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Opposite effect of ERK1/2 and JNK on p53-independent p21$^{WAF1/CIP1}$ activation involved in the arsenic trioxide-induced human epidermoid carcinoma A431 cellular cytotoxicity

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Summary

While arsenic trioxide (As_2O_3) is an infamous carcinogen, it is also an effective chemotherapeutic agent for acute promyelocytic leukemia and some solid tumors. In human epidermoid carcinoma A431 cells, we found that As_2O_3 induced cell death in time- and dose-dependent manners. Similarly, dependent regulation of the p21$^{WAF1/CIP1}$ (p21) promoter, mRNA synthesis, and resultant protein expression was also observed. Additionally, transfection of a small interfering RNA of p21 could block the As_2O_3-induced cell growth arrest. The As_2O_3-induced p21 activation was attenuated by inhibitors of EGFR and MEK in a dose-dependent manner. Using a reporter assay, we demonstrated the involvement of the EGFR-Ras-Raf-ERK1/2 pathway in the promoter activation. In contrast, JNK inhibitor enhanced the As_2O_3-induced p21 activation, also in a dose-dependent fashion. Over-expression of a dominant negative JNK plasmid likewise also enhanced this activation. Furthermore, MEK inhibitor attenuated the anti-tumor effect of As_2O_3. In contrast, in combination with JNK inhibitor and As_2O_3 enhanced cellular cytotoxicity. Therefore, we conclude that in A431 cells the ERK1/2 and JNK pathways might differentially contribute to As_2O_3-induced p21 expression and then due to cellular cytotoxicity.

Introduction

Arsenic exists ubiquitously in our environment, and various forms of arsenic circulate in soil, water, air, and living organisms. It has been reported that high arsenic levels in drinking water (0.35–1.14 mg/l) will induce neurotoxicity, liver injury, peripheral vascular disease (known as blackfoot disease), and increase risks of cancer in skin, bladder, kidney, lung and colon [1]. Paradoxically, arsenic has also been used for medicinal purposes for more than 2400 years. Two arsenic compounds, including arsenic trioxide (As_2O_3) and arsenic disulfide, used in some traditional Chinese remedies are very effective in the treatment of acute promyelocytic leukemia (APL), syphilis and psoriasis [2]. However, the mechanistic basis for the carcinogenic or therapeutic effects

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of arsenic is still poorly unclear. As_2O_3 may act on APL cells through several mechanisms, including induction of differentiation, growth inhibition, apoptosis, and angiogenesis inhibition [2]. It seems that As_2O_3 exerts its effects against APL by triggering apoptosis, possibly through induction of reactive oxygen species [3, 4]. Studies in culture cells show that it inhibits growth and promotes apoptosis not only in myeloid leukemia, multiple myeloma, lymphoma, and lymphocytic leukemia, but also in solid tumor cell lines including prostate, cervical, bladder, ovarian, colon, and gastric cancer cell lines [5]. Recently, the National Cancer Institute Cooperative Research and Development Agreement (NCICRADA) wonder to determine whether the preclinical anti-tumor effects of As_2O_3 can be translated into significant clinical benefit.

The cell cycle is mainly controlled by a series of regulatory proteins called cyclins and cyclin-dependent kinase (CDKs). The CDKs are activated by cyclin binding and inhibited by CDK inhibitors (CDKIs) to regulate cell cycle [6]. The control of cell cycle is predominantly performed through checkpoints in the G1/S transition, G2 and M phases. $p21^{WAF1/CIP1}$ (p21) is regarded as a CDKI of G1 cyclin-CDK complexes, which keeps retinoblastoma tumor suppressor protein (RB) in its active hypophosphorylated form and results in an arrest of cells in the G1 phase of the cell cycle [7]. p21 was cloned independently by two laboratories as a CDK-binding protein by yeast two-hybrid analysis (hence Cip1 for CDK-interacting protein-1) [8], then as a gene activated by wild-type p53 (thus WAF1 for wild type p53-activated factor) [9]. Others have shown that p21 plays an important role in the regulation of G2-M phase transition via its inhibitory effect on Cdc2-cyclin B complex accumulation [10]. Under some conditions, low levels of p21 plays a role of assembly factor for cyclin D-Cdk4 complex formation to facilitate S-phase entry by phosphorylating the pRb [11]. p21 gene expression is regulated by two distinct mechanisms. One is the p53-dependent mechanism, which is activated by DNA damage [12]. The other is the p53-independent mechanism, which is activated by mitogenic growth factors [13] or oxidative stress [14].

It seems that As_2O_3 damages DNA and induces cell cycle arrest at G1 or at G2-M phase [15]. However, the molecular mechanisms of arsenic's effects have not been fully elucidated. Yih and Lee [16] have proved that arsenic induce cell cycle arrest at the G2-M phase through an ATM $\rightarrow$ p53 $\rightarrow$ p21 signaling pathway in human fibroblast (HFW) cells. Recently, they also demonstrate that arsenic-induced c-anaphases mainly exit from mitosis without cell division and become tetraploid in the subsequent cell cycle [17]. In cancer cell lines, another group also suggests that As_2O_3 treatment, similar to most anti-tubulin agents, leads to accumulation of cells in mitosis, and then induction of apoptosis [18].

In response to extracellular stimuli, three distinct MAPK pathways have been characterized completely in cells. The extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (Raf $\rightarrow$ MEK1/2 $\rightarrow$ ERK1/2) is activated by mitogens via Ras and by phorbol esters via PKC. The stress-activated MAPK pathways, JNK/SAPK (MEK kinase1,3 $\rightarrow$ MKK4,7 $\rightarrow$ JNK1,2,3) and p38 (MAPKKK $\rightarrow$ MKK3,6 $\rightarrow$ p38 $\alpha, \beta, \gamma, \delta$), are activated by cellular stress, such as UV light, oxidative stress, and inflammatory cytokines [19]. So far, the signal transduction pathways regulated by arsenic include JNK/SAPK [20], p38 [21], and Ras-Raf-ERK1/2 via the EGF receptors [22].

