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Abbreviations: MM, multiple myeloma; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PS, phosphatidylserine; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; TMRE, tetramethylrhodamine ethyl ester.

Abstract

The proteasome inhibitor bortezomib is currently an important drug for treatment of relapsed and refractory multiple myeloma (MM) and for elderly patients. However, cells from some patients show resistance to bortezomib. We have evaluated the possibility of improving bortezomib therapy with Apo2L/TRAIL, a death ligand that induces apoptosis in MM but not in normal cells. Results indicate that cotreatment with low doses of bortezomib significantly increased apoptosis of MM cells showing partial sensitivity to Apo2L/TRAIL. Bortezomib treatment did not significantly alter plasma membrane amount of DR4 and DR5 but increased Apo2L/TRAIL-induced caspase-8 and caspase-3 activation. Apo2L/TRAIL reverted bortezomib-induced upregulation of β-catenin, Mcl-1 and FLIP, associated with the enhanced cytotoxicity of combined treatment. More important, some cell lines displaying resistance to bortezomib were sensitive to Apo2L/TRAIL-induced apoptosis. A cell line made resistant by continuous culture of RPMI 8226 cells in the presence of bortezomib (8226/7B) was highly sensitive to Apo2L/TRAIL-induced apoptosis. Moreover, RPMI 8226 cells overexpressing Mcl-1 (8226/Mcl-1) or Bcl-x_L (8226/Bcl-x_L) also showed enhanced resistance to bortezomib, but co-treatment with Apo2L/TRAIL reverted this resistance. These results indicate that Apo2L/TRAIL can cooperate with bortezomib to induce apoptosis in myeloma cells and can be an useful adjunct for MM therapy.

Keywords: bortezomib; TRAIL; Mcl-1; Bcl-x_L; apoptosis; myeloma

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (Apo2L/TRAIL) is a death messenger that induces apoptosis in many types of human hematological neoplasia [1] including multiple myeloma (MM) [2, 3]. An improved recombinant form of Apo2L/TRAIL (Apo2L/TRAIL.0), which exerts no toxicity in normal human cells [4], is being currently evaluated in clinical trials for non-Hodgkin's lymphoma [5] and could also be useful as an anti-myeloma weapon. We have recently evaluated the factors determining sensitivity of human MM cells to this recombinant form of Apo2L/TRAIL [3]. Expression in plasma membrane of at least one of the two death receptors for Apo2L/TRAIL (DR4 or DR5) and a significant expression level of caspase-8 are essential to elicit apoptosis. In some myeloma cells fulfilling these requirements, clustering of DRs in lipid rafts is also necessary for an efficient signaling [3]. However, data from a study with plasma cells from a significant number of MM patients indicate that only about 30% of samples undergo apoptosis upon TRAIL exposure [6].

On the other hand, the proteasome inhibitor bortezomib (Velcade) has emerged in the last years as a very useful drug for the treatment of relapsed and refractory MM [7], as well as in elderly patients not suitable for bone marrow transplantation [8]. The overall response rate to bortezomib in phase III trials was 43% [8], indicating that some patients show resistance to bortezomib or develop secondary resistance, not responding when they are rechallenged after relapsing from an earlier response. Despite of its undoubted benefits, bortezomib may cause thrombocytopenia [8], toxicity on activated T cells [9] and significantly impairs the immunostimulatory capacity of human dendritic cells [10]. The combined use of Apo2L/TRAIL with lower doses of bortezomib could be useful to alleviate the mentioned problems. Indeed,

bortezomib potentiates TRAIL-induced apoptosis in some types of solid tumors and this has been attributed to the increase of Bik [11, 12] or the decrease of FLIP [13]. In human hepatoma and human non–small cell lung cancer cells, bortezomib enhanced TRAIL-induced apoptosis in spite of increased expression of FLIP [14, 15] and this was associated in the latter case to DR5 up-regulation [15]. Data concerning the combined use of bortezomib and TRAIL in hematological neoplasias are scarce. Bortezomib sensitized some primary effusion lymphoma cells to TRAIL-induced apoptosis [16] and cooperated with TRAIL to induce apoptosis in Jurkat cells overexpressing Bcl-2 [17]. In a recent study, bortezomib potentiated apoptosis in TRAIL-sensitive but not in TRAIL-resistant mantle cell lymphoma [18]. In a single experiment, bortezomib potentiated TRAIL-induced apoptosis in MM.1S myeloma cells [2] though the TRAIL recombinant form used (LZ-TRAIL) has shown to be toxic for keratinocytes [19] and hepatocytes [14], and not suitable for *in vivo* use. The problem of toxicity of recombinants forms of TRAIL for normal human cells has been discussed in detail elsewhere [3].

In the present work we have evaluated the ability of Apo2L/TRAIL to cooperate with bortezomib in apoptosis induction in myeloma, as well as the mechanism underlying their cooperation. Results indicate that the apoptotic signals elicited by each of these agents mutually reinforce and that Apo2L/TRAIL can revert several antiapoptotic outcomes of bortezomib treatment in myeloma cells.

2. Materials and Methods

2.1 Materials

Recombinant Apo2L/TRAIL lacking exogenous sequence tags (Apo2L/TRAIL.0, residues 114-281) was kindly provided by Genentech (South San Francisco, CA, USA). The proteasome inhibitor bortezomib (Velcade) was provided by Millenium Pharmaceuticals (Cambridge, MA, USA). MTT was from Sigma (Madrid, Spain).

2.2 Cell cultures

All cells were routinely cultured at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine and penicillin/streptomycin (hereafter, complete medium).The following human multiple myeloma (MM) cell lines were used: MM.1S, kindly provided by Dr. Atanasio Pandiella (CIC, Salamanca, Spain), RPMI 8226 from Dr. Martine Amiot (INSERM, Nantes, France) and NCI-H929 from the ATCC. IM-9, a human B-leukemia from a patient with multiple myeloma was from the ATCC. SUDHL-4 was from DSMZ (Germany). A bortezomib-resistant MM cell subline (8226/7B) was generated from RPMI 8226 cells by serial cultivation over 18 months with increasing concentrations of bortezomib (from 2 to 100 nM) and recovery of surviving cells. For the generation of Mcl-1-overexpressing RPMI 8226 cells (8226/Mcl-1), the cDNA encoding human Mcl-1 (kindly provided by Dr. Xiandong Wang, University of Texas, USA) was cloned into a retroviral expression vector containing GFP as a selectable marker (pLZR-IRES-GFP-Mcl-1). RPMI 8226 cells were transfected by retroviral infection as described previously [20]. Clones derived from single cells were obtained by limiting dilution and cells expressing significant amounts of GFP were expanded. Clones were tested for the expression of Mcl-1 by Western blotting, and those exhibiting increased expression of Mcl-1 relative to

controls were selected for experimental use. Cells containing empty vector (pLZR-IRES-GFP) were used as suitable controls. Bcl-x_L-overexpressing cells were generated from the retroviral construct MMP-1-AU1-Bcl-x_L, generously provided by Dr. X Pimentel-Muiños (CIC, Salamanca). Forty-eight hours after retroviral infection of RPMI 8226 cells, overexpression of Bcl-x_L was analyzed by Western blotting.

