Y27632 attenuates the aristolochic acid-promoted invasion and migration of human urothelial cancer TSGH cells \textit{in vitro} and inhibits the growth of xenografts \textit{in vivo}

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\textbf{Abstract}

\textbf{Background.} Aristolochic acid I (AAI) has been implicated in urothelial cell carcinoma (UCC) in humans. However, whether AAI promotes invasion/migration of UCC has not been established.

\textbf{Methods.} A study of human UCC TSGH cells cultured with AAI was conducted. Cell viability, the effects of AAI on the activity of matrix metalloproteinase (MMP)-9, the abilities of invasion/migration and the migration-related proteins (Ras, RhoA, ROCK1, PI-3K, pAkt and nuclear factor-kappaB) of the TSGH cells were assessed. The TSGH cells were subcategorized to 1-day or 30-day AAI exposure. An \textit{in vivo} study using a nude mice xenograft model was employed to test the antitumor effects of Rho kinase inhibitor or Y27632.

\textbf{Results.} A time- and dose-dependent increase in both activity and messenger RNA (mRNA) level of MMP-9 were demonstrated. The mRNA level of urokinase-type plasminogen activator was increased and tissue inhibitor of metalloproteinase-1 was decreased in the cells with 30-day but not 1-day AAI exposure. A dose-dependent enhancement in wound-healing rate and cell migration was demonstrated, especially in the 30-day AAI-exposed cells. Expressions of Ras/RhoA and other migration-related proteins were increased after AAI treatment, which could be inhibited by Y27632. The \textit{in vivo} results demonstrated that Y27632 was able to attenuate the speed of growth of the inoculated tumors in nude mice.

\textbf{Conclusion.} Clinically, the patients with prolonged AAI exposure are highly associated UCC, our results provided \textit{in vitro} and \textit{in vivo} evidence that prolonged AAI exposure enhances invasion and migration of human TSGH cells.

\textbf{Keywords:} aristolochic acid; invasion; matrix metalloproteinase; migration; urothelial cell carcinoma

\textbf{Introduction}

Chinese herb nephrotoxicity has been traced to aristolochic acid (AA), which was the major alkaloid extracted from \textit{Aristolochia fangchi} \cite{1, 2}. AA is a mixture of structurally related nitrophenanthrene carboxylic acids, with aristolochic acid I (AAI) and AAlI being major components.
Toxicological studies of AA have shown that AAI was a major component of the carcinogenic plant extract AA and exposure to AA was strongly associated with human urothelial malignancy [3, 4], which includes moderate atypia and atypical hyperplasia of the urothelium [5]. The prevalence rate of urothelial cell carcinoma (UCC) after exposure to AA has been reported to range from 39 to 46% [6–9] and, recently, the estimated cumulative dose of AA showed a statistically significant linear dose response [AA: at 151–250 mg, odds ratio (OR) = 1.4, 95% confidence interval (CI) = 1.1–1.8 and at >500 mg, OR = 2.0, 95% CI = 1.4–2.9] with the risk of UCC (P < 0.001) [10].

The molecular changes that occur in human UCCs are numerous and can be categorized into several mechanisms: (i) chromosomal alterations leading to carcinogenesis, (ii) loss of cell cycle regulation accounting for cellular proliferation, (iii) metastasis, guided by events such as the extracellular matrix (ECM) and angiogenesis [11]. The mutagenic and carcinogenic properties of AA have been shown to be based on the formation of DNA adducts [12, 13], which were also demonstrated to be associated with cell proliferation, tumor induction [14, 15], activation of the H-ras oncogene and overexpression of the p53 [7, 16]. Activation of Ras proteins has been reported to induce the constitutive activation of downstream kinase cascades, which results in continuous mitogenic signaling and transformation of immortalized cells into human bladder cancer [17]. Recently, we reported that human bladder cancer tissue expressed increased Ras, PI-3K, Akt, nuclear factor-kappaB (NF-κB), RhoA and that human UCCs transfected with Ras would enhance expression of invasion/migration-promoting proteins [18]. Because UCC is the major cancer in the dialysis population and kidney transplant recipients in Taiwan [19, 20], we designed this study with the aim to test whether AA has a role enhancing invasion and migration of UCC cells and whether AA-associated urothelial tumorigenesis is also involved in the Ras/RhoA, PI-3K, Akt and NF-κB signaling [18].

