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Antiproliferative activity of the isoindigo 5′-Br in HL-60 cells is mediated by apoptosis, dysregulation of mitochondrial functions and arresting cell cycle at G0/G1 phase

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ABSTRACT

Our new compound, 5′-Br [(*E*)-1-(5′-bromo-2′-oxoindolin-3′-ylidene)-6-ethyl-2,3,6,9-tetrahydro-2,9-dioxo-1*H*-pyrrolo[3,2-*f*]quinoline-8-carboxylic acid], had shown strong, selective antiproliferative activity against different cancer cell lines. Here, we aim to comprehensively characterize the mechanisms associated with its cytotoxicity in the human promyelocytic leukemia HL-60 cells. We focused at studying the involvement of apoptotic pathway and cell cycle effects. 5′-Br significantly inhibited proliferation by inducing caspase-dependent apoptosis. Involvement of caspase independent mechanism is also possible due to observed inability of z-VAD-FMK to rescue apoptotic cells. 5′-Br was found to trigger intrinsic apoptotic pathway as indicated by depolarization of the mitochondrial inner membrane, decreased level of cellular ATP, modulated expression and phosphorylation of Bcl-2 leading to loss of its association with Bax, and increased release of cytochrome *c*. 5′-Br treated cells were found arrested at G0/G1 phase with modulation in protein levels of cyclins, dependent kinases and their inhibitors. Expression and enzymatic activity of CDK2 and CDK4 was found inhibited. Retinoblastoma protein (Rb) phosphorylation was also inhibited whereas p21 protein levels were increased. These results suggest that the antiproliferative mechanisms of action of 5′-Br could involve apoptotic pathways, dysregulation of mitochondrial functions and disruption of cell cycle checkpoint.

Introduction

Acute myeloid leukemia (AML) is hematopoietic malignancy characterized by uncontrolled proliferation and accumulation of myeloblasts in the bone marrow, blood, and other organs [1]. This complex disease involves multiple genetic and molecular alterations causing cellular transformation, deregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis [1]. AML patients typically respond to initial treatment with anthracycline and cytarabine (1-β-*D*-Arabinofuranosylcytosine, Ara-C)-based chemotherapy, however, the response is poor or short-lived and often associated with relapse and resistance. The inadequacy of conventionally available therapies in AML has fueled the quest for finding new molecules that can be used in chemotherapy with better selectivity and efficacy.

The bis-indole containing alkaloids indigo, indirubin and isoindigo have been employed in treating myeloid leukemia. The clinical application of these drugs in treating myeloid leukemia is hampered by their potential side effects, poor water solubility, bone marrow suppression and drug-resistance in prolonged treatments [2]. In addition, the limited water solubility of these isoindigos hinders the

Abbreviations: 5′-Br, [(*E*)-1-(5′-bromo-2′-oxoindolin-3′-ylidene)-6-ethyl-2,3,6,9-tetrahydro-2,9-dioxo-1*H*-pyrrolo[3,2-*f*]quinoline-8-carboxylic acid]; ΔΨm, difference in mitochondrial transmembrane potential; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra-large; Bax, Bcl-2-associated X protein; Bak, Bcl-2 homologous antagonist killer; PARP, poly (ADP-ribose) polymerase; CDK, cyclin dependent kinase; CKI, cyclin kinase inhibitor; Rb, retinoblastoma protein; z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; Ac-LEHD-pNA, *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide; Ac-IETD-pNA, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide; Ac-DEVD-*p*NA, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; Ac-VDVAD, *N*-acetyl-Val-Asp-Val-Ala-Asp-p-nitroanilide; Ac-VEID-pNA, *N*-acetyl-Val-Glu-Ile-Asp-p-nitroanilide; pNA, p-nitroanilide; DMSO, dimethyl sulfoxide.

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detailed characterization of their antiproliferative signaling pathways. Thus, extensive efforts have been employed to synthesize novel indirubin and isoindigo derivatives with increased bioavailability and bioactivity. In spite of the extensive investigations for the mode of action of isoindigos in myeloid leukemia and other cancers, there are gaps in our understanding of their cellular targets and mechanism of action.

