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DNA damage, lysosomal degradation and Bcl-xL deamidation in doxycycline- and minocycline-induced cell death in the K562 leukemic cell line

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ABSTRACT

We investigated mechanisms of cytotoxicity induced by doxycycline (doxy) and minocycline (mino) in the chronic myeloid leukemia K562 cell line. Doxy and mino induced cell death in exposure-dependent manner. While annexin V/propidium iodide staining was consistent with apoptosis, the morphological changes in Giemsa staining were more equivocal. A pancaspase inhibitor Z-VAD-FMK partially reverted cell death morphology, but concurrently completely prevented PARP cleavage. Mitochondrial involvement was detected as dissipation of mitochondrial membrane potential and cytochrome C release. DNA double strand breaks detected with γH2AX antibody and caspase-2 activation were found early after the treatment start, but caspase-3 activation was a late event. Decrement of Bcl-xL protein levels and electrophoretic shift of Bcl-xL molecule were induced by both drugs. Phosphorylation of Bcl-xL at serine 62 was ruled out. Similarly, Bcr/Abl tyrosine kinase levels were decreased. Lysosomal inhibitor chloroquine restored Bcl-xL and Bcr/Abl protein levels and inhibited caspase-3 activation. Thus, the cytotoxicity of doxy and mino in K562 cells is mediated by DNA damage, Bcl-xL deamidation and lysosomal degradation with activation of mitochondrial pathway of apoptosis.

1. Introduction

Inhibition of cell death can contribute to the resistance of leukemic cells to chemotherapy [1]. Cell death may be divided into distinct entities based on the morphological and biochemical features [2]. Apoptosis may be processed through an extrinsic or intrinsic pathway. The extrinsic pathway is initiated by extracellular stressors and propagated by the trans-membrane receptors in a caspase-dependent manner. The intrinsic apoptosis is a response to intracellular stress such as DNA damage or oxidative stress and is mediated by the mitochondrial outer membrane permeabilization (MOMP) in either the caspase-dependent or -independent pathway [2]. The loss of mitochondrial membrane potential ($\Delta\psi m$) with subsequent drop in ATP production is considered to be the point of no return along the apoptotic pathway [3]. Necrosis may be uncontrolled or programmed. The former is characterized by early loss of membrane integrity and dilation of organelles, the latter by receptor-interacting protein kinase (RIP)-1 and RIP-3 [4]. The lysosomes can contribute to any type of cell death [5].

Doxycycline (doxy) and minocycline (mino) are semi-synthetic derivatives of tetracyclines that exhibit anti-bacterial effects and antitumor activities in several cancer cell lines [6,7]. Doxy-induced apoptosis was associated with loss of $\Delta\psi m$, cytochrome C (cytC) release, Bcl-xL inhibition, caspase activation and intracellular ROS formation in colorectal and pancreatic cancer cell lines [6,7]. Mino-induced apoptosis was associated with caspase-3 activation and PARP-1 inhibition in ovarian cancer [8].

The leukemic K562 cell line established from a patient in blastic crisis of chronic myeloid leukemia (CML) possesses characteristics of CML [9] as cells express the p210 Bcr/Abl fusion protein and overexpress the antiapoptotic Bcl-xL protein [10]. The aim of our study was to assess the mechanisms of doxy- and mino-induced cytotoxicity in K562 cells.

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2. Materials and methods

2.1. Chemicals and reagents

Doxycycline hyclate, minocycline hydrochloride, chloroquine diphosphate, vinblastine sulfate and bongkrekic acid were purchased from Sigma Aldrich (Stockholm, Sweden); etoposide from Bristol-Myers Squibb (Bromma, Sweden); resazurin from R&D systems Inc. (Minneapolis, MN, USA); Z-VAD-FMK from Bachem AG (Bubendorf, Switzerland); cOmplete mini protease inhibitor cocktail from Roche AB (Stockholm, Sweden); RPMI 1640 media, Dulbecco's phosphate-buffer saline (PBS) and fetal bovine serum (FBS) from Invitrogen AB, (Stockholm Sweden).

2.2. Cell culture

The human myeloid HL-60 and lymphoblastic Jurkat cell lines were purchased from DSMZ (Braunschweig, Germany), the chronic myeloid K562 cell line from ATCC (LGC Promochem Ab, Borås, Sweden). Cells were seeded at concentrations of 2×10^5 cells/ml and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (complete medium) at 37 °C in 95% humidified 5% CO_2 atmosphere. All experiments were performed in exponentially growing cells.

2.3. Treatment

Cells were treated with drugs in final concentrations of 0.5–500 μg/ml in complete medium. Cells treated in 0.02% dH_2O served as controls for solvent toxicity. Cells treated with etoposide (6 or 40 μg/ml) or vinblastine (Vb; 10 or 50 μg/ml) served as positive controls for cell death. Cells incubated in complete media served as controls.

Cells were treated with pan-caspase inhibitor Z-VAD-FMK in a concentration of 100 μM for 1 h. Then doxy or mino were added in a final concentration of 50 μg/ml and cells were incubated for 6, 24 and 48 h. Fresh Z-VAD-FMK was added to the cultures every 24 h.

