Doxorubicin Overcomes Resistance to Drozitumab by Antagonizing Inhibitor of Apoptosis Proteins (IAPs)

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Abstract. *Background/Aim: Drozitumab is a fully human agonistic monoclonal antibody that binds to death receptor DR5 and induces apoptosis. However, drozitumab resistance is a major obstacle limiting anticancer efficacy. Materials and Methods: We examined the potential for the chemotherapeutic agent doxorubicin to overcome resistance against drozitumab-resistant breast cancer cells both in vitro and in vivo. Results: Treatment with doxorubicin increased cell surface expression of DR5, reduced levels of Inhibitors of Apoptosis Proteins (cIAPs) and re-sensitised cells to drozitumab-induced apoptosis. Animals implanted with resistant breast cancer cells into the mammary fat pad and treated with a combination of drozitumab and doxorubicin showed inhibition of tumor growth and a substantial delay in tumor progression compared to untreated controls and mice treated with each agent alone. Conclusion: These results suggest that combination of drozitumab with chemotherapy and agents that modulate IAP levels could potentially be a useful strategy in the treatment of breast cancer.*

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Drozitumab is a fully human agonistic monoclonal antibody designed to specifically bind to Apo2L/TRAIL death receptor DR5 and induce apoptosis by activating the extrinsic apoptotic pathway (1). Drozitumab mediates clustering of the receptor, which leads to the recruitment of the cytoplasmic adaptor protein, Fas-associated death domain (FADD) that binds to the receptor by its corresponding death domain. Consequently, the apoptosis-initiating, caspase-8 and/or caspase-10, bind to FADD and form a death-inducing signaling complex (DISC), which leads to the activation of these initiator caspases. Activation of these caspases at the DISC is followed by the activation of effector caspases, including caspase-3, caspase-6 and caspase-7. Eventually, the initiation of this intracellular caspase cascade leads to apoptotic (2). Drozitumab can also activate the intrinsic apoptosis pathway *via* caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member Bid, which eventually amplifies apoptosis (3-5).

Previous studies demonstrated the ability of drozitumab to induce apoptosis of a wide-variety of cancer cell types while sparing normal cells (1, 6, 7, 8). *In vivo*, drozitumab displays tumor suppressive activity in various xenograft models of cancer, including colorectal, lung, pancreatic and rhabdomyosarcoma (1, 6, 7). Data from phase I studies examining the safety, pharmacokinetic profile and antitumor efficacy in a cohort of patients with solid and haematological malignancies, showed drozitumab to be well-tolerated and capable of inducing prolonged stable disease (9, 10). Phase II clinical trials, evaluating the efficacy of drozitumab as a single agent and in combination with chemotherapy in a variety of malignancies, are ongoing.

We have previously assessed the cytotoxic effect of drozitumab against breast cancer cells *in vitro* and evaluated its antitumor activity in murine models of breast cancer development and progression in both the orthotopic mammary gland and in bone (8). *In vitro*, drozitumab induced apoptosis in a panel of breast cancer cell lines but was without effect on normal human mammary epithelial cells, primary osteoblasts or fibroblasts. *In vivo*, drozitumab exerted a remarkable tumor suppressive activity as a single agent, leading to complete regression of well-advanced tumors within the mammary tissue. In a model of osteolytic breast cancer, drozitumab reduced tumor burden within the bone marrow cavity and protected the bone from breast cancer-induced osteolysis (8).

Despite its potent apoptotic activity, some cancer cells remain resistant to drozitumab-induced apoptosis. The mechanisms for this resistance are poorly-understood and multiple factors have been proposed, including reduced cell surface expression of DR5, high expression of c-FLIP that may compete with the recruitment of caspase-8 and -10 at the DISC, increased levels of Inhibitors of Apoptosis Proteins (cIAPs) that antagonise caspase activity and other critical factors involved in the extrinsic and intrinsic apoptotic signalling pathways (2, 5, 11-13).

Previous studies have demonstrated that Apo2L/TRAIL or drozitumab resistance can be reversed by combination with chemotherapeutic agents (6, 14-17). Anthracyclines, such as doxorubicin (DOX), are commonly used chemotherapeutics for treating breast cancer, especially in the adjuvant setting (18). In this study we developed a drozitumab-resistant subline of MDA-MB-231-TXSA breast cancer cells. While normally highly sensitive to drozitumab-induced apoptosis, prolonged exposure of these cells to drozitumab led to the outgrowth of a resistant cell line (MDA-MB-231-TXSA-droz-R). We examined the potential for the chemotherapeutic drug doxorubicin (DOX) to overcome resistance to drozitumab-induced apoptosis *in vitro* and to enhance anticancer efficacy in an orthotopic model of breast cancer. Our *in vitro* results demonstrated that DOX resensitised drozitumab-resistant cells to drozitumab-induced apoptosis and identified Inhibitors of Apoptosis Proteins (cIAPs) as major determinants for drozitumab's resistance. *In vivo*, doxorubicin (DOX) cooperated with drozitumab to inhibit mammary tumor growth and to increase survival, suggesting that this combination could provide a promising therapeutic approach for patients with breast cancer.

