Combination treatment with arsenic trioxide and sulindac enhances apoptotic cell death in lung cancer cells via activation of oxidative stress and mitogen-activated protein kinases

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Abstract. Arsenic trioxide (As_2O_3) has been introduced to the treatment of acute promyelocytic leukemia (APL), and has also been shown to induce apoptosis in a variety of solid tumor cell lines, including non-small cell lung cancer. However, the prohibitively high concentration required for the induction of apoptotic cell death in many solid tumor cells is unacceptable for clinical utilization due to the excessive toxicity associated with this dose. Sulindac is known to enhance the cellular responsiveness of tumors toward chemotherapeutic drugs. Herein, we demonstrated that combination treatment with As_2O_3 and sulindac resulted in a synergistic augmentation of cytotoxicity in H157 lung cancer cells, which was revealed by apoptotic induction as demonstrated by an increase in the sub-G_0/G_1 fraction. In addition, combination treatment with As_2O_3 and sulindac increased reactive oxygen species (ROS) and oxidative stress, as evidenced by the heme oxygenase-1 (HO-1) expression and mitogen-activated protein kinase (MAPK) phosphorylation. MAPK inhibitors blocked the induction of HO-1 by combination treatment. Inhibitors of p38 and JNK partially inhibited the augmented cell death whereas the ERK inhibitor showed poor inhibition. Combination treatment with As_2O_3 and sulindac induced oxidative DNA damage in a time-dependent fashion, which was evaluated by H2AX phosphorylation along with HO-1 induction.

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Introduction

Although many new therapeutic approaches have been developed for the treatment of different cancers, improvement of disease outcome in lung cancer patients has remained a major challenge, and there is a high mortality rate associated with this form of cancer (1). Therefore, new therapeutic strategies are needed. While a number of new agents have become available in the treatment of lung cancer, none of the chemotherapy regimens offer a significant advantage over the others (2). Moreover, the drugs in use today are relatively non-selective and sometimes manifest dose-limiting systemic toxicity in normal tissue. A major challenge of combination treatment for improving the chemotherapeutic index is by increasing the cancer-killing action of the drug and, at the same time, reducing its systemic toxicity.

Arsenic trioxide (As_2O_3) has long been used as an anticancer agent in traditional Chinese medicine (3) and currently is used in the treatment of refractory and relapsed acute promyelocytic leukemia (APL) without severe marrow suppression (4). It has become evident that the apoptotic effects of As_2O_3 are not only restricted to APL cells but can also be observed in various solid tumor cell lines, including carcinomas of the lung, esophagus, stomach, prostate and ovary as well as neuroblastoma cells (5-9). However, higher concentrations of As_2O_3 are required to induce apoptosis in non-APL tumor cells, suggesting that higher, more toxic doses may be needed for clinical efficacy.

Sulindac, a structural isoform of indomethacin, exerts antiproliferative and apoptotic effects, which eventually leads cancer cells to regress (10,11). Non-steroidal anti-inflammatory drugs (NSAID) are known to enhance the cellular responsiveness of tumors toward chemotherapeutic drugs through the induction of apoptosis, or through antiangiogenic effects (12). Studies have shown that chemotherapeutic drugs in combination with sulindac synergistically inhibit the growth of lung cancer cells (13). We previously demonstrated that combination treatment with As_2O_3 and sulindac augments their apoptotic potential in lung cancer cells through activation of the caspase cascade and mitochondrial dysfunction (5).

Considerable evidence suggest that As_2O_3 induces the accumulation of reactive oxygen species (ROS), subsequently inducing oxidative stress (14,15). The intracellular redox status has been shown to be important in predicting whether a cell will respond to arsenic (16). We report that combination treatment with As_2O_3 and sulindac shows an increase in ROS generation, oxidative stress and oxidative DNA damage. Our data suggest that combination treatment with As_2O_3 and sulindac may have practical applications in the treatment of lung cancer as an alternative anticancer strategy, by circumventing the serious general toxicity of As_2O_3.

