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P-glycoprotein enhances TRAIL-triggered apoptosis in multidrug resistant cancer cells by interacting with the death receptor DR5

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Abbreviations:
DOX, doxorubicin
FADD, Fas associated death domain
MDR, multidrug resistance
P-gp, P-glycoprotein
ROS, reactive oxygen species
TNF-α, tumor necrosis factor-α
TNFR1, tumor necrosis factor-α receptor 1
TRAIL, tumor necrosis factor-related apoptosis-inducing ligand

ABSTRACT

The death-inducing cytokine TRAIL is a promising agent for anticancer therapy since it preferentially kills cancer versus normal cells; however, some cancer cells are TRAIL-resistant. We initially explored whether overexpression of the *MDR1* gene product P-glycoprotein (P-gp), which causes multidrug resistance (MDR) in cancer cells, also contributes to TRAIL-resistance. Surprisingly, our results revealed that P-gp-overexpression enhances TRAIL-induced apoptosis not only in neoplastic cells transfected with the *MDR1* gene but also in MDR variants selected with cytotoxic anticancer agents. Mechanistic analysis of TRAIL-induced apoptosis in the *MDR1*-transfected MCF-7 breast cancer cell line BC-19 revealed that TRAIL-triggered significantly more apoptosis in these cells compared with parental MCF-7 cells by binding to the TRAIL receptor DR5. DR5 but not DR4 engagement by TRAIL attenuated cellular ATP levels by robustly stimulating P-gp ATPase activity, and thus triggered P-gp-dependent apoptosis by depletion of the cellular ATP pool. In addition to hyperactive P-gp-mediated ATP hydrolysis, TRAIL-induced, P-gp-potentiated apoptosis was associated with activation of caspases-6, -7, -8, and -9; Bid cleavage; and mitochondrial depolarization. P-gp interacted with the TRAIL receptors DR4, DR5, and DcR1 in plasma membranes and enhanced TRAIL binding to DR5. Interestingly, the decreased level of the decoy TRAIL receptor, DcR1, in BC-19 cells further sensitized these cells to TRAIL. Therefore, both extrinsic and intrinsic apoptosis pathways are involved in this process. These findings for the first time reveal that TRAIL treatment preferentially causes apoptosis in P-gp-overexpressing MDR cells, and suggests significant clinical implications for the use of TRAIL in treating neoplasms that have failed chemotherapy.

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1. Introduction

Drug resistance is the major reason for the ultimate failure of chemotherapy in the majority of human cancers. Certain types of cancer display intrinsic resistance to multiple chemotherapeutic drugs. Many other cancers acquire multidrug resistance (MDR) during chemotherapy. MDR is frequently associated with the overexpression of P-glycoprotein (P-gp), a 170 kDa ATP-dependent transmembrane protein encoded by the *MDR*1 gene. P-gp is capable of pumping a number of structurally unrelated chemotherapy drugs and other compounds out of the cell by utilizing the energy of ATP hydrolysis [9,30,34]; this results in decreased intracellular accumulation of the compounds and hence resistance to drug cytotoxicity. Introducing the *MDR*1 gene exogenously through a gene transfer approach [18] has established a direct association between P-gp expression and the MDR phenotype. In addition to its efflux pump activity, in some cell lines P-gp has also been shown to have anti-apoptotic function by inhibiting anticancer drug-, Fas ligand-, tumor necrosis factor (TNF-α)-, or UV irradiation-mediated activation of caspase pathways [17,26–29]. Many cellular proteins are involved in regulating apoptosis, including members of the tumor necrosis factor (TNF) receptor superfamily of death receptors such as TNF-α receptor 1, Fas, and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 [37]. When productively bound by their respective ligands (TNF-α, FasL, and TRAIL) on the cell surface, the death receptors trimerize and a caspase-dependent apoptotic pathway is initiated [8,16,17,33]. While TNF-α and FasL effectively cause apoptosis in various tumor cell types, they also cause significant apoptosis in normal tissues. However, administration of recombinant human TRAIL to mice causes no toxicity in normal tissues [2,41], supporting the potential value of TRAIL in cancer therapy. TRAIL binds to four membrane receptors including Death Receptor 4 (DR4, TRAIL-R1), KILLER/DR5 (TRAIL-R2, TRICK2), DcR1 (TRID, TRAIL-R3), DcR2 (TRUNDD or TRAIL-R4), and a soluble receptor, osteoprotegerin (OPG). Only DR4 and DR5 contain cytoplasmic death domains and mediate apoptosis upon binding to TRAIL in sensitive target cells [1]. In contrast, neither DcR1 nor DcR2, which contains a truncated cytoplasmic death domain, mediates apoptosis after binding to TRAIL; DcR1 and DcR2 thus serve as decoy receptors that sequester TRAIL at the cell surface [1].

TRAIL is the most promising cytokine for anticancer therapy since its cytotoxic activity is relatively selective to cancer cells compared to normal cells. However, despite early promising results, recent studies have identified several TRAIL-resistant cancer cell lines [6,10,36,38]. In this paper, we explored whether overexpression of P-gp in MCF-7 breast cancer cells transfected with *MDR*1 cDNA or MDR cells selected with various anticancer agents causes resistance to TRAIL. Surprisingly, our results revealed that P-gp-overexpression actually enhances the sensitivity of cancer cells to TRAIL-induced apoptosis. Mechanistic analysis established that TRAIL engagement of DR5 but not DR4 induces hyperactive ATP hydrolysis by P-gp, and that the consequent depletion of cellular ATP potentiates death induction by canonical TRAIL/DR5 signaling.

2. Materials and methods

2.1. Cell lines, culture conditions and cell survival assay

The MCF-7 human breast cancer cell line, and MCF-7 cells transfected with the human P-glycoprotein gene *MDR*1 (BC-19) [44], were obtained from Dr. Kenneth H. Cowan (University of Nebraska Medical Center, Omaha, Nebraska). The human epidermoid carcinoma cell line KB-3-1, the P-gp-overexpressing MDR variants KB-V1 and KB-8-5, and the *MDR*1-transfected KB-GSV2 and KB-VSV1 cell lines were obtained from Dr. Igor Roninson (University of Illinois, Chicago, IL). The SW620 human colon carcinoma cell line and its P-gp-overexpressing MDR variant SW620/Ad300 [5] were obtained from Dr. Tito Fojo (Cancer Therapeutics Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland). The human breast cancer cell line MDA-MB-231 and its P-gp-overexpressing MDR variant, MDA-MB-231A1 [4] were obtained from Dr. Michael W. DeGregorio (Division of Hematology and Oncology, University of California, Davis, CA). Cells were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS) and 100 ng/ml each of penicillin and streptomycin (Life Technologies, Grand Island, NY) at 37 °C in 5% CO_2. For the methylene blue cell survival assay, cells seeded on 96-well plates (1×10^3 cells plated/well) were cultured in 100 μl of the growth medium in the presence or absence of increasing concentrations (0.001–5 μM) of doxorubicin (DOX) or 10 and 50 ng/ml TRAIL (R&D Systems, Minneapolis, MN), respectively, at 37 °C in 5% CO_2 for 48 h. The cells were fixed with 70% ethanol, stained with methylene blue, and the absorbance was measured on an automated scanning photometer at a wavelength of 630 nm. The concentration of drug that inhibited cell survival by 50% (IC_{50}) was determined from cell survival plots. For each drug-resistant cell line, fold resistance to drugs was determined by calculating the ratio of the IC_{50} value for the drug-resistant cells to the IC_{50} value for the parental, drug-sensitive counterpart.

2.2. Colony forming assay

To determine the cytotoxic effects of TRAIL on the cells, a clonogenic assay was performed as we previously described [31]. Briefly, cells plated in duplicate wells were treated with or without 10 or 50 ng/ml TRAIL, respectively; 48 h later the media was replaced with fresh medium without TRAIL. The number of colonies in each control and treated well were counted after 6 days using a Zeiss inverted microscope.

2.3. Annexin V binding assay to detect apoptotic cells

Following treatment with 10 or 50 ng/ml TRAIL, the cells (5×10^5 cells/treatment group) were analyzed for translocation of phosphatidylserine to the outer surface of the plasma membrane, which is a marker of apoptosis, using the human phospholipid-binding protein annexin V conjugated with fluorescein (Molecular Probes, Eugene, OR) by flow cytometry as we previously described [43]. Apoptosis and necrosis were analyzed by quadrant statistics on the propidium iodide (PI)-negative, fluorescein isothiocyanate (FITC)-positive cells, and PI-positive cells, respectively.

2.4. Hoechst staining

To visualize nuclear morphology in MCF-7 and BC-19 cells by DNA staining, 2×10^5 cells were incubated with 10 or 50 ng/ml recombinant human TRAIL (R&D Systems, Minneapolis, MN) in 3 ml growth medium for 24 h. Hoechst 33342 (1 mM, Sigma, St. Louis, MO) staining dye was added to the cell culture (10 μl/ml of media) and incubated at 37 °C for 30 min. Without washing out the Hoechst dye, cells were viewed on a fluorescence microscope (inverted Nikon epifluorescence microscope, Diaphot 200) with a magnification of 20×, and images were captured using a SPOT color camera.

2.5. Treatment of cells with neutralizing antibodies to the death receptors, and detection of apoptosis

To dissect the roles of the TRAIL receptors DR4, DR5, and DcR1 in TRAIL-induced, P-gp-potentiated apoptosis in drug-resistant cancer cells, the cells were pretreated with neutralizing polyclonal goat antibodies raised against extracellular domains of TRAIL-R1 (anti-DR4), TRAIL-R2 (anti-DR5), and TRAIL-R3 (DcR1) (20 μg/ml, R&D Systems, Minneapolis, MN) 3 h prior to treatment with 10 and 50 ng/ml TRAIL, respectively, for 24 h. In control experiments, cells were treated with normal goat serum prior to TRAIL treatment. Apoptosis was measured by the annexin V assay described above.