2.3 Cell proliferation and apoptosis assays

For toxicity assays, cells (3-5x10^5 cells/ml) were treated in flat-bottom, 24-well (1 ml/well) or 96-well plates (100 μl/well) with different concentrations of Apo2L/TRAIL (2–200 ng/ml), bortezomib (2-80 nM) or both, in complete medium, for the indicated times. In combination experiments, MM cells were preincubated with bortezomib for 1 h prior to treatment with Apo2L/TRAIL. Cell proliferation was determined by a modification of the MTT-reduction method [21] and apoptosis by determining PS exposure and $\Delta\Psi_m$ loss by flow cytometry, as indicated later. The cytotoxic effect of combinations of Apo2L/TRAIL and bortezomib was analyzed by calculating the combination indexes (CI) by the Chou-Talalay method [22], using the CalcuSyn 2.0 software (Biosoft, Great Shelford, UK). Briefly, data from cell toxicity assays were expressed as fraction of cells affected by the dose in drug-treated cells compared with untreated cells (controls). CalcuSyn program is based on the Chou-Talalay method according to the following equation: CI = (D)1/(Dx)1 + (D)1(D)2/(Dx)1(Dx)2, where (D)1 and (D)2 are the doses of drug 1 and drug 2 that have the same x effect when used alone, and CI is the combination index. CI < 1.0 indicates synergism; CI ≈ 1.0 indicates an additive effect and CI > 1.0 an antagonistic effect. MM.1S and H929 cells were treated for 24 h with a constant concentration of bortezomib and increasing concentrations (0-100 ng/ml) of Apo2L/TRAIL and the apoptosis evaluated

by flow cytometry as described below. CI values for each combination were obtained from three different experiments fulfilling experimental prerequisites of the Calcusyn program.

2.3 Flow cytometry analysis

Plasma membrane expression of TRAIL receptors was analyzed by sequential incubation of cells (2 x 10^5 cells in 200 μl) at room temperature for 30 min, with either mouse anti-DR4 (clone DJR1) or anti-DR5 (clone DJR2-4), both from eBioscience (USA), and rabbit anti-mouse IgG-Alexa 488 (Invitrogen). In cell lines transfected with pLZR-GFP constructs, the secondary antibody used was a goat anti-mouse IgG-PE (Invitrogen). Quantitative determination of apoptosis was performed by the simultaneous determination of phosphatidylserine (PS) exposure and mitochondrial membrane potential ($\Delta\Psi_m$) in the same cells, as indicated [23]. Briefly, cells (2.5x10^5 in 200 μl) were incubated with 2 nM $DiOC_6(3)$ (Invitrogen) at 37°C for 10 min in annexin-binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.4). Then, 0.5 μl annexin V-PE (Invitrogen) were added and incubated at room temperature for 15 min. In cells showing non-specific binding of annexin-PE, annexin V-FITC was used and $\Delta\Psi_m$ determined separately with tetramethylrhodamine ethyl ester (TMRE, Invitrogen). In GFP-expressing cells, $\Delta\Psi_m$ was also determined with TMRE [24]. Cells were incubated with 60 nM TMRE at 37ºC for 20 min in complete medium. In all cases, cell suspensions were diluted to 0.5 ml with annexin-binding buffer or culture media and analyzed in a flow cytometer (FACScan, BD Biosciences). Intracellular amount of active caspase-3 was determined by immunolabeling with an anti-active caspase-3-FITC mAb (clone C92-605, BD

Biosciences). After the different treatments, cells (1x 10^6) were fixed in 4% paraformaldehyde in PBS (15 min, 4ºC), permeabilized with 0.1% saponin in PBS containing 5% goat serum (15 min) and incubated with the mAb at 20ºC for 30 min. Cells were resuspended in 500 μl PBS and analyzed by flow cytometry.

2.4 Western blot analysis

The amount of several cytosolic proteins involved in apoptosis was determined by Western blotting of cell extracts. MM cells were lysed in a 50 mM Tris/HCl pH 7.4 buffer containing 1% Triton X-100 [3]. Solubilized proteins from equal numbers of Trypan-blue negative cells (1x10^6 cells/lane) were resolved by SDS-12%PAGE, transferred to nitrocellulose membranes and incubated with one of the following antibodies: anti-caspase-8 (clone 5F7, MBL), anti-c-FLIP (NF6, Alexis), anti-Mcl-1 (sc-819) and anti- Bcl-x_L (sc-1041) both from Santa Cruz Biotechnology, anti-XIAP (clone 28), anti-β-catenin (clone 14) both from BD Biosciences, and anti-β-actin (AC-15, Sigma). Membranes were next incubated with the corresponding phosphatase alkaline-labeled secondary antibody (Sigma) and revealed with BCIP/NBT, as described [25].

3. Results

3.1 Cooperation between bortezomib and Apo2L/TRAIL against MM cells

We have previously shown that the sensitivity of MM cells to Apo2L/TRAIL depends on a sufficient amount of plasma membrane DR4 or DR5 receptors and of cytosolic caspase-8 [3]. According to their respective DR4 and DR5 expression levels, RPMI 8226 cells are highly sensitive and H929 and MM.1S partly sensitive to Apo2L/TRAIL. IM-9 cells, which express low levels of caspase-8 [2, 3, 26], the main initiator caspase in TRAIL pathway [26], are barely sensitive to Apo2L/TRAIL-induced apoptosis. All of these cell lines were sensitive to bortezomib, though they displayed significant differences among their corresponding IC_{50} values (Fig. 1), being H929 the most sensitive and RPMI 8226 the most resistant. We first evaluated if bortezomib could improve the apoptotic-inducing ability of Apo2L/TRAIL in MM cells showing partial sensitivity to Apo2L/TRAIL. H929 and MM.1S cells were incubated with increasing concentrations of Apo2L/TRAIL in the presence of a concentration of bortezomib equal or lower than their IC_{50} (Fig. 1) and apoptosis evaluated by the simultaneous determination of $\Delta\Psi_m$ loss (Fig. 2) and PS exposure (which give comparable results). In these conditions, apoptosis induced by Apo2L/TRAIL in both H929 and MM.1S cells was greatly increased by bortezomib (Fig. 2). The application of Chou-Talalay method indicated that, under these conditions, the combination of TRAIL and bortezomib had an additive to slightly synergic effect (combination indexes raging from 1 to 0.71). However, bortezomib was unable to sensitize IM-9 cells to Apo2L/TRAIL (Fig. 2c). Therefore, bortezomib can cooperate with Apo2L/TRAIL to increase apoptosis in MM cells showing partial sensitivity to this death ligand.

