Leukemia Research 59 (2017) 124–135

Contents lists available at ScienceDirect

Leukemia Research

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

Research paper

Antileukemic activity and cellular effects of the antimalarial agent artesunate in acute myeloid leukemia

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ARTICLE INFO

Keywords:
Artimisinin
Artesunate
Acute myeloid leukemia
BCL-2
Reactive oxygen species
Iron
Chemotherapy
ABT-199
Venetoclax

ABSTRACT

The artimisinins are a class of antimalarial compounds whose antiparasitic activity is mediated by induction of reactive oxygen species (ROS). Herein, we report that among the artimisinins, artesunate (ARTS), an orally bioavailable compound has the most potent antileukemic activity in AML models and primary patients' blasts. ARTS was most cytotoxic to the *FLT3-ITD*+ AML MV4-11 and MOLM-13 cells (IC_{50} values of 1.1 and 0.82 μM respectively), inhibited colony formation in primary AML and MDS cells and augmented cytotoxicity of chemotherapeutics. ARTS lowered cellular BCL-2 level via ROS induction and increased the cytotoxicity of the BCL-2 inhibitor venetoclax (ABT-199). ARTS treatment led to cellular and mitochondrial ROS accumulation, double stranded DNA damage, loss of mitochondrial membrane potential and induction of the intrinsic mitochondrial apoptotic cascade in AML cell lines. The antileukemic activity of ARTS was further confirmed in MV4-11 and *FLT3-ITD*+ primary AML cell xenografts as well as *MLL-AF9* syngeneic murine AML model where ARTS treatment resulted in significant survival prolongation of treated mice compared to control. Our results demonstrate the potent preclinical antileukemic activity of ARTS as well as its potential for a rapid transition to a clinical trial either alone or in combination with conventional chemotherapy or BCL-2 inhibitor, for treatment of AML.

1. Introduction

The artemisinins are a family of antimalarial compounds derived from the sweet wormwood *Artmisia Annua*. Among the artemisinins, artesunate (ARTS) appears to be the most active agent with regard to the antimalarial activity and has optimal water solubility and oral bioavailability [1,2]. The antimalarial activity of artemisinins has been attributed to the generation of reactive oxygen species (ROS) occurring via cleavage of the endoperoxide bond in their structure [3]. Given a relatively high iron content of the cancer cells [4], the iron-catalyzed lysosomal ROS generation appears to be one of the main pro-apoptotic mechanisms mediating the artemisinins' cytotoxicity [5]. Although artemisinins have shown strong cytotoxic activity against a variety of cancer cell lines in vitro, their mechanisms of action remain to be fully dissected [5–9].

Although up to 80% of younger patients with acute myeloid leukemia (AML) achieve complete remission with conventional chemotherapy, their 5-year survival is only around 40% [10]. Outcome is particularly poor for older patients as well as those with adverse cytogenetics and molecular abnormalities like *FLT3* internal tandem duplication (*FLT3-ITD*) [10]. Treatment refractoriness and disease relapse are thought to be due to persistence of quiescent leukemia stem cells (LSC) which remain resistant to conventional chemotherapy [11].

Such functionally defined LSCs have been shown to have low levels of ROS as well as overexpression of BCL-2 [12]. Maintenance of low ROS levels appears to be critical to survival and persistence of LSC. Therefore, inducing ROS generation has the potential to selectively target LSC given the sensitivity of the latter to ROS levels, thereby providing the rationale for testing artimisinins in AML. We therefore examined the antineoplastic activity as well as mechanism of activity of artimisinins against AML cell lines and primary cells both in vitro and in animal models. In this paper, we demonstrate that among the artimisinins tested, the compound ARTS has the most potent antileukemic activity that is synergistic with chemotherapeutic agents. We further show that ARTS treatment also led to decrease in BCL-2 expression and strong synergy with the selective BCL-2 inhibitor venetoclax (ABT-199).

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http://dx.doi.org/10.1016/j.leukres.2017.05.007
Received 8 November 2016; Received in revised form 4 May 2017; Accepted 8 May 2017
Available online 10 May 2017

2. Materials and methods

2.1. Patient samples

Bone marrow (BM) samples from health donors, myelodysplastic syndrome (MDS) patients and AML patients were obtained under a specimen banking protocol approved by the Institutional Review Board of City of Hope Medical Center, in accordance with assurances filed with and approved by the Department of Health and Human Services, and meeting all requirements of the Declaration of Helsinki. Mononuclear cells were isolated using Ficoll (Stem Cell Technologies Inc., Vancouver, BC) density gradient separation as previously reported. CD34+ cells were isolated with 95% purity using immunomagnetic column separation per manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

2.2. Reagents

Artemisinin (ARTM), Artesunate (ARTS), dihydroartimisinin (DHA), Deferoxamine, daunorubicin and Cytosine arabinoside were purchased from Sigma (St. Louis, MO). ABT-199 (venetoclax) was purchased from Selleckchem (Houston, TX). Deferasirox was provided by Novartis (Basel, Switzerland). Growth factors for colony forming assay including erythropoietin (EPO), stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and interleukin-3 (IL-3) were obtained from Peprotech (Rocky Hill, NJ). Carboxyfluorescein succinimidyl ester (CFSE), MitoProbe JC-1 assay kit, Carboxy-H2DCFDA and MitoSox Red used for cellular ROS and mitochondrial ROS detection respectively were obtained from Invitrogen (Carlsbad, CA). Antibodies used for flow cytometry were obtained from BioLegend (San Diego, CA).

