**Suberoylanilide Hydroxamic Acid Can Re-sensitize a Cisplatin-Resistant Human Bladder Cancer**

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Cisplatin chemotherapy is the standard treatment for metastatic urothelial carcinoma. Although there are second-line chemotherapeutic agents approved by the U.S. Food and Drug Administration (FDA) such as those targeting programmed death-ligand 1 (PD-L1), more effective pharmacotherapy is required for cisplatin-resistant bladder cancer due to its limited overall survival and progression-free survival. The synergistic anti-cancer effect of cisplatin and suberoylanilide hydroxamic acid (SAHA) in cisplatin-resistant bladder cancer cells (T24R2) was examined. Tumor cell proliferation and cell cycle was examined using the cell counting kit (CCK)-8 assays and flow cytometry, respectively. Synergism was examined using the combination index (CI). CCK-8 assay and CI test were used to observe the strong synergistic anti-cancer effect between SAHA and cisplatin. Activation of caspase mediated apoptosis, down-regulated expression of the anti-apoptotic B-cell lymphoma-2 (Bcl-2) and up-regulated expression of pro-apoptotic B-cell lymphoma-2 (Bcl-2)-associated death promoter (BAD) were observed in Western blot. SAHA synergistically could partially re-sensitize cisplatin-resistant bladder cancer cells (T24R2) through the cell cycle arrest and induction of apoptosis pathway. SAHA-based treatment could be a potential treatment regimen in patients with cisplatin resistant bladder cancer.

**Key words** cisplatin resistance; suberoylanilide hydroxamic acid; bladder cancer

**INTRODUCTION**

Urinary bladder cancer is the second most common urologic cancer after prostate cancer and the seventh most common cancer worldwide. Approximately 30% of patients are initially diagnosed with muscle-invasive bladder cancer, which is treated with cisplatin-based combination chemotherapy followed by radical cystectomy. Nevertheless, half of these patients eventually develop incurable metastatic disease. Cisplatin-based chemotherapy is the most widely used first-line regimen for advanced or metastatic bladder cancer. However, cotreatment with cisplatin and other cancer drugs is considered a novel therapeutic approach because of its chemoresistance and undesirable side effects.

Histone deacetylase (HDAC) is a major epigenetic regulator in gene transcription. HDAC modulates cell differentiation, cell cycle, and apoptosis by regulating the acetyl group, which binds to the histone protein of DNA. Several studies have explored the synergistic anti-cancer effect of combination treatment with HDAC inhibitors and DNA-targeting agents in several malignant diseases. HDAC inhibitors downregulate the constitutive activation of nuclear factor (NF)-κB by generating reactive oxygen species (ROS), which causes conformational changes in B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax) protein, leading to the loss of mitochondrial membrane potential and the release of cytochrome c into the cytosol. The release of cytochrome c then activates caspase-9, caspase-3, and poly(ADP)-ribose polymerase (PARP) cleavage, leading to caspase-dependent apoptosis. Therefore, the anti-cancer effects of HDAC inhibitors suggest that they might be useful chemotherapy agents for bladder cancer.

Recently, suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor, was approved for the treatment of lymphoma by the U.S. Food and Drug Administration (FDA). In cervical cancer, the combination of cisplatin and SAHA has shown synergistic effects on HeLa cell viability by downregulating Bcl-2. In breast cancer cell lines, cotreatment with SAHA, claritromycin, and bortezomib potently enhanced the apoptosis-inducing effect compared to treatment using each agent alone based on the overexpression of endoplasmic reticulum (ER)-stress-related genes and enhanced apoptosis.

In this study, we investigated the synergistic anti-cancer effect of SAHA and cisplatin in cisplatin-resistant T24R2 bladder cancer cells by analyzing the expression of cell-cycle modulating proteins. This study will provide basic information for the development of new treatment strategies for patients with cisplatin-resistant bladder cancer.

**MATERIALS AND METHODS**

**Cell Lines and Chemotherapeutic Agents** Cisplatin-sensitive T24 bladder cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). The cisplatin-resistant bladder cancer cell line T24R2 was generated using serial desensitization. Cells were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen, Carlsbad, CA, U.S.A.) and 50 U/mL penicillin/50 mg/L streptomycin (Gibco/Invitrogen). SAHA and cisplatin were obtained from Sigma and Pfizer Korea Ltd., Seoul, Korea, respectively.

**Cell Proliferation Assay** Cells in 96-well plates were treated with variable cisplatin (0.05 to 100.0 µg/mL) or SAHA (0.25 to 500.0 µM) for 48 and 72 h. Ten microliters of cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, U.S.A.) solution was added to each well con-
taining 100 µL medium. After 4 h incubation, the absorbance was measured at a wavelength of 450 nm.

