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Characterization of the enhanced apoptotic response to azidothymidine by pharmacological inhibition of NF-kB

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ABSTRACT

Aims: The present study addresses the issue of enhanced apoptotic response to AZT following co-treatment with an NF-kB inhibitor.
Main methods: To investigate this issue, different cell lines were assayed for susceptibility to AZT-mediated apoptosis without or with the addition of the NF-kB inhibitor Bay-11-7085. For further investigation, U937 cells were selected as good-responder cells to the combination treatment with 32 or 128 μM AZT, and 1 μM Bay-11-7085. Inhibition of NF-kB activation by Bay-11-7085 in cells treated with AZT was assayed through Western blot analysis of p65 expression and by EMSA. Involvement of the mitochondrial pathway of apoptosis in mechanisms underlying the improved effect of AZT following Bay-11-7085 co-treatment, was evaluated by assaying the cytochrome *c* release and the mitochondrial membrane potential (MMP) status using the JC-1 dye. Moreover, the transcriptional activity of both anti- and pro-apoptotic genes in U937 cells after combination treatment was quantitatively evaluated through real-time PCR.
Key findings: We found that the combined treatment induced high levels of cytochrome *c* release and of MMP collapse in association with evident changes in the expression of both anti- and pro-apoptotic genes of the Bcl-2 family. Overexpression of Bcl-2 significantly suppressed the sensitization of U937 cells to an enhanced apoptotic response to AZT following co-treatment with the NF-kB inhibitor.
Significance: The new findings suggest that a combination regimen based on AZT plus an NF-kB inhibitor could represent a new chemotherapeutic tool for retrovirus-related pathologies.

Introduction

The nucleoside inhibitor of reverse transcriptase (NRTI) 3′-azido-3′-deoxythymidine (AZT), or zidovudine, is still a backbone of the standard antiretroviral therapy (ART) in a large part of patients affected by the acquired immunodeficiency syndrome. A number of studies have shown that AZT, although showing limited toxicity at pharmacological and supra-pharmacological concentrations, is endowed with pleiotropic effects that are, in part, related to its side effects. These include induction of metabolic stress, impairment of adipocyte function through inhibition of autophagy [38], mitochondrial dysfunction through depletion of mtDNA [9,36], inhibition of the glutathione-mediated antioxidant pathway and induction of reactive oxygen species in macrophages [1,41]. The ultimate result of all these events is cellular genetic instability and DNA damage [11,23]. AZT affects also the growth of cancer cells by inducing telomere shortening [16,25], through inhibition of telomerase reverse transcriptase activity [15,20] and causing cell cycle arrest. The global result is a remarkable antiproliferative effect, leading in some instances to senescence [10,19]. Moreover, only when utilized in combination with other agents, AZT was shown to exert a higher cytotoxic activity on tumor cell lines of different origins [2,3,8,30]. Indeed, all these data show that AZT may potentially cause major DNA damage, although the capability of the drug to act as a cell death inducer by itself is limited. In agreement with this, we found that AZT can induce a sort of pre-commitment towards apoptosis, but only low levels of apoptotic cell death [28] and that low levels of apoptosis in cells treated with AZT might depend on enhanced levels of the NF-kB activation. In fact, AZT exerted a potent pro-apoptotic effect in cells in which NF-kB activation was hindered by stable transfection with a dominant negative IkBα[29].

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The NF-kB system plays a complex role as first line of defense in infection, inflammation, DNA damage and tumorigenesis. NF-kB activity also plays an important role in response to chemotherapy, since it modulates chemoresistance to programmed cell death [13], through induction of cell proliferation by upregulating expression of cyclins and proto-oncogenes and by inhibiting p53, a well-known tumor-suppressor protein [21]. Moreover, resistance to apoptosis mainly occurs through regulation of anti-apoptotic genes of the Bcl-2 family, [37]. As a consequence, targeting NF-kB has been considered a novel strategy to improve cancer chemotherapy [12,17,18,24,42].

Based on our above reported observations we wanted to explore the potential therapeutic application of sensitization to AZT-induced apoptosis by NF-kB inhibition. To this purpose in the present study we addressed the issue of the mechanisms underlying the enhanced apoptotic response to AZT following co-treatment with the pharmacological NF-kB inhibitor Bay-11-7085. We demonstrated that the combined treatment promoted the release of cytochrome *c*, collapse of mitochondria potential and changes in both anti- and pro-apoptotic gene expression. Overexpression of Bcl-2 significantly suppressed the susceptibility of monocytic cells to the enhancement of apoptotic response to AZT by combined treatment with an NF-kB inhibitor.