Materials and Methods

Cell lines and tissue culture. The MDA-MB-231-TXSA cell line was kindly provided by Dr. Toshiyuki Yoneda (formely at University of Texas Health Sciences Centre, San Antonio, Texas). T47-D cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2 mM glutamine, 100 IU/ml penicillin, 160 μg/ml gentamicin, Hepes (20 mM) and 10% fetal bovine serum (Biosciences, Sydney, Australia) in a 5% CO_2-containing humidified atmosphere. The generation of luciferase-tagged MDA-MB-231-TXSA-TGL human breast cancer cells were previously described (25). The MB-231-TXSA-TGL human breast cancer cell line has been tested and authenticated by CellBank Australia (Wentworthville, NSW, Australia) using short tandem repeat (STR) profiling (REPORT NO. 13-163).

Reagents. Drozitumab (1) and BV6-pan-IAP inhibitor (22) were a kind gift from Genentech, Inc. (South San Francisco, CA, USA). Affinity pure goat anti-human IgG Fc_γ fragment was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). Doxorubicin (DOX) was purchased from Ebewe Pharma (A-4866; Unterach, Austria).

Generation of drozitumab-resistant MDA-MB-231-TXSA-TGL cells in vitro. MDA-MB-231-TXSA-TGL cells (25) were seeded in a T75 flask until 75% confluent. Cells were exposed to drozitumab at 100 ng/ml plus anti-human IgG Fc_γ for 8-10 weeks. During the selection period, cell debris was removed every three days and cells were incubated with fresh media containing drozitumab. By week 8, in the continual presence of drozitumab, clones that tolerated drozitumab treatment emerged. Thereafter, the newly generated cell line, denoted MDA-MB-231-TXSA-droz-R, was cultured in drozitumab-free media until used for further experiments.

Cell viability assay. To determine the cytotoxic effects of drozitumab on cell growth, 1×10^4 cells per well were seeded in 96-well microtiter plates and allowed to adhere overnight. Cells were then treated with increasing concentrations of drozitumab alone (0-1,000 ng/ml) or with increasing concentrations of DOX (0-6.25 μM) or BV6 (0-0.5 μM) in combination with drozitumab at 100 ng/ml for 24 h. Prior to treatment, drozitumab was cross-linked with an anti-human IgG Fc_γ for 30 min at 4˚C. Cell viability was assessed using the Cell Titer Blue Cell Viability Assay (Promega, Madison, WI, USA). Experiments were performed in triplicate and repeated at least 3 times. Results of representative experiments are presented as the mean +standard deviation (SD).

Flow cytometry analysis (FACs) for cell surface expression of DR4 and DR5. MDA-MB-231-TXSA and MDA-MB-231-TXSA-droz-R cells were seeded in T75 flasks and either left untreated or treated with DOX at 5 μM for 24 h. Cells were then washed with phosphate buffered saline (PBS) and detached using 2 mM EDTA in PBS at 37˚C for 5 min. For flow cytometric analysis, all subsequent incubation steps were performed on ice and centrifugation steps performed at 4˚C (to achieve optimal receptor binding). For the analysis of cell surface Apo2L/TRAIL receptor expression, cells were washed twice in protein-free clear PBS, resuspended in wash buffer (1% BSA + 0.1% sodium azide in PBS) at 1×10^6 cells/ml and washed by centrifugation. Cells were then resuspended in 50 μl blocking buffer (5% normal goat serum, 1% BSA in PBS + 0.1% sodium azide). Monoclonal antibodies against TRAIL-R1 (DR4; R&D Systems, Minneapolis, MN, USA) and TRAIL-R2 (DR5; Immunex, San Francisco, CA, USA) were diluted in blocking buffer at 20 μg/ml and added (150 μl) to 50 μl aliquots of cell suspension and incubated for 45 min. Cells were then washed three times with 2-ml of wash buffer and collected by centrifugation. A phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody

(50 μl) was added (aIgG-PE; Southern Biotech, Birmingham, Alabama, USA) to the resuspended cell pellets, diluted 1:50 in wash buffer and the cells were incubated for a further 45 minutes in the dark. After the last incubation step, cells were then washed three times as above, then re-suspended and fixed in fluorescent-activated cell sorting fix (FACs fix) solution (10 ml of 37% formalin stock, 20 g glucose, 2 ml 10% sodium azide stock, 988 μl PBS). The tubes were placed in 4˚C until analysis.

