Biochemical Pharmacology 86 (2013) 1430–1440

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

OSU-A9, an indole-3-carbinol derivative, induces cytotoxicity in acute myeloid leukemia through reactive oxygen species-mediated apoptosis

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ARTICLE INFO

Article history:
Received 16 July 2013
Accepted 4 September 2013
Available online 13 September 2013

Keywords:
OSU-A9
Indole-3-carbinol
Acute myeloid leukemia
Reactive oxygen species
Glutathione

ABSTRACT

Indole-3-carbinol (I3C) is a broadly targeted phytochemical shown to prevent carcinogenesis in animal studies and to suppress the proliferation of cancer cells of human breast, colon, prostate, and endometrium. Here we demonstrate that OSU-A9, an I3C derivative with improved anticancer potency, induces cytotoxicity in acute myeloid leukemia (AML) cell lines (HL-60 and THP-1) and primary leukemia cells from AML patients in a dose-responsive manner. Normal human bone marrow cells were less sensitive to OSU-A9 than leukemia cells. OSU-A9 induces caspase activation, PARP cleavage, and autophagy but not autophagic cell death. Interestingly, pretreatment of AML cell lines and primary AML cells with *N*-acetylcysteine or glutathione rescues them from apoptosis (and concomitant PARP cleavage) and Akt hypophosphorylation, implicating a key role of reactive oxygen species (ROS) in OSU-A9-related cytotoxicity. Importantly, the anticancer utility of OSU-A9 is extended *in vivo* as it, administered intraperitoneally, suppresses the growth of THP-1 xenograft tumors in athymic nude mice without obvious toxicity. This study shows that ROS-mediated apoptosis contributes to the anticancer activity of OSU-A9 in AML cell lines and primary AML cells, and thus should be considered in the future assessment of its translational value in AML therapy.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematological malignancy characterized by the proliferation of clonal neoplastic hematopoietic cells, with diverse clinical presentations. Chemotherapy with or without hematopoietic stem cell transplantation has been the mainstay AML treatment. However, although advances in medicine and supportive care have led to complete remission for 70–80% of adult AML patients, only 20–30% of these patients have long term disease-free survival [1]. The major cause of this discrepancy is the occurrence of refractory or relapsed AML that inevitably develops with the onset of chemoresistance. Alternative compounds or strategies are therefore needed to effectively manage patients with AML.

Indole-3-carbinol (I3C), a natural phytochemical found in the vegetables of the cruciferous family, has been shown to be beneficial for chemoprevention in animal models of human cancer [2–4]. Moreover, experiments have demonstrated the ability of I3C to suppress the proliferation of cancer cells of breast, colon, prostate, and endometrium by targeting multiple signaling pathways [5,6]. Despite these advances in translational research, several factors limit the clinical utility of I3C as an anticancer agent. First, the effective antitumor concentration of I3C, between 50 and 100 μmol/L, is not practical *in vivo*. Second, the chemical instability of I3C and vulnerability to acid-catalyzed conversion into a variety of derivatives in the stomach decrease its antitumor activity [7]. Third, the difficulty in monitoring I3C concentration in plasma limits its pharmacokinetic analysis [8]. Consequently, structural modification of I3C or its metabolite 3,3′-diindolylmethane has been carried out to develop novel indole derivatives with improved potency and stability [6,9]. Among them, OSU-A9, an acid-stable derivative with higher apoptosis-inducing potency, is active against cancers of prostate, breast and liver *in vitro*, and has inhibited tumor growth of these cancers in xenograft animal models. Importantly,

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http://dx.doi.org/10.1016/j.bcp.2013.09.002

OSU-A9 administrated daily to nude mice in these experiments was well tolerated without significant toxicities [10–13].

In the present study, we investigate the anticancer activity and possible underlying mechanisms of OSU-A9 against two AML cell lines and primary leukemia cells from patients with AML. In addition, the ability of OSU-A9 to inhibit leukemia growth is demonstrated in athymic nude mice bearing THP-1 xenografts.

2. Materials and methods

2.1. Cells and culture conditions

Bone marrow from patients was obtained under a protocol approved by the China Medical University Hospital internal review board. Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. AML cells were isolated from freshly collected bone marrow using Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions if the leukemia cells accounted for more than 90% of non-erythroid mononucleated cells of bone marrow. Normal bone marrow nucleated cells were harvested using Ficoll-Paque™ PLUS from patients with treatment-naive non-Hodgkin's lymphoma for whom bone marrow examination for lymphoma staging was performed but determined to be normal. Human AML cell lines HL-60 (ATCC CCL-240) and THP-1 (ATCC TIB-202) were from American Type Culture Collection (ATCC, Manassas, VA). All cells were incubated in RPMI-1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and penicillin (100 U/mL)/streptomycin (100 μg/mL) (Invitrogen) at 37 °C in the presence of 5% CO_2.