Materials and methods

Chemicals

Tris (hydroxymethyl)-aminoethane, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride, bovine serum albumin, leupeptin, Nonidet P-40, deoxycholic acid, sodium orthovanadate, phallolidin–fluorescein isothiocyanate (FITC) and AA1 were purchased from Sigma–Aldrich (St Louis, MO). Phosphate buffer saline (PBS), trypsin–EDTA and powered RPMI 1640 medium, fetal bovine serum (FBS), 100 U/mL penicillin G and 100 mg/mL streptomycin were purchased from Gibco/BRL (Gaithersburg, MD). Antibody against Akt and MAPK/ERK1/2, phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). PI-3K (p85), NF-κB (p65), Ras, RhoA and ROCK1 antibodies were purchased from BD Transduction Laboratories (San Diego, CA).

Cell culture

TSGH 8301 cells, established from a well-differentiated human UCC of the urinary bladder (Grade II, Stage A), were cultured in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum, 100 U/mL penicillin G and 100 mg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The medium was changed twice a week, and the cells were subcultured when confluence was achieved.

Determination of cell viability [3-(4, 5-cimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay]

Cells were seeded in a 100 mm dish at a density of 3.5 × 104 cells/dish and treated with concentrations of AA from 0 to 50 μM at 37°C for 5 days. After the exposure period, the medium was removed and the cells were washed with PBS, then treated with AAI every day. At days 6, 11, 16 and 21, the cells were subcultured and 3.5 × 104 cells were seeded. Then the cells were harvested to detect the viability using an [3-(4, 5-cimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay] assay after 30 days of AA exposure. To evaluate the cytotoxicity of AAI, an MTT colorimetric assay was performed to determine the cell viability. Cells were seeded in 24-well plates at a density of 3.5 × 104 cells/well and treated with concentrations of AA from 0 to 50 μM at 37°C for 24 h. After the exposure period, media were removed, followed by washing of the cells with PBS. Thereafter, the medium was changed and incubated with 20 μL MTT (5 mg/mL) for 4 h. The viable cell number per dish was directly proportional to the production of formazan, which, following solubilization with isopropanol, can be measured spectrophotometrically at 563 nm.

Determination of matrix metalloproteinase-9 by zymography

The levels of matrix metalloproteinase (MMP)-9 released in the cultured medium were detected by gelatin zymography assays as previously described [21]. First, serum-free conditioned medium was prepared with a 5% loading buffer containing 0.01% SDS without β-mercaptoethanol. The prepared samples were subjected to electrophoresis with 8% SDS polyacrylamide gels containing 0.1% gelatin. Electrophoresis was performed at 140 V for 3 h in an ATTO apparatus. Gels were washed twice with 50 mL distilled water containing 2% Triton X-100 on a gyratory shaker for 30 min at room temperature to remove the SDS after electrophoresis. The gel was then incubated in 50 mL reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM CaCl2, 0.02% NaN3) overnight at 37°C, stained with Coomassie brilliant blue R-250 and destained with methanol–acetic acid–water (5, 7.5 and 87.5%, vol/vol/vol).