To define the role of P-gp function in TRAIL-induced, P-gp-potentiated apoptosis of BC-19 cells, the cells were incubated with or without the anti-P-gp mouse monoclonal antibody (IgG2a) MRK-16 (Kamiya Biomedical Co., Seattle, WA), which neutralizes the transporter function of P-gp, for 3 h prior to treatment with 0, 10, and 50 ng/ml TRAIL for 24 h. Subsequently, apoptosis was quantitated by annexin V flow cytometric analysis as described above. IgG2a was used as an isotype control.

2.6. Flow cytometric analysis of DR4, DR5 and DcR1 expression

MCF-7 and BC-19 cells (5×10^6) were spun down at $500 \times g$, washed with phosphate-buffered saline (PBS) and resuspended in 500 μl PBS. The cells were then incubated with 10 μl goat serum, anti-DR4, anti-DR5, or anti-DcR1 polyclonal goat antibody (1:100, R&D, Minneapolis, MN), respectively, for 1 h. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma Chemical Co.) was added to the cell suspension and incubated for 1 h on ice followed by washing with PBS. After rinsing, the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

2.7. Cytofluorometric analysis of mitochondrial transmembrane potential (Ψ_m)

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) during apoptosis in MCF-7 and BC-19 cells were studied using 5,5′,6,6,-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine (JC-1; Molecular Probes, Inc., Eugene, OR) as a probe as described previously [14]. The cytofluorometric analysis of mitochondrial $\Delta\Psi_m$ was performed in duplicate.

2.8. Measurement of total cellular ATP

MCF-7 and BC-19 cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After 24 h incubation, the cells were exposed to 10 and 50 ng/ml TRAIL, respectively, with or without 20 μg/ml of the anti-P-gp (MDR1) monoclonal antibody MRK-16 for 3 h before adding TRAIL. The levels of ATP were detected by an ATPlite luminescence ATP detection assay system (Perkin-Elmer, Boston, MA) as described by the manufacturer.

2.9. P-gp ATPase activity assay

P-gp ATPase activity was determined by quantifying the release of inorganic phosphate from ATP [21]. Purified plasma membranes were prepared as previously described [19]. MCF-7 and BC-19 plasma membrane samples were diluted to 20 μg/ml in ice-cold ATPase assay medium (3 mM ATP, 100 mM KCl, 10 mM $MgCl_2$, 4 mM DTT, 100 mM Tris pH 8.0, 4 mM EGTA, 2 mM ouabain, and 10 mM NaN_3). Each series of experiments was conducted in a 96-well plate, with a reaction volume of 50 μl/well corresponding to 1 μg of protein per well. To selectively block P-gp ATPase activity, plasma membranes were incubated with the P-gp ATPase inhibitor PSC 833 (2 μM) for 30 min before adding 10 and 50 ng/ml TRAIL, respectively, at 37 °C for 1 h. The reaction was terminated by adding 200 μl of ice-cold stopping medium (0.2%, w/v ammonium molybdate, 1.3%, v/v sulfuric acid, 0.9%, w/v SDS, 2.3%, w/v trichloroacetic acid and 1%, w/v ascorbic acid) to each well. After 75 min incubation at room temperature, the released phosphate was quantitated colorimetrically in an MRX microplate reader (Dynatech Laboratories Inc.) at 630 nm. To detect the effects of ATP on TRAIL-induced apoptosis in MCF-7 and BC-19 cells, the cells were incubated with or without 0.2 μM rotenone, an inhibitor of ATP synthesis (Sigma), for 3 h prior to TRAIL treatment for 24 h. Annexin V flow cytometric analysis was performed as described above. Total cellular ATP was measured by the ATPlite luminescence ATP detection assay system as described above.

2.10. TRAIL binding assay

MCF-7 and BC-19 cells (5×10^6) were incubated with 0, 10 and 50 ng/ml FLAG-tagged TRAIL, respectively, for 24 h. After being spun down and washed three times with PBS, the cells were incubated with 5 μg/ml of FITC-conjugated anti-FLAG monoclonal antibody (Sigma) at room temperature for 1 h followed by rinsing with PBS. The fluorescence intensities of the samples were then measured by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

2.11. Western blot analysis

Western blot analysis was performed as we described elsewhere [43] using several antibodies. In short, 50 μg protein/lane were separated by 5–15% SDS-PAGE, blotted onto a PVDF

Immobilon membrane, and then the protein levels were detected using the dilutions of the antibodies and peroxidase-conjugated secondary anti-rabbit, anti-mouse or anti-goat antibodies (1:2000, v/v, Amersham, Arlington Heights, IL) as described by the manufacturer. The membranes were then exposed to Kodak X-Omat film for various times. The human MDR1 P-gp-specific polyclonal antibody MDR-7 was produced in rabbits using a peptide sequence obtained from the deduced amino acid sequence of the *MDR1* gene. The MDR-7 antibody was used at a concentration of 1:2000 (v/v). The anti-P-gp mouse monoclonal antibody MRK-16 (IgG2a) was obtained from Kamiya Biomedical Company (Seattle, WA). The goat anti-caspase-7 polyclonal antibody (1:1000, v/v) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mouse anti-caspase-8 polyclonal antibody (1:1000, v/v) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). The anti-caspase-9 polyclonal antibody (1:1000, v/v) was provided by Chemicon International (Temecula, CA). The anti-caspase-6 mouse monoclonal antibody (1:1000, v/v) was purchased from Medical and Biological Laboratories Co., Ltd. (Watertown, MA). The neutralizing polyclonal goat antibodies raised against extracellular domains of TRAIL-R1 (DR4), TRAIL-R2 (DR5), and TRAIL-R3 (DcR1) (20 μg/ml) were purchased from R&D Systems (Minneapolis, MN). The anti-cytochrome *c* mouse monoclonal antibody (1:1000) was purchased from BD Pharmingen (San Diego, CA).

2.12. *Immunoprecipitation*

Cells (10^7) were treated with or without 10 and 50 ng/ml TRAIL for 24 h. The cells were then lysed for 30 min on ice with lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 5 mM EDTA and protease inhibitor cocktail) and centrifuged at 15,000 × *g* for 15 min at 4 °C. The supernatants were precleared with normal mouse IgG and protein A/G agarose (Santa Cruz, CA) and incubated with C219 P-gp-specific monoclonal antibody in combination with protein A/G agarose at 4 °C for 16 h. After seven washes with lysis buffer, the protein complexes were separated by SDS-PAGE and transferred to PVDF membranes for Western blotting with the indicated antibodies.

3. Results

3.1. *TRAIL induces preferential apoptosis of P-gp-overexpressing human breast carcinoma BC-19 cells*

To elucidate in-depth the molecular mechanism of TRAIL-induced apoptosis in P-gp-overexpressing drug-resistant cancer cells, we used MCF-7 breast carcinoma cells transfected with the human *MDR1* gene (BC-19). BC-19 cells display 65, 709, and 1033-fold resistance to doxorubicin (DOX), vinblastine (VBL), and Taxol (TAX), respectively, compared to MCF-7 cells (Table 1). As shown in Fig. 1A, Western blot analysis using the monoclonal antibody for P-gp showed that BC-19 cells robustly overexpressed P-gp, while no P-gp was detected in parental MCF-7 cells. To examine TRAIL-induced growth arrest in MCF-7 and BC-19 cells, we assessed the effects of 10 and 50 ng/ml TRAIL on the survival and proliferation of these cells by methylene blue cell survival and clonogenic assays. The results presented in Fig. 1B revealed that in the methylene blue cell survival assay, TRAIL reduced the proliferation and survival of BC-19 cells in a dose-dependent fashion, and that these effects were significantly more pronounced in the drug-resistant cells compared to sensitive MCF-7 cells ($^{*}P < 0.05$, $^{**}P < 0.01$). We also investigated the effects of TRAIL on long-term cell growth by determining the colony-forming capacity of TRAIL-treated

Table 1 – TRAIL-induced apoptosis in MDR cell lines

Cell line	Relative drug resistance			% TRAIL-induced apoptosis	
	DOX	VBL	TAX[a]	10 ng/ml	50 ng/ml[b]
MCF-7	1	1	1	8.67 ± 0.2	14.4 ± 0.8
BC-19	65	709	1033	28.9 ± 0.5	30.44 ± 0.5
MDA-MB-231	1	1	1	11.14 ± 0.3	12.11 ± 0.9
MDA-MB-231/A1	450	294	130	18.52 ± 0.5	20.15 ± 1.6
KB-3-1	1	1	1	15.99 ± 0.1	25.51 ± 1.6
KB-8-5	3.2	6.3	12.7	24.50 ± 1.4	35.70 ± 2.9
KB-V1	428	1120	1067	31.31 ± 2.0	50.80 ± 4.0
KB-GSV2	6.1	42	85	31.31 ± 2.8	48.51 ± 3.8
KB-VSV1	12.3	24	37	89.96 ± 5.9	93.25 ± 7.5
SW620	1	1	1	7.92 ± 0.6	13.08 ± 0.6
SW620/Ad300	18.2	14	35	64.99 ± 3.0	69.11 ± 4.8

[a] Relative degrees of resistance to doxorubicin (DOX), vinblastine (VBL) and Taxol (TAX) were obtained by dividing the IC_{50} values (the concentration of the drug that reduced cell survival by 50%). IC_{50} values for these drugs for MCF-7 and SW620 cells were obtained by methylene blue cytotoxicity assay after 96 h treatment with increasing concentrations of the drugs. The IC_{50} values for DOX, VBL, and TAX treatments for MCF-7 cells were 20, 1.2, and 0.6 nM, respectively, the IC_{50} values for these drugs for MDA-MB-231 cells were 4, 5.1, and 5.45 nM, respectively, and the IC_{50} values for these drugs for SW620 cells were 11, 20, and 5.4 nM, respectively. The IC_{50} values for these drugs for KB-3-1 cells were obtained by clonogenic assay as described previously [36]. The IC_{50} values for DOX, VBL, and TAX treatment for KB-3-1 cells were 3.4, 0.35, and 0.3 nM, respectively.