Synergism Determination The synergism of the two drugs was determined based on the combination index (CI), which was calculated using CalcuSyn version 2.1 (Biosoft®). CI values <1.0, >0.1, and =1 indicate synergism, antagonism, and additive effects, respectively.

Clonogenic Assay The cisplatin-resistant bladder cancer cells T24R2 were incubated with serial concentrations of cisplatin and SAHA. The cells were incubated for another 2 weeks in chemotherapeutic agent-free condition. The colonies formed were stained with 0.1% crystal violet solution. The number of colonies >0.2 mm in diameter was quantified.

Cell Cycle Analysis Using Flow Cytometry T24R2 cells were treated with cisplatin (2.5 µg/mL), SAHA (2.5 µM), or both for 48 h, followed by flow cytometric analyses using a previously described method.14) Cell cycle distribution was determined using the fluorescence-activated cell sorting (FACS) Calibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, U.S.A.).

Synergism-Related Protein Expression Using Western Blotting T24R2 cells were treated with cisplatin (2.5 µg/mL), SAHA (2.5 µM) or both for 48 h and then the proteins were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Rockford, IL, U.S.A.). After separation using sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.), which were blocked with Tris-buffered saline plus Tween (TBS-T) containing 5% milk for 1 h, followed by incubation with primary antibodies at 4°C overnight. The following primary antibodies were used: cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, PARP, Bcl-2, Bcl-2-associated death promoter (BAD), cytochrome c, β-actin, p21, cyclin A2, cyclin D1, cyclin E1, phosphorylated-AKT (p-AKT), total AKT, p-extracellular signal-regulated kinase (ERK), and total ERK (Cell Signaling Technology®, Beverly, MA, U.S.A.). After incubation with secondary antibodies, the protein expression was detected using an enhanced chemiluminescence Western blot substrate kit (Pierce, Rockford, IL, U.S.A.).

Statistical Analysis Unless otherwise specified, results are presented as the means ± standard deviation (S.D.) after a minimum of three repeated experiments. Tukey’s multiple range test was used to assess the statistical significance. p-Values <0.05 were considered statistically significant. All analyses were conducted using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Anti-cancer Effect of SAHA on Cisplatin-Sensitive (T24) and Cisplatin-Resistant (T24R2) Bladder Cancer Cells Cisplatin showed significant anti-cancer effects against T24 bladder cancer cells in a concentration- and time-dependent manner (Fig. 1). At a concentration range of 0.05 to 100 µg/mL of cisplatin with 48 and 72 h exposure, cisplatin treatment suppressed the proliferation of T24 cells by up to almost 90%, including a mean of 86.67 ± 0.08% at 48 h and 91.92 ± 0.11% at 72 h. At a concentration range of 0.25 to 500 µM of SAHA, SAHA suppressed the proliferation of T24 cells by up to 87%, including a mean of 78.48 ± 0.60% at 48 h and 87.12 ± 0.72% at 72 h. However, T24R2 cells virtually showed no response to cisplatin treatment at a concentration of up to 2.5 µg/mL (91.62 ± 9.44%, Fig. 1). The mean cisplatin IC50 of T24R2 cells at 48 and 72 h of exposure was 6.19 and

Fig. 1. Effect of Cisplatin and Suberoylanilide Hydroxamic Acid (SAHA) on Bladder Cancer Cell Survival

Cell survival was determined using cell counting kit (CCK)-8 assay experiments. Cisplatin concentrations of 0.05 to 100 µg/mL for 48 and 72 h. Anti-proliferative effect of cisplatin determined in T24R2 cells. Asterisks (*) indicate significantly higher survival of T24R2 than T24 cells, which were treated with SAHA at 0.25 to 500 µM for 48 and 72 h. Values are means ± standard deviation (S.D., n = 5). *p < 0.05 compared to T24 cells.
5.56 µg/mL, respectively. Compared to the sensitivity of the parent T24 cells, T24R2 cells were significantly more resistant to cisplatin treatment after 48 and 72 h exposure. However, significant anti-cancer effects against T24R2 bladder cancer cells in a concentration-dependent and time-dependent manner were observed after 48 and 72 h exposure to SAHA treatment (Fig. 1).

