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ANTICANCER EFFICACY OF As_4O_6 IN 5637 HUMAN BLADDER CANCER CELL LINE

Ajou University.
Graduate School of Pharmaceutical Industry and Clinical Pharmacy.

Global Pharmaceutical Clinical Pharmacy

WU ZHAOYAN

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-Abstract-

Among bladder cancer, non-invasive bladder cancer is commonly recurs after trans urethral resection of bladder tumor, some of them develop invasive bladder cancer and then distant metastasis occurs. Bacillus Calmette-Guérin (BCG) is injected into the bladder to remove cancer cells, but patients who subsequently relapse are more difficult to treat. As_4O_6 has been reported to have a certain inhibitory effect on cancer, such as esophageal cancer, head and neck cancer, cervical cancer, gastric cancer, neuroblastoma and breast cancer. Therefore, it is planned to observe the effects of As_4O_6 on the growth, migration, and invasion in 5637 bladder cancer cell line, and to study its possible mechanism of action.

As experimental methods, Cell migration assay and MTT assays were used to determine the inhibition of migration and growth in 5637 cells by different concentrations of As_4O_6, and Western blotting was used to detect EGFR, p-EGFR (phosphorylation of EGFR) and HER-2 and MMP-2 protein expression and to observe the mechanism of As_4O_6 on the metastasis and growth inhibition in 5637 human bladder cancer cell lines.

As_4O_6 effectively inhibited the migration and growth of 5637 human bladder cancer cell line. The mechanism has shown that the anticancer efficacy of As_4O_6 is related to the HER-2/EGFR pathway. As_4O_6 showed an inhibitory effect on migration and growth in 5637 human bladder cancer cell line by down-regulating EGFR, p-EGFR and HER-2. In addition, the possibility of inhibition of bladder cancer metastasis associated with 5637 human bladder cancer cell line was observed by down-regulating the protein MMP-2 which plays an important role in cancer metastasis.

As_4O_6 significantly inhibited the migration and growth of 5637 human bladder cancer cell lines, EGFR, HER-2, p-EGFR, and MMP-2 might be involved as a mechanism. These results suggest that As_4O_6 can be used for the development of anticancer agents or metastasis inhibitors for bladder cancer. In the future, it is necessary to clarify the mechanism through experiments with more cells and in PDX animal models and to advance to the possibility of clinical application through clinical trials.

Key words: As_4O_6, bladder cancer, 5637 cell, migration, invasion, signaling pathway.

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TABLE

I. INTRODUCTION

Bladder cancer is one of the most frequent malignances among urological cancer. As of 2015, bladder cancer has affected about 3.4 million persons worldwide, with new 430,000 cases arising each year.[1][2][5][9] In 2018, the incidence of new cases is predicted to amount to 549,400, with the most thereof found in males. Incidence rates internationally differs by about 15 folds, reaching peaks in Europe, North America, West Asia, and North Africa and the lowest in Middle Africa and Mid-South Asia. As of 2018, about 200,000 persons would die of bladder cancer[3], with the incidence four fold higher in males than females and two fold higher in white males than black males . In 2019, 80,470 new cases will be presumably diagnosed, with the bladder cancer-related death toll rising to 17,670.[4] According to cancer survey data in 2015 China, the incidence of bladder cancer in China increasingly increased every year, with a frequent tendency in youth.[5]

Transitional cell carcinoma is the most common type of bladder cancer[6] while squamous cell carcinoma and adenocarcinoma account for other types. Bladder cancer is treated by surgery, radiotherapy, chemotherapy, or immunotherapy according to the progression stages of cancer. For surgical therapies, transurethral resection, partial or radical cystectomy, or urinary diversion is employed.[5] Immunotherapy is conducted by transferring Bacillus Calmette-Guérin (BCG) into the bladder to remove cancer cells.[7] However, patients who subsequently relapse are more difficult to treat.[8] Bladder cancer is diagnosed to be non-muscle invasive in 70%-75% of the cases at the first diagnosis, with a local recurrence rate amounting to 70%. Non-muscle invasive bladder cancer frequently recurs after urethral resection and about 20% of the bladder cancer diagnosed to be non-invasive at the first diagnosis develops into invasive bladder cancer and then undergo distant metastasis.[9][10]

