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Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cells

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ABSTRACT

Salinomycin is a polyether antibiotic isolated from *Streptomyces albus* that acts in different biological membranes as a ionophore with a preference for potassium. It is widely used as an anticoccidial drug in poultry and is fed to ruminants to improve nutrient absorption and feed efficiency. Salinomycin has recently been shown to selectively deplete human breast cancer stem cells from tumorspheres and to inhibit breast cancer growth and metastasis in mice. We show here that salinomycin induces massive apoptosis in human cancer cells of different origin, but not in normal cells such as human T lymphocytes. Moreover, salinomycin is able to induce apoptosis in cancer cells that exhibit resistance to apoptosis and anticancer agents by overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with enhanced proteolytic activity. Salinomycin activates a distinct apoptotic pathway that is not accompanied by cell cycle arrest and that is independent of tumor suppressor protein p53, caspase activation, the CD95/CD95L system and the proteasome. Thus, salinomycin should be considered as a novel and effective anticancer agent that overcomes multiple mechanisms of apoptosis resistance in human cancer cells.

Salinomycin is a 751 Da monocarboxylic polyether antibiotic isolated from *Streptomyces albus* (strain No. 80614) that exhibits a unique tricyclic spiroketal ring system and an unsaturated six-membered ring in the molecule. It is an anionic and weakly acidic compound with the molecular formula $C_{42}H_{70}O_{11}$ [1,2]. Salinomycin acts in different biological membranes including cytoplasmic and mitochondrial membranes as a ionophore with strict selectivity for alkali ions and a great preference for potassium [3,4]. Salinomycin exhibits antimicrobial activity against gram-positive bacteria including mycobacteria, some filamentous fungi, *Plasmodium falciparum*, and *Eimeria* spp., parasites responsible for the poultry disease coccidiosis [2,5,6]. Therefore, salinomycin is used as an anticoccidial drug in poultry and is furthermore fed to ruminants to improve nutrient absorption and feed efficiency [7,8].

In addition to these well-known properties, salinomycin has recently been shown to selectively deplete breast cancer stem cells from tumorspheres and to inhibit breast tumor growth in mice by unknown mechanisms [9], strongly suggesting that salinomycin can be used as an anticancer drug. Therefore it is mandatory to investigate in detail the effects of salinomycin on different human cancer cells. Here we show for the first time that salinomycin induces apoptosis selectively in human cancer cells, including those that display wild-type p53 or p53 mutations [10–12] and multidrug resistance due to overexpression of Bcl-2 [13], P-glycoprotein [14–17] or 26S proteasomes with deregulated proteolytic activity [17,18]. Salinomycin activates a distinct apoptotic pathway in cancer cells that is not accompanied by cell cycle arrest and that is independent of p53, caspase activation, the CD95/CD95L system and the 26S proteasome.

Materials and methods

Antibodies and reagents. Anti-β-actin antibody (mouse monoclonal, AC-15) was purchased from Sigma (Taufkirchen, Germany), anti-p27 (mouse monoclonal, DSC-72) and anti-p53 (mouse monoclonal, DO-1) were purchased from Santa Cruz (Heidelberg, Germany).

The proteasome inhibitor bortezomib (PS-341, Velcade®) was purchased from Millennium Pharmaceuticals (Cambridge, USA). Salinomycin was purchased from Sigma. The biotinylated proteasome-specific affinity probe AdaK(Bio)AhX_3L_3VS (Bio-ALVS) was purchased from Calbiochem (Darmstadt, Germany). Z-VAD-fmk and pifithrin-α were purchased from Bachem (Heidelberg, Germany) and Sigma, respectively, and were dissolved in DMSO. Salinomycin and bortezomib were dissolved in DMSO. Salinomycin, bortezomib, Z-VAD-fmk and pifithrin-α were stored in stock solutions at −20 °C.

