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Original Contribution

A novel small molecule that induces oxidative stress and selectively kills malignant cells

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ABSTRACT

We have synthesized a novel molecule named XB05 (1-bromo-1,1-difluoro-non-2-yn-4-ol) and evaluated its effects in a variety of human cell lines. XB05 displayed potent antiproliferative activity against cell lines derived from leukemia or solid tumors, but had less effect on nonmalignant cells. To identify factors that contribute to the cancer selectivity of XB05, we chose three cell lines that had high sensitivity to XB05 (U937 leukemia), moderate sensitivity (A549 lung cancer), or low sensitivity (Hs27 nonmalignant skin fibroblasts), and proceeded to assess cell death and oxidative stress in these cells. XB05 was found to induce cell death via both apoptotic and nonapoptotic mechanisms in U937 and A549 cells, whereas it had no cytotoxicity against Hs27 cells at comparable concentrations. Treatment with XB05 caused an increase in reactive oxygen species in all cell lines tested, but levels were higher in malignant compared to nonmalignant cells. XB05 treatment also induced DNA damage exclusively in the malignant cells. Differences in antioxidant responses were observed between cell lines. For example, XB05 caused a decrease in levels of glutathione and nuclear Nrf2 in the most sensitive cells (U937), whereas the least sensitive cells (Hs27) displayed increased glutathione levels and no change in nuclear Nrf2. XB05 could react *in vitro* with cysteine and glutathione, but had much lower reactivity compared to typical thiol-reactive electrophiles, diethyl maleate and maleimide. In summary, XB05 is a novel compound that selectively kills malignant cells, most likely by disrupting cellular redox homeostasis, making it a promising candidate for development as a chemotherapeutic agent.

Introduction

XB05 is a synthetic small molecule that was originally generated as a building block to introduce fluorine-containing groups into organic molecules [1]. Intrigued by its novel chemical structure and resemblance to marine-derived natural products with antitumor activity [2], we screened XB05 for biological activity in human cell lines. Although XB05 was not originally intended as an anticancer agent, our studies have revealed significant activity against cancer cells. Here we describe for the first time the cancer-selective antiproliferative and cytotoxic effects of XB05, and propose an oxidative stress-based mechanism to explain its activity against cancer cells.

Materials and methods

Materials

XB05 was synthesized using a previously described method [1]. Further details are provided in Supplementary Information. *S*-(+)-Camptothecin, Z-VAD-fmk, L-buthionine-sulfoximine, antimycin A, diethyl maleate, maleimide, reduced L-glutathione, *N*-acetylcysteine, L-cysteine, and L-ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against cleaved caspase-3 (No. 9661), γ-H2AX (No. 9718), phosphorylated (Ser 51) eIF2α (No. 9721), eIF2α (No. 9722), and BiP (No. 3177) were purchased from Cell Signaling Technology, Inc. (Danvers, MA).

Abbreviations: AA, L-ascorbic acid; BSO, buthionine sulfoximine; carboxy-H_2DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DCF, dichlorofluorescein; DEM, diethyl maleate; DSB, double-strand breaks; DTNB, dithiobis-2-nitrobenzoic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetylcysteine; Nrf2, NF-E2-related factor 2; PI, propidium iodide; ROS, reactive oxygen species; TBHP, *tert*-butyl hydrogen peroxide

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Thapsigargin, anti-rabbit and anti-mouse antibodies linked to horseradish peroxidase, and antibodies against PARP-1 (sc-8007), Nrf2 (sc-365949), Ku-70 (sc-5309), and GAPDH (sc-47724) were purchased from Santa Cruz Biotech (Santa Cruz, CA).

Cell culture and treatments

Cell lines were either recently purchased from the American Type Culture Collection (ATCC) or verified by short tandem repeat (STR) analysis (IDEXX Laboratories, Westbrook, ME). Cells were grown in the appropriate medium supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT), 62.5 μg/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY) in a humidified incubator at 37 °C with 5% CO_2. The media were as follows: Dulbecco's modified Eagle's medium (DMEM) for A549, MDA-MB-231, DU145, and Hs27 cells; RPMI 1640 for U937 cells; Eagle's minimal essential medium (EMEM) supplemented with Eagle's balanced salt solution (EBSS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and nonessential amino acids (Lonza, Walkersville, MD) for IMR-90 cells; mammary epithelial growth medium (MEGM) supplemented with all of the components of the MEGM SingleQuots kit except for GA-1000 (Lonza) for MCF-10A cells. Cells were treated by direct addition of XB05 solutions into the culture medium to give the final concentrations indicated in the figures. Unless otherwise stated, cells were at approximately 40% confluence at the start of treatment. XB05 solutions were freshly prepared from stock solutions of 2 mM in 100% DMSO by dilution with cell culture medium. Final DMSO concentrations were 0.05% in both vehicle control and XB05-treated cells. As a positive control for apoptosis, cells were treated with camptothecin (6 μg/mL) for the times indicated in the figure legends. As a positive control for DNA damage, cells were irradiated with 800 μJ in a UV Stratalinker 2400 (Stratagene, Santa Clara, CA), and then allowed to grow in culture for an additional 2 h.