[b] Percent apoptosis was determined by annexin V binding assay as described in Section 2.

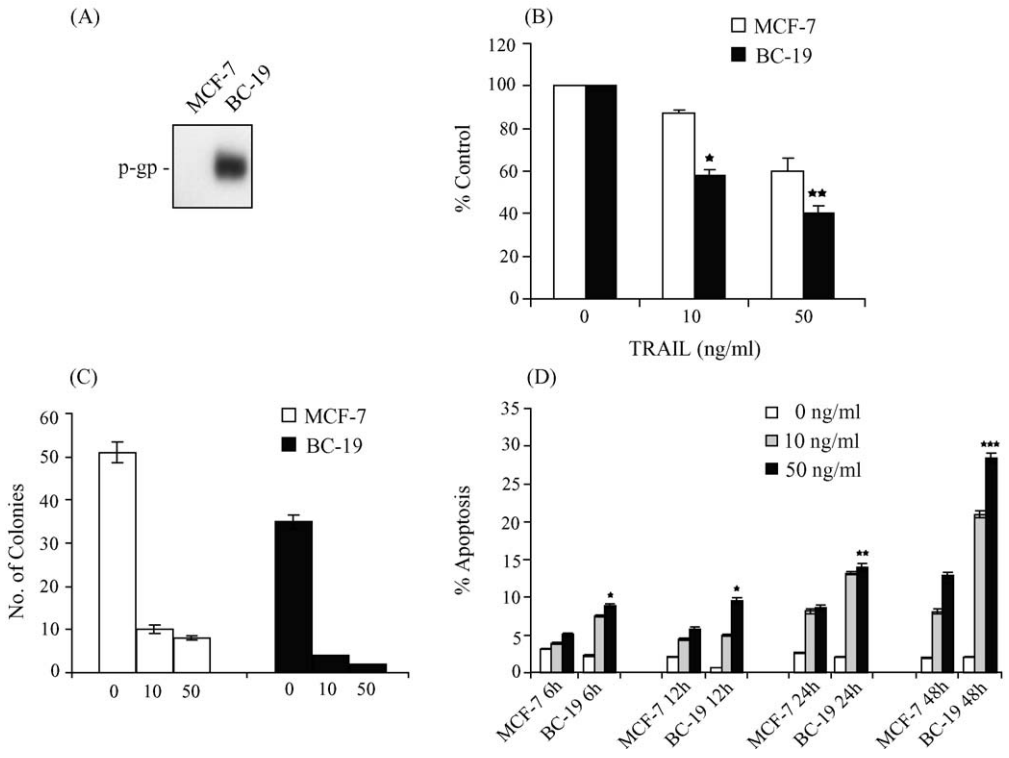

Fig. 1 – TRAIL inhibited cell proliferation and induced apoptosis in drug-sensitive MCF-7 cells and its P-gp-expressing MDR variant BC-19 cells. (A) Western blot analysis of endogenous P-gp levels in MCF-7 and BC-19 cells. Western blotting was performed as described in Section 2. (B) Effect of TRAIL on cell survival using methylene blue cytotoxicity assay as described in Section 2. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. MCF-7 cells. (C) Effect of TRAIL on long-term cell growth using clonogenic assay as described in Section 2. (D) Time coursed effects of TRAIL on apoptotic cell death in MCF-7 and BC-19 cells. 5×10^5 cells were treated with 10 and 50 ng/ml TRAIL for 6, 12, 24 and 48 h, and apoptosis was measured by annexin V binding assay. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. % apoptosis of MCF-7 cells at the same time point.

MCF-7 and BC-19 cells. The results presented in Fig. 1C show that TRAIL (10 and 50 ng/ml) reduced the number of colonies by greater than 90% in both cell lines.

Early in the apoptotic process, phospholipid asymmetry in the plasma membrane is disrupted, leading to exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. Hence, early apoptosis can be measured based on the detection of cell-surface PS with fluorescein-conjugated annexin V (FITC-annexin V). We employed this method to measure apoptotic cell death in MCF-7 and BC-19 cells treated with TRAIL. The data presented in Fig. 1D revealed that treating the cells with TRAIL (10 and 50 ng/ml) for 6, 12, 24, and 48 h induced apoptosis in both MCF-7 and BC-19 cells, and that the levels of TRAIL-induced apoptosis in BC-19 cells treated for 12, 24, and 48 h were significantly greater than those of MCF-7 cells ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

In contrast with a previous report which suggested that P-gp transports endogenous PS in a human gastric carcinoma cell line from the inner leaflet to the cell surface [24], our fluorescence microscopy analysis revealed that annexin V did not interact with the cell surface of control untreated MCF-7 or BC-19 cells. This is illustrated by the results shown in Fig. 2D. Therefore, the detection of enhanced TRAIL-induced apoptosis in BC-19 cells with FITC-annexin V is not the result of an apoptosis-independent display of PS at the cell surface of this P-gp-overexpressing cell line. To test the validity of the FITC-annexin V-based measurements of apoptosis in Fig. 1D, which show that TRAIL induces more apoptosis in BC-19 cells than MCF-7 cells, we also measured apoptosis based on nuclear morphology by light-phase contrast microscopy and fluorescence microscopy of Hoechst 33342-stained cells. TRAIL-induced typical apoptotic changes in the nuclear morphology, with pronounced condensation of nuclei and nuclear fragmentation in both MCF-7 and BC-19 cells (Fig. 2, Panels A and B). Quantification of apoptosis as determined by Hoechst 33342 staining revealed that TRAIL caused substantial apoptosis in these cells lines, but the levels of TRAIL-induced apoptosis at 24 h were significantly greater in BC-19 cells than MCF-7 cells (Fig. 2C) ($^{*}P < 0.01$, $^{**}P < 0.05$).

In order to determine whether enhanced TRAIL sensitivity is also a feature of other P-gp-overexpressing MDR human carcinoma cells, we investigated several established P-gp-bearing drug-resistant variants as well as drug-sensitive cell lines transfected with *MDR1* full-length cDNA. We treated the cells with 10 and 50 ng/ml TRAIL for 24 h, and compared the levels of TRAIL-induced apoptosis in these cell lines versus their parental drug-sensitive counterparts. The results shown in Table 1 revealed that TRAIL induced more apoptosis in each P-gp-expressing MDR cell line versus its drug-sensitive counterpart regardless of the level of MDR.

In the mechanism of P-gp-catalyzed, ATP-dependent drug transport, the amino-acid residue P-gp Gly185 is pivotal for