Arsenic and arsenic-derived compounds have long been used as drugs for various diseases such as cancer, psoriasis, rheumatoid arthritis, etc. in the East.[11] In the 1970s, China started to apply arsenic trioxide (As_2O_3), which had been used in the Chinese traditional medicine, to the treatment of various kinds of cancer. In 1992, arsenic trioxide was reported to effectively induce the complete remission of recurrent or acute promyelocytic leukemia (APL). Since then, there have been experimental reports on the induction of apoptosis in cell lines of esophageal cancer, head and neck cancer, uterine cervical cancer, bladder cancer, gastric cancer, glioblastoma, and the like.[12][13][14][15] Exhibiting its ability to induce apoptosis, arsenic trioxide was approved by the FDA for a therapeutic agent for leukemia in 2000 and sold under the brand name of Trisenox. However, the molecular mechanism of arsenic trioxide for inducing apoptosis solid cancer has

remained unproved up to date.[16]

Tetraarsenic oxide (As_4O_6) (Fig.1), which is an orally administrable, stable trivalent inorganic arsenic compound, has physicochemical properties different from those of arsenic trioxide. Having greatly mitigated toxicity compared to As_2O_3, As_4O_6 was administered at a dose of up to 50mg/kg/day to mice, with no side effects observed. The trivalent arsenic compound was reported to have a median lethal dose (LD_{50}) of 120-180 mg/kg in the animal experiments.[17][18][19] In addition, there are various articles about the therapeutic effectiveness of tetraarsenic oxide on cancer such as leukemia,[20][21] uterine cervical cancer,[22][23][24] gastric cancer,[25][26] colorectal cancer,[27] glioblastoma,[28] and breast cancer.[29][30][31] It was reported to the Korean Food and Drug Administration that phase I of clinical trials had been done for the anticancer effect and pharmacological action of As_4O_6. In this study, we investigated the effects of As_4O_6 on the growth, migration, and invasion of 5637 bladder cancer cell line and revealed a possible mechanism of action thereof, aiming to develop As_4O_6 to an anticancer agent for bladder cancer.

II. MATERIALS AND METHODS

A. Experimental Materials and Reagents

1. Cells: 5637 human bladder cancer cell line was purchased from the CAS Shanghai Life Science Cell Resource Center.

2. Reagent and Chemical Treatment

As_4O_6 with a purity of 99.99%, provided from Chemas Co. Ltd., was dissolved in water to prepare a 5mmol/L stock which was then filtered and stored at room temperature until use at different dilution concentrations in media.

A purchase was made of fetal bovine serum (FBS), 0.25% trypsin, and MEM from Hyclone, Beijing; rabbit anti-EGFR monoclonal antibody, rabbit anti-PEGFR monoclonal antibody, rabbit anti-HER2 monoclonal antibody, rabbit anti-MMP2 monoclonal antibody, and sheep anti-rabbit IgG (immunoglobulin G) from CST China Ltd.; mouse anti-β-Actin monoclonal antibody and sheep anti-mouse IgG (immunoglobulin G) from Trans; ECL reagent capable of detection of antigens at a ng level and high-sensitivity ECL reagent capable of detection of antigens at a pg level from Sangon Biotech (Shanghai) Co., Ltd. Other necessary materials include TBS: 100mM Tris, 150mM NaCl (pH7.5), a transfer buffer: 25mM Tris, 192mM glycine, 5% methanol, TBST: 0.1% Tween-20 in TBS, and a blocking buffer: 5% skim milk in TBST.

