and -resistant (T24R2) human bladder cancer cell lines.

Methods: Cell Counting Kit-8 (CCK-8) and clonogenic assays were performed to assess the anti-proliferative effect of TH588 or TH287 on T24 and T24R2 cells. The generation of reactive oxygen species (ROS) in T24 and T24R2 cells was evaluated with 2',7'-dichlorofluorescein diacetate using the IncuCyte® ZOOM System. Flow cytometry was performed to analyze the changes in cell cycle and apoptosis. The expression of proteins related to apoptosis and cell cycle was determined by western blotting.

Results: The CCK-8 and clonogenic assays demonstrated the dose-dependent antitumor effects of TH588 or TH287 on T24 and T24R2 cells. Treatment with TH588 or TH287 increased the relative level of reactive oxygen species in both cell lines. MTH1 expression was not dependent on the treatment dose of TH588 or TH287. TH588 or TH287 treatment induced apoptosis via increased expression of fragmented poly (ADP-ribose) polymerase, caspase-3, -8, -9, and cytochrome c. Cell cycle arrest induced by TH588 or TH287 was accompanied by decreased expression of cyclin A.

Conclusions: Our results suggest that TH588 or TH287 may induce cancer cell suppression by off-target effects rather than MTH1 inhibition in cisplatin-sensitive and –resistant bladder cancer cells. Further investigations or clinical trials are needed to confirm the antitumor effects and underlying molecular mechanisms of MTH1 inhibitors in human bladder cancer cells.

Key words: bladder cancer, cisplatin, MutT homolog 1, drug resistance, antitumor effect

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μM) treatment of T24 cells. *b*, TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *c*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. *d*, TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. (B) Cell cycle distribution was measured quantitatively. *a*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *b*, TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *c*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. *d*, TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

Figure 5. Effect of treatment with MTH1 inhibitors for 24 h on the expression of apoptosis-related proteins in bladder cancer cells. (a) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (b) TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (c) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. (d) TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

Figure 6. Effect of treatment with MTH1 inhibitors for 24 h on the expression of cell cycle-related proteins in bladder cancer cells. (a) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (b) TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (c) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. (d) TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

Introduction

Cisplatin-based combination chemotherapy is currently the first-line treatment for advanced or metastatic bladder cancer. Although bladder cancer is a chemosensitive disease, approximately 30% of patients do not respond to initial cisplatin-based therapy and most patients eventually progress to refractory or recurrent disease within the first year of treatment [1]. As a standard second-line regimen has not been established for patients who are refractory to or have relapsed with cisplatin-based chemotherapy, development of an effective class of novel chemotherapeutic agents is needed for the treatment of cisplatin-resistant bladder cancer.

Reactive oxygen species (ROS), which are by-products of dysfunctional redox regulation, play an important role in cancer cell survival, proliferation, and metastasis [2]. However, high levels of ROS trigger apoptosis and inhibition of proliferation via incorporation of oxidized deoxyribonucleotide-triphosphates into the genomic DNA, resulting in tumor growth suppression [3,4]. The MutT homolog 1 (MTH1) is overexpressed under increased oxidative stress in cancer cells [5]. This enzyme, which converts oxidized deoxyribonucleotide-triphosphates into monophosphates, prevents their incorporation into the DNA and inhibits cell death.[6)] Recent studies have demonstrated that the MTH1 enzyme is essential for cancer cell survival regardless of the tissue of origin [5,7-9]. Accordingly, MTH1 has been considered as a novel treatment strategy for cancer.

Several types of small molecules, such as (S)-crizotinib, SCH51344, TH588, and TH287 have

been reported as MTH1 inhibitors, and have demonstrated selective suppression of tumor growth in a number of different human cancer xenograft models, including osteosarcoma, non-small cell lung cancer, and colorectal cancer [5,7,8]. However, two recent studies reported that certain types of MTH1 inhibitors do not suppress survival and proliferation in human cancer cell lines [10,11]. A study by Kawamura *et al*. described that MTH1 is not absolutely necessary for cancer cell survival and the cytotoxicity of MTH1 inhibitors is attributed to off-target effects [12].

Few studies have investigated the cytotoxic effects of MTH1 inhibitors on human bladder cancer cells. The aim of this study was to investigate the antitumor effects and the underlying molecular mechanisms of MTH1 inhibitors in cisplatin-sensitive (T24) and -resistant (T24R2) human bladder cancer cell lines.

