Toxicology and Applied Pharmacology 334 (2017) 35–46

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Quinacrine induces the apoptosis of human leukemia U937 cells through FOXP3/miR-183/β-TrCP/SP1 axis-mediated BAX upregulation

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ARTICLE INFO

Keywords:
Quinacrine
p38 MAPK
ERK
FOXP3/miR-183/β-TrCP/SP1 axis
BAX upregulation

ABSTRACT

Quinacrine, which is clinically used as an antimalarial drug, has anti-cancer activity. However, mechanism underlying its cytotoxic effect remains to be completely elucidated. In the present study, we investigated the cytotoxic effect of quinacrine on human leukemia U937 cells. Quinacrine-induced apoptosis of U937 cells was accompanied with ROS generation, mitochondrial depolarization, and BAX upregulation. Quinacrine-treated U937 cells showed ROS-mediated p38 MAPK activation and ERK inactivation, which in turn upregulated FOXP3 transcription. FOXP3-mediated miR-183 expression decreased β-TrCP mRNA stability and suppressed β-TrCP-mediated SP1 degradation, thus increasing SP1 expression in U937 cells. Upregulated SP1 expression further increased BAX expression. BAX knock-down attenuated quinacrine-induced mitochondrial depolarization and increased the viability of quinacrine-treated cells. Together, our data indicate that quinacrine-induced apoptosis of U937 cells is mediated by mitochondrial alterations triggered by FOXP3/miR-183/β-TrCP/SP1 axis-mediated BAX upregulation.

1. Introduction

Genetic mutations and aberrations are associated with the pathogenesis of acute and chronic myeloid leukemias (Dohner et al., 2015; Talati et al., 2015). Targeted therapies based on mechanism-based strategies have been developed for treating acute and chronic myeloid leukemias (Dohner et al., 2015; Shah et al., 2016; Komanduri and Levine, 2016). However, defects in apoptotic pathways and resistance to multiple anticancer drugs cause treatment failure in patients with these leukemias (Testa and Riccioni, 2007; Rumjanek et al., 2013; Bhola et al., 2016). Some studies indicate that dysregulation of BCL2 expression contributes to the chemotherapeutic resistance of chronic and acute myeloid leukemias (Tzifi et al., 2012; de Necochea-Campion et al., 2016). Therefore, drugs that overcome defects in apoptotic pathways by downregulating the expression of antiapoptotic BCL2 family proteins or upregulating the expression of proapoptotic BCL2 family proteins may help in improving the treatment of myelogenous leukemias.

Drug repositioning is an important pharmaceutical strategy for drug development because it reduces drug-related adverse effects. Drug repositioning allows rapid development of anticancer drugs (Wurth et al., 2016). Quinacrine (6-chloro-9-[4-diethylamino-1-methylbutylamino]-2-methoxyacridine) is a 9-aminoacridine derivative clinically used as an antimalarial drug and is reported to have anticancer activity (Ehsanian et al., 2011; Preet et al., 2012). Several studies suggest that 9-aminoacridine derivatives exert anticancer effects by intercalating with DNA. However, the anticancer activity of quinacrine is not closely associated with its DNA-binding ability (Ehsanian et al., 2011). Quinacrine exerts anticancer effects by suppressing PI3K/AKT/mTOR and NF-κB survival pathways (Wang et al., 2005; Gurova et al., 2005; Guo et al., 2009; Ehsanian et al., 2011; Gallant et al., 2011). Moreover, quinacrine functions as a chemosensitizer to enhance chemotherapeutic drug-induced apoptosis of cancer cells (Friedman et al., 2007; Jani et al., 2010; Wang et al., 2010; Gallant et al., 2011; Wang et al., 2011; Wu et al., 2012; Khurana et al., 2015). Interestingly, some studies indicate that quinacrine-induced chemosensitization is primarily attributed to altered expression of BCL2 family proteins (Jani et al., 2010; Wang et al., 2010, 2011; Gallant et al., 2011). Other studies indicate that the anticancer activity of quinacrine is associated with the downregulated expression of antiapoptotic proteins, including BCL2, BCL2L1 (also known as Bcl-xL), and MCL1, or upregulated expression of proapoptotic proteins such as BAX (Wang et al., 2010; Preet et al., 2012; Das et al.,