Western blot analysis. Cells were seeded in T25 flasks (2×10^6 cells /flask), treated as indicated (drozitumab 100 ng/ml, DOX 5 μM) and incubated at 5% CO_2–containing humidified atmosphere at 37˚C overnight. Cells were then lysed in buffer containing 10 mM Tris HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 2 mM sodium vanadate and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and stored at –70˚C until required. Anti-caspase-8 and pAb anti-caspase-9 were purchased from Cell Signaling Technology (Beverly, MA, USA), mAb anti-caspase-10 from MBL (Naka-ku Nagoya, Japan), mAb anti-caspase-3 from Transduction Laboratories (Lexington, KY, USA), pAb anti-Bid from Chemicon International (Temecula, CA, USA), mAb anti-Bcl-2 and anti-Bax from Santa Cruz (Santa Cruz, CA, USA), anti-cIAP1, anti-cIAP2 and anti-XIAP from R&D systems and pAb anti-Poly (ADP-ribose) polymerase (anti-PARP) from Roche Diagnostics (Mannheim, Germany). Anti-actin mAb (Sigma, Saint Louis, MO, USA) was used as a loading control. Membranes were then rinsed several times with PBS containing 0.1% Tween-20 and incubated with 1:5,000 dilution of anti-mouse, anti-goat or anti-rabbit alkaline phosphatase-conjugated secondary antibodies (Pierce, Rockford, IL, USA) for 1 h. Visualization and quantification of protein bands was performed using the ECF substrate reagent kit (GE Healthcare, Buckinghamshire, UK) on a FluorImager (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Apoptosis analysis - 4,6-Diamidine-2-phenylindole staining of nuclei (DAPI stain). Cells were seeded onto plastic chamber slides at 1×10^4/well and either left untreated or treated with drozitumab alone at a 100 ng/ml, DOX alone at 5 μM or with the combination of drozitumab and DOX. Cells were fixed in ethanol:acetic acid (6:1) for 10 minutes, washed twice with PBS and incubated with 0.8 mg/ml 4,6-diamidine-2-phenylindole (Roche Diagnostics GmbH, 68298 Mannheim, Germany) in methanol for 5 min at room temperature. After several washes in PBS, the coverslips were mounted with the Prolong Gold Anti-fade reagent with DAPI (Life Technologies, Grand Island, NY, USA). 4,6-Diamidine-2-phenylindole staining was visualized by fluorescence microscopy.

Measurement of DEVD-caspase activity. DEVD-caspase activity was assayed by cleavage of zDEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP-ribose) polymerase. Cells (1×10^4/well) grown in 24-well plates were treated as indicated, washed once with PBS and re-suspended in 30 μl Igepal lysis buffer containing 5 mmol/l Tris-HCl, 5 mmol/l EDTA, and 10% Igepal (pH 7.5). Cell lysate (15-20 μg of protein) was added to each assay tube containing 8 μmol/l substrate in 1 ml flurometric protease buffer (50 mmol/l HEPES, 10% sucrose, 10 mmol/l DTT, 0.1% CHAPS (pH 7.4)). After 4-5 hours at room temperature, fluorescence was quantified (Ex 400 and Em 505) in a Perkin-Elmer LS50 fluorescence spectrometer (Waltham, Massachusetts, USA).

Animals. Female athymic nude mice at 8 weeks old (Institute of Medical and Veterinary Services Division, Gilles Plains, SA, Australia) were acclimatized to the animal housing facility for a minimum period of 1 week prior to the commencement of experimentation. The general physical well-being and weight of animals were monitored continuously throughout the experiments. All mice were housed under pathogen-free conditions and all experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research and were approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science, Adelaide, SA, Australia.

Mammary fat pad injections of breast cancer cells. The MDA-MB-231-TXSA-TGL-droz-R human breast cancer cells were cultured as described above until they reached 70-80% confluency. Adherent cells were removed from flasks with 2 mM EDTA and re-suspended in 1 × PBS at 0.5×10^6 cells/10 μl and kept on ice in an Eppendorf tube. An equal volume of Matrigel™-HC (BD Biosciences, Bedford, MA, USA) was added to the cells and re-suspended. Mice were anaesthetised by isoflurane (Faulding Pharmaceuticals, SA, Australia), the mammary fat pad area of the mice was wiped with ethanol and the skin was lifted over the left outermost nipple. Finally, 20 μl of cells were injected into the mammary fat pad using a 25G needle. Mice were allowed to recover under a heat lamp before being transferred into cages.