Materials and Methods

Cell Lines and Chemicals

The human bladder cancer cell line T24 (grade 3) was obtained from ATCC® (Manassas, VA, USA). The cisplatin-resistant bladder cancer cell line T24R2 was established by serial desensitization of T24 cells to increasing concentrations of cisplatin.[13] T24 and T24R2 cells were cultured in RPMI supplemented with 10% fetal bovine serum (Gibco®) and penicillin (100 U/mL)/streptomycin (100 mg/L) (Gibco®). TH588 and TH287 were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Cell Proliferation Assay

T24 and T24R2 cells were seeded in 96-well plates and treated with TH588 or TH287 for 24 and 48h, respectively. Following this, 10 μL of the Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added to each well containing 100 μL medium. Cell proliferation was measured by CCK-8 assay according to the manufacturer's instructions. After 4 h of incubation, the absorbance was measured at 450 nm with a plate reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was determined as the percentage of viable cells in the total population.

Clonogenic Assay

T24 cells were treated with TH588 (0, 1, 2.5, 5 or 10 μM) or TH287 (0, 1, 2.5, 5 or 10 μM) for 24 h. T24R2 cells were treated with TH588 (0, 1, 2.5, 5 or 10 μM) or TH287 (0, 0.5, 1, 2.5 or 5 μM) for 24 h. The cells were maintained for an additional 2 weeks in drug-free medium. The colonies formed were stained with 0.4% crystal violet. Plates were photographed and the number of colonies greater than 0.2 mm in diameter was counted.

ROS Assay

T24 and T24R2 cells treated with TH588 or TH287 for 24 h were incubated for 20 min with 20 mM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA) in a CO_2 incubator. DCF fluorescence was measured at 485 nm (excitation) and 530 nm (emission) wavelengths using the IncuCyte® ZOOM System (Essen Bioscience, Ann Arbor, Michigan, USA).

Flow Cytometry

T24 and T24R2 cells were treated with TH588 or TH287 for 24 h, fixed in 70% ethanol, and stained with propidium iodide (Sigma-Aldrich) solution for 30 min at 37°C. The DNA profile was measured using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA).

Apoptosis and Cell Cycle-Related Protein Expression

T24 and T24R2 cells were treated with TH588 or TH287 for 24 h. Protein was extracted using RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mM phenylmethylsulfonyl fluoride, fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Millipore^{TM}). The separated proteins were subsequently blotted using specific antibodies against poly (ADP-ribose) polymerase (PARP), caspase-3, -8, -9, cytochrome c, bcl-2, bad, cyclin A, E1, p-Akt, Akt, β-actin. Protein expression was detected with an enhanced chemiluminescence western blot substrate kit (Pierce, Rockford, IL, USA).

Statistical Analysis

Three independent experiments were performed in triplicate. Data analyses were performed using the statistical software SPSS ver. 20.0 (IBM, Armonk, NY, USA). All data are presented as mean ± standard deviation (S.D.). Statistical differences were determined by Tukey's multiple range test. $P < 0.05$ was considered statistically significant.

Results

Antitumor Effects of MTH1 Inhibitors on Human Bladder Cancer Cells

T24 and T24R2 cells were treated to increasing doses of TH588 or TH287 for 24 and 48 h. Based on the CCK-8 assay, the viability of T24 and T24R2 cells was significantly reduced by treatment with TH588 or TH287 in a dose-dependent manner (Fig. 1).

Colony formation assay was performed to evaluate the anti-proliferative effect of TH588 or TH287 on T24 and T24R2 cells. T24 cells were treated with different concentrations of TH588 (0, 1, 2.5, 5, or 10 μM) or TH287 (0, 1, 2.5, 5, or 10 μM) for 24 h. The number of T24 cell colonies was significantly reduced by treatment with 10 μM of TH588 or ≥ 5 μM of TH287 (Fig. 2, A & B). T24R2 cells were treated for 24 h with different concentrations of TH588 (0, 1, 2.5, 5, or 10 μM) or TH287 (0, 0.5, 1, 2.5, or 5 μM). Treatment with TH588 at a concentration of ≥ 5 μM or TH287 at a concentration of ≥ 2.5 μM significantly reduced the number of T24R2 colonies (Fig. 2, C & D).

Effects of MTH1 Inhibitors on the generation of ROS

After treatment with TH588 or TH287 for 24 h, ROS generation in T24 and T24R2 cells was evaluated with 2',7'-DCFH-DA using the IncuCyte® ZOOM System. Treatment of T24 cells with both TH588 and TH287 at a concentration of ≥ 1 μM each significantly increased the relative levels of ROS (Fig. 3, A & B). The relative ROS level in T24R2 cells was increased by

treatment with TH588 or TH287 in a dose-dependent manner (Fig. 3, C & D).

Effect of MTH1 Inhibitors on Cell Cycle Alteration

To evaluate the effect of TH588 or TH287 on cell cycle progression in T24 and T24R2 cells, we determined cell cycle distribution via flow cytometry. Exposure of T24 and T24R2 cells to TH588 or TH287 for 24 h resulted in a dose-dependent decrease in the number of cells in G1 phase. Depending on the dose of TH588 and TH287, the distribution of cells in the phases of cell cycle was shifted toward the sub-G1 and G2/M phases in both cell lines (Fig. 4, A & B).

