Free Radical Biology & Medicine 51 (2011) 2195–2209

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Combination of arsenic trioxide and BCNU synergistically triggers redox-mediated autophagic cell death in human solid tumors

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ARTICLE INFO

Article history:
Received 7 December 2010
Revised 20 September 2011
Accepted 21 September 2011
Available online 29 September 2011

Keywords:
Arsenic trioxide
BCNU
Synergism
Redox
Autophagy
Cancer therapy
Free radicals

ABSTRACT

Arsenic trioxide (As_2O_3) is an effective treatment for relapsed or refractory acute promyelocytic leukemia (APL). After the discovery of As_2O_3 as a promising treatment for APL, several studies investigated the use of As_2O_3 as a single agent in the treatment of solid tumors; however, its therapeutic efficacy is limited. Thus, the systematic study of the combination of As_2O_3 with other clinically used chemotherapeutic drugs to improve its therapeutic efficacy in treating human solid tumors is merited. In this study, we demonstrate for the first time, using isobologram analysis, that As_2O_3 exhibits a synergistic interaction with *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU). The synergistic augmentation of the cytotoxicity of As_2O_3 with BCNU is in part through the autophagic cell death machinery in human solid tumor cells. As_2O_3 and BCNU in combination produce enhanced cytotoxicity via the depletion of reduced glutathione (GSH) and augmentation of reaction oxygen species (ROS) production. Further analysis indicated that the extension of GSH depletion by this combined regimen occurs through the inhibition of the catalytic activity of glutathione reductase. Blocking ROS production with antioxidants or ROS scavengers effectively inhibits cell death and autophagy formation, indicating that redox-mediated autophagic cell death involves the synergism of As_2O_3 with BCNU. Taken together, this is the first evidence that BCNU could help to extend the therapeutic spectrum of As_2O_3. These findings will be useful in designing future clinical trials of combination chemotherapy with As_2O_3 and BCNU, with the potential for broad use against a variety of solid tumors.

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Arsenic is a natural carcinogen that has been used medicinally for many years [1]. Recently, arsenic trioxide (As_2O_3) has been found to cause complete remission of acute promyelocytic leukemia (APL)[1] [2]. Investigators introduced As_2O_3 alone and in combination with chemotherapeutics as a cancer therapy for patients with APL and achieved notable rates of complete remission [3,4]. Although the mechanism is not completely understood, As_2O_3 seems to exert its antileukemic effect, at least in part, through the degradation of the promyelocytic leukemia (PML)–retinoic acid receptor α fusion oncoprotein and/or PML proteins [5].

The therapeutic potential of As_2O_3 is not restricted to APL cells but has also been observed in various solid tumor cells, including neuroblastoma, head and neck cancer, and prostate cancer [6]. However, most studies showed that the antitumor effects of As_2O_3 in solid tumor cells were not comparable with those seen in APL. Even though As_2O_3 was reported to induce apoptosis or growth inhibition in various tested cell lines, the As_2O_3 concentrations required were higher than those in hematologic malignancies and, thus, not clinically achievable without the risk of As_2O_3-mediated side effects [4,6–8]. Therefore, other therapeutic strategies are required to enhance the efficacy of As_2O_3 against human solid malignancies. Combination therapy with multiple drugs is a common practice in the treatment of cancer. The promising anticancer activity of As_2O_3 has promoted considerable interest in combining this drug with cisplatin [9,10], L-buthionine sulfoximine (BSO) [11,12], docosahexaenoic acid (DHA) [13], sulindac [14], and anthraquinones [15]. However, the relationship between

Abbreviations: APL, acute promyelocytic leukemia; AVO, acidic vesicular organelles; BCNU, *N,N′*-bis(2-chloroethyl)-*N*-nitrosourea; BSO, L-buthionine sulfoximine; CPT, camptothecin; DHA, docosahexaenoic acid; 5-FU, 5-fluorouracil; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; LC3, microtubule-associated protein 1 light chain 3; 3-MA, 3-methyladenine; NAC, *N*-acetyl-L-cysteine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PML, promyelocytic leukemia; ROS, reactive oxygen species; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid; VP-16, etoposide.

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doi:10.1016/j.freeradbiomed.2011.09.023

As_2O_3 and clinically used chemotherapeutic drugs in human solid tumor models has not been systematically studied.

In this study, we used isobologram analysis to evaluate the synergism and additive, or the antagonistic, effects in human solid tumor cells of As_2O_3 with several anticancer agents, including *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (BCNU), camptothecin (CPT), paclitaxel, cisplatin, etoposide (VP-16), and 5-fluorouracil (5-FU). Herein, we demonstrate that a significant potentiation of cytotoxicity and synergy was obtained with a combination of As_2O_3 and BCNU. The aim of this investigation was to elucidate a possible cytotoxic enhancing effect in human solid tumor cells by combining As_2O_3 with BCNU to obtain initial preclinical evidence for the potential efficacy of using these two drugs in combination therapy to treat human solid malignancies.