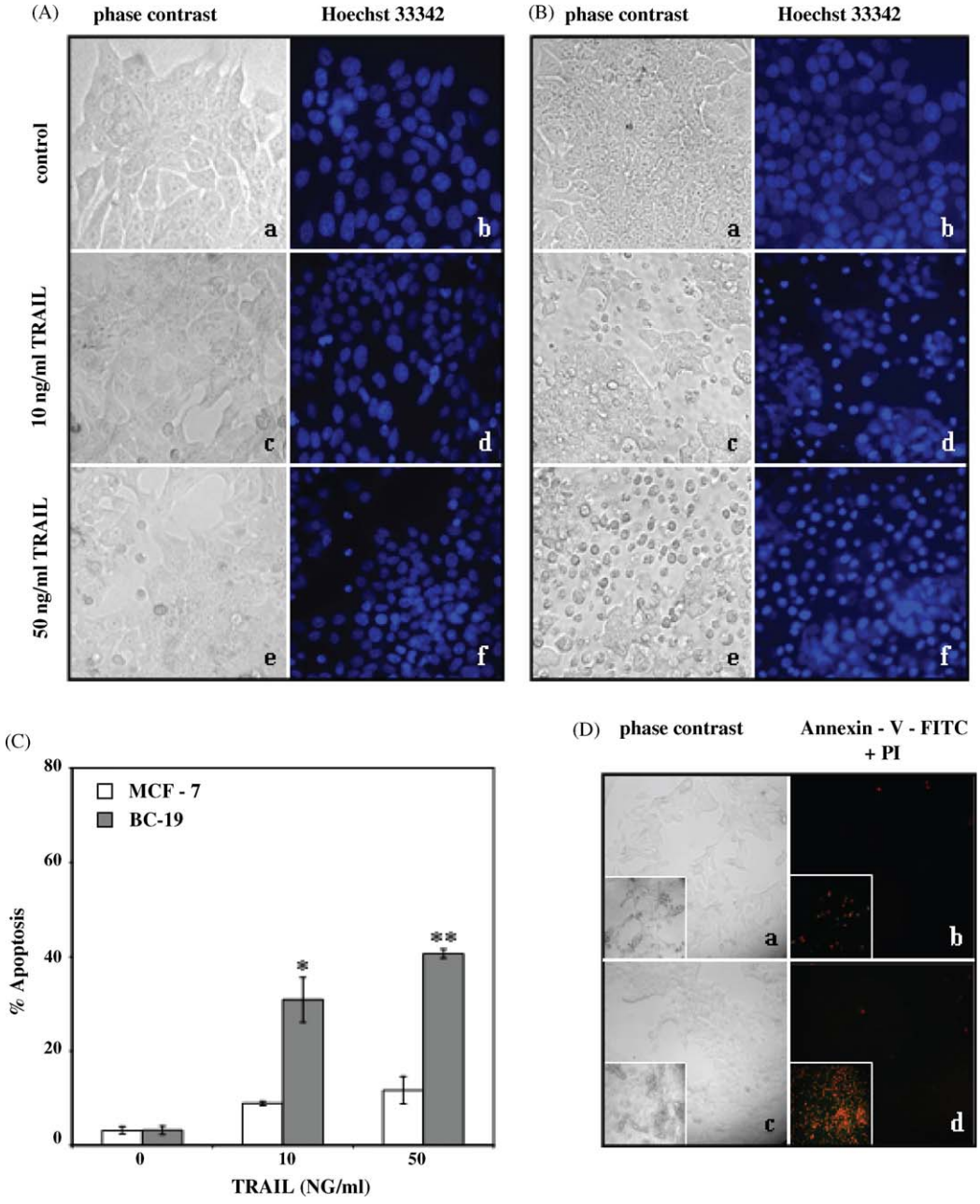

Fig. 2 – Apoptosis induction mediated by TRAIL in MCF-7 and BC-19 cells. (A and B) Phase contrast (a, c, e) and fluorescence microscopy images (b, d, f) after staining for DNA using Hoechst 33342. BC-19 cells (B) treated with 10 ng/ml (c and d) and 50 ng/ml TRAIL (e and f) showed significantly higher numbers of fragmented nuclei than TRAIL-treated MCF-7 (A) and control cells (a and b) that received 0.1% of DMSO. (C) Quantification of apoptosis was carried out by counting the fragmented nuclei stained with Hoechst 33342 among 500 cells. Shown are the representative apoptosis rates from three independent counts ($^{*}P < 0.01$, $^{**}P < 0.05$). (D) Control MCF-7 and BC-19 cells received 0.1% DMSO after staining with annexin V-FITC and propidium iodide (PI). MCF-7 as well as BC-19 cells treated with 50 ng/ml TRAIL for 24 h were stained with annexin V-FITC and PI and shown in the insets as positive controls.

transmitting conformational changes between the catalytic sites and the drug binding domains of P-gp [22,31]. It is therefore intriguing that TRAIL induced much more apoptosis in the KB-3-1 transfectant KB-VSV1, which expresses the mutant G185V P-gp, than in the wt P-gp transfectant KB-GSV2 (Table 1) (see Section 4).

3.2. *TRAIL-induced apoptosis is blocked by neutralizing antibodies for TRAIL receptor DR5 but not DR4 in BC-19 cells*

We then explored whether enhanced TRAIL-induced apoptosis in P-gp-overexpressing MDR cells requires DR4 or DR5 death receptors containing a cytoplasmic death domain. The

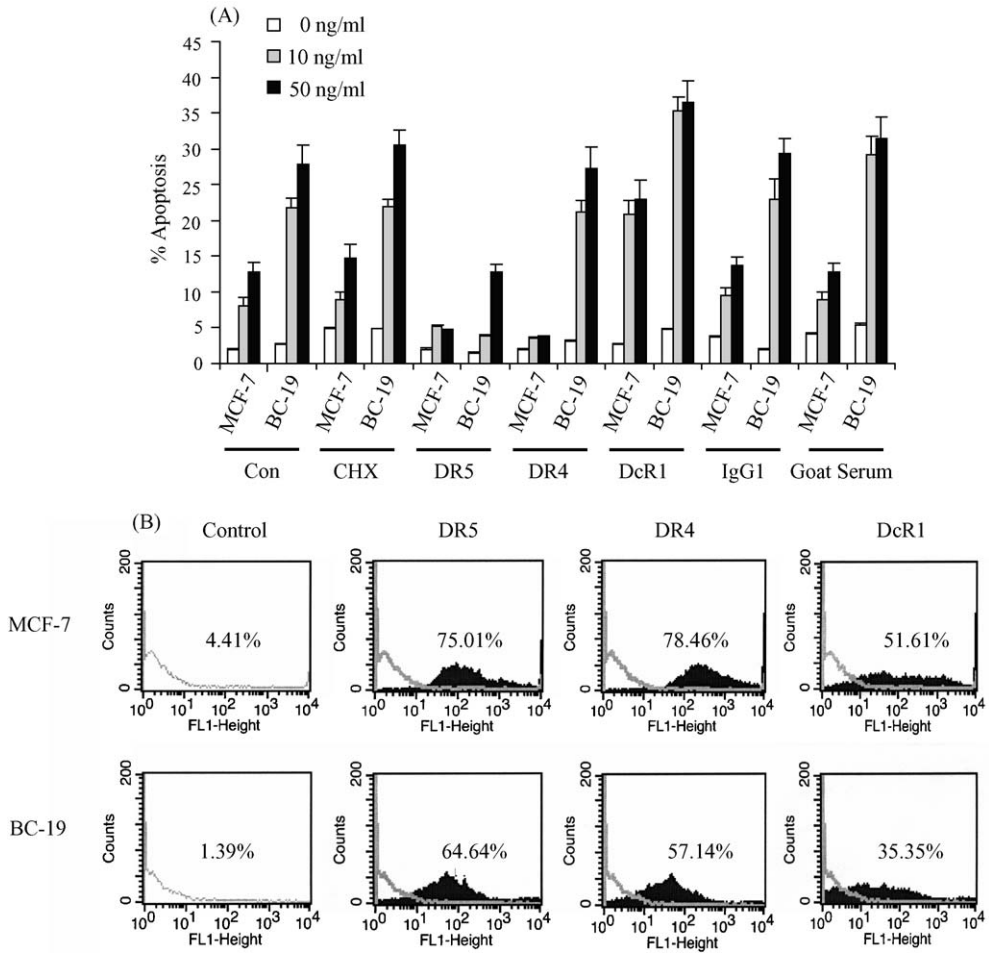

Fig. 3 – Analysis of TRAIL receptors and their role in TRAIL-induced apoptosis in MCF-7 and BC-19 cells. (A) Effects of neutralizing antibodies to TRAIL receptors on TRAIL-induced apoptosis. MCF-7 and BC-19 cells were pretreated with the neutralizing polyclonal goat antibodies raised against extracellular domains of TRAIL-R1 (anti-DR4), TRAIL-R2 (anti-DR5), and TRAIL R3 (DcR1) (20 μg/ml) 3 h prior to the treatment with 10 and 50 ng/ml TRAIL, respectively, for 24 h. In control experiments, cells were treated with normal goat serum prior to TRAIL treatment. Apoptosis was the measured by annexin V assay as described above. (B) Analysis of cell surface expression of DR4, DR5 and DcR1 TRAIL receptors in MCF-7 and BC-19 cells. MCF-7 and BC-19 cells (5×10^6) were incubated with 10 μl of goat serum, or anti-DR4, anti-DR5, or anti-DcR1 polyclonal goat antibody (1:100), respectively, for 1 h after washing. Then cells were rinsed and incubated with FITC-conjugated rabbit anti-goat polyclonal antibody as described in Section 2. Ten thousand cells were analyzed for each sample. The open peak represents non-specific background fluorescence as detected using goat serum as the control primary antibody. The black peaks indicate the surface expression of the DR4, DR5 and DcR1 receptors. Note that fluorescence intensity is shown on a logarithmic scale.

levels of TRAIL-induced apoptosis in the parental MCF-7 and the P-gp-overexpressing BC-19 cell lines were not altered by treatment with cycloheximide (CHX), a protein synthesis inhibitor, revealing that TRAIL causes apoptosis of both cell lines independently of protein synthesis and endogenous TRAIL (Fig. 3A). Moreover, as shown in Fig. 3A, treating MCF-7 cells with neutralizing antibodies to either TRAIL receptor DR4 or DR5 prior to TRAIL treatment similarly inhibited TRAIL-induced apoptosis. However, only treatment with the DR5 antibody inhibited TRAIL-induced apoptosis in BC-19 cells; DR4 neutralization had no effect on TRAIL-induced apoptosis in these P-gp-overexpressing cells (Fig. 3A). Interestingly, treating MCF-7 or BC-19 cells with the DcR1 neutralizing antibody prior to TRAIL treatment enhanced TRAIL-dependent apoptosis, consonant with the decoy function of this TRAIL receptor. Attribution of the apoptosis-inhibitory activities of the antibodies to specific interactions with the cognate receptor was validated by negative control experiments using isotype-specific IgG1 or normal goat serum (Fig. 3A).

