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DOWNREGULATION OF THE ANTIAPOPTOTIC MCL-1 PROTEIN AND APOPTOSIS IN MA-11 BREAST CANCER CELLS INDUCED BY AN ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR–*PSEUDOMONAS* EXOTOXIN A IMMUNOTOXIN

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***Pseudomonas* exotoxin (PE)–containing immunotoxins (ITs) act by arresting protein synthesis and promoting apoptosis, but the mechanisms of the induced apoptosis and the relationship to protein synthesis inhibition is not well elucidated. We studied these effects in MA-11 human breast cancer cells treated with 425.3PE, an unmodified PE covalently linked to the 425.3 antibody, which targets the EGF receptor. This IT induced efficient inhibition of protein synthesis with simultaneous induction of apoptosis. Thus, treatment of cells with 10 ng/ml of IT for 5 hr caused 85% inhibition of protein synthesis in parallel with caspase-3, -8 and -9 activation and PARP inactivation. Even after 72 hr of IT treatment, preincubation with the broad-spectrum caspase inhibitor z-VAD-FMK caused a significant increase in cell survival without affecting IT-induced protein synthesis inhibition. Interestingly, a combination of z-VAD-FMK and the cathepsin B/L inhibitor z-FA-FMK prevented completely IT-induced cell death in MA-11 cells after 24 hr, indicating that cathepsin activation may be important for optimal induction of IT-induced cell death. IT treatment caused after 2.5 hr a significant decrease in the level of the antiapoptotic protein Mcl-1 but not of Bcl-2 and Bcl-X_L. Furthermore, Mcl-1 expression was not sensitive to caspase inhibitors but was totally prevented by the lactacystin proteasome inhibitor, suggesting that IT-induced apoptosis may be triggered by a reduction in the Mcl-1 level. Mitochondrial membrane potential ($\Delta\Psi$ mito) decreased concurrently with caspase activation, showing the involvement of $\Delta\Psi$ mito as a regulator of IT-induced apoptosis. Our results demonstrate that 425.3PE-mediated cell death involves simultaneous induction of apoptosis and protein synthesis inhibition in MA-11 cells, thus contributing to an understanding of the mechanisms involved in IT-induced apoptosis.**

Key words: *immunotoxin; apoptosis; Mcl-1; breast cancer*

ITs represent one group of targeted therapeutics that have shown promising efficacy in patients for whom standard treatment is no longer an option.[1,2] ITs are dimeric proteins consisting of a targeting moiety linked to a toxin (ricin, PE, DT), which induce arrest of protein synthesis and result in cell death.[1] The targeting moiety binds to molecules expressed on cancer cells, theoretically leaving normal cells unaffected.

The mechanism of action of such toxins and ITs is not, as previously believed, merely inhibition of protein synthesis in the cell. Thus, ricin, PE and DT may also induce apoptosis, a feature of major importance in cancer therapy.[3,4]

Two common features of apoptotic cell death are activation of a group of cysteine proteases called caspases and caspase-catalyzed cleavage of so-called death substrates, such as the nuclear repair enzyme PARP.[5] Caspases are present in cells as inactive precursors, which are proteolytically activated upon induction of apoptosis. The protein substrates of caspases include proteins of regulatory or structural function in cell life. Caspases play a central role in the apoptotic program, functioning as initiators and executors of the apoptotic pathway. Caspase-3, *e.g.,* is directly involved in apoptosis induced by both toxins and ITs in several human tumor cell lines.[6–8] The Bcl-2 protein family is a major molecular modulator of apoptosis.[9,10] These proteins can be divided into 2 groups, including antiapoptotic (Bcl-2, Mcl-1, Bcl-X_L) and proapoptotic (Bax, Bak, Bad) molecules that regulate cellular sensitivity to drugs at the mitochondrial level. Pro- and antiapoptotic family members can heterodimerize and titrate each other's function, implying that their relative concentration may act as a balance in the suicide program.[11] Therapeutic drugs can modulate the expression of Bcl-2 family members, their activity and their subcellular localization.

We have previously shown that the 425.3PE IT increased symptom-free survival in a human breast cancer model in nude rats[12] and wanted to investigate the mechanisms underlying the IT-induced cell death. This IT consists of the 425.3 MAb, which targets the EGFr and covalently links to the bacterial toxin PE.[12] It is well known that PE-containing ITs induce protein synthesis arrest by inactivation of EF-2, but it remains to be elucidated how this is linked to apoptosis.

In the present study, we demonstrate simultaneous induction of apoptosis and protein synthesis inhibition by the holo-PE-containing IT in MA-11 breast cancer cells. Thus, upon IT treatment, expression of the antiapoptotic protein Mcl-1 was quickly and markedly reduced. The IT also induced loss of $\Delta\Psi$ mito and activation of the caspase cascade, followed by DNA fragmentation and proteolytic cleavage of essential cell survival proteins.

MATERIAL AND METHODS

The anti-EGFr antibody 425.3 was a gift from Dr. R. Reisfeld (Scripps Institute, La Jolla, CA).[13] The antibody was conjugated to PE (obtained from Dr. D. Galloway, University of Ohio, Columbus, OH) by a thioether bond formed with the reagent sulfo-SMCC (Pierce, Rockford, IL) as described previously.[14]

Abbreviations: $\Delta\Psi$ mito, mitochondrial membrane potential; DT, diphtheria toxin; ECL, enhanced chemiluminescence; EF-2, elongation factor 2; EGFr, epidermal growth factor receptor; HRP, horseradish peroxidase; HSA, human serum albumin; IC_{50}, concentration that inhibits 50%; IT, immunotoxin; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide; MAb, monoclonal antibody; Mcl-1, myeloid cell leukemia 1; PARP, poly(ADP-ribose) polymerase; PE, *Pseudomonas* exotoxin A; pNA, *p*-nitroanilide; PVDF, polyvinylidene difluoride; sulfo-SMCC, sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; TCA, trichloroacetic acid; TNF, tumor necrosis factor; z-FA-FMK, benzoxycarbonyl-Phe-Ala-fluromethyl-ketone; z-VAD-FMK, benzoxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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The broad-spectrum caspase inhibitor z-VAD-FMK, the cathepsin B/L inhibitor z-FA.FMK, the caspase inhibitor set II and the proteasome inhibitor lactacystin (Calbiochem, San Diego, CA) were resuspended in DMSO (Sigma, St. Louis, MO).

Cell culture

Establishment and characterization of the MA-11 breast cancer cell line has previously been described.[15,16] Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and glutamax (Life Technologies, Paisley, UK) and kept in a standard tissue culture incubator at 37°C.

Measurement of protein synthesis inhibition

Protein synthesis inhibition caused by IT was measured using the [^{3}H]leucine incorporation assay.[17] Cells (3 to 4 $\times$ 10^4/well) were seeded in 48-well plates and allowed to grow overnight before addition of different concentrations of IT. After 2.5, 5, 16, 24 and 48 hr incubation, cells were washed twice with cold PBS, 0.1% FCS and incubated with [^{3}H]leucine (2 μCi/ml) in leucine-free medium for 45 min at 37°C. Cells were washed twice with 5% TCA, for 10 and then 5 min, and dissolved in 0.1 M KOH for at least 5 min. The resultant solution was transferred to the liquid scintillator Aquasafe 300 Plus (Zinsser Analytic, Frankfurt/Main, Germany). Sample counts were determined in a liquid scintillation counter (LKB Wallac, Perkin Elmer, Boston, MA). Assays were performed in duplicate and repeated at least 3 times.

