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RESEARCH

Autophagy Interplays with Apoptosis and Cell Cycle Regulation in the Growth Inhibiting Effect of Trisenox in HEP-2, a Laryngeal Squamous Cancer

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Abstract Laryngeal squamous cell carcinoma (LSCC) is the most common among several types of head and neck cancers. Current treatments have a poor effect on early and advanced cases, and further investigations for novel agents against LSCCs are desirable. In this study, we elucidate the cytotoxic enhancing effect of arsenic trioxide (As_2O_3) combined with L-buthionine sulfoximine (BSO) in LSCC. The effect of BSO with As_2O_3 or Cisplatin (CDDP) on cell viability was examined using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The reactive oxygen species (ROS) levels, cell cycle, and apoptosis were measured by flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), propidium iodide (PI) and annexin V/PI. The acidic vacuolar organelles were visualized by fluorescence microscope and quantified using flow cytometry. Neither CDDP nor As_2O_3 when used alone reduced the cell viability. BSO was found to enhance only As_2O_3 sensitivity, leading to G2/M arrest and autophagy with no correlation of ROS induction. This result suggests that modulation of glutathione enhances autophagy, which interplays with apoptosis. In this study, we obtained initial preclinical evidence for the potential efficacy of these drugs in a combined therapy protocol.

Keywords Laryngeal squamous cancer · Trisenox · L-buthionine sulfoximine · Autophagy · Apoptosis

Abbreviations

HNSCC	Head and neck squamous cell carcinomas
LSCC	Laryngeal squamous cell carcinoma
CDDP	Cisplatin
As_2O_3	Arsenic Trioxide
BSO	L-buthionine sulfoximine
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide
DCFH-DA	2′,7′-dichlorodihydrofluorescein diacetate
PI	Propidium iodide
DMSO	Dimethylsulfoxide
3-MA	3-methyladenine
AO	Acridine orange
H_2O_2	Hydrogen peroxide
ROS	Reactive oxygen species
AVO	Acidic vacuolar organelle

Introduction

Head and neck squamous cell carcinomas (HNSCCs) are a group of cancers that originate from epithelial cells of the oral cavity, pharyngeal, and laryngeal regions, sharing alcohol and tobacco as their common carcinogens [1–4]. Worldwide, laryngeal squamous cell carcinoma (LSCC) is the second common respiratory tract cancer and the most common among several types of head and neck cancers, representing 25 % of

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malignant tumors that affect this area. The incidence is higher in men over the age of 40 years [5]. The most recent global estimate revealed around 57,000 new cases per year and approximately 26,000 deaths annually [6]. Current treatments, including surgical intervention, radiation therapy, and chemotherapy have a moderate effect on early-stage cases, but are less effective in more advanced cases [7]. As a result, the 5-year overall survival for LSCC patients is poor [8]. The morbidity of LSCC is the third largest among HNSCC, comprising almost 50 % of all malignancies in some developing nations [5, 8]. Despite the well-known etiology, the disease often recurs locally and/or as distant metastasis, leading to an unfavorable prognosis and failure in treatment [5–8]. Therefore, further investigations for novel agents and protocol strategies against LSCCs are desirable.

The harmony between the positive and the negative (*ying* and *yang*) forces is the principle concept of using a controlled dose of poison to overcome an ailment in Traditional Chinese Medicine [9, 10]. Arsenic trioxide (As_2O_3) is among the natural poisons used as medicine against different types of infections and malignancies for over 5,000 years. During its golden age, from the 1830s to 1930s, As_2O_3 gained fame in western medicine as a broad spectrum antiparasitic drug and became the first effective chemotherapy for acute promyelocytic leukemia (APL) a special subtype of acute myeloid leukemia (AML) at median clinical daily dosage of 0.16 mg per kilogram (range, 0.06–0.2) in patients [11] and at final concentration as low as 2 μM for myeloid cell lines such as NBA, HL60 and K562 [12–14]. Very recently, however, it has become evident that the antitumoral effect of As_2O_3 is not restricted to these type of cancer cells, but have also been observed in preclinical studies in other solid malignant cell lines such as glioma, U118-MG and U87 [15, 16], ovarian [17, 18] and head and neck carcinomas [19, 20]. On the other hand, most of the studies in vitro have shown that the As_2O_3 concentrations required for its antitumor effect in solid tumor cells lines are higher (up to 25 μM) than those used in acute promyelocytic leukemia. Therefore, other therapeutic strategies are required to enhance the efficacy of As_2O_3 against human solid malignancies without the risk of side effects [21, 22]. Combination therapy with multiple drugs is a common practice in cancer treatment. The promising anticancer activity of As_2O_3 has promoted considerable interest in combining it with traditional chemotherapy treatment, such as cisplatin (CDDP) [23], anthraquinones [24] or with L-buthionine sulfoximine (BSO), an inhibitor of glutathione (GSH) synthesis [25]. Elevated GSH levels are observed in many types of tumors, and this makes the neoplastic tissues more resistant to chemotherapy such as As_2O_3 or Trisenox [26]. Among the HNSCC cell lines, the As_2O_3-combined therapeutic strategies were mostly described for nasopharyngeal and tongue carcinoma [27–29], but in LSCC, they have been poorly understood. Very recently, studies have explored strategies of individually inhibiting GSH metabolism in addition to new chemotherapy agents, with variable cell death pathways inductions trigged or not by oxidative stress in human squamous cells carcinoma [30–32].