In vivo Bioluminescence Imaging (BLI). Non-invasive, whole body imaging to monitor luciferase-expressing MDA-MB-231-TXSA-droz-R cells in mice was performed weekly using the IVIS 100 Imaging system (Xenogen, Alameda, CA, USA). Mice were injected *i.p.* with 100 μl of the D-Luciferin solution at final dose of 3 mg/20 g mouse body weight (Xenogen) and then gas-anaesthetized with isoflurane (Faulding Pharmaceuticals). Images were acquired for 0.5-30 sec (images are shown at 1 second) from the front angle and the photon emission transmitted from mice was captured and quantitated in photons/sec/cm^2/sr using the Xenogen Living image (Igor Pro version 2.5) software (Waltham, Massachusetts, USA).

Histology. Mammary tumors were fixed in 10% (v/v) buffered formalin (24 h at 4˚C) and were then paraffin embedded. Five micron sections were prepared and stained with Hematoxylin and eosin stain (H&E) and additional sections were used for Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Promega, Madison, WI, USA), following the manufacture's protocol. Analysis was performed on an Olympus CX41 microscope and images were taken using the Nanozoomer Digital Pathology (NDP-Hamamatsu, Hamamatsu City, Shizuoka, Japan). Tumor area measured in mm^2 was assessed using the Nanozoomer software.

Data analysis and statistics. Experiments were performed in triplicate and data presented as mean±standar error of the mean (SEM). All statistical analyses were performed using the GraphPad Prism (San Diego, CA, USA) using the unpaired Student's *t*-test. Comparisons between groups were assessed using a one way analysis of variance (ANOVA) test and *p*-values were obtained by using the unpaired Student's *t*-test. In all cases, $p<0.05$ was considered statistically significant.

Results

Prolonged exposure of MDA-MB-231-TXSA breast cancer cells to drozitumab selects for resistant clones overexpressing IAPs. We have previously shown that MDA-MB-231-TXSA breast cancer cells are highly sensitive to drozitumab-induced apoptosis *in vitro* and *in vivo* (25). In this study, we developed drozitumab-resistant clones from this highly sensitive parental cell line by continuous and prolonged *in vitro* exposure of these cells to drozitumab and investigated the potential for the chemotherapeutic drug, DOX, to reverse resistance. Fc crosslinked drozitumab, induced a dose- dependent increase in apoptosis in the parental-sensitive MDA-MB-231-TXSA cell line after a 24-h treatment, reaching a maximum of 90% cell death at a dose of 100 ng/ml of drozitumab (Figure 1A). In contrast, the resistant, denoted MDA-MB-231-TXSA-droz-R, was completely refractory to the apoptotic effects of the antibody at the same doses and remained resistant, even at the highest dose of 1 μg/ml (data not shown). Drozitumab treatment induced a dose-dependent activation of caspase-3 only in the sensitive cells (Figure 1B) and morphological changes characteristic of apoptosis, including chromatin condensation and DNA fragmentation that were clearly evident following treatment with drozitumab only in the parental cells but not in the resistant subline (Figure 1C). These cells remained resistant to drozitumab indefinitely, even in the absence of the antibody and also after multiple cycles of freezing and thawing.

To define the molecular determinants of drozitumab resistance, we compared the expression profile of various components of death receptor signaling between sensitive and resistant cells. Initially, we compared the cell surface expression profile of Apo2L/TRAIL death receptors using flow cytometry. The results demonstrated that the parental (sensitive) MDA-MB-231-TXSA cells express high levels of both cell surface DR4 and DR5. While cell surface expression of DR5 appears to have decreased in the newly developed MDA-MB-231-TXSA-droz-R cells, when compared to the parental cell line, nonetheless these levels remained high so that reduced DR5 levels were thought unlikely to contribute to drozitumab resistance (Figure 2A). In addition to DR4 and DR5 cell surface expression, immunoblots assessing intracellular factors, involved in apoptotic signalling, were carried out with antibodies against caspases-8,-10,-9, -3, FADD, FLIP, Bid, Bcl-2, Bax, cIAP1/2 and XIAP. The most remarkable observation was a marked increase in the level of cIAP2 protein in the drozitumab-resistant cells when compared to the sensitive parental cells. No significant differences were seen with any of the other pro- and anti-apoptotic proteins examined (Figure 2B). These results suggest that increased expression of cIAP2 may be an important determinant of acquired resistance to drozitumab-induced apoptosis in these cells.

