Combination of arsenic trioxide and chemotheraphy in small cell lung cancer

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A B S T R A C T

Introduction: Small cell lung cancer (SCLC) carries high mortality despite standard chemotherapy. Arsenic trioxide (ATO) has demonstrated clinical efficacy in leukemia and in vitro activity in various solid tumors. This study was conducted to determine the in vitro and in vivo combination effects of ATO and chemotherapy in SCLC.

Materials and methods: The in vitro model consisted of 5 SCLC cell lines (H187, H526, H69, H841 and DMS79) and the anti-proliferative effects of ATO, cisplatin, etoposide or combinations thereof were measured. Synergism was determined by calculation of the combination index (CI) according to Chou and Talalay. Assays for apoptosis, intracellular glutathione (GSH) content, and mitochondrial membrane depolarization (MMD) were performed. Arsenic content was measured by inductively coupled plasma-mass spectrometry. Expression level of MRP1, MRP2 and pH2AX was detected by Western blot while cellular pH2AX level was monitored by immunofluorescent staining. An in vivo xenograft model in nude mice was established with a H841 cell line to test the effects of drug combinations.

Results: All 5 SCLC cell lines were sensitive to ATO, with IC50 values (48 h) 1.6–8 μM. Synergistic or additive effects were obtained by combining cisplatin with ATO in all 5 cell lines. Combination of etoposide with ATO resulted in antagonistic or barely additive effects. Apoptotic assays and pH2AX immunofluorescent staining corroborated the synergistic combination of ATO and cisplatin. In addition, the ATO/cisplatin combination enhanced MMD, depleted GSH, downregulated MRP2 and elevated intracellular ATO content compared with either ATO or cisplatin alone. In vivo combination of ATO and cisplatin also demonstrated synergism in the H841 xenograft model.

Conclusions: There was clinically relevant in vitro activity of ATO in a panel of 5 SCLC cell lines. Significant synergism was demonstrated with the ATO/cisplatin combination, while antagonism was noted with the ATO/etoposide combination in both in vitro and in vivo models.© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Small cell lung cancer (SCLC) accounts for 12–15% of all newly diagnosed cases of lung cancer [1] and a combination of cisplatin and etoposide remains the cornerstone first-line therapy [1–4]. Concurrent chemo–radiotherapy is feasible only for medically fit patients with limited-stage disease [5]. Upon disease relapse, salvage chemotherapy is often disappointing with short-lasting benefits [6]. The overall prognosis of SCLC thus remains extremely poor [2]. There is clearly a pressing need for novel effective combination treatments in SCLC.

In the past decade, arsenic trioxide (ATO) has emerged as one of the most effective treatments for acute promyelocytic leukemia (APL), leading to FDA approval for its clinical use [7]. There has also been accumulating evidence of in vitro activity of ATO in other cancers [8–10]. Data on its effect in SCLC are nonetheless scant.

Combination therapy with ATO and all-trans retinoic acid (ATRA) is a highly efficacious regimen in APL [11,12]. The in vitro combination of ATO with cisplatin has been shown to be synergistic in ovarian cancer cells [13,14] and NSCLC A549 cells [15]. The potential benefits of combining ATO with MAPK inhibitors [16], suberoylanilide hydroxamic acid [17], and bithionine sulfoximine [18] have also been elucidated.

In this study, we demonstrated the in vitro activity of ATO in a panel of SCLC cell lines and explored the potential interaction of ATO with cisplatin and etoposide. A combination of ATO with cisplatin or etoposide was also studied in an in vivo SCLC tumor xenograft model.
2. Materials and methods

2.1. Chemicals and reagents

ATO (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in 1.65 M NaOH. Cisplatin and etoposide (Ebewe Pharma, Untersch, Austria) were obtained from the Pharmacy of Queen Mary Hospital, Hong Kong SAR.

2.2. Cell lines and culture

Five SCLC cell lines (H187, DMS79, H526, H69 and H841) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in specific culture medium and conditions according to ATCC instructions.

2.3. Cell proliferation assay and drug interaction

Following exposure to ATO, cisplatin, or etoposide for 48 h, 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added and further incubated for 3 h at 37 °C followed by addition of detergent solution. Optical density was measured at 570 nm using a microplate reader (FLUO star OPTIMA, Bmg Labtec GmbH, Ortenberg, Germany).

When studying the effect of drug combinations, cells were incubated with a series dilution of drug mixtures. The potential interaction between ATO and cisplatin or etoposide was evaluated using CalcuSyn software (Version 2.0, Biosoft, Cambridge, UK) [19]. The combination index (CI) method of Chou and Talalay was adopted to define synergism (CI < 0.9), additivity (CI = 0.9–1.1) or antagonism (CI > 1.1) [19].