Here we evaluated a possible cytotoxic enhancing effect of As_2O_3/BSO combination in human LSCC cell line, HEp-2. Our results implicated GSH as a potential target for therapy, since its depletion was related to autophagy enhancement that interplays with cell cycle arrest and apoptosis in HEp-2 cell line. Thus, in the present study, we obtained initial preclinical evidence for the potential efficacy of As_2O_3/BSO combination in the treatment of LSCC.

Material and Methods

Reagents

Cisplatin (CDDP) was purchased from Accord farmacêutica, LTDA (São Paulo, Brazil), Trisenox (As_2O_3) was purchased from Cell Therapeutics (Washington, USA), and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from GE Healthcare (Uppsala, Sweden). Reduced GSH, RNAse, BSO, dimethylsulfoxide (DMSO), 3-methyladenine (3-MA), and Acridine orange (AO) were purchased from Sigma-Aldrich Chemical (St. Louis, USA). Fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) assay (Apoptosis Detection Kit) were purchased from Genzyme Diagnostics (Cambridge, MA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Oregon, USA) and hydrogen peroxide (H_2O_2) was purchased from Merck (Darmstadt, Germany).

Cell Culture and Treatments

The human LSCC cell line HEp-2 was kindly provided by Dr. Cerli R. Gatass (Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). The lineage was mycoplasma negative and used within 6 months from source. Authentication of the cell line was confirmed by short tandem repeat (STR) profile. All culture materials were purchased from Gibco Laboratories (Grand Island, USA). The cells were cultured in Dulbecco's Modified Eagle Medium high glucose supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin and 0.1 % amphotericin B at 37 °C under humidified atmosphere of 5 % CO_2. The inhibitors BSO (25 μM) and/or 3-MA (2.0 mM) were added 24 h and 2 h before As_2O_3 treatment, respectively.

Cell Viability

Initially, cell viability was determined by measuring the mitochondrial conversion of MTT to a colored product [33]. HEp-2 cells were seeded at a concentration of 1×10^4 cells/well in 96-well plates in the culture medium supplemented with 10 % fetal bovine serum. The cells were pretreated during 24 h with a specific inhibitor of γ-glutamylcysteine synthetase, BSO (25 μM), the rate-limiting enzyme in GSH synthesis [34], and then treated with As_2O_3 (1.0–10 μM) or CDDP (1.0–10 μM). Afterwards, to verify the autophagic potential of As_2O_3, we also incubated the cells with an important autophagic inhibitor, 3-MA (2.0 mM), for 2 h before the treatments with 1.0 and 4.0 μM As_2O_3 with or without BSO. Four hours before the end of each incubation period (24, 48, and 72 h), the MTT reagent (0.5 mg/ml) was added to each well. The formazan crystals were solubilized with DMSO and measured using a spectrophotometer at 570 nm. All data were expressed as a percentage, considering untreated cells as 100 %.

Drug Interaction Analysis

Combination index calculations were carried out following the procedure developed by Fischel et al. [35], adapted from Chou and Talalay method [36]: R=Survival (BSO + As_2O_3)/Survival (BSO) × Survival (As_2O_3). According to Fischel et al. [35]: If (1) $R<0.8$, the association is considered to be synergistic; (2) $0.8<R<1.2$, the association is considered to be additive; (3) $R > 1.2$, the association is considered to be antagonistic.

