의학박사학위논문

Anaysis of anti-cancer effect of suberoylanilide hydroxamic acid (SAHA) in cisplatin resistant human bladder cancer cell-lines

cisplatin 저항성 인간 방광암 세포주에서 suberoylanilide hydroxamic acid (SAHA)의 항암 효과 기전 분석

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의학과 비뇨기과학 전공
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Abstract

Anaysis of anti-cancer effect of suberoylanilide hydroxamic acid (SAHA) in cisplatin resistant human bladder cancer cell-lines

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Introduction and Objective: Cisplatin-based chemotherapy remains first-line treatment for advanced bladder cancer. No standard chemotherapeutic agent has been established for patients with cisplatin resistant bladder cancer. We investigated the synergistic antitumor effect of suberoylanilide hydroxamic acid (SAHA) and cisplatin in cisplatin resistant bladder cancer cells.

Methods: The cisplatin resistant human bladder cancer cell line (T24R2) was treated with cisplatin and/or SAHA. Tumor cell proliferation was assessed by cell counting kit-8 assay and clonogenic assay. Synergism was determined by combination index. Changes in cell cycle were determined by flow cytometry. Expression of caspase-3, 8 and 9, PARP,

cytochrome c, p21, Bcl-2, Bad, p27, cyclin A, cyclin D1, and cyclin E were analyzed by Western blotting.

Results: Synergistic antitumor effect between cisplatin and SAHA was observed by cell counting kit-8 assay, clonogenic assay and confirmed with combination index less than 1.0. The underlying mechanism could be synergistic cell cycle arrest, activation of apoptotic pathway including capase-3, -8, -9 and fragmented PARP or decreased expression in anti-apoptotic Bcl-2 and increased expression in pro-apoptotic BAD.

Conclusions: SAHA may synergistically enhance the antitumor effect of cisplatin and re-sensitize cisplatin resistant bladder cancer cells. These findings suggest the potential use of SAHA as a combination agent to enhance the antitumor effect of cisplatin in patients with advanced bladder cancer.

Keywords: cisplatin resistance, SAHA, T24R2 cell

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Introduction

Bladder cancer is one of the most common urinary tract cancers and the eleventh most common cancer in the world. (Park J 2010) At diagnosis, approximately 70% of cancers are non-muscle invasive tumors while the remaining 30% are muscle invasive. About 10% of patients diagnosed with these muscle invasive tumors have metastatic feature with poor prognosis. (Arantes-Rodrigues R 2013) Bladder cancer is considered a chemosensitive disease. The prognosis of patients with metastatic disease is still poor, with a median survival of approximately 12 to 14 months. (Calabro F 2012) Because the 5-year cancer specific mortality rate is over 50%, metastatic bladder cancer is an important theme for cancer investigators. (Castillo-Martin M 2010) Cisplatin based combination therapies have been established as standard therapy for advanced or metastatic bladder cancer. (Kaufman DS 2006) For several decades, the following two regimens have been widely used: combination treatment with cisplatin and gemcitabine, and the MVAC regimen (i.e., Methothrexate, Vinblastine, Adriamycin, and Cisplatin). (Vaishampayan U 2009) In these regimens, cisplatin has a key role in treating bladder cancer. However, cisplatin is also responsible for chemo-resistant feature of bladder cancer following chemotherapy.

Resistance mechanisms of cisplatin of bladder cancer cells include decreased intracellular drug concentration, direct inactivation of cisplatin due to increased molecules containing sulfur (glutathione,

metallothionein), repairing of cell damages by DNA topoisomerase, and recruitment of apoptotic signals. (Drayton RM 2012) Although DNA damage is a major mechanism by with cisplatin induces cytotoxicity, the efficacy of cisplatin is often decreased by cell resistance which is mostly associated with glutathione based detoxification and efflux system. (Choudhary S 2013)

Bcl-2 oncoprotein as an apoptosis inhibitor is widely expressed in various cancers such as breast cancer, prostate cancer, and head and neck carcinoma. (Bolenz C 2007) The expression of Bcl-2 is known to have correlation with multi-drug resistance and poor prognosis. (Karam 2007) In cisplatin resistant bladder cancer cell lines, over-expression of Bcl-2 and Bcl-xL in addition to inactivation of Bax as a proapoptotic factor has been reported. (Cho HJ 2006) Furthermore, overexpression of translocation in the nuclear factor kappa B (NF-kB) as a regulator of Bcl-2 expression has been confirmed in cisplatin resistant cell lines. (Hong 2002)

In the last three decades, many trials have been conducted to elicit and overcome cisplatin resistance in numerous cancer cases. There has been a rapid progress in the understanding of cisplatin resistance using microarray analysis that permits simultaneous and quick expression profiling of tens of thousands of genes, leading to the identification of genes that might be significant in cisplatin resistance. (Choi MK 2006, Lee S 2013)

The cytotoxic effects of cisplatin are thought to be mediated by

irreversible DNA damage. (Stewart DJ 2007) Most human DNA is tightly compacted around histones, limiting the access of transcriptional factors and RNA polymerase. The same is true with DNA targeting drugs such as cisplatin, etoposide, 5-fluorouracil, and doxorubicin. Their antitumor action are usually limited by their access to DNA due to tight DNA coiling. (Rikiishi H 2007) These findings suggest that unwinding tight DNA coiling to facilitate the access of DNA targeting agents such as cisplatin to DNA might be a possible strategy to enhance their antitumor effect. Acetylated histones are prone to transcription with open structure. In contrast, deacetylated histones make closed DNA structure. (Khan AN 2008)