2.4. Apoptosis assay

Treated cells were stained for 15 min at room temperature with phycoerythrin (PE)-annexin V (Ex/Em = 488 nm/578 nm)/7-AAD (Ex/Em = 546 nm/647 nm) (BD Biosciences, CA, USA), then read by Cytomics FC 500 analyzer with FL2/FL4 channels (Beckman Coulter, CA, USA).

2.5. Measurement of mitochondrial membrane depolarization (MMD)

Treated cells were stained for 15 min at 37 °C with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetracyclohexylbenzimidazolylcarbocyanine iodide (JC-1) (5 µg/ml) before reading with FL1/FL2 channels of Cytomics FC 500 analyzer (Beckman Coulter).

2.6. Detection of glutathione (GSH)

Treated cells were incubated at 37 °C for 30 min with 5 µM 5-chloromethylfluorescein diacetate (CMFDA) (Ex/Em = 522 nm/595 nm) (Invitrogen, CA, USA), followed by incubation of complete medium at 37 °C for 40 min. 5-Chloromethylfluorescein (CMF) fluorescence intensity was determined using a Cytomics FC500 flow cytometer (Beckman Coulter) [20].

2.7. Intracellular arsenic detection with inductively coupled plasma-mass spectrometry (ICP-MS)

Treated cells were separated into nuclear and cytosolic fractions using a NE-PER® Nuclear and Cytoplasmic Extraction kit (Pierce Biotechnology, Rockford, USA). Arsenic content of the nucleus and cytoplasm were measured by ICP-MS with the ELAN DRCplus 6100 analyser (PerkinElmer-SCIEX, Canada). Samples were digested with nitric acid and diluted in ultra-pure water, with yttrium as internal standard. The nebulized sample was then heated at 6000 °C to create positively charged ions that were detected by mass spectrometer. The readouts were normalized with cell number to obtain cellular arsenic content.

2.8. Western blot for protein expression

Cell lysates were subjected to 7.5–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK), then incubated overnight at 4 °C with primary antibody (MRP1, MRP2 [Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] and pH2AX [Cell Signaling Technology, Danvers, MA, USA]). The membranes were then incubated with corresponding horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for a further 2 h at 4 °C. Bands were visualized using enhanced chemiluminescence (ECL) kit (GE Healthcare) following exposure to X-ray film.

2.9. Immunofluorescent staining

Cells (1 × 10^5/slip) were seeded on 18 mm coverslips. Treated cells were fixed for 10 min with 3.7% formalin and penetrated for 20 min with 100% methanol. After washing with phosphate-buffered saline (PBS), coverslips were incubated with pH2AX antibody (Cell Signaling Technology) overnight at 4 °C. Coverslips were incubated at room temperature for 1 h with FITC-conjugated anti-rabbit antibody (Sigma–Aldrich) and counterstained with DAPI (Santa Cruz Biotechnology). Images were obtained using a microscope (Eclipse E-800; Nikon, Tokyo, Japan).

2.10. In vivo effect of ATO/cisplatin or etoposide combination

A tumor xenograft model was established with nude mice (female, 4–6-week-old, 10–12 g, BALB/cAnN–nu, Charles River Laboratories, Wilmington, USA). Prior to treatment, 5 × 10^6 H841 cells were inoculated subcutaneously into the right flank of each mouse, resulting in a palpable tumor after 3 days of incubation. The mice were then allocated to 6 treatment groups: control (with PBS once/day), cisplatin (2.5 mg/kg/once/week), ATO (7.5 mg/kg/once/day), etoposide (10 mg/kg thrice/week), combined ATO/cisplatin, and combined ATO/etoposide. All drug combinations were administered intraperitoneally (IP). Our preliminary explorations revealed that the cytotoxic effects were more potent in vitro when ATO was administered following cisplatin or etoposide (data not shown); for humane reasons and to minimize the pain by reducing injection volume, ATO was administered 3 h following cisplatin or etoposide. Body weight and tumor size (using a standard caliper) were measured every 3 days. Tumor volumes (V) (before and during treatment) were calculated according to the formula V = (length × width × depth)/2. Mice were sacrificed after 4 weeks of treatment or upon reaching humane endpoints. The weight of tumor xenografts following treatment was then measured. The study protocol was approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (CULATR 2665–12).