Analysis of Intracellular Reactive Oxygen Species Levels

The production of reactive oxygen species (ROS) was measured with DCFH-DA, as described previously [37]. Briefly, HEp-2 cells (10^6/ml) pretreated or not with BSO were treated with As_2O_3 at 1.0 and 4.0 μM for 72 h. Then, the cells were collected, washed, and suspended in 1.0 ml of DMEM with 10 % fetal bovine serum containing 300 mM of DCFH-DA. To compare the effect of As_2O_3 on intracellular ROS levels in HEp-2 cells, the cells were treated with H_2O_2 (1.0 μM) as a positive control. The samples were incubated during 30 min in the dark at 37 °C and 1 mg/ml of PI was added before acquisition to exclude dead cells. Samples were analyzed by flow cytometry at 530/40 nm (FITC channel-DCF) and 575/25 nm (PE channel-PI) (CyAn ADP flow cytometer, Beckman-Coulter, CA, USA). In all the experiments, the mean fluorescence intensity from 10.000 events was accessed and analyzed using Summit v4.3 software (Beckman-Coulter, CA, USA).

Cell Cycle Analysis

HEp-2 cells (3×10^5/well) were seeded in 24-well plates, followed by BSO and/or As_2O_3 treatment. After 48 h of incubation, the cells were washed with PBS, harvested and incubated with a citrate buffer solution (pH 6.0) containing PI (50 μg/ml), 0.3 % Triton X-100, and RNAse (100 μg/ml) for 30 min in the dark at room temperature. The DNA content was analyzed by flow cytometry at 575/25 nm (PE channel-PI) (CyAn ADP flow cytometer, Beckman-Coulter, CA, USA). In all the experiments, the mean fluorescence intensity from 10.000 events was accessed and analyzed using Summit v4.3 software (Beckman-Coulter, CA, USA). In all the experiments, the mean fluorescence intensity of DNA content from 10.000 cells was analyzed as the percentage of cell population in each cell cycle phase.

Visualization and Quantification of Acidic Vacuolar Organelles by AO Staining

AO is a marker of acidic vacuolar organelles (AVOs) that fluoresces green in the whole cell, except in acidic compartments, where it fluoresces red. Development of AVOs is a typical feature of autophagy, because only mature/late autophagosomes are acidic [38]. The cells were plated at a concentration of 5.8×10^4 cells/well in 24-well plates and pre-incubated or not with BSO, followed by As_2O_3 (1.0 and 4.0 μM) treatments during 72 h. Subsequently, the cells were incubated with AO (3.0 μg/ml/well) in the dark for 15 min at room temperature, followed by visualization in a fluorescence microscope and Image Pro Express software (Nikon Eclipse TE300). To quantify the percentage of cells with AVOs, the treated and untreated cells were marked with AO (3.0 μg/ml/well) in the dark for 15 min at room temperature, removed from the plate and analyzed by flow cytometry at 530/40 nm (FITC channel-green viable cells) and 613/20 nm (PE Texas Red channel-AVOs) (CyAn ADP flow cytometer, Beckman-Coulter, CA, USA), as described previously [39, 40].

In all the experiments, the mean fluorescence intensity from 10.000 events was accessed and analyzed using Summit v4.3 software (Beckman-Coulter, CA, USA) as the percentage of cell population in each condition (viable cells, autophagic cells or unlabeled/dead cells).

Apoptosis Analysis

In general, 3×10^5 HEp-2 cells were pre-incubated or not with BSO, followed by treatment with As_2O_3 (1.0 and 4.0 μM) for 72 h. After incubation, the cells were washed twice with PBS and labeled with FITC-labeled annexin V and PI, according to the manufacturer's instructions. This assay distinguishes viable cells (annexin V−/PI−) from cells in early apoptosis (annexin V+/PI−), late apoptosis/secondary necrosis (annexin

V+/PI+), or those undergoing necrosis (annexin V−/PI+). The samples were incubated during 30 min in the dark at 37 °C and analyzed by flow cytometry at 530/40 nm (FITC channel-annexin) and 575/25 nm (PE channel-PI) (CyAn ADP flow