Histone deacetylase (HDAC) is a major epigenetic regulation factor in transcription. (Glaser 2003) HDAC involves cell differentiation, cell cycle, and apoptosis via regulating the acetyl group which bonds to histone protein of DNA. (Yamashita 2003) For this purpose, histone deacetylase (HDAC) inhibitor is considered a promising candidate since it leads to less compacted but transcriptionally more active chromatin formation by histone hyperacetylation. (Suzuki M 2009) Based on these concepts, several studies have explored the synergistic antitumor effect of combination treatment with HDAC inhibitors and DNA targeting agents for some malignant diseases. (Yang YT 2009)

HDAC inhibitors have shown ability in clinical cancer therapy. They can consistently induce p21WAF1/CIP1 expression in a p53-independent manner and increase the acetylation of chromatin in p21WAF1/CIP1

promoter region. (Li 2004) HDAC inhibitors result in the down-regulation of constitutive activation of NF-kB via generating reactive oxygen species which causes conformational changes in Bax protein, leading to loss of mitochondrial membrane potential and the release of cytochrome c to the cytosol. The release of cytochrome c then activates caspase-9, caspase-3, and poly(ADP)-ribose polymerase cleavage, leading to caspase-dependent apoptosis. (Hussain 2008) HDAC inhibitors also promote chromatin remodeling, potentially making DNA more accessible to chemotherapy. (Rundall 2005) In this notion, the anti-tumor effects of HDAC inhibitors suggest that they might be useful chemotherapy agents for bladder cancer. Furthermore, they might be candidates to overcome the resistance of cisplatin or gemcitabine via regulating the expression of Bcl-2, Bax, and NF-κB. (Galluzzi L 2012)

Recently, suberoylanilide hydroxamic acid (SAHA) as one of HDAC inhibitors has been approved for lymphoma by the Food and Drug Administration (FDA). (Zhang et al., 2006; Law et al., 2008; Roy et al., 2005; Duan et al., 2005) In cervical cancer, the combination of cisplatin and SAHA has shown synergistic effect on HeLa cell viability by down-regulating Bcl-2. (Jin 2010) In breast cancer cell lines, combined treatment of SAHA, clarithromycin, and bortezomib can potently enhance the apoptosis-inducing effect compared to treatment using each reagent alone based on the overexpression of ER-stress-related genes and enhanced apoptosis. (Komatsu 2013)

Purpose of Investigation

In this study, we investigated the synergistic antitumor effect of suberoylanilide hydroxamic acid (SAHA) and cisplatin in cisplatin resistant bladder cancer cells T24R2. The synergistic effect of cisplatin and SAHA on T24R2 cell line was established by analyzing the expression of cell-cycle modulating proteins. This study will provide basic information of gene expression in specimen of bladder cancer patients.

Materials and Methods

(1) Cell Lines and Chemicals

Bladder cancer cells T24 and T24R2 were obtained from ATCC®. The cisplatin resistant T24R2 cell line was generated by serial desensitization. Cells (T24 and T24R2) were maintained in RPMI media supplemented with 10% fetal bovine serum (FEB, Mediatech, Herndon, Virginia) and 50 U/ml penicillin/50 mg/l streptomycin (Gibco®). SAHA and cisplatin were obtained from Sigma® and Pfizer Korea Ltd., Seoul, Korea, respectively.

(2) Cell Proliferation Assay

Cells in 96-well plates were treated with cisplatin (1.25 to 20.0 μg/ml) or SAHA (0.05 to 1.0 μM) for 48 and 72 h. At the end of drug exposure, 10 μl of Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, Maryland) solution was added to each well containing 100 μl medium. After 4-hour of incubation, absorbance was measured at wavelength of 450 nm.

(3) Synergism Determination

The synergistic effect between the two drugs was determined based on combination index (CI) using CalcuSyn version 2.1 (Biosoft®). CI value less than 1.0 indicates synergism. CI value greater than 1.0 indicates antagonism. CI value equal to 1.0 indicates additive effect.

(4) Clonogenic Assay

T24R2 cells were treated with cisplatin (2.5 μg/ml) and/or SAHA (2.5

μM) for 48 hours. Cells were maintained for another two weeks in drug-free condition. The colonies formed were stained with 0.4% crystal violet. The number of colonies greater than 0.2 mm in diameter was quantified.

(5) Cell cycle analysis by flow cytometry

T24R2 cells were treated with cisplatin (2.5 μg/ml) and/or SAHA (2.5 μM) for 48 h. Flow cytometric analyses were conducted as previously described. (Yoon CY 2011) Cell cycle distribution was determined using FACS Calibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

(6) Synergism related protein expression (Western blot)

T24R2 cells were treated with cisplatin (2.5 μg/ml) and/or SAHA (2.5 μM) for 48 h. Protein was extracted using RIPA lysis buffer and Mitochondrial Isolation Kit (Thermo Scientific, Rockford, Illinois). After fractionation by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, Massachusetts). After blocking with Tris-buffered saline-Tween containing 5% milk for 1 hour, membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-caspase 3, anti-caspase 8, anti-caspase 9, anti-PARP, anti-Bcl-2, anti-Bad, anti-cytochrome C, anti-β-Actin, anti-p21, anti-p53, anti-cyclin D1, anti-cyclin E1, anti-pERK, anti-p-Akt, and anti-PTEN (Cell Signaling Technology®). After incubation with secondary antibodies, protein

expression was detected with an enhanced chemoiluminescence Western blot substrate kit (Pierce, Rockford, Illinois).

(7) Statistical analysis

Unless otherwise specified, results are presented as mean ± SD after a minimum of three repeated experiments. Tukey's multiple range tests were used to assess the statistical significance. P values of less than 0.05 were considered statistically significant. All analyses were conducted using IBM SPSS version 21.0 (SPSS Inc., Chicago, IL).