To clarify the As_2O_3-induced p53-independent pathway, we utilized the human epidermoid carcinoma A431 cell line, which carries a mutant and inactive p53 protein (G $\rightarrow$ A at codon 273) resulting in an arginine to histidine substitution [23]. Presently, we demonstrate that As_2O_3 could up-regulate p21 and then induce cellular cytotoxicity. However, As_2O_3 up-regulated p21 expression through at least two opposing pathways. One is the EGFR-Ras-Raf-ERK1/2 pathway, which is involved in the As_2O_3-induced p21 activation. The other is the JNK pathway, which inhibits As_2O_3-induced p21 activation. Furthermore, combining As_2O_3 and inhibitors for MEK and JNK also showed opposing effects to cell viability. Therefore, we conclude that the opposing effects of these two pathways are involved in As_2O_3-induced cellular cytotoxicity.

Materials and methods

Reagents

As_2O_3 and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MI, USA).

PD153035, SB203580, PD98059 and SP600125 were purchased from Calbiochem (San Diego, CA, USA). U0126, Wizard Plus MiniPreps DNA purification system, pfu polymerase, DNase I, luciferase plasmid pGL-3 and luciferase assay system were from Promega (Madison, WI, USA). Qiagen-tip 100 was from Qiagen (Hilden, Germany). GENECLEAN III kit was from BIO 101 (La Jolla, CA, USA). β-Galactosidase plasmid (CMV β) was from Clontech Laboratories (Palo Alto, CA, USA). LipofectAMINE^{TM} 2000 reagent was from Invitrogen (Carlsbad, California, USA). Dulbecco's modified Eagle's medium, ribonuclease inhibitor and Opti-MEM medium were from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT, USA). Restriction enzymes and T_4-polynucleotide kinase were obtained from New England Biolabs (Beverly, MA, USA) or TaKaRa (Kyoto, Japan). Titan^{TM} One Tube RT-PCR System and Rapid DNA ligation kit were from Roche Molecular Biochemicals (Mannheim, Germany). Antibody against p21 was obtained from Transduction Laboratories (Lexington, KY, USA). Antibodies against serine phosphorylated and nonphosphorylated ERK, JNK, and p38 were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture

The human epidermoid carcinoma A431 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were grown at 37 °C in a humidified atmosphere of air/CO_2 (19:1, v/v). The reporter of p21 promoter, WWP-luc, was kindly provided by Dr. Bert Vogelstein [9].

MTT assay

The effect of As_2O_3 on A431 cell survival was determined using the MTT reduction assay, as described previously [24] with minor modifications. In brief, 5×10^3 cells were plated in 96-well plates in 100 μl of regular medium per well. After 24 h growth, medium with various concentrations of As_2O_3 was added to each well without changing the medium; 10 μl of MTT (5 mg/ml in serum-free medium) were added at 24 and at 48 h after the initial addition of As_2O_3. After incubation for 4 h at 37 °C, wells were added 50 μl of lysis solution (10%SDS in 0.01 M HCl) and incubated for 24 h. The cell survival fraction was measured at optical density (O.D.) 490 nm on a TECAN microplate ELISA reader (TECAN, Crailsheim, Germany) using MagellCE software. Assays were performed in triplicate. Percentage survival was defined as:

$$\%\,\text{survival} = 100\% \times (\text{O.D. of test sample}/\text{O.D. of control}).$$

Western blot

An analytical 10% SDS-PAGE gel was prepared. The cell lysates (50 μg protein of each) prepared from control and As_2O_3-treated cells were analyzed. For immunoblotting, proteins in the SDS-PAGE gel were transferred to a polyvinylidene difluoride membrane by a Semidry apparatus. Antibodies against human p21, β-actin, phspho-MAP kinase, phospho-SAPK/JNK, phospho-p38 MAP kinase, ERK protein, JNK1 protein, and p38 protein were employed as the primary antibodies. Immunoblot analysis was carried out with mouse or rabbit lgG antibody coupled to horseradish peroxidase. An enhanced chemiluminescence kit (Amersham) was used for detection. The density of the immunoblots was determined by an image analysis system installed with a software BIO-ID (Vilber Lourmat, France).

Transfection and reporter gene assays

The transfection method was performed with LipofectAMINE^{TM}2000 reagent according to the manufacturer's instruction with a slight modification. A431 cells were subcultured for 24 h before transfection at a density of 3×10^5 cells in 2 ml of fresh culture medium in a 6-well plate per well. For use in transfection, 1 μl of LipofectAMINE^{TM} 2000 reagent was mixed with 0.5 μg of p21 luciferase plasmid or other plasmids in 1 ml of Opti-MEM medium and incubated for 30 min at room temperature. Cells were transfected by changing the medium to 1 ml of culture medium containing the plasmids and LipofectAMINE^{TM}2000 reagent. Following incubation at 37 °C in a humidified atmosphere of air/CO_2 (19:1) for 48 h, cells were treated according to experimental designs and lysed. Then the luciferase activity was measured as described previously [25].

In addition, a p21 small interfering RNA (siRNA) (Santa Cruz, CA) was used for the p21 gene knockdown in A431 cells according to the manufacturer's recommendations. After transfection, the cells were subjected to MTT assay and Western blot as described above. Each transfection was normalized with appropriate empty vector plasmids or random sequence of p21 siRNA.

RT-PCR

Total RNA of human A431 cells was used for RT-PCR amplification of p21 expression. A sense primer (5′-GCCGAAGTCAGTTCCTTGTGGA-3′) and an antisense primer (5′-GTGGGCGGAT TA GGGCTT-3′), corresponding to the nucleotide sequence from nucleotide 1 to 22 and that from nucleotide 562 to 579, respectively, were prepared. The RT-PCR method was performed with Titan^{TM} One Tube RT-PCR System according to the manufacturer's instruction with a slight modification. The RT-PCR mixture contained 3 μg of the total RNA, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 mg/ml bovine serum albumin, 1 unit of DNA polymerase, and a 200 μM concentration of each of dATP, dCTP, dGTP, and dTTP in a total volume of 50 μl. The RT-PCR was carried out in a Gene Amp PCR System model 2400 (Applied Biosystems, Foster, CA, USA). The first RT-PCR cycle consisted of a reverse transcription step (50 °C, 30 min), and a denature step (95 °C, 5 min). In the subsequent 30 cycles consisted of the denature step (95 °C, 30 s), the annealing step (60 °C, 30 s), and the elongation step (68 °C, 1 min). For the final step, the duration of the elongation was 7 min. Finally, samples were mixed with loading buffer [50 mM Tris, 10 mM EDTA, 0.25% (w/v) bromophenol blue] and loaded onto a 1% agarose gel containing 0.1 μg/ml ethidium bromide. The agarose gels were run at 50 V for 90 min in a TAE buffer. The gels were observed and photographed under UV light.