Materials and methods

Reagents. RPMI-1640, fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL Co. (Grand Island, NY). Arsenic trioxide, sulindac, methylthiazol-2-yl-2,5-diphynyl, tetrazolium bromide (MTT), bicinchoninic acid (BCA), dimethyl sulfoxide (DMSO), n-acetylcysteine (NAC) and glutathione (GSH) were bought from Sigma Chemical Co. (St. Louis, MO). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG conjugated horseradish-radish peroxidase antibody and the enhanced chemiluminescent (ECL) kit were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and viability test. NCI-H157 human lung cancer cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and grown in RPMI-1640 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% FBS. Cells ($5x10^4$ cells/each well of a 24-well plate) were cultured in a humidified atmosphere of 5% CO_2 in air at 37˚C and maintained in log phase. Cell viability was determined by MTT assay. To determine the cell viability, MTT (0.5 mg/ml) was added to 1 ml of cell suspension for 4 h. After three washes of cells with phosphate-buffered saline (PBS, pH 7.4), the insoluble formazan product was dissolved in DMSO. The optical density (OD) of each culture well was then measured using a microplate reader (Titertek Multiskan, Flow Laboratories, North Ryde, Australia) at 590 nm. The OD of formazan formed in the control cells was taken as 100% of viability, and the positively stained cells with MTT were expressed as a percentage (%), compared to the control cells.

Flow cytometry. The degree of apoptosis was determined by measuring the number of cells showing DNA content below the G_0/G_1 phase from flow cytometry, after staining of the cells with propidium iodide (PI), as originally described by Crissman and Steinkamp (17). The cell cycle analysis was performed with FACScan equipped with Cell Quest Software (Becton-Dickinson, San Jose, CA).

Determination of the H_2O_2 generation. The generation of intracellular H_2O_2 was determined as described by Root *et al* (18) by monitoring the decrease in fluorescence of scopoletin during its oxidation catalyzed by horseradish peroxidase (HRP). The H157 cells were harvested and resuspended at $1x10^6$/ml in PBS without Ca^{++} and Mg^{++} in the presence of 5 μM scopoletin and 1 U/ml HRP in the dark. Cells were added to the wells of the microplate, which was pre-warmed at

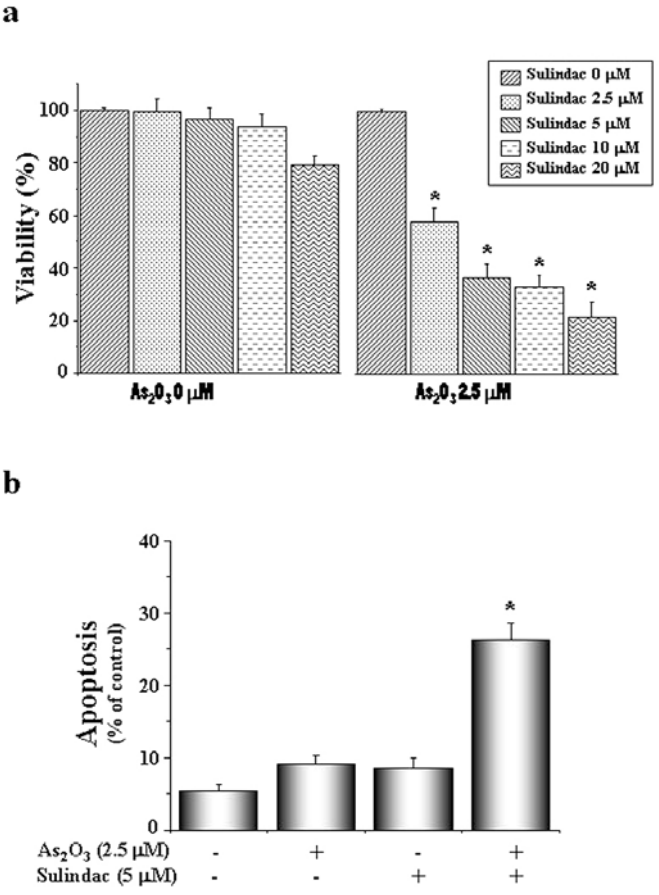

Figure 1. Combination treatment with As_2O_3 and sulindac enhanced apoptotic cell death in H157 cells. (A) Cells were treated with different concentrations of sulindac in the absence or presence of 2.5 μM As_2O_3 for 48 h and viability was determined by MTT assay. The data represent the mean ± SD of three independent experiments. *P<0.05 vs. the control. (B) Cellular DNA was stained with PI and the cell cycle was analyzed by flow cytometry. The data represent the means ± SD of triplicates. *P<0.05 vs. the control.