Results

(1) Antitumor effect of Cisplatin and SAHA on bladder cancer cells

Cisplatin produced antitumor effects against all cells in both dose and time dependent manner. In concentration range from 0.05 to 100 μg/ml with 48-h and 72-hour of exposure, cisplatin treatment suppressed the proliferation of T24 cells up to almost 90%. In the concentration range from 0.05 to 500 μM, SAHA suppressed the proliferation of T24 cells up to 80%. However, T24R2 cells virtually had no response to cisplatin treatment (mean, 1.6% ± 1.1%) at a concentration up to 5.0 μg/ml. (Figure 1) The mean IC50 values of cisplatin on T24R2 cells at 48 and 72 hours of exposure were 11.17 ± 5.93 and 8.66 ± 4.49 μg/ml, respectively. The mean IC50 values of SAHA on T24R2 cells at 48 and 72 hours of exposure were 5.42 ± 2.15 to 6.24 ± 2.26 μM, respectively. Compared to the sensitivity of the parental T24 cell line, T24R2 was 12.7- and 36.2- fold more resistant to cisplatin at 48 and 72 hours of exposure, respectively. In each cell line, 48 and 72-hour exposure to SAHA significantly suppressed cell proliferation in both dose and time dependent fashion. (Figure 1)

(2) SAHA mediates T24R2 cell resensitization

The combined treatment with cisplatin and SAHA resulted in significantly greater growth inhibition than either agent alone. Concomitant treatment with SAHA in the dose range of greater than 0.5

μM potentiated and restored the antitumor effect of cisplatin on T24R2 cells to almost the same level as that on T24 cells. (Figure 2) The combination index of cisplatin and 50 μM of SAHA after 48 h and 72 h of exposure was less than 1.0, indicating synergism between the two agents. (Figure 2. B, Table 1)

When data were analyzed based on CI, strong synergism was found with CI less than 1 for most dose combinations tested. (Figure 2. B) Since the combination of cisplatin and SAHA indicated synergism, we determined the CI values at different fraction affected (fas). Results revealed that the two drugs acted synergistically over a wide range of fractions affected (20% to 100% cell death). The correlation coefficient r was greater than 0.9 (range, 0.909 to 0.971), indicating that the data confirmed to the median effect principle. The combined treatment with cisplatin and SAHA yielded a favorable dose reduction along a wide fas range. Results also indicated synergistic effects on T24R2 cells across a broad range of concentrations for cisplatin and SAHA.

Clonogenic assays were performed to determine the effects of 48 h of treatment with cisplatin alone, SAHA alone, or in combination of the two on T24R2 cells. Although there was a considerable decrease in colony number when T24R2 cells were exposed to cisplatin (mean 66.1% ± 7.8% of control) or SAHA (61.4% ± 19.1% of control) alone, the overall decrease in clonogenicity was significantly higher when T24R2 cells were exposed to both cisplatin and SAHA (mean 6.7% ± 16.6%, p = 0.012, Figure 3).

(3) Potentiated cisplatin induces cell cycle alterations

T24R2 cisplatin exposure resulted in dose dependent S phase cell cycle arrest (mean 7.9% ± 5.2% to 48.7% ± 1.1%) and G2/M phase cell cycle arrest (5.2% ± 2.8% to 25.5% ± 1.3%) while causing an increase in the subG1 population (0.7 ± 0.2% to 4.73% ± 1.1%, Figure 4 *A*). A similar effect was exerted by SAHA which showed a dose dependent increase in the subG1 phase (mean, 0.71% ± 0.2% to 6.94% ± 1.3%), S phase (7.9% ± 2.4% to 24.3% ± 4.9%), and G2/M phase (5.2% ± 1.9% to 33.5% ± 2.4%, fig. 4, *A*). The combination treatment at suboptimal doses of cisplatin (2.5 μg/ml) and SAHA (2.5 μM) caused a significant increase in subG1 phase (mean, 1.3% ± 0.7% to 7.6% ± 3.5%, $p = 0.048$), S phase (9.5% ± 5.2% to 48% ± 1.9%, $p = 0.016$), and G2/M phase (6.2% ± 2.8% to 19.9% ± 1.2%, $p = 0.024$) compared to untreated controls (Figure 4 *A*). Compared to the cisplatin only treated group, the combination treatment group showed significant increases in both subG1 and S phases ($p = 0.054$ and $p = 0.078$, respectively, Figure 4 *B*).

(4) Cisplatin and SAHA Mediate changes in apoptosis and cell cycle regulator expression

Treatment of T24R2 cells with 2.5 μg/ml cisplatin resulted in increased expression of cleaved caspase- 3, 8, and 9. Concomitant treatment with 2.5 μM of SAHA further enhanced the expression of caspases,

especially cleaved caspase- 3, 8, and 9, fragmented PARP, and cytochrome C (Figure 5). Western blot analysis showed similar results. The activities of caspase- 3, 8, and 9 activities in cells treated with cisplatin only were increased (mean, 145.3% ± 4.2%, 127.9% ± 3.8%, and 123.9% ± 4.6%, respectively, of the control), which was further enhanced in the presence of SAHA (192.3% ± 8.3%, 136.3% ± 1.2%, and 157.2% ± 4.6%, respectively, of the control, Figure 5). The expression of anti-apoptotic Bcl-2 was decreased, whereas the activity of pro-apoptotic Bad was increased following the combined treatment with cisplatin and SAHA. Thus, activation of the intrinsic apoptotic pathway was associated with the synergism of the two agents.

Exposure to cisplatin alone or in combination with SAHA resulted in increased expression of p21WAF1/CIP1 in T24R2 cells (Figure 6A). Combination treatment markedly suppressed the expression of cyclin D1, E1, pERK, and p-AKT, but up-regulated the expression of pro-apoptotic Bad. The combination treatment also almost completely abolished MTH1 expression in T24R2 cells (Figure 6B).

