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Deglycosylated bleomycin induces apoptosis in lymphoma cell via c-jun NH_2-terminal kinase but not reactive oxygen species

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ABSTRACT

Bleomycin (BLM) has demonstrated potent activity in treating malignant lymphomas but its therapeutic efficacy is hampered by induction of lung fibrosis. This side effect is related to the ability of the drug to generate reactive oxygen species in lung cells. In the present study, we evaluated the consequences of deglycosylation of BLM in term of cytotoxic activity and generation of reactive oxygen species. When tested on U937 human lymphoma cells, both compounds generated a typical apoptotic phenotype. Cell death induction was associated with Bax oligomerization, dissipation of the mitochondrial membrane potential, release of cytochrome c, caspase activation, chromatin condensation and internucleosomal degradation. Whereas both reactive oxygen species and c-jun NH_2-terminal kinase (JNK) inhibitors prevented BLM-induced U937 cell death, only JNK inhibition prevented deglycosylated BLM-mediated cell death. Both compounds induced clustering of TRAIL receptors (DR4 and DR5) and Fas at the cell surface but neither a chimeric soluble DR5 receptor that inhibits TRAIL-induced cell death nor a dominant negative version of the adaptor molecule Fas-associated death domain prevented BLM-induced cytotoxicity. These observations indicate that deglycosylation of BLM does not impair the ability of the drug to trigger cell death through activation of the intrinsic pathway but prevents induction of reactive oxygen species. This observation suggests that deglycosylated BLM could exhibit less toxic side effects and could warrant its use in clinic.

1. Introduction

Bleomycins (BLM) are a family of glycopeptides isolated from *Streptomyces verticullis* in 1966 [1] which exhibit antibiotic properties (for review [2]). They are attractive therapeutic drugs hardly induce myelosuppression [3] or immunosuppression [4]. They are commonly included in chemotherapy regimens used to treat patients with Hodgkin's or non Hodgkin's malignant lymphoma [5,6], squamous-cell carcinoma [7] or germ-cell tumor [8]. Their therapeutic use is

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limited by a dose-dependent lung toxicity that eventually leads to fibrosis [9,10].

The clinically administrated form of BLM, Bleonoxane (Bristol-Meyers Squibb), is essentially composed of two molecules, BLM-A2 (~60%) and BLM-B2 (~40%), which differ in their positively charged tail. The anti-neoplastic properties of BLM were attributed to their ability to link metals including iron and to form a complex that reduces molecular oxygen to superoxide and hydroxyl radicals. This causes single- and double-stranded DNA breaks and ultimately leads to cell death [11,12], the extent of which depends on drug concentration and incubation [12,13]. At low doses, BLM arrests cells in the G2/M phase of the cell cycle, which possibly ends with mitotic catastrophe. Extensive DNA double stranded-breaks induced by BLM at higher doses can trigger apoptosis [14], as observed in alveolar epithelial cells [15,16] as well as in limited number of tumor cells [17,18].

Deglycosylation of BLM was proposed as a potential mean to reduce the toxicity of the molecule [19] but whether deletion of the carbohydrate moieties of BLM affected the ability of the compound to trigger apoptosis remained obscure.

A series of experimental evidences indicates that while most anti-tumor drugs activate the intrinsic death pathway [20–23], death receptor extrinsic pathway could contribute to the cytotoxic activity of a limited number of specific anticancer drugs [24–26]. The intrinsic pathway, also called mitochondria-dependent pathway, requires sentinel, such as BH3-only proteins of the Bcl-2 family that bind to and inhibit anti-apoptotic members of this family in order to activate multi-domain pro-apoptotic members such as Bax and Bak. The outer mitochondrial membrane (OMM) then becomes permeable to cytochrome *c* that, in the cytosol, enables the assembly of the apoptosome in which caspase-9 is activated, leading to subsequent activation of a caspase cascade and cell demise [27]. The death receptor-mediated pathway, also known as the extrinsic pathway, is activated upon engagement of death receptors such as Fas and TNF-related apoptosis inducing ligand (TRAIL) receptors DR4 and DR5 at the cell surface. Interaction of these receptors to their cognate ligands results in the recruitment of the adaptor molecule Fas-associated death domain protein (FADD) that, in turn, recruits the initiator caspase-8 within the Death inducing Signaling Complex (DISC) in which the protease is activated. Depending on the cell type, caspase-8 either directly activates downstream effector caspases such as caspase-3 or cleaves the sentinel BH3-only protein Bid to connect the extrinsic to the intrinsic pathway to death [28].

The present study was undertaken to determine the influence of deglycosylation on the ability of BLM to trigger cell death by apoptosis. We show that both parental drug and its derivative activate the intrinsic pathway through the activation of the JNK pathway. Death receptors, although redistributed at the cell surface upon drug exposure are, however, unlikely to be involved in the cytotoxic effect. Whereas BLM also induces the formation of ROS, the deglycosylated derivative does not, which might indicate a less toxic profile.

2. Materials and methods

2.1. Drugs and reagents

Lyophilized BLM (kindly provided by Nippon Kayaku (Tokyo, Japan)) was dissolved in sterile water and stored at −20 °C. Nonglycosylated form of BLM (D-BLM) was obtained by β-elimination under mild alkaline conditions, and by solvolysis with hydrogen fluoride as previously described [19]. The recombinant soluble FasL was collected from the supernatant of FasL-transfected Neuro2A cells (Dr. Fontana, Lausanne, Switzerland). A unique pool of sFasL supernatant was used throughout the study. The Ig TRAIL R2-Fc and the his-tagged TRAIL was produced and used as described previously [29]. The blocking CD95/Fas (ZB4) was purchased from Immunotech (Marseille, France), the broad spectrum caspase inhibitor Z-(Benzyloxycarbonyl)-Val-Ala-Asp(OMe)-CH2F (fluoromethylketone) (Z-VAD-fmk) from Bachem (Weil am Rhein, Germany). The caspase-3 inhibitor Z-(Benzyloxycarbonyl)-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (fluoromethylketone) (Z-DEVD-fmk) from Calbiochem (Meudon, France). The caspase-8 inhibitor Z-(Benzyloxycarbonyl)-lle-Glu(OMe)-Thr-Asp(OMe)-CH_2F(fluoromethylketone) (Z-IETD-fmk) and the caspase-9 inhibitor Z-(Benzyloxycarbonyl)-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F(fluoromethylketone) (Z-LEHD-fmk) from R&D Systems (Abinqton, England). The JNK1, 2, 3 inhibitor from Biomol (tebubio, PA), the antioxidant N-acetylcysteine (NAC), and all other chemicals and reagents from Sigma (Saint Quentin Fallavier, France) or other local sources.

2.2. Antibodies

For Western blotting, antibodies used include monoclonal antibodies against human caspase-8 (Immunotech), -9 (Upstate, Virginia, USA), FADD (BD Pharmingen, San Diego, CA), HSC 70 (Santa Cruz Biotechnology, Santa Cruz, CA). Polyclonal antibodies against human caspase-3 active form (Cell Signaling, Beverly, MA), horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies from Jackson Immunoresearch Laboratories (West Grove, USA). For immunofluorescence studies, we used antibodies against DR4/TRAIL-R1 (Immunex Corp, USA), DR5/TRAIL-R2 B-L27 (Diaclone, Besançon, France), Fas/CD95 (BD Pharmingen, San Diego, CA), Bax N20 (Santa Cruz, CA), cytochrome *c* (Pharmingen, San Diego, CA), 488-alexa goat anti-mouse and 568-alexa goat anti-rabbit antibodies from Molecular Probes (Cergy Pontoise, France).