DOX overcomes resistance to drozitumab-induced apoptosis in vitro by modulating IAP levels. We and others have previously shown that certain conventional chemotherapeutic drugs in combination with pro-apoptotic receptor agonists, including Apo2L/TRAIL and agonistic antibodies to DR4 and DR5, cooperate to induce apoptosis *in vitro* and potentiate anticancer efficacy in various cancer xenograft models (6-9, 12, 13, 16, 18, 24). To determine whether the MDA-MB-231-TXSA-droz-R cells could be re-sensitised to drozitumab-induced apoptosis, we used the chemotherapeutic agent DOX, which is commonly used for the treatment of patients with breast cancer. Dose escalation treatment of MDA-MB-231-TXSA-droz-R cells with DOX showed a maximum 30% cell death only at the highest dose of 6.25 μM, demonstrating that these cells are relatively resistant to DOX alone. However, the combination of DOX with drozitumab, at 100 ng/ml, induced a dose-dependent increase in apoptosis. As shown in Figure 3A, approximately 30% cell death was achieved at a dose of 2 μM and reached 70% death at the maximum dose of 6.25 μM over a 24-h period (Figure 3A). The loss in cell viability with the combination treatment was accompanied by a significant increase in caspase-3 activity, whereas DOX treatment alone had no effect on caspase-3 activation, even at the highest dose (Figure 3B). In addition, DAPI staining of drozitumab-resistant cells, treated with a combination of DOX and drozitumab, exhibited all the hallmarks of apoptosis induction, including membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (Figure 3C).

While the basal cell surface expression of DR4 and DR5 was intrinsically high in the resistant cells, treatment with DOX lead to a substantial increase in cell surface expression of both DR4 and DR5 (Figure 4A). Western blot analysis performed on cell lysates, treated with DOX and drozitumab alone or in combination, showed that reversal of resistance was associated with processing and activation of the initiator caspase-8 and cleavage of pro-caspase-3, which was associated with cleavage of the apoptosis target protein PARP (Figure 4B). Engagement of the intrinsic apoptotic signaling pathway was also observed in cells treated with the combination of DOX and drozitumab with evidence of cleavage of the Bcl-2 protein family member Bid and enhanced processing and activation of caspase-9. Interestingly, cIAP1 and 2, but not XIAP, levels were significantly decreased by DOX treatment alone and more so when in combination with drozitumab, suggesting that IAPs may play a significant role in inhibiting drozitumab-induced apoptosis in these resistant cells, an effect which could be reversed following DOX treatment (Figure 4B).

To assess the role of IAPs in inhibiting drozitumab-induced apoptosis in the MDA-MB-231-TXSA-droz-R cells, we investigated the potential of the small-molecule IAP antagonist, BV6, to overcome resistance to drozitumab-induced apoptosis (21). Cells were treated with