3.2 Characteristics of apoptosis induced by the combination of Apo2L/TRAIL and bortezomib

To gain insight into the mechanism of cooperation in apoptosis, we analyzed the effect of bortezomib and TRAIL on the relative amounts of some proteins, relevant to their respective mechanisms of apoptosis, in MM cells. Bortezomib treatment for 6, 12 or 24 h (Fig.3), did not significantly modify the plasma membrane expression of DR4 and DR5 in H929 and MM.1S cells. Caspase-8, the main initiator caspase of extrinsic route, was strongly activated in cells incubated with TRAIL alone or combined with bortezomib, as assessed by the disappearance of its specific substrate, Bid (Fig. 4). The amount of c-FLIP$_L$, the main endogenous regulator of caspase-8 activation, increased with bortezomib treatment but the combination of Apo2L/TRAIL and bortezomib led to a decrease of both FLIP isoforms (Fig. 4). Combined treatment with TRAIL and bortezomib also increased processing of the endogenous inhibitor of apoptosis XIAP (Fig. 4). Apo2L/TRAIL reverted bortezomib-induced upregulation of the antiapoptotic protein Mcl-1, as well as of the prosurvival protein β-catenin, associated with the enhanced cytotoxicity of combined treatment (Fig. 4). The mutual potentiation of apoptosis by the simultaneous treatment with Apo2L/TRAIL and bortezomib resulted in the increased generation of active caspase-3 in both H929 and MM.1S cells (Fig. 5). Taken together, these results suggested that cotreatment with Apo2L/TRAIL reverted some of the prosurvival or antiapoptotic side effects elicited by bortezomib resulting in a reinforcement of the apoptotic signal.

3.3 Apo2L/TRAIL induces apoptosis in bortezomib-resistant cell lines

We next analyzed if Apo2L/TRAIL could be useful to boost apoptosis in myeloma cells showing enhanced resistance to bortezomib. First, we generated a resistant cell

line (8226/7B) by continuous culture of RPMI 8226 cells in the presence of increasing concentrations of bortezomib. As shown in Fig. 6, the DL_{50} of bortezomib increased around 5-fold in these cells (15 nM in parental RPMI 8226 cells versus 75 nM in 8226/7B cells). Resistance of 8226/7B cells to bortezomib was greater than that described for 8226/R5 cells (IC_{50}, 42 nM), another 8226-derived cell line showing resistance to this drug [27] and also greater than the resistance of SUDHL-4 lymphoma cells (Fig. 6), previously used in several works as a model of bortezomib resistance [28-30]. In spite of their resistance to bortezomib, Apo2L/TRAIL efficiently induced apoptosis in 8226/7B cells (Fig. 6), which still expressed significant plasma membrane amounts of DR4 and DR5 (Fig. 7B). Other possible cause of bortezomib resistance could be the increased expression of Mcl-1 protein. Bortezomib treatment increases the intracellular amount of the anti-apoptotic protein Mcl-1 (Fig. 4) and this has been interpreted as a restraint reducing its apoptotic potency [31, 32]. To better evaluate the possible contribution of the increased expression of Mcl-1 to bortezomib resistance, we generated Mcl-1-overexpressing cells (8226/Mcl-1) by retroviral infection of RPMI 8226 cell line. 8226/Mcl-1 cells showed a lower sensitivity to Apo2L/TRAIL than vector-transfected cells (Fig. 7) and this was associated with a reduced expression of DR4 and DR5 in their plasma membrane (Fig. 7). 8226/Mcl-1 cells also displayed increased resistance to bortezomib compared to vector-transfected cells (Fig. 8). However, when Apo2L/TRAIL and bortezomib were combined a significant increase in the amount of apoptotic 8226/Mcl-1 cells occurred (Fig. 8B). Moreover, myeloma resistance to chemotherapeutic agents has been also linked to Bcl-x_L expression [33, 34]. The expression of this antiapoptotic protein may largely vary among MM cell lines [35]. To evaluate if Apo2L/TRAIL could be useful to improve the apoptotic-inducing ability of bortezomib in MM cells expressing high

amounts of Bcl-x_L, we generated a RPMI 8226-derived cell line overexpressing this antiapoptotic protein (8226/Bcl-x_L). Results indicated that 8226/Bcl-x_L were indeed highly resistant to bortezomib treatment (Fig. 8). However, these cells were still sensitive to Apo2L/TRAIL (Fig. 7), though less than vector-transfected cells, and the combination of TRAIL with bortezomib greatly increased this cytotoxicity (Fig. 8).

4. Discussion

The proteasome inhibitor bortezomib is an important drug for the treatment of relapsed and refractory myeloma and is also useful in first-line therapy [36]. However, the appearance of a significant number of patients with MM cells resistant to bortezomib, its immunosuppressive effect on activated T cells and other side effects support its combination with another drug to improve its clinical benefits. A recombinant form of the death ligand Apo2L/TRAIL (Apo2L/TRAIL.0) may induce apoptosis in human myeloma cells while preserving normal cells. This feature makes Apo2L/TRAIL an attractive candidate to improve or complement therapy with bortezomib. However, there are virtually no data concerning the potential usefulness of this combination for myeloma therapy. There is a single report describing an additive effect of TRAIL when combined with bortezomib in the MM.1S cell line [2]. However this result was obtained with LZ-TRAIL, a recombinant form toxic for normal human keratinocytes [19, 37] and hepatocytes [14, 15], and thus not suitable for its *in vivo* use. With regard to the possible mechanism of cooperation, it has been described that bortezomib potentiates TRAIL-induced apoptosis in a variety of adherent tumor cell lines. This effect has been attributed to either DR5 up-regulation or FLIP down-regulation [14, 15].

We show here that Apo2L/TRAIL and bortezomib indeed cooperate in apoptosis induction in MM cells provided that they display a measurable sensitivity to TRAIL. Consequently, bortezomib did not increased TRAIL-induced apoptosis in the insensitive cell line IM-9. These results agree with those obtained in other hematological neoplasias. Bortezomib potentiated the toxicity of TRAIL in two primary effusion B-lymphoma cell lines showing partial sensitivity to TRAIL, but not in Jurkat T-cells insensitive to TRAIL [16]. Bortezomib also potentiated apoptosis in TRAIL-

sensitive but not in TRAIL-resistant lines of mantle cell lymphoma [18]. Cooperation was not due to a strengthening of initial steps of TRAIL signaling since bortezomib does not alter DR4 and DR5 receptor expression. The effect seems to be rather due to the reversion by TRAIL of some anti-apoptotic side effects that occur during apoptosis elicited by bortezomib. In particular, TRAIL reverted the bortezomib-induced accumulation of c-FLIP, the main endogenous regulator of caspase-8 activation, and that of β-catenin, a transcription factor of the Wnt pathway which controls growth, survival, and migration of MM cells [38] and is up-regulated by inhibition of its proteasomal degradation [39]. In its turn, bortezomib could improve the apoptotic efficiency of TRAIL by blocking NF-κB activation, a transcription factor involved in the resistance to TRAIL in myeloma [40].