2.3. Cell culture

The human lymphoma U937 cells were purchased from the American Type collection (Rockville, MD). Part of them were stably infected with a lentiviral construct containing a dominant negative mutant deleted of most the death effector domain of FADD (FADD-DN) or with the corresponding empty vector (EV) [30,31] or were stably transfected with a vector encoding Bcl-2 (U937/Bcl2). These cells were cultured in RPMI 1640-glutamax (Gibco BRL, Cergy-Pontoise, France) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker, Vervier,

Belgium) in an atmosphere of 95% air and 5% CO_2 at 37 °C.

2.4. Clonogenic assays

Cells were incubated for 2 h in the presence or absence of various concentrations of BLM and D-BLM. U937 cells were then plated at various densities in semisolid medium by the methylcellulose technique [32]. Cultures were incubated in a humidified 5% CO_2 incubator at 37 °C, and colonies were scored at 11 days.

2.5. Cytotoxicity analysis

Cells (5×10^5/mL) were plated in 96-well plates and incubated with various concentrations of BLM or D-BLM for various time points. Cell viability was measured by using the cell titer 96 aqueous non-radioactive cell proliferation assays, according to the manufacturer's instructions (Promega, Charbonnières, France).

2.6. Identification of apoptotic cells

Apoptosis was analyzed by staining the nuclear chromatin with Hoechst 33342 dye. Briefly, untreated and treated cells (5×10^5/mL) were collected, washed with PBS, stained with 1 μg/mL Hoechst 33342 dye for 15 min at 37 °C, mounted on glass slides, and observed under microscope. The percentage of apoptotic cells (chromatin condensation and nuclear fragmentation) was determined by counting 300 cells in each sample.

2.7. DNA fragmentation analysis by gel electrophoresis

Cells were collected, washed in PBS, and DNA was isolated using the Wizard SV genomic DNA purification system kit according to the manufacturer's instructions (Promega). The DNA samples were mixed with 5 μL of loading buffer (0.25% bromophenol blue, 40% glycerol) and loaded onto 1.8% agarose gel containing 5 μg/mL of ethidium bromide. Electrophoresis was performed at 10 V/cm for 2 h. DNA was visualized under UV light.

2.8. Caspase activity measurement

Cells were incubated in lysing buffer (150 mM NaCl, 50 mM Tris–HCl [pH 8.0], 0.1% SDS, 1% Nonidet P-40 [NP-40], 0.5% sodium deoxycholate) for 30 min at 4 °C and centrifuged (13,000 × *g*, 30 min, 4 °C). Proteins of the supernatant (50 μg) were incubated in buffer assay (100 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid] [pH 7.0], 1 mM ETDA, 0.1% CHAPS [3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfate], 10% glycerol, 20 mM dithiothreitol) in the presence of 100 μM fluorogenic peptide substrate (Ac-DEVD-AMC [7-amino-4-methylcoumarin], Ac-IETD-AMC, or Ac-LEHD-AFC (Biomol, Le Perray-en-Yvelines, France). Released AMC and AFC were excited at 380 and 400 nm to measure emission at 460 and 505 nm, respectively. Fluorescence was monitored continuously at 37 °C for 30 min in a dual luminescence fluorimeter (MicroTek OS; Bio-Tek Kontron Instruments, Winooski, VT).

2.9. Western blot analyses

Cells were washed twice with PBS, lysed in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8.0, 0.1% SDS, 0.5% sodium desoxycholate) in the presence of a cocktail of protease inhibitors (Roche, Meylan, France) for 30 min, then centrifuged for 30 min at 13,000 × *g*. Fifty micrograms of supernatant proteins were mixed (v/v) with loading buffer (125 mM Tris–HCl, pH 6.8, 10% ß-mercaptoethanol, 4.6% SDS, 20% glycerol and 0.003% bromophenol blue), boiled for 5 min, separated by SDS-PAGE and transferred onto polyvinyl difluoride (PVDF) membranes (BioRad, Ivry sur Seine, France). After blocking of non-specific binding sites overnight by 5% non-fat milk in PBS containing 0.1% Tween 20 (TPBS), membranes were probed with primary antibodies and immuno-reactive proteins were visualized using horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescent peroxidase substrate (Santa Cruz).

2.10. Flow cytometry studies

Mitochondrial membrane potential was assessed by the retention of 3,3′-diethyloxacarbo-cyanine DiOC6 (3) (Molecular Probes, Leiden, The Netherlands) measured by flow cytometry as described [33]. Cell surface expression of cell death receptors (Fas, DR4, and DR5) was determined by flow cytometry as described [33]. For ROS measurements, cells were incubated for 15 min at 37 °C in the presence of 6.6 μM dihydro-ethidium (Sigma) and analyzed by flow cytometry.

2.11. Death receptor clustering and immunofluorescence analysis

U937 cells (5×10^5/mL) were treated with 100 μM BLM and D-BLM for 24 and 72 h, respectively, then fixed with 2% paraformaldehyde (Sigma) in PBS for 10 min at 4 °C, washed twice with PBS, and incubated with primary antibody diluted 1:100 in PBS-1% bovine serum albumin for 2 h at room temperature. Samples were then washed with PBS–sodium azide, incubated 30 min with appropriate 568- or 488-alexa-conjugated antibodies, washed twice with PBS-sodium azide, fixed with 2% paraformaldehyde in PBS for 10 min at 4 °C, mounted on glass slides, and observed under microscope (Nikon). Non-relevant isotype-matching antibodies were used as negative controls.

3. Results

3.1. BLM and D-BLM-induced apoptosis in U937 human lymphoma cells is mediated by caspases

Exposure of U937 human lymphoma cells to BLM and D-BLM decreased both cell viability (Fig. 1A and B) and clonogenic survival (Fig. 1C and D) in a concentration- and time-dependent manner. This cytotoxic effect was observed at low concentrations, e.g. exposure of U937 cells to 1 μM BLM or D-BLM induced a 60 and 40% decrease in their clonogenicity, respectively (Fig. 1C and D). The cytotoxic effect of BLM and its