Cells and cell lines. Human CD4+ T-cell leukemia cells were obtained from three different individuals with acute T-cell leukemia after informed consent. Cells were isolated from heparinized peripheral venous blood by Ficoll-Hypaque (Winthrop Pharmaceu-

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ticals, New York, USA) density gradient centrifugation at 700*g* for 40 min. In all patients, more than 92% of the peripheral blood leucocytes were CD4+ leukemia cells, as determined by flow cytometry analysis of specific cell surface markers. Human CD4+ T-cell leukemia cells, Molt-4 cells, Jurkat cells, MES-SA/Dx5 cells, Namalwa cells (ATCC, Manassas, USA) and Namalwa cells adapted to bortezomib were maintained in culture medium (CM) consisting of RPMI1640 (Gibco-Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine (Sigma), 100 IU penicillin and 100 μg/ml streptomycin (Gibco-Invitrogen) supplemented with 10% fetal calf serum (FCS, Gibco-Invitrogen). Jurkat cells stably transfected with the plasmid SFFV-human bcl-2 (Jurkat-Bcl-2) or the empty control plasmid SFFV-neo (Jurkat-neo) were cultured in CM supplemented with 2% (v/v) geneticin and 1% (v/v) Hepes. Bortezomib-adapted Namalwa cells were generated by continuous exposure of Namalwa cells to 12.5 nM bortezomib. The viable and growing cells adapted to bortezomib were termed Namalwa^{adBor} cells. Namalwa^{adBor} cells were maintained in CM supplemented with 12.5 nM bortezomib that was exchanged every 48 h.

Isolation of CD4+ cells. $CD4^+$ T cells were isolated from heparinized peripheral venous blood from human healthy donors as described previously [19]. Cells were maintained in CM for a maximum of 48 h.

Detection of apoptotic cells. Cells were incubated for 24 h at 37 °C in CM containing different concentrations of salinomycin, bortezomib, Z-VAD-fmk, pifithrin-α or 5 μl/ml DMSO as a control. Staining of apoptotic cells was performed using the annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis assay kit (BD Pharmingen, Heidelberg, Germany) according to the manufacture's instructions. In all experiments, apoptotic cells were also detected by flow cytometry analyses of nuclear hypodiploid DNA content after incubation of the cells for 30 min at 4 °C in a staining solution consisting of PBS supplemented with 50 μg/ml propidium iodide, 0.1% natrium citrate-2-hydrate and 0.1% Triton X-100. Determination of apoptotic cells was performed by flow cytometry using a FACScan® flow cytometer and the Cellquest® software (Becton Dickinson, Heidelberg, Germany).

Calculation of specific apoptosis. Specific apoptosis (SA) was calculated as described previously [20] using the following formula:

$$SA(\%) = 100 \times \frac{A_E - A_C}{100 - A_C}$$

where A_E equals % of apoptotic cells in the experimental group and A_C equals % of apoptotic cells in the control group.

Western blot analysis. Whole cell lysates were prepared by pelleting and lysing cells in immunoprecipitation assay (RIPA) buffer in the presence of a protease inhibitor mixture (Roche, Penzberg, Germany). Protein content was quantified using the D_C-Protein assay kit. Samples with equivalent amounts of total protein were boiled in SDS–PAGE sample buffer, separated on 12% Tris–HCl gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Life Science, Braunschweig, Germany). After blocking the membrane with a solution of PBS containing 5% (v/v) bovine serum albumin and 0.1% Tween 20 for 1 h at room temperature, the membrane was incubated for 1 h with the respective specific antibody followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz). Signals of targeted proteins were detected by the Super Signal West Pico Chemiluminescence reagent (Pierce, Bonn, Germany) and recorded on ECL Hyperfilm (Amersham Life Science) in the linear detectable range. Equal protein loading was confirmed by blotting β-actin. All Western blots were performed in triplicate with similar results.

Measurement of proteasome activity. The proteolytic activity of the proteasomal subunits β1, β2 and β5 were measured and calculated in Namalwa cells and Namalwa^{daBor} cells as described previously [17].

Determination of P-glycoprotein expression and rhodamine 123 efflux. Cell surface expression of P-glycoprotein and rhodamine 123 efflux of MES-SA/Dx5 cells were determined by flow cytometry as described previously [17].

Cell cycle analysis. Cells cycle analysis was performed as described previously [17]. Cells were washed once with PBS, pelleted by centrifugation and resuspended in PBS containing 50 μg/ml propidium iodide, 0.1% natrium citrate-2-hydrate and 0.1% Triton X-100. Samples were stored at 4 °C for 30 min and gently vortexed before analysis by flow cytometry.