Discussion

Recent studies have shown that HDAC inhibitors have antitumor activity for various neoplasms. (Zhang et al., 2006; Law et al., 2008; Roy et al., 2005) Compared to other conventional chemotherapeutic agents which usually affect tumor and normal tissues simultaneously, HDAC inhibitors have relatively high tumor selectivity with less toxicity to normal tissue. (Mei 2004) In addition, HDAC inhibitors can synergistically enhance the antitumor effect of certain agents, especially drugs or enzymes that act on DNA. (Shen 2007) Although the underlying mechanisms have not been fully elucidated, HDAC inhibitor might be able to neutralize the positively charged histone proteins through hyper-acetylation and cause the relaxation of compacted chromatin. Relaxed chromatin can permit more efficient access to DNA and increase the antitumor efficiency of DNA targeting drugs. Thus, HDAC inhibitors are potent candidates for combination chemotherapy to enhance the antitumor effect of DNA targeting agents such as cisplatin, doxorubicin, and etoposide with a relatively good safety profile. We presented evidence that SAHA as a HDAC inhibitor had synergistic antitumor effect with cisplatin. It could resensitize cisplatin resistant human bladder cancer cell-line by enhancing cisplatin mediated cell cycle arrest and caspase dependent apoptosis.

Exposure of T24R2 cells to cisplatin resulted in dose dependent cell cycle arrest at the S and G2/M phases. Concomitant HDAC inhibitor

treatment with cisplatin in T24R2 cells has predominantly caused G2/M phase arrest. (Byun 2009) The G1/S checkpoint is a crucial part of the DNA damage surveillance system that interrupts cell cycle progression and allows time for DNA repair. Failure to arrest in G1 usually results in apoptotic cell death via overexpression of cell cycle inhibitors. For instance, CCRF-CEM (acute T-cell leukemia cell line) cells only in the G2/M phase are destined to apoptosis by HDAC inhibitors. This can be prevented by G1 phase cell cycle arrest. (Lee EJ 2008) The absence of G1 phase arrest and the accumulation of T24R2 cells in the G2/M phase in response to SAHA treatment suggest that T24R2 is more vulnerable to apoptosis in the presence of SAHA than its parental T24 cells. This assumption is compatible with other studies in which HDAC inhibitors have induced cell cycle arrest in cisplatin resistant ovarian cancer cells at the G2/M phase followed by apoptosis, while cisplatin sensitive parental cells have shown predominantly G1 phase arrest without detectable apoptosis. (Strait 2005) Cell cycle arrest in G2/M phase in T24R2 cells has been found in the investigation with trichostatin A (TSA, a HDAC inhibitor). (Yoon CY 2011)

In the current study, combination with SAHA significantly enhanced cisplatin mediated S and G2/M phase cell cycle arrest accompanied by marked suppression of cyclin E1, cyclin D1, pERK, and p-AKT. Combination treatment also caused significant increase in subG1 population with increased expression of cleaved caspase- 3, 8, and 9 accompanied by PARP cleavage. This suggests that cyclin related cell

cycle arrest and caspase dependent apoptosis play important roles in the combination treatment.

Cyclin D and cylcin E are known as G1/S-phase regulators. (Pestell RG 2013) Combination treatment in this study showed decreased expression of cylcin D1 and cyclin E1. Our results suggest that cisplatin and SAHA combination treatment inhibited cell proliferation by down-regulating cyclin D1 and cyclin E1, leading to S-phase cell-cycle arrest. Thus, cisplatin and SAHA combination treatment significantly inhibited T24R2 cell proliferation, which was partially related to cell cycle arrest.

HDAC inhibitors have resulted in increased expression of p21WAF1/CIP1 in several types of transformed cells. HDAC inhibitor-induced increase in p21WAF1/CIP1 expression appears to play a major role in arresting transformed cell growth. (Ocker M 2007) The increase in p21WAF1/CIP1 transcription is associated with accumulation of acetylation of the gene-associated histones. (Vrana JA 1999) The expression of p21WAF1/CIP1 in our study was compatible with previous literatures. In this pathway, SAHA rapidly induced changes of p21WAF1/CIP1 gene and associated proteins, increased the sensitivity of gene encoding DNase I, and increased its accessibility to restriction enzymes. (Huang L 2000)

SAHA and combination therapy can decrease the expression of Akt, a survival regulator. (Xu J 2002) When Akt signaling is attenuated, cisplatin sensitivity is restored, suggesting a potential therapeutic strategy

for preventing tumor recurrence by resensitizing bladder cancer. (Tatokoro M 2011) A reciprocal expression was found between caspases (up-regulated) and Akt (down-regulated) in this study. The expression of Akt and caspases (-3, -8, -9) represented the environment for apoptotic cell death of T24R2 cell in combination therapy.

In previous studies, the expression of abnormal Bcl-2 family members was closely related to cisplatin resistance in T24R2 cells. (Drayton RM 2012) Bcl-2 protein is an integral outer mitochondrial membrane protein that prevents apoptotic cell death. (Bolenz 2007) Bcl-2 mRNA and protein levels are increased in cisplatin-resistant bladder cancer cells. Treatment with antisense Bcl-2 oligonucleotide has significantly enhanced the cytotoxicity of cisplatin. (Hong 2002) However, Bad, a pro-apoptotic protein, is known to interfere with the function of Bcl-2. (Le Bras M 2006) These findings are compatible with our data showing that Bcl-2 expression was up-regulated in T24R2 cells in response to cisplatin treatment. The combination of cisplatin and SAHA in T24R2 caused down-regulated expression of Bcl-2 whilst Bad was up-regulated.

The increase of Bad and the decrease of Bcl-2 with simultaneous increase of cytochrome C after the combined treatment of SAHA and cisplatin suggest that the intrinsic apoptotic pathway is activated which might be the underlying mechanism. Western blotting showed increased activities of proteins including cytochrome C and caspase-3 known to be involved in the intrinsic apoptotic pathway through altering the expression of Bcl-2 family proteins. (Cho 2006) The presentation of

these molecules associated with intrinsic apoptotic pathway in our results implicated their role in the synergistic effect of SAHA and cisplatin on apoptosis.