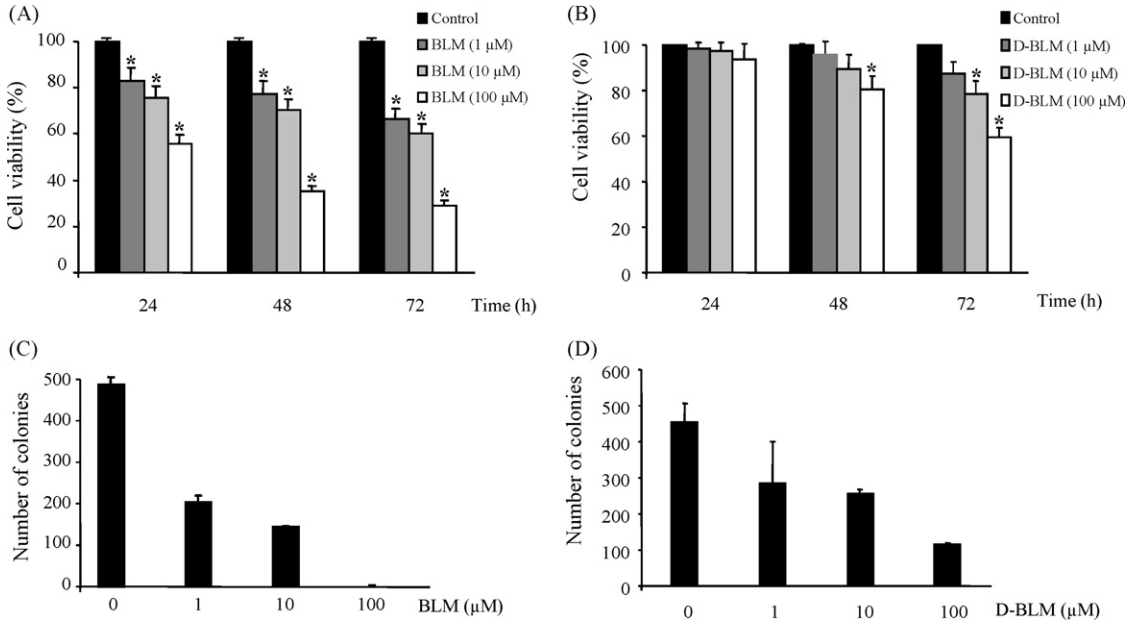

Fig. 1 – Cell death induction by bleomycin (BLM) and deglycosylated bleomycin (D-BLM). (A and B) U937 cells were plated in RPMI supplemented with 10% FCS at a density of 5×10^5 per mL and incubated with the indicated concentrations of BLM (A) or D-BLM (B) and at the indicated time intervals. Cell death was measured with the cell titer 96 aqueous non-radioactive cell proliferation assays. Results are the mean ± S.D. (bars) of three independent experiments, $^*P < 0.05$. (C and D) The cytotoxic activity of BLM (C) or D-BLM (D) was measured in U937 cells by use of a clonogenic assay in methylcellulose. Colonies were scored at day 11 with an inverted microscope. Data are representative of two independent experiments carried out in triplicate.

deglycosylated form appeared to be apoptotic as demonstrated by the condensation of nuclear chromatin (Fig. 2A and C) and the internucleosomal fragmentation of the DNA (Fig. 2B and D). Again, apoptosis induction increased with drug concentration and exposure duration (Fig. 2A and C). This cell death was prevented by co-treatment with cell permeant caspase inhibitors, i.e. the pan-caspase inhibitor z-VAD-fmk induced a 75% decrease in the number of cells with nuclear chromatin condensation upon exposure to 100 μM BLM or D-BLM whereas other more specific caspase inhibitors induced a

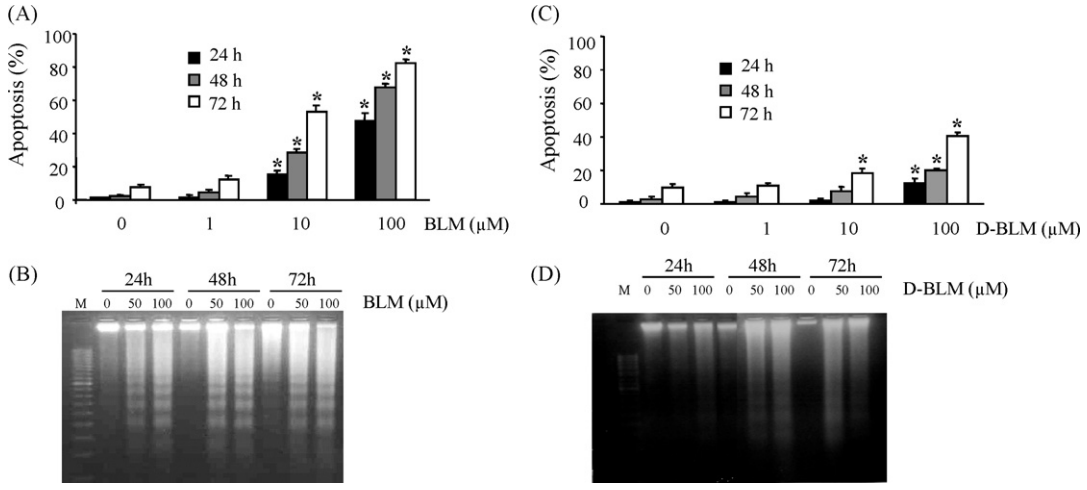

Fig. 2 – Apoptosis-associated DNA fragmentation in U937 cells treated with BLM and D-BLM. (A and C) U937 cells were exposed to various concentrations of BLM or D-BLM for the indicated times. Apoptosis is analyzed by cell nuclear staining with Hoechst 33342 dye. In untreated cells, the percentage of apoptosis was always lower than 5%. Data represent mean ± S.D. (bars) of three independent experiments, $^*P < 0.05$. (B and D) Detection of oligonucleosomal fragments was determined by submitting the DNA to agarose gel electrophoresis. Results are representative of three independent experiments.