On the other hand, our results indicate that Apo2L/TRAIL may be a useful adjunct in the therapy of some myeloma plasma cells displaying enhanced resistance to bortezomib. Apo2L/TRAIL efficiently killed MM cells made resistant to bortezomib by prolonged exposition to this drug. Apo2L/TRAIL may be also useful in bortezomib resistance due to increased expression of Mcl-1 or Bcl-x_L. Mcl-1 is an antiapoptotic protein from Bcl-2 family, highly expressed in MM cells [41] and critical for their survival [42, 43]. The intracellular amount of Mcl-1 increase during bortezomib treatment and this has been interpreted as a hindrance to the apoptotic effect of the drug [31, 32]. We confirm here that enhanced expression of Mcl-1 in RPMI 8226 cells is associated to increased resistance to bortezomib. However, these cells were still sensitive to Apo2L/TRAIL and the combination of TRAIL and bortezomib overcame bortezomib resistance. We previously reported that another MM cell line overexpressing Mcl-1 (U266/Mcl-1) was also sensitive to Apo2L/TRAIL [3]. Cotreatment with Apo2L/TRAIL and bortezomib also enhanced apoptosis in Bcl-x_L

overexpressing cells (8226/Bcl-x_L), highly resistant to bortezomib.

In summary, Apo2L/TRAIL could be a useful adjunct for bortezomib in multiple myeloma therapy. Low doses of bortezomib can potentiate Apo2L/TRAIL-induced apoptosis in cells partly sensitive to this death ligand. More important, Apo2L/TRAIL can induce apoptosis in MM cells displaying resistance to bortezomib. This resistance may be due to either repeated exposure to bortezomib or to overexpression of the antiapoptotic proteins Mcl-1 or Bcl-x_L. Apo2L/TRAIL could therefore improve the therapeutic efficiency of bortezomib towards myeloma without eliciting side effects.

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Legend of Figures

Fig. 1. Effect of bortezomib treatment on proliferation of MM cells. Cells were incubated with increasing concentrations of bortezomib for 24 h and cell viability determined by the MTT-reduction assay. Results are expressed as percent of cell growth relative to untreated cells and are the mean of four replicates from three different experiments (bars, SD).

Fig. 2. Cooperation of TRAIL and bortezomib in apoptosis induction in MM cells. A) H929, B) MM.1S and C) IM-9 cells were treated for 24 h with increasing concentrations of Apo2L/TRAIL in the presence or absence of 4 nM bortezomib (H929, MM.1S) or 10 nM bortezomib (IM-9). Apoptosis was quantified by determining $\Delta\Psi_m$ loss by flow cytometry with $DiOC_6(3)$. Data points are the mean of 3-4 triplicate determinations; bars, SD. Numbers on each point indicate combination indexes (CI), calculated with the CalcuSyn software.

Fig. 3. Bortezomib does not modify plasma membrane amount of cytotoxic TRAIL receptors. Cells were incubated with 4 nM bortezomib for 6h and plasma membrane expression of DR4 and DR5 determined by FACS analysis. Similar results were obtained after 12 or 24 h incubation with bortezomib. Numbers on histograms indicate the mean fluorescence intensity (MFI) of each peak. Solid line, isotypic control, dotted line, anti-DR4 or DR5 labelling.

Fig. 4. Effect of Apo2L/TRAIL and bortezomib, alone or in combination, on caspase-8, Bid, FLIP, XIAP, β-catenin and Mcl-1 protein expression. H929 and MM.1S cells were treated with either 4 nM bortezomib (B), 100 ng/ml Apo2L/TRAIL (T) or both

(B+T) for 24 h and protein expression analysed by Western blotting with specific antibodies.

Fig. 5. Combination of Apo2L/TRAIL and bortezomib increases caspase-3 activation in MM cells. A) H929 and MM.1S cells were incubated for 24 h with either 100 ng/ml Apo2L/TRAIL, 4 nM bortezomib or both, as indicated, and the amount of active caspase-3 determined by flow cytometry with a FITC-labeled anti-active caspase-3 antibody. A representative experiment is shown. B) Mean ± SD values of caspase-3 activation from the independent experiments for each cell line.

Fig. 6. Apo2L/TRAIL induces apoptosis in MM cells resistant to bortezomib. A) Effect of bortezomib on growth of SUDHL-4 and 8226/7B cells. Cells were incubated for 24 h with increasing concentrations of bortezomib and cell viability determined by the MTT reduction assay. B) Apo2L/TRAIL-induced apoptosis in 8226/7B cells. Cells were incubated with the indicated concentrations of Apo2L/TRAIL and apoptosis evaluated by annexin V-FITC binding and flow cytometry. Data points are the mean of quadruplicate determinations on three different experiments; bars, SD.

Fig. 7. Effect of Apo2L/TRAIL in RPMI 8226 cells overexpressing Mcl-1 or Bcl-x_L. A) Induction of apoptosis by Apo2L/TRAIL in 8226/Mcl-1 and 8226/Bcl-x_L cells. Cells were incubated with the indicated concentrations of Apo2L/TRAIL for 24 h and apoptosis determined by measuring $\Delta\Psi_m$ loss with TMRE by flow cytometry. Points are the mean of four replicate determinations on three different experiments; bars, SD. B) Flow cytometry analysis of DR4 and DR5 expression in plasma membrane of

RPMI 8226, 8226/7B, 8226/Mcl-1 and 8226/Bcl-x_L cells. Solid line, isotypic control; dotted line, anti-DR4 or anti-DR5. Insert numbers indicate MFI for each peak.

Fig. 8. Cooperation between TRAIL and bortezomib in apoptosis induction in Mcl-1 or Bcl-x_L-overexpressing cells. A) Toxicity of bortezomib in 8226/Mcl-1 and 8226/Bcl-x_L cells. Cells were incubated with increased concentrations of bortezomib for 24 h and apoptosis determined by measuring $\Delta\Psi_m$ loss with TMRE by flow cytometry. B) Effect of combination of bortezomib with Apo2L/TRAIL in apoptosis induction in 8226/Mcl-1 and 8226/Bcl-x_L cells. Cells were incubated or not (controls, C) with the indicated concentration of bortezomib, Apo2L/TRAIL or both for 24 h and apoptosis determined by measuring $\Delta\Psi_m$ loss with TMRE by flow cytometry. Similar results were obtained when measuring PS exposure with annexin V-PE. Points and columns are mean of quadruplicate (A) or triplicate (B) determinations; bars, SD. $*$ P <0.05; $**$ P < 0.01; $***$ P < 0.001.