Probing of proteasome β-subunit activity. Affinity labeling of active proteasome subunits β1, β1i, β2, β2i, β5 and β5i using the proteasome-specific affinity probe Bio-ALVS was performed as described previously [21]. Briefly, cells were incubated with salinomycin or bortezomib for 1 h. Cells were washed twice and pelleted with ice-cold PBS. A volume of glass beads (⩾106 μm, acid-washed, Sigma) equivalent to the volume of the pellet was added, followed by a similar volume of homogenization buffer (50 mM Tris–HCl, pH 7.4, 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose). Cells were vortexed at high speed for 1 min, and the beads and cell debris were removed by centrifugation at 10,000*g* for 5 min at 4 °C. The resulting supernatant was centrifuged at 10,000*g* for 20 min at 4 °C to remove intact cells and nuclei. Protein concentration was determined using the D_C-Protein assay kit (Bio-Rad, Munich, Germany). Equal amounts of cell lysates (approximately 25 μg) were incubated with 3 μg Bio-ALVS for 2 h at 37 °C. Subsequently, proteins were denatured by boiling in reducing 4× sample buffer, separated on 12% sodium dodecyl sulfate (SDS) polyacrylamide Tris–HCl gels and electrotransferred

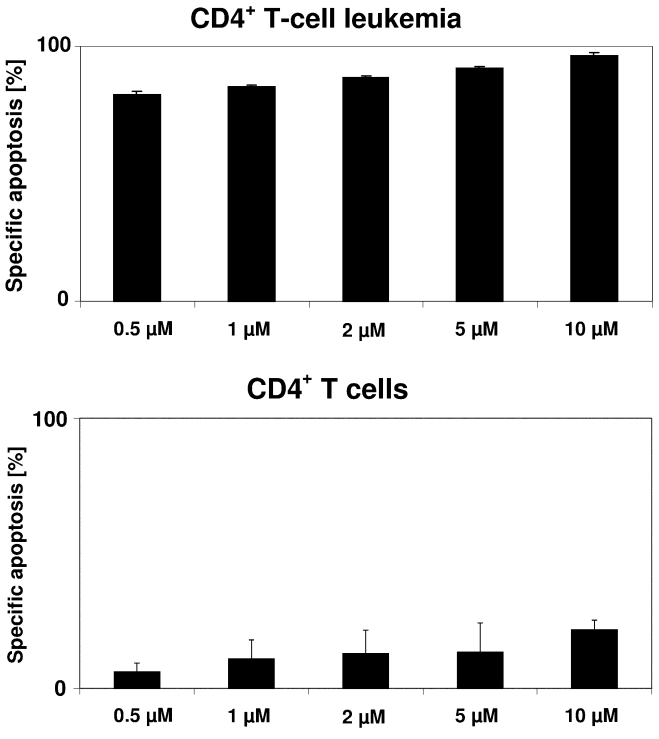

Fig. 1. Salinomycin induces apoptosis in human CD4+ T-cell leukemia cells, but not in normal CD4+ T cells. (A) Apoptosis induced by salinomycin in CD4+ T-cell leukemia cells obtained from a 71 year old male patient with acute T-cell leukemia. Similar results were obtained with CD4+ T-cell leukemia cells from two other patients. (B) Salinomycin failed to induce marked apoptosis in normal CD4+ T cells obtained from a healthy human. Similar results were obtained with CD4+ T cells from two other healthy humans. In all experiments, salinomycin was added to the cells for 24 h. All data are given as mean values ± SD of three independ experiments in triplicate.

onto PVDF membranes. Immunoblotting was performed using Vectastain ABC Kit (Linaris, Wertheim, Germany) followed by enhanced chemiluminescence (ECL, Pierce). Experiments were performed in triplicate with similar results.