Effect of IT on breast cancer cell viability

The IT effect on cell viability was measured using CellTiter 96 Aqueous One Solution (MTS assay; Promega, Madison, WI). Cells were seeded in 96-well plates at 5,000–8,000/well and grown to about 60–80% confluence (depending on incubation time with IT); old medium was replaced with new medium containing IT and incubated at 37°C for 5–72 hr. CellTiter 96 Aqueous One Solution was then added to wells, and absorbance was measured 2–4 hr later at a wavelength of 490 nm. Values for total viability of treated cells were compared to values generated for untreated control cells and reported as the percentage cell viability. Assays were performed in triplicate and repeated at least 3 times.

For determination of the involvement of caspase family members in reducing cell viability, cells were incubated with different caspase inhibitors (z-VAD-FMK, z-DEVD-FMK, z-LEHD-FMK, z-IETD-FMK and z-WEHD-FMK), cathepsin inhibitor (z-FA-FMK) or vehicle alone (DMSO 0.1–0.25%) for 1 hr prior to addition of IT.

DNA fragmentation analysis

Cells (5×10^6) were seeded into flasks and incubated with IT (10 ng/ml). After the indicated treatment period, floating and adherent cells were collected. DNA was extracted by the salting-out technique with minor modifications.[18] Cells were lysed in 1 ml lysing buffer containing 10 mM TRIS-HCl (pH 7.4), 10 mM EDTA, 10 mM NaCl, 0.5% SDS and 0.5 μg/ml proteinase K for 1 hr at 50°C. High m.w. DNA was precipitated by addition of 1 M NaCl, with overnight storage at 4°C. Samples were centrifuged for 30 min at 2,700*g*. Supernatants were collected, 2.5 vol 95% ethanol was added and DNA was precipitated overnight at –20°C, followed by centrifugation at 6,000*g* for 10 min at 4°C. The pellet was washed in 70% ice-cold EtOH and centrifuged at 6,000*g* for 10 min at 4°C. The pellet was dried and dissolved in 20 μl of 10 mM TRIS-HCl (pH 7), 15 mM NaCl, 1 mM EDTA and 5 μl of 1 mg/ml RNase A for 1 hr at room temperature. DNA was run in a 1.5% agarose gel containing ethidium bromide and photographed under UV illumination. With this method, high m.w. DNA (>20 kbp) remains trapped in or near the well, while digested smaller fragments (down to 180 bp) are resolved in the gel.

Western blot analysis

After IT stimulation for the indicated time period, both adherent and floating cells were lysed by an SDS-boiling method. Cell pellets were resuspended in lysate buffer [2% SDS, 1 mM Na_3VO_4 and 10 mM TRIS-HCl (pH 7.6)], which was held at 100°C when added, and the lysates boiled for 5 min. After 6 passages through a 20-gauge syringe on ice, lysates were cleared by centrifugation. Protein concentrations were then determined using the BCA protein assay (Pierce). Lysates were snap-frozen in liquid N_2 and kept at –70°C.

A portion (20 μg protein) of each lysate was fractionated by 8–12% SDS-PAGE, depending on the protein weight, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA) by electroblotting. Filters were probed with a designated primary antibody. Anti-caspase-3, -caspase-8, -caspase-9 and -Bcl-2 were purchased from R&D Systems (Minneapolis, MN). Anti-α-tubulin and -LaminB were from Oncogene (SanDiago, CA). Anti-PARP antibody was from Roche (Mannheim, Germany) and anti-Mcl-1 antibody, from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-X_L was purchased from Cell Signaling Technology (Beverly, MA). Immune complexes were detected with appropriate HRP-coupled secondary antibodies. Peroxidase activity was visualized with enzyme-linked ECL (Amersham, Aylesbury, UK) and quantified by densitometry (Imagemaster, Amersham). All Western blots were stained with amidoblack and probed with anti-α-tubulin to confirm equal loading and transfer of samples.

Caspase activity assays

Caspase activity was determined in 96-well plates using cell lysate from 2×10^6 MA-11 cells for each measurement. Caspase activities were measured with the designated caspase colorimetric protease assay (BioSource, Camarillo, CA) in MA-11 cells treated with 425.3PE (10 ng/ml) for 24 hr prior to preparation of the cell lysate.

Cell extracts were incubated for 2 hr at 37°C with 200 μM of the respective tetrapeptide substrate (DEVD, caspase3/7; IETD, caspase-8; LEHD, caspase-9) coupled to the chromophoric pNA. The color of free pNA, generated as a result of cleavage of the substrate by caspase, was quantified using a microplate reader at 405 nm. Background readings from cell lysates and buffers were subtracted from the readings of both IT-induced and control samples before calculating the increase in caspase activity.

ΔΨm

ΔΨmito was measured by flow cytometry and fluorescence microscopy using the fluorescent probe JC-1 (Molecular Probes, Eugene, OR), which produces green fluorescence in the cytoplasm and red–orange fluorescence when concentrated in intact mitochondria with a negative internal potential.

Cells stained with JC-1 were viewed with the fluorescence microscope Axioplan 2 (Zeiss, Oberkochen, Germany) and the ×63 Plan Apochromat lens (Zeiss) using the rhodamine and fluorescein filter.

For flow cytometry, cells were plated at 2.1×10^6/culture flask (25 cm^2) overnight. Medium was decanted and fresh medium containing IT (10 ng/ml) added. After the appropriate incubation time, cells were trypsinized, rinsed once with PBS+HSA (0.1%), resuspended in the same buffer, treated and incubated with JC-1 at a concentration of 1 μM for 15 min at 37°C. Cells were then washed twice with PBS and held on ice prior to FACS analysis. Fragmented cells and debris were excluded from measurements by gating the remaining intact cells in a forward and side scatter analysis. The FACStar flow cytometer (Becton Dickinson, San Jose, CA) was set up to measure forward and side scatter, green fluorescence (FL1, 525 nm bandpass) and red fluorescence (FL2, 590 nm bandpass) for the change from JC-1 aggregate (red) to monomer (green). FACS analysis was carried out on 1×10^4 cells.

RESULTS

IT 425.3PE induced dose- and time-dependent inhibition of protein synthesis, followed by decreased MA-11 cell viability

The time- and concentration-dependent effects of 425.3-PE on protein synthesis were examined first. IC_{50} values were 8.2 ng/ml at 2.5 hr, 1.5 ng/ml at 5 hr, 0.42 ng/ml at 16 hr, 0.085 ng/ml at 24 hr and

0.062 ng/ml at 48 hr, reflecting a high sensitivity of MA-11 cells to the IT (Fig. 1*a*). When the effect of the IT on cell survival was measured using the MTS assay, it was found that cell viability after 5 hr of IT (10 ng/ml) treatment decreased by only 5% (Fig. 1*b*), with close to 90% inhibition of protein synthesis obtained after the same period of incubation (Fig. 1*a*).

The amino-terminal cell-binding domain of PE is intact in our IT, making the IT able to bind also to the α-macroglobulin receptor on cells. We therefore included as controls both free PE and anti-425.3 antibody. Even at concentrations of 100 ng/ml for 24 hr, close to 1,000-fold higher than the IC_{50} for the IT, no effect on protein synthesis and cell viability was observed with any of the molecules alone (not shown).