Materials and methods

Cell culture

Human acute lymphoblastic T MOLT-3 cells, Jurkat cells and human monocytic U937 cells, originally derived from a patient with histiocytic lymphoma (Zooprofilactic Institute, Brescia, Italy), were passaged in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 50 U/ml streptomycin, 50 U/ml penicillin and 2 mM glutamine (all from Hyclone, Cramlington, England, UK), in a humidified incubator at 37 °C and 5% CO_2. U937 cells stably transfected with a vector bearing the murine *bcl-2* gene (U937-Bcl-2) or with the pMEP control vector (U937-pMEP) were previously described [5]. These cells were grown in the same conditions as other cells, but under hygromycin B (50 mg/ml) selection. Where appropriate, Bay-11-7085, up to 2.5 μM (Sigma-Aldrich, St. Louis, MO, USA), was added to the cells 1 h before treatment with AZT (Wellcome Research Labs, Beckenham, Kent, UK) up to 128 μM at different times. The concentrations of Bay-11-7085 and AZT utilized were based on the results of our previous studies and preliminary experiments showing that: a) AZT from 32 μM to 128 μM was an appropriate range for a dose–response effect on apoptosis, without any sign of necrotic cell death; and b) concentrations of Bay-11-7085 higher than 2.5 μM can exert direct pro-apoptotic effects. Following incubation under various experimental conditions, cells were harvested and washed three times by 5 min centrifugation at 1000 *g* with cold phosphate-buffered saline (PBS), before they were processed for successive experimental phases.

Apoptosis assay

Apoptosis was assessed by flow cytometry analysis of isolated nuclei after staining with propidium iodide, as previously described by some of us [27]. Briefly, cells for analysis were treated with a mixture consisting of 2% Triton X-100 (Sigma-Aldrich), 25 μg/ml propidium iodide (Sigma-Aldrich) and 0.05% sodium citrate (Sigma-Aldrich). Samples were mixed by gentle inversions for 30 min and then placed at 4 °C, until isolated nuclei were analyzed by fluorescence and by forward- and side-angle-scatter multiparameter analyses using a Becton Dickinson FACS analyzer. A minimum of 5000 events per sample were analyzed.

JC-1 assay

For studying mitochondrial membrane potential (MMP) in U937 cells subjected to different treatments the MitoProbe™ JC-1 Assay Kit for Flow Cytometry (Molecular Probes Europe BV, Leiden, Netherlands) was utilized, as previously described [31].

Quantitative detection of human cytochrome c

Release of cytochrome *c* was evaluated by means of a commercial ELISA (human cytochrome *c* ELISA; BioVendor GmbH, Heidelberg, Germany). Briefly, after different times in culture (from 30 min up to 48 h) with control vehicle or Bay-11-7085 (1 μM) and with or without AZT 128 μM, cells were processed according to the manufacturer's instructions for quantifying cytochrome *c* levels in cell extracts. Absorbance values in a 96-well microplate were detected by means of a Labsystem Multiskan Bichromatic Microplate Reader (Helsinki, Finland).

Western blot analysis

Aliquots of 10^7 cells subjected to different experimental conditions were suspended in buffers for cytoplasmic and nuclear protein extraction and processed for Western blot analysis as previously described [29]. The primary antibodies used were the rabbit monoclonal antibody phosphoNF-kB p65 Ser (536) (Cell Signaling Technology, Beverly, MA, USA) and the mouse monoclonal antibody human beta-actin (Novus Biologicals, Littleton, CO, USA). The secondary antibodies used were anti-mouse (Bio-Rad Laboratories, Richmond, CA, USA) and anti-rabbit IgG chain specific conjugated to peroxidase (Calbiochem, Merck Millipore, Darmstadt, Germany).

NF-kB-binding assays

Activation of NF-kB was assayed by detecting binding of nuclear extracts to NF-kB-specific DNA-probe by non-radioactive EMSA, as previously described [29]. Specificity of the binding was confirmed in all the experiments by adding an excess of kB-specific, unlabeled DNA-probe (200×) in one of the samples.

Real-time quantitative reverse transcription PCR

The transcriptional activity of the genes of interest was evaluated by real-time quantitative reverse transcription PCR (RQ-PCR), using a CFX-96 real-time instrument (Bio-Rad) and cDNA specific primers purchased from Primm (Milan, Italy). The list of genes assayed and the corresponding sequences of the primers utilized are reported in Table 1. For reverse-transcription, 0.25 μg of total RNA extracted from each experimental sample were processed for cDNA generation using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. RQ-PCR was performed and relative mRNA levels were calculated as previously described in detail [31]. As a housekeeping gene, glucuronidase beta (GUSB) was used. To compare the expression of each gene in the different treatment conditions, ΔC_t values for each gene after treatment were compared to vehicle control using the formula: $2^{-\Delta\Delta Ct} = 2^{(-\Delta Ct(\text{gene of interest}) - \text{value of } \Delta Ct \text{ from the vehicle expressed gene})}$. The obtained relative $2^{-\Delta\Delta Ct}$ values of the genes of interest were then compared to evaluate the treatment effect.

Statistical analysis

Each experiment was performed at least in triplicate. All data were presented as mean ± SD or SE and indicated in the text. Data analysis was performed using the SPSS statistical software system (version 17.0 for Windows, Chicago, IL). A comparison of the means of apoptosis levels, JC-1 expression, cytochrome *c* release, real time gene expression in response to AZT and Bay-11-7085 treatment was carried out using Bonferroni's post-hoc multiple comparison ANOVA test.