Results and discussion

Salinomycin induces apoptosis and overcomes multiple mechanisms of apoptosis resistance in human cancer cells

In view of recent findings that salinomycin depletes human breast cancer stem cells in tumorspheres and inhibits the growth and metastasis of human breast cancer cells injected into NOD/SCID mice [9], we investigated the effects of salinomycin on different human cancer cells, including those that display anticancer drug resistance by different mechanisms. As shown in Fig. 1A, salinomycin induces massive apoptosis in human CD4+ T-cell leukemia cells isolated from the peripheral blood of a patient with acute T-cell leukemia (Fig. 1A). Similar results were obtained with CD4+ T-cell leukemia cells from two other patients (not shown). By contrast, salinomycin failed to induce marked apoptosis in normal CD4+ T cells isolated from the peripheral blood of a healthy human (Fig. 1B).

Furthermore, salinomycin induces apoptosis in a dose-dependent manner in human Molt-4 CD4+ T-cell leukemia cells (Fig. 2A), which express wild-type tumor suppressor protein p53 [10,11]. Since induction of apoptosis by many anticancer agents essentially depends on the expression of a functional (wild-type) p53 protein [22,23], we tested the effect of salinomycin on human Jurkat CD4+ T-cell leukemia cells which fail to express p53 protein due to a homozygous nonsense mutation in codon 196 of the p53 gene [12]. Similar to Molt-4 cells, Jurkat cells undergo apoptosis in a dose-dependent manner in response to exposure to salinomycin (Fig. 2C), indicating that salinomycin-induced apoptosis is independent of the p53 status of the cell. Moreover, Jurkat cells, which display resistance to apoptosis induced by cytostatic drugs and