Conclusions

SAHA may synergistically enhance the antitumor effect of cisplatin and re-sensitize cisplatin resistant bladder cancer cells. These findings suggest the potential use of SAHA as a combination agent to enhance the antitumor effect of cisplatin in patients with advanced bladder cancer.

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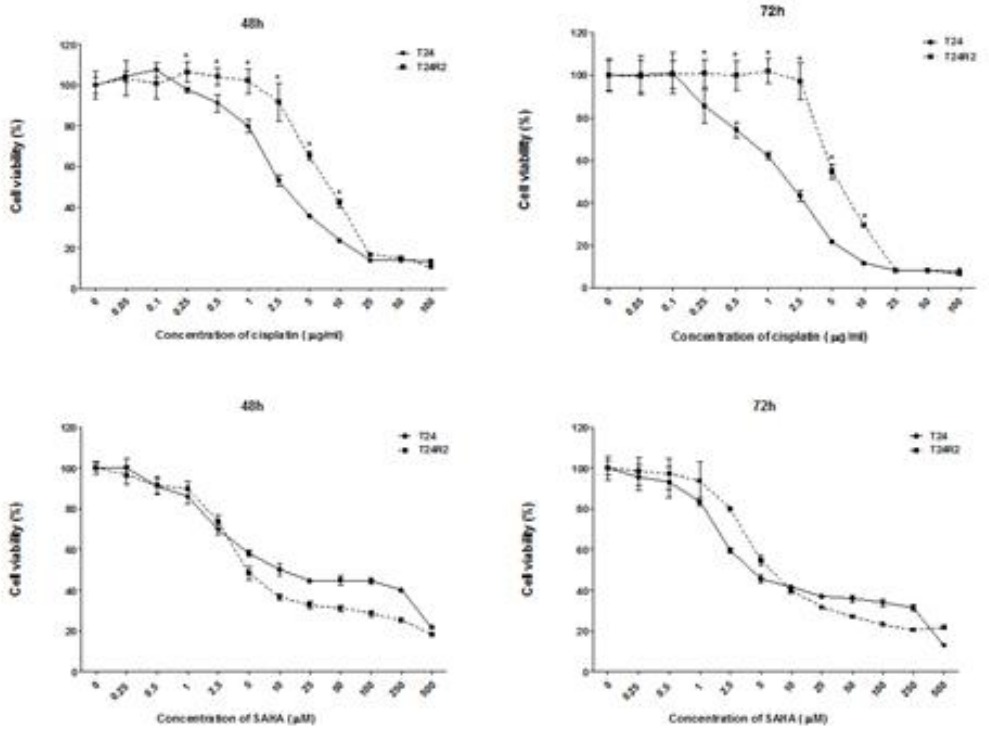

Figure 1. Cisplatin and SAHA effect on bladder cancer cell survival. Cell survival was determined by CCK-8 assay experiments. Cisplatin doses of 0.05 μg/ml to 100 μg/ml for 48 and 72 hours. Anti-proliferative effect of cisplatin determined in T24R2. Asterisks indicate significantly higher survival of T24R2 than T24 cells. T24 and T24R2 cell lines were treated with dose of SAHA of 0.25 to 500 μM for 48 and 72 hours. Values represent mean ± S.D (n=5). *P<0.05 compared to T24 cells.

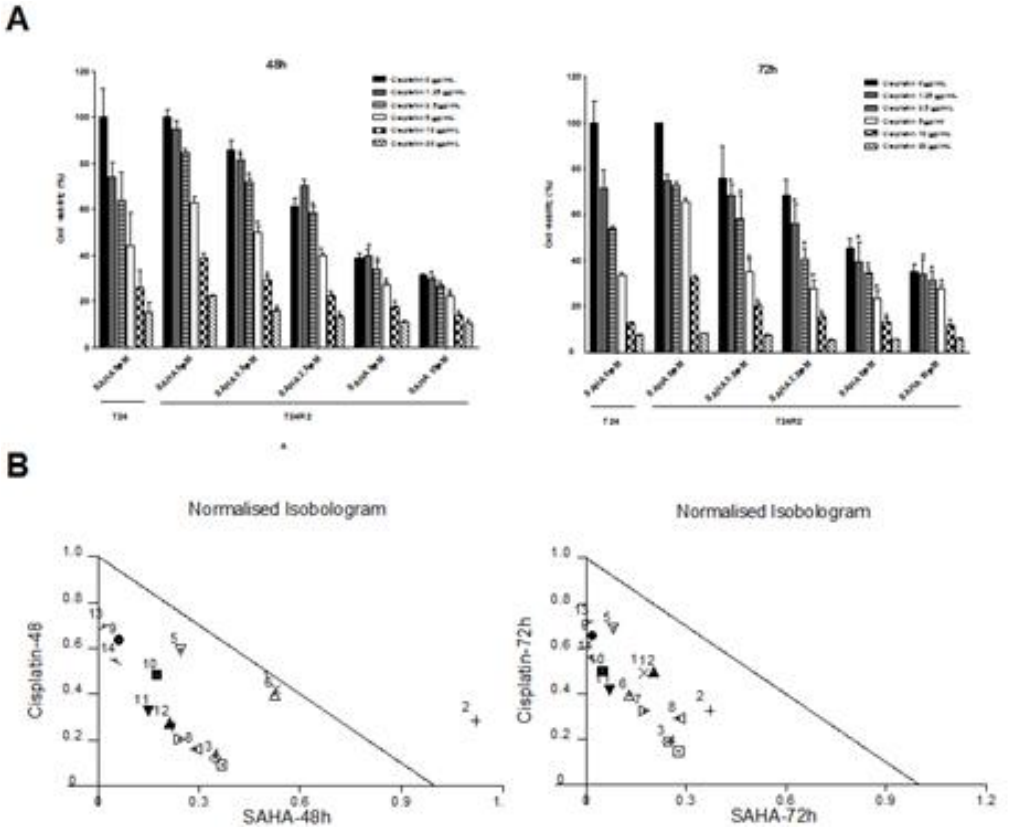

Figure 2. Cisplatin and SAHA combination treatment effect on T24R2 cell viability. (A) cells were treated with cisplatin of 1.25μg/ml to 20μg/ml alone or with 0.05μM to 1μM of SAHA for 48 and 72 hours, and cell viability was evaluated by CCK-8 assay. (B) combination index of cisplatin and SAHA was less than 1.0, revealing the synergism. Values Represent mean ± SD (n=5). *P<0.05 compared to single treatment.