3. Instrument

A BCA protein concentration assay kit was purchased from Beijing Solar bio Science & Technology Co., Ltd., a blood cell counter from Shanghai Chemical Laboratory Equipment Co., Ltd., an auto BDS200 inverted biological microscope from Chongqing Auto Optical Instrument Co., Ltd., an ultraclean bench (YT-CJ-2ND) from Beijing Yatai Cologne Instrument Technology Co., Ltd., a CO_2 incubator (SANYO) from Beijing Dongxun Tiandi Medical Instrument Co., Ltd., and a low-speed centrifuge (SC-3616) from Anhui Zhongke Zhongjia Scientific Instrument Co., Ltd. A camera manufactured by Cannon, Japan (Canon EOS 600D) was used.

B. Experimental Methods

1. Cell migration assay

The 5637 cell line in log phase growth was harvested and assigned 2 ml each well at a density of $5x10^5$ cells/well to 6-well plates before incubation at 37℃ in a 5% CO_2 incubator maintained at saturated humidity. Thereafter, the supernatant was aspirated, and the cells in each

well of the 6-well plates were divided in half by making a horizontal scratch thereon with a 20 μL pipette tip and then washed twice with PBS. As_4O_6 dilutions in 1% FBS (0 μmol/L, 0.39 μmol/L, 0.78 μmol/L, and 1.56 μmol/L) were added in triplicate to the wells. Images were taken after 0 and 24 hours using a 100x inverted microscopes. Recovery rate (%) = (pre-drug width – post-drug width)/pre-drug width x 100%

2. Cell proliferation assay (MTT assay)

The 5637 cell line in the log phase growth was treated with 0.25% trypsin, diluted in 10% FBS, and seeded at a density of $5x10^5$ cells/well into 96-well plates. After 24 hours of incubation at 37℃, As_4O_6 solutions with concentrations of 0 μmol/L, 0.78 μmol/L, 1.56 μmol/L, 3.13 μmol/L, 6.25 μmol/L, 12.5 μmol/L, 25 μmol/L, and 50 μmol/L were added in an amount of 100 μL per well to 6-well plates and incubated for 24, 48, and 72 hrs, together with untreated, control wells. Subsequently, the cells were treated at 37℃ for 4 hrs with 20μL of MTT (0.5 mg/well). The medium was aspirated and 100μL of DMSO was added to each well to reduce MTT to formazan. Absorbance (OD) at 570 was read on a microplate reader to obtain cell viability (= OD value of treated group ÷ OD value of control x 100%).

3. Western blot

The 5637 cell line in the log phase growth was harvested and suspended at a density of 1.0 x 10^8 cells/ml before being cultured in 75-cm^2 flasks. The culture was added in a volume of 1 ml to each well. The As_4O_6-treated group was set forth to have concentrations of 0 μmol/L, 0.5 μmol/L, and 1 μmol/L and then incubated at 37℃, together with the blank control, in a humidified, 5% CO_2 incubator. After 48 hrs of incubation, the medium was discarded from each flask and the cells were washed three times with 2 ml of PBS. When the cells were made morphologically round and increased in intercellular gap after 2-3 min of digestion with 0.25% trypsin, the trypsin was removed and a 1640 medium supplemented with 10% FBS was added to the cells to terminate the digestion. The cells were completely dislodged from the walls of the flask bottles by gently hitting or blowing the walls with a pipette. The floating cells were harvested and proteins were obtained therefrom.

Protein concentrations were determined using the BCA method. Eluted fractions were separated on SDS-PAGE. The, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane which was then blocked with 5% skim milk powder in TBST at room temperature (25℃) for 1 hr. The blocked membrane was washed with TBST, incubated with antibodies (EGFR, p-EGFR, HER-2, and MMP-9) at 4℃, and then washed again with TBST. After incubation at room temperature for 1 hr, the supernatant was aspirated and the membrane was washed with TBST and

then with TBS. ECL solutions A and B were mixed. After 1 min, a film was positioned on a wrap and the membrane was overlaid so as to allow the protein surface to face upwardly. The film was uniformed covered with the A and B mixture, shielded from light for 3 min, and transferred to another wrap for packaging. Then, the film was placed in a cassette and exposed in a dark room. Using Gel-Pro analyzer 4.0 software, gray level analysis was made of ECL color rendering results.