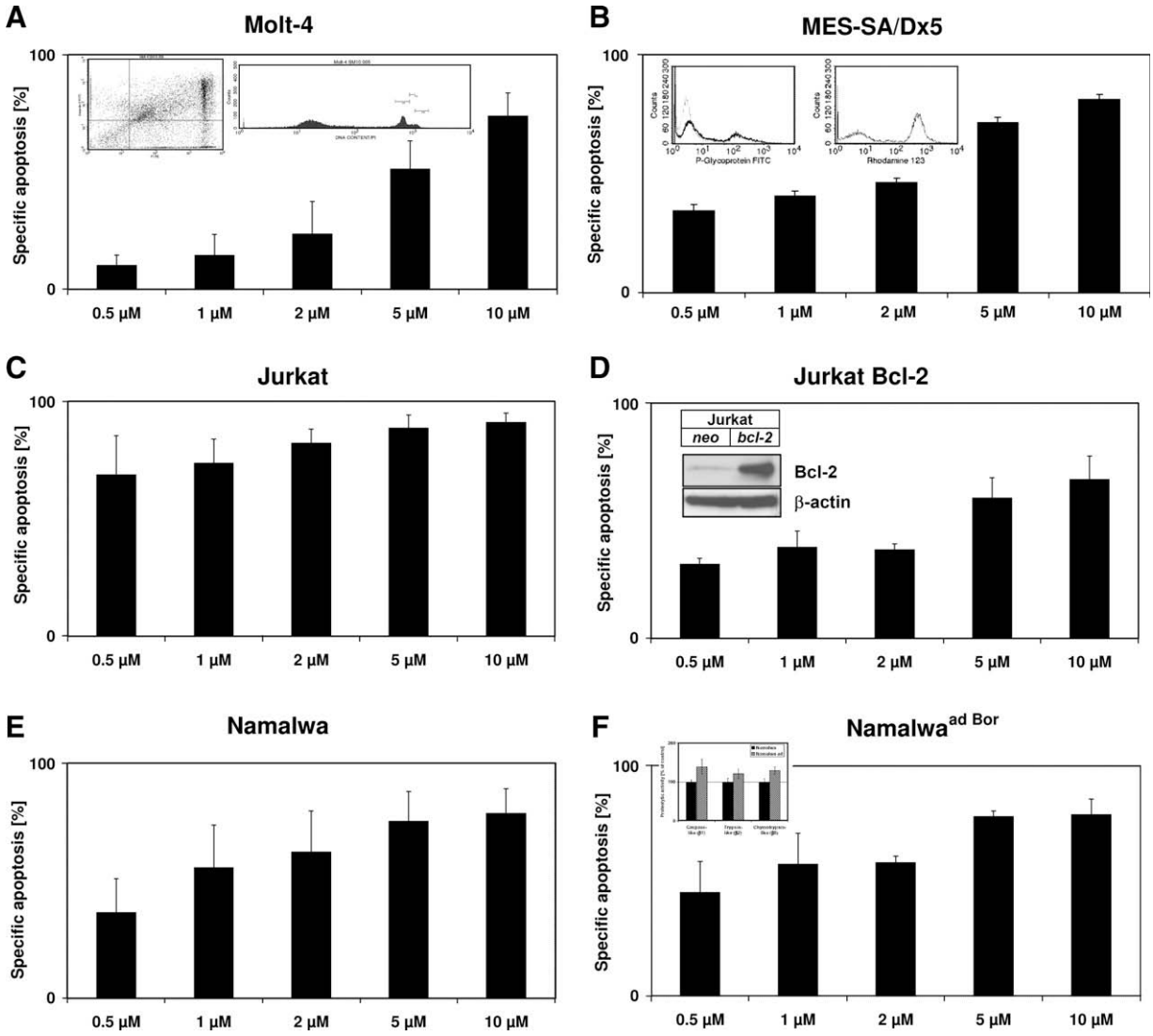

Fig. 2. Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cell lines. (A) Salinomycin-induced apoptosis in Molt-4 T-cell leukemia cells. Inserts show flow cytometric detection of apoptosis by annexin V bindings and propidium iodide uptake (left) and nuclear hypodiploid DNA content (right). (B) Salinomycin-induced apoptosis in MES-SA/Dx5 uterine sarcoma cells expressing P-glycoprotein (detected by flow cytometry, insert left) and eliminating rhodamine 123 by P-glycoprotein-mediated efflux (detected by flow cytometry, insert right). (C) Salinomycin-induced apoptosis in Jurkat T-cell leukemia cells, and (D) in Jurkat cells overexpressing Bcl-2 (demonstrated by Western blot, see insert). (E) Salinomycin-induced apoptosis in Namalwa Burkitt lymphoma cells, and (F) in Namalwa^{adBor} cells adapted to bortezomib. Insert shows the proteolytic activity of proteasomal subunits β1, β2 and β5 in Namalwa cells (black bars) and Namalwa^{adBor} cells (grey bars). In all experiments, salinomycin was added to the cells for 24 h. All data are given as mean values ± SD of three independ experiments in triplicate.

proteasome inhibitors due to induced overexpression of the anti-apoptotic protein Bcl-2 [13], undergo marked apoptosis in response to exposure to salinomycin (Fig. 2D). We next tested the ability of salinomycin to induce apoptosis in human Namalwa Burkitt lymphoma cells, which exhibit general apoptosis resistance and hyperproliferation due to increased expression and proteolytic activity of 26S proteasomes in response to adaptation of the cells to lethal concentrations of the proteasome inhibitor bortezomib [17,18]. Similar to their non-adapted counterparts, Namalwa^{adBor} cells undergo massive apoptosis in response to exposure to salinomycin (Fig. 2E and F), demonstrating that salinomycin can overcome apoptosis resistance due to overexpression and enhanced proteolytic activity of 26S proteasomes. Finally we demonstrate that salinomycin induces apoptosis in human MES-SA/Dx5 uterine sarcoma cells (Fig. 2B), which display resistance to a large panel of anticancer drugs by virtue of expression of the *mdr-1*-encoded P-glycoprotein, a 170 kDa transmembrane efflux pump that eliminates various drugs and small molecules from the cytosolic compartment [14–17]. Thus, salinomycin induces apoptosis in human cancer cells, but not in their non-malignant counterparts. Most importantly, salinomycin also induces apoptosis in cancer cells that exhibit resistance to apoptosis by multiple mechanisms.

Salinomycin belongs to a small group of *Streptomyces spp.*-isolated polyether antibiotics, including nigericin, monensin and boromycin that act as ionophores with a selectivity for monovalent cations and a preference for potassium [3,4,24]. Notably, nigericin and monensin have been shown to induce apoptosis in human lung cancer and lymphoma cells, respectively [25,26], suggesting that the common mechanism of polyether ionophore antibiotics to release potassium from the cytoplasm and/or mitochondria [3,4,24] contributes to the induction of apoptosis in cancer cells. This hypothesis is supported by previous data showing that a decrease in the intracellular potassium concentration is essential for induction of apoptosis [27,28].