Reverse transcription–polymerase chain reaction

Total RNA was isolated from cells using a guanidinium chloride procedure and complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) amplification were performed as previously described [22]. For reverse transcription, 4 μg of total cellular RNA was used as templates in the reaction buffer containing 4 μL deoxynucleoside triphosphate (2.5 mM), 5 μL oligo dT20 (10 pmol/mL) and 1 μL R Tase (200 U/mL), and the reaction was performed at 42°C for 1 h. Then, the cDNA was stored at 4°C after reaction for 5 min at 99°C. Afterward, 10 μL cDNA product was amplified by PCR with the following primers: urokinase-type plasminogen activator (uPA) (314 bp), 5′-CAGCAGAAGGGGAGATGAAA-3′ (sense) and 5′-ACAAGTTTGTGGTTGACTT-3′ (antisense); MMP-9 (263 bp), 5′-CAGCTGTCACCCCTTCAGAGC-3′ (sense) and 5′-GCCACTTTGTCGCGATAAGG-3′ (antisense); tissue inhibitor of metalloproteinase-1 (TIMP-1) (481 bp), 5′-CTGGTTTGTGTCTGGTCTGATA-3′ (sense) and 5′-CCGTCCACAAAGCATATCG-3′ (antisense) and glyceraldehyde 3-phosphate dehydrogenase (462 bp), 5′-GAAAGTGAGAGGTCGGAGTC-3′ (sense) and 5′-GAAGATGCTGTGGGTACCTT-3′ (antisense). PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.

Wound-healing assay

The TSGH 8301 cells were cultured in one well of a six-well culture dish. A line was drawn on the underside of the well with a yellow P200 pipette tip. These lines served as fiducial marks for the wound areas to be analyzed. The growth medium was replaced by calcium-free PBS, then the cells were washed with PBS. Thereafter, the medium was changed and incubated with 20 μL MTT (5 mg/mL) for 4 h. The viable cell number per dish was directly proportional to the production of formazan, which, following solubilization with isopropanol, can be measured spectrophotometrically at 563 nm.
surface of the membranes were fixed with methanol and acetate (3:1), stained with Giemsa and counted under a light microscope.

Electrophoresis and immunoblotting
Analysis of Ras, PI-3K, pAkt, Nf-κB, RhoA and ROCK1 was performed using SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The medium was removed and washed with PBS. Then 0.5 mL of cold RIPA buffer (1% NP-40, 50 mM Tris–base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) with fresh leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL) were added. Scraping of cells and transfer of the lysate into an Eppendorf tube were performed prior to a 30-min incubation on ice incubation with the addition of 5 μL of 10 mg/mL phenylmethlysulfonyl fluoride stock. The cell lysate was centrifuged (10 000 g) for 10 min at 4°C. Cell lysate (50 μg purified protein) was mixed with an equal volume of electrophoresis sample buffer and then boiled for 10 min, followed by analysis using SDS–PAGE and transfer of protein from the gel to nitrocellulose membranes (Millipore, Bedford, MA) using an electroblotting apparatus. Nonspecific binding was blocked by incubation of the membrane with Tris-buffered saline (TBS) containing 1% (wt/vol) nonfat dry milk and 0.1% (vol/vol) Tween-20 (TBST) for >2 h. Membranes were washed with TBST three times for 10 min and incubated with an appropriate dilution of primary antibody in TBST for 2 h. Membranes were then extensively washed with TBST before being incubated with an appropriate amount of horseradish peroxidase-conjugated secondary antibody for 1 h. After washing the membrane three times for 10 min in TBST, detection was performed using ECL reagents for 1 min and exposed to ECL hyperfilm in a darkroom. Protein expression was determined by quantitative densitometry using an Alphalmager Series 2200 software.