C. Statistical Analysis

Statistical analyses were performed using SPSS 17.0 software. Measured data are expressed as mean ± standard error of the mean ($\bar{x}$ ±s). Multivariate data was analyzed by one way ANOVA, with the application of LSD for multiple comparison. Linear regression analysis was performed to test for linear trend. $p<0.05$ was considered statistically significant.

III. RESULTS

A. Effect of As_4O_6 on Migration of 5637 Human Bladder Cancer Cell Line

To investigate the effect of As_4O_6 on the migration of the 5637 human bladder cancer cells, a wound healing assay was conducted. After 24 hrs, as shown in Fig. 2, the gap between cell groups was reduced in the control in which As_4O_6 had not intervened, but under the action of As_4O_6, the gap reduction was decreased depending on the concentrations. Here, a remarkable difference in the gap was observed between the group treated with 1.56 μmol/L of As_4O_6 and the control after 24 hrs. The result indicates that As_4O_6 effectively inhibits cell migration in a dose-dependent manner.

B. Inhibitory Activity of As_4O_6 against 5637 Human Bladder Cancer Cell Line

Investigation was made of the viability of 5637 human bladder cancer cells under the action of As_4O_6. To this end, the cells were incubated with different concentrations of As_4O_6, i.e., 0 μmol/L, 0.78 μmol/L, 1.56 μmol/L, 3.13 μmol/L, 6.25 μmol/L, 12.5 μmol/L, 25 μmol/L, and 50 μmol/L of As_4O_6 for 24 hrs, 48 hrs, and 72 hrs, followed by analyzing the cells for cell viability according to drug concentrations and times. The cell viability significantly decreased with increasing of As_4O_6 concentration. Particularly at 1.56 μmol/L, the cell viability was already 74.09% after 24 hrs and declined to 67.72% after 48 hrs and to 54.45% after 72 hrs. (Fig. 3) As a consequence, As_4O_6 was observed to significantly inhibit the growth of 5637 human bladder cancer cells in a dose-dependent manner. (Table 1)

C. As_4O_6-Controlled Signaling Pathway in 5637 Human Bladder Cancer Cell line

Expression levels of the apoptosis-related proteins EGFR, p-EGFR, and HER-2 and the metastasis-related protein MMP-2 were analyzed using Western blotting. At 0 μmol/L, 0.5 μmol/L, and 1 μmol/L, the expression levels of EGFR, p-EGFR, HER2, and MMP2 were measured. Compared to the control, all the four proteins were expressed at significantly reduced levels at 1 μmol/L as shown in Fig. 4, but with no dose dependency observed.

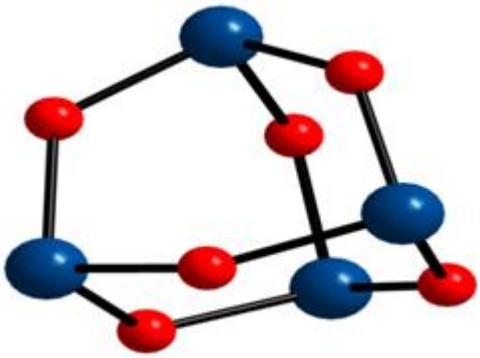

Fig.1 Chemical structure of tetraarsenichexoxide. tetraarsenichexoxide(2,4,6,8,9,10-Hexaoxa-1,3,5,7-tetraarsatricyclo [3.3.1.1$^{3.7}$]decane,Tetraarsenichexoxide; As_4O_6)