Effect of MTH1 Inhibitors on Expression of Apoptosis Related Proteins

We performed western blot analysis to evaluate the changes in expression of apoptosis-related proteins following treatment of bladder cancer cells with MTH1 inhibitors. The expression of MTH1 was not dependent on the treatment dose of TH588 or TH287 in both T24 and T24R2 cells (Fig. 5). In both T24 and T24R2 cells, exposure to TH588 or TH287 for 24 h resulted in increased expression of fragmented PARP, caspase-3, -8, -9, and cytochrome c in a dose-dependent manner. The expression of Bcl-2 was decreased, whereas Bad level was increased depending on the incremental dose of TH588 or TH287 in T24 cells (Fig. 5, A & B).

Effect of MTH1 Inhibitors on Expression of Cell Cycle Related Proteins

The changes in expression of cell cycle-related proteins following treatment of T24 and

T24R2 cells with TH588 or TH287 for 24 h were examined by western blotting. Exposure to TH588 or TH287 for 24 h resulted in a decreased expression of cyclin A in both T24 and T24R2 cells. Depending on the dose of TH287, the expression of p-Akt was decreased in both cell lines (Fig. 6).

Figure 1. Cell Counting Kit-8 assay in T24 and T24R2 bladder cancer cells treated with MTH1 inhibitors TH588 (a) and TH287 (b) for 24 and 48 h. Asterisk indicates significant difference compared to control ($P < 0.05$).

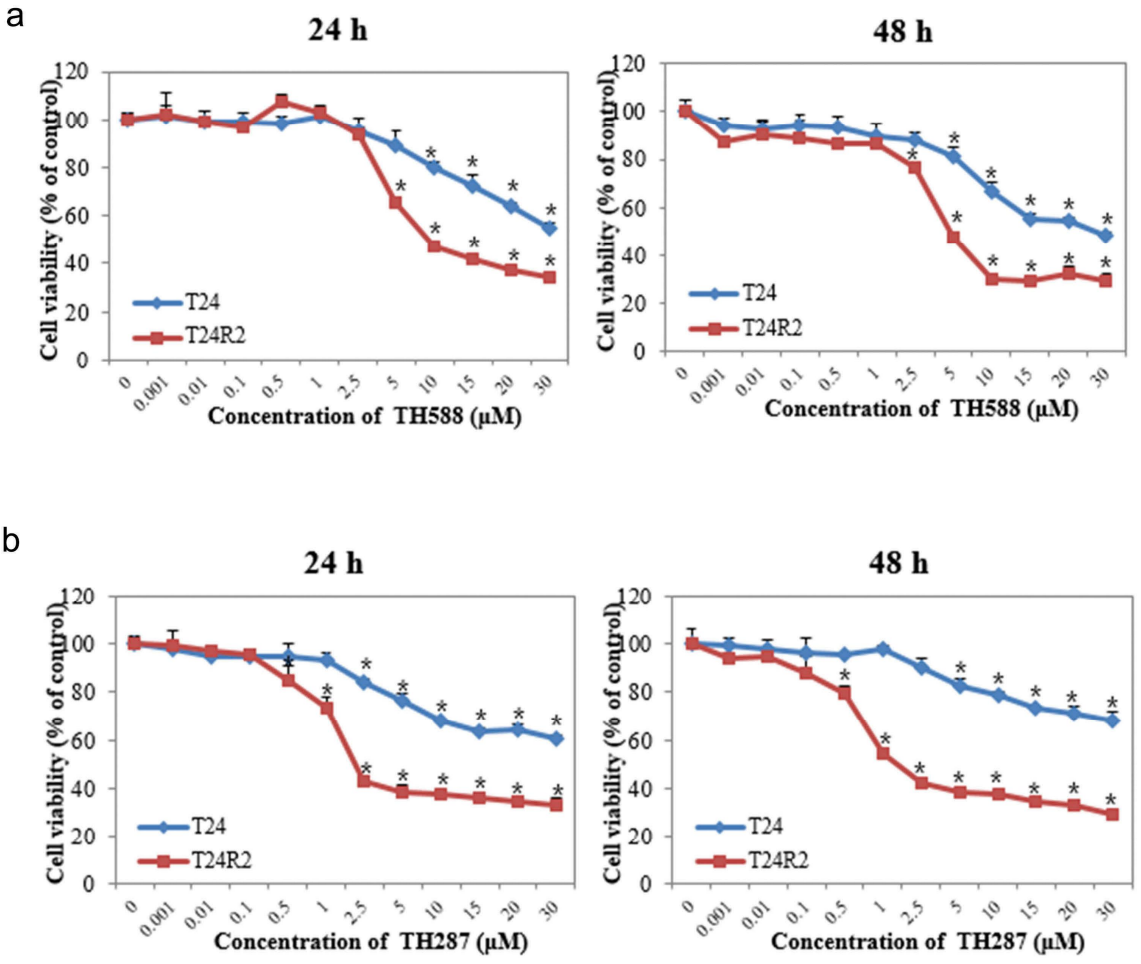


Figure 2. Clonogenic assay in bladder cancer cells treated with MTH1 inhibitors for 24 h. (a) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (b) TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (c) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. (d) TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