Fig. 1

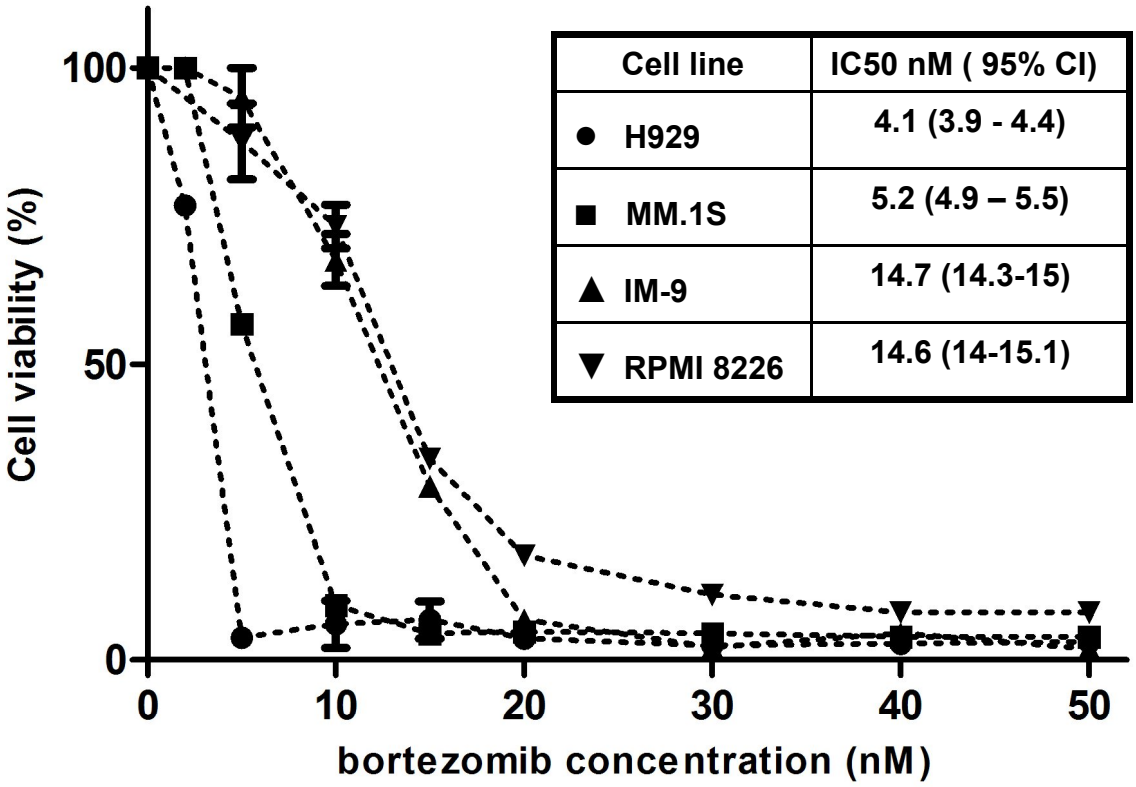
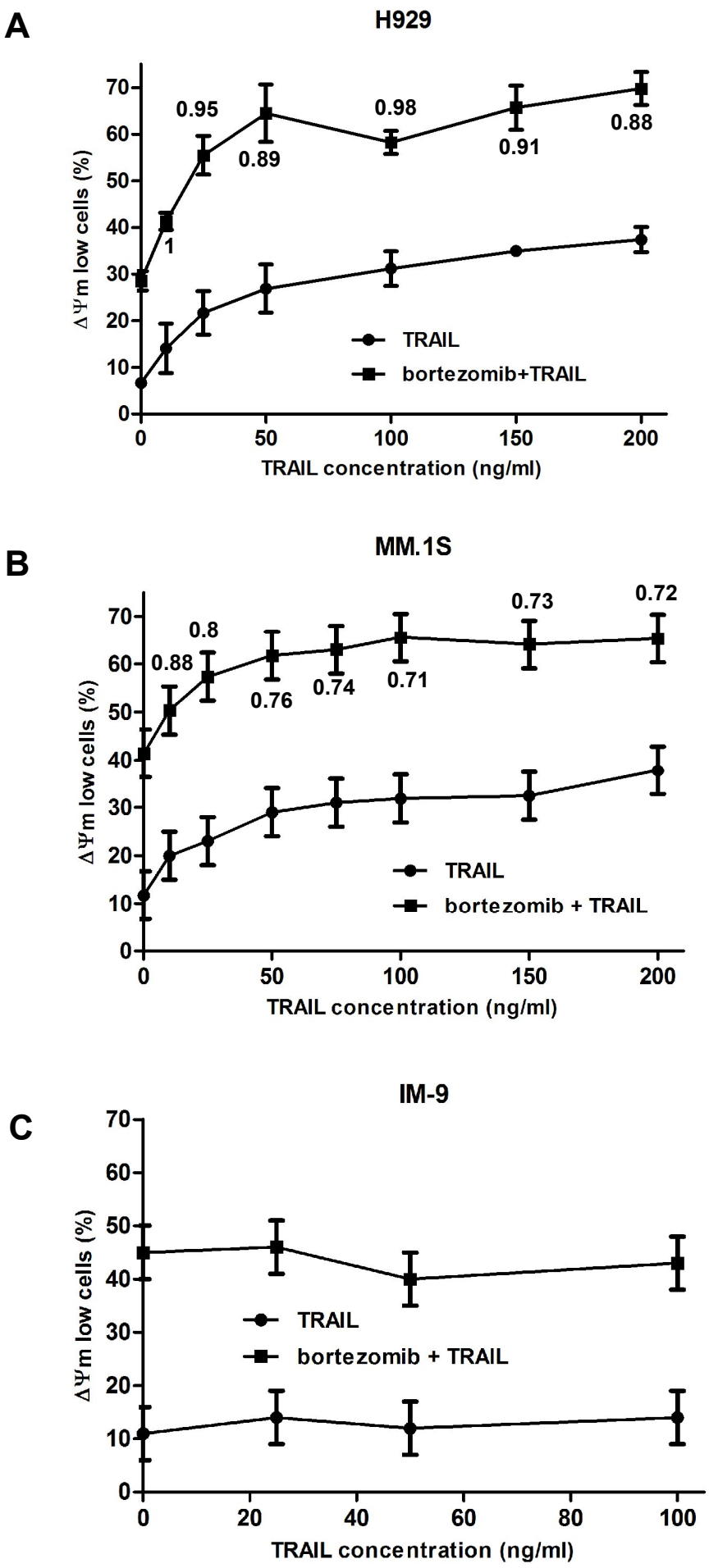