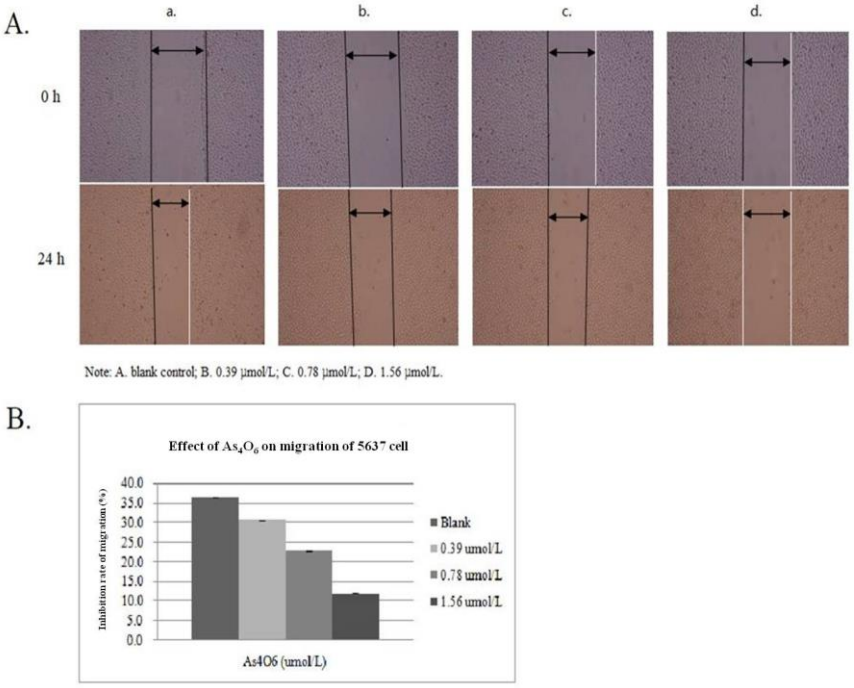

Fig. 2 Migration assay in 5637 human bladder cancer cell line. The wound was created by scratching the cells with a sterile 20-μL pipette tip and incubated with As_4O_6(0, 0.39, 0.78, 1.56 μmol/L) for 24h. *$p<0.05$, compared with the 0μmol/L group.

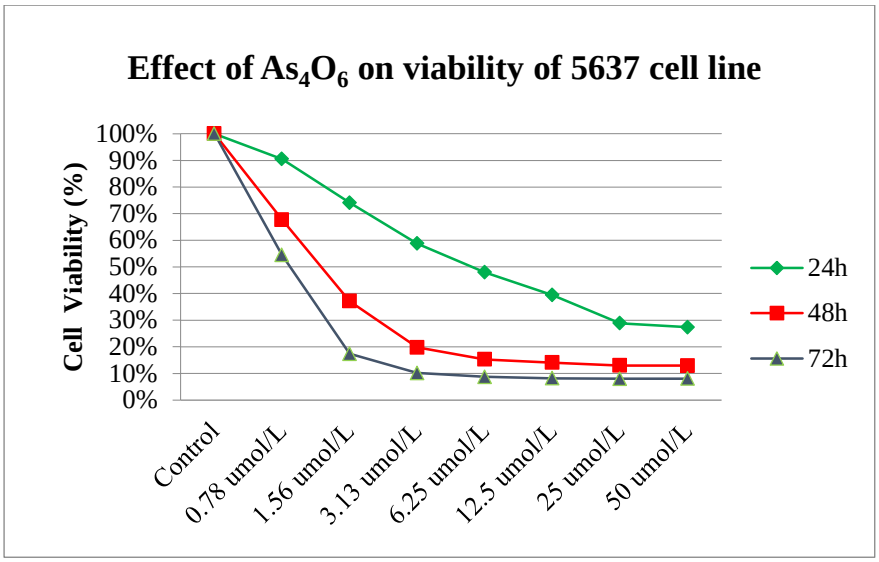

Fig. 3 Cell proliferation assay in 5637 human bladder cancer cell line. In 24hrs, 48hrs, 72hrs after the treatment of As_4O_6, the curves after 24hrs showed rapid decline near concerntration of 25 μmol/L；after 48hrs showed 3.13 μmol/L; after 72hrs showed 1.56 μmol/L.