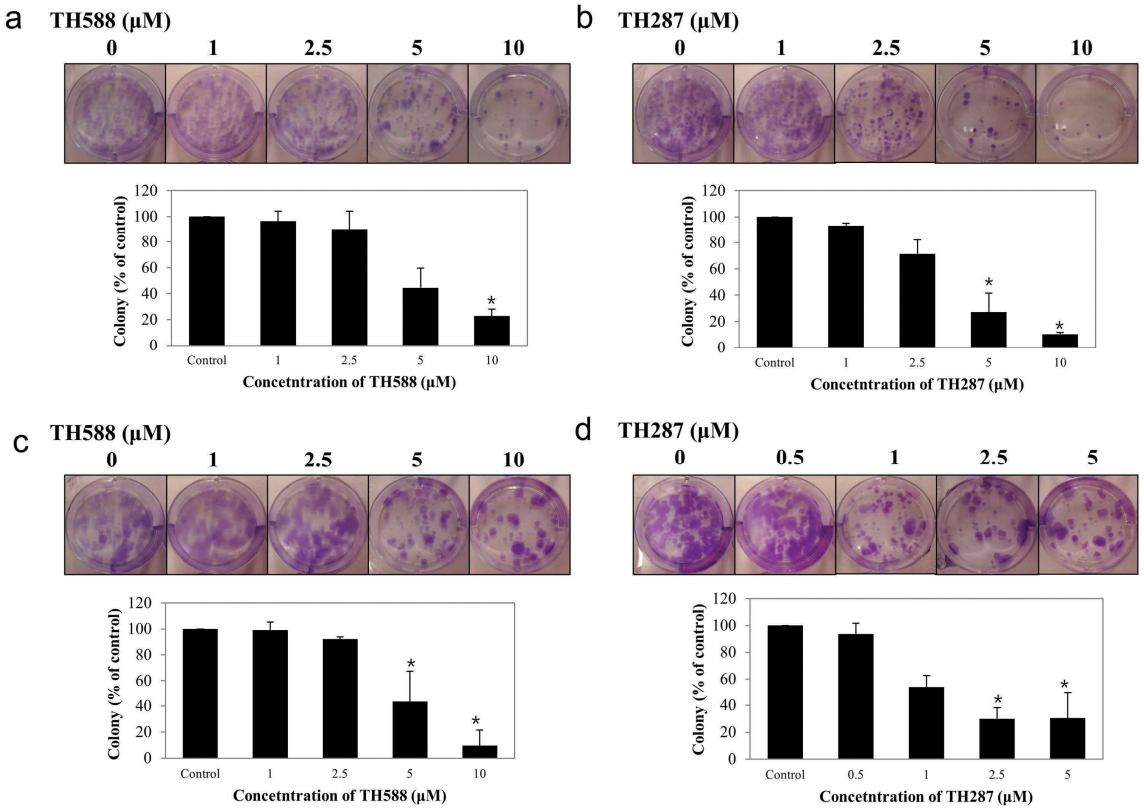


Figure 3. ROS levels in bladder cancer cells treated with MTH1 inhibitors for 24 h. (a) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (b) TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (c) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. (d) TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