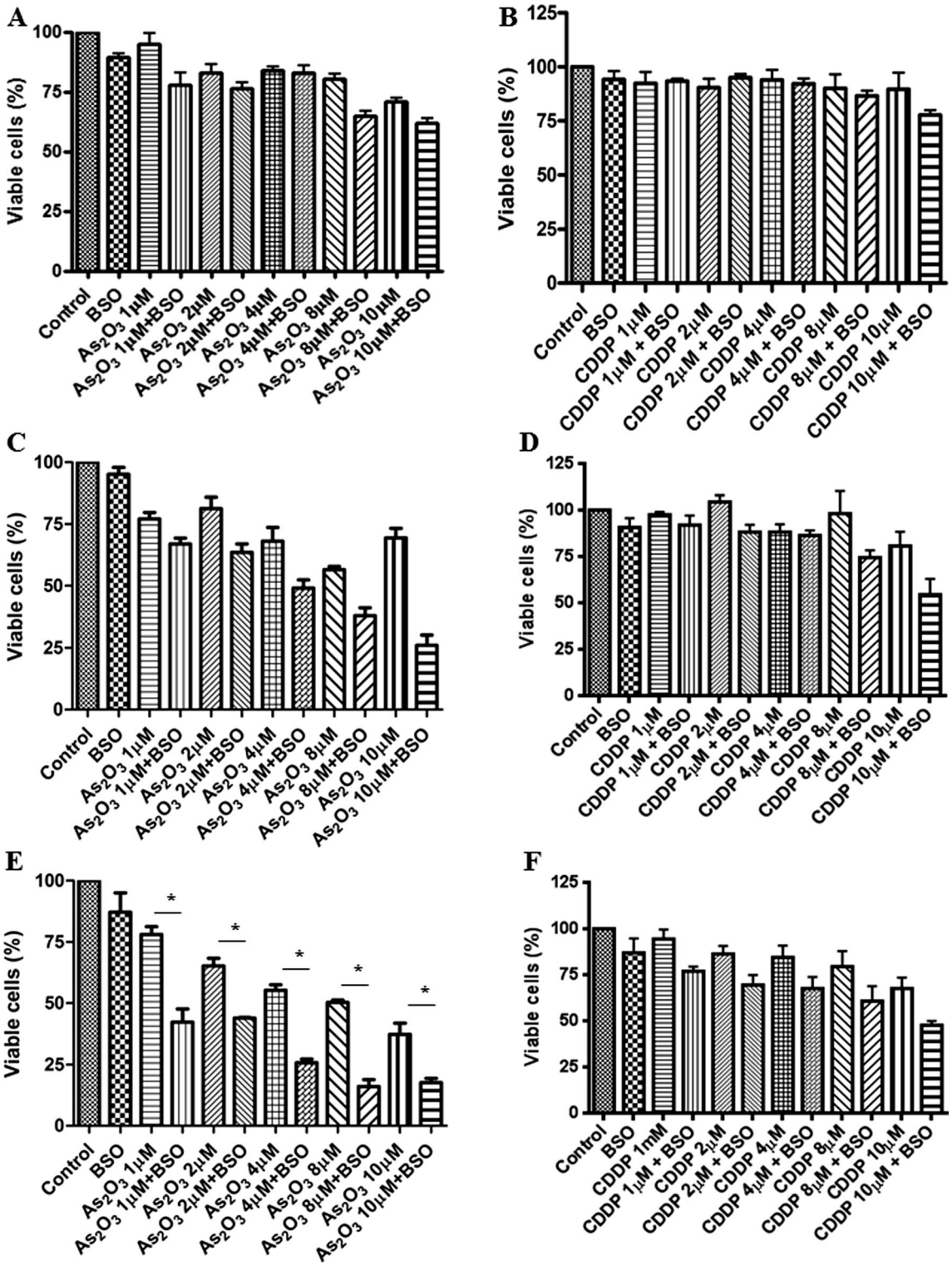

Fig. 1 BSO potentiates cytotoxic effect of As_2O_3, but not of CDDP. HEp-2 cells were pre-incubated with or without BSO and treated with increasing concentrations of As_2O_3 (**a**, **c**, **e**) or CDDP (**b**, **d**, **f**) for 24 h, 48 h, and 72 h. Cell viability was determined by MTT assay and expressed by percentage. The values are mean ± SEM of three independent experiments performed in triplicate. *$P<0.05$, comparing As_2O_3 treated cells with or without BSO pretreatment

cytometer, Beckman-Coulter, CA, USA). In all the experiments, the mean fluorescence intensity from 10.000 events was accessed and analyzed using Summit v4.3 software (Beckman-Coulter, CA, USA) as the percentage of cell population in each condition.

Statistical Analysis

In all experiments, the values were expressed as mean ± standard error of the mean (SEM), considering at least three independent experiments in triplicate. All data were analyzed using two-way analysis of variance (ANOVA) or unpaired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

Results

BSO is Capable of Enhancing As_2O_3 and CDDP Sensitivity

To evaluate the capability of BSO to enhance the cytotoxic effect of As_2O_3 and CDDP, we performed MTT assays (Fig. 1a–c). The viability of HEp-2 cells reached 50 % after 48 h of incubation with the highest concentrations (8.0 and 10 μM) of As_2O_3 (Fig. 1b), however, the same viability effect was not observed for CDDP at the highest concentrations. After 72 h, with 30 % of the intracellular levels of GSH reduced by BSO (data not shown), the viability of HEp-2 cells significantly ($P<0.05$) decreased at all concentrations of As_2O_3 combined with BSO pretreatment, when compared with As_2O_3 isolated treatment (Fig. 1c). In contrast, the CDDP did not show the same reduction of cell viability, even combined with BSO ($P>0.05$). Therefore, the study was directed to As_2O_3 at concentrations of 1.0 and 4.0 μM.