2.3. Cell culture

CD34+ cells from healthy donors or AML patients were cultured in StemSpan serum-free medium (Stem Cell Technologies, Vancouver, BC) supplemented with low concentrations of growth factors (granulocyte-macrophage colony stimulating factor (GM-CSF)200 pg/mL, granulocyte colony-stimulating factor(G-CSF) 1 ng/mL, stem cell factor (SCF) 200 pg/mL, leukemia inhibitory factor (LIF)50 pg/mL, macrophage inflammatory protein-1α (MIP-1α) 200 pg/mL, and interleukin-6 (IL-6) 1 ng/mL) at 37 °C with 5% CO_2 and high humidity. KG1a, OCI-AML3, MOLM-13 and MV4-11 cells were cultured in IMDM supplemented with 10% FBS and 1% penicillin/streptomycin. CD34+ cells from MDS patients were cultured in IMDM with 30% FBS and GFs [EPO (3 u/mL); SCF (5 ng/mL); GM-CSF (20 ng/mL); G-CSF (20 ng/mL) and IL-3 (5 ng/mL)] at 37 °C in 5% CO_2.

2.4. Proliferation and Apoptosis assays

Cell proliferation was measured by CellTiter-Glo® Luminescent Cell Viability Assay as per manufacturer's instructions (Promega, Madison, WI). Briefly, human AML cell lines were seeded in 96 well plates at density of 2×10^5 cells/mL. Cells were cultured under different treatment conditions for 48 h. The Cell Titer-Glo substrate and buffer reagents were added into each well and mixed to obtain cell lysis. Plates were read on a Microplate reader (Beckman Coulter DTX880, Brea,CA). Data from three replicates were expressed as percentage of treated cells with respect to untreated controls.

To measure apoptosis, cells treated with different drugs for 48–72 h were labeled with Annexin V (BioLegend) and Propidium Iodide (Invitrogen). Fluorescence was measured by flow cytometry in order to determine percentage of apoptotic cells. For proliferation assay, 2×10^5 cells/mL cells were labeled with 5 μM of CFSE and cultured for 24 h under different treatment conditions and analyzed using LSRII flow cytometer (BD Biosciences, San Jose, CA).

2.5. Measurement of ROS

KG1a, MOLM-13 and MV4-11 cells were treated with drugs of interest in IMDM containing 10% Fetal Bovine Serum for 24 h. Following collection, cells were incubated with 5 μM Carboxy-H2DCFDA and 3 μM MitoSox Red at 37 °C for 30 min to stain for cellular and mitochondrial ROS, respectively. Cells were then labeled with Annexin V and analyzed by flow cytometry. Cellular ROS and mitochondrial ROS levels in CD34+ cells were analyzed with BD FlowJo (Ashland,OR) software version 9.6.1.

2.6. Flow cytometry for BCL-2 expression

MOLM-13 and MV4-11 cells were plated at 2×10^5 cells/mL density and treated with drugs of interest in IMDM containing 10% FBS for 24 h.Cell were washed with PBS and resuspended in 2% paraformaldehyde for 1 h at −4 °C followed by cell washing and permeablized with 70% ethanol at −20 °C overnight. Intracellular BCL2 (BioLegend) staining was performed and analyzed using LSRII flow cytometer.

2.7. RT-PCR for BCL-2

The RNA expression levels of the BCL2 were analyzed by SYBR green real time PCR. The RNA isolated from control and treated cells were reverse transcribed to cDNA using Superscript ™ II Reverse Transcriptase (Invitrogen). The primers for real time PCR amplification and conditions have been previously described [13]. The primer sequences for human *BCL2* were: forward 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′ and reverse 5′-GGTGCCGGTTCAGGTACTCAGTCA-3′. The SYBR green real time Quantitect PCR kit was purchased from Qiagen (Valencia, CA, USA). GAPDH was used as control in separate reactions.

2.8. Western blot

1×10^6 cells were cultured in IMDM containing 10% FBS with or without relevant drugs for 24 h. The cells were washed with PBS and lysed by resuspending in 100 μl RIPA buffer (ThermoFisher Scientific, Waltham, MA) supplemented with protease and phosphatase inhibitor cocktail. Proteins were resolved on 4–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. Membranes were sequentially probed with γ-H2AX, cleaved caspase 3, uncleaved Caspase 3, c-Myc, c-Src (Tyr416), pJNK (Tyr183/Tyr185), BCL-2, β-Actin and GAPDH antibodies and horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc, Danvers, MA). Detection was performed using the SuperSignal West Dura extended duration substrate kit (Thermo Fisher Scientific).

2.9. Mitochondrial membrane potential (MMP) measurement

The effect of drug treatment on MMP was analyzed using MitoProbe JC-1 Assay kit (Molecular Probes, Eugene, OR). The collapse in the electrochemical gradient across the mitochondrial membrane was measured using a fluorescent cationic dye 5,5′,6,6,-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolo-carbocyanin iodide, also known as JC-1. This dye exhibits potential dependent accumulation in the mitochondrial matrix. 1×10^6 cells were incubated with 1 μM JC-1 for 15 min at room temperature and analyzed on LSRII flow cytometer.

2.10. Colony forming cell (CFC) assays

Human CD34 + cells from health donors or AML or MDS patients were treated with drugs of interest for 48 h at 5 μM. Cells were washed and transferred into colony formation culture containing IMDM, 30% FBS, GFs [EPO (3 u/mL); SCF (5 ng/mL); GM-CSF (20 ng/mL); G-CSF