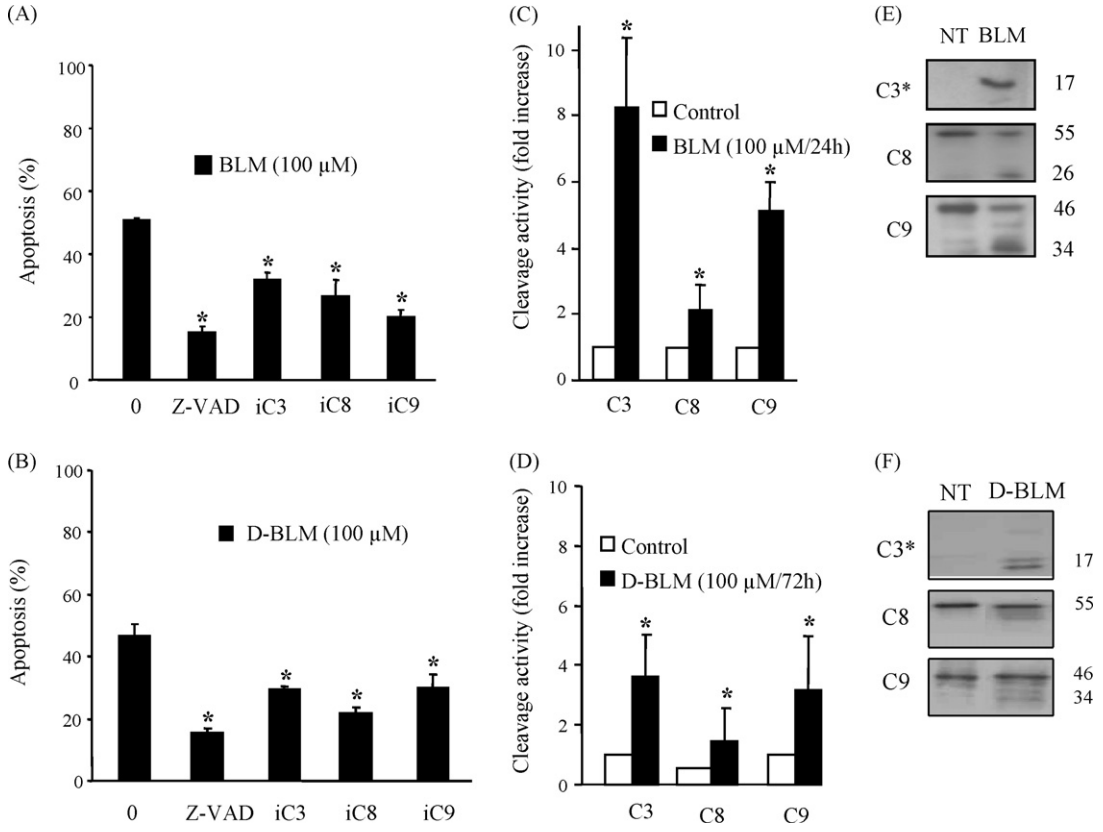

Fig. 3 – Involvement and activation of caspases in BLM- and D-BLM-induced apoptosis. (A and B) U937 cells were treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h, alone or in the presence of 100 μM pan caspase (Z-VAD) or 100 μM specific caspase inhibitors (iC). Apoptotic cells were counted after Hoechst 33342 staining. Results are the mean ± S.D. (bars) of at least three independent experiments. (C and D) U937 cells were treated with 100 μM BLM for 24 h (C) or with 100 μM D-BLM for 72 h (D) before measuring caspase-3 (C3), -8 (C8), and -9 (C9) activities. Mean ± S.D. (bars) of at least three independent experiments, $^{*}P < 0.05$. (E and F) Western blot analysis of indicated proteins in no treated U937 cells (NT) or in U937 cells exposed to 100 μM BLM for 24 h (E) or to 100 μM D-BLM for 72 h (F). Results are representative of three independent experiments. C3* represents the active form of caspase-3.

30–50% decrease in the percentage of apoptotic cells (Fig. 3A and B). Accordingly, the use of peptide substrates that mimic the preferred target site of each caspase detected Ac-DEVD-AFC, Ac-EHD-AFC, and Ac-IETD-AFC cleavage activities in lysates of U937 cells exposed to BLM for 24 h and D-BLM for 72 h, indicating that caspase-3, caspase-9, and caspase-8 were activated (Fig. 3C and D). Immunoblot analysis of these cell lysates further demonstrated the activation of these enzymes upon drugs exposure as indicated by a decrease in their pro-form or the appearance of their active cleavage fragments (Fig. 3E and F).

3.2. *BLM- and D-BLM-induced apoptosis involves the mitochondria*

Exposure of U937 cells to BLM and D-BLM induced a decrease in the mitochondrial transmembrane potential ($\Delta\psi_m$) attested by (i) a decrease in cell labelling with $DiOC_6$ (3) (Fig. 4A), (ii) conformational changes of Bax as determined by fluorescence microscopy using a conformational-specific antibody (Fig. 4B) and (iii) the mitochondrial release of cytochrome *c* from the intermembrane space of the mitochondria to the cytosol (Fig. 4C). Overexpression of Bcl-2, known to inhibit BH3-only proteins pro-apoptotic activity, hampered BLM and D-BLM-induced apoptosis in U937 cells (Fig. 4D). Collectively, these results indicate that BLM and its deglycosylated form induce lymphoma cell apoptosis through the mitochondrial pathway.

3.3. *BLM- and D-BLM-induced apoptosis depends on JNK activation but partially or not on ROS production*

BLM was previously shown to trigger the production of ROS [11,34]. Accordingly, by using dihydro-ethidium and a flow cytometry assay, we observed that exposure of U937 cells to different concentrations of BLM for 24 h increased U937 cell content in ROS, in a dose-dependent manner. ROS production was prevented by co-treatment with 10 mM NAC, a ROS scavenger [35] (Fig. 5A). By contrast, D-BLM failed to increase ROS (Fig. 5A). Accordingly, NAC-induced inhibition of ROS production protected U937 cells from BLM- but not from D-BLM-induced apoptosis in dose-dependent manner (Fig. 5B). Pretreatment of U937 cells with phosphatidylinositol 3-kinase

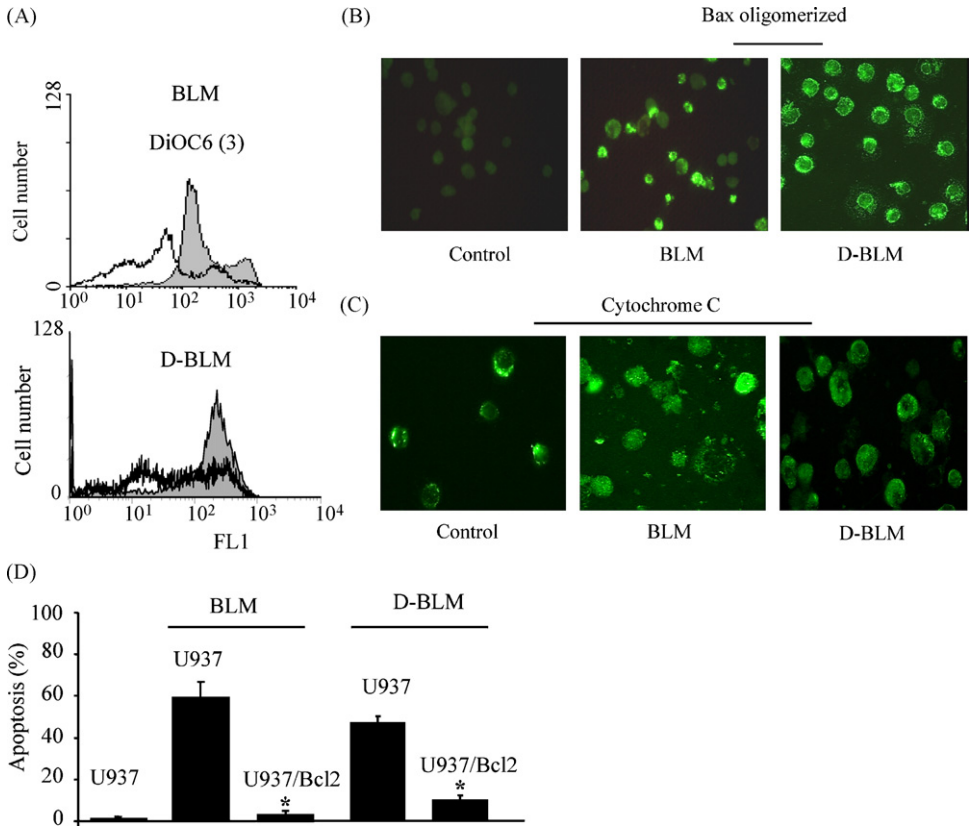

Fig. 4 – Mitochondrial changes during BLM- and D-BLM-induced apoptosis. (A) U937 cells were treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h, then stained with 1 nM $DioC_6$ (3) probe before measuring cell fluorescence by flow cytometry (FL-1). Diminished mitochondrial membrane bound $DiOC_6$ (3) (reduced FL1 intensity) indicates a decrease in the mitochondrial transmembrane potential $\Delta\psi m$. (B) U937 cells demonstrate Bax oligomerization upon treatment with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h, as identified by fluorescence microscopy using a conformation-specific antibody. (C) U937 cells treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h demonstrate cytochrome c diffuse staining, suggesting release from mitochondria to cytosol, as identified by fluorescence microscopy. Results are representative of three independent experiments. (D) U937 cells, stably transfected with a vector encoding Bcl-2 (U937/Bcl2), were treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h, and apoptotic cells were counted after Hoechst 33342 dye staining. Results are the mean ± S.D. (bars) of three independent experiments, $^*P < 0.05$.