Morphological assessment of cellular cytotoxicity

Cells were pretreated with or without PD98059 or SP600125 for 1 h, respectively, and then treated with As_2O_3 at the concentration of 20 μM for 24 h. Images were examined in a Nikon Eclipse TE2000-U inverted phase-contrast microscope (Nippon Kogaku KK, Tokyo, Japan) at 400× magnification to observe the morphological changes of the cells and photos were taken.

Flow-cytometric analysis

A431 cells were pretreated with or without PD98059 or SP600125 for 1 h, respectively, and then treated with As_2O_3 at the concentration of 20 μM for 24 h. Trypsinized cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20 °C for at least 1 h. After fixation, cells were washed twice and incubated in 0.5 ml of 0.5% Triton X-100/PBS at 37 °C for 30 min with 1 μg/ml of RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide for 10 min. Fluorescence emitted from the propidium iodide-DNA complex was quantified after excitation of the fluorescent dye by fluorescence-activated cell sorter (FACscan, Becton Dickinson, Mountain View, CA, USA).

Results

Reduction of cell survival and induction of p21 by As_2O_3

Figure 1a showed that A431 cells were sensitive to different concentrations of As_2O_3 for 24 and 48 h by using the MTT assay. In cells treated with 20 μM As_2O_3, p21 promoter activity was up regulated in a time-dependent manner (Figure 1b). Similarly, the time-dependent regulation of p21 mRNA expression was confirmed using RT-PCR (Figure 1c). The level of GAPDH verified loading of an equivalent amount of RNA (Figure 1c). Finally, protein production also showed a time-dependent behavior (Figure 1d). Dose-dependent effects on As_2O_3-induced p21 expression were also revealed (data not shown).

p21 siRNA attenuated the As_2O_3-induced cell growth arrest

To further investigate the role of p21 in the As_2O_3-induced cell growth arrest, p21 siRNA transfected-cells were left untreated or were exposed to 20 μM As_2O_3 for 24 h prior to the determination of p21 protein expression by means of Western blotting and the percentage of cell survival by the MTT

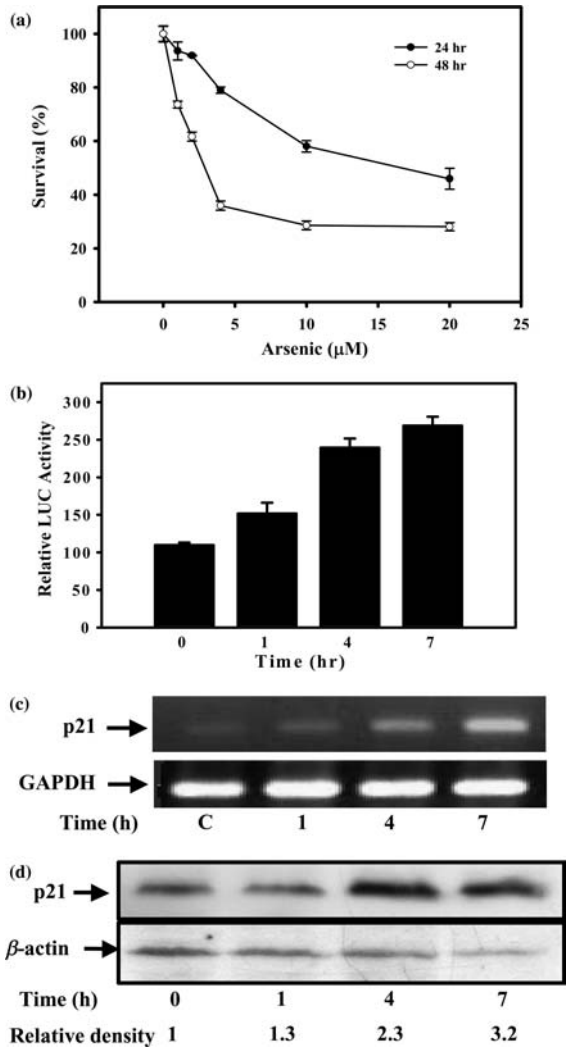

Figure 1. Effects of As_2O_3 on A431 cell survival and p21 expression. (a) Cells were exposed to various concentrations of As_2O_3 for different time courses. After treatment, cultures were assessed by MTT assay for cell survival determination as described in "Materials and methods". The experiment was repeated twice with similar results. Each point represents the mean of triplicate samples. Bars indicate standard error. (b) A mixture of p21 luciferase plasmid (0.5 μg) was transfected into A431 cells by using the transfection method as shown in "Materials and methods". Then cells were treated with 20 μM As_2O_3 for various time periods. The luciferase activity was determined and normalized with the amount of total protein. Values are means ± S.E.M. of triplicate measurements and expressed as "Relative Luc Activity" (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with untreated control). (c) Three μg of total RNA was analyzed by RT-PCR method to determine the expression of As_2O_3-treated p21 expression (upper). The level of GAPDH mRNA (bottom) verified loading of an equivalent amount of RNA. (d) Fifty μg of As_2O_3-treated cell lysates of A431 cells was analyzed by immunoblot using anti-p21 antibody.

assay. As shown in Figure 2a, p21 siRNA blocked the As_2O_3-induced p21 expression in a dose-dependent manner. Additionally, cells transfected with p21 siRNA were more resistant to As_2O_3-induced arrest than the control (Figure 2b).

Activation of EGFR-Ras-Raf pathway in As_2O_3-induced p21 expression

Arsenite usurps the EGFR-Ras-ERK1/2 signaling pathway to activate ERK1/2 phosphorylation [22]. We used specific inhibitors and dominant positive or negative plasmids to determine whether EGFR-Ras-Raf pathway was responsible for As_2O_3-induced p21 expression. The experiments showed that pretreatment of cells with the EGFR inhibitor, PD153035, inhibited As_2O_3-induced p21 promoter activation (Figure 3a) and protein expression (Figure 3b), and another inhibitor (AG1478) also attenuated protein expression (data not shown). The expression vector of the Ras dominant positive mutant (pSV2-ras) and Ras dominant negative mutant (pMM-rasDN) were used to determine whether As_2O_3-induced p21 expression was mediated through Ras activation. As shown in Figure 3c, transfection of pSV2-ras activated p21 promoter expression in a dose-dependent manner. Conversely, transfection of pMM-rasDN inhibited As_2O_3-induced p21 promoter activation, also in a dose-dependent manner (Figure 3d). Transfection with the expression vectors of Raf dominant negative mutant (Raf-C4B) also inhibited the promoter activation (Figure 3e).