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http://dx.doi.org/10.1016/j.taap.2017.08.019
Received 6 June 2017; Received in revised form 21 August 2017; Accepted 25 August 2017
Available online 01 September 2017
0041-008X/

2017). Nevertheless, the molecular mechanism responsible for quinacrine-induced change in the expression of BCL2 family proteins is not well addressed in these studies. Because a previous study suggested the repositioning potential of quinacrine for treating acute myeloid leukemia (AML) (Eriksson et al., 2015), we investigated the effect of quinacrine on the expression of BCL2 family proteins in AML cell line U937 in the present study. Our data showed that quinacrine-induced apoptosis of U937 cells was mediated by mitochondrial alterations triggered by FOXP3/miR-183/β-TrCP/SP1 axis-mediated BAX upregulation. Understanding the signaling pathway associated with quinacrine-induced apoptosis of AML cells might afford the benefit of searching effective strategies in improving AML treatment and overcoming therapy resistance.

2. Materials and methods

Quinacrine, *N*-acetylcysteine (NAC), digitonin, SB202190 (p38 MAPK inhibitor), mithramycin A (SP1-specific chemical inhibitor), diphenylene iodonium, *tert*-butyl hydroperoxide, MTT, cycloheximide, actinomycin D, horseradish peroxidase-conjugated secondary antibodies and anti-β-actin antibody were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), and dichlorodihydrofluorescein diacetate (H_2DCFDA), annexin V-FITC kit and rhodamine-123 were purchased from Molecular Probes (Carlsbad, CA). Anti-MCL1, anti-SP1 (H-255), anti-β-TrCP (H-85) and anti-FOXP3 (H-190) antibodies were obtained from Santa Cruz (Santa Cruz, CA). Anti-caspase-3 antibody, Z-DEVD-FMK (caspase-3 inhibitor), and Z-VAD-FMK (pan-caspase inhibitor) were obtained from Calbiochem (San Diego, CA). Anti-cytochrome *c* and anti-BCL2L1 antibodies were obtained from BD Biosciences (San Jose, CA), anti-caspase-9, anti-PARP, anti-BAX, anti-BCL2, anti-BAK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-ERK, anti-phospho-ERK, anti-JNK and anti-phospho-JNK antibodies were the products of Cell Signaling (Danvers, MA). Cell culture supplies were purchased from GIBCO/Life Technologies (Carlsbad, CA). Unless otherwise specified, all other reagents were of analytical grade.

2.1. Cell culture and cell viability assay

Human acute myeloid leukemia U937 cells were obtained from ATCC (Manassas, VA, USA). Human acute myeloid leukemia HL-60 cells were obtained from BCRC (Hsinchu, Taiwan). U937 cells and HL-60 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM glutamine and penicillin (100 units/ml)/streptomycin (100 μg/ml) in an incubator humidified with 95% air and 5% CO_2. Exponentially growing cells (2×10^4) were plated in 96-well plates and treated with a series of concentrations of quinacrine in serum-free medium without phenol red after 24 h of growth. For pharmacological experiments, culture cells were pre-treated with 10 μM SB202190 (in DMSO), 2 mM NAC (in DMSO), 10 nM mithramycin A (in DMSO) or vehicle control (an equal volume of DMSO) for 1 h before quinacrine was added. At suitable time intervals, MTT solution was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h. Plates were then centrifuged at 400 × *g* for 10 min, and supernatants were removed from the wells. Formazan crystals resulting from MTT reduction were dissolved by addition of 100 μl DMSO per well. The absorbance was detected at 595 nm using a plate reader.