37˚C and contained 100 μl of reaction mixture with 10 μl of buffer. The data were analyzed by flow cytometry (Molecular Devices Co.) with an excitation and emission spectrum of 350 and 460 nm, respectively.

Western blotting. The cell extract was separated by 10% SDS-PAGE under reduced conditions and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBST (25 mM Tris, pH 7.6, 138 mM NaCl and 0.05% Tween-20) for 1 h and probed with primary antibodies (1:1,000-1:5,000). After a series of washes, the membrane was further incubated with secondary antibody (1:2,000-1:10,000) and conjugated with horseradish peroxidase (HRP). The immunoreactive signal was detected with the enhanced chemiluminescent (ECL) detection system.

Statistical analysis. Each experiment was performed at least three times, and all values are represented as means ± SD of triplicates. Student's t-test was used to analyze a statistical significance of the results. Values of $p<0.05$ were considered as statistically significant.

Results

Combination treatment with As_2O_3 and sulindac synergistically increased the apoptotic activity of H157 cells. To test the synergism in cytotoxicity, H157 cells were treated with As_2O_3 and sulindac, and then cell viability was measured by MTT assay. In contrast to various concentrations of sulindac alone, combinations of 2.5 μM As_2O_3 with varying doses of sulindac from 2.5 to 20 μM resulted in a significant decrease of the viability of H157 cells in a dose-dependent fashion ($p<0.05$) (Fig. 1A).

We then examined whether combination treatment with As_2O_3 and sulindac may induce apoptosis in H157 cells. DNA fragmentation by apoptosis was analyzed as the sub-G_0/G_1 fraction of cell cycle analysis by flow cytometry in H157 cells stained with PI. In contrast, in the control culture, the sub-G_0/G_1 fraction after combination treatment with As_2O_3 and sulindac significantly increased, up to 26.2% (Fig. 1B). These data indicate that combined treatment with As_2O_3 and sulindac significantly increased apoptosis compared with their individual treatments.

Combination treatment with As_2O_3 and sulindac increased ROS and the oxidative stress in H157 cells. As_2O_3 induces apoptosis, mediated by an increase of ROS generation (19), and several groups have reported on ROS generation in NSAID-treated cells (20,21). We measured the generation of intracellular hydrogen peroxide (H_2O_2) by scopoletin-horseradish peroxidase (HRP) assay. The data revealed that a combination treatment with As_2O_3 and sulindac resulted in a significant increase in the generation of ROS (Fig. 2A). Oxidative damage has been postulated to be a key mechanism by which arsenic initiates the apoptotic process and it is possible that the combination treatment increases cellular oxidative stress. Therefore, we determined whether As_2O_3 and sulindac affected heme oxygenase-1 (HO-1), which has been widely described as a stress-response protein (22). The data showed that the expression of HO-1 was slightly increased in cells treated with As_2O_3 only, and that the sulindac-induced HO-1 expression was decreased, whereas combination treatment with As_2O_3 and sulindac further increased the expression of HO-1 in a time-dependent fashion (Fig. 2B).

Combination treatment with As_2O_3 and sulindac activates phosphorylated MAPKs in H157 cells. Previous reports suggest that oxidative stress introduces positive signals toward the mitogen-activated protein kinase (MAPK) pathway, and the activation is crucial for HO-1 induction (23). We thus examined whether combination treatment with As_2O_3 and sulindac affects the expression of MAPKs in H157 cells. The levels of the protein expression of ERK1/2, p38 and JNK in H157 cells treated with combination treatment were assessed by Western blotting, using the corresponding phospho-specific and non-phosphospecific antibodies. The data showed that combination treatment with As_2O_3 and sulindac resulted in an increase in the expression of phosphorylated MAPK proteins in a time-dependent fashion (Fig. 3).