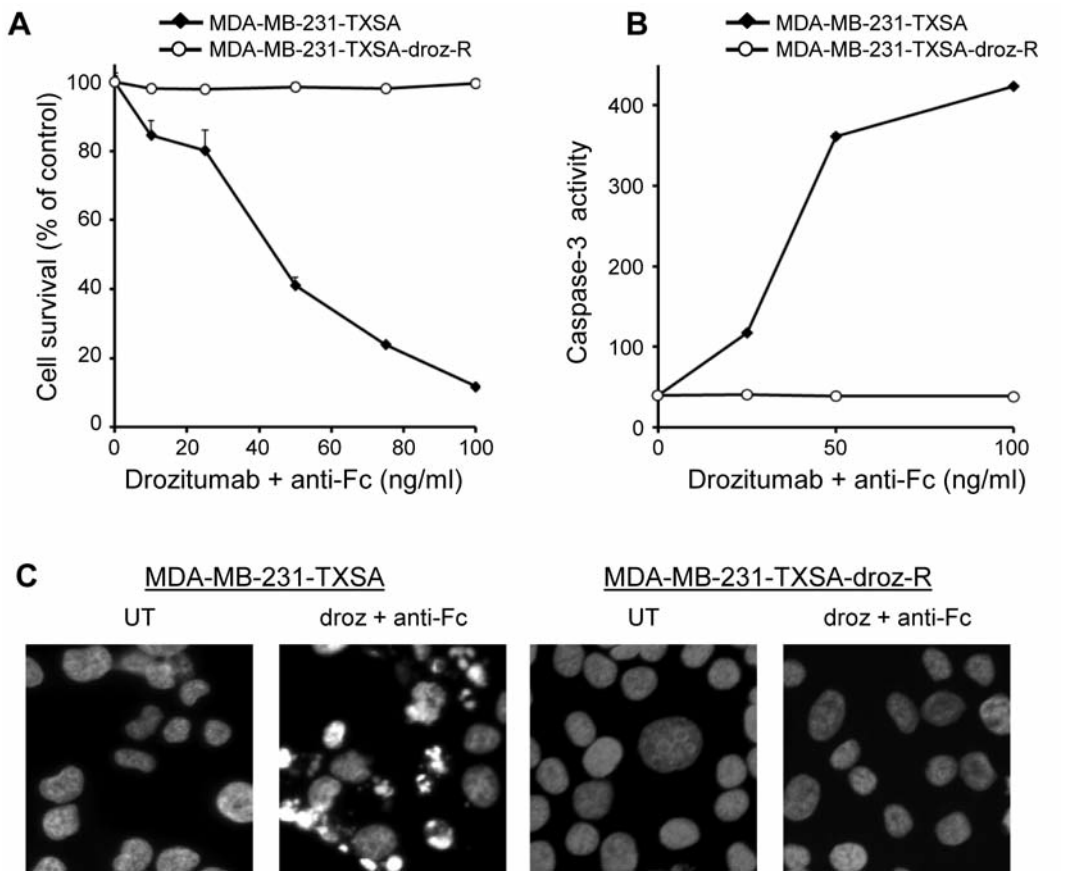

Figure 1. *Development of drozitumab-resistant MDA-MB-231-TXSA breast cancer cells with prolonged treatment in vitro. A. The newly-generated resistant MDA-MB-231-TXSA-droz-R cells and the parental cells (MDA-MB-231-TXSA) were treated with increasing doses of drozitumab plus anti-human IgG Fc$_\gamma$, as indicated, and cell viability was assessed. MDA-MB-231-TXSA-droz-R cells were refractory to drozitumab-induced apoptosis showing 90% viability, even at the highest dose of 1μg/ml (data not shown) when compared to the parental-sensitive cell line. Data are presented as the mean±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells. B. MDA-MB-231-TXSA-droz-R and MDA-MB-231-TXSA cells were treated with increasing doses of drozitumab, as indicated and caspase-3-like activity was determined as described in the Materials and Methods. MDA-MB-231-TXSA-droz-R showed lack of drozitumab-induced caspase-3 activity compared to the parental cell line C. DAPI staining on MDA-MB-231-TXSA-droz-R and MDA-MB-231-TXSA cells following treatment with drozitumab at 100 ng/ml plus anti-Fc for 24 h.*

increasing concentrations of BV6 alone or in combination with drozitumab at 100 ng/ml and cell viability was assessed 48 h after treatment. Treatment with drozitumab alone or BV6 alone had minimal effect on cell viability of MDA-MB-231-TXSA-droz-R cells, showing only a 9% cell death at the highest dose after 48 hours (Figure 5A). In contrast, co-administration of drozitumab and BV6 resulted in a synergistic increase in cell death, reaching 48% cell death at the higher dose when compared to drugs used alone. As expected, treatment with BV6 resulted in the rapid degradation of cIAP1/2 and also XIAP (Figure 5B).

Similar observations were made using the T47-D breast cancer line previously shown to be intrinsically resistant to apoptosis induction by drozitumab. In this setting, the combination of DOX and drozitumab reversed the innate resistance of T47-D breast cancer cells to drozitumab-induced apoptosis (Figure 6A). This effect was again associated with degradation of cIAP1/2 and XIAP in the combined treatment, which was concomitant with caspase-3, caspase-9, Bid and PARP cleavage similarly to what has been observed in the MDA-MB-231-TXSA-droz-R cells (Figure 6B). Similarly, treatment with the IAP antagonist BV6 in T47-D cells also resulted in the robust degradation of both cIAP1/2 and XIAP and sensitised the intrinsically resistant cells to drozitumab-induced apoptosis (Figure 6C and 6D). Taken together, these data identify IAP proteins as important molecular determinants likely partly contributing to drozitumab resistance.

