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Heat shock protein inhibitors, 17-DMAG and KNK437, enhance arsenic trioxide-induced mitotic apoptosis

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ABSTRACT

Arsenic trioxide (ATO) has recently emerged as a promising therapeutic agent in leukemia because of its ability to induce apoptosis. However, there is no sufficient evidence to support its therapeutic use for other types of cancers. In this study, we investigated if, and how, 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG), an antagonist of heat shock protein 90 (HSP90), and KNK437, a HSP synthesis inhibitor, potentiated the cytotoxic effect of ATO. Our results showed that cotreatment with ATO and either 17-DMAG or KNK437 significantly increased ATO-induced cell death and apoptosis. siRNA-mediated attenuation of the expression of the inducible isoform of HSP70 (HSP70i) or HSP90α/β also enhanced ATO-induced apoptosis. In addition, cotreatment with ATO and 17-DMAG or KNK437 significantly increased ATO-induced mitotic arrest and ATO-induced BUBR1 phosphorylation and PDS1 accumulation. Cotreatment also significantly increased the percentage of mitotic cells with abnormal mitotic spindles and promoted metaphase arrest as compared to ATO treatment alone. These results indicated that 17-DMAG or KNK437 may enhance ATO cytotoxicity by potentiating mitotic arrest and mitotic apoptosis possibly through increased activation of the spindle checkpoint.

Introduction

Arsenic trioxide (As_2O_3, ATO), a trivalent inorganic arsenite, has been proved to be an effective therapeutic agent against acute promyelocytic leukemia (Soignet et al., 1998). Numerous reports have revealed that arsenite exerts its therapeutic activity by induction of apoptosis (Miller et al., 2002; Douer and Tallman, 2005). It also induces apoptosis in a variety of cancer cells over a wide dose range, but its therapeutic efficacy in the treatment of these cancers is minimal (Evens et al., 2004; Gazitt and Akay, 2005). The establishment of methods to increase the susceptibility of cancer cells which are relatively resistant to arsenite is of critical importance to improve its therapeutic potential.

Trivalent arsenite has pleiotropic effects on many biological systems and induces complex toxicopathological injuries, including the generation of reactive oxygen species, induction of DNA damage, disruption of mitochondrial function, modification of gene and/or protein expression and intracellular signal transduction pathways, alteration of cell cycle progression, and induction of cytogenetic aberrations and cellular transformation (Simeonova et al., 2000; Kitchin, 2001; Miller et al., 2002; Yih et al., 2002). These deleterious effects trigger arsenite-induced apoptosis. However, the use of ATO as a single agent in clinical trials against solid tumors refractory to current therapies was found to be mostly not effective or too toxic (Dilda and Hogg, 2007). Combination of ATO with other chemotherapeutic agents may increase its therapeutic effects, while reducing its toxic side-effects and therefore expand its potential in cancer therapy.

HSP70 and HSP90 are molecular chaperones involved in protein folding, stability, and turnover (Picard, 2002; Arndt et al., 2007). Many of their client proteins play critical roles in signal transduction and cell cycle progression (Caplan et al., 2007). Their function as protein chaperones helps cells recover from thermal-, radio-, or chemical-induced injuries (Brodsky and Chiosis, 2006; Camphausen and Tofilon, 2007). In addition, HSP70 and HSP90 interact with, and inhibit, apoptosis proteins, thus protecting cells from anti-cancer drugs (Beere, 2005) and are therefore potential targets for cancer therapy. Numerous reports have shown that inhibition of HSP70i and HSP90 enhances radiosensitivity of tumors or sensitizes them to other chemotherapeutic agents (Brodsky and Chiosis, 2006; Camphausen and Tofilon, 2007).