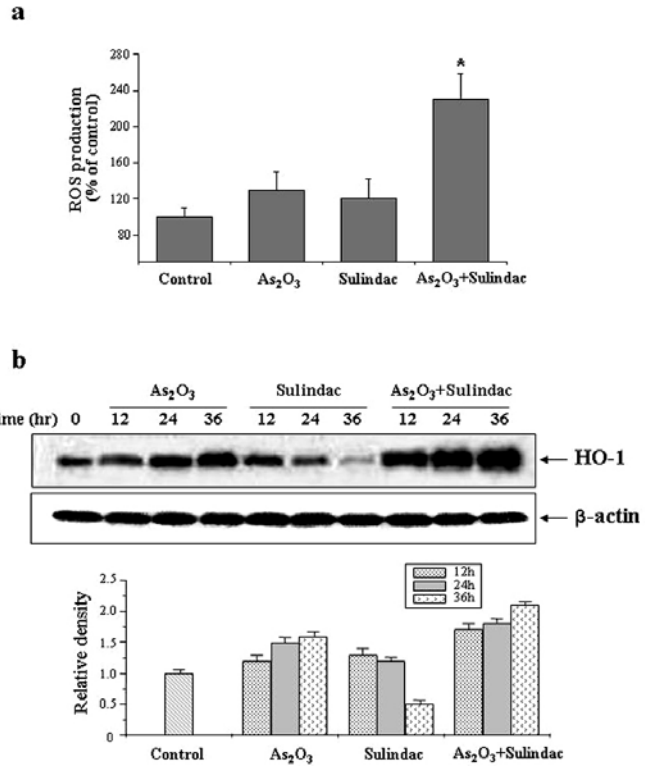

Figure 2. Combination treatment with As_2O_3 and sulindac increased the intracellular ROS and oxidative stress in H157 cells. (A) The measurement of intracellular H_2O_2 was determined fluorimetrically using scopoletin-horseradish peroxidase assay. The data represent the means ± SD of three independent experiments. *P<0.05 vs. the control. (B) Cells were treated with 2.5 μM As_2O_3 and 5 μM sulindac for up to 36 h and the lysate was subjected to 12.5% SDS-PAGE to measure the expression of HO-1 protein.

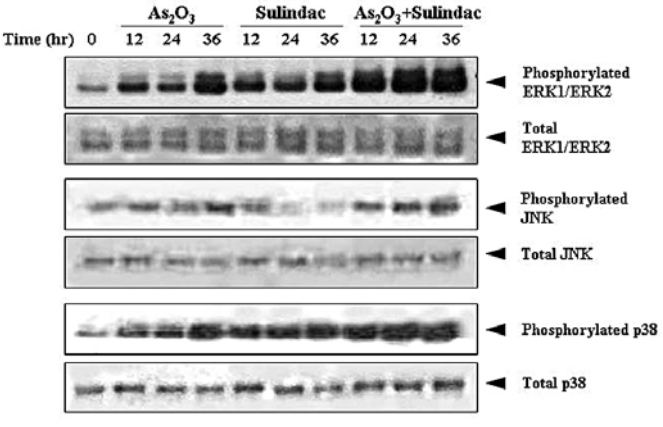

Figure 3. Combination treatment with As_2O_3 and sulindac augmented MAPK activation in H157 cells. The activation of MAPKs was evaluated by Western blotting using phospho-specific antibodies to ERK1/ERK2, p38 and JNK. As controls, the same cell lysates were subjected to Western blotting using corresponding non-phosphospecific antibodies to detect total ERK1/ERK2, total p38 or total JNK.

MAPK inhibitors blocked the induction of HO-1 by combination treatment, and partially inhibited the augmented cell death. The possibility that the MAPK signaling pathway