BSO/As_2O_3 is a Synergic Combination

Through the combination index calculations (R) developed by Fishel et al. [35], the effect of the combination BSO/As_2O_3 was found to be synergistic ($R<0.8$) after 48 h of incubation with 4.0–10 μM As_2O_3 and in all As_2O_3 concentrations after 72 h (Table 1).

BSO/As_2O_3 Sensitivity is Not Correlated to ROS Induction

We subsequently investigated the mechanisms involved in the activation of BSO/As_2O_3 sensitivity pathway. Through DCFH-DA probe by flow cytometry, we observed that BSO isolated or combined with As_2O_3 at different concentrations has no correlation with ROS production, suggesting involvement of other pathways in this process (Fig. 2 and Suppl. Fig. 1).

Table 1 Drug interaction analysis

[μM]	BSO/As_2O_3 (R value)		
	24 h	48 h	72 h
1.0	0.92	0.89	0.60
2.0	1.11	0.80	0.80
4.0	1.13	0.76	0.52
8.0	0.91	0.71	0.36
10.0	0.98	0.40	0.45

Combination index calculations were analyzed by the equation R=Survival (BSO + As_2O_3)/Survival (BSO) × Survival (As_2O_3). If $R<0.8$, the association is considered to be synergistic; if $0.8<R<1.2$, the association is considered to be additive; and if $R>1.2$, the association is considered to be antagonistic

BSO/As_2O_3 Combined Treatment Promotes G2/M Arrest

Most of the chemotherapeutic agents are capable of inducing cell cycle arrest. Although many mechanisms of cell death induction have already been described for As_2O_3, the effect of its combination with BSO on cell cycle is poorly understood. In the present study, we demonstrated that 4.0 μM As_2O_3 with or without BSO pretreatment promotes significant ($P<0.05$) G2/M arrest after 48 h (Fig. 3 and Suppl. Fig. 2).

Autophagy Evaluation in HEp-2 Cells Incubated with BSO/As_2O_3

We subsequently determined whether the combined treatment of cells with BSO/As_2O_3 results in an induction of autophagy in HEp-2 cells. For that purpose, we determined the

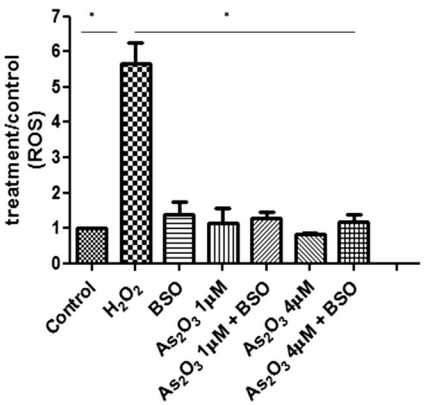

Fig. 2 ROS production in HEp-2 treated cells. HEp-2 cells were pre-incubated with or without BSO and treated with As_2O_3 (1.0 and 4.0 μM) for 72 h. Positive control comprised cells that were separately incubated with H_2O_2. ROS production was determined by DCFH-DA fluorescence probe using flow cytometry. The values are mean ± SEM of three independent experiments. *$P<0.05$, comparing treated cells and untreated cells (control). Representative cytometry graphic analysis in supplementary figure 1

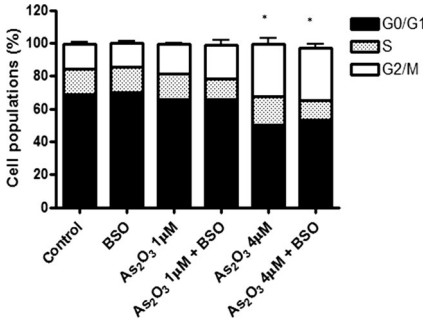

Fig. 3 Cell cycle arrests by BSO/As_2O_3 treatment. HEp-2 cells were pre-incubated with or without BSO and treated with As_2O_3 (1.0 and 4.0 μM) for 48 h. The cell cycle phases were determined by PI fluorescence using flow cytometry. The values are mean ± SEM of five independent experiments. *$P<0.05$, comparing treated cells and untreated cells (control). Representative cytometry graphic analysis in supplementary figure 2