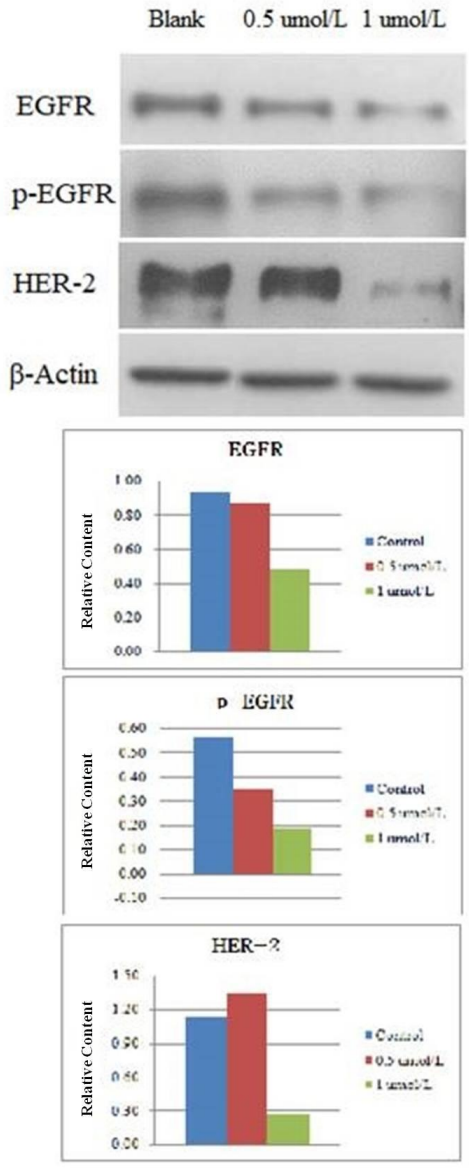

Fig 4. Effects of As_4O_6 on the protein levels of EGFR, p-EGFR and HER-2 in 5637 human bladder cancer cell line. We performed western blotting to detect the expressions of proteins related to the EGFR/HER-2 signaling pathway of migration and invasion.

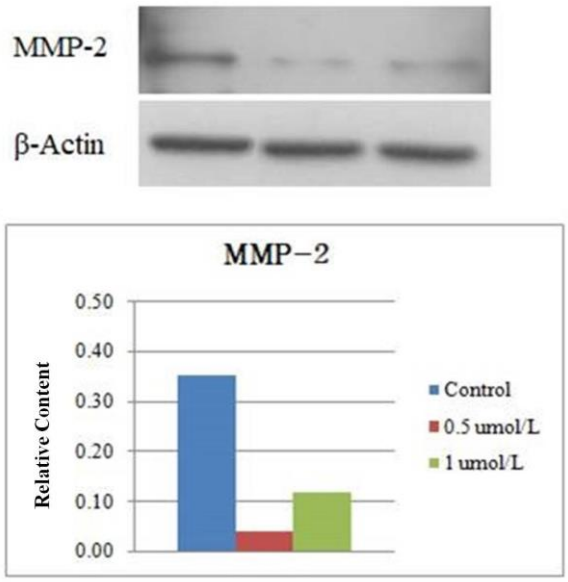

Fig 5. Effects of As_4O_6 on the protein level of MMP-2 in 5637 human bladder cancer cell line. We performed western blotting to detect the expressions of protein related MMP-2 signaling pathway of metastasis.

Table 1. IC_{50} value of As_4O_6 inhibiting proliferation in 5637 human bladder cancer cell line.