Involvement of ERK1/2 activation in As_2O_3-induced p21 expression

The EGFR inhibitor, PD153035, inhibited As_2O_3-induced ERK1/2 phosphorylation in 5 and 10 min (Figure 4a), consistent with the involvement of As_2O_3-induced ERK1/2 activation in the EGFR-Ras-Raf pathway. Pretreatment of 20 μM MEK inhibitor, PD98059, could reduce As_2O_3-induced ERK1/2 activation (Figure 4b, lane 3) and p21 promoter activity (Figure 4c, column 5) to the basal level (Figure 4b, lane 1 and 4C, column 5). Interestingly, 20 μM PD98059 could not completely inhibit As_2O_3-induced p21 protein expression (Figure 4b). We conclude that the EGFR-Ras-Raf-ERK1/2 pathway is involved in As_2O_3-induced p21 expression. However, other

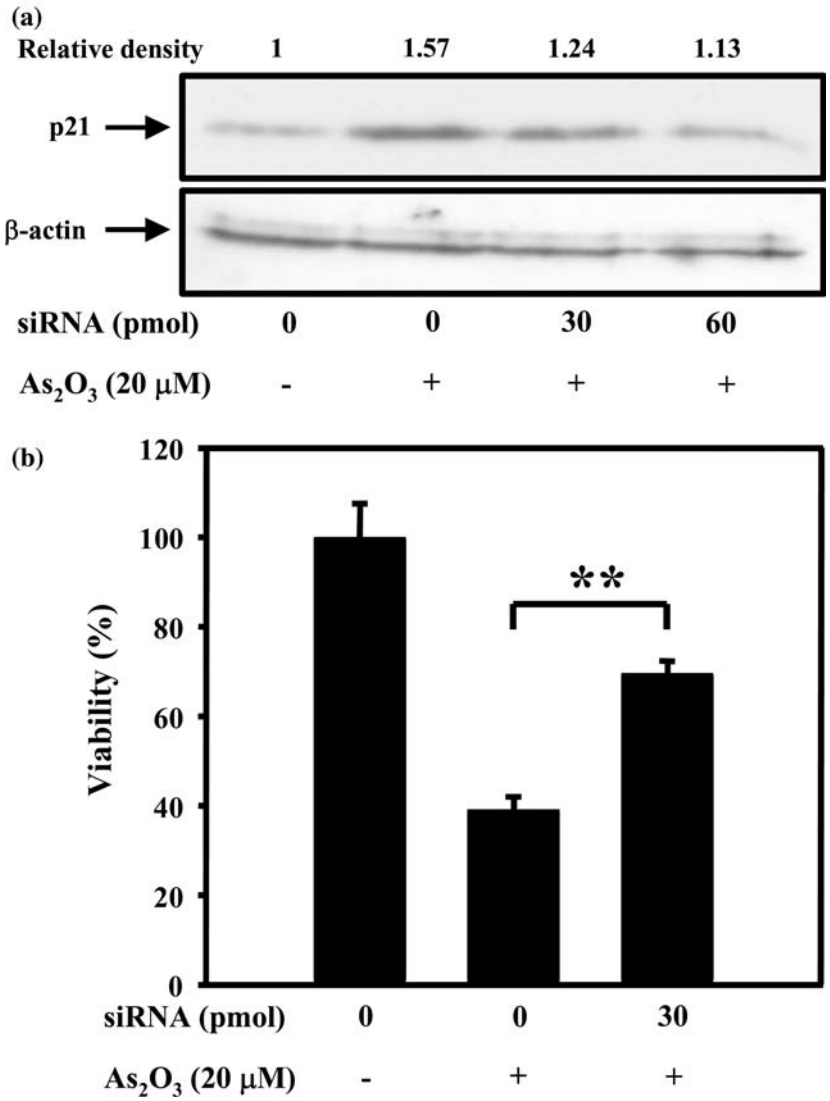

Figure 2. Reduction of the effects of As_2O_3-induced p21 expression and cytotoxicity of A431 cells by transfection of p21 siRNA. A431 cells were transfected with control or various amounts of p21 siRNAfor 40 h and followed by treatment with As_2O_3 (20 μM) for 24 h. p21 mass (a) in cell lysates and cell survival assay (b) were determined and analyzed by Western blot and MTT assay, respectively, as described in the "Materials and Methods". Values are means ± S.E.M. of triplicate measurements (*$p < 0.05$; **$p < 0.01$ compared with untreated control). The relative density of blots was quantified as indicated.

pathways or mechanisms involving in As_2O_3-induced p21 expression should not be ruled out.

Enhancement of As_2O_3-induced p21 expression by blockage the JNK activation

On the other hand, we observed that As_2O_3 activated JNK phosphorylation in a time-dependent manner (Figure 5a upper). Pretreatment of the JNK inhibitor, SP600125, absolutely inhibited As_2O_3-induced JNK activation, and the total level of JNK1 was not affected by the treatment (Figure 5a middle). Surprisingly, pretreatment of SP600125 enhanced the As_2O_3-induced p21 protein expression (Figure 5a bottom) and promoter activity (Figure 5b). To study the role of Rac-MEKK-JNK (Rac-Mitogen activated protein kinase kinase kinase-c-JUN N-terminal protein kinase) signaling pathway in As_2O_3-induced p21 expression, we transfected a dominant positive