Fig. 1. Effects of different durations and concentrations of AAI exposure on the viability of TSGH cells by MTT assay. TSGH cells (1 × 10⁵ cells/mL) were treated with various concentrations of AAI for (a) 1–3 days (b) or 30 days, respectively. The viability of the cells was determined by MTT assay. The survival cell number was directly proportional to formazan, which was measured spectrophotometrically at 563 nm. Values were expressed as the mean ± SD of three independent experiments. *P < 0.01 compared with the untreated controls, **P < 0.001 compared with the untreated controls.
Electrophoretic mobility shift assay (EMSA) was carried out by a LightShift EMSA Optimization and Control Kit and Chemiluminescent Nucleic Acid Detection Modules (Pierce, Rockford, IL). Binding reactions containing 10 μg of nuclear protein, 2 μl of 100× binding buffer, 1 μl poly-(dl-dC), 12.5 μl poly-l-lysine and 2 pmol of oligonucleotide probe were incubated for 20 min at room temperature. Protein-DNA complex was separated by electrophoresis on a 6% nondenaturing acrylamide gel, transferred to positively charged nylon membranes and then cross-linked in a Stratagene cross-linker. Gel shifts were visualized by streptavidin–horseradish peroxidase and followed by chemiluminescent detection.

Animal and tumor xenograft study

BALB/c nude mice (male, 5-week old) were purchased from the National Taiwan University Animal Center, Taiwan. The mice were randomly selected to be placed in three treatment groups (six in each group). The first group of mice (group T, controls) was subcutaneously (s.c.) injected with 2 × 106 TSGH cells mixed with an equal volume of Matrigel (BD Biosciences) in the left groin. The second and third groups (TA and TAY groups, respectively) were s.c. injected with 2 × 106 30-day 50 μM AAI-treated TSGH cells mixed with an equal volume of Matrigel in the left groin. Meanwhile, the TAY group was injected with Y27632 (25 mg/kg) in the left groin near the tumor cells three times a week. During the 4-week feeding period, all mice used were handled according to the guidelines of the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals. The mice were housed with a regular 12 h light/12 h dark cycle. After 28 days, the mice were sacrificed for the assay of tumorigenicity (e.g. tumor weight and tumor inhibition).

Statistics

Data were shown as the mean ± SD. Statistical comparisons were performed using Student’s t-tests. P-values <0.05 were considered to be statistically significant.

Results

Effect of AAI on the viability of TSGH cells

Prior to the investigation of the pharmacological potential of AAI on TSGH cell viability, we initially determined the time and dose dependence of the cytotoxic effects of AAI in TSGH cells by means of MTT assay. Unexpectedly, AAI showed a significant dose- and time-dependent inhibition, but no promotive effect on the growth of the TSGH cells (Figure 1a). Given that AAI, at a concentration <50 μM and exposure for 1 day, had modest cytotoxic effect on the cells, the cell viability of 30-day exposure to AAI was examined. TSGH cells in 30-day AAI exposure revealed a dose-dependent induction on the growth of TSGH cells, especially treatment with 50 μM AAI (P < 0.01) (Figure 1b). These results demonstrated that AAI increased cell survival after long-term incubation, whereas short-term AAI exposure resulted in loss of TSGH cell viability.

Effect of AAI on MMP-9 activity using gelatin zymography assay and on MMP-9, up A, TIMP-I messenger RNA level using reverse transcription–PCR of TSGH cells

In addition to facilitating tumor migration, extracellular products such as MMPs can modulate migration, cancer cell proliferation and metastasis [25]. The levels of MMP-9 were assayed by gelatin zymography. TSGH cells with 30-day AAI exposure displayed a dose-dependent increase
of MMP-9 (AAI 6.25 μM, P < 0.01; AAI 12.5, 25 and 50 μM, P < 0.001) (Figure 2). However, the expression of MMP-9 after 1-day AAI exposure was without significant variation. Furthermore, to explore whether AAI-regulated MMP-9, TIMP-1 and uPA on the transcriptional level, a semiquantitative reverse transcription (RT)–PCR analysis was performed. The 30-day AAI exposure stimulated uPA, MMP-9 messenger RNA (mRNA) levels but inhibited TIMP-1 mRNA level dose dependently (Figure 3), which indicated that TIMP-1 was inhibited and uPA was activated by 30-day AAI treatment in early stage and then promoted the formation of pro-MMP-9 to MMP-9.