The antileukemic effects of these compounds are mediated through multi-signaling pathways including inhibition of DNA biosynthesis and assembly of microtubules, arresting cells at G1 phase of the cell cycle, interaction with the aryl hydrocarbon receptor (AhR) triggering cell differentiation and maturation leading to complete inhibition of cell growth, and down-regulation of *c-myb* gene expression [2–6]. Several of these compounds have been shown to inhibit cyclin-dependent kinases (CDKs) and glycogen-synthase kinase (GSK-3β), and induce apoptosis with varying degrees of potency [2–5,7–12]. Recently, a novel 7-azaisoindigo derivative [namely N(1)-(*n*-butyl)-7-azaisoindigo] has been shown to trigger apoptosis through reactive oxygen species (ROS), deregulation of the mitochondrial functions and activation of caspases [13].

Successful chemotherapeutics are able to trigger death of cancer cells mainly through intrinsic/extrinsic apoptotic pathways [14]. Apart from extrinsic apoptotic pathway that is dependent on a receptor-mediated activation of caspase-8, these drugs may stimulate the intrinsic (mitochondria-dependent) pathway which is evoked by the release of mitochondrial apoptogenic factors such as cytochrome c to the cytosol allowing activation of caspase-9 [15].

Tumor cells are characterized by having a deregulated cell cycle, which contributes to their uncontrolled proliferation (reviewed in Refs. 16 and 17). The molecular mechanisms of cell cycle arrest by many anticancer agents involve modulation of several cell cycle regulatory proteins. Although human cells highly express the D type cyclins (D1, D2 and D3) in early and late G1 phase [18–20], proper execution of later phases (S and G2-M) require the subsequent activation of other CDK-cyclin complexes: CDK2/cyclin E, CDK2/cyclin A, CDK1/cyclin A and CDK1/cyclin B [16,21]. CDK activity can be regulated by cell cycle inhibitory proteins (CKI), which bind to CDK alone or to the CDK/cyclin complex [16,17].

In line with the efforts aiming to synthesize more soluble and effective anticancer isoindigo derivatives, we have identified a compound [(*E*)-1-(5′-bromo-2′-oxoindolin-3′-ylidene)-6-ethyl-2,3,6,9-tetrahydro-2,9-dioxo-1*H*-pyrrolo[3,2-*f*]quinoline-8-carboxylic acid] (known here as 5′-Br, Fig. 1A) with increased solubility (up to 25 mM) in 25% aqueous DMSO (dimethyl sulphoxide) [22]. 5′-Br effectively inhibited the proliferation of several human hematological and solid tumor cell lines at low doses in a selective manner [22].

The acute promyelocytic leukemia cell line HL-60 is a subtype of AML, which accounts for approximately 10% of all AML cases [1]. Therefore, it is an ideal cell line to investigate novel potential chemotherapeutic agents for this subtype of AML. In this report, we studied the effect of 5′-Br in triggering apoptosis and cell cycle effects in HL-60 cells. Evidence suggests that 5′-Br induces mitochondrial apoptosis in HL-60 cells. 5′-Br triggers depolarization of mitochondria in HL-60 cells, decreases the expression of the anti-apoptotic protein Bcl-2 and promotes its hyperphosphorylation leading to loss of functional association with the proapoptotic factor Bax. The antiproliferative effect is also shown to be through G0/G1 phase arrest, which is mediated by modulating the expression and functions of the G1 phase-related proteins. 5′-Br inhibited expression of cyclin D1 and D2, and reduced Rb phosphorylation. It also significantly upregulated expression of p21 and inhibited expression levels as well as activities of CDK2 and CDK4. These results suggest that the cytotoxic and antiproliferative effects of 5′-Br are mediated by apoptosis, dysregulation of mitochondria functions and cell cycle checkpoint regulation.

Materials and methods

Reagents

The pyridone-annelated isoindigo 5′-Br compound [(*E*)-1-(5′-bromo-2′-oxoindolin-3′-ylidene)-6-ethyl-2,3,6,9-tetrahydro-2,9-dioxo-1*H*-pyrrolo[3,2-*f*]quinoline-8-carboxylic acid] was previously synthesized and chemically characterized in details in our recent publication [22]. Other reagents and experimental protocols used in this study are provided in Supplementary Methods.