Cell	Hours	IC_{50}（umol/L）
5637	24	6.11
	48	1.12
	72	0.81

IV. DISCUSSION

Among urological malignancies, bladder cancer still takes the lead in incidence rate worldwide. Principal therapy in current use is surgery or BCG injection. However, the 5-year recurrence rate, even though lowered by post-surgical BCG injection, amounts to as high as 24%-84%.[32]

According to research reports, As_4O_6 exhibits anti-tumor effects in various patterns including induction of tumor apoptosis, inhibition of tumor angiogenesis, increase of sensitivity of solid cancer to radiation upon radiotherapy. However, the mechanism of As_4O_6 still remains uncertain for bladder cancer. Accordingly, we examined whether or not As_4O_6 can inhibit cell migration and growth in the 5637 human bladder cancer cell line and further investigated the mechanism therefor.

Epidermal growth factor (EGF) is known to incite cell proliferation by activating the four EGFR family members including EGFR/ErbB-1/HER1, HER2/ErbB-2, HER3/Erb B-3, and HER4/ErbB-4. In the human body, the EGFR family consisting of 30 or more ligands and four receptors is at the head of complex, hierarchical signaling networks involved in various oncogenic pathways. EGFR is known to increase in activity in many tumors such as breast cancer, colorectal cancer, ovarian cancer, prostate cancer, pancreatic cancer, and the like as well as being essential for normal physiological activities. This would be attributed to the synthesis or overexpression of EGF or an increase in the mutation of EGFR.[33][34] Complete transformation in vivo and in vitro requires coexpression of various ErbB receptors. As much as 50-70% of cases with lung cancer, colon cancer, and breast cancer were found to express EGFR or ErbB3.[34] Gain-of-function genetic alterations in EGFR are observed in up to 30% of solid tumors.[35][36] EGFR, although expressed in normal urothelial cells, is overexpressed, which is associated with the grading and progression stages of bladder cancer.[37]

ErbB2 (HER2) is also named neu because it was derived from a rodent glioblastoma cell line, a type of neural tumor. ErbB(/EGF) receptor is located in the Basolateral membrane of epithelial cells interacting with a ligand in an interstice, mediating signaling between the epithelium and the extracellular matrix. ErbB2 is a receptor observed to have the highest alteration ability. Although other factors such as pregnancy and hormone changes have an influence on the induction of tumor, aErbB receptors makes oncogenic induction more effectively.[34] It is reported that HER-2 is also overexpressed in bladder cancer[38][39] in proportion to the grading and progression stage of bladder cancer.[40] The expression of HER-2 will be related to the prognosis of bladder cancer. The amplification of HER-2 increases the risk of death in patients with bladder cancer while the overexpression of HER-2 is correlated with poor prognosis of bladder cancer and is an important

index for the prediction of disease-free survival (DFS) and progression-free survival (PFS).[41][42]

Expression of EGFR and ErbB2 is associated with poor prognosis. Overexpression of EGFR is correlated with decreased survival rates against head and neck cancer, uterine cervical cancer, esophageal cancer, ovarian cancer, etc. Analysis of the processes of controlling EGFR signal transduction, such as receptor heterodimerization and endocytosis, revealed the mechanisms contributing to opportunities of new therapies and efficacy of existing anticancer therapies.[34] Co-expression of EGFR and HER-2 is histologically associated with grading and bladder cancer grades and disease stages, accounting for biological invasion behaviors.[32]