425.3PE-induced activation of procaspase-3

We investigated by Western blotting whether a key effector molecule in apoptosis, the specific cysteine protease caspase-3, participates in 425.3PE-induced cell death. As shown in Figure 2*a,* procaspase-3 was proteolytically cleaved in a dose-dependent manner in IT-treated MA-11 cells (5 hr), followed by the appearance of the active caspase-3 fragment (17 kDa). In untreated control cells, no caspase-3 was detected. To further investigate the role of caspases in IT-induced apoptosis, MA-11 cells were preincubated for 1 hr with the cell-permeable, broad-spectrum caspase inhibitor z-VAD-FMK (50 μM) and the specific caspase-9 inhibitor z-LEHD-FMK, 50 μM, followed by IT treatment for 5 hr. z-VAD-FMK partially prevented processing of procaspase-3, though the inhibitor efficiently blocked further cleavage of inactive caspase-3 (p20) to active caspase-3 (p17) (Fig. 2*b*). z-LEHD-FMK had no effect on the processing of procaspase-3, though it partially blocked IT-induced caspase-3 activation (p20 to p17) (Fig. 2*b*). With a fixed IT dose (10 ng/ml), we observed an initial slight increase followed by a gradual reduction in the level of the inactive procaspase-3 (Fig. 2*c*). After 24 hr of IT treatment, all procaspase-3 was processed and the caspase inhibitors z-VAD-FMK and z-LEHD-FMK could partially block cleavage, suggesting an amplification loop in caspase-3 activation mediated by caspase-9 and other caspases (Fig. 2*c*). However, the active caspase-3 (p17) did not accumulate with time, suggesting instability of the active p17 (data not shown). The Western blot probed with anti-α-tubulin

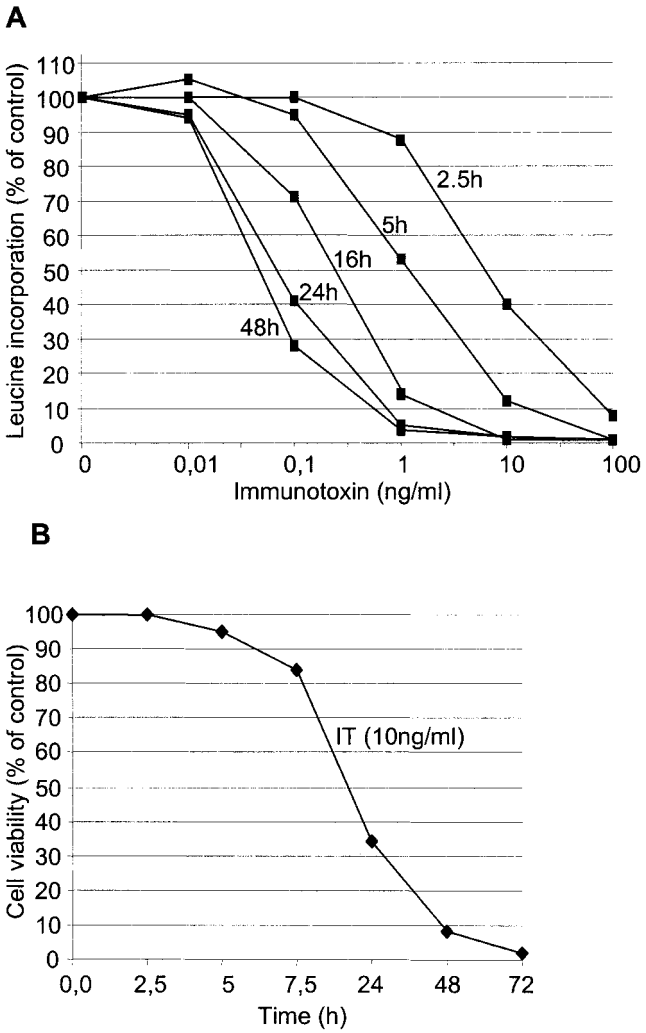

FIGURE 1 – Dose- and time-dependent effects of 425.3PE on protein synthesis and MA-11 cell viability. *(a)* Cells were incubated with different concentrations of 425.3PE for the indicated time periods. The rate of protein synthesis in each culture was expressed as a percentage of the value obtained in control cultures without 425.3-PE. Data were expressed as the mean of triplicates, representative of 3 independent experiments. *(b)* Cells were incubated with 10 ng/ml 425.3PE for the indicated time periods before the MTS assay was performed. Results represent means of 5 determinations, and the assay was repeated at least 3 times.

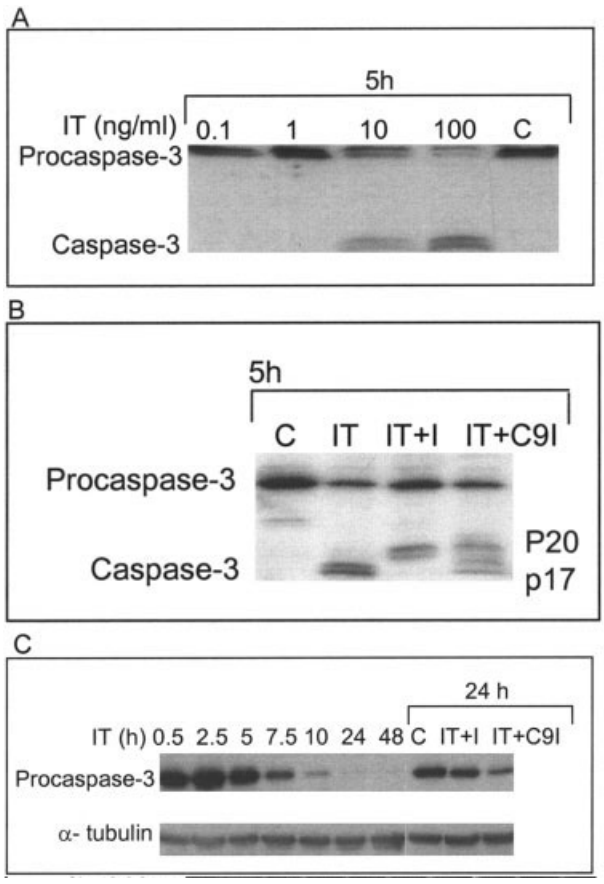

FIGURE 2 – Time- and dose-dependent 425.3PE induction of caspase-3 cleavage. MA-11 cells were treated with increasing amounts (0.1–100 ng/ml) of 425.3PE for 5 hr *(a),* 425.3PE (10 ng/ml) for 5 hr with the indicated inhibitors *(b)* or 425.3PE (10 ng/ml) for the indicated time periods (0–48 hr) *(c).* Cleavage of caspase-3 was determined by Western blotting, as described in Material and Methods. The broad-spectrum caspase inhibitor z-VAD-FMK (I) or the caspase-9 inhibitor (C9I) z-LEHD-FMK was added 1 hr prior to the IT at a final concentration of 50 μM. C, control; IT, 425.3PE. Anti-α-tubulin detection was used to confirm equal loading of proteins.

demonstrated an unchanged α-tubulin level, confirming that the protein samples were equally loaded (Fig. 2*b*).