(LY294002), p38 MAPK (SB-239063) or ERK (PD98059) inhibitors did not influence the cytotoxic activity of BLM and D-BLM whereas pretreatment with an inhibitor of JNK (SP-600125) significantly reduced the percentage of cell dying upon exposure to these both drugs (Fig. 5C). Altogether these results argue for a substantial role of ROS and JNK in BLM-induced cell death while JNK but not ROS plays a role in D-BLM-induced apoptosis.

3.4. *BLM-induced apoptosis does not involve death receptors*

Having identified some of the molecular mechanisms involved in both BLM- and D-BLM-induced apoptosis of U937 cells, we wondered whether death receptors might also play a role in the process. Exposure of U937 cells to BLM and to D-BLM did not modulate the expression of DR4, DR5, or Fas at their surface (Fig. 6A). However, both drugs triggered the redistribution of these receptors at the cell surface, as demonstrated by immunofluorescence microscopy. While untreated U937 cells exhibited a diffuse membrane distribution of DR4, DR5, and Fas, in cells exposed to BLM or D-BLM at 10 or 100 μM these receptors yet formed aggregates at the cell surface (Fig. 6B). Neither the TRAIL antagonistic fusion protein Fc-TRAIL-R2 (Fc-R2) (Fig. 6C) nor the blocking anti-Fas antibody (ZB4) (Fig. 6D) prevented U937 cells from BLM- and D-BLM-induced cell death. In line with this, U937 cells expressing a dominant negative mutant version of FADD (FADD-DN), which were protected from Fas ligand- and TRAIL-induced apoptosis (Fig. 6E), remained as sensitive to BLM-induced apoptosis as those expressing the empty vector (EV) and non transfected cells (U) (Fig. 6F). Altogether, these observations suggested that the death receptor pathway was not involved in BLM- and D-BLM-induced apoptosis of lymphoma cells.

3.5. *BLM and D-BLM sensitize lymphoma cells to TRAIL- but not to FasL-mediated cell death*

To determine whether BLM- and D-BLM-triggered death receptor clustering would sensitize U937 cells to death

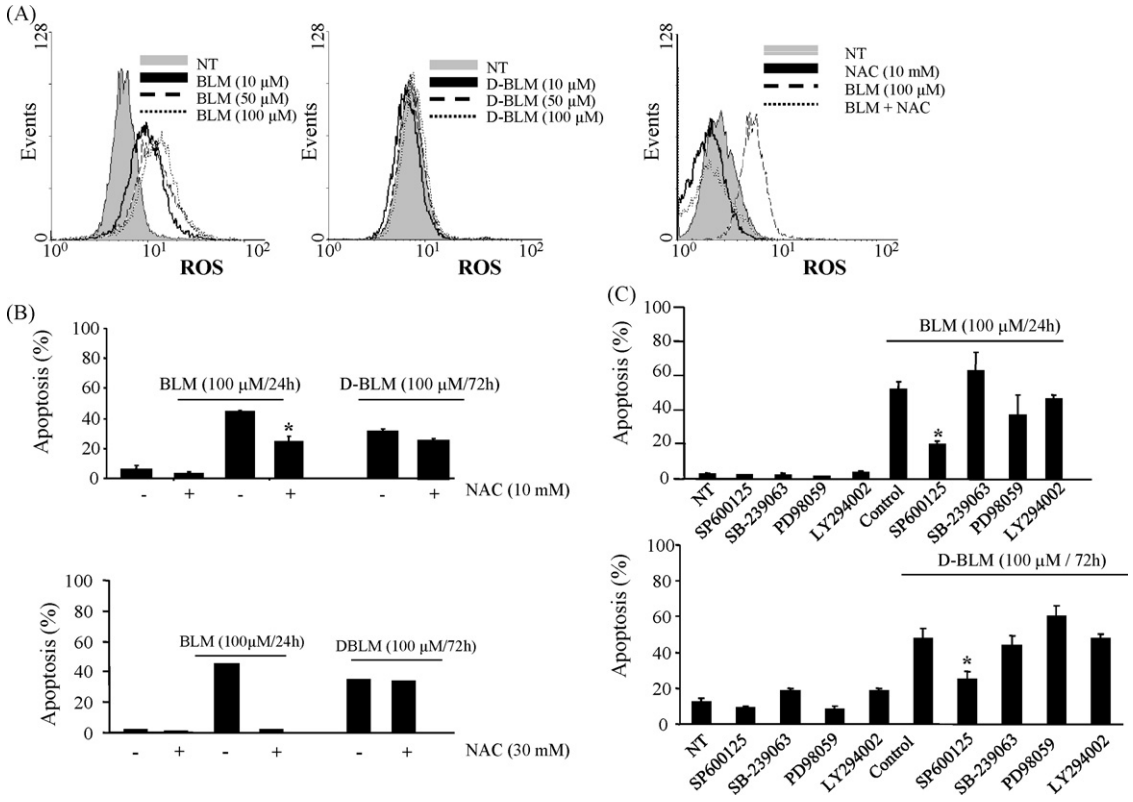

Fig. 5 – Role of ROS and JNK for BLM- and D-BLM-induced apoptosis. (A) Analysis by flow cytometry of ROS levels using dihydro-ethidium in U937 treated with different concentrations of BLM or D-BLM alone or in the presence of 10 mM NAC. Results are representative of three independent experiments. (B and C) Apoptotic cell analysis after Hoechst 33342 staining of U937 cells treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h alone (−) or in the presence (+) of 10 and 30 mM (NAC) (B), or in the presence of various protein kinase inhibitors: the JNK SP600125 inhibitor, the p38 SB-239063 inhibitor, the ERK PD98059 inhibitor, and the Pi3K/Akt Ly294002 inhibitor (C). Results are the mean ± S.D. (bars) of three independent experiments, $^{*}P < 0.05$. Results with 30 mM NAC represent one experiment.

receptor ligand-induced cell death, leukemia cells were treated with BLM or D-BLM alone or in combination with TRAIL or FasL and apoptosis was analyzed by the cell nuclear staining with Hoechst 33342 dye. Exposure of U937 cells to BLM or D-BLM modestly enhanced TRAIL-induced apoptosis in U937 cells (Fig. 7A, B) but failed to sensitize these cells to FasL-induced cell death (Fig. 7C and D).