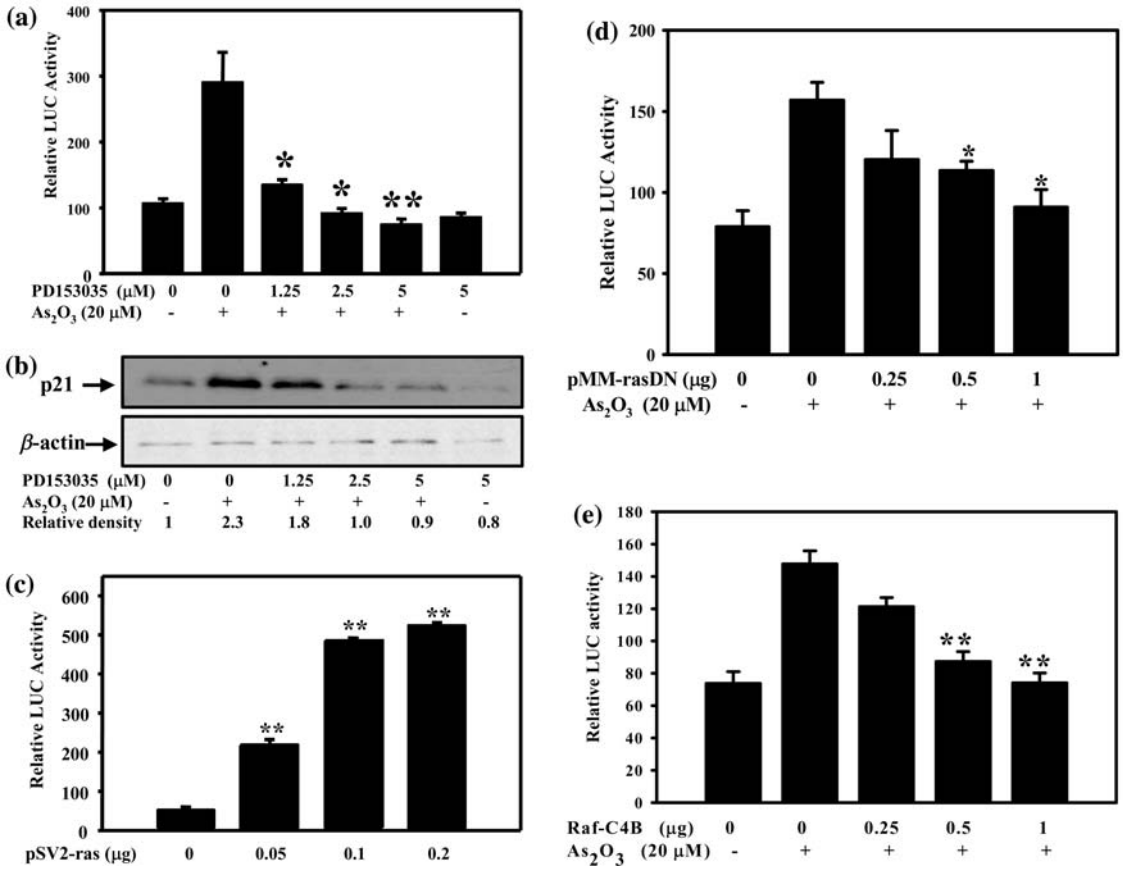

Figure 3. EGFR-Ras-Raf pathway was involved in As_2O_3-induced p21 expression. (a) A mixture of p21 luciferase plasmid (0.5 μg) was transfected into A431 cells by using the transfection method. Cells were pretreated with different doses of PD153035 for 1 h and further treated with 20 μM As_2O_3 for 4 h. The luciferase activity was determined and normalized with the amount of total protein. (b) Cells were pretreated with different doses of PD153035 for 1 h and then further treated with 20 μM As_2O_3 for 4 h. Western blot was performed by probing with anti-p21 antibody. (c) Cells were transiently cotransfected with 0.5 μg p21 luciferase plasmid and different amounts of pSV2-ras plasmids by using transfection method. The luciferase activity was determined and normalized with the amount of total protein. (d) Cells were cotransfected with p21 luciferase plasmid and different amounts of pMM-rasDN plasmids using transfection method and then treated with 20 μM As_2O_3 for 4 h. The luciferase activity was determined and normalized with the amount of total protein. (e) Cells were cotransfected with p21 luciferase plasmid and different amounts of Raf-C4B plasmids by using transfection method and then treated with 20 μM As_2O_3 for 4 h. The luciferase activity was determined and normalized with the amount of total protein. Values are means ±S.E.M. of triplicate measurements (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with untreated control).

plasmid of MEKK (pFC-MEKK) and a dominant negative plasmid of JNK (mJNK) into A431 cells and performed the p21 promoter assays. As shown in Figure 5c, transfection of pFC-MEKK inhibited promoter activation of p21 in a dose-dependent manner. Conversely, transfection of mJNK increased As_2O_3-induced p21 promoter activity, also in a dose-dependent manner (Figrue 5d). We conclude that the As_2O_3-induced p21 expression is enhanced by blockage the JNK pathway.

Opposing effects of combination As_2O_3 and inhibitors for MEK or JNK on cellular cytotoxicity

To assess the roles of As_2O_3-induced ERK1/2 and JNK pathways in cellular cytotoxicity, we combined As_2O_3 with inhibitors of MEK and JNK, respectively, to treat A431 cells and observed the cytotoxicity. Using the inverted phase-contrast microscope (400×), As_2O_3-treated A431 cells became round and began to detach from the dish