SAHA Induces Re-sensitization of Cisplatin-Resistant (T24R2) Bladder Cancer Cells Combined treatment with SAHA and cisplatin resulted in significant inhibition of growth than either monotherapy. Co-treatment with SAHA at concentrations greater than 0.5 µM potentiated and restored the anti-cancer effect of cisplatin on T24R2 cells to almost the similar level as that on T24 cells (Fig. 2). When the data were analyzed based on the CI, significant synergism was found with a CI < 1 for most concentration tested with SAHA and cisplatin. These results showed strong synergistic effects on T24R2 cells at a broad range of concentrations of cisplatin and SAHA (Fig. 2B). The CI of 2.5 µg/mL cisplatin and 2.5 µM SAHA after 48 (0.526) and 72 h (0.526) exposure was < 1.0, indicating the adequate synergism between the two agents (Fig. 2B). Clonogenic assays were also performed to determine the anti-cancer effects of 48 h treatment with 2.5 µg/mL cisplatin and 2.5 µM SAHA alone or in combination on cisplatin resistant T24R2 cells. The clonogenicity of T24R2 cells was markedly inhibited by cisplatin (mean 66.00 ± 2.51% of control) or SAHA (63.13 ± 12.12%) as a single agent. Significant decrease in clonogenicity was observed when T24R2 cells were exposed to both cisplatin and SAHA (mean 4.55 ± 7.88%, Fig. 3) than either agent alone like the result of CI test.

SAHA and Cisplatin Combination Treatment Induce Cell Cycle Alterations S phase cell cycle arrest (13.11 ± 4.76 to 19.37 ± 4.09%), G2/M phase cell cycle arrest (20.13 ± 4.79 to 28.15 ± 7.95%) and an increase of the subG1 population (0.49 ± 0.13 to 1.79 ± 0.30%) in T24R2 cells were observed after exposure to cisplatin single treatment (Fig. 4). The increase in the G1 phase arrest (66.57 ± 9.57 to 72.52 ± 2.84%), were observed after exposure to SAHA single treatment. Combined treatment at suboptimal concentrations of cisplatin (2.5 µg/mL) and SAHA (2.5 µM) significantly
increased the subG1 phase (0.49 ± 0.13 to 4.22 ± 0.33%) and S phase (13.11 ± 4.76 to 42.06 ± 3.81%) compared to the untreated control cells.

SAHA and Cisplatin Combination Treatment Induce Changes in Apoptosis-Related Expression

Single treatment with 2.5 µg/mL cisplatin or 2.5 µM SAHA in T24R2 cells increased the expression of caspase-3 and caspase-9. Combined treatment with 2.5 µM SAHA and 2.5 µg/mL cisplatin significantly induced the up-regulated expression of the cleaved caspase-3, cleaved caspase-8, and cleaved caspase-9, as well as fragmented PARP and cytochrome C (Figs. 5A, B). The down-regulated expression of anti-apoptotic Bcl-2 and up-regulated expression of pro-apoptotic BAD and BAX were observed following dual treatment with cisplatin and SAHA (Figs. 5A, B). The ratio of Bcl-2/BAX was also significantly reduced by combination treatment with 2.5 µM SAHA and 2.5 µg/mL cisplatin compared with the untreated control cells (1.00 ± 0.02 vs. 0.26 ± 0.03). These results suggested that combination treatment with 2.5 µM SAHA and 2.5 µg/mL cisplatin could promote apoptosis of bladder cancer cells via mitochondria-mediated apoptosis pathway. Thus, activation of the intrinsic apoptotic pathway in cisplatin resistant bladder cancer cells could be associated with the synergism of the dual treatment with cisplatin and SAHA.

DISCUSSION

The current standard first-line therapy for advanced urothelial carcinoma including bladder cancer is cisplatin-based chemotherapy. In addition, for cisplatin-ineligible patients, immunotherapeutic agents such as pembrolizumab and atezolizumab have recently been recommended as second-line treatments in muscle-invasive bladder cancer. However, the overall survival rate with pembrolizumab and atezolizumab is still limited to 10.3 and 7.9 months, respectively, and the efficacy of these drugs is reported to decrease in patients with...
low programmed death-ligand 1 (PD-L1) expression levels.\textsuperscript{15,16} Considering the numerous side effects and resistance mechanism of cisplatin-based chemotherapy, research into the development of ideal chemotherapeutic agents in muscle-invasive bladder cancer is ongoing.