4. Discussion

BLM-containing regiments remain commonly used to treat malignant lymphomas [36], but the use of BLM exposes patients to lung fibrosis [37,38], side effects related to the ability of the molecule to generate free radicals such as ROS [16,39]. Here, we show that deglycosylation of the molecule generates a compound that remains able to trigger apoptosis in a lymphoma cell line but does not generate ROS.

When studied in a lymphoma cell line, this pathway appears to involve Bax, which is in accordance with the fact that fibroblasts from Bax−/− bak−/− mice resist to BLM-induced cell death [15]. Cell death triggered by BLM also involves the production of ROS as shown using NAC. ROS generation upon BLM exposure might act early in the death pathway activated by BLM since NAC prevents BLM-induced Bax oligomerization and subsequent release of cytochrome c from the mitochondria (data not shown). Interestingly, D-BLM did not generate production of ROS but triggered cell death that was not affected by NAC. Therefore, deglycosylation poorly affect the cytotoxic properties of the molecule. The failure of D-BLM to produce ROS may be related to its subcellular distribution. Accordingly, we have demonstrated [40] that both drugs reveal significantly different intracellular distribution, with low and high accumulation into the cell nuclei for BLM and D-BLM, respectively. The nuclear location of D-BLM may limit its interaction with mitochondria, where ROS production is thought to occur, without affecting its ability to activate caspases. Alternatively and although, procaspases are mainly localized in the cytosol, procaspase-3 and procaspase-9 have been shown in the nucleus [41]. Their activation by D-BLM could change either caspase target availability or death signaling pathways triggered by D-BLM compared to BLM, explaining the reduced toxicity of the deglycosylated variant.

In rat primary alveolar type II cells, BLM activates the stress kinase JNK and its cytotoxic effect is strongly reduced when

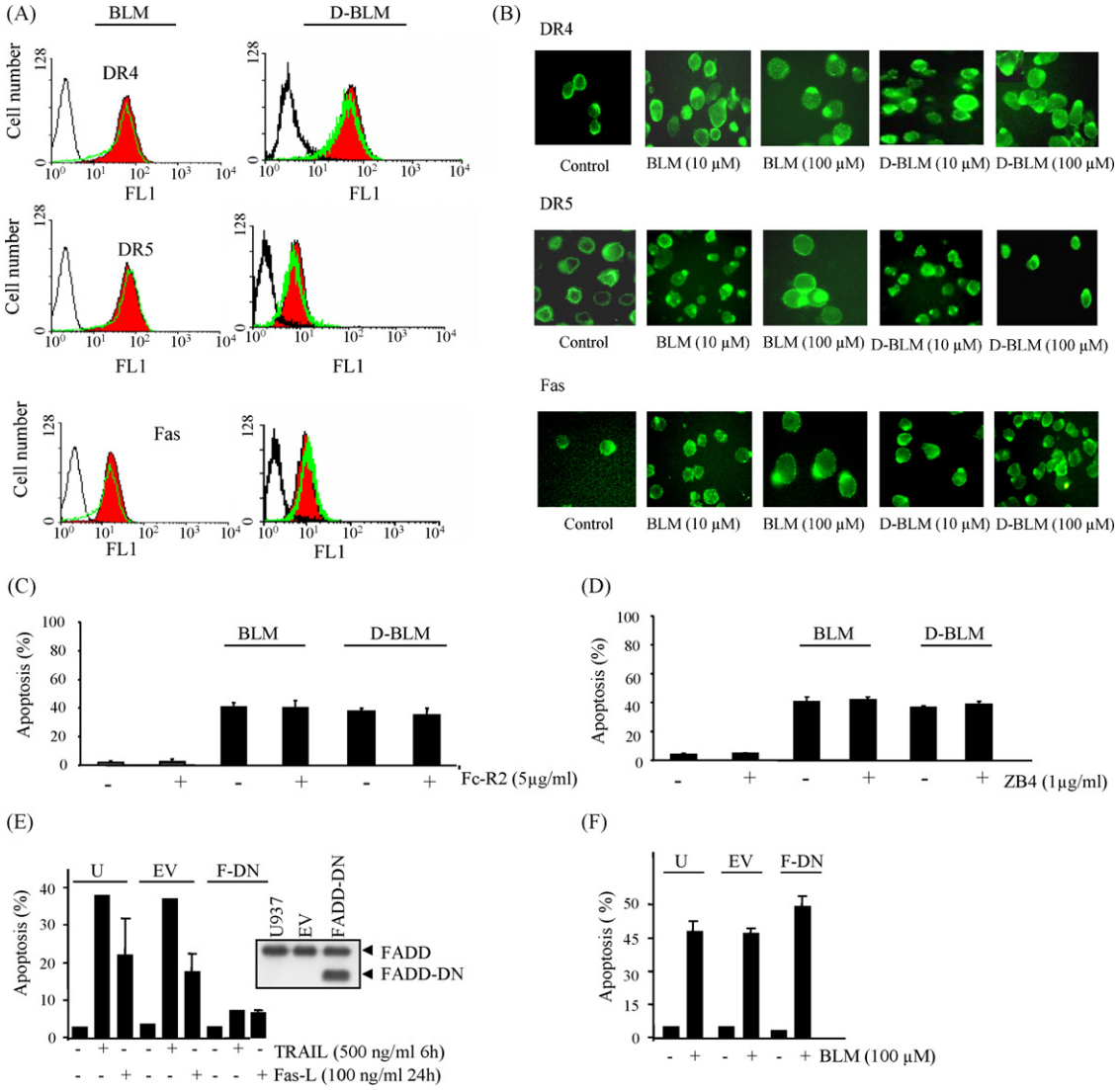

Fig. 6 – Death receptor-independent apoptosis induced by BLM and D-BLM. (A) Flow cytometry analysis of TRAIL receptors (DR4 and DR5) and Fas expression at the surface of U937 cells left untreated (red curve) or treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h (green curve). Non-immune mouse IgGs were used as controls (black curve). One representative of three experiments is shown. (B) Immunostaining analysed by fluorescence microscopy of DR4, DR5, and Fas in U937 cells exposed to vehicle (control), or to BLM for 24 h or to D-BLM for 72 h at the concentration of 10 and 100 μM. Results are representative of three independent experiments. (C and D) U937 cells were treated with 100 μM BLM for 24 h or with 100 μM D-BLM for 72 h alone (−) or in the presence (+) of a TRAIL receptor antagonist, Fc-R2 (C), and a Fas antagonist antibody, ZB4 (D). Apoptotic cell percentage was determined after Hoechst 33342 nuclear staining. Results are the mean ± S.D. (bars) of three independent experiments. (E and F) U937 cells were transfected with a empty vector (EV) or a vector encoding the dominant negative form of FADD (F-DN) (E, insert: Western blot analysis of the expression of the F-DN in U937 cells), then left untreated (−) or treated (+) with 100 ng/mL Fas ligand (Fas-L) for 24 h, 500 ng/mL TRAIL for 6 h (E) or 100 μM BLM for 24 h (F). Results are the mean ± S.D. (bars) of three independent experiments.

these cells are engineered to express a dominant negative version of JNK [15]. Here we show that an inhibitor of JNK prevents apoptosis induced by either BLM or D-BLM, which suggest that this kinase plays an important role in the death pathway activated by the two drugs [42–44]. On the other hand, death domain containing receptors are unlikely involved in BLM-induced lymphoma cell death since neither inhibition of TRAIL- and Fas-induced cell death by the use of a chimeric soluble DR5 receptor or a Fas antagonistic antibody nor the expression of a dominant negative version of FADD prevented BLM- and D-BLM-induced apoptosis. The role of death receptors clearly depends on cell types since BLM-induced apoptosis in lung epithelial cells is independent of the Fas pathway both in human [15,16] and mouse [45] fibrosis models but involves Fas in some other cell types, both *in vitro* [46,47] and *in vivo* [48]. In this latter case, BLM-induced apoptosis was proposed to proceed through a p53-dependent up-regulation of Fas expression [47].