Characterization of salinomycin-induced apoptosis

Since many apoptosis-inducing agents trigger intracellular accumulation and stabilization of p53 protein [29,30], we determined by immunoblot analysis the intracellular content of p53 in Molt-4 cells exposed to salinomycin. In contrast to the proteasome inhibitor bortezomib, salinomycin failed to induce accumulation and stabilization of p53 in Molt-4 cells (Fig. 3A and B), excluding the possibility that salinomycin induces p53-mediated apoptosis. This was further confirmed by demonstrating that pifithrin-α, a small molecule inhibitor of p53-dependent transcriptional activation and apoptosis [31], failed to inhibit salinomycin-induced apoptosis in Molt-4 cells (Fig. 3C). We next demonstrate that salinomycin fails to induce a cell cycle arrest in Molt-4 cells (Fig. S1). Many anticancer drugs including microtubule targeting agents, histone deacetylase inhibitors and proteasome inhibitors induce cell cycle arrest which proceeds or is accompanied by the induction of apoptosis [32]. Accumulation and stabilization of p53 and/or the cyclin-dependent kinase inhibitor p27 is one important mechanism for the induction of cell cycle arrest at the G1 phase [33,34], and we show that salinomycin, in contrast to bortezomib, does not induce an accumulation and stabilization of p53 and p27 leading to a G1 cell cycle arrest (Figs. 3A, 3B, S1). We next investigated the involvement of caspases in salinomycin-induced apoptosis. As shown in Fig. 4A, preincubation of Molt-4 cell with the general caspase inhibitor *N*-benzyloxycarbonyl-L-valyl-L-alanyl-L-aspartate fluoromethylketone (Z-VAD-fmk), which inhibits the activation of multiple caspases required for the initiation and execution of extrinsic and intrinsic apoptosis [35,36], failed to inhibit salinomycin-induced apoptosis. By contrast, proteasome inhibitor-induced apoptosis, which is known to