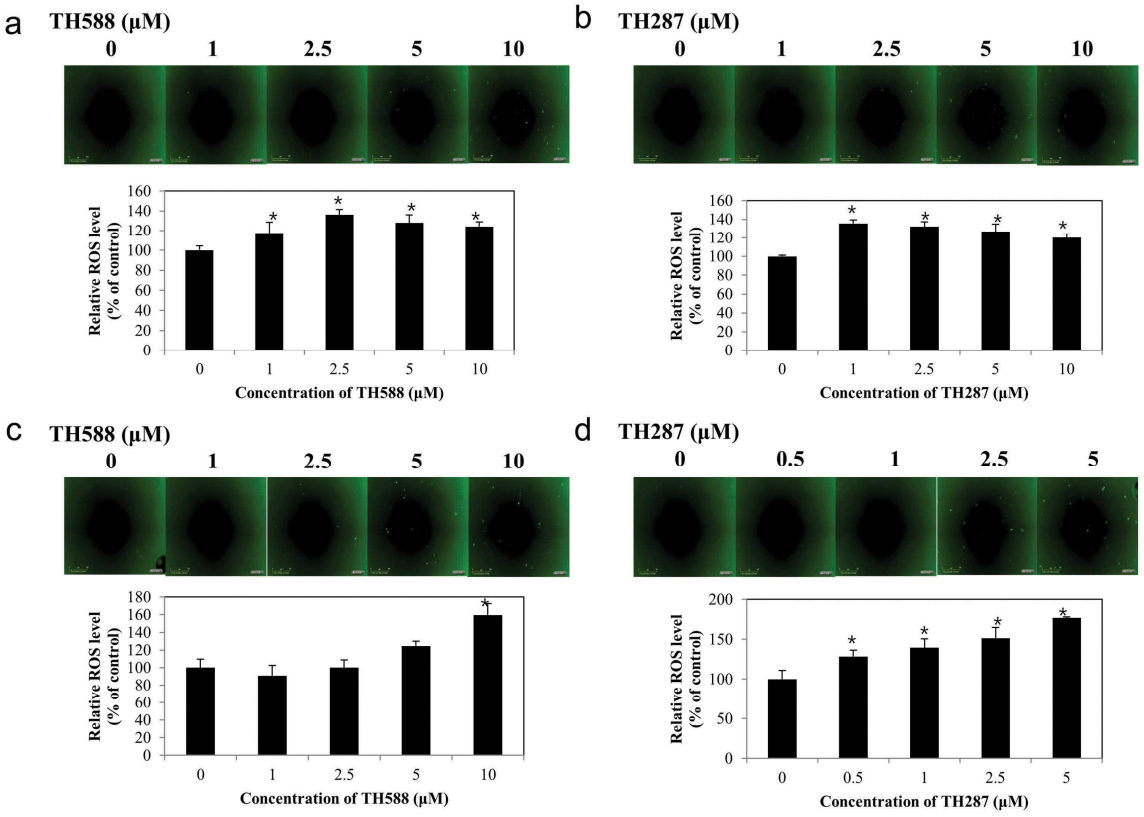


Figure 4. Flow cytometric analysis. (A) Flow cytometric DNA content histogram of T24 and T24R2 bladder cancer cells treated with MTH1 inhibitors for 24 h. *a*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *b*, TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *c*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. *d*, TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. (B) Cell cycle distribution was measured quantitatively. *a*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *b*, TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. *c*, TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. *d*, TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).

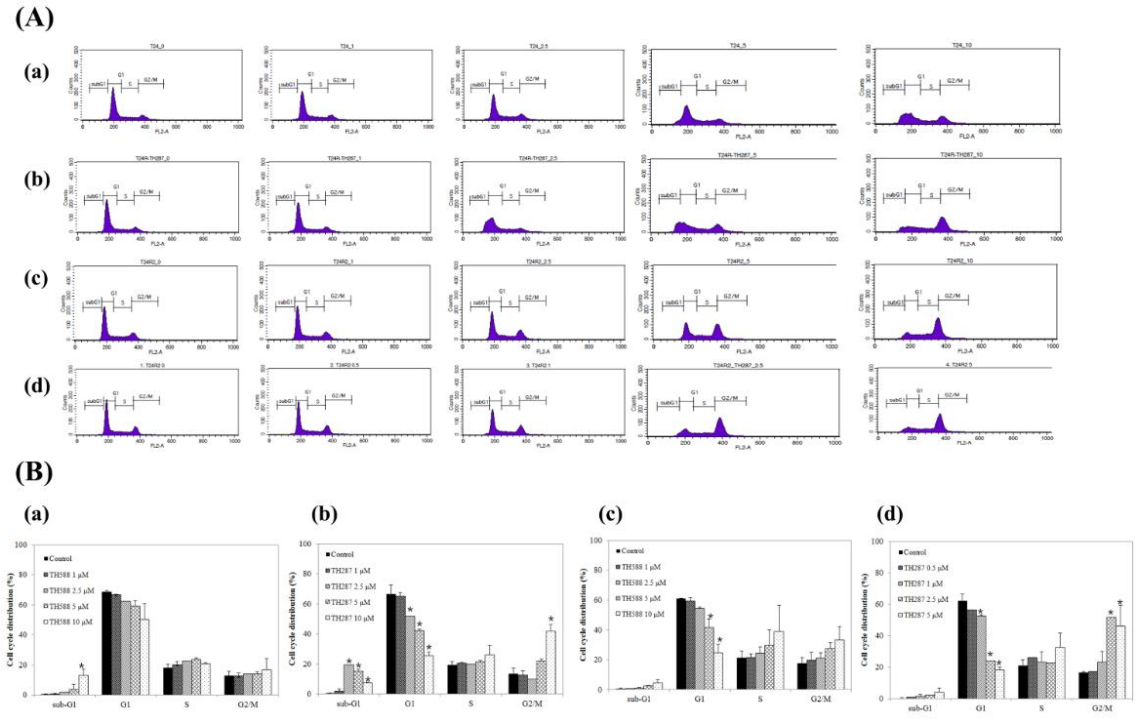
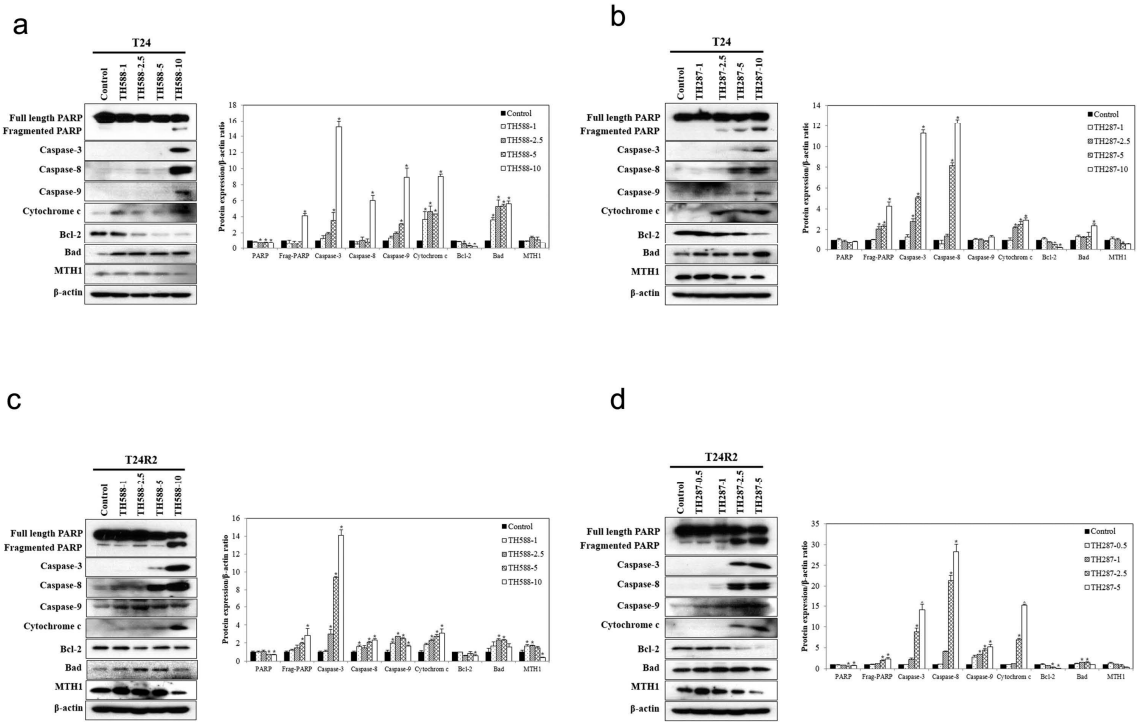