IT-induced PARP inactivation, LaminB cleavage and DNA fragmentation in MA-11 cells

Activation of effector caspases induces proteolysis of a variety of cytosolic and nuclear target proteins, leading to cellular changes associated with apoptosis. Our Western blot analysis showed that in MA-11 cells cleavage of PARP correlated well with caspase-3-dependent generation of the 85 kDa inactive fragment (Fig. 3*a*). Time-course experiments showed initiation of PARP cleavage at 5 hr and that cleavage was complete within 7.5 hr of IT treatment.

DNA fragmentation, a typical hallmark of apoptosis, was detected already after 7.5 hr of IT treatment, and after 24 hr all cellular DNA showed the characteristic DNA ladder of 180 bp fragments (Fig. 3*b*). We also investigated cleavage of LaminB as this protein is critical in maintaining the integrity of the nuclear envelope and cellular morphology. As shown in Figure 3*c,* 425.3PE induced LaminB cleavage in a time-dependent manner.

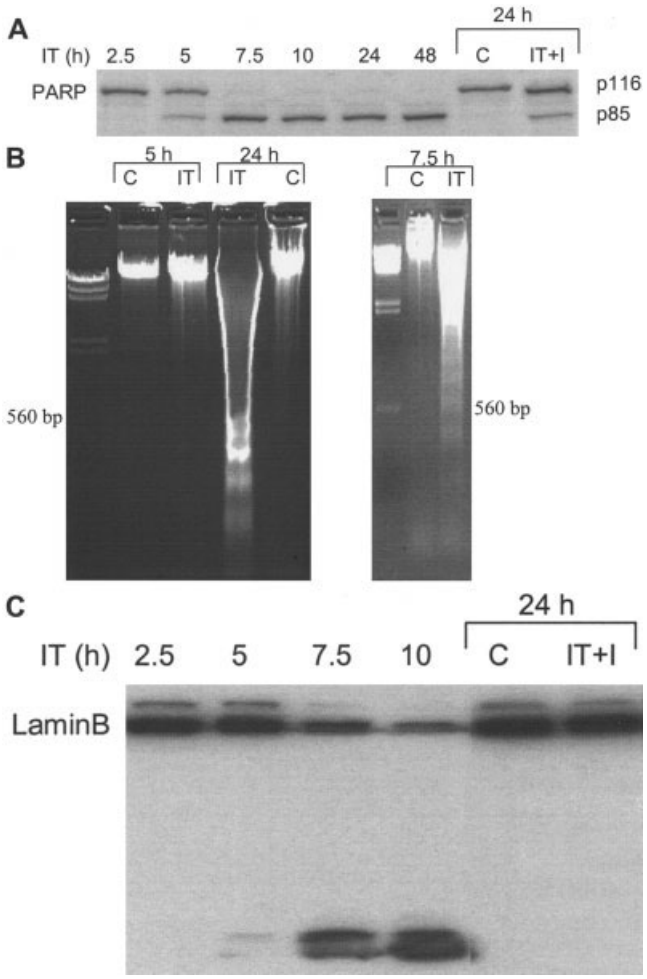

FIGURE 3 – Effect of 425.3PE on PARP cleavage, DNA laddering and LaminB cleavage. After exposure to 10 ng/ml 425.3-PE for the time periods indicated, cells were lysed and DNA and protein were isolated as described in Material and Methods. Agarose gel/Western blot is representative of at least 2 independent experiments. The peptide inhibitor z-VAD-FMK (I) was added 1 hr prior to the IT at a final concentration of 50 μM. *(a,c)* Western blots incubated with anti-PARP or anti-LaminB, respectively. *(b)* Isolated DNA was subjected to electrophoresis in agarose gel (1.5%) and stained with ethidium bromide. C, control; IT, 10 ng/ml 425.3PE.

The 32 kDa LaminB fragment was indicative of proteolytic digestion and visible after 7.5 hr incubation with the IT.

Whereas degradation of PARP was only partially inhibited by preincubation with the broad-spectrum caspase inhibitor (Fig. 3*a*), both the IT-induced LaminB cleavage (Fig. 3*c*) and DNA fragmentation (not shown) were completely prevented. The specific caspase-9 inhibitor had no effect on IT-induced PARP inactivation (not shown). No PARP inactivation, LaminB cleavage or DNA fragmentation was evident in cells treated with 425.3 alone or PE alone (not shown).

Cleavage of caspase-8 and caspase-9 is involved in 425.3PE-mediated apoptosis

Caspase-3 can be activated by the so-called initiator caspases: caspases 8 and 9. Western blot analysis showed that the activation kinetics of caspase-9 cleavage in 425.3PE (10 ng/ml)–treated MA-11 cells (Fig. 4*a*) was similar to that of caspase-3 activation (Fig. 2*c*), whereas cleavage of caspase-8 appeared to be induced later (Fig. 4*b*).

In experiments with the caspase-9 inhibitor and the broad-spectrum caspase inhibitor, both inhibited IT-mediated caspase-8 cleavage (Fig. 4*b*). Our results indicated that caspase-8 activation is downstream of at least caspase-9 activation. The z-VAD-FMK broad-spectrum inhibitor had a minor effect on caspase-9 activation (Fig. 4*a*), suggesting that caspase-9 cleavage is more independent of activation of the other caspases. To examine whether caspase-9 activation is necessary for IT-induced caspase-3 activation, MA-11 cells were pretreated with the caspase-9 inhibitor for 1 hr prior to IT treatment. The caspase-9 inhibitor only partially inhibited caspase-3 activation (p17) (Fig. 2*b*), suggesting that, besides that of mitochondria-dependent caspase-9 activation, other pathways may be involved in activation of caspase-3.

To confirm that the activated forms of procaspases 3, 8 and 9 had enzymatic activity, whole-cell lysates from IT-treated and untreated cells were examined using specific substrates, DEVD, IETD and LEHD, which are the recognition sites for caspase-3/-7, caspase-8 and caspase-9, respectively. There were significant increases in caspase-3, - 8 and –9 activity (7.5-, 1.5- and 2.5-fold, respectively) at 24 hr of IT treatment (Fig. 4*c*).

Effect of caspase inhibitors and cathepsin B/L inhibitor on IT-induced decrease in cell viability

To determine the importance of apoptosis in 425.3PE-induced cell death, MA-11 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD-FMK for 1 hr and thereafter incubated for 24, 48 and 72 hr with 10 ng/ml of the IT (Fig. 5*a*). At 24 hr of incubation, about 35% of IT-treated control cells were viable, but for cells preincubated with the caspase inhibitor the viable fraction increased to 85%. Interestingly, the effect of the inhibitor did not decrease with time, and after treatment with IT for 72 hr a high fraction of the cell population was still viable.

To further elucidate the role of the different caspases on cell viability, the IT-mediated effect of several specific caspase inhibitors was examined. As shown in Figure 5*b,* caspase inhibitors z-LEHD-FMK, z-IETD-FMK and z-DEVD.FMK demonstrated approximately the same effect, increasing the IT-induced decrease in cell viability from 35% to 60%. Since the broad-spectrum inhibitor made the cells even more resistant (about 85%) (Fig. 5*a,b*), the results support the conclusions that IT activation of apoptosis occurs also through pathways other than the caspase-9-dependent one and that activation of caspase-3 (Figs. 2*a,b,* 4*c*) and caspase-8 (Fig. 4*b,c*) is involved in IT-induced apoptosis. That the caspase-5-specific inhibitor z-WEHD.fmk had no effect on IT-induced cell death demonstrates further the specificity of IT-induced caspase activation. No effect on cell viability was observed for cells treated only with caspase inhibitors or with 0.1–0.25% DMSO (not shown).