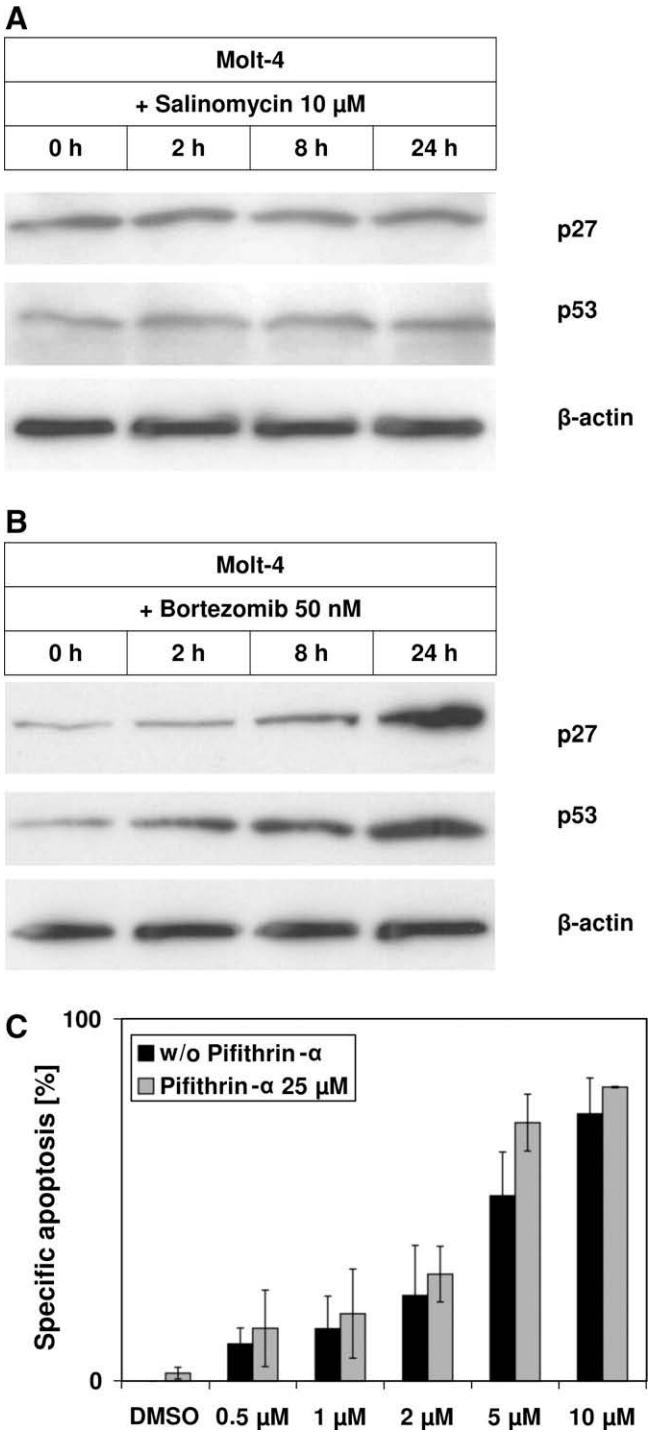

Fig. 3. Salinomycin-induced apoptosis is p53-independent and not accompanied by accumulation and stabilization of p53 and p27 leading to a G1 cell cycle arrest. Immunoblot analyses of accumulation of p53 and p27 in Molt-4 cells exposed to (A) salinomycin or (B) bortezomib. (C) Salinomycin-induced apoptosis is not inhibited by pifithrin-α. Molt-4 cells were incubated with pifithrin-α 30 min. before exposure for 24 h to salinomycin. Data are given as mean values ± SD of three independ experiments in triplicate.

be mediated by activation of caspases [37], was markedly inhibited by Z-VAD-fmk (Fig. 4B). Because apoptosis induced by various anticancer drugs is mediated by binding of cell membrane-bound and soluble CD95 ligand (CD95L) to its cognate receptor CD95 in an autocrine and paracrine fashion [38,39], we investigated the involvement of the CD95/CD95L system in salinomycin-induced