Figure 5. Effect of treatment with MTH1 inhibitors for 24 h on the expression of apoptosis-related proteins in bladder cancer cells. (a) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (b) TH287 (0, 1, 2.5, 5, or 10 μM) treatment of T24 cells. (c) TH588 (0, 1, 2.5, 5, or 10 μM) treatment of T24R2 cells. (d) TH287 (0, 0.5, 1, 2.5, or 5 μM) treatment of T24R2 cells. Asterisk indicates significant difference compared to control ($P < 0.05$).



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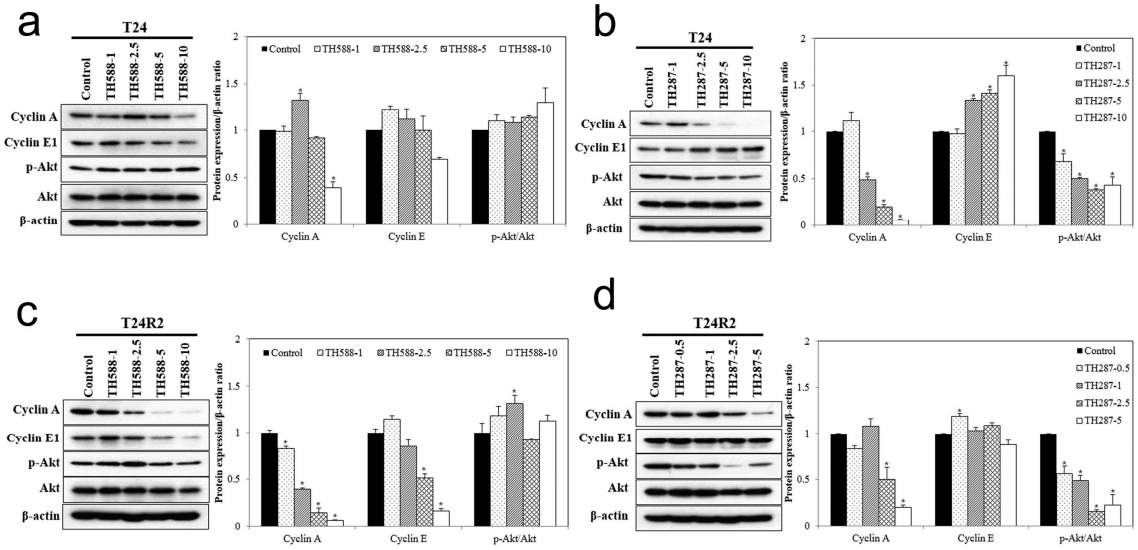