Fig.2



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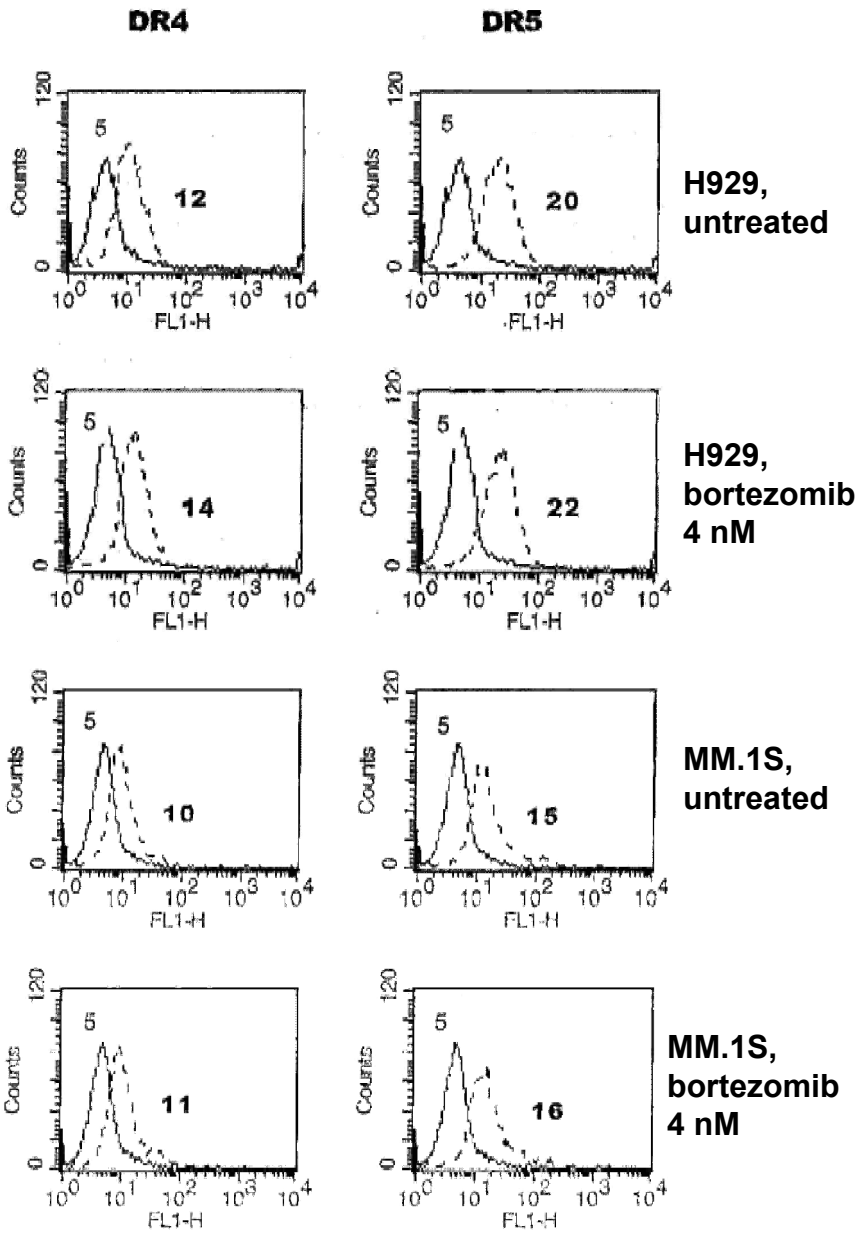