HDAC inhibitors have a main mechanism composed of cell cycle arrest, apoptosis, autophagy, anti-angiogenic effects and anti-cancer effects, and SAHA is known to be a pan-HDAC inhibitor class associated with all mechanisms of anti-cancer effects such as CDKN1A/p21 cell cycle arrest, apoptosis through intrinsic pathway, autophagy, etc.\textsuperscript{17,18} Unlike chemotherapeutic agents that also affect normal cells, HDAC inhibitors have been reported to induce highly selective anti-cancer effects with less toxicity to normal cells.\textsuperscript{19} The evidence for mechanisms of HDAC inhibitors action has not yet been established, but it is presumed that the cause is the relative specificity of epigenetic regulatory mechanisms between normal and cancer cells. HDACs are an essential factor in the growth and survival of cancer cells, but not in normal cells. Also, depending on the type of cancer cells, the mechanisms of anti-cancer effects can be completely different.\textsuperscript{20}

HDAC inhibitors have been reported to have synergistic anti-cancer effects with DNA damaging agents, and SAHA is known to cause anti-cancer effects in combination with carfilzomib and bortezomib in T-cell leukemia and myeloma. Cisplatin has been reported to have synergistic effects with valproic acid and belinostat in neuroblastoma and lung cell models, but no studies have been conducted on HDAC inhibitor combinations in bladder cancer cells.\textsuperscript{21,22} In this study, we demonstrated that combination of SAHA as a HDAC inhibitor with cisplatin had synergistic anti-cancer effects on advanced bladder cancer cells. We have shown that the cisplatin-resistant human bladder cancer cell-line was re-sensitized by cell cycle arrest and induction of caspase-dependent apoptosis.

Deregulation of cell cycle is one of the critical events of cancer progress.\textsuperscript{23} In the current study, exposure of T24R2 cells to cisplatin and SAHA (pan HDAC inhibitor) treatment resulted in cell cycle arrest at the subG1 phase and S phase, although an valproic acid (HDAC inhibitor type I, IIa) induced G1 phase cell cycle arrest in time- and dose-dependent manners in muscle-invasive human bladder cancer cell lines.\textsuperscript{24} Cyclins A, D1, and E are known as G1/S-phase regulators,\textsuperscript{25,26} while p21 is also a crucial regulator of cell cycle progression in the G1 and S phases and a directly inhibit DNA synthesis by binding to proliferating cell nuclear antigen (PCNA).\textsuperscript{23,27} The present study revealed combination treatment with SAHA and cisplatin significantly reduced the expressions of S phase-related proteins including cyclins A2, D1, and E1, resulting in S phase cell cycle arrest. P21 overexpression also promotes S phase cell cycle arrest. Dual treatment with SAHA and cisplatin in cisplatin-resistant bladder cancer cells significantly improved cisplatin-mediated S phase cell cycle arrest with outstanding suppression of cyclins A2, D1, and E1 and induction of p21. Dual treatment with SAHA and cisplatin also significantly increased the subG1 population with increased expression of cleaved caspases-3, -8, and -9 accompanied by PARP cleavage. These findings imply that induction of caspase-dependent apoptosis may partially play important roles in these synergistic anti-cancer effects and re-sensitization of cisplatin resistance mechanism of bladder cancer cells.

SAHA based treatment resulted in the down-regulated expression of AKT, a survival regulator.\textsuperscript{28} Attenuation of AKT signaling restores cisplatin sensitivity, suggesting this could be a potential therapeutic strategy for preventing tumor recurrence by re-sensitizing advanced bladder cancer.\textsuperscript{29}

Bcl-2 protein is known to be an integral outer mitochondrial membrane protein that prevents apoptotic cell death.\textsuperscript{30} However, BAD, a pro-apoptotic protein, is reported to play a role in interfering with the function of Bcl-2.\textsuperscript{31} Dual treatment of T24R2 cells with cisplatin and SAHA downregulated the
expression of Bcl-2 while that of Bad was upregulated. The up-regulated expression in cytochrome c with a simultaneous up-regulated expression in pro-apoptotic BAD and down-regulated expression of the anti-apoptotic in Bcl-2 after combination treatment with cisplatin and SAHA could suggest that the intrinsic apoptotic pathway is activated, which might be the underlying mechanism of these synergistic anti-cancer effects. Up-regulated activities of proteins such as cytochrome c and caspase-3 were known to be involved in the intrinsic apoptotic pathway through alteration of Bcl-2 family protein expression.\textsuperscript{22} The identification of these molecules related with the intrinsic apoptotic pathway in our study could contribute to their role in the synergistic effect of cisplatin and SAHA in terms of apoptosis pathway.

SAHA synergistically could partially re-sensitize cisplatin-resistant bladder cancer cells (T24R2) through the cell cycle arrest and induction of apoptosis pathway. These findings suggest that SAHA-based treatment could be the potential treatment regimen in patients with cisplatin resistant bladder cancer.

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Conflict of Interest The authors declare no conflict of interest.

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