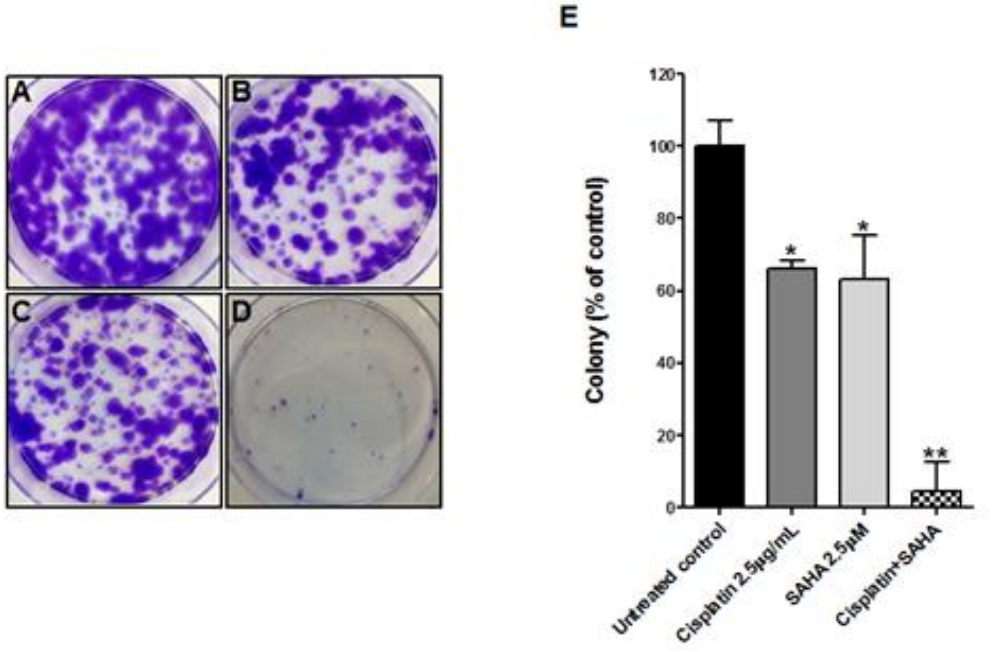

Figure 3. Clonogenic assays shows effect in T24R2 cells treated with cisplatin alone, SAHA alone or combination for 48hours. Cell cultured in fresh medium for 11days to form colonies. Colony formation was assessed by crystal violet staining. A, untreated control. B, 2.5μg/mL cisplatin. C, 2.5μM SAHA D, combination were cisplatin 2.5μg/mL plus SAHA 2.5μM. E, number of colonies was counted and percentile change from untreated controls, considered 100%, was calculated. Values Represent mean ± S.D. **P*<0.05 and ***P*<0.001 compared to untreated control by t-test (n=3).