autophagic response through the analysis of AVOs formation. As shown in Fig. 4a, As_2O_3-HEp-2-treated cells displayed a large number of fluorescent AVOs in the cytoplasm, when compared with the control or BSO-treated cells or even BSO/As_2O_3-HEp-2-treated cells. Moreover, BSO/As_2O_3-HEp-2-treated cells showed less AVOs and a significant vacuolar morphology. Flow cytometry analysis demonstrated that BSO treatment potentiates autophagy induction at low concentrations of As_2O_3. After 72 h, we observed a significant ($P<0.05$) increase in AO-positive cells among HEp-2 cells subjected to 1.0 μM BSO/As_2O_3 combined treatment and 4.0 μM As_2O_3, as shown in Fig. 4b.

3-MA Inhibited Autophagy Induction by BSO/As_2O_3 Treatment in HEp-2 Cells

To confirm autophagy induction by BSO/As_2O_3 treatment, we used a well-known autophagy inhibitor, 3-MA. We examined the cytotoxic effect of the combined treatment with or without 3-MA using MTT cell viability assay. After 72 h of incubation, 3-MA treatment at different concentrations and combined with BSO and/or As_2O_3 showed no significant decrease in cell viability (Fig. 5a–b).

Evaluation of BSO/As_2O_3 Treatment Autophagic Effect on Cell Death

We evaluated whether BSO/As_2O_3-autophagy induction in HEp-2 cells interplays with apoptosis through annexin V/PI assay. As shown in Fig. 6 and Suppl. Fig. 3, the percentage of unlabeled cells in AO assay corresponded to the percentage of apoptotic cells following As_2O_3 and BSO/As_2O_3 treatments. We observed that BSO pretreatment significantly enhances the apoptotic effect of As_2O_3 ($P<0.05$).