The wound-healing assay and the invasion ability of TSGH cells treated with AAI

Cancer cell migration can be viewed as a process regulated by matrix-degrading proteinases, integrins, other cell adhesion molecules and the healing of a wound [26, 27]. The impact of AAI on wound healing and cell invasion was then studied (Figures 4 and 5). The results demonstrated that 30-day exposure of AAI exhibited prominent promotion on cell mobility/migration. In addition, the cell amount of invasion was dose-dependently increased when the cells were with 30-day AAI exposure compared with those with only 1-day exposure (AAI 12.5, 25 and 50 μM, P < 0.001) (Figure 5). Therefore, prolonged exposure with higher dose of AAI enhanced the capability of wound healing and invasion of TSGH cells.

Effect of AAI on migration-related proteins of TSGH cells treated with or without Y27632

Stimulated Ras could promote many Ras-related effectors. We investigated whether different duration and concentration of AAI exposure could affect the interaction of Ras and downstream Ras-dependent signalings. The TSGH cells with 1-day AAI exposure did not display significant changes in the levels of RhoA, ROCK1, PI-3K, pAkt and NF-κB compared to those of the control cells on different AAI concentrations. However, after 30-day exposure, TSGH cells showed significantly dose-dependent increased levels in RhoA, ROCK1, PI-3K, pAkt, and NF-κB compared to those of the controls, suggesting that prolonged AAI exposure promoted both the PI-3K and RhoA pathways (Figure 6). Additionally, the addition of Y27632, a Rho kinase inhibitor, caused a significant reduction in the levels of ROCK1, PI-3K and PI-3K-related downstream proteins. A significant decrease in the levels of PI-3K and PI-3K-related proteins were found after adding wortmannin, a PI-3K inhibitor. This demonstrated that ROCK1 was an intermediate factor regulating PI-3K and its related protein expression (Figure 7). Figure 8 demonstrated the increased phalloidin binding and cellular F-actin levels by AAI treatment in the TSGH cells. The amount of F-actin could be inhibited by the Rho kinase
inhibitor or Y27632 but not PI-3K inhibitor. In addition, nuclear extracts were analyzed for NF-κB DNA-binding ability; AAI treatment increased NF-κB DNA binding ability which could be inhibited by Y27632 but not PI-3K inhibitor (Figure 9).

Y27632 decreased the speed of growth of the inoculated tumors in nude mice

To further test the antitumor effect of Y27632, xenograft assays were employed. After inoculating human bladder cancer AAI-treated TSGH cells into the nude mice s.c., Y27632 was simultaneously injected into the mice. Afterward, the same amount of Y27632 was injected into the mice three times a week. The tumor volume was calculated (1/2 × length × height × width) every 4 days and the tumor weights were detected after sacrifice (Figure 10 and Table 1). The tumors in the Y27632-treated mice were much smaller than in the TA group. We also measured the diameters of the inoculated tumors 1 week after the inoculation when all of the mice started to show signs of the formation of solid tumor masses. The measurements were taken every 4 days for 28 days. At Day 28, the tumor masses from the mice inoculated with AAI-treated TSGH cells were ~18.5-fold or bigger than those treated with Y27632. A marked reduction (95.3%) in tumor formation was obtained after the s.c. inoculation of Y27632-treated TA cells as compared with the untreated TA cells. Interestingly, the tumor weight and tumor volume of the TAY group were also smaller than the TSGH-inoculated alone mice (P < 0.001). This in vivo study suggests that Y27632 is able to attenuate the speed of growth of inoculated tumors.
tumors in nude mice. Taken together, a proposed model for the AAI-mediated invasion/migration of human bladder cancer cells is summarized in Figure 11.

**Discussion**

Our results demonstrated that AAI promoted invasion and migration of TSGH UCC cells via dual induction of MMP-9 enzyme activity and gene transcription. The migration and invasion abilities of AAI on TSGH cells were significantly enhanced in accordance with longer duration and higher concentration of AAI. MMP-9 gene expression can be activated via a signal transduction pathway through Ras, RhoA, PI-3K and Akt, which were the upstream modulators of NF-κB [28]. This study revealed that 30-day AAI exposure stimulated the Ras/RhoA signaling and expressions of Ras, RhoA, ROCK1, PI-3K/pAkt and NF-κB in TSGH cells, and that these promoting effects of invasion/migration were time- and dose-dependent.