Recent studies have demonstrated that arsenite induces disorganized mitotic spindles and abnormal chromosome segregation (Huang and Lee, 1998; Li and Broome, 1999; Ling et al., 2002). The

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aberrant mitosis induced by arsenite is followed by intensive induction of mitosis-mediated apoptosis (Huang and Lee, 1998; Cai et al., 2003; Yih et al., 2005). Moreover, induction of mitotic arrest was recently shown to be one of the major mechanisms for arsenite-induced apoptosis of cancer cells (Li and Broome, 1999; Huang et al., 2000; Park et al., 2001; Ling et al., 2002; Cai et al., 2003; Liu et al., 2003; Yih et al., 2005) and may contribute to its therapeutic effect (Cai et al., 2003; Gazitt and Akay, 2005). Alternatively, arsenic compounds are known to be HSP inducers, and overexpression of HSP70 and HSP90 has been reported to protect cells from arsenite insults (Khalil et al., 2006; Pelicano et al., 2006). Abrogation of the function of HSP70 or HSP90 may therefore be a potential strategy for improving the therapeutic efficacy of arsenite. In this study, we investigated whether benzylidine lactam (KNK437) an inhibitor of HSP induction and thermotolerance (Yokota et al., 2000), and 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG), an HSP90 antagonist, could potentiate the cytotoxicity of the trivalent arsenite drug ATO.

Materials and methods

Cell culture. HeLa-S3 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in monolayer and maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 0.37% sodium bicarbonate, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in an humidified incubator in air and 10% CO_2 and were passaged twice per week.

Drug treatment. Logarithmically growing cells were left untreated or were treated with 1–4 μM ATO (Sigma, St. Louise, MO), 10–50 nM 17-DMAG (Calbiochem, San Diego, CA), or 15–50 μM KNK437 (Calbiochem) alone or cotreated with ATO and 17-DMAG or KNK437 for the indicated time. They were then harvested and used for cytotoxicity, cell cycle distribution, apoptosis, immunofluorescence staining, or immunoblot studies. An ATO stock solution (10 mM) was freshly prepared in 0.1 N NaOH and diluted in culture medium before use. Aliquotes of 10 mM 17-DMAG or KNK437 stocks were prepared in DMSO and stored at −20 °C.

Cytotoxicity assay. Cytotoxicity was determined with a viability assay in which cells were stained with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) (Dojindo Molecular Technologies, Inc., Gaithersburg, MD), which produces a water-soluble formazan dye upon reduction in the presence of an electron carrier of viable cells. Cells were seeded in a 96-well plate (5000/well), then, 24 h later, were treated with drugs for 72 h. At the end of treatment, WST-8 was added to the medium and the plates incubated at 37 °C for 1 h before cell viability was determined by the optical absorption of the reduced formazan at 450 nm. Cell viability was determined as percent of absorption (450 nm) of the untreated control versus drug concentration. The concentration of ATO to induce 50% inhibition on cell viability (IC_{50}) either alone or in combination with 17-DMAG or KNK437 was calculated by using GraphPad PRISM version 4 (GraphPad Software, Inc., San Diego, CA).

RNA interference. RNA interference was carried out as described previously (Elbashir et al., 2001). The small interfering RNAs (siRNAs) were synthesized by Proligo Singapore Pte Ltd (Singapore). Cells were plated at a density of $0.5–1\times10^5$ per 35 mm dish one day before transfection. siRNA was transfected into the cells using Lipofectamine (Invitrogen) at a final concentration of 0.1 μM. Twenty-four hours after transfection, the medium were replaced with fresh medium and the cells treated with ATO. The inducible HSP70 (HSP70i) was depleted by two double-stranded siRNAs which respectively targeted at 5′-GGACAUCAGCCAGAACAAG-3′ and 5′-GCGAGAGGGUGUCAGCCAA-3′ of the HSPA1A (NM_005345) and HSPA1B (NM_005346) genes (Aghdassi et al., 2007). The HSP90α/β was depleted by doubled-stranded siRNAs which respectively targeted at 5′-AACCUUAUACCGA-ACAAACAA-3′ of HSP90AA1 gene (NM_005348) and 5′-UCCACGAAG-ACUCCACUAA-3′of HSP90AB1 gene (NM_007355). A double-stranded RNA targeting luciferase (5′-cguacgcggaauacuucgadTdT-3′) was used as a control. Cellular expression of HSP70i and HSP90α/β was examined by immunoblot analysis at 24 and 72 h after ATO treatment.