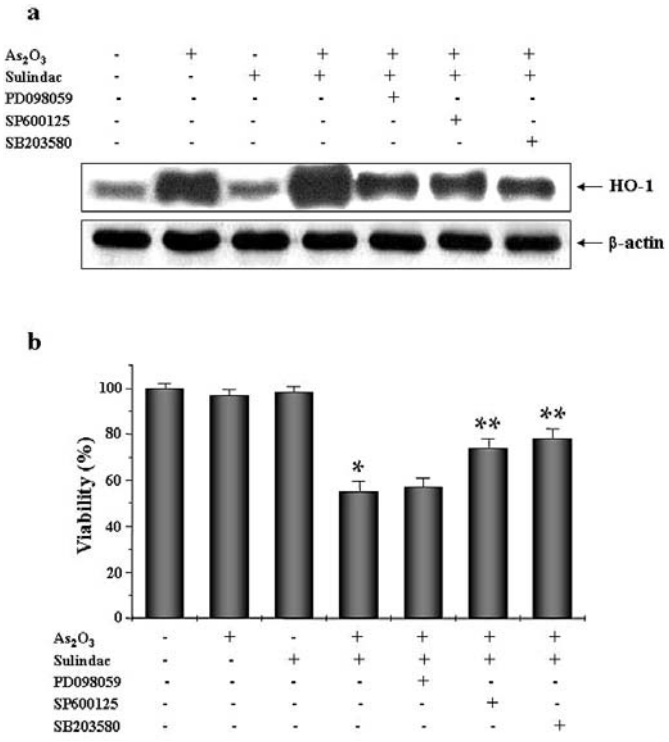

Figure 4. Pharmacological inhibition of MEK1, p38 and JNK blocked the induction of HO-1 by combination treatment and the p38 and JNK inhibitors partially inhibited the augmented cell death. (A) H157 cells were pretreated with or without MAPK inhibitors including, the MEK1 inhibitor PD098059, p38 inhibitor SB203580 and JNK inhibitor SP600125 and further treated with As_2O_3 and/or sulindac. The cell lysate was used to measure the expression of HO-1. (B) Cells were pretreated with or without MAPK inhibitors, followed by the addition of As_2O_3 and sulindac for 48 h. Viability was measured by MTT assay. The data represent the means ± SD of three independent experiments. $^*P<0.05$ vs. control, $^{**}P<0.05$ vs. As_2O_3 and sulindac.

mediated the induction of HO-1 expression by combination treatment was examined using selective chemical inhibitors of MAPKs, including PD098059, a specific MEK1 inhibitor of ERK; SB203580, a selective chemical inhibitor of p38 and SP600125, a specific inhibitor of JNK. The H157 cells were pretreated with or without inhibitors, and then treated with As_2O_3 or sulindac alone. This combination was incubated for 38 h, and the cell lysate was used to carry out Western blotting for HO-1. The data revealed that combination treatment with As_2O_3 and sulindac resulted in a 2.7-fold increase of HO-1 expression when compared to the unstimulated control cells, and MAPK inhibitors decreased the cisplatin-induced HO-1 expression from 42 to 48% (Fig. 4A). We examined MAPK inhibitors on the combination treatment-mediated augmented cell death. The cells were pretreated with or without MAPK inhibitors, followed by the addition of As_2O_3 and sulindac for 48 h, and then viability was measured by MTT assay. As shown in previous data, combination treatment with As_2O_3 and sulindac resulted in a significant decrease in cell viability (58% of control cells). Pretreatment with the p38 and JNK inhibitors partially inhibited the augmented cell death. However, the ERK inhibitor showed only slight inhibition (Fig. 4B).

Combination treatment with As_2O_3 and sulindac induced oxidative stress-mediated DNA damage. Oxidative stress is

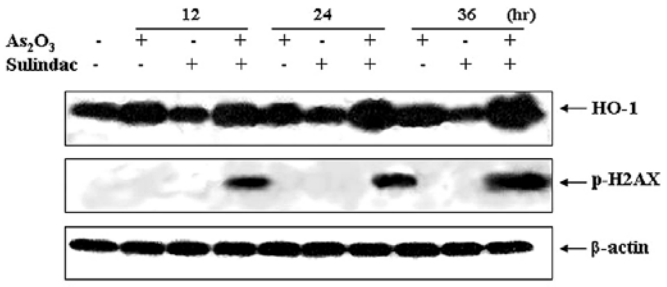

Figure 5. Combination treatment with As_2O_3- and sulindac-mediated oxidative DNA damage in H157 cells. Cells were treated with 2.5 μM As_2O_3 and/or 5 μM sulindac for up to 36 h and the lysate was subjected to 12.5% SDS-PAGE to measure the expression of HO-1 or phospho-H2AX proteins. ß-actin was used as a loading control.

well known to cause DNA damage (24). Thus, we examined whether combination treatment with As_2O_3 and sulindac causes DNA damage. H2AX phosphorylation is tightly linked to DNA damage (25,26), and combination treatment strongly induced H2AX phosphorylation along with strong HO-1 induction in a time-dependent fashion, but no phosphorylation was observed in cells treated individually (Fig. 5). These data indicated that any increased DNA damage by combination treatment may be mostly caused by oxidative stress.