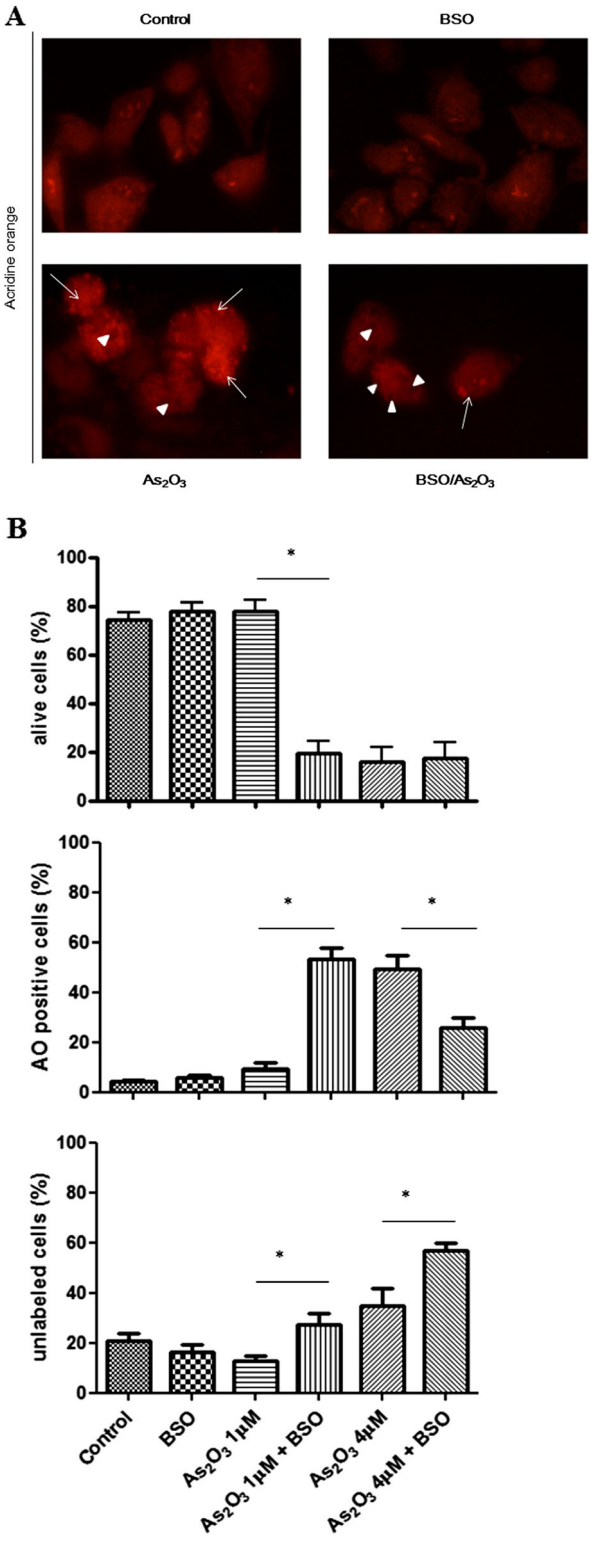

Fig. 4 Autophagy induction by BSO/As_2O_3 treatment in HEp-2 cells. The cells were pre-incubated with or without BSO and treated with As_2O_3 (1.0 and 4.0 μM) for 72 h. AVOs formation was documented by fluorescence microscope (**a**, 100× of magnification) and quantified by flow cytometry (**b**). The values are mean ± SEM of three independent experiments. The *arrows* indicate AVOs positivity and arrowheads vacuolar morphology. *$P<0.05$, comparing As_2O_3 treated cells with or without BSO pretreatment

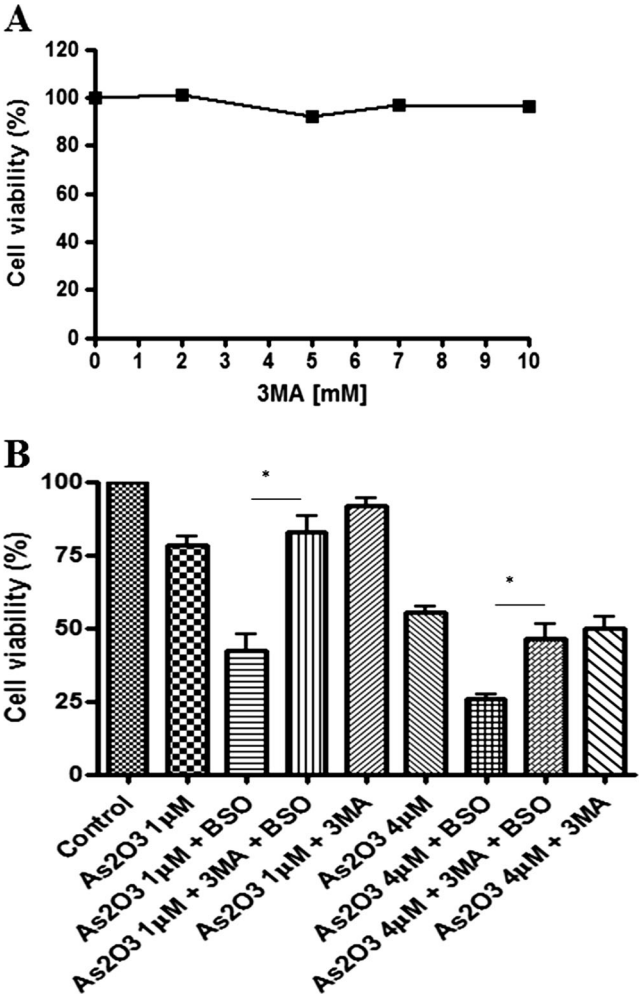

Fig. 5 3-MA inhibited BSO/As_2O_3 effect in HEp-2 cells. The cells were pre-incubated with or without 3-MA and BSO and treated with As_2O_3 (1.0 and 4.0 μM) for 72 h. 3-MA cytotoxicity (**a**) and cell viability of treatments (**b**) were determined by MTT assay and expressed in percentage. The values are mean ± SEM of three independent experiments performed in triplicate. *$P<0.05$, comparing As_2O_3 treated cells with or without BSO pretreatment

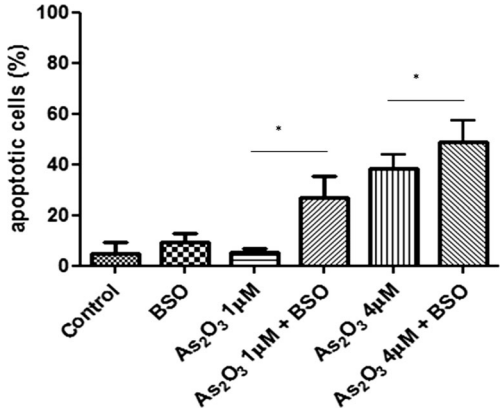

Fig. 6 Effect of BSO on As_2O_3-induced apoptosis. HEp-2 cells were pre-incubated with or without BSO and treated with As_2O_3 (1.0 and 4.0 μM) for 72 h. Annexin+/PI− and Annexin+/PI + were considered total apoptotic cells by flow cytometry. The values are mean ± SEM of five independent experiments. * $P<0.05$, comparing As_2O_3 treated cells with or without BSO pretreatment. Representative cytometry graphic analysis in supplementary figure 3