Several reports have shown that moderate to high concentrations of z-VAD-FMK inhibitor are not as specific as originally accepted and

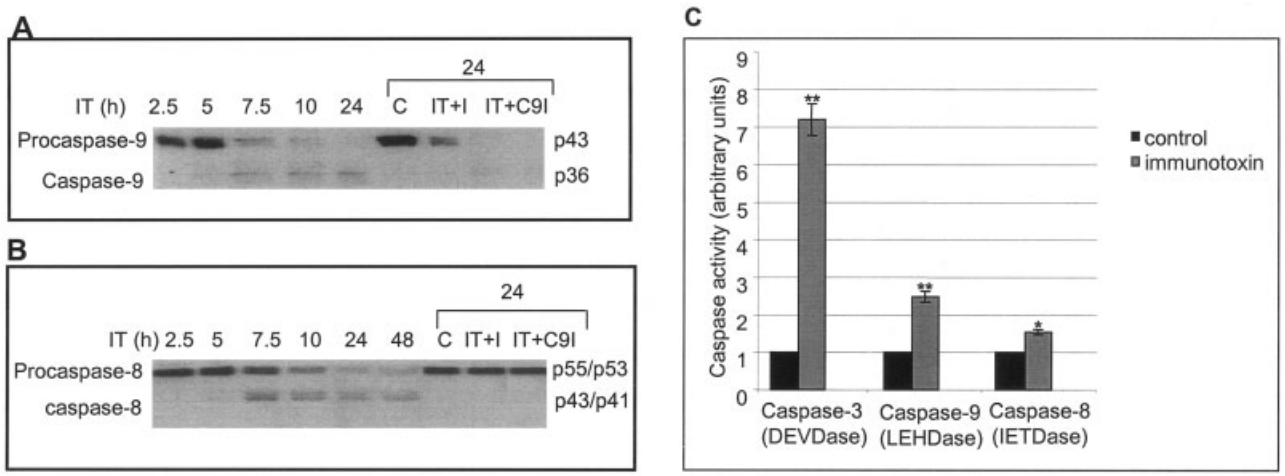

FIGURE 4 – Western blot analyses of caspase-8 and caspase-9 cleavage. After treatment with 10 ng/ml 425.3PE for the time indicated, cell lysates were subjected to Western blotting. Filters were incubated with anti-caspase-9 *(a)* or anti-caspase-8 *(b)* as described in Material and Methods. Gels represent at least 3 independent experiments. Peptide inhibitors z-VAD-FMK (I) and z-LEHD-FMK (C9I) were added 1 hr prior to the IT at a final concentration of 50 μM. Caspase-3, caspase-8 and caspase-9 protease activity induced by 425.3PE was measured with the designated caspase colorimetric protease assay *(c)* (BioSource) as described in Material and Methods. Control activity was designated as 1.0. Data represent means ± SE from 2 separate experiments (triplicates). $*p < 0.01$, $**p < 0.001$ compared to control.

may also inhibit the activity of cathepsin B.[19] The inhibitor of cathepsin B/L, z-FA-FMK, was included in our system and partially blocked 425.3PE-induced cell death (about 60% survival), suggesting involvement of cathepsin B in this process (Fig. 5*b*). To further analyze the possible differential involvement of the caspases and cathepsin B/L, the effect of low concentrations of z-VAD-FMK (1–10 μM) and z-FA-FMK (5–25 μM) on IT-induced cell death was examined on Western blots and in the MTS assay. A dose–response effect on cell viability, PARP and caspase-3 was obtained with z-VAD-FMK (Fig. 5*c*). Even the lowest z-VAD-FMK concentration used (1 μM) increased the survival of IT-treated cells (24 hr, from 31% to 51 %). With z-FA-FMK we found a dose-dependent reduction in IT-induced cell death, about 40% survival at 5 μM (not shown) and 55% at 25 μM (Fig. 5*c*), arguing for cathepsin B/L as mediators in IT-mediated death. z-FA-FMK (25 μM) had no detectable effect on IT-induced PARP inactivation or caspase-3 activation (Fig. 5*c*). Interestingly, the combination of both inhibitors (z-VAD-FMK, 5 or 10 μM, and z-FA-FMK, 25 μM) caused complete resistance to IT-induced cell death after 24 hr treatment, with a decrease in active caspase-3 and less inactive PARP (Fig. 5*c*), demonstrating that initial IT-induced cell death is dependent on both caspase and cathepsin B/L activation. The protective effect of the inhibitor combination was more effective than z-VAD-FMK (50 μM) alone for 48 hr IT treatment; thereafter, the effect was equal to that of z-VAD (50 μM) alone (Fig. 5*d*). The caspase contribution to IT-induced cell death was high even after a long period without protein synthesis (nearly 72 hr), whereas cathepsinB/L appears to play an initial and accelerative role (Fig. 5*d*). The cathepsin inhibitor pepstatin A (100 μM) and E64 (100 μM) had no effect on IT-induced cell death (not shown), suggesting specific cathepsin B/L involvement. The combination of inhibitors had no or minor effects on untreated cells, even at high concentration and with long incubation time (100 ± 5% of control).

Mitochondrial membrane depolarization is an early event in 425.3PE-induced MA-11 cell death

To characterize the upstream signaling pathways through which the IT may regulate activation of the caspase cascade, we analyzed the depolarization of $\Delta\Psi$ mito. To detect these early changes in mitochondrial function, we used the fluorescent cation JC-1, which emits a red color when sequestered in the mitochondria of healthy cells and a green color when located in the cytoplasmic compartment of cells undergoing apoptosis. At 0 hr, MA-11 cells sequestered >95% of the dye in the mitochondria, which dropped to 81% after 5 hr of IT treatment (Fig. 6) and to 55% after 24 hr. After 5 hr of IT treatment, no cells were dead, whereas at 24 hr (not shown) a large fraction of the total number of cells was dead and excluded from measurement. This finding was in good agreement with results obtained in the MTS assay (Fig. 1*b*). In untreated cells, 95% of the JC-1 dye remained in the mitochondria throughout the experiment (not shown).

The effects were visualized by fluorescent microscopy, showing high $\Delta\Psi$ mito in control cells, as demonstrated by the aggregated form of JC-1 (punctuated orange–red fluorescence) and low membrane potential with the monomer of JC-1 (loss of punctated orange–red fluorescence), in IT-treated cells (Fig. 6).

These data suggest that mitochondrial dysfunction is one of the primary mechanisms whereby the IT exerts its cell-killing activity. Moreover, the changes in $\Delta\Psi$ mito are in agreement with activation of caspase-9, a protease known to be activated by mitochondrial disarrangement.