Discussion

Defects in apoptotic pathways play a crucial role in tumorigenesis and drug resistance. Reactivation of the apoptotic pathway bypassing the defects associated with resistance to therapy is currently the main focus of drug development in cancer therapy [14,15]. Cisplatin is the first platinum-based DNA-targeting anticancer drug that is used to treat a number of cancers, including lung, colorectal, ovary, and bladder cancers [16]. Cisplatin-based combination chemotherapy is the first-line treatment for advanced or metastatic bladder cancer. The efficacy of cisplatin, which is associated with apoptosis and cell cycle alterations in the G2/M phase, is often limited by the development of drug resistance [16]. No standard second-line chemotherapeutic regimen has been established for patients with cisplatin-resistant bladder cancer. Therefore, it is necessary to develop novel chemotherapeutic agents effective against cisplatin-sensitive and –resistant bladder cancer cells. The present study, to our knowledge, is the first to investigate the antitumor effects and underlying molecular mechanisms of MTH1 inhibitors (TH588 and TH287) in human bladder cancer cells.

It has been reported that the MTH1 enzyme, which is not normally essential, is necessary for the survival of cancer cells regardless of the tissue of origin. This enzyme prevents tumor suppression induced by high levels of ROS [5,7-9]. MTH1 inhibitors, such as TH287 and its metabolically more stable analog TH588, suppress tumor growth and induce impressive therapeutic responses in a variety of human cancers [5,7]. In contrast, recent studies have

demonstrated that certain classes of MTH1 inhibitors exhibit limited cytotoxicity in human cancer cells and questioned whether MTH1 is a therapeutic target [10,11]. In a study by Kawamura *et al*. [12], TH588 and TH287 induced phosphorylation of Bcl-2 and cell cycle arrest at the G2/M phase but suppressed *in vitro* tubulin polymerization in HeLa cells. These findings suggest that the therapeutic target of TH588 and TH287 could be tubulin, which is responsible for the cytotoxic effects, rather than MTH1.

Based on CCK-8 and colony formation assays, the present study revealed the dose-dependent antitumor effects of TH588 and TH287 on both T24 and T24R2 cells. After exposure to TH588 or TH287 for 24 h, the relative ROS level was increased in T24 and T24R2 cells. However, MTH1 expression was not dependent on the treatment dose of TH588 or TH287 in both cell lines. These results suggest that TH588 and TH287 may induce cancer cell suppression by off-target effects rather than MTH1 inhibition in cisplatin-sensitive and -resistant bladder cancer cells. (S)-crizotinib, which is an MTH1 inhibitor, can induce oxidative stress in several cancer cell types [17]. In gastric cancer cells, (S)-crizotinib increases the intracellular levels of ROS in a dose-dependent manner, which causes ROS-induced apoptosis [17]. The therapeutic target of (S)-crizotinib was independent of MTH1 [17]. In a study by Aristizabal Prada *et al.* [18], TH588 efficiently suppressed the neuroendocrine cancer cells by downregulating the PI3K-Akt-mTOR pathway and increasing oxidative stress and apoptosis.

Cisplatin mainly kills tumor cells by apoptosis via caspase activation at the beginning of apoptosis. Activation of caspase-8 and -9 induces the activation of downstream caspases, such

as caspase-3 and -7 [19]. The major pathways of apoptotic cell death include the extrinsic death receptor and intrinsic mitochondrial pathways. Resistance to cisplatin might be mediated via loss of or decreased expression of pro-apoptotic factors or increased expression of anti-apoptotic proteins [20,21].

Our data showed that treatment of both T24 and T24R2 cells with TH588 or TH287 increased the expression of fragmented PARP, caspase-3, -8, -9, and cytochrome c in a dose-dependent manner. These results suggest that the cytotoxic effects of TH588 and TH287 might be associated with an altered apoptotic pathway in both T24 and T24R2 cells.

Bcl-2 is an anti-apoptotic protein that interferes with the apoptotic death of cancer cells [22], and Bad is a known pro-apoptotic protein that blocks the anti-apoptotic function of Bcl-2 in the mitochondria [23]. According to the incremental dose of TH588 or TH287, the expression of Bcl-2 was decreased, whereas that of Bad was increased in T24 cells. In T24R2 cells, Bcl-2 expression tended to decrease, whereas Bad expression increased as compared with the control. Akt is a serine/threonine-specific protein kinase that plays an important role in various cellular processes, such as survival, apoptosis, and cell proliferation. In this study, the level of Akt phosphorylation was remarkably decreased in both T24 and T24R2 cells, depending on the dose of TH287. These findings demonstrated that treatment with TH588 or TH287 inhibits cell proliferation via alterations in the expression of apoptosis- and cell survival-related proteins in both T24 and T24R2 cells.