2.11. Statistical analysis

Data are presented as mean ± standard deviation (SD), with between-group differences analyzed by two-tailed Student t-test. A p-value < 0.05 was considered to be statistically significant. Linear regression was used to calculate IC_{50} values. All statistical
Fig. 1. Effect of ATO combined with cisplatin or etoposide in SCLC cell lines. Dose–effect curves of combining ATO with (A) cisplatin or (B) etoposide at fixed ratio of concentrations. (C) Combination index (CI) at ED50, indicating synergistic (CI < 0.9), additive (CI = 0.9–1.1) and antagonistic (CI > 1.1) drug interactions.
analyses were performed using Prism 5 (GraphPad Software, La Jolla, Southern California, USA).

3. Results

3.1. Cytotoxic effect of ATO, cisplatin and etoposide

All SCLC cell lines were considered sensitive to ATO, with $IC_{50}$ values at 48 h (1.6–8 μM) that could be clinically achievable (around 7 μM) [21]. DMS79 cells were the least sensitive to ATO. As indicated by the pattern of chemosensitivity to cisplatin and etoposide, H187 and H526 cells were the most sensitive, DMS79 cells were the most resistant, and H69 and H841 cells were moderately resistant (Table 1).

3.2. In vitro combination effects of ATO and cisplatin or etoposide

Analysis of the dose-effect curve revealed that a combination of ATO and cisplatin conferred significantly more cell inhibition than ATO or cisplatin alone across a wide range of drug concentrations (Fig. 1A). There was no similar benefit with a combination of ATO and etoposide among our panel of SCLC cell lines (Fig. 1B). Based on CI value in defining the mode of drug interaction [22], moderate (CI = 0.6–0.8) to strong (CI = 0.2–0.6) synergism was observed for a combination of ATO and cisplatin in all 5 SCLC cell lines at half the maximal effective dose (ED50) (Fig. 1C). Significant (at least moderate) antagonism (CI > 1.1) was demonstrated with the ATO/etoposide combination in both chemosensitive (H187, H526) and resistant (DMS79) cell lines, while a borderline additive (CI = 0.9–1.1) effect was seen in moderately resistant cell lines (H69, H841).

3.3. Apoptotic effects of drug combinations in H841

H841 was selected as the only adherent cell line in our model to investigate the apoptotic effects of ATO/cisplatin or etoposide combinations. The proportions of cells that underwent apoptosis was 17.4 ± 5.9%, 24.2 ± 7.8%, and 45.2 ± 14.2% with 2.5 μM ATO, 10 μM cisplatin and 10 μM etoposide treatment for 48 h, respectively (Fig. 2A and B). Combination of ATO and cisplatin achieved a significantly higher degree (55.5 ± 10.9%) of apoptotic cell death than either ATO or cisplatin alone (p < 0.05) (Fig. 2B). Nonetheless, combining ATO with etoposide attained only a similar degree (40.7 ± 10.6%) of apoptosis compared with etoposide alone (Fig. 2B).

3.4. MMD induced by drug combinations in H841 cells

Treatment with 2.5 μM ATO, 10 μM cisplatin and 10 μM etoposide for 48 h resulted in 8.8 ± 3.4%, 11.8 ± 5.4%, and 21.7 ± 4.1% of MMD by JC-1 staining in H841 cells, respectively (Fig. 2C). Combining ATO with cisplatin significantly enhanced the extent of MMD to 30.8 ± 11.0%, while ATO/etoposide combination achieved a similar degree (23.0 ± 2.8%) of MMD to etoposide alone (Fig. 2D).
3.5. **Cellular GSH levels with drug combinations in H841 cells**

GSH is related to sensitivity of ATO and cisplatin in cancer cells, thus cellular GSH content was measured. With 2.5 μM ATO, 10 μM cisplatin and 10 μM etoposide treatment for 48 h, cellular GSH was decreased to 23.4 ± 1.9%, 16.5 ± 1.9%, and 7.4 ± 1.9% respectively compared with control (39.9 ± 5.1%) (Fig. 3A and B). A combination of ATO and cisplatin could further deplete GSH content (8.6 ± 1.3%) compared with single arm treatment, while ATO/etoposide could attain only a similar level (9.7 ± 2.4%) to etoposide alone (Fig. 3B).

3.6. **Effects of drug combination on DNA damage**

The amount of DNA double strand breaks (DBS) was not significant with ATO/cisplatin single treatment while the ATO/cisplatin combination induced DBS with a stronger pH2AX staining signal and more nuclear DNA damage foci (Fig. 4A). Expression of pH2AX was upregulated in the ATO/cisplatin combination arm when compared with single treatment groups while there was no difference with etoposide and ATO/etoposide combination (Fig. 4B).