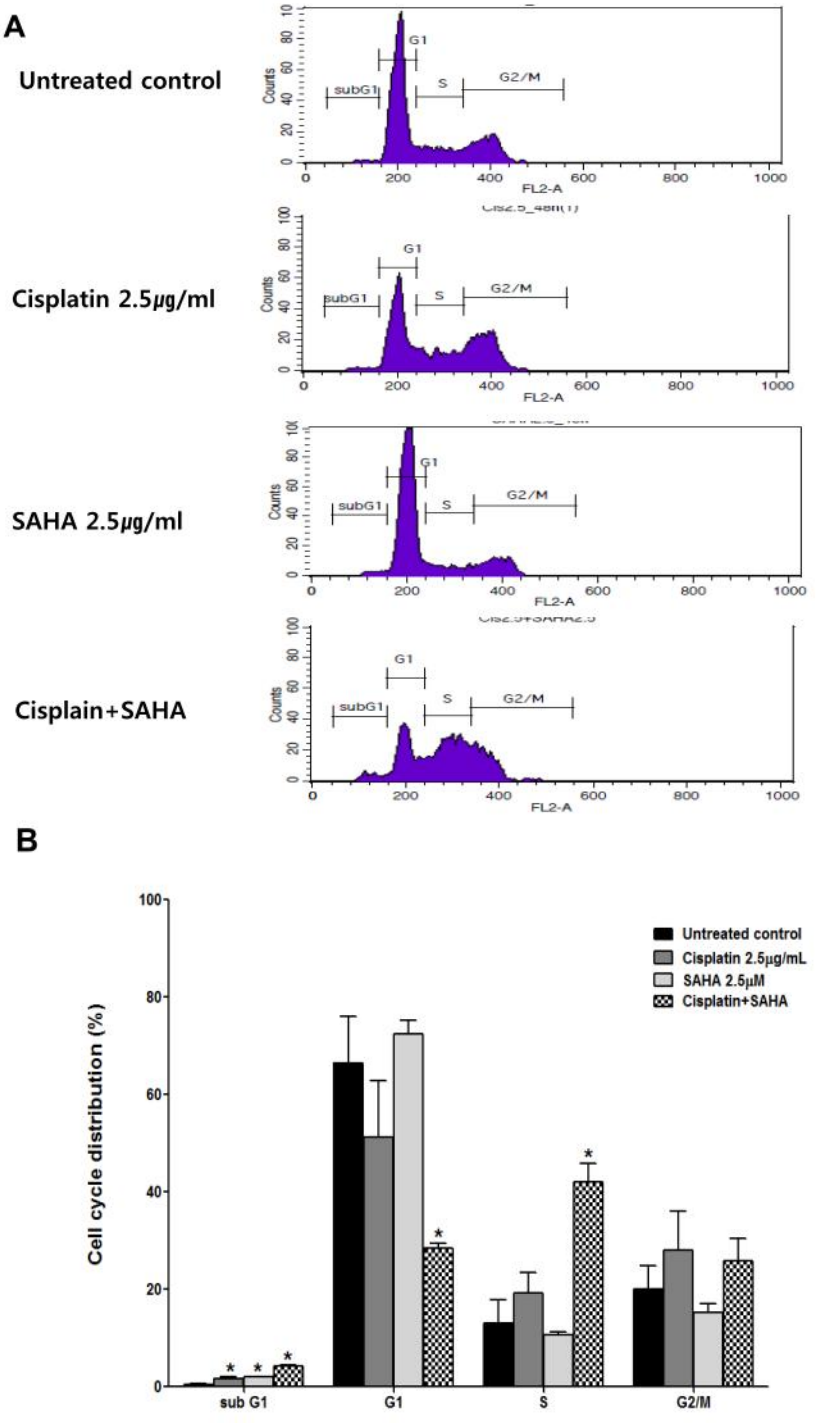

Figure 4. Analysis of cell cycle in T24R2 cells for 48hours. Cellular

DNA was stained with propium iodied. A, Flow cytometric DNA content histogram of T24R2 cells after 48 hours of treatment with single or co-treated with combined 2.5μg/ml cisplatin and 2.5μM of SAHA. B, Cell cycle distribution was measured quantitatively and plotted according to the type of treatments and the phase of cell cycle. Values represent mean ± S.D of duplicate experiments. *P<0.05 compared to untreated control.

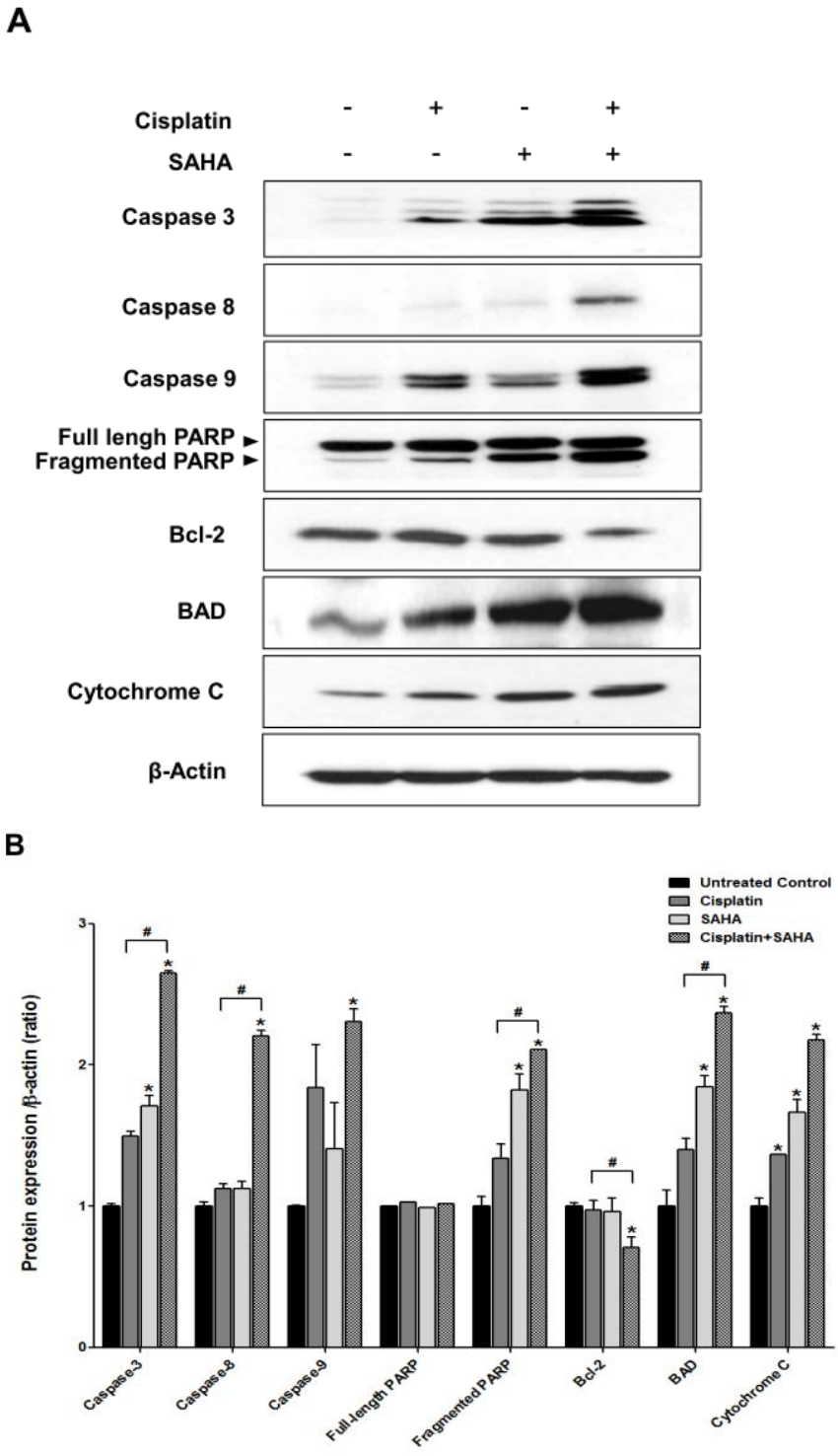

Figure 5. Effect of combination treatment on apoptosis related protein

expression in T24R2 cells. A, cells were treated with 2.5μg/ml cisplatin alone or co-treated with 2.5 uM SAHA for 48 hours. Protein expression was detected by western blot. B, densitometric measurement of each protein. Values represent mean ± S.D of duplicate experiments. *P<0.05 compared to untreated control. #P<0.05 vs cisplatin