Discussion

Head and neck cancer has the distinction of being the first human cancer site for which successful combined therapy, such as using selective epidermal growth factor receptor inhibitor, cetuximab, with platinum chemotherapy, has significantly prolonged progression-free survival in patients at the late stage of the disease [41–43]. Despite many mechanistically targeted biological therapy applications successfully described in different types of head and neck cancers, further improvement of the therapeutic ratio for laryngeal cancer still remains to be elucidated. Owing to the decreasing 5-year overall survival rates, the current standard of care for laryngeal cancer needs to be re-examined [8]. Therefore, many attempts have been made to enhance the effectiveness of chemotherapeutics, simultaneously reducing its toxicity. The As_2O_3 antineoplasm, as a single agent, is effective for relapsed or refractory acute promyelocytic leukemia with the induction of programmed cell death type I, apoptosis. A promising As_2O_3 treatment in solid tumors has also been described; however, the molecular mechanism of cell death induction depends on the cellular context. Nevertheless, its therapeutic efficacy is limited. Therefore, the systematic study of the combination of As_2O_3 with other clinically used chemotherapeutic drugs and the understanding of their cell death mechanism induction to improve As_2O_3 therapeutic efficacy in treating human solid tumors is beneficial. Many strategies of inhibiting the chemotherapy resistance ability of GSH with BSO have been explored with variable pathways of death induction [30–32]. The use of BSO has already been performed in clinical trials [44–47]. However, the lack of tumor specificity is a potential problem [48]. The GSH resistance system functions via ROS inactivation [49] and/or chemotherapeutic conjugation [50]. Intracellular GSH levels modulation to enhance chemosensitivity in HEp-2 cells has been well documented [32]. HEp-2 cell line remained CDDP resistant, even combined with BSO. In an effort to search for strategies that could enhance As_2O_3-induced cancer cell death, we reported the activation of the pro-survival autophagy process as the main pathway mediated by the combination of BSO and As_2O_3 in HEp-2 cell lines. The synergistic As_2O_3 cytotoxic effects observed could be achieved by diminishing GSH intracellular levels through inhibition of γ-glutamylcysteine synthetase activity by BSO and by triggering G2/M cell cycle arrest in HEp-2 cells and is correlated with the up-regulation of survivin (data not shown), an important inhibitor of apoptosis family member. We also provided evidence that BSO/As_2O_3 combination was capable to increase characteristic AVOs cytoplasmic formation, confirming the autophagy induction. Through cell viability assay with cells previously treated with

the autophagy inhibitor, 3-MA, the As_2O_3-cytotoxic induction with or without BSO was suppressed. Originally, autophagy is considered to be cytoprotective; however, depending on the type of tumor, it is also an alternative route of programmed cell death, known as type-2 programmed cell death or autophagic cell death, which can interplay with apoptosis [51]. More recently, high Beclin-1 expression was described to be a favorable prognostic indicator for LSCC patients [52], making autophagy a good pathway target for death induction in LSCC. Survivin up-regulation is near-universal in solid tumors, and is associated with reduced apoptosis as well as resistance to therapy and poor prognosis. Survivin expression is dependent on cell cycle, increasing during G1 phase and peaking at G2/M phase [53]. However, although the enhanced expression of survivin is related to apoptosis resistance, the increase in survivin levels was not observed to be related to death resistance. Thus, these results indicate that autophagy is the main cell death pathway induction by BSO/As_2O_3, and highlight the interesting correlations whereby GSH is the main arsenic-resistant molecule. In fact, we infer that the role of GSH on arsenic resistance might be in the intracellular arsenic detoxification pathway, which is the complex of arsenic (+3 oxidation state) by GSH to form arsenic triglutathione and its efflux through ABC (ATP-binding cassette) transporters [54]. This detoxification pathway, first described in chemically defined systems and later demonstrated in vivo, could explain the arsenic resistance in HEp-2 cell lines. Another alternative in progress is the development and optimization of GSH analogues that inhibits the enzyme glutathione-S-transferase (GST) responsible for the detoxification overcoming, therefore, chemoresistance [48]. Among the GSH analogues developed, one (TLK 286) which is in clinical trial phase 3 settings for non-small cell lung and ovarian cancer, appears to sensitize these tumors to cytotoxic chemotherapies [48]. Nevertheless, intracellular-level GSH inhibition represents a useful strategy to overcome clinical arsenic resistance in HEp-2 cells, a unique LSCC cellular model commercially available for in vitro studies.

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Competing Interests The authors have declared that no competing interest exists.

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