3.7. **Subcellular arsenic distribution and expression of multi-drug resistant proteins (MRP1 and MRP2)**

After exposure for 48 h to ATO, both nuclear and cytoplasmic arsenic content was increased after ATO single treatment and ATO/cisplatin combination group, mainly localized in the cytoplasm. In the ATO/cisplatin combination, both nuclear and cytoplasmic arsenic content was increased notably compared with ATO treatment alone (Fig. 5A).

MRP1 and MRP2 are related to drug resistance of many chemotherapeutic drugs. The baseline MRP1 level was undetectable in SCLC cell line H841 (data not shown). ATO or cisplatin single treatment did not induce downregulation of MRP2 while combination treatment did (Fig. 5B).

3.8. **Drug combinations in H841 xenograft model**

In H841 xenograft model, exponential tumor growth was observed by 14 days following inoculation (control arm) (Fig. 6A). Different treatments were started 3 days following inoculation with a palpable tumor size of 22.4 ± 6.8 mm³. Single treatment with ATO (7.5 mg/kg once/day) or cisplatin (2.5 mg/kg once/week) resulted in a similar degree of tumor growth compared with control (Fig. 6A).

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<td>Half-maximal inhibitory concentration (IC50 in μM) for drug treatment (48 h) in small cell lung cancer cell lines.</td>
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<tr>
<td><strong>Drugs</strong></td>
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<tr>
<td>Arsenic trioxide</td>
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<td>Cisplatin</td>
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<td>Etoposide</td>
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*Fig. 3.* GSH content in H841 cells treated with ATO, cisplatin, etoposide or combinations. (A) Representative CMFDA staining plot. Right-sided peak represents the CMF-fluorescent GSH content. (B) CMF-fluorescent GSH content with different treatments. Statistical comparison was made between treatment groups and control, unless otherwise indicated. *p<0.05; **p<0.01; ns, not significant.
Fig. 4. DNA damage in ATO/cisplatin combination treatment. (A) DNA damage was analyzed by immunofluorescent staining following 48 h of drug treatment. (B) Downregulation of pH2AX expression in different treatment groups by Western blotting. *p < 0.05; **p < 0.01; ns, not significant.
Combination ATO and cisplatin synergistically suppressed tumor growth compared with other treatment arms (Fig. 6A). There was no weight loss with the combination arm (ATO/cisplatin). There were otherwise no observed detrimental health effects to the mice during treatment. The in vivo H841 model was rather sensitive to etoposide (10 mg/kg thrice/week), as obvious tumor suppression was evident after one cycle (1 week) of treatment (Fig. 6B). There was no significant weight loss in different treatment arms. Combination of ATO with etoposide attained a similar degree of tumor suppression to etoposide alone (Fig. 6B). Mice in the etoposide-containing arms suffered loss of appetite and diarrhea, and one mouse in ATO/etoposide arm died with marasmus by the end of the second cycle of treatment. The overall antitumor effects with different treatment arms were also evidenced by the weight of tumor xenografts upon treatment completion (Fig. 6C).

4. Discussion

We have demonstrated clinically relevant cytotoxic activity of ATO in a panel of SCLC cell lines with different chemosensitive patterns to standard chemotherapy (cisplatin and etoposide). Our drug combination experiments have further supported a synergistic interaction between ATO and cisplatin, at least mediated via suppression of intracellular GSH, while antagonism was observed with a combination of ATO and etoposide. The in vitro findings were confirmed by a tumor xenograft model using a H841 cell line in nude mice.

The potential benefit of combining ATO and platinum has been shown in vitro in ovarian cancer [14,23], head and neck cancer [24], hepatocellular carcinoma [25], and non-small cell lung cancer [15]. Since platinum remains a fundamental component of chemotherapy for SCLC, our finding of a synergistic combination of ATO with cisplatin in SCLC, irrespective of platinum sensitivity, is clinically relevant. In general, synergistic combination of anticancer drugs allows reduction of individual drug concentrations by 2–8-fold [19]. Isobologram analysis revealed that combination of ATO and cisplatin could achieve above 80% cell inhibition with individual drug concentrations at ¼ to 1-fold of IC50 values across our panel of SCLC cell lines. As ATO and cisplatin have largely non-overlapping toxicities, their combination would appear to be a promising regimen for the treatment of SCLC.