Cells were incubated with the adenine nucleotide (ADP/ATP) translocator (ANT) inhibitor bongkrekic acid (BKA) in a final concentration of 100 μM for 4 h and then treated concomitantly with BKA (50 μM) and doxy or mino (50 μg/ml) for 48 h.

Cells were treated with the lysosomal inhibitor chloroquine (CQ) in final concentrations of 25 or 50 μM for 1 h followed by treatment with doxy or mino in concentrations of 50 μg/ml for 48 h.

2.4. Cell viability assay

Ten thousands cells per well were seeded in triplicate on 96 wells black microplates and incubated with doxy or mino (0.5–500 μg/ml) for 24 h. Then resazurin was added in a final concentration of 10% and cells were further incubated for 2 h at 37 °C. Fluorescence was read using FLUOstar Optima (BMG Labtech GmbH, Offenburg, Germany) at a wavelength of 590 nm. Viability of treated cells was expressed as a percentage of the viability of controls. The 50% inhibitory concentration (IC_{50}) was calculated as the drug concentration inducing 50% reduction in cell viability using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

2.5. Assessment of cell death

100,000 cells were stained with annexin V (Becton Dickinson (BD), San Jose, CA, USA) in a concentration of 125 ng/ml and propidium iodide (PI) (Sigma Aldrich, Stockholm, Sweden) in a concentration of 5 μg/ml in 100 μl of annexin V binding buffer for 15 min at room temperature (RT) in the dark. Samples were analyzed using FACScan flow cytometer, CELL Quest software (BD) and free Flowing Software ver. 2.5.1 (created by Perttu Terho, Cell Imaging Core, Turku Centre for Biotechnology, Finland, http://www.flowingsoftware.com/).

Cell death morphology consistent with apoptosis (condensed chromatin and fragmented nuclei) or necrosis (increased cell size and loss of cellular architecture) was assessed using May-Grünwald-Giemsa staining on cytospun slides and expressed as a percentage of a minimum of 400 cells counted per slide.

2.6. Assessment of mitochondrial membrane potential ($\Delta\psi m$)

Cells were incubated with the mitochondrial probe tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Carlsbad, CA, USA) in a final concentration of 25 nM in PBS for 30 min at 37 °C. After washing, cells were re-suspended in PBS and analyzed using flow cytometry.

2.7. Western blot (WB) analysis

Cells were lysed in Tris-NaCl buffer (50 mM Tris pH 7.4, 150 mM NaCl, 25 mM EDTA, 1 mM NaF, cOmplete mini protease inhibitor cocktail, 1 mM PMSF and 1% Triton × 100) on ice for 30 min, then centrifuged at 10,000 g for 10 min at 4 °C.

To assess poly (ADP-Ribose) polymerase (PARP), cells were incubated in PARP extraction buffer (62.5 mM Tris pH 6.8, 6 M urea, 2% SDS, 10% Glycerol and 0.001% bromophenol blue) for 15 min at RT, then for another 5 min at 95 °C.

For subcellular fractionation, the cells were incubated in digitonin cytosolic buffer (5 mM Tris–HCl pH 7.4, 5 mM succinic acid pH 6.3, 10 mM $MgCl_2.6H_2O$, 0.5 mM EDTA, 147.5 mM KCl, 5 mM KH_2PO_4 and 0.005% digitonin) for 30 min on ice and then centrifuged at 10,000 g for 10 min at 4 °C. The supernatant (cytosolic fraction) was collected and the pellet was re-suspended in PBS containing cOmplete mini protease inhibitor (pellet fraction).

Protein concentrations were assessed using Pierce® BCA or 660 nm® protein assay kits (Pierce, Rockford, IL, USA) according the manufacturer's recommendations.

For protein separation, equal volumes of samples and 2x Laemmli buffer supplemented with 5% 2-mercaptoethanol were boiled for 5 min at 95 °C. Protein samples (10 or 20 μg) were separated on 12 or 15% SDS-PAGE and transferred to a PVDF or to nitrocellulose membranes, whichever appropriate. The membranes were blocked in 5% non-fat dry milk solution for 2 h at RT and incubated overnight with primary antibodies (Supplementary Table 1) at 4 °C. Then the membranes were rinsed and incubated with secondary antibodies conjugated with peroxidase (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or IRDye®800CW or IRDye®680CW (LI-COR, Lincoln, NE, USA) for 1 h at RT. The proteins were visualized using SuperSignal West Pico Chemiluminescent substrate (Pierce) or ODYSSEY imaging system (LI-COR). Actin was used as a marker of equal protein loading.

2.8. Assessment of intracellular reactive oxygen species (ROS)

The intracellular ROS levels were assessed using OxiSelect™ Intracellular ROS assay Kit with green fluorescence (Cell Biolabs, San Diego, CA, USA). Following the treatment with doxy or mino, cells were incubated with 100 μM of 2′-7′-dichlorodihydrofluorescin (DCFH-DA) in media for 1 h at 37 °C. Then the cells were washed in PBS and the DCF fluorescence measured using FLUOstar Optima at wavelengths 485/520 nm.