Downregulation of Mcl-1 in MA-11 cells

Expression of 3 antiapoptotic (Bcl-2, Bcl-X_L and Mcl-1) members of the Bcl-2 protein family was examined by Western blotting. Levels of Bcl-2 and Bcl-X_L were not considerably altered after 425.3PE treatment (10 ng/ml, 5 hr) compared to untreated control cells (Fig. 7*a*). By contrast, a major decrease in Mcl-1 protein expression was found after 2.5 hr IT treatment, presumably reflecting either downregulation in Mcl-1 protein expression or a decrease in Mcl-1 protein stability. The time-dependent loss of Mcl-1 protein expression by IT treatment was not caspase- or cathepsin-dependent, as shown by the lack of clear response to the inhibitors z-VAD-FMK, z-LEHD-FMK and z-FA-FMK (Fig. 7*b*). However, z-VAD-FMK had a minor effect on Mcl-1 expression, suggesting that a feed-forward amplification loop in caspase-3 activation and caspase-mediated cleavage of Mcl-1 are involved in the acceleration of apoptosis. The combination of z-VAD-FMK and z-FA-FMK had no additional effect on Mcl-1 downregulation (not shown). The proteasome inhibitor lactacystin protected cells against IT-induced downregulation of Mcl-1, caspase-3 activation and PARP inactivation (Fig. 7*c*). The rapid turnover of Mcl-1 protein caused by proteasome degradation contributed at least to 2 of the hallmarks of apoptosis, caspase-3 activation and PARP inactivation, supporting the importance of Mcl-1 downregulation resulting from IT treatment.

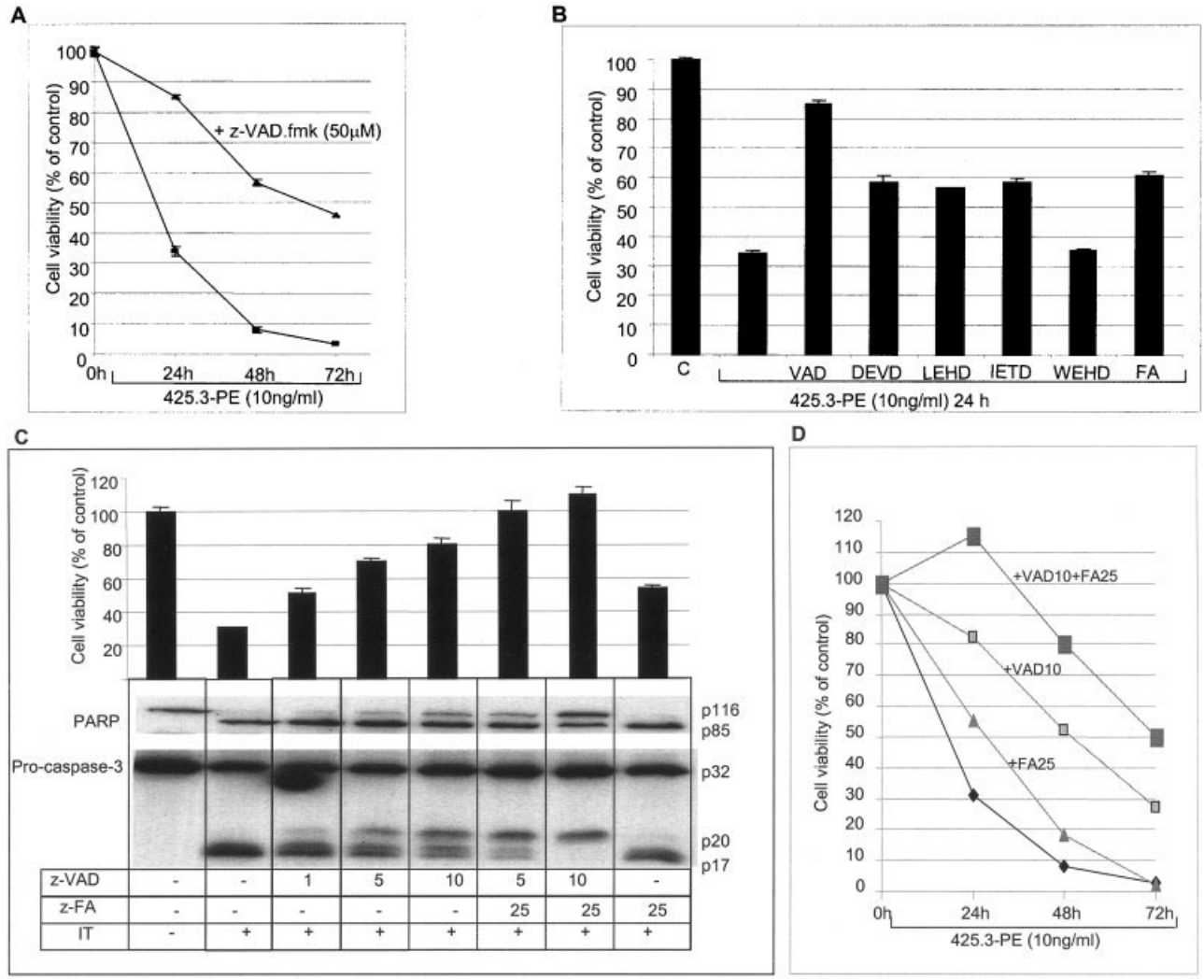

FIGURE 5 – Influence of different caspase inhibitors and cathepsin B/L inhibitor on 425.3PE-induced reduction in MA-11 cell viability. Cells were seeded in a 96-well plate the day before treatment was started with 425.3PE (10 ng/ml) with or without 1 hr pretreatment with different caspase or cathepsin B/L inhibitors. After the indicated treatment periods, CellTiter 96 Aqueous One Solution was added to the wells and the absorbence, measured 4 hr later. *(a)* Cells exposed to 425.3PE with or without 1 hr pretreatment with z-VAD-FMK (50 μM) for 24, 48 or 72 hr. *(b)* Cells exposed to 425.3PE with or without different caspase inhibitors (50 μM; VAD, z-VAD-FMK; DEVD, z-DEVD-FMK; LEHD, z-LEHD-fmk; IETD, z-IETD-FMK; WEHD, z-WEHD-FMK) or the cathepsin inhibitor (50 μM; FA, z-FA-FMK) for 24 hr. *(c)* Cells exposed to 425.3PE with or without 1 hr pretreatment with z-VAD-FMK (1–10 μM) and/or z-FA.FMK (25 μM) for 5 hr (Western blot) or 24 hr (cell viability). PARP and caspase-3 cleavage was determined by Western blotting as described in Material and Methods. *(d)* Cells exposed to 425.3PE with or without 1 hr pretreatment with z-VAD-FMK (10 μM) and/or with z-FA.FMK (25 μM) for 24, 48 or 72 hr. Data points represent the mean ± SE ($n = 5$) of a representative experiment of 3. Gels represent 3 independent experiments.

DISCUSSION

ITs induce cell death by 2 mechanisms, inhibition of protein synthesis and apoptosis.[7,8] We have previously shown that the 425.3-PE IT is active against breast cancer in experimental animal models,[12] and these findings prompted us to investigate the mechanism involved in 425.3PE-induced effects.