In order to understand the mechanisms that lead to synergism between ATO and cisplatin in SCLC, H841 cells were chosen as an appropriate model. First, H841 cells were relatively resistant to cisplatin but sensitive to etoposide: this could facilitate study of synergism with cisplatin and possibly antagonism with etoposide at clinically relevant concentrations. Second, H841 was the only predominantly adherent cell line among our panel, and
was technically more feasible for mechanistic exploration. In H841 cells, our findings suggested that a combination of ATO and cisplatin could enhance apoptosis through MMD and reduction of intracellular GSH. Notably, ATO has been shown to be a mitochondrial toxin that can induce loss of mitochondrial membrane potential and subsequently increase oxidative stress in target cells [26,27]. Nonetheless, cisplatin also works through formation of platinum–DNA adducts [28], initiating a series of biological activities that result in apoptosis [29]. With the combination of ATO and cisplatin in our cell line model, a significantly greater degree of MMD could be attained, suggesting that the synergistic interaction was mediated through the mitochondrial pathway. Cellular GSH content has been implicated as a determining factor for cisplatin chemosensitivity [30–32]. Thus our finding of significantly more profound depletion of GSH with combined ATO/cisplatin treatment may explain the enhanced cytotoxicity compared with cisplatin alone.

H2AX is a histone protein required for DNA repair. Phosphorylation of H2AX on Ser 139 is an unique reaction to cellular exposure to DNA damaging agents and has been well established as a DNA double strand break (DSB) marker [33–35]. In our study, combination of ATO and cisplatin further increased H2AX phosphorylation. This may serve as a novel mechanism to explain the synergistic effect of ATO and cisplatin.

There are also alternative mechanisms that may account for the synergism between ATO and cisplatin seen in other cancer types. In neuroblastoma cell lines, co-administration of ATO and cisplatin led to sustained cellular Ca2+ increase that resulted in enhanced cytotoxicity and apoptosis [36]. Alternatively, multidrug resistant protein 1 (MRP1) has been shown to serve as a competitive transporter for both cisplatin and glutathione-conjugated trivalent form of arsenic within cancer cells. Based on a kidney cell line model, co-application of ATO could compete with cisplatin for binding to MRP1, thereby reducing the efficiency of efflux of cisplatin, resulting in increased intracellular cisplatin concentration and formation of cisplatin–DNA adducts [37,38]. In our study we detected MRP2 expression only in H841 cells. MRP 1 and 2 belong to the MRP family with similar substrate specificity and resistance profile [39]. MRP2 has been demonstrated to confer cisplatin resistance in vivo [40] and in vitro by effluxing GSH conjugated cisplatin [41,42] and regarded as a GS-X pump for the elimination of cisplatin [43]. It has been demonstrated that MRP2 interacted with cisplatin and ATO by calcein efflux studies and GSH was required in the process [43]. In the present study, MRP2 protein level decreased significantly in the combination of ATO and cisplatin treated cells. This means that less drugs were pumped out of cells and is in line with a higher intracellular arsenic content. Although the exact underlying mechanism of MRP2 downregulation remains unclear, such downregulation may play an important role in the synergistic effect of ATO and cisplatin.

To the best of our knowledge, this is the first time that antagonism has been reported with the combination of ATO and etoposide in SCLC: previous studies have reported potentially beneficial effects of ATO and traditional chemotherapeutic agents including etoposide in ovarian and prostate cancer cell lines [44]. Our finding of an antagonistic combination of ATO and etoposide based on proliferation (MTT) assay was supported by the lack of additional mitochondrial membrane depolarization and GSH depletion compared with etoposide alone. The exact mechanism of this antagonistic phenomenon has not been elucidated. Interestingly, ATO could induce apoptosis via inhibition of topoisomerase II, which is also the main target of etoposide, in an ovarian cancer cell line [38]. Drug combinations of agents that effectively work on the same therapeutic target (e.g. ATO and etoposide on topoisomerase II) may obviate synergism.

Consistent with our in vitro findings, we have established a tumor xenograft model with H841 cell line that confirmed the
synergistic effect of a combination of ATO and cisplatin. Notably, there was a significant tumoricidal effect with ATO/cisplatin combination after around 3–4 weeks of treatment with good tolerance.

In conclusion, ATO has demonstrated clinically relevant in vitro activity in a panel of SCLC cell lines. A synergistic interaction of a combination of ATO and cisplatin in SCLC has been demonstrated in vitro and in vivo. Nonetheless a combination of ATO and etoposide appears to be antagonistic. Further exploration of the clinical application of combination treatment of ATO and cisplatin in SCLC is warranted.

Conflict of interest statement

All authors in this manuscript have no financial and personal relationships with other people or organizations that could inappropriately influence our work. None of the authors have conflict of interest to declare.

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