Fig. 4

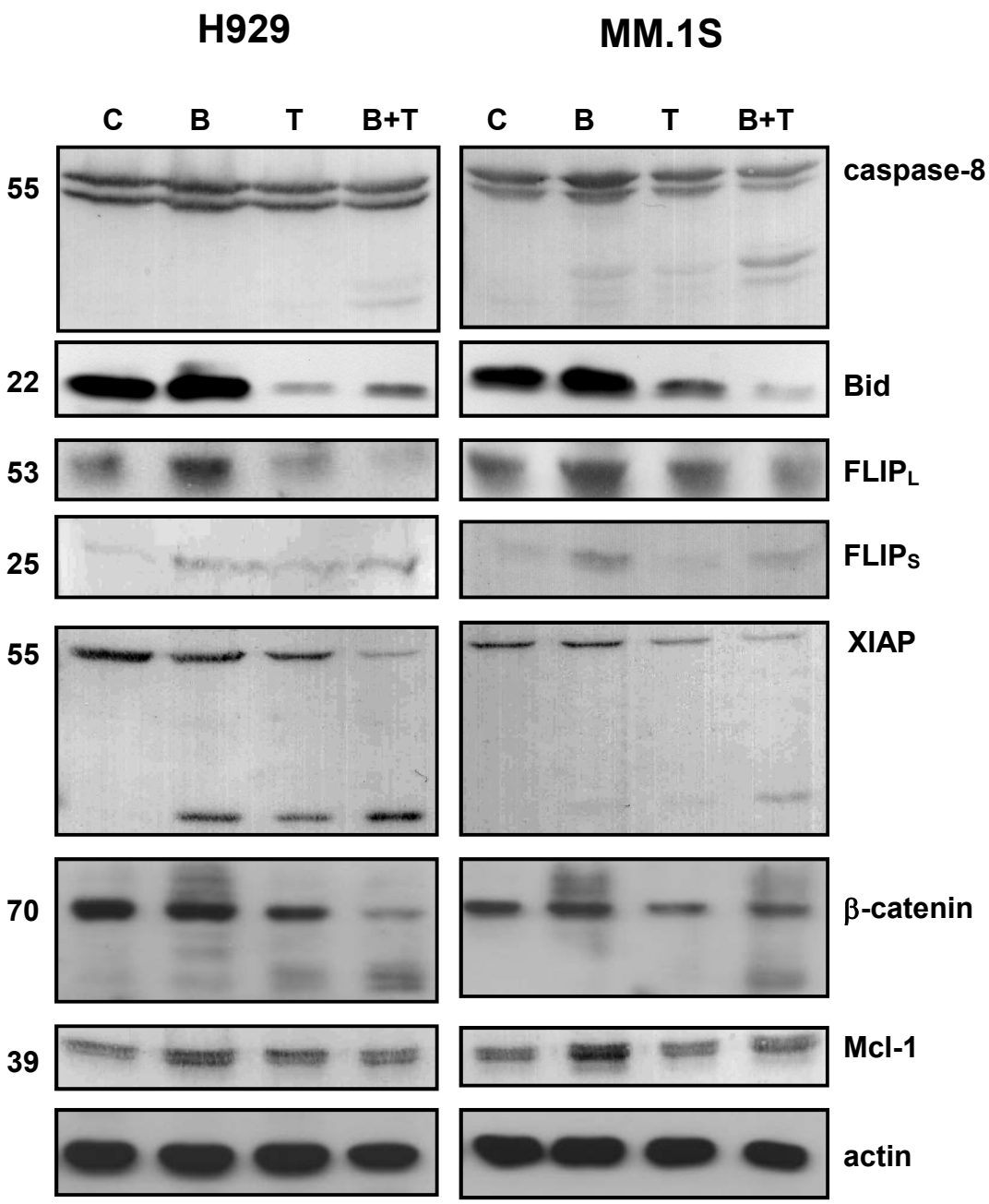
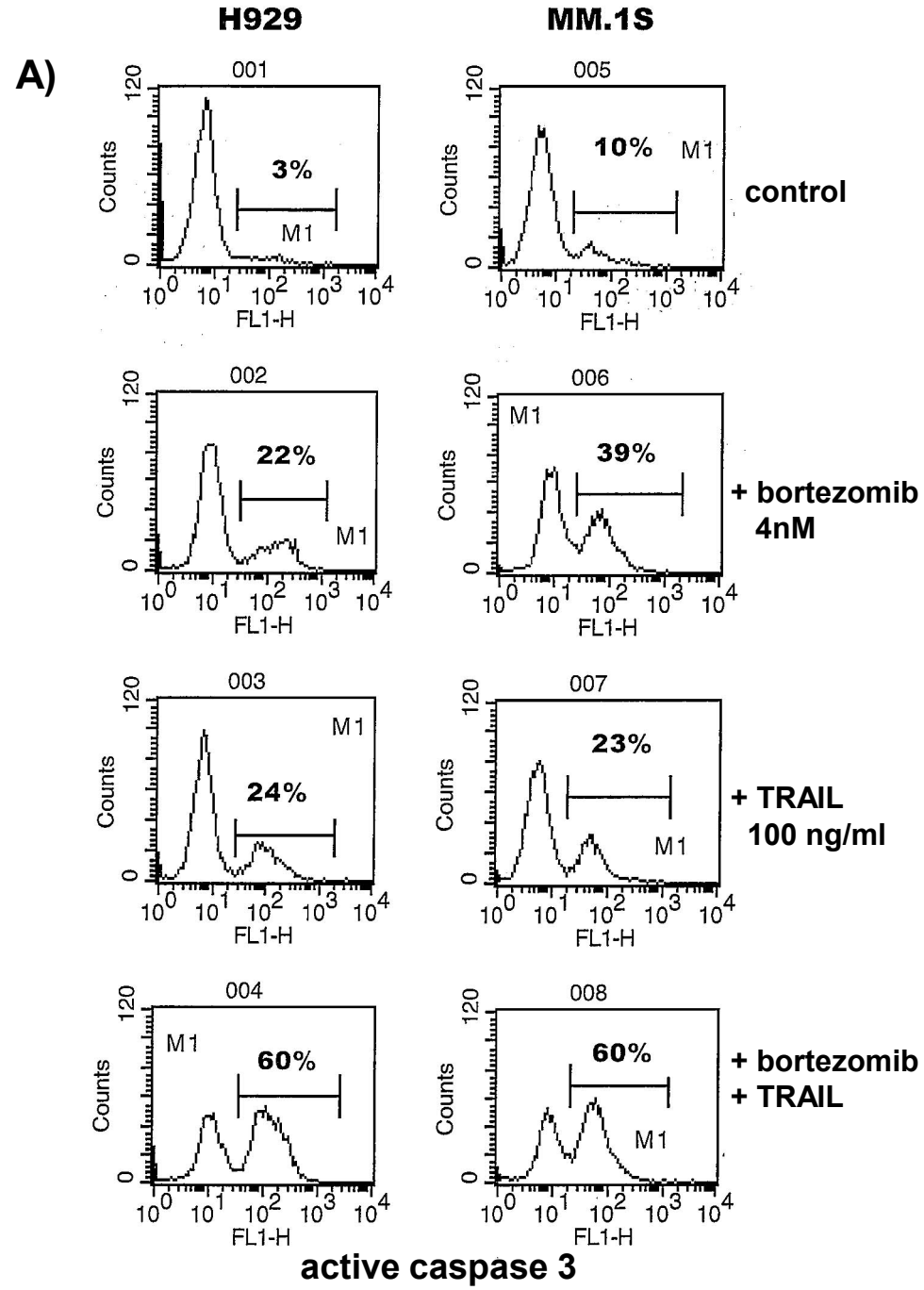