Cell proliferation assays

The antiproliferative activity of XB05 was tested using a previously published 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay protocol [3]. Briefly, cells were seeded in quadruplicate wells in 96-well plates and allowed to adhere overnight. To account for intrinsic differences in growth rates, cells were plated at the following densities so as to achieve comparable MTT absorbance values (between 1 and 2) in untreated samples after 72 h: A549, DU145, and MDA-MB-231, 1000 cells/well; Hs27 and U937, 1500 cells/well; IMR-90 and MCF-10A, 5000 cells/well. Plates were incubated with XB05 for 72 h, during which the cell culture medium was not changed. Cell viability was determined and the background corresponding to medium alone (no cells) was subtracted. Data were analyzed as described in figure legends.

Trypan blue exclusion assay and microscopy

Cells were treated as indicated in the figures. After 72 h, cells were trypsinized (in the case of adherent cells) and stained with trypan blue solution (0.4%) (BioRad, Hercules CA), and cell number and percentage viability determined on a TC10 automated cell counter (BioRad) using triplicate measurements for each sample. Phase contrast microscopy images were obtained on an EVOSfl digital inverted microscope (Advanced Microscopy Group, Bothell, WA).

Flow cytometric assays

Analyses were performed using a FACScalibur flow cytometer (BD Biosciences, Mountain View, CA) and FlowJo software (Tree Star, Inc., Ashland, OR). Cells were treated as indicated in the figures and harvested by using TrypLE Cell Dissociation Reagent (Life Technologies). For cell death analyses, cells (adherent and detached cells) were stained with annexin-V-FITC and propidium iodide (PI) using an apoptosis detection kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. For cell-cycle distribution, cells were fixed, and stained with PI using the Cycle Test Plus kit (Becton Dickinson, Franklin Lakes, NJ). For detection of γ-H2AX, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min at 37 °C. Cells were then permeabilized in 90% methanol for 30 min on ice, blocked in 2% BSA (w/v in PBS) for 1 h, and incubated with γ-H2AX antibody or rabbit IgG isotype control (Santa Cruz, sc-2027) (0.3 μg/mL in 1% BSA) for 1 h. After washing, cells were incubated with goat anti-rabbit IgG antibody conjugated to Alexa Fluor-488 (Life Technologies) for 1 h at room temperature. For reactive oxygen species (ROS) detection, cells (1×10^6 cells/mL) were stained with 25 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Life Technologies) in PBS for 30 min at 37 °C. After washing with PBS, cells were resuspended in PBS containing 1 μg/mL PI to allow exclusion of nonviable cells. For assays using MitoSOX Red (Life Technologies), cells were harvested, washed with PBS, and stained with 5 μM MitoSOX Red in warm PBS for 10 min at 37 °C. Cells were then washed twice with PBS, resuspended at a density of 1×10^6 cells/mL in PBS, and analyzed by flow cytometry.

Clonogenic assays

Cells were plated at low density (300 cells/well) in 6-well tissue culture plates and allowed to adhere overnight. Where indicated, the medium was then replaced with fresh medium containing antioxidants or vehicle (sterile ultrapure H_2O). XB05 or vehicle control (DMSO) was then added directly to the medium at the concentrations indicated. After 10 days, cells were fixed with 4% paraformaldehyde in PBS, stained with Accustain Crystal Violet Solution (Sigma Aldrich), washed, and air-dried. Colonies were counted using the cell counter feature in the Image J software, available from the National Institute of Health (rsbweb.nih.gov/ij/download.html).

Cell extracts

After treatment as indicated in figure legends, cells were washed twice with ice-cold PBS. Nuclear extracts were isolated from cells using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Rockford, IL), according to the manufacturer's protocol. For total cell extracts, cells were lysed in RIPA buffer (Thermo Scientific) containing protease inhibitor cocktail III and phosphatase inhibitor cocktail (Calbiochem, Billerica, MA) for 5 min at 4 °C, and then cleared by centrifugation at 16,000 *g* for 10 min at 4 °C. All protein concentrations were determined using the DC Protein Assay kit (BioRad, Hercules, CA).

Western blotting

Equal amounts of protein per sample (typically, 50 μg) were resolved by SDS–Tris polyacrylamide gel electrophoresis and then electrotransferred onto polyvinylidine fluoride membranes (Millipore, Bedford, MA) in Tris–glycine buffer containing 20% methanol. Membranes were blocked with 5% milk or 5% BSA (for phosphoprotein detection) in TBS/0.01% Tween. Proteins were detected using the following primary antibody concentrations: PARP-1