Detection of apoptosis. The number of apoptotic cells was determined using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Calbiochem) as described previously (Yih et al., 2005). After drug treatment, the cells were trypsinized, washed once with phosphate-buffered saline, pH 7.4, (PBS), and resuspended in 100 μl of binding buffer containing 5 μl of a 200 μg/ml solution of FITC-conjugated Annexin V and 5 μl of a 30 μg/ml solution of propidium iodide (PI). After 10 min incubation at room temperature, FITC binding was analyzed using a fluorescence activated cell sorter (Epics®XL/MCL, Beckman Coulter) and the percentage of apoptotic cells (FITC-positive) per 10,000 cells calculated. It is recognized that, during apoptosis progression, cells were first PI-negative/Annexin V-positive due to phosphatidylserine exposure to the outer leaflet of the cell membrane (lower right quadrant, Fig. 2A). Then these cells start loosing their membrane integrity, hence become PI-positive/Annexin V-positive and shift from the lower right quadrant to upper right quadrant (Fig. 2A) (Krysko et al., 2008). Since induction of apoptosis may not be a synchronous event, both PI-negative/Annexin V-positive cells and PI-positive/Annexin V-positive cells were included in our results. Apoptosis was also determined by detecting cleaved-poly (ADP-ribose) polymerase (c-PARP) in cells with the aid of a flow cytometer (Li and Darzynkiewicz, 2000). After drug treatment, cells were fixed with ice-cold 70% ethanol for 16 h, and immunostained for 3 h at room temperature with c-PARP-specific antibody (#9541, Cell Signaling Technology, Danvers, MA), followed by incubation for 1 h at room temperature with allophycocyanin-conjugated secondary antibody (Invitrogen). The level of c-PARP of individual cells was analyzed using a fluorescence activated cell sorter (Epics®XL/MCL, Beckman Coulter, Fullerton, CA).

Analysis of cell cycle distribution. Cell cycle progression was monitored using DNA flow cytometry. DNA was stained with PI and mitotic cells quantified by measuring the expression of the mitosis-specific marker, phospho-histone H3 (Yih et al., 2006). After drug treatment, the cells were trypsinized, washed once with PBS, fixed with ice-cold 70% ethanol for 16 h, and immunostained for 3 h at room temperature with mouse anti-phospho-histone H3 (serine 10) antibody (#9706, Cell Signaling Technology), followed by incubation for 1 h at room temperature with FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were then stained with 4 μg/ml of PI in PBS containing 1% Triton X-100 and 0.1 mg/ml of RNase A. Phospho-histone H3 levels and the DNA content of individual cells were analyzed using a fluorescence activated cell sorter (Epics®XL/MCL, Beckman Coulter, Fullerton, CA), and the cell cycle distribution of the cells determined using a computer program provided by Beckman Coulter, as described previously (Yih et al., 2005).

Immunoblots. Levels of cellular proteins in cell extracts were examined by immunoblot analysis, as described previously (Yih et al., 2005). Briefly, the treated cells were either scraped off or shaken off, washed twice with ice-cold PBS, collected in a 1.5 ml vial, and boiled in reducing SDS-PAGE sample buffer (Laemmli, 1970). Samples containing equal amounts of cellular proteins (10–30 μg) were resolved by 8 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Health Care, Piscataway, NJ), which were then blocked for 1 h at room temperature with 5% skimmed milk in PBS containing 0.2% Tween 20 (PBST) and incubated overnight at 4 °C with primary antibodies diluted in PBST, then for 1 h at room