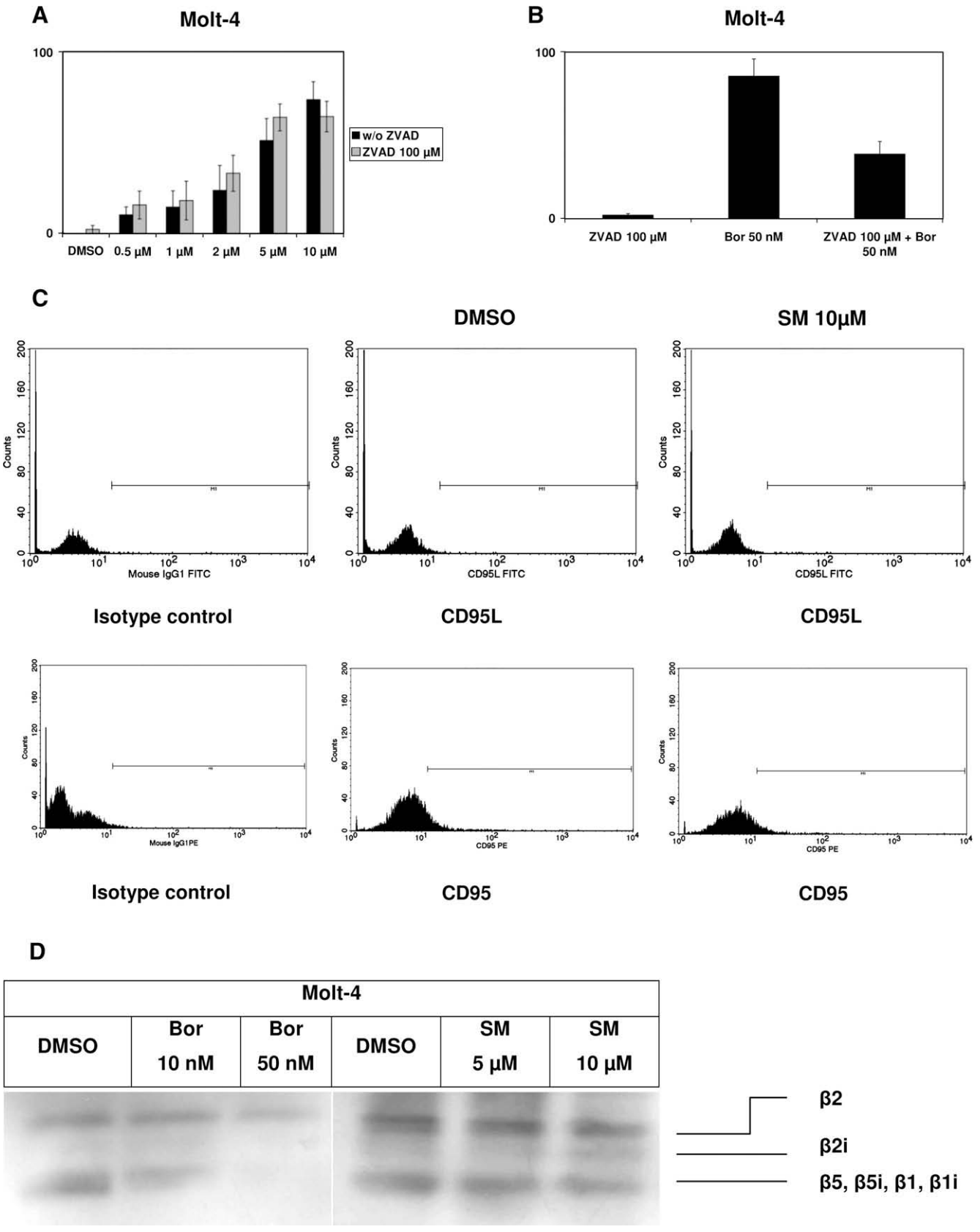

Fig. 4. Salinomycin-induced apoptosis is independent of caspase activation, the CD95/CD95L system and the proteasome. Molt-4 cells were incubated with Z-VAD-fmk 30 min. before exposure for 24 h to (A) salinomycin or (B) bortezomib. Data are given as mean values ± SD of three independ experiments in triplicate. (C) Flow cytometry analyses of cell surface expression of CD95L and CD95 on Molt-4 cells exposed for 8 h to salinomycin. Similar results were obtained after exposure of the cells for 12 and 16 h to salinomycin. One representative experiment out of four similar experiments is shown. (D) Affinity labeling of active proteasome subunits β1, β1i, β2, β2i, β5 and β5i after exposure of Molt-4 cells for 1 h to salinomycin or bortezomib.

apoptosis. As determined by flow cytometry, Molt-4 cells exhibit a low cell surface expression of CD95 that is not enhanced after exposure to salinomycin (Fig. 4C). Moreover, cells surface expression of CD95L was not observed in response to exposure to salinomycin (Fig. 4C), indicating that salinomycin-induced apoptosis is not mediated by paracrine or autocrine interactions of CD95L with CD95 and finally excluding an involvement of the CD95/CD95L system in salinomycin-induced apoptosis. In view of the fact that a considerable number of proteasome inhibitors constitute natural products isolated from bacteria and fungi [18,40], we tested the possibility that the *Streptomyces* product salinomycin is a proteasome inhibitor and induces apoptosis by inhibiting the proteasome. Immunoblot analysis of affinity-labeled proteolytically active proteasome subunits β1, β1i, β2, β2i, β5 and β5i revealed that salinomycin has no effect on the proteolytic activity of the proteasome in contrast to the specific proteasome inhibitor bortezomib (Fig. 4D), demonstrating that apoptosis induced by salinomycin does not occur as a result of proteasome inhibition.

We show here that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest and independent of p53, caspase activation, the CD95/CD95L system and the proteasome. Although the exact mechanism of salinomycin-induced apoptosis remains still unclear and needs further investigation, we show in this study that salinomycin is able to induce apoptosis in human cancer cells that exhibit resistance to apoptosis by overexpression of Bcl-2, P-glycoprotein or 26S proteasomes with enhanced proteolytic activity. However, many human cancer cells harbor or acquire such mechanisms of apoptosis resistance [17,18,41–43]. Therefore, salinomycin should be considered as a novel anticancer agent that not only depletes cancer stem cells [9], but also induces apoptosis and overcomes multiple mechanisms of resistance to apoptosis in human cancer cells. In view of some reported cases of accidental toxicity of salinomycin in animals and humans [44–46], future studies with salinomycin in humans should be designed carefully.

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