Matrix metalloproteinase-2 (MMP-2), which is an endopeptidase bearing a metal ion, digests (together with MMP-9) type IV collagen, which is most abundantly found in the stratum basale, allowing tumor cells to penetrate into the stratum basale and finally make metastasis.[43] The stratum basale plays an important role in maintaining tissue structures, structurally supporting cells, and having influences on cellular signaling and initiative. Digestion of the stratum basale is an essential step for the progression of most cancer metastases.[44] In addition, MMP mainly acts to degrade extracellular matrix (ECM), helping cancer cells undergo distant metastasis. Invadopodia have a protrusive and adhesive structure formed on cancer cells which is associated with degradation of the extracellular matrix in cancer invasiveness and metastasis. For the local secretion and activation thereof, invadopodia concentrate MMPs (inclusive of MT1-MMP, MMP-2, and MMP-9).[45] In addition, the degradation product resulting from the action of MMP may further promote the formation of invadopodia and the activity of MMP.[46] Finally, MMP-2 and other several MMPs proteolytically activate TGF-β, promoting the epithelial mesenchymal transition (EMT), which is an important process in cancer metastasis.[47] Generally by degrading ECM, MMP allows the release of growth factors bound to ECM so that the factors bind to cellular receptors to influence cellular signaling. Furthermore, various MMPs activate, together with growth factors, other pro-MMPs. MMP-2 also cleaves non-ECM substrates including TGF-β, FGF receptor-1, pro TNF, IL-1β, and growth factors such as various chemokines.[48] For example, MMP-2, as well as MMP-9, is involved in the cleavage of latent TGF-β which makes complex interaction with cancer cells. Generally, TGF-β plays a role in maintaining the homeostasis of tissues and preventing the progression of tumor.[49] MMP-2 shows a close relation with various tumors including bladder cancer and promotes the invasion and metastasis of bladder cancer cells. Because a high expression level thereof is closely associated with poor prognosis of bladder cancer, MMP-2 plays a critical role in the invasion of bladder cancer.[50][51]

Bladder cancer are being under active study worldwide. However, although arsenic compounds were found to have anti-tumor effects in various patterns such as tumor apoptosis, anti-

angiogenetic activity, etc., not much research has been conducted into the influence of arsenic compounds on bladder cancer. As_4O_6 is orally administerable and was observed to have almost no particular side effects even at a dose of 15 mg/day thanks to the greatly mitigated toxicity thereof compared to As_2O_3. In this study, we conducted experiments to investigate the influence of As_4O_6 on 5637 human bladder cell line.

In the migration assay, the gap between the cell groups divided in half was increasingly closed in the presence of As_4O_6 in a dose-dependent manner. The gap in the cell groups at a concentration of 1.56 μmol/L significantly differed from that in the control, indicating that As_4O_6 effectively suppresses cell migration. In the MTT assay, As_4O_6 reduced cell proliferation in a dose- and time-dependent manner. According to the cell growth curve, As_4O_6 was observed to remarkably inhibit the cell growth until a concentration of 25 μmol/L 24 hrs after treatment therewith and until a concentration of 3.13 μmol/L 48 hrs after treatment therewith, and until a concentration of 1.56 μmol/L 72 hrs after treatment therewith. The data implicate that As_4O_6 inhibits the growth of 5637 human bladder cancer cell line, with significance.

Furthermore, a Western blot assay in which EGFR (p-EGFR) and HER-2, which were related to cell apoptosis, were analyzed for expression at the protein level in the presence of As_4O_6 showed that the two proteins were significantly reduced at 1 μmol/L. Another Western blot assay for MMP-2, which is related to cancer metastasis, revealed a reduction of the protein at 1 μmol/L of As_4O_6. Taken together, the data suggest that As_4O_6 downregulates the expression of the four proteins, thereby showing inhibitory effects on the migration, growth, and metastasis of 5637 human bladder cell line.

V. CONCLUSIONS

Through this study, we reached that conclusion that As_4O_6 has inhibitory activity at a concentration of 1.56 μmol/L against the migration and growth of 5637 human bladder cancer cell line, targeting the epithelial factor receptors EGFR, p-EGFR, and HER-2. In addition, it is considered that As_4O_6 might make a potential action through the downregulation of MMP-2 during the process of inhibiting the invasion and metastasis of 5637 human bladder cancer cell line.

Accordingly, there is a need for research into additional mechanisms for anti-bladder cancer effects by genetic analysis in animal PDX models on the basis of this study.

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