To correlate the inhibitory effects of the anti-DR4 and anti-DR5 neutralizing antibodies on TRAIL-induced apoptosis with cell surface expression of these receptors, flow cytometric quantitation of these receptors was carried out as described in Section 2 using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Results presented in Fig. 3B show that while similar levels of DR5 expression were seen in MCF-7 and BC-19 cells, DR4 and particularly DcR1 cell surface expression in BC-19 cells were less than in MCF-7 cells. To

Table 2 – Relative expression of DR4, DR5 and DcR1 receptors before and after treatment with TRAIL[a]

Cell line	TRAIL (ng/ml)	DR4	DR5	DcR1
MCF-7	0	63.22 ± 2.072	81.74 ± 3.29	45.87 ± 0.99
	10	76.18 ± 3.64	79.84 ± 2.62	56.12 ± 1.27
	50	78.65 ± 3.11	80.30 ± 2.11	62.58 ± 1.05
BC-19	0	52.48 ± 2.26	72.27 ± 2.09	30.08 ± 0.54
	10	49.96 ± 1.44	67.51 ± 1.25	24.70 ± 0.70
	50	52.58 ± 1.07	66.06 ± 2.13	26.87 ± 0.33

[a] Relative expression levels of DR4, DR5, and DcR1 in MCF-7 and BC-19 cells were obtained from the % of FITC intensity count through flow cytometry. MCF-7 and BC19 cells were treated with or without 10 and 50 ng/ml TRAIL, respectively, for 24 h. Cell were then rinsed with PBS and incubated with monoclonal anti-DR4, anti-DR5 and anti-DCR1 antibodies (1:200) or isotype-specific antibody IgG2a for 1 h. After washing, cells were incubated with FITC-conjugated anti-mouse antibody for 45 min followed by washing with PBS. Then the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer and the CellQuest program. Expression levels of DR4, DR5, and DcR1 in duplicate samples were normalized against the values obtained using IgG2a as the isotype control.

determine whether TRAIL treatment affects the cell surface expression of DR4, DR5 and DcR1, the levels of these receptors in MCF-7 and BC-19 cells before and after treatment with 10 and 50 ng/ml TRAIL for 24 h were determined by flow cytometric analysis as described in Section 2. As shown in Table 2, the effects of TRAIL treatment on cell surface expression of the DR4, DR5 and DcR1 receptors were modest in MCF-7 cells and negligible in BC-19 cells. Similar results were obtained by Western blot analysis (data not shown). These results clearly establish that the DR4-independence of TRAIL-induced apoptosis of BC-19 cells (Fig. 3A) occurs in the context of abundant DR4 expression (Fig. 3B).

3.3. *TRAIL preferentially triggers initiator caspase-8 and executioner caspase activation in BC-19 cells*

To determine whether the enhanced TRAIL-induced apoptotic response of the P-gp-overexpressing BC-19 cell line compared with parental MCF-7 cells involved distinct patterns of caspase activation, we performed Western blotting of the executioner caspases (caspases-6 and -7) and initiator caspases (caspases-8 and -9) using antibodies capable of recognizing both the pro-forms and activated forms of these caspases. As shown in Fig. 4A, treatment of the MCF-7 cells with 10 and 50 ng/ml TRAIL for 24 h caused processing and activation of the 34 kDa procaspase-6 to its active 20 kDa form. Consistent with the more vigorous apoptotic response of BC-19 cells to TRAIL, cleavage and processing of procaspase-6 to its active forms occurred to a greater degree in BC-19 cells compared to MCF-7 cells (Fig. 4A). Similarly, TRAIL-induced conversion of procaspase-7 to its active forms was more efficient in BC-19 cells compared to MCF-7 cells (Fig. 4B). MCF-7 cells do not express executioner caspase-3. Therefore, our results demonstrate that the enhanced apoptotic response of drug-resistant BC-19 cells to TRAIL is associated with enhanced executioner caspase activation.

TRAIL at 10 and 50 ng/ml caused cleavage of procaspase-8 to its 43 and 41 kDa activated fragments in BC-19 cells vigorously and to a greater extent compared with MCF-7 cells (Fig. 4C; the 43 and 41 kDa active forms are seen as a doublet).

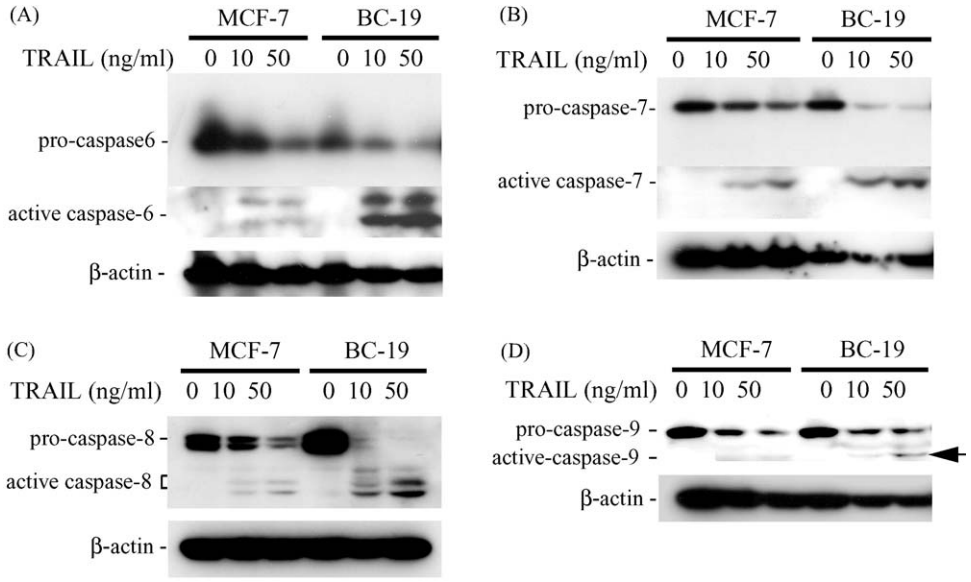

Fig. 4 – TRAIL triggers caspase activation in MCF-7 and BC-19 cells. (A) Caspase-6, (B) Caspase-7, (C) Caspase-8 and (D) Caspase-9. Aliquots (50 μg of protein/lane) were separated by 5–15% SDS-PAGE, and subjected to Western blot analysis as described in Section 2.

In contrast, TRAIL at 10 and 50 ng/ml induced only modest activation of procaspase-9 to its 37 kDa active form in both MCF-7 and BC-19 cells (Fig. 4D). These results strongly implicate a role for initiator caspase-8 in the differential apoptotic responses of MCF-7 and BC-19 cells to TRAIL.

3.4. *TRAIL preferentially triggers decreased mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome c release in BC-19 cells*

While the detection of only a modest amount of activated caspase-9 in TRAIL-treated MCF-7 and BC-19 cells argued against vigorous activation of the mitochondrial amplification-loop in TRAIL-induced apoptosis of these cell lines, the marked TRAIL-induced loss of procaspase-9 (Fig. 4D) argued otherwise. We next pursued this question by exploring whether TRAIL triggers in MCF-7 and BC-19 cells (a) altered mitochondrial membrane potential ($\Delta\Psi_m$) (Fig. 5A), and (b) the release of cytochrome c from mitochondria (Fig. 5B).

The JC-1 mitochondrial potential sensor is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). Therefore, mitochondrial depolarization is indicated by a decrease in the red (FL2)/green (FL1) fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. As shown in Fig. 5A, BC-19 cells underwent a dramatic decrease in mitochondrial membrane potential compared to MCF-7 after treatment with 10 and 50 ng/ml TRAIL for 24 h. (The cells with depolarized mitochondria are those with a loss of red fluorescence (in FL2) and a gain of green fluorescence (in FL1)). In corroboration of these results, treatment with 10 and 50 ng/ml TRAIL resulted in the release of cytochrome c from the mitochondria robustly and more efficiently in BC-19 cells compared to MCF-7 cells (Fig. 5B). Moreover, in both MCF-7 and BC-19 cells TRAIL-triggered cleavage of Bid to its truncated form tBid (data not shown) known to translocate to mitochondria and release cytochrome c. Our results strongly implicate involvement of the mitochondrial amplification-loop in the enhanced apoptotic response of BC-19 cells to TRAIL.

3.5. *P-gp plays an indispensable role in enhancing TRAIL-induced apoptosis in BC-19 cells*

Seeking proof of principle that overexpression of P-gp plays a major role in the enhanced TRAIL sensitivity of drug-resistant human cancer cells (Table 1), we exposed MCF-7 and BC-19 cells to 10 and 50 ng/ml TRAIL, after a 3 h preincubation with 20 μg/ml of the anti-P-gp (MDR1) monoclonal antibody MRK-16, known to inhibit P-gp function, or IgG2a control antibody. Apoptosis was then quantitated by annexin V binding assay.