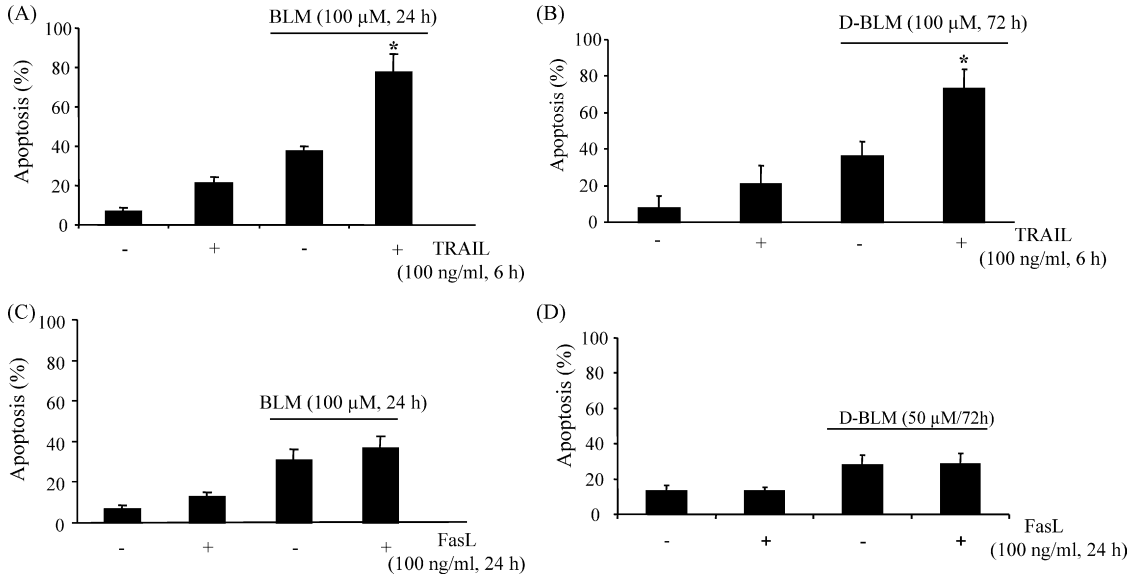

Fig. 7 – BLM and D-BLM sensitize leukemia cells to TRAIL- but not to FasL-mediated cell death. Apoptotic U937 cells were determined by Hoechst 33342 staining after a co-treatment with 100 μM BLM for 24 h (A and C) or with 50–100 μM D-BLM for 72 h (B and D) and 100 ng/mL of recombinant soluble TRAIL for 6 h (A and B) or 100 ng/mL FasL for 24 h (C and D). Results are the mean ± S.D. (bars) of three independent experiments. Sensitization obtained with TRAIL is statistically significant (*$P < 0.05$), because the student test was calculated by a comparison of the sum of results obtained with compounds used alone to results obtained with combinations.

Interestingly, both BLM and D-BLM efficiently induced Fas, DR4 and DR5 clustering at the plasma membrane level, without modifying the expression level of these proteins. Redistribution of these receptors occurred independently of apoptosis since low doses of BLM and D-BLM (10 μM) induced the clustering of death receptors in non-apoptotic cells. Receptor aggregation might be at the origin of TRAIL-induced apoptosis sensitization. Inhibition of JNK activation, however, decreased the clustering of DR4, DR5 and Fas (data not shown), indicating that the JNK pathway could regulate the process. Whether the tested drugs alter plasma membrane phospholipids e.g. by increasing of ceramide level [49] remains a matter of speculation.

In conclusion, our data provide two important insights into BLM molecular pharmacology. First, we show that both BLM and D-BLM can activate a mitochondrial-dependent cell death pathway to apoptosis that relies on JNK activation in lymphoma cells. Second, BLM deglycosylation suppresses its ability to trigger ROS formation without hampering its cytotoxic properties. Lastly, by inducing the clustering of TRAIL agonist receptors at the cell surface, BLM and D-BLM could act synergistically with TRAIL receptor agonists in inducing lymphoma cell death.

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REFERENCES

[1] Umezawa H, Maeda K, Takeuchi T, Okami Y. New antibiotics, bleomycin A and B. J Antibiot (Tokyo) 1966;19:200–9.
[2] Chen J, Stubbe J. Bleomycins: towards better therapeutics. Nat Rev Cancer 2005;5:102–12.
[3] Boggs SS, Sartiano GP, DeMezza A. Minimal bone marrow damage in mice given bleomycin. Cancer Res 1974;34:1938–42.
[4] Lehane DE, Hurd E, Lane M. The effects of bleomycin on immunocompetence in man. Cancer Res 1975;35:2724–8.
[5] Bayer RA, Gaynor ER, Fisher RI. Bleomycin in non-Hodgkin's lymphoma. Semin Oncol 1992;19:46–52 [discussion 52–53].
[6] Bonadonna G, De Lena M, Monfardini S, Bartoli C, Bajetta E, Beretta G, et al. Clinical trials with bleomycin in lymphomas and in solid tumors. Eur J Cancer 1972;8:205–15.
[7] Hamakawa H, Bao Y, Takarada M, Tanioka H. Histological effects and predictive biomarkers of TPP induction chemotherapy for oral carcinoma. J Oral Pathol Med 1998;27:87–94.
[8] Levi JA, Raghavan D, Harvey V, Thompson D, Sandeman T, Gill G, et al., Australasian Germ Cell Group. The importance of bleomycin in combination chemotherapy for good-prognosis germ cell carcinoma. J Clin Oncol 1993;11:1300–5.
[9] Martin WG, Ristow KM, Habermann TM, Colgan JP, Witzig TE, Ansell SM. Bleomycin pulmonary toxicity has a negative impact on the outcome of patients with Hodgkin's lymphoma. J Clin Oncol 2005;23:7614–20.