2.2. Detection of ROS generation and mitochondrial membrane potential

Quinacrine-treated cells were incubated with 10 μM H_2DCFDA for 20 min at room temperature. ROS was measured using Beckman Coulter Paradigm™ Detection Platform with excitation at 485 nm and emission at 530 nm. Alternatively, quinacrine-treated cells were incubated with 20 nM rhodamine-123 for 20 min prior to harvesting, and then washed with PBS. Rhodamine-123 intensity was determined by flow cytometry with excitation at 511 nm and emission at 534 nm. Cells with reduced fluorescence (less rhodamine-123) were counted as having lost their mitochondrial membrane potential.

2.3. Separation of cytosolic and mitochondrial fractions

Following induction of apoptosis, cytosolic and pellet (mitochondrial) fractions were generated using a digitonin-based subcellular fractionation technique according to the method described in Chen et al. (2010). Cytochrome *c* and BAX were detected by western blot analysis.

2.4. Protein turnover assay

Cells were incubated with 5 μM quinacrine for 24 h, and then treated with 10 μM cycloheximide for indicated time periods. SP1 protein expression was detected by western blot analyses.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from untreated control cells or quinacrine-treated cells using the RNeasy minikit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed with 2 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's recommendations. PCR amplification was performed using GoTaq Flexi DNA polymerase (Promega) followed by 30 cycles at 94 °C for 60 s, 55 °C for 60 s, and 72 °C for 60 s. After a final extension at 72 °C for 5 min, PCR products were resolved on 2% agarose gels and visualized by ethidium bromide transillumination under UV light. Primer sequences were as follows:

BAX-forward: 5′-TGCTTCAGGGTTTCATCCAGG-3′
BAX-reverse: 5′-TGGCAAAGTAGAAAAGGGCGA-3′
SP1-forward: 5′-ATGGGGGCAATGGTAATGGTGG-3′
SP1-reverse: 5′-TCAGAACTTGCTGGTTCTGTAAG-3′
β-TrCP-forward: 5′-CACTTAGACAGACATACAACA-3′
β-TrCP-reverse: 5′-TCTGCAACATAGGTTTAAGAT-3′. Each reverse-transcribed mRNA product was internally controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR using primers 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TGTGGTCATGAGT CCTTCCA-3′ (reverse). The BAX, SP1 and β-TrCP reverse transcriptase-PCR products were subsequently confirmed by direct sequencing.

2.6. Real-time RT-PCR

Quantitative PCR was performed using iQ5 System (Bio-Rad, Hercules, CA). Reactions were performed using GoTaq qPCR Master mix (Promega, Madison, WI). The PCR arbitrary units of each gene were defined as the mRNA levels normalized to the GAPDH expression in each sample. Specificity was verified by melting curve analysis and agarose gel electrophoresis. Primer sequences used are listed in following: BAX, 5′-GGGGACGAACTGGACAGTAA-3′ (forward), 5′-CAGTT GAAGTTGCCGTCAGA-3′ (reverse); β-TrCP, 5′-GTTCAGTCCTCTGTCT CCAGTATGATG-3′ (forward), 5′-CAAGTGCAGAACTGCTTCACAATGGT G-3′ (reverse); FOXP3, 5′-GAAACAGCACATTCCCAGAGTTC-3′ (forward), 5′-ATGGCCCAGCGGATGAG-3′ (reverse); GAPDH, 5′-GAAATC CCATCACCATCTTCCAGG-3′ (forward), 5′-GAGCCCCAGCCTTCTCCAT G-3′ (reverse). Stem-loop reverse transcription and real-time PCR for detecting miR-183 was performed essentially according to the method described in Liu and Chang (2012). To examine β-TrCP mRNA stability, actinomycin D (10 μg/ml) was added directly to cell cultures that were already treated with quinacrine without removal of the original stimulant. Cells were collected at 1, 2 and 4 h after the addition of actinomycin D.