Cell viability assay

The quantitative determination of viable cells after various treatments was performed by using the dual DNA intercalating fluorescent dyes kit from EMD Millipore Bioscience (Muse™ Cell Count & Viability Assay). Briefly, 3×10^5 of HL-60, in a 1.0 mL of RPMI-1640 medium, was seeded in each well of a 24 well-plate. After 24 hr, cells were exposed to increasing concentrations of 5′-Br (0.0–16.0 μM) for additional 24 hr. Alternatively, HL-60 cells were incubated with 8.0 μM 5′-Br for different time-points (0–72 hr) before analysis. Cytarabine (0.01–0.03 μM) was added to HL-60 cells as positive controls.

Analysis of apoptosis by flow cytometry

The percentage of cells undergoing apoptosis after treatment with 5′-Br was determined using the Muse™ Annexin-V and Dead Cell Assay kit (EMD Millipore Bioscience) and Muse™ cell analyzer according to the manufacturer's protocol. The kit utilizes a fluorescent dye (FITC) conjugated to Annexin-V to detect phosphatidylserine (PS) on the external membrane of apoptotic cells and 7-AAD (7-amino-actinomycin D) as a dead cells marker. When z-VAD-FMK was used, cells were incubated with the desired concentration of this caspase inhibitor for 4 hr before addition of 5′-Br. HL-60 cells were also treated with 0.02 μM cytarabine as positive control.

Analysis of changes in mitochondrial transmembrane potential

Measurement of changes in mitochondria membrane potential ($\Delta\Psi m$) was performed with the Muse™ MitoPotential Assay kit (EMD Millipore Bioscience). This flow cytometry-based assay differentiates 4 populations of cells: live cells with depolarized mitochondrial membrane; MitoPotential$^-$/7-AAD$^-$, live cells with intact mitochondrial membrane; MitoPotential$^+$/7-AAD$^-$, dead cells with depolarized mitochondrial membrane; MitoPotential$^+$/7-AAD$^+$ and dead cells with intact mitochondrial membrane; MitoPotential$^-$/7-AAD$^+$. After treatment with 5′-Br, HL-60 cells were incubated with the fluorescent dyes and the percentage of depolarized cells (depolarized alive + depolarized dead) were determined by Muse™ Cell Analyzer.

Quantitation of ATP levels in HL-60 treated Cells

ATP contents were determined using the ATP Colorimetric/Fluorometric Assay Kit (BioVision, Inc.) according to the manufacturer's instructions. After treatment with 8.0 μM 5′-Br for increasing time points, HL-60 (1×10^6) cells were lysed in a lysis buffer, deproteinized and 10 μL of the supernatant was added to a 50 μL of the assay reaction mixture. Absorbance was measured at OD 570 nm in a Spectra Max™ micro-plate reader. All samples were measured in triplicates and the values were expressed relative to the untreated control.

Release of cytochrome c *from mitochondria to the cytosol of HL-60 cells*

Mitochondrial and cytosolic fractions from HL-60 cells treated with 5′-Br were prepared by differential centrifugation at 4 °C as described in our recent publication [23]. The release of cytochrome *c* from mitochondria into the cytoplasm of 5′-Br-treated HL-60 cells was detected by Western blotting of mitochondrial (30 μg) or cytosolic (50 μg) fractions as previously described [23].

Immunoprecipitation and western blot analyses

For immunoprecipitation, 25×10^6 HL-60 were treated with or without 5′-Br for 24 hr. Cells were lysed in ice-cold lysis buffer containing 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitors [2 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μg/mL pepstatin A and 1 mM PMSF]. After centrifugation at 14,000 *g* for 20 min at 4 °C, lysates (300 μg) were pre-cleared by incubating (2 hr at 4 °C) with 25 μL protein A/G plus-agarose beads (Santa Cruz Biotechnology). Pre-cleared lysates were incubated overnight with 5 μg of specific anti-Bax or anti-Bcl-2 antibodies. Immunocomplexes were captured with 25 μL protein A/G plus-agarose, and the presence of Bcl-2 in these complexes was determined by Western blot analysis.

Samples preparation and Western blot analysis were performed as previously described [23]. Primary antibodies against caspase-2 [(C2) Mouse mAb 2224], caspase-8 [(1C12) Mouse mAb 9746], caspase-9 [(C9) Mouse mAb 9508], cleaved caspase-3 [(Asp175) (5A1E) Rabbit mAb 9664], cleaved caspase-6 [(Asp162) Antibody 9761], phospho-Bcl-2 [antibody 2875] and PARP [antibody 9542] were Obtained