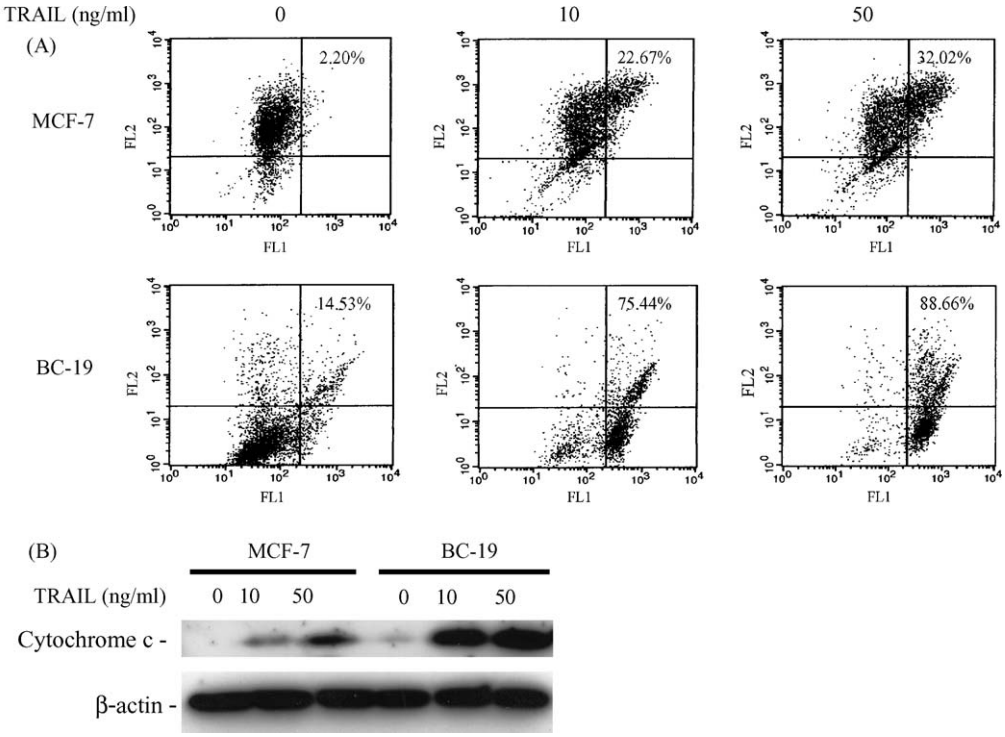

Fig. 5 – Effects of TRAIL on the mitochondrial potential and release of cytochrome c in MCF-7 and BC-19 cells. (A) TRAIL-induced $\Delta\Psi_m$. 5×10^5 cells were treated with or without 10 and 50 ng/ml TRAIL in culture media for 24 h. 5,5′,6,6,-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine (JC-1; Molecular Probes, Inc., Eugene, OR) was used as a probe as described previously. JC-1 membrane potential-related fluorescence was recorded using an FL1 photomultiplier tube (PMT) by a FACSCalibur flow cytometer. The numbers in the upper right corners are the percentages in $\Delta\Psi_m$ in both cell lines in the absence or presence of 10 and 50 ng/ml TRAIL. This experiment was performed in triplicate. (B) Cytochrome c release. The cytosol fractions were isolated as described under Section 2. Western blot analysis was performed as described in Section 2.

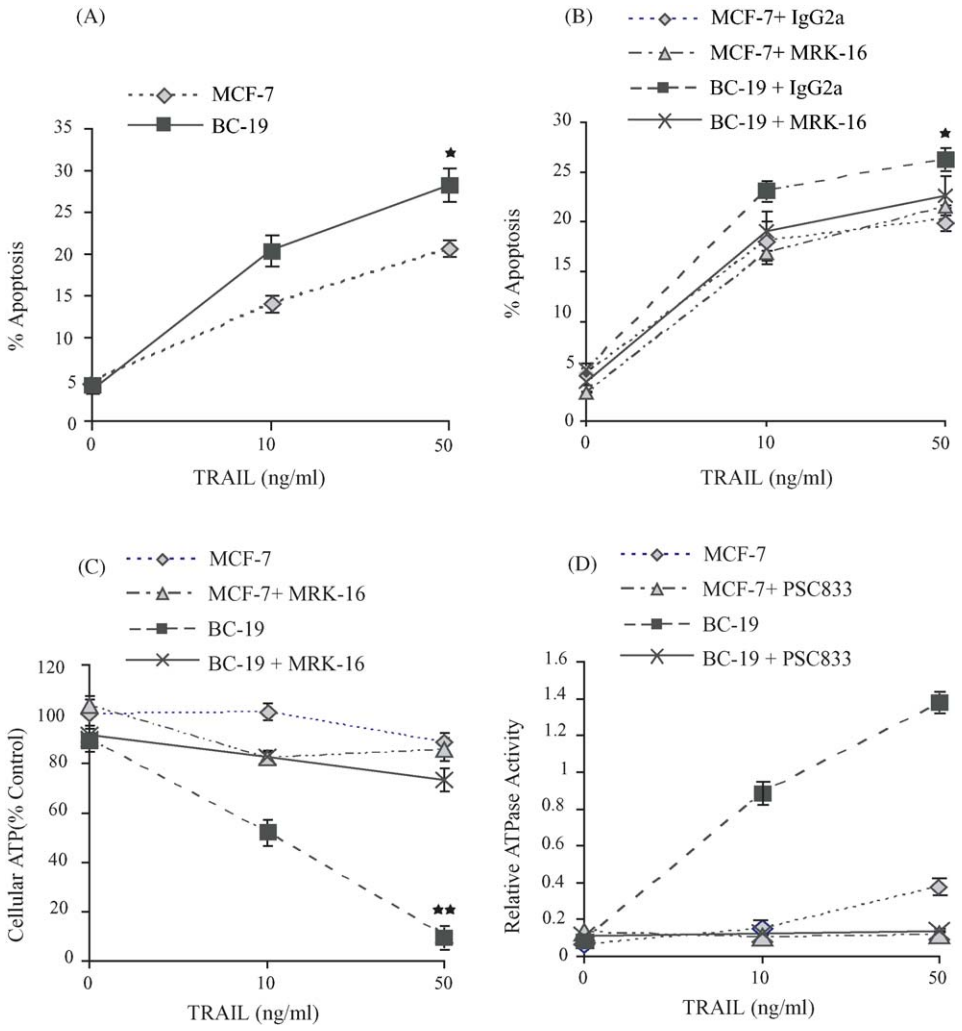

Fig. 6 – TRAIL-induced ATP depletion and apoptosis in P-gp-expressing BC-19 cells via increased P-gp ATPase activity. (A and B) Effect of P-gp-specific MRK-16 monoclonal antibody on TRAIL-induced apoptosis in MCF-7 and BC-19 cells. The cells (5×10^5 cells/treatment) were incubated with 10 μg/ml MRK-16 (IgG2a) mouse monoclonal antibody (Kamiya Biomedical Co., Seattle, WA) prior to treatment with 10 and 50 ng/ml TRAIL for 24 h. Apoptosis was measured by annexin V binding assay using fluorescein-conjugated annexin V (Molecular Probes, Eugene, OR) by flow cytometry as described under Section 2. (C) Analysis of total cellular ATP. MCF-7 and BC-19 cells were seeded in 96-well plates at a density of 5×10^4 cells/well. After 24 h growth, the cells were exposed to 10 and 50 ng/ml TRAIL, respectively, with or without 20 μg/ml of the anti-P-gp (MDR1) monoclonal antibody MRK-16 for 3 h before adding TRAIL. The ATP levels were detected by an ATPlite luminescence ATP detection assay system as described under Section 2. (D) Analysis of P-gp ATPase activity. MCF-7 and BC-19 plasma membrane samples were diluted to 20 μg/ml in ice-cold ATPase assay medium. Each series of experiments was conducted in a 96-well plate, with a reaction volume of 50 μl/well corresponding to 1 μg of protein per well. To block the ATPase activity, plasma membranes were incubated with 2 μM of the P-gp ATPase inhibitor PSC 833 for 30 min before adding 10 and 50 ng/ml TRAIL, respectively, at 37 °C for 1 h. The reaction was terminated by the adding 200 μl of ice-cold stopping medium to each well. After 75 min incubation at room temperature, the released phosphate was quantitated colorimetrically as described in Section 2. $^{*}P < 0.05$, $^{**}P < 0.01$.

Results shown in Fig. 6A and B demonstrate that the level of TRAIL-induced apoptosis in BC-19 cells treated with MRK-16 decreased to the level of apoptosis in MCF-7 cells which lack endogenous P-gp, whereas MRK-16 had no effect on TRAIL-induced apoptosis of MCF-7 cells. The effect of the anti-P-gp monoclonal antibody was clearly due to specific interactions with P-gp, since the isotype-specific IgG2a normal immunoglobulin did not affect TRAIL-induced apoptosis in either cell line (Fig. 6B). These results indicate that cell-surface expression of P-gp is indispensable for the enhanced apoptotic response of the drug-resistant MCF-7 subline BC-19 to TRAIL.

P-gp-mediated active efflux of chemotherapy drugs and other structurally unrelated substrates is driven by the energy of ATP hydrolysis [19]. To test the hypothesis that P-gp boosts TRAIL-induced apoptosis in drug-resistant cancer cells by depleting the cellular ATP pool, we measured the effect of

TRAIL on ATP levels in MCF-7 and BC-19 cells in the absence or presence of the anti-P-gp antibody MRK-16 (Fig. 6C). The results in Fig. 6C clearly show that TRAIL sharply decreased the ATP level in BC-19 cells but was without effect in MCF-7 cells, and that pretreatment with the anti-P-gp antibody MRK-16 prevented the reduction in the cellular ATP level of BC-19 cells. These results indicate that the P-gp-dependent enhancement of TRAIL-induced apoptosis in BC-19 cells is associated with TRAIL-stimulated, P-gp-dependent ATP hydrolysis and consequent cellular ATP depletion. Furthermore, the results constitute strong evidence that TRAIL stimulates P-gp ATPase activity in drug-resistant cancer cells.

Some chemosensitizers with tight binding affinity to P-gp, such as cyclosporine A and its non-immunosuppressant analog PSC 833, are potent P-gp ATPase inhibitors [35]. Therefore, in order to directly test the hypothesis that TRAIL enhances the ATPase activity of P-gp in BC-19 cells, we treated isolated plasma membranes prepared from BC-19 cells with 2 μM PSC 833 for 30 min followed by a 1 h treatment at 37 °C with 10 and 50 ng/ml TRAIL and measured P-gp ATPase activity. Fig. 6D clearly shows that TRAIL induced a dose-dependent increase in the ATPase activity of P-gp in isolated BC-19 plasma membranes. These results established that TRAIL signals to hyperactive P-gp ATPase catalysis in BC-19 cells.