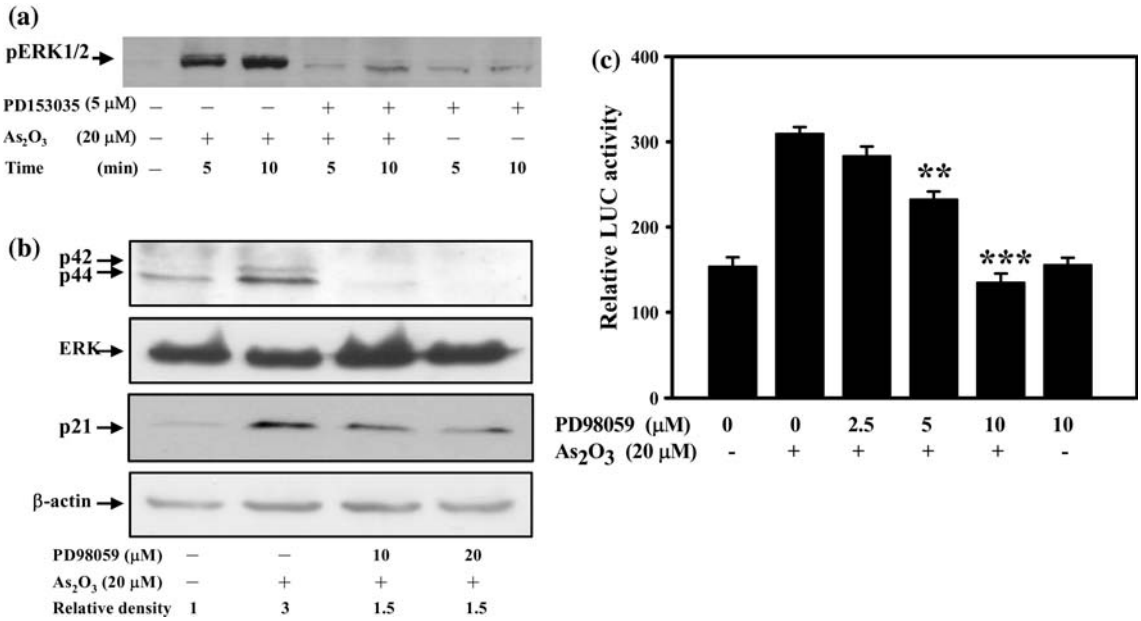

Figure 4. ERK1/2 activation was involved in As_2O_3-induced p21 expression. (a) A431 cells were pretreated with or without 5 μM PD153035 for 1 h and then treated with 20 μM As_2O_3 for 5 and 10 min. Western blot was performed by probing with anti-phospho ERK1/2 antibody. (b) A431 cells were pretreated with different doses of PD98059 and then treated with 20 μM As_2O_3 for 4 h. Western blot was performed by probing with anti-p21 antibody. (c) Reporter assay of p21 promoter activity was performed. Cells were pretreated with different doses of PD98059 for 1 h, and then treated with 20 μM As_2O_3 for 4 h. The luciferase activity was determined and normalized with the amount of total protein. Values are means ±S.E.M. of triplicate measurements (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with untreated control).

(Figure 6a, right) in comparison with the control (Figure 6a, left). In Figure 6b, cells pretreated with 20 μM PD98059 seemed to reduce As_2O_3-induced cytotoxicity. The number of round and detached cells was reduced. However, cells treated with As_2O_3 combining with 20 μM SP600125 seemed to change to spindle shape, appear vacuoles in cytoplasm (Figure 6c, middle and right). The cellular cytotoxicity induced by combination As_2O_3 and inhibitors of MEK or JNK in A431 cells was further explored by flow cytometry. Here, we showed that the cell population after treatment with combination with As_2O_3 and PD98059 showed a decrease in numbers in the sub-G1 phase (Figure 6d). When co-treated with As_2O_3 and SP600125, the cell population showed an increase in numbers in the sub-G1 phase (Figure 6e). The observation of sub-G1 population in the As_2O_3-treated cells suggests that apoptosis is the main case of cell death. Therefore, we suggest that ERK1/2 and JNK pathways play opposing roles in As_2O_3-induced cytotoxicity.

Discussion

In the past, a solution of potassium arsenite (Fowler's solution) is acknowledged to be successful in the treatment of psoriasis [2]. A major component of the psoriatic phenotype is epidermal keratinocyte hyper-proliferation. A431 cells are keratinocytic carcinoma cells, and one of our aims was to investigate the mechanism of anti-proliferative response of arsenic against psoriasis. In addition, the tumor suppressor gene p53 plays a key role in maintaining a balance between cell growth and cell death [26]. In a number of human cancers, inactivation of the gene is associated with unlimited cellular proliferation. Thus, study of the mechanisms by which As_2O_3 imparts anti-tumor effects in the absence of wild-type p53 is of compelling importance.

Presently, we have demonstrated that As_2O_3 reduced A431 cells survival and induced p21 promoter expression and resultant protein production (Figure 1). In a clinical setting, the real-