Fig. 5



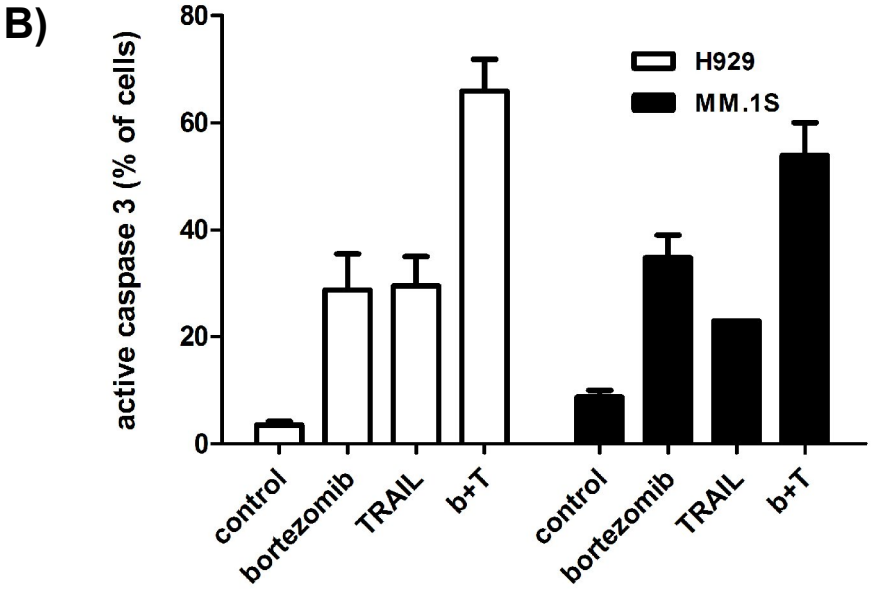


Fig. 6

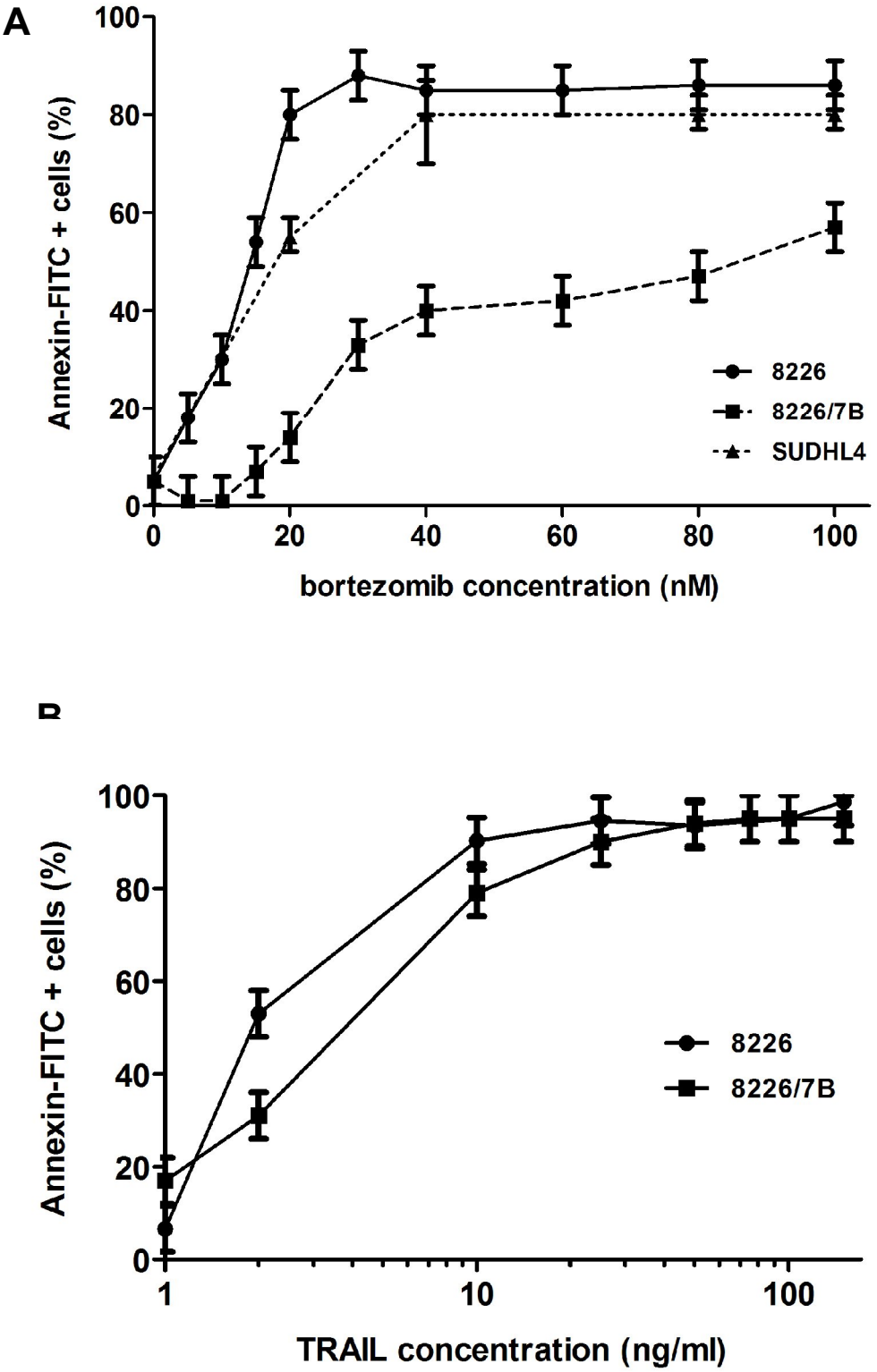


Fig. 7

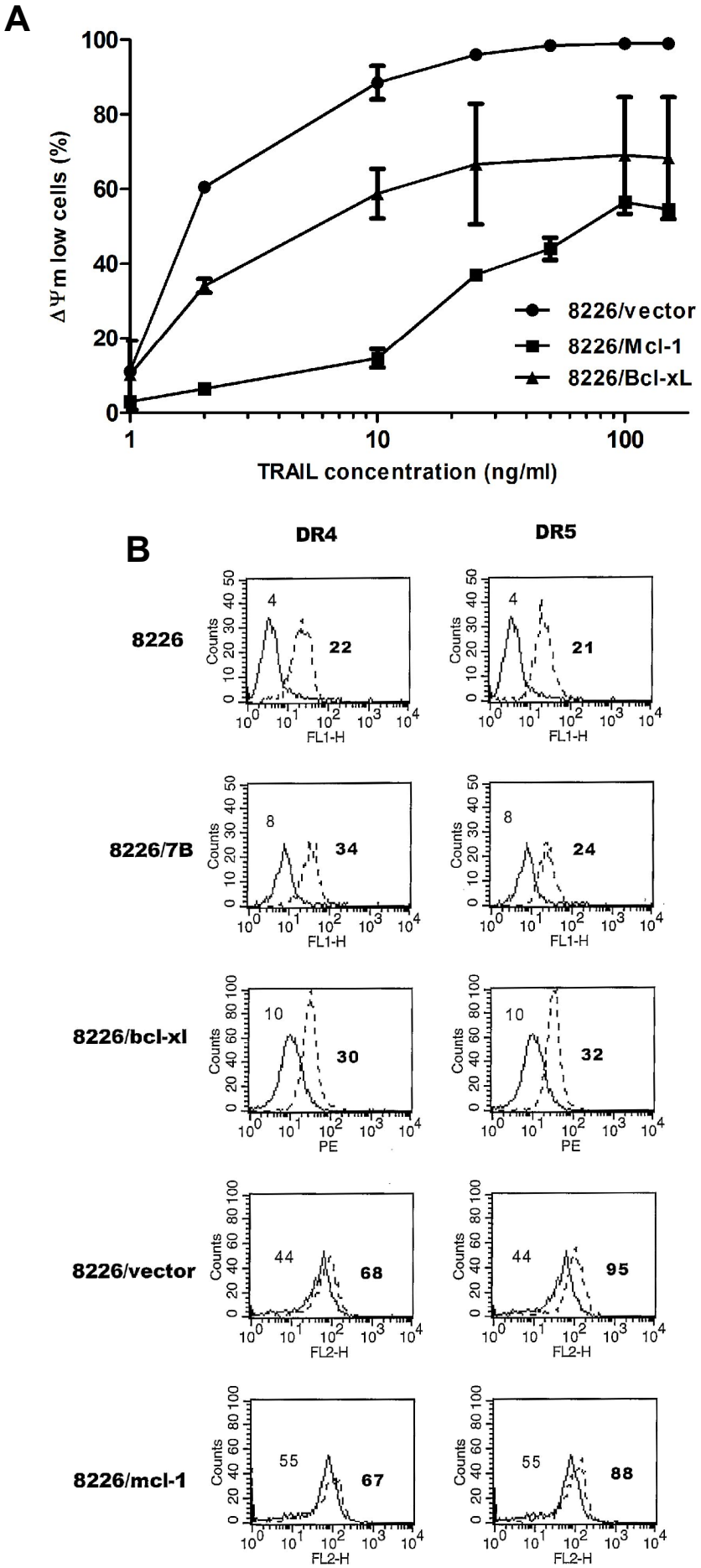


Fig. 8