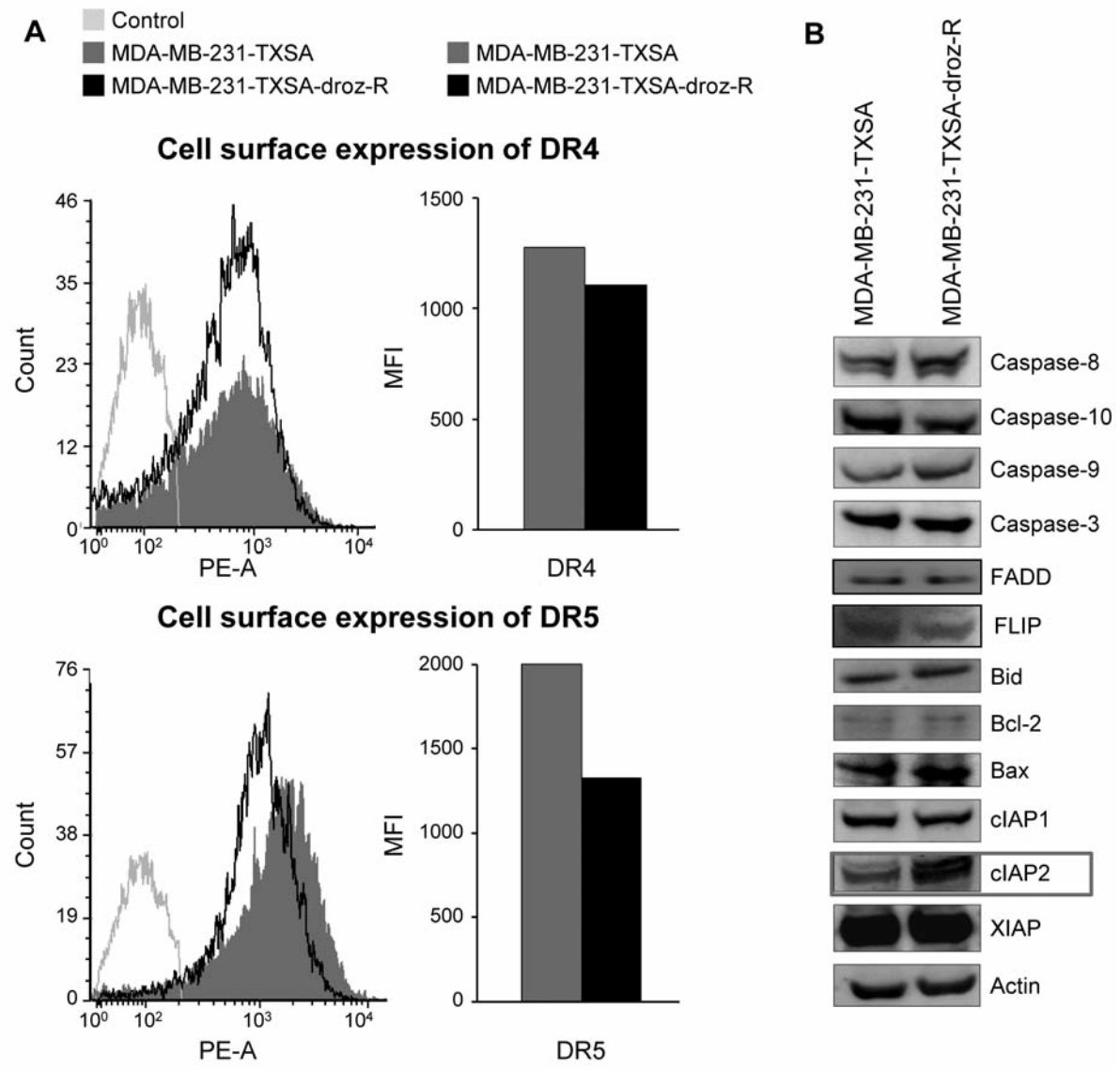

Figure 2. *Comparison of cell surface expression of death receptors do not explain the resistance of MDA-MB-231-TXSA-droz-R cells to drozitumab. A. Flow Cytometry analysis of cell surface expression of Apo2L/TRAIL death receptors in drozitumab-sensitive MDA-MB-231-TXSA cells versus resistant MDA-MB-231-droz-R cells. Graphs were obtained after staining with anti human DR4 and DR5 monoclonal antibodies as described in the Materials and Methods. B. Western Blot analysis on MDA-MB-231-TXSA and MDA-MB-231-droz-R cell lysates. There was no detectable difference in the basal levels of anti- and pro-apoptotic proteins between the two cell lines. However, cIAP2 levels were extremely high in the drozitumab resistant cells compared to the sensitive parental cell line.*

Anticancer efficacy of drozitumab and DOX on the growth of mammary tumor xenografts. To examine if the cooperative activity of drozitumab and DOX seen *in vitro* could be translated into *in vivo* efficacy, we assessed the anticancer potential of each drug when administered alone and in combination against mammary tumors, which develop following direct transplantation of MDA-MB-231-TXSA-droz-R cells into the mammary fat pad of athymic female nude mice. For non-invasive bioluminescence imaging (BLI) of tumor growth, breast cancer cells were retrovirally infected with a triple-fusion protein reporter construct encoding herpes simplex virus thymidine kinase (TK), green fluorescent protein (GFP) and firefly luciferase (Luc) (17, 25). Following cancer cell transplantation, tumors were allowed to establish in the mammary gland of mice for two weeks before treatment was initiated. At day 14 mice were randomized into 4 groups and treated with drozitumab or DOX as single agents or in combination. Tumors in the