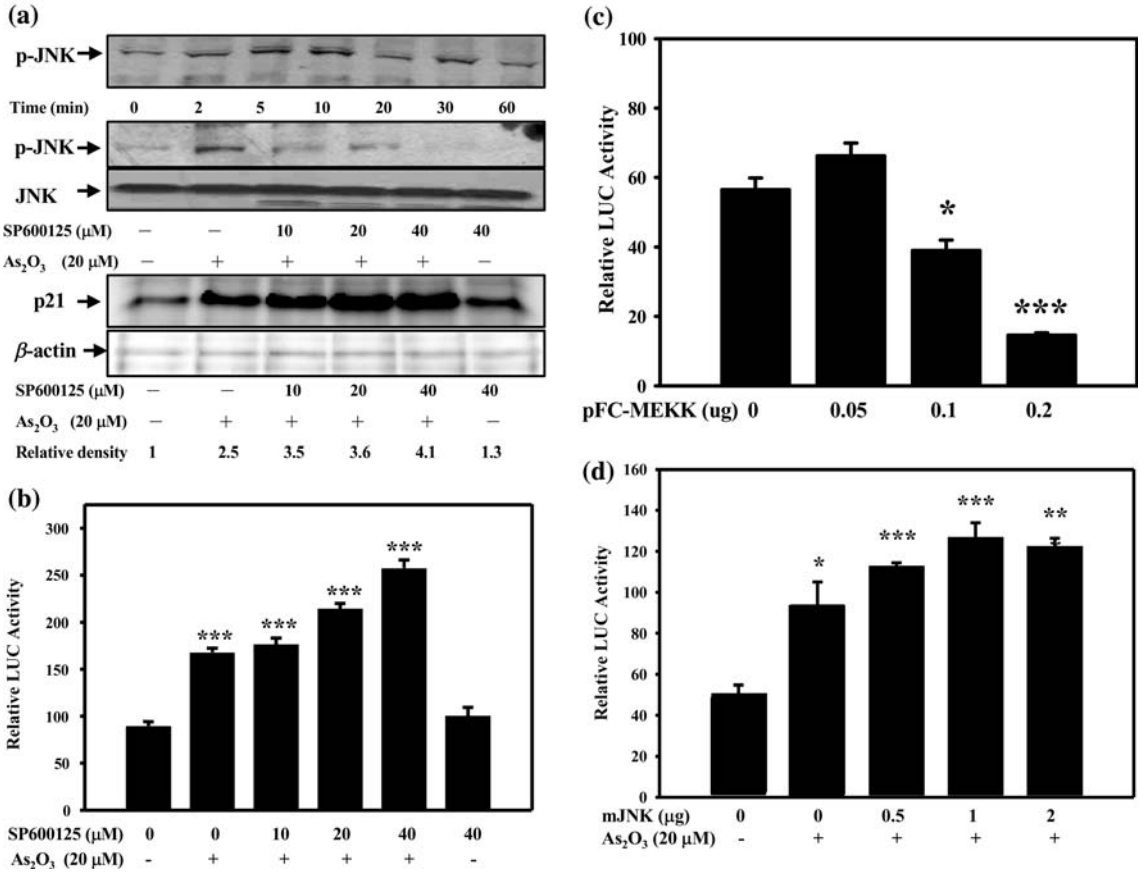

Figure 5. JNK inactivation enhanced As_2O_3-induced p21 expression. (a) Cells were treated with 20 μM As_2O_3 for different time points, and JNK activation was detected by Western blot (upper). Cells were pretreatment with different doses of SP600125 for 1 h, and then treated with 20 μM As_2O_3 for 10 min. The inhibitory effect of SP600125 on As_2O_3-induced JNK phosphorylation was shown (middle). The total level of JNK was used as a loading control. Cells were pretreatment with different doses of SP600125 for 1 h, and then treated with 20 μM As_2O_3 for 4 h. The enhanced effect of SP600125 on As_2O_3-induced p21 expression was shown (bottom). (b) Reporter assay of p21 promoter was performed. Cells were pretreatment with different doses of SP600125 for 1 h, and then treated with 20 μM As_2O_3 for 4 h. p21 promoter plasmid was cotransfected with different amount of pFC-MEKK (c) or mJNK (d) plasmids into A431 cells by the transfection method. Cells were treated with 20 μM As_2O_3 for 4 h. The luciferase activity was determined and normalized with the amount of total protein. Values are means ±S.E.M. of triplicate measurements (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with untreated control).

istic concentration of As_2O_3 that can be achieved ranges from about 0.5–2 μM to trigger apoptosis in NB4 cells (human leukemia cell line) [27]. We observed that cell survival was compromised beginning at low concentrations (1–2 μM) of As_2O_3 (Figrue 1a). Chronic exposure to low levels of As_2O_3 may eventually result in effects similar to those induced by acute exposure to higher concentration of As_2O_3 [28]. A high concentration of As_2O_3 was also used for a short time period to explore the signal transduction pathway of As_2O_3-induced NIH3T3 cell cycle arrest [29]. Therefore, we performed the assay with a high concentration (20 μM) of As_2O_3 in a short time period (4 h) to treat the A431 cells to elucidate the intracellular signals.

Three interesting findings are provided by this study. Firstly, As_2O_3 induces p21 activation via the EGFR-Ras-Raf-ERK1/2 pathway. In A431 cells, we found As_2O_3 phosphorylated ERK1/2 (Figure 4), JNK (Figrue 5), and the p38 protein (data not shown), but only ERK1/2 protein

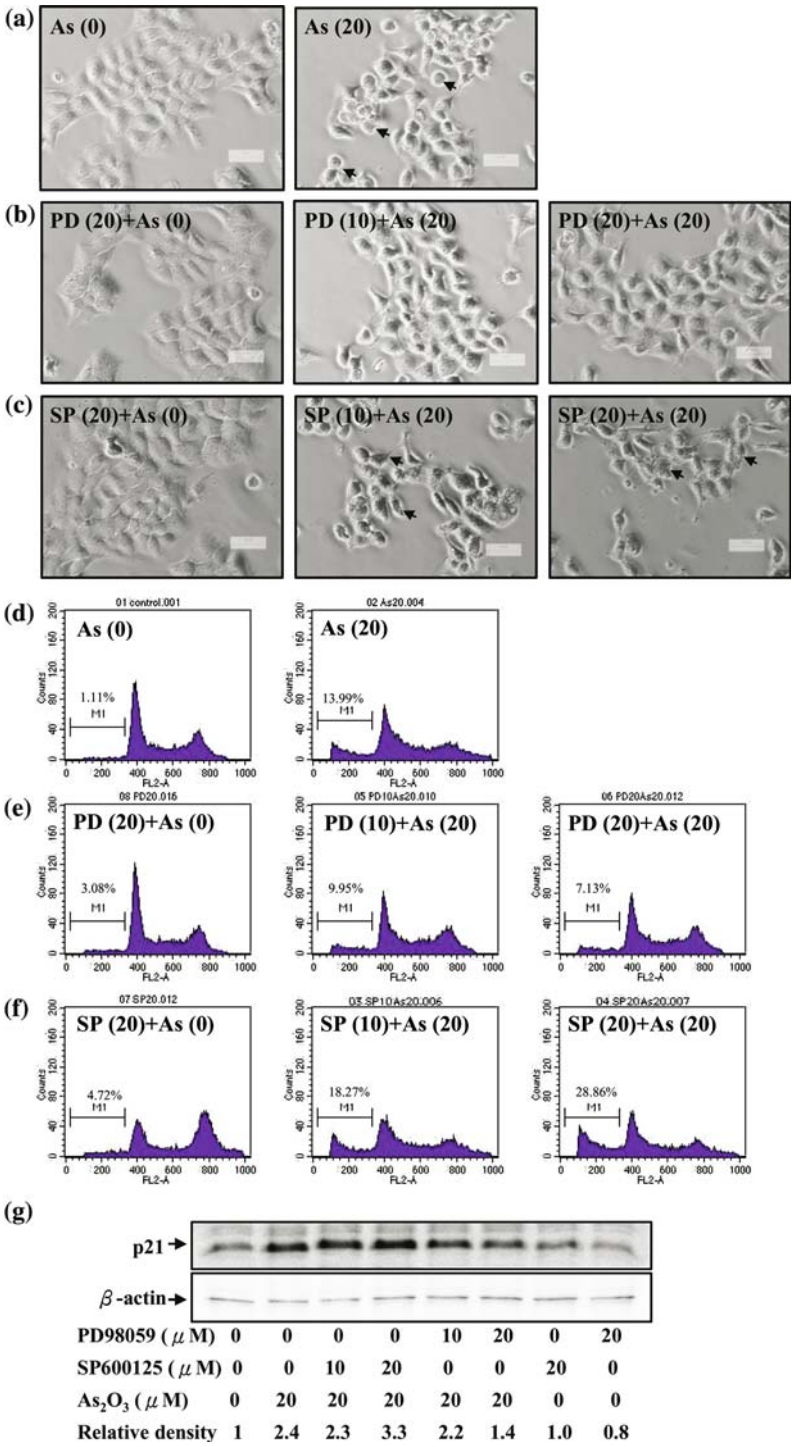

Figure 6. Opposing effects of combination As_2O_3 and PD98059 or SP600125 on cellular cytotoxicity. A431 cells were treated with As_2O_3 at the concentration of 20 μM for 24 h. The morphological changes (a, b and c) and flow-cytometric analysis (d, e and f) of the A431 cells were shown. The combination of As_2O_3 plus MEK inhibitor partially prevented the As_2O_3-induced p21 activation and cellular cytotoxicity (b, e and g). The combination of As_2O_3 plus JNK inhibitor enhanced the As_2O_3-induced p21 activation and cellular cytotoxicity (c, f and g). A magnification ''bar'' on the photo pictures indicates the original length, 20 μm.