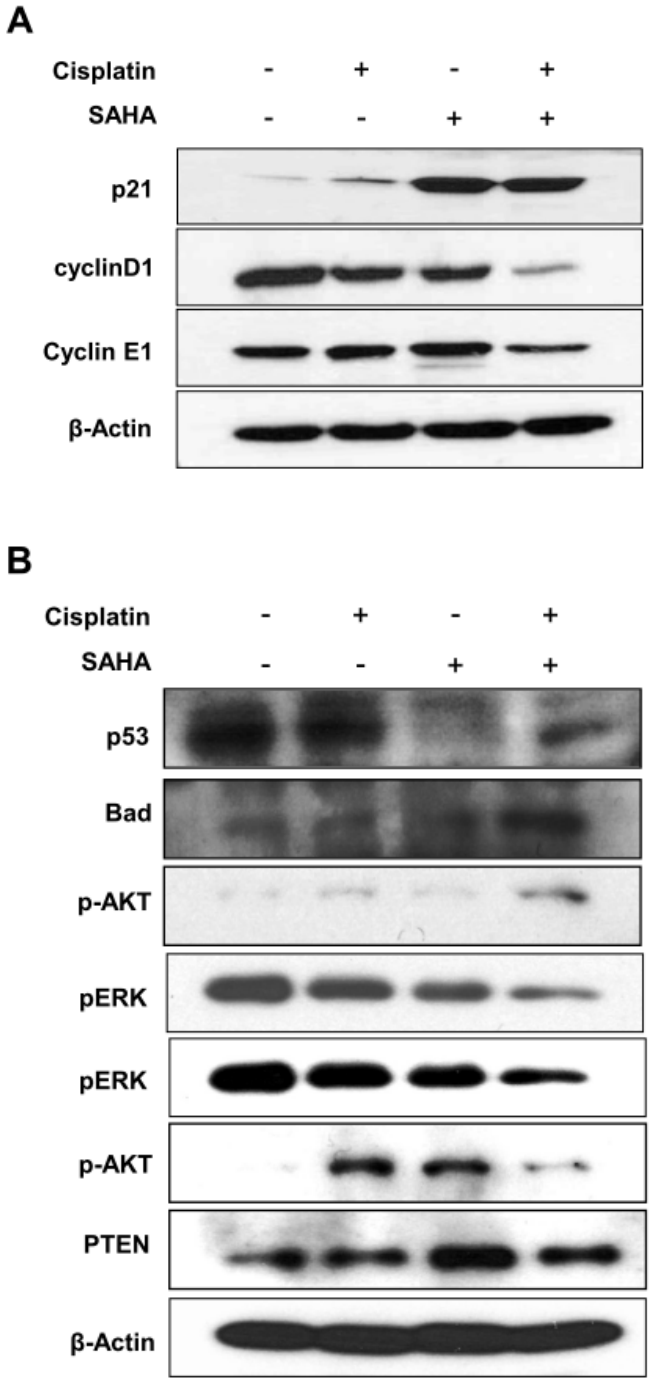

Figure 6. Effect of combination treatment on cell cycle related protein expression in T24R2 cells. Cells were treated with 2.5μg/ml cisplatin

alone or co-treated with 2.5 uM SAHA for 48 hours. Protein expression was detected by western blot. **A**, Cell cycle related protein expression was shown. **B**, Cell survival pathway related protein expression.

국문초록

cisplatin 저항성 인간 방광암 세포주에서 suberoylanilide hydroxamic acid (SAHA)의 항암 효과 기전 분석

진행성 방광암에 대한 일차 항암화학제는 cisplatin을 기초로 하나, 현재까지 cisplatin에 저항성을 가진 방광에 대한 치료 요법은 확립되지 않았다. 이 연구에서는 histone deacetylase inhibitor인 suberoylanilide hydroxamic acid (SAHA)와 cisplatin을 cisplatin에 저항성을 가진 인체 방광암 세포주 (T24R2)에 투여하여 그 효과 및 관련 유전자를 분석하였다.

T24R2 세포주에 cisplatin과 SAHA를 단독 또는 복합 투여 하여, CCK-8 assay를 통하여 세포 분화도 및 항암효과 상승 작용을 분석하였다. Western blot 분석을 이용하여 유전자 발현 변화를 분석하였다.

CCK-8 assay를 통하여 cisplatin과 SAHA의 항암효과 상승작용을 확인하였으며 그 combination index는 1.0보다 작았다. 이러한 상승작용의 작용기전은 cisplatin과 SAHA의 동시 투여를 통해 T24R2 세포내에서 세포 주기 중단, caspase-3, -8, -9 및 PARP 분절에 의한 세포고사 기전의 활성화, 항세포고사 인자인 Bcl-2의 저발현 등으로 설명할 수 있다.

SAHA는 복합투여로 인한 상승작용으로 cisplatin의 항암효과를

증대 시키며, cisplatin에 저항성이 있는 방광암 세포를 재감작시키는 것으로 보인다. 이러한 소견은 SAHA를 cisplatin과 복합투여를 통하여 진행성 방광암 환자의 항암화학 효과를 증대시킬 것으로 기대된다.

주요어: 방광암, cisplatin, suberoylanilide hydroxamic acid (SAHA)
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