[10] Sleijfer S. Bleomycin-induced pneumonitis. Chest 2001;120:617–24.
[11] Sausville EA, Peisach J, Horwitz SB. Effect of chelating agents and metal ions on the degradation of DNA by bleomycin. Biochemistry 1978;17:2740–6.
[12] Iqbal ZM, Kohn KW, Ewig RA, Fornace Jr AJ. Single-strand scission and repair of DNA in mammalian cells by bleomycin. Cancer Res 1976;36:3834–8.
[13] Moore CW, Malcolm AW, Tomkinson KN, Little JB. Ultrarapid recovery from lethal effects of bleomycin and gamma-radiation in stationary-phase human diploid fibroblasts. Cancer Res 1985;45:1978–81.
[14] Tounekti O, Kenani A, Foray N, Orlowski S, Mir LM. The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway. Br J Cancer 2001;84:1272–9.
[15] Lee VY, Schroedl C, Brunelle JK, Buccellato LJ, Akinci OI, Kaneto H, et al. Bleomycin induces alveolar epithelial cell death through JNK-dependent activation of the mitochondrial death pathway. Am J Physiol Lung Cell Mol Physiol 2005;289:L521–8.
[16] Wallach-Dayan SB, Izbicki G, Cohen PY, Gerstl-Golan R, Fine A, Breuer R. Bleomycin initiates apoptosis of lung epithelial cells by ROS but not by Fas/FasL pathway. Am J Physiol Lung Cell Mol Physiol 2006;290:L790–6.
[17] Yang LC, Yang SH, Tai KW, Chou MY, Yang JJ. MEK inhibition enhances bleomycin A5-induced apoptosis in an oral cancer cell line: signaling mechanisms and therapeutic opportunities. J Oral Pathol Med 2004;33:37–45.
[18] Gimonet D, Landais E, Bobichon H, Coninx P, Liautaud-Roger F. Induction of apoptosis by bleomycin in p53-null HL-60 leukemia cells. Int J Oncol 2004;24:313–9.
[19] Kenani A, Bailly C, Helbecque N, Catteau JP, Houssin R, Bernier JL, et al. The role of the gulose-mannose part of bleomycin in activation of iron-molecular oxygen complexes. Biochem J 1988;253:497–504.
[20] Filomenko R, Prevotat L, Rebe C, Cortier M, Jeannin JF, Solary E, et al. Caspase-10 involvement in cytotoxic drug-induced apoptosis of tumor cells. Oncogene 2006.
[21] Decaudin D, Marzo I, Brenner C, Kroemer G. Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (review). Int J Oncol 1998;12:141–52.
[22] Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997;275:1132–6.
[23] Perkins C, Kim CN, Fang G, Bhalla KN. Overexpression of Apaf-1 promotes apoptosis of untreated and paclitaxel- or etoposide-treated HL-60 cells. Cancer Res 1998;58:4561–6.
[24] Fulda S, Meyer E, Friesen C, Susin SA, Kroemer G, Debatin KM. Cell type specific involvement of death receptor and mitochondrial pathways in drug-induced apoptosis. Oncogene 2001;20:1063–75.
[25] Lacour S, Hammann A, Wotawa A, Corcos L, Solary E, Dimanche-Boitrel MT. Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res 2001;61:1645–51.
[26] Micheau O, Solary E, Hammann A, Dimanche-Boitrel MT. Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. J Biol Chem 1999;274:7987–92.
[27] Green DR. Apoptotic pathways: paper wraps stone blunts scissors. Cell 2000;102:1–4.
[28] Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998;17:1675–87.
[29] Schneider P. Production of recombinant TRAIL and TRAIL receptor: Fc chimeric proteins. Methods Enzymol 2000;322:325–45.
[30] Claessens YE, Park S, Dubart-Kupperschmitt A, Mariot V, Garrido C, Chretien S, et al. Rescue of early-stage myelodysplastic syndrome-deriving erythroid precursors by the ectopic expression of a dominant-negative form of FADD. Blood 2005;105:4035–42.
[31] Rebe C, Cathelin S, Launay S, Filomenko R, Prevotat L, L'Ollivier C, et al. Caspase-8 prevents sustained activation of NF-{kappa}B in monocytes undergoing macrophagic differentiation. Blood 2006.
[32] Aye MT, Seguin JA, McBurney JP. Erythroid and granulocytic colony growth in cultures supplemented with human serum lipoproteins. J Cell Physiol 1979;99:233–8.
[33] Millet A, Bettaieb A, Renaud F, Prevotat L, Hammann A, Solary E, et al. Influence of the nitric oxide donor glyceryl trinitrate on apoptotic pathways in human colon cancer cells. Gastroenterology 2002;123:235–46.
[34] Galvan L, Huang CH, Prestayko AW, Stout JT, Evans JE, Crooke ST. Inhibition of bleomycin-induced DNA breakage by superoxide dismutase. Cancer Res 1981;41:5103–6.
[35] Gillissen A, Nowak D. Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy. Respir Med 1998;92:609–23.
[36] Canellos GP, Anderson JR, Propert KJ, Nissen N, Cooper MR, Henderson ES, et al. Chemotherapy of advanced Hodgkin's disease with MOPP, ABVD, or MOPP alternating with ABVD. N Engl J Med 1992;327:1478–84.
[37] Hagimoto N, Kuwano K, Nomoto Y, Kunitake R, Hara N. Apoptosis and expression of Fas/Fas ligand mRNA in bleomycin-induced pulmonary fibrosis in mice. Am J Respir Cell Mol Biol 1997;16:91–101.
[38] Duggan DB, Petroni GR, Johnson JL, Glick JH, Fisher RI, Connors JM, et al. Randomized comparison of ABVD and MOPP/ABV hybrid for the treatment of advanced Hodgkin's disease: report of an intergroup trial. J Clin Oncol 2003;21:607–14.
[39] Hagiwara SI, Ishii Y, Kitamura S. Aerosolized administration of N-acetylcysteine attenuates lung fibrosis induced by bleomycin in mice. Am J Respir Crit Care Med 2000;162:225–31.
[40] Kenani A, Bailly C, Houssin R, Henichard JP. Comparative subcellular distribution of the copper complexes of bleomycin-A2 and deglycobleomycin-A2. Anticancer Drugs 1994;5:199–201.
[41] Sordet O, Rebe C, Dubrez-Daloz L, Boudard D, Solary E. Intracellular redistribution of procaspases during TPA-induced differentiation of U937 human leukemic cells. Leukemia 2002;16:1569–70.
[42] Saeki K, Kobayashi N, Inazawa Y, Zhang H, Nishitoh H, Ichijo H, et al. Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase pathways for apoptosis in human leukaemic cells stimulated by epigallocatechin-3-gallate (EGCG): a distinct pathway from those of chemically induced and receptor-mediated apoptosis. Biochem J 2002;368:705–20.
[43] Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, et al. The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Mol Cell Biol 2002;22:4929–42.
[44] Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000;288:870–4.
[45] Aoshiba K, Yasui S, Tamaoki J, Nagai A. The Fas/Fas-ligand system is not required for bleomycin-induced pulmonary fibrosis in mice. Am J Respir Crit Care Med 2000;162:695–700.
[46] Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, et al. p53 activates the CD95 (APO-1/Fas) gene in

response to DNA damage by anticancer drugs. J Exp Med 1998;188:2033–45.
[47] Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, et al. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest 1997;99:403–13.
[48] Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, et al. Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. J Clin Invest 1999;104:13–9.
[49] Grassme H, Cremesti A, Kolesnick R, Gulbins E. Ceramide-mediated clustering is required for CD95-DISC formation. Oncogene 2003;22:5457–70.