Experimental procedures

Materials

As_2O_3 and BCNU were purchased from Sigma–Aldrich (St. Louis, MO, USA). Monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody was purchased from Trevigen (Gaithersburg, MD, USA). Monoclonal antibody for α-tubulin and polyclonal antibody for microtubule-associated protein 1 light chain 3 (LC3) were purchased from Sigma–Aldrich. Atg7-targeted small interfering RNA (siRNA) and scramble control siRNA were purchased from Invitrogen (Carlsbad, CA, USA). Horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell culture reagents were obtained from Gibco BRL Life Technologies (Gaithersburg, MD, USA). All other chemicals were from E. Merck Co. (Darmstadt, Germany) or Sigma–Aldrich and were standard analytic grade or higher.

Cell culture

Human solid tumor cell lines derived from nasopharyngeal carcinoma (HONE-1), glioblastoma multiforme (DBTRG-05MG), colorectal carcinoma (HT-29), gastric carcinoma (TSGH), and non-small-cell lung cancer (H460) were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum. The human melanoma (A2058) and breast carcinoma (MCF-7) cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. All cell lines were grown in a 5% CO_2 humidified atmosphere at 37 °C.

Cell viability assay

Cells in logarithmic phase were seeded into 96-well plates to adhere overnight. They were then exposed to various concentrations of test drugs in quadruplicate for the indicated times and then incubated in a serum-free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/ml for 2 h. The conversion of MTT to formazan by metabolically viable cells was measured by the absorbance at 570 nm in a 96-well microtiter plate reader.

Analysis of combined drug effects

Isobologram plots [16] were constructed using IC_{50} values obtained from MTT assays of the two agents alone or in combination. Single-agent IC_{50} values were interpolated from the plots of fractional growth versus drug concentration. An isobole of IC_{50}, defined as the line joining all dose pairs inducing the same specified effect, was generated by plotting combination doses of each dose pair expressed as fractions of the single-agent IC_{50} values. An isobole is represented algebraically by the sum of the ratios of combination to single-agent doses of each drug producing an isoeffect, or $(A_{combination}/A_{single}) + (B_{combination}/B_{single})$, where *A* and *B* denote the two drugs being combined. When this sum is equal to 1, the interaction is said to be additive. A synergistic interaction appears below the line of additivity, whereas an antagonistic interaction appears above this line.

Annexin V/propidium iodide (PI) binding assay

A FITC Annexin-V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) was used according to the manufacturer's instruction. Briefly, cells were trypsinized and collected in phosphate-buffered saline (PBS) by centrifugation and resuspended in 1× Binding buffer, then FITC–annexin V and PI were added. After incubation at room temperature for 15 min in the dark, the cells were analyzed by flow cytometry. Control cells stained with annexin V or PI alone were used to compensate for the flow cytometric analysis.

Determination of caspase-3 activity

Caspase-3 activity was measured with the CaspACE Assay System–Fluorometric Kit (Promega, Madison, WI, USA). Cells were initially seeded at a density of 1×10^6 in 100-mm^2 dishes. After treatment with test drugs for the indicated times, caspase-3 activity was measured by the cleavage of the fluorometric substrate Ac-DEVD-AMC according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay

To determine whether a test drug could induce apoptosis, TUNEL assay using the *In Situ* Cell Death Detection Kit–Fluorescein (Roche Applied Science, Mannheim, Germany) was performed according to the manufacturer's instructions.

Western blot analysis

Cells were initially seeded at a density of 1×10^6 in 100-mm^2 dishes. After treatment with test drugs for the indicated times, adherent cells were washed twice with PBS, gently scraped from the dishes, centrifuged, and lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 0.8 M NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitors aprotinin, leupeptin, and pepstatin 20 mg/ml each). Lysates were centrifuged at 12,000×*g* for 15 min and the supernatants were collected and quantified. Equal amounts of lysate (on a protein basis) were separated by SDS–PAGE, blotted on polyvinylidene difluoride membranes, conjugated with various specific primary antibodies, and then probed with appropriate secondary antibodies. The immunoreactive bands were detected with the ECL method and visualized on Kodak Bio-MAX MR film.

Detection of acidic vesicular organelles (AVOs) with acridine orange staining

To detect the presence of AVOs, the cells were stained with the vital dye acridine orange (1 μg/ml) and then examined under a fluorescence microscope. To quantify the formation of AVOs, cells were harvested and stained with 1 μg/ml acridine orange for 10 min and immediately analyzed by flow cytometry with the detection of emission wavelengths of green (510–530 nm) and red (650 nm). The percentage of AVOs was calculated from the amount of cells present in both upper-left and upper-right quadrants.

Transfection of the GFP-LC3B expression plasmid and visualization of the expressed GFP-LC3B in cells

The green fluorescent protein (GFP) and GFP-LC3B expression plasmids were kindly donated by Dr. Noboru Mizushima. Plasmids were transfected into HONE-1 cells by using Lipofectamine LTX

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