2.2. Reagents

OSU-A9 {[1-(4-chloro-3-nitrobenzene-sulfonyl)-1*H*-indol-3-yl]-methanol} was synthesized as previously described [10], with identity and purity (≥99%) verified by proton nuclear magnetic resonance, high-resolution mass spectrometry, and elemental analysis. For *in vitro* experiments, OSU-A9 was dissolved in dimethyl sulfoxide (DMSO), and added to the culture medium with a final DMSO concentration less than 0.1%. The pharmacological agents were purchased from the respective vendors: *N*-acetylcysteine (NAC; Sigma–Aldrich, St. Louis, MO); L-glutathione (Cayman Chemical, Ann Arbor, MI); chloroquine (Sigma–Aldrich); 3-methyladenine (3-MA; Sigma–Aldrich).

2.3. MTS assay

Measurement of cell growth was performed using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit purchased from Promega (Madison, WI). Cells (0.25×10^6/mL) were placed in 200 μL volume in 96-well microtiter plates with the indicated reagent and incubated at 37 °C. MTS solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium] and PMS (phenazine methosulfate) solution were mixed 20:1 by volume. The colorimetric measurements were performed 4 h later at 490 nm wavelength by a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA). The cell viability was expressed as a percentage of absorbance value in treated samples compared to that observed in control vehicle-treated samples (subtract the blank in both conditions).

2.4. Cell viability and apoptosis assay by flow cytometry

Cell viability was assessed by dual staining with annexin V conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI). Cells (0.5×10^6) were stained by annexin V-FITC (BD Pharmingen, San Diego, CA) and PI (BD Pharmingen) according to the manufacturer's instructions. Cells were analyzed by a flow cytometer BD FACSCanto II (BD, Franklin Lakes, NJ). Annexin V-FITC positive cells were identified as apoptotic cells. Viable cells were those with both annexin V-FITC negative and PI negative staining.

2.5. Comet assay

OSU-A9-treated or etoposide (positive control)-treated cells (0.5×10^6) were pelleted, resuspended in ice-cold PBS, and mixed with 1.5% low-melting point agarose. The mixture was then placed on a frosted slide that had been precoated with 0.7% agarose, and covered by a coverslip. The slides were submerged in prechilled lysis buffer (1% Triton X-100, 2.5 mol/L NaCl, and 10 mmol/L EDTA, pH 10.5) for 1 h at 4 °C. After the slides had been soaked with prechilled electrophoresis buffer (0.3 mol/L NaOH and 1 mmol/L EDTA) for 20 min, they were subjected to electrophoresis for 30 min at 50 V/m (20 mA). Finally, the slides were stained with porpidium iodide (PI) and visualized at 200× magnification under a fluorescence microscope.

2.6. Western blotting

Cell lysates were prepared using RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris pH 8.0, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor (Sigma–Aldrich) and phosphatase inhibitor cocktail (Calbiochem). Antibodies against various proteins were obtained from the following sources: poly-ADP-ribose polymerase (PARP), p^{Thr308}-Akt, caspase-3, caspase-9, cyclin B1, LC3B (Cell Signaling, Danvers, MA); Akt, cyclin A, p21, p27 (Santa Cruz Biotechnology, Santa Cruz, CA); procaspase-8 (Millipore, Billerica, MA); β-actin (Sigma–Aldrich). The goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates and goat anti-mouse IgG-HRP conjugates were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA).

2.7. Autophagy detection with acridine orange staining

Cells (0.25×10^6/mL) were treated with DMSO or indicated concentrations of OSU-A9 for 24 h. The cells were washed with phosphate-buffered saline (PBS) twice, suspended in PBS and stained by acridine orange (1 μg/mL, Sigma–Aldrich) at 37 °C for 15 min; then the cells were washed with PBS and resuspended in 0.5 mL of PBS. For visual examination of autophagosomes, cells were analyzed under a fluorescence microscope. For quantification of acridine orange positive, red color (650 nm) fluorescence emission illuminated with blue (488 nm) excitation light was measured with a flow cytometer BD FACSCanto II (BD). In the inhibitor study, cells were pretreated with 3-MA (1 mmol/L) or chloroquine (10 μmol/L) for 1 h, followed by incubation with DMSO or OSU-A9.

2.8. Reactive oxygen species (ROS) generation

Briefly, cells (0.25×10^6/mL) were treated with DMSO or indicated concentrations of OSU-A9 with or without antioxidant pre-treatment (1 mmol/L NAC or 2.5 mmol/L L-glutathione) at 37 °C for 15 min. At the indicated time point, the cells were washed with PBS twice and then stained by H_2DCFDA (5 μmol/L, Invitrogen) at 37 °C for 30 min. After washing with PBS, fluorescence intensity for ROS determination was assessed using a flow cytometer BD FACSCanto II (BD).