To test the hypothesis that depletion of cellular ATP is sufficient to boost TRAIL-induced apoptosis of BC-19 cells, we also examined the effects of rotenone, an inhibitor of oxidative phosphorylation known to deplete ATP, on this process. MCF-7 and BC-19 cells were exposed to rotenone for 3 h prior to treatment with TRAIL for 24 h as described in Section 2. As shown in Fig. 7A, the addition of rotenone depleted ATP by several fold in untreated MCF-7 and BC-19 cells. The sensitivity of these ATP-depleted cells to TRAIL was determined by annexin V binding assay. The results in Fig. 7B show that depletion of ATP had only a minor effect on apoptosis induced by 50 ng/ml TRAIL in MCF-7 cells, but clearly increased apoptosis triggered by TRAIL at 10 and 50 ng/ml in the P-gp-overexpressing BC-19 cells. These results and our data discussed above reveal that the cellular ATP supply is insufficiently impacted by rotenone in MCF-7 cells to boost TRAIL-induced apoptosis whereas the P-gp-dependent boost in TRAIL-induced BC-19 apoptosis is additive with the effect of further ATP depletion by rotenone.

To this point, our results established that TRAIL treatment stimulated P-gp ATPase activity in BC-19 cells with consequent ATP depletion (Fig. 6), and that ATP depletion by rotenone enhanced TRAIL-induced BC-19 apoptosis (Fig. 7). To test whether TRAIL-induced stimulation of P-gp ATPase activity is mechanistically linked to the hypersensitivity of BC-19 cells to TRAIL compared to the parental drug-sensitive counterpart MCF-7, we inactivated P-gp function and/or prevented interaction of TRAIL with DR4, DR5 or DcR1 by treating the P-gp-overexpressing cells with or without the anti-P-gp monoclonal antibody MRK-16 and/or with the neutralizing goat anti-DR4, anti-DR5, or anti-DcR1 antibody for 3 h prior to TRAIL treatment for 24 h. As negative controls, the cells were incubated with the isotype-specific IgG2a, normal goat serum, or a combination of these two. The data in Fig. 8A show that treating BC-19 cells with anti-DR5 or MRK-16 decreased TRAIL-induced apoptosis, and the combination of these antibodies further reduced apoptosis. In negative control experiments, no change in TRAIL-induced apoptosis was observed. Interestingly, treating the cells with the DR4 neutralizing antibody did not decrease TRAIL-triggered apoptosis. Treatment with the anti-P-gp antibody MRK-16 inhibited apoptosis, but the combination of MRK-16 and the DR4 antibody had only a little more effect on apoptosis than MRK-16 alone (Fig. 8B).

The additive inhibition of BC-19 apoptosis in Fig. 8A by the P-gp Ab that blocked P-gp ATPase activity in the cells (MRK-16) (Fig. 6C) and the neutralizing DR5 antibody, taken together with the stimulation of P-gp ATPase activity in BC-19 cells by TRAIL treatment and rotenone enhancement of TRAIL-

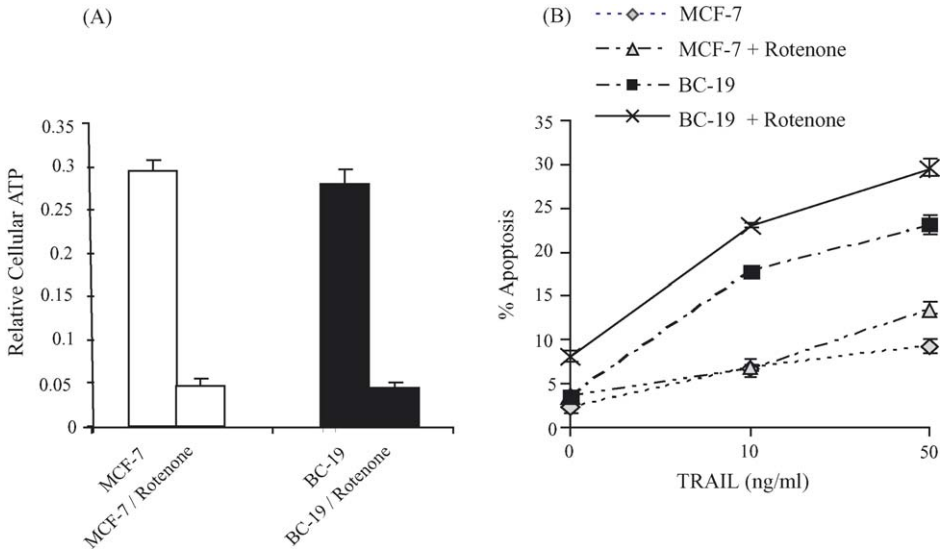

Fig. 7 – Effect of ATP inhibitor on the TRAIL-induced apoptosis. (A) Total ATP is abolished after treatment 0.2 μM rotenone for 3 h. (B) MCF-7 and BC-19 cells were incubated with or without 0.2 μM rotenone for 3 h, then treated with or without 10 and 50 ng/ml TRAIL for 24 h, and apoptosis was measured by annexin V binding assay as described in Section 2.

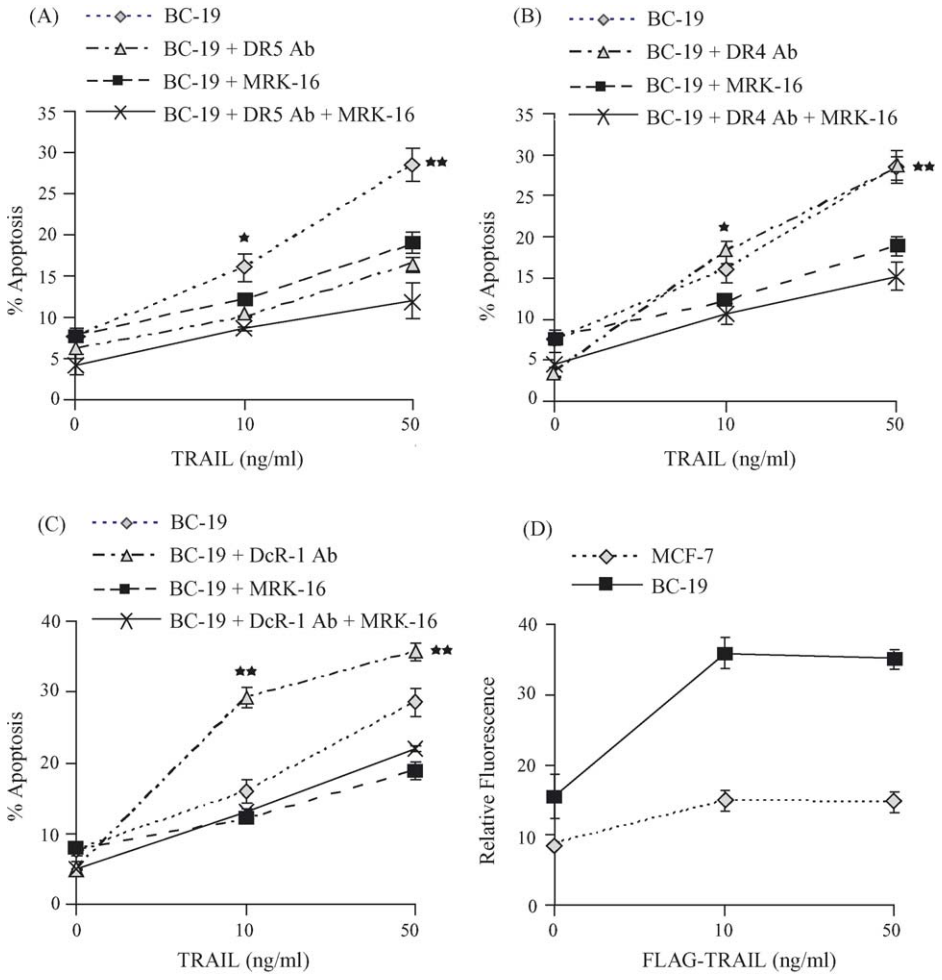

Fig. 8 – Analysis of the effects of anti-TRAIL receptors antibodies and anti-P-gp antibody on TRAIL-induced-apoptosis and the binding of FLAG-tagged TRAIL to MCF-7 and BC-19 cells. 5×10^5 cells were incubated with anti-DR5 (A), anti-DR4 (B), anti-DcR1 (C) or/and MRK-16 antibody for 3 h prior to the treatment with or without 10 and 50 ng/ml TRAIL for 24 h, and apoptosis was measured by annexin V binding assay as described in Section 2. Goat serum and IgG2a were used as the isotype controls and had no significant effects on TRAIL-induced apoptosis. (D) FLAG-tagged TRAIL binding to the TRAIL receptors of MCF-7 and BC-19 cells. These cells were incubated with 10 and 50 ng/ml of FLAG-tagged-TRAIL, respectively, for 24 h. After washing, cells were incubated with 5 μg/ml of FITC-conjugated anti-FLAG monoclonal antibody at room temperature for 1 h. The samples were then analyzed by flow cytometry using a FACSCalibur flow cytometer with the CellQuest program. Ten thousand cells were analyzed for each sample. Data are average of duplicate experiments ($^*P < 0.05$, $^{**}P < 0.01$).

induced BC-19 apoptosis, intimately ties the potentiation of TRAIL-induced apoptosis by P-gp to hyperactive P-gp ATPase catalysis. Moreover, even more striking results were obtained when the BC-19 cells were treated with the neutralizing antibody to the DcR1 receptor. As shown in Fig. 8C, this antibody was able to increase TRAIL-induced BC-19 apoptosis, and the P-gp antibody MRK-16 abrogated the response. These data indicate that decreasing the level of unoccupied DcR1 in P-gp-overexpressing cells as shown in Fig. 8C enhances TRAIL sensitivity in BC-19 cells. The implication of this is that reducing unoccupied DR4 or DcR1 expression would render more TRAIL available to interact with DR5, and thereby cause more apoptosis in BC-19 cells.