phosphorylation was involved in As_2O_3-induced p21 activation (Figure 4). Moreover, As_2O_3-induced ERK1/2 protein phosphorylation was mediated by the EGF receptor (Figure 3A,B and 4A) and 20 μM PD98059 could not completely inhibit As_2O_3-induced p21 protein expression (Figure 4b). These results are in agreement with previous studies showing that arsenite usurps the EGFR-Ras-ERK1/2 signaling pathway to activate ERK1/2 protein phosphorylation in rat PC12 cells [22]. Johannessen et al. also demonstrate that the mechanisms by which EGF increases p21 protein levels in A431 cells are post-transcriptional and post-translational stabilization [30]. Whether As_2O_3 via the same mechanisms to stabilize p21 protein in A431 cells should be further elucidated.

Secondly, we found that ERK1/2 and JNK may differentially contribute to p21 expression in response to As_2O_3 in A431 cells via an As_2O_3 induction of p21 activation through the EGFR-Ras-Raf-ERK1/2 pathway (Figure 7). In contrast, we found JNK might serve as a negative regulator on p21 expression in response to As_2O_3 (Figure 5). To explain how a relatively small number of seemingly linear, intracellular signaling pathways can mediate a large various response to a large variety of stimuli, the cross-talk relationship between these pathways may be a prudent avenue of exploration. Some of the biological functions of JNK activators, such as TNF- and ceramide, may negatively regulate the ERK1/2/MAPK pathway by uncoupling ERK1/2 activation from MEK via activation of Jun expression [31]. However, we did not observe a change of ERK1/2 phosphorylation in response to As_2O_3 by pretreatment with the JNK inhibitor, SP600125 (data not shown). Therefore, we suggest the opposite effect of ERK1/2 and JNK on p21 expression might be through the transcriptional factors binding to the responsive elements at the p21 promoter. Studies are underway to address this suggestion.

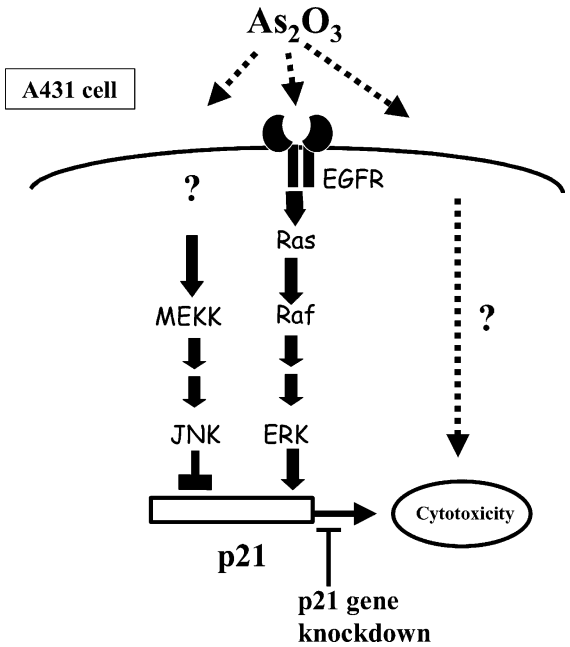

Figure 7. Summary of signal transduction pathways activated by As_2O_3 in A431 cells. We demonstrate As_2O_3 induced p21 activation and then leading to cell death through EGFR-Ras-Raf-ERK1/2 pathway. In contrast, MEKK-JNK pathway might serve as a negative regulator on p21 expression in response to As_2O_3. We conclude that ERK1/2 and JNK pathways differentially contribute to p21 expression and cellular cytotoxicity in response to As_2O_3 in A431 cells.

Thirdly, ERK1/2 and JNK pathways play opposing roles in As_2O_3-induced cytotoxicity. p21 is regarded as a universal inhibitor of cyclin-cdk complexes [7], which results in cell-cycle arrest. In our previous results, we suggest that arsenite-treated A431 cell finally results in apoptosis [25]. Presently, according to the MTT assay, we demonstrated that transfection of p21 siRNA reduced As_2O_3-induced A431 cell growth arrest (Figure 2b). MEK inhibitor, PD98059, partially blocked As_2O_3-induced p21 activation and cellular cytotoxicity (Figure 6b, e and g). In contrast, JNK inhibitor, SP600125, enhanced As_2O_3-induced p21 activation and cellular cytotoxicity (Figure 6c, f and g). As_2O_3 inhibits proliferation of human myeloma cells via cell-cycle arrest in association with induction of p21 and apoptosis [32]. However, little is known about the molecular mechanism of As_2O_3-induced apoptosis or As_2O_3-induced modulation of cell cycle-regulatory proteins. More studies are needed to clarify this. As_2O_3 inhibits cell growth and induces apoptosis in gastric cancer cells, in processes involving p53 over-expression and activation of caspase-3 [33]. The A431 cells, utilized in our study, have mutant p53 protein [23]. Therefore, we conclude the induction of p21 and cellular cytotoxicity by As_2O_3 appears to be independent of wild-type p53.

Exogenous induction of CDKIs induces growth arrest or apoptosis in cancer cells, indicating the potential use of CDKIs as a therapeutic

tool [34]. According to our results, the observations concerning the opposite effect of ERK1/2 and JNK on As_2O_3-induced, p53-independent p21 expression not only provide the mechanism of As_2O_3 effects, but may also be helpful to develop new strategies for the therapeutic use of As_2O_3 in certain types of tumor. Therefore, the therapeutic use of As_2O_3 may require strategies for the simultaneous inhibition of JNK. The combination may potentiate the anticancer effects of As_2O_3 by increasing the activation of p21 that either arrests cell cycle progression or facilitates cancer cell cytotoxicity.

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