The herbal drug AA, in which the major components are nitrophenanthrene carboxylic acids, after metabolic activation via cytochrome P450 1A1, 1A2, NAD(P)H: quinone oxidoreductase (NQO1) [29, 30] and DT diaphorase [31], were genotoxic mutagens. Moreover, AA exerted genotoxicity via nitric oxide and its derivative peroxynitrite in a dose-dependent manner (0–200 μM) in human HepG2 cells [32]. After 48 h of treatment, mouse embryonic fibroblasts showed decreased cell survival, from 80% at 20 μM to <20% at 100 μM concentrations of AAI [33].

In rats, short-term toxicity (3 days) of AA could cause dysfunction of both kidney and liver [34, 35]. Oral or intravenous administration of AA in rats and mice caused death from acute renal failure within 15 days with lethal dose, 50% ranged from 56 to 203 mg/kg orally or 38 to 83 mg/kg intravenously [36]. In mice, AA treatment in daily doses of 5.0 mg/kg for 3 weeks resulted in squamous cell carcinoma of the forestomach, adenocarcinoma of the glandular stomach, kidney adenomas, lung carcinomas and uterine hemangiomas [37]. Big Blue rats treated with AAI at concentrations of 0.1, 1 and 10 mg/kg five times per week for 12 weeks disclosed a strong linear dose–response for mutation frequency inductions for AA-induced DNA adducts, which suggested that the mutagenic effects of AA were associated with the formation of AA–DNA adducts [38]. The AA–DNA adducts had been identified and

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Fig. 5. Effects of AAI on the invasive ability of TSGH cells. (a) The cells (1 × 10^5 cells/mL) were treated with different concentration and duration of AAI and migrated cells were analyzed using a modified Boyden chamber. Cells in serum-free RPMI medium were added to the upper chamber and allowed to migrate for 5 h through an 8-mm porous membrane toward the lower chamber to conditioned medium. Motility was quantified by counting the number of cells that migrated to the undersides of the membrane under microscopy (100×). (b) The results are shown as means ± SDs of eight independent experiments. *P < 0.01 compared with the untreated controls, **P < 0.001 compared with the untreated controls.
detected in experimental animals exposed to AA or botanical products containing AA and in urothelial tissues from AA nephropathy patients [6, 8, 37].

From 1998 to 2002, there were 949 new cases of end-stage renal disease in Taiwan in which the patients had consumed herbal products containing AA before the diagnosis of chronic kidney disease, representing 3.7% of all new patients with end-stage renal disease [39]. Chang et al. [20] reported that chronic tubulointerstitial nephritis is the most likely underlying renal disease in hemodialysis patients with UCC, and a high percentage of the chronic tubulointerstitial nephritis related to the usage of Chinese herbs or compound analgesics may contribute to the development of UCC. In addition, from 2001 to 2002, there were 118 new cases of UCC in Taiwan associated with the ingestion of >60 g of the Chinese herb Mu Tong (containing significant AA), representing 3% of all new patients with UCC [10]. Nortier et al. conducted regular cystoscopic examinations and the prophylactic removal of native kidneys and ureters in patients with end-stage Chinese herb nephropathy. Among 39 patients who agreed to undergo prophylactic surgery, there were 18 cases of urothelial carcinoma (prevalence, 46%; 95% CI, 29 to 62%), 17 cases of carcinoma of the ureter, renal pelvis or both and 1 case of a papillary bladder tumor [7]. The prevalence rate of UCC after exposure to AA has been reported to range from 39 to 46% [6–9]. AA correlated with a higher risk of UCC if total cumulative doses of AA were >200 g reported from Belgium [7] and 150 g reported from Taiwan [10].