We next investigated whether P-gp enhances binding of TRAIL to its cognate receptors. The binding of FLAG-tagged TRAIL to MCF-7 and BC-19 cells at 24 h was quantitated by flow cytometry. The data in Fig. 8D show that indeed more TRAIL was bound to BC-19 cells compared to MCF-7 cells. This cannot be accounted for by the differences in TRAIL receptor expression levels in BC-19 versus MCF-7 cells shown in Table 2.

To determine whether P-gp exerts its effect on TRAIL-induced apoptosis by physically interacting with the TRAIL receptors, we performed co-immunoprecipitation experiments as described in Section 2 (Fig. 9). The data presented clearly show that P-gp forms complexes with DR4 (A), DR5 (B), and DcR1 (C), and that TRAIL enhances these binding interactions. How P-gp expression enhances the ability of TRAIL to interact with its receptors and whether P-gp favors TRAIL interactions with DR5 are interesting questions that remain to be addressed.

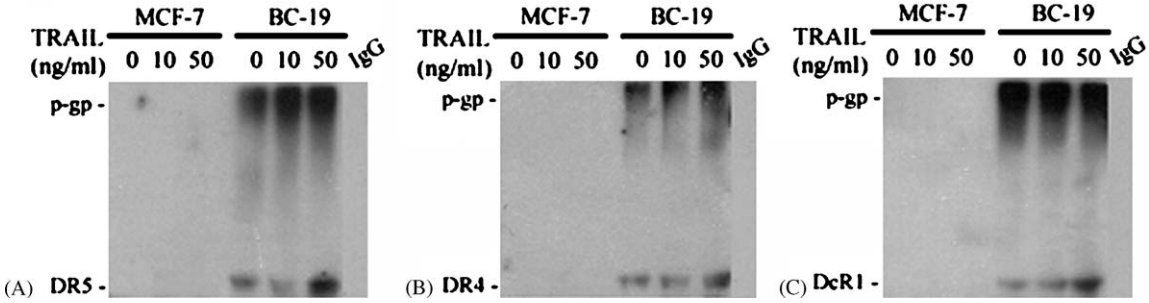

Fig. 9 – P-gp association with TRAIL receptors. Lysates from MCF-7 and BC-19 treated with or without 10 and 50 ng/ml TRAIL were immnoprecipitated with anti-P-gp monoclonal antibody C219 and subjected to Western blot analysis. The immunoblot was probed for the presence of (A) DR5, (B) DR4, (C) DcR1, and P-gp as described in Section 2.

4. Discussion

It is well documented that P-gp-overexpression causes MDR in cancer cells. In this report, we explored whether overexpression of P-gp in MDR cancer cells also causes resistance to TRAIL. Surprisingly, our data demonstrated that P-gp-overexpressing MDR cells are more susceptible to TRAIL than their drug-sensitive counterparts. While this work was in progress, Cenni et al. [7] also reported that the osteosarcoma MDR-U2OS subline exhibited more TRAIL sensitivity than its U2OS drug-sensitive counterpart [7]. Our data clearly show for the first time that enhanced TRAIL sensitivity is a common property shared among diverse drug-selected and MDR1-transfectant human cancer cell lines that overexpress P-gp as compared with parental, drug-sensitive counterparts (Table 1). Furthermore, through in-depth mechanistic analysis comparing TRAIL-induced apoptosis of P-gp-overexpressing BC-19 MDR1 transfectants versus parental, drug-sensitive MCF-7 cells in this report, we elucidate the mechanism of enhanced TRAIL-induced apoptosis of the drug-resistant cells. Specifically, our analysis demonstrates that the enhancement of TRAIL-triggered apoptosis in P-gp-overexpressing BC-19 cells was associated with the conversion of the DR4/DR5-dependent mechanism of MCF-7 cell apoptosis by TRAIL to a DR5-dependent, DR4-independent mechanism of TRAIL-induced apoptosis. Furthermore, the enhanced apoptotic response of BC-19 cells to TRAIL entailed DR5-dependent, marked stimulation of P-gp ATPase activity and consequent depletion of cellular ATP, in association with enhanced activation of the executioner caspases (caspases-6 and -7) and initiator caspases (caspases-8 and -9) expressed by the cells, Bid cleavage, and increased mitochondrial depolarization.

It is well documented that caspase-8 activation results in truncation of the proapoptotic protein, Bid, and that the translocation of truncated Bid (tBid) to the mitochondria is associated with subsequent release of cytochrome *c*, Omi/HtrA2 and Smac/DIABLO into the cytosol [11]. Our results showed that Bid degradation was also associated with the release of cytochrome *c* from the mitochondria to cytosol in TRAIL-treated MCF-7 and BC-19 cells. Considering that decreased mitochondrial membrane potential has been described as an event occurring in apoptosis induced by a variety of agents, including TRAIL [39], we hypothesize a direct correlation between the increased apoptosis induced by TRAIL in BC-19 cells, more efficient release of cytochrome *c*, and decreased $\Delta\Psi_m$.

The general consensus is that apoptosis is an active energy-requiring process, and it has been proposed that the cellular ATP level is an important factor in apoptosis [25]. Furthermore, a decreased ATP/ADP ratio or decreased ATP concentration has been proposed to enhance apoptosis [40]. Moreover, a recent report showed that Bid is activated in kidney cells following ATP depletion in vitro and ischemia in vivo [15]. In addition, treatment with small cell-permeable non-peptide inhibitors of Bcl-2 reduced $\Delta\Psi_m$, generated reactive oxygen species (ROS), increased mitochondrial respiration, and decreased ATP synthesis in CEM leukemia cells [42]. These inhibitors per se did not induce apoptosis, but rather sensitized CEM cells to TRAIL-induced apoptosis by uncoupling mitochondrial respiration.

Our results also showed that the decoy TRAIL-R3 receptor (DcR1) may prevent the P-gp enhanced paradigm of TRAIL-induced apoptosis, and that by neutralizing this receptor, TRAIL-induced apoptosis is enhanced. These results corroborate and extend the proposed function for DcR1 in protecting cells from TRAIL-induced apoptosis [2].

The results of co-immunoprecipitation experiments revealed that there is molecular proximity between P-gp, DR4, DR5, and DcR1. Recently data demonstrated that a significant fraction of P-gp resides in lipid raft microdomains [3]. Moreover, recent data clearly showed that lipid raft microdomains are closed chambers in which death receptors, including DR4 and DR5, as well as procaspase-8, procaspase-10, FADD, and Bid reside, and that death-mediated apoptotic signaling mechanisms are dependent upon lipid rafts [13,20,12]. Based on these results and our data, it is plausible that cross-talk between P-gp and TRAIL receptors may take place in lipid raft microdomains, and tempting to speculate that P-gp may favor DR5 versus DR4 incorporation into lipid rafts, which would account for the absence of a role for DR4 in TRAIL-induced BC-19 cell apoptosis. Furthermore, our data suggest that the physical interaction of P-gp with DR5 results in enhanced TRAIL binding to DR5.

Based on the above results, several lines of evidence support a role for P-gp in enhancing TRAIL-induced apoptosis: (1) TRAIL induced preferential apoptosis in P-gp-bearing cells selected for resistance to several anticancer agents, as well as

in cells transfected with the *MDR1* and overexpressing P-gp; (2) treating P-gp-expressing BC-19 cells with the anti-P-gp monoclonal antibody MRK-16 decreased TRAIL-induced apoptosis to similar levels induced in their drug-sensitive counterparts; (3) TRAIL induced preferential apoptosis in P-gp-overexpressing cells by depleting ATP and increasing the ATPase activity of P-gp; (4) KB-VSV1 transfectants with P-gp mutated at position 185 (glycine-185 to valine, G185V) underwent much more TRAIL-induced apoptosis than the transfectants expressing wild-type P-gp. The superior TRAIL sensitization elicited by G185V P-gp strengthens the linkage between P-gp transporter function and enhancement of TRAIL-induced apoptosis, because thermodynamic analysis has revealed that G185V P-gp is more efficient in the ATP-dependent transport of substrates than wt P-gp, owing to a lower Arrhenius activation energy for the rate-limiting step of ATP hydrolysis-coupled substrate transport [31].

P-gp is expressed normally in the epithelial cells of the liver, kidney, small and large intestine, and capillary endothelial cells in the brain, ovary and testis, where it functions both as a barrier to the uptake of xenobiotics and causes their excretion [32]. However, it has been shown that TRAIL does not induce apoptosis in normal cells [23], indicating that despite the presence of P-gp in some normal tissues, other components of the signaling pathways of TRAIL-induced apoptosis may differ between normal tissues and MDR cancer cells.

In conclusion, for the first time, we have shown in this report that P-gp-overexpressing cells are more susceptible to TRAIL-induced apoptosis than their drug-sensitive counterparts. These results have important clinical implications for cancer therapy since (1) sensitivity to TRAIL-induced apoptosis is a major factor affecting the efficacy of TRAIL treatment and (2) P-gp-overexpressing MDR tumor cells can be eliminated by TRAIL treatment. These results offer a wholly novel paradigm for P-gp targeted cancer therapy. Rather than combination therapy regimens of cytotoxic chemotherapy and a P-gp inhibitor intended to facilitate drug retention in P-gp expressing cancer cells, our results suggest that the upregulation of P-gp in neoplasms that have failed chemotherapy can be exploited as an Achilles heel that sensitizes the cancer cells to TRAIL therapy. Further understanding of the molecular mechanisms of TRAIL-induced apoptosis in drug-sensitive and -resistant cells should provide insights and approaches useful for the development of more effective therapeutic strategies to eliminate cancer cells.

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