Flow cytometry revealed that exposure to TH588 or TH287 results in a decrease in the

number of cells in G1 phase and an increase in the number of cells in sub-G1 and G2/M phase in both T24 and T24R2 cell lines. Cyclin A expression in both cell lines was decreased depending on the dose of TH588 and TH287. Our results suggest that TH588 and TH287 suppress cell proliferation by downregulating cyclin A expression, leading to G2/M phase cell cycle arrest and apoptosis.

MTH1 inhibitors, such as TH588 and TH287, have potent antitumor effects on cisplatin-sensitive and cisplatin-resistant bladder cancer cells. Hence, our results suggest that MTH1 inhibitors are an attractive novel class of chemotherapeutic agents for the treatment of not only patients with advanced bladder cancer, but also patients who are refractory to or have relapsed with cisplatin-based combination chemotherapy. Further investigations or clinical trials are needed to confirm the antitumor effects and underlying molecular mechanisms of MTH1 inhibitors in human bladder cancer cells.

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국문초록

인간 방광암 세포주에서 MutT homolog 1 inhibitors 의 항암 효과

서론: 인간 방광암 세포주에서 MutT homolog (MTH)1 inhibitor 의 항암효과와 그 기전을 분자생물학적 수준에서 분석함으로써 전이성 방광암에 대한 새로운 항암 후보물질로서의 MTH1 inhibitor 를 규명하고, 나아가 cisplatin 내성 방광암에 대한 2 차 항암화학요법의 근간으로써 MTH1 inhibitor 의 임상적 활용에 대한 기초자료를 확보하고자 하였다.

방법: 침윤성 방광암 세포주 T24 와 cisplatin 내성 방광암 세포주 T24R2 에 대한 MTH1 inhibitors (TH588, TH287)의 항암효과를 확인하기 위해 Cell Counting Kit-8 (CCK-8)과 클론원성 분석 (clonogenic assay)을 시행하였다. T24 와 T24R2 에 TH588 또는 TH287 을 용량별로 처리한 후 IncuCyte® ZOOM System 을 이용하여 reactive oxygen species (ROS)의 발현을 분석하였다. TH588 과 TH287 처리 후 T24 와 T24R2 의 세포주기 변화를 확인하기 위해 유세포 분석 (flow cytometry)을 시행하였고, 세포주기 및 세포고사 관련 단백질 발현의 변화를 측정하기 위해 Western blot 을 시행하였다.

결과: CCK-8 과 클론원성 분석 결과 TH588 과 TH287 은 T24 와 T24R2 에서 용량 의존적 항암 효과를 보여주었다. T24 와 T24R2 에 TH588 또는 TH287 을

처리하였을 때, 두 세포주 모두에서 ROS 가 증가하였다. 유세포 분석 결과 24 시간 동안 TH588 또는 TH287 을 처리하였을 때 T24 와 T24R2 두 세포주에서 용량 의존적으로 G1 phase 는 감소하였고, sub-G1 과 G2/M phase 는 증가하였다. Western blot 결과, MTH1 발현은 TH588 또는 TH287 의 처리 용량에 의존하지 않았다. TH588 과 TH287 은 fragmented poly (ADP-ribose) polymerase, caspase-3, -8, -9, cytochrome c 의 발현을 증가시켜 세포사멸을 유도하였고, cyclin A 의 발현 감소를 통해 세포주기 정지를 유도하였다.

결론: 본 연구결과 MTH1 inhibitor 인 TH588 과 TH287 은 시스플라틴 민감성 및 내성 방광암 세포에서 MTH1 억제가 아닌 표적 외 효과로 항암효과를 유도하였다. 그 기전을 확인하기 위한 추가 연구가 필요하다.

주요어: 방광암, 시스플라틴, MutT homolog 1, 약제 내성, 항암효과

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감사의 글

이 논문이 완성될 때까지 도와주신 모든 분들께 진심으로 감사드립니다.

저를 현재의 비뇨의학과 의사로 학문적으로나 인간적으로 아낌없는 가르침을 주시는 지도 교수님이신 이은식 교수님과 서울의대 비뇨의학교실 모든 교수님들께 깊은 감사를 드립니다. 그리고 연구를 진행하고 논문을 완성하는데 많은 도움을 주신 호진녕 박사님께 진심으로 감사드립니다.

바쁘신 중에도 이 논문을 성심껏 심사해주신 분당서울대학교병원 변석수 교수님과 이상철 교수님, 서울대학교병원 병리학교실 문경철 교수님, 그리고 동국대학교 일산병원 이해원 교수님께도 감사를 드립니다.

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