Ras oncogenes played a key role in the initiation of human UCC carcinogenesis [40, 41]. AA-induced UCC was associated with the formation of DNA adducts and mutations in H-ras [42, 43] and p53 [12]. The carcinogenesis of human UCC was a multistep process and the rate-limiting step of cancer cell invasion was the breakdown of connective tissue barriers, ECM, which comprised collagens, proteoglycan, elastin, laminin and fibronectin [44]. The main groups of proteolytic enzymes involved in the ECM degradation were MMPs and zinc-dependent proteinases. The degradation of ECM by MMP-2 and -9 had been shown to be an important biological process in the metastasis of cancer cells [45, 46]. MMP-9 had increased expression in UCC when compared to normal urothelium and also correlated with increased tumor stage [47]. A specific inhibitor of the Rho kinase, Y27632 [48, 49] had been reported to block both Rho-mediated activation of actomyosin, the invasive activity of cultured rat MM1 hepatoma
cells and prevented Ras-induced migration-related proteins of TSGH human bladder cancer cells [18]. Continuous treatment with this inhibitor reduced dissemination of MM1 hepatoma cells implant into the peritoneal cavity of syngeneic rats [51]. In this study, Y27632 could inhibit RhoA, ROCK1, PI-3K, pAkt and NF-κB expression of AAI-treated TSGH cells and the growth of xenografts in vivo.

In summary, our study showed that 1-day AAI exposure caused cytotoxicity on TSGH cells and 30-day exposure of AAI promoted the expressions of Ras/RhoA, PI-3K, pAkt,

Table 1. Y27632 attenuated AAI-treated TSGH cells to form tumors in nude mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor weight (g)</th>
<th>Tumor volume (mm³)</th>
<th>Tumor inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>T</td>
<td>0.11 ± 0.027</td>
<td>94.11 ± 16.4</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>0.74 ± 0.054*</td>
<td>504.11 ± 56.4</td>
<td></td>
</tr>
<tr>
<td>TAY</td>
<td>0.04 ± 0.016#</td>
<td>23.7 ± 5.16**</td>
<td>95.3#</td>
</tr>
</tbody>
</table>

*P < 0.001 versus T (control) group; #P < 0.0001 between TA (AAI-treated) group and TAY (AAI-treated plus Y27632) group.

Fig. 7. Effects of AAI (50 μM) on the expression of migration-related proteins of the TSGH cells treated with Y27632 and Wortmannin. Total cell lystate was extracted and the expression of Ras, RhoA, ROCK1, PI-3K, and NF-κB were assayed by western blotting. Actin was used as an internal control.

Fig. 8. The increased phalloidin binding and cellular F-actin levels by AAI treatment in the TSGH cells. The AAI-treated cells (1 × 10⁵ cells/mL) that were pretreated Y27632 or Wortmannin for 1 h were seeded in a six-well plate. F-actin was stained with rhodamine phalloidin (red). The F-actin staining was more intense in the AAI-treated groups than the control cells and the amount of F-actin could be inhibited by the Rho kinase inhibitor but not PI-3K inhibitor.

Fig. 9. The DNA-binding ability of NF-κB. Nuclear extracts were analyzed for NF-κB DNA-binding ability using biotin-labeled NF-κB-specific oligonucleotide by EMSA. Three independent experiments were conducted, all showing similar patterns of change.
NF-κB, ROCK1 and enhanced invasion/migration of TSGH cells. Moreover, RhoA kinase inhibitor could inhibit AAI-induced migration-related proteins. Our results provide in vitro and in vivo evidence that prolonged and accumulated exposure of AAI enhances UCC invasion and migration and the promotion was through Ras and/or RhoA pathways. This might offer hope with regard to the possibility of developing new treatment strategies for interventions in AA-associated UCCs.

Conflict of interest statement. None declared.

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Received for publication: 15.10.10; Accepted in revised form: 30.5.11