In the MA-11 breast cancer cell line, 425.3PE induced rapid inhibition of protein synthesis with simultaneous induction of apoptosis. Treatment of MA-11 cells with 425.3PE (10 ng/ml) caused >85% protein synthesis inhibition as early as 5 hr post-treatment. This dose, which is about 6.7-fold higher than the IC_{50}, was used in most of the experiments based on the criterion of having a near maximal effect on protein synthesis but being sufficiently low to permit observations of the initial effects on the apoptotic machinery. The short treatment period caused induction of several classical key events in apoptosis, including caspase-3-, -8 and –9 activation and PARP inactivation with minor changes in cell viability at 5 hr. The protein synthesis inhibition and induction of apoptotic markers occurred at similar kinetics, suggesting a close connection between the 2 mechanisms. In comparison, for some protein synthesis inhibitors, such as cycloheximide and didemnin, the maximal inhibition of protein synthesis appeared to be insufficient to trigger apoptosis.[7,20]

Caspases are crucial mediators of apoptosis, and a rapid IT-induced decrease of procaspases 3, 8 and 9 was detected on Western blots. With this method it is difficult to dissect which caspase is activated first due to differences in antibody sensitivity. However, caspase-8 activation was downstream of caspase-3 and caspase-9 activation as the caspase-8 inhibitor had no effect on the activation of the other 2. Caspase-9 can function as an initiator when mitochondrial dysfunction is the primary event in apoptosis, whereas under conditions in which disruption of mitochondria is a

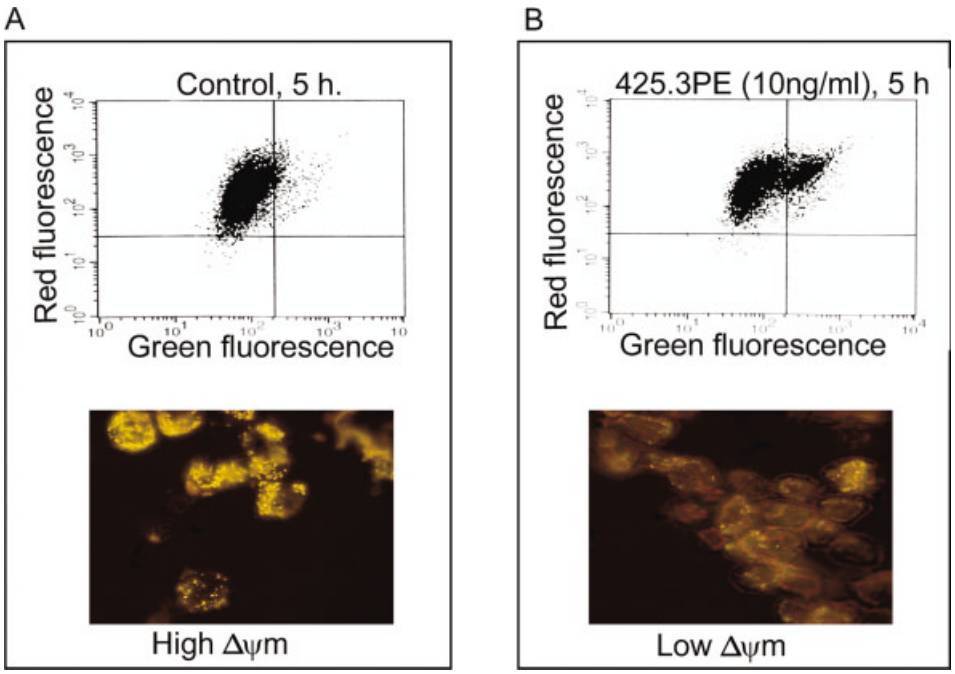

FIGURE 6 – Effect of 425.3PE IT treatment on $\Delta\Psi$ mito analyzed by flow cytometry and fluorescence microscopy. MA-11 cells were treated with or without 425.3PE (10 ng/ml) for 5 hr and then stained with JC-1 as described. *(Upper panel)* Distinct populations of cells with different extents of mitochondrial depolarization are seen in IT-treated cells *(b)* compared to untreated control cells *(a)*. *(Lower panel)* Reduction in $\Delta\Psi$ mito using the JC-1 dye was also observed with fluorescence microscopy. High $\Delta\Psi$ mito is marked by punctuate orange–red fluorescence staining *(a)*. Low $\Delta\Psi$ mito is shown by loss of punctuate orange–red fluorescence staining *(b)*. Data are representative of 2 separate experiments performed in duplicate.

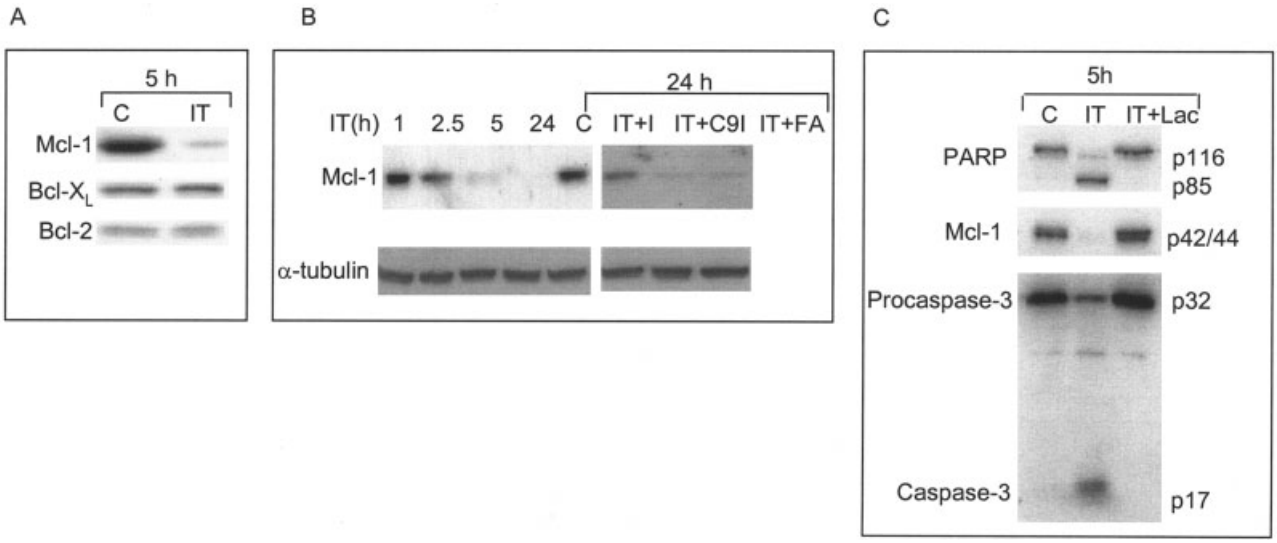

FIGURE 7 – Western blot analyses of Bcl-2 family members (Bcl-2, Bcl-X_L and Mcl-1) in MA-11 breast cancer cells treated with 425.3PE. After treatment with 425.3PE for the times indicated, cell lysates were Western-blotted. *(a)* Filters were incubated with primary antibody to Mcl-1, Bcl-X_L or Bcl-2 as described in Material and Methods. *(b)* Time-dependent downregulation of Mcl-1 protein in MA-11 cells treated with 425.3PE. Peptide inhibitor z-VAD-FMK (I), z-LEHD-FMK (C9I) or z-FA-FMK (FA) was added 1 hr prior to 425.3PE at a final concentration of 50 μM. *(c)* Inhibition of Mcl-1 degradation with proteasome inhibitor lactacystin. MA-11 cells treated with 425.3PE with or without lactacystin (Lac). Lactacystin was added 1 hr prior to 425.3PE at a final concentration of 10 μM. Filters were incubated with primary antibody to PARP, Mcl-1 or caspase-3 as described under Material and Methods. C, control; IT, 10 ng/ml 425.3PE. Gels are representative of no fewer than 3 independent experiments.