Discussion

ROS generated in response to As_2O_3 leads to the accumulation of intracellular hydrogen peroxide and subsequent induction of oxidative stress. Intracellular oxidative status has been shown to be important for As_2O_3 sensitivity (27,28). Sulindac also elevated ROS generation more than the selective COX-2 inhibitor (29). Increased ROS induces apoptosis by activating the MAPKs and caspase cascades, and/or by disrupting the mitochondrial membrane potential (30).

MAPKs, including ERK, JNK and p38, are components of signaling cascades that respond to extracellular stimuli by targeting transcriptional factors, resulting in the modulation of gene expression. ERK is involved in cellular responses such as cell proliferation and survival, whereas JNK and p38 are involved in proapoptotic events (31,32). Reports suggest that MAPKs are activated by As_2O_3 and sulindac individually (33,34). In this study, we showed that combination treatment with As_2O_3 and sulindac resulted in a significant increase in the generation of ROS, and the activation of MAPK proteins.

Many oxidative and chemical stressors lead to the activation of MAPK signaling pathways and the subsequent activation of Nrf2, which binds to the antioxidant response element (ARE) of the HO-1 gene, causing an increase in its transcription. To confirm the possibility that a MAPK signaling pathway mediated the induction of HO-1 expression by combination treatment, we examined the process using MAPK inhibitors. Our data showed that the induction of HO-1 by combination treatment with As_2O_3 and sulindac requires the activation of a MAPK signaling pathway.

HO-1 is a member of the family of heat shock proteins (HSP32). HO-1 expression is triggered by diverse stress-

inducing stimuli (35,36). The biological roles of HO-1 are believed to be associated with fundamental adaptive and innate defense responses against various stress conditions in cells, including oxidative stress. The enhanced resistance of certain cell types to the cytotoxic effects of arsenic appears to be related to relatively high levels of HO-1 in these cells (37). Conversely, the free iron and carbon monoxide (CO) released during HO-1-mediated heme catabolism may exacerbate intracellular oxidative stress, and promote injury to the mitochondria and other subcellular compartments (38). Thus, it is conceivable that HO-1 induction may mediate, rather than protect against, the cytotoxic effects of drugs under certain experimental and clinical conditions.

ROS induces cell injury, and acts on many different cellular targets, such as DNA, proteins and membrane lipids. ROS may cause DNA-protein cross-links and alterations (23,39). This DNA damage can lead to translocations and chromosomal instability in the generation of malignancy (40-42). A sensitive means of detecting the formation of DNA damage is to assess the phosphorylation of histone H2AX, a variant of a family of at least eight protein species of the nucleosome core histone H2A (43,44). The phosphorylated form of H2AX appears during apoptosis concurrently with the initial appearance of high molecular weight DNA fragments (25). In this study, we showed that combination treatment with As_2O_3 and sulindac induced phosphorylation of the histone H2AX along with strong HO-1 induction. Inhibitors of p38 and JNK failed to rescue the augmented cell death. Therefore, oxidative DNA damage appears to be primarily important.

In conclusion, we demonstrated that combination treatment with As_2O_3 and sulindac strongly increased the accumulation of ROS, and subsequently induced the activation of MAPK pathways, and the increase of oxidative stress. Moreover, combination treatment resulted in oxidative stress-induced DNA damage. Up to 10-fold higher concentrations of As_2O_3 are required to induce apoptosis in non-APL tumor cells, which is generally unacceptable in the clinic due to its higher toxicity. Our data suggest that the combination treatment with As_2O_3 and sulindac may be useful in practical applications for the treatment of non-small cell lung cancer.

Acknowledgements

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