late event, it serves to amplify the apoptotic signaling initiated by other caspases.[21] We conclude that the IT induced the former event as the caspase-9-specific inhibitor could partially rescue IT-treated cells, partially inhibit caspase-3 activation and fully inhibit caspase-8 activation. The caspase-9 inhibitor did not prevent the processing of procaspase-3 to the p20 intermediate, indicating that other caspases are involved, whereas cleavage of caspase-3 p20 to p17 (active) was partially blocked. In other systems, z-VAD-FMK did not block the processing of procaspase-3 to inactive p20 but did block further processing to p17,[22] supporting our findings using the z-VAD-FMK and caspase-9 inhibitors. In the present study, several different caspase inhibitors had a rescuing effect on 425.3PE-induced cell death, thus promoting cell survival. The half-life of the caspase inhibitors has been proposed to be around 12 hr in culture medium. Therefore, addition of fresh caspase inhibitor over the experiment period was included, with only minor effects on cell viability compared to the culture given a single dose. Induction of cell death was more effectively delayed in the presence of the broad-spectrum z-VAD-FMK than in the presence of any of the other specific caspase inhibitors, suggesting that several caspase pathways are involved in 425.3PE-mediated apoptosis.

z-VAD-FMK improved cell viability even after a long IT treatment period (72 hr), without any simultaneous effect on protein synthesis inhibition, which was maximal already 5 hr posttreatment. Therefore, inhibition of protein synthesis cannot be the sole cause of MA-11 cell death within this period. These findings indicate that early induction of apoptosis plays a significant, but not exclusive, role in IT-mediated cell death but in cells resistant to apoptosis the inhibition of protein synthesis will be crucial for IT-induced cytotoxicity.

The broad-spectrum caspase inhibitor z-VAD-FMK may also inhibit proteases other than caspases, such as calpain[23] and cathepsin.[19] Indeed, the cathepsin B/L inhibitor z-FA-fmk made IT-treated cells partially resistant to cell death, revealing a possible role of cathepsin B in the process. z-FA-fmk not only inhibits cathepsinB/L but also might inhibit caspase activity *in vitro*.[24] Interestingly, however, in our study the cathepsin inhibitor affected neither IT-induced caspase-3/-8/-9 cleavage, Mcl-1 downregulation nor protein synthesis (unpublished data), indicating that IT-induced caspase activation is independent of cathepsin activation. Interestingly, by simultaneous inhibition of caspase and cathepsin B/L activity, MA-11 cells were desensitized for 24 hr IT-induced cell death, though the 90% protein synthesis inhibition was unaffected (not shown). This suggests that lysosomal and mitochondrial factors might sustain and augment each other.[25,26] The cell viability data also suggest a role of cathepsin B in IT-induced cell death, and the factor(s) responsible for cathepsin B/L–mediated facilitation of IT-induced cell death will be further examined. In our cell system, even the highest z-FA-FMK concentration (50 μM) had no effect on caspase-3 processing or Mcl-1 downregulation, indicating that cathepsin B/L functions downstream of or in parallel with the Mcl-1 pathway. Keppler-Hafkemeyer *et al.*[7] showed that, with a recombinant modified IT, PARP cleavage occurred first after 16 hr in MCF-7 cells, similar to the time needed for TNF-α-induced PARP cleavage in the same breast cancer cell line. In our MA-11 breast cancer cells, onset of PARP cleavage by 425.3PE was seen already after 5 hr, possibly reflecting the faster kinetics of holo-IT cell killing. Whereas caspase-3 cleavage was present at 5 hr, no DNA fragmentation was detected this early, in agreement with results showing that DNA fragmentation is mostly mediated by caspase-3 cleavage.[27] That PARP inactivation also preceded IT-induced DNA fragmentation is in line with the DNA repair function of active PARP.

The Bcl-2 protein family (both anti- and proapoptotic members) are important regulators of the apoptotic signaling pathway by acting as a checkpoint at the mitochondrial level where the ratio of the family members determines the fate of the cell after an apoptotic stimulus.[9,10] For the modified PE-based IT (IL-13-PE38QQR), the effect on apoptosis has been reported to result in a lowered Bcl-2 level and increased Bax expression.[28] Similarly, the Shiga toxin induces apoptosis in epithelial cells with enhanced expression of the pro-apoptotic protein Bax[29] and in endothelial cells with decreased expression of the antiapoptotic protein Mcl-1.[30]

In our case, the antiapoptotic protein Mcl-1 was markedly decreased in MA-11 cells whereas expression of the antiapoptotic Bcl-2 and Bcl-X_L proteins was not changed. Interestingly, the kinetics of Mcl-1 protein downregulation was somewhat faster than that of caspase-3 and caspase-9 activation, and the respective inhibitors had no effect on the IT-induced decrease in Mcl-1 protein level. Similar to other Bcl-2 family members, Mcl-1 localizes to the mitochondria as well as to other intracellular membranes and can associate with proapoptotic Bcl-2 family members, thereby improving cell viability under various cytotoxic conditions.[31] Mcl-1 differs from Bcl-2 and Bcl-X_L in structure and in its very short half-life caused by the presence of a PEST sequence and R:R motifs, favoring its proteolysis.[32] In the absence of the Mcl-1 protein, the stable death proteins dominate and promote apoptosis, suggesting that loss of Mcl-1 is sufficient to trigger apoptosis.[33,34] By proteasome inhibition by lactacystin, which restored Mcl-1 protein expression in our system, caspase-3 activation and PARP inactivation were inhibited. It may be speculated, therefore, that the disappearance of Mcl-1 in 425.3PE-treated MA-11 cells could be the key factor responsible for the induced apoptosis.

Activated caspases cleave antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-X_L and Mcl-1) through a positive feedback loop, further strengthening the importance of Bcl-2 family proteins in apoptosis.[35,36] However, the Bcl-2 and Bcl-X_L proteins were not processed in our system and the Mcl-1 disappearance was mostly caspase-independent, though a fraction was z-VAD-FMK-sensitive. The antiapoptotic effect of Bcl-2, Bcl-X_L and Mcl-1 occurs at the mitochondrial level, preventing the loss of $\Delta\Psi$ mito and release of cytochrome *c*. That the changes in $\Delta\Psi$ mito were seen 5 hr posttreatment whereas the decline in Mcl-1 protein started already after 2.5 hr and prior to the onset of other apoptotic changes supports the importance of Mcl-1 in inducing apoptosis of MA-11 cells. Another IT, MOC31-PE,[37] also induced apoptosis with Mcl-1 downregulation and caspase-3 activation in MA-11 and T47D cells (not shown), suggesting that the induction of IT-mediated cell death is not specific for one IT and one breast cancer cell line.

The present results offer insight into the cell death mechanisms of an IT consisting of a MAb chemically conjugated to holo-PE in MA-11 human breast cancer cells. The results reveal a signal-transduction pathway in 425.3PE-induced apoptosis involving *(i)* a strong decrease in expression of the antiapoptotic Mcl-1 protein, *(ii)* decreased $\Delta\Psi$ mito, *(iii)* caspase-3 activation mainly by caspase-9, *(iv)* cathepsin B/L activation and *(v)* PARP inactivation and fragmentation of DNA.

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