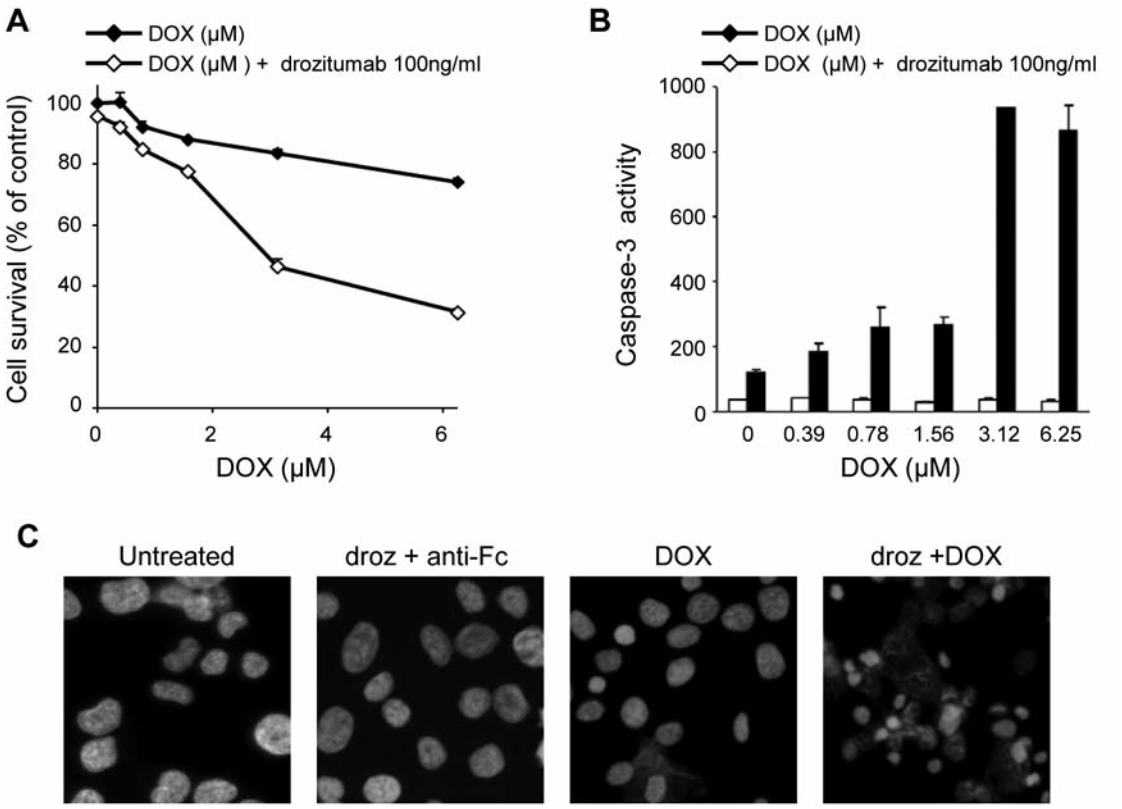

Figure 3. *Cooperation of drozitumab and DOX in apoptosis induction of resistant cells in vitro. A. MDA-MB-231-TXSA-droz-R cells were treated with increasing concentrations of DOX (0-6.25 μM) in combination with drozitumab plus anti-human IgG Fc$_\gamma$ at 100ng/ml, as described in the Materials and Methods. DOX-alone resulted in a 30% cell death only at the highest dose of 6.25 μM, whereas the combined treatment with drozitumab led to a significant loss in cell viability. B. MDA-MB-231-TXSA-droz-R cells were treated as above and caspase-3-like activity was determined, as described in the Materials and Methods. The combined treatment resulted in a dramatic increase in caspase-3 activation. Data are presented as the mean±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells. C. DAPI staining on MDA-MB-231-TXSA-droz-R cells following treatment with DOX at 5 μM or drozitumab plus anti-human IgG Fc$_\gamma$ at 100 ng/ml alone or in combination for 24 h.*

vehicle-treated group showed an exponential increase of mean photon emission associated with a rapid increase in tumor burden, which was clearly evident from day 14 onwards and continued to increase until day 28 at which point mice were humanely killed due to the high tumor load (Figure 7A). Animals treated with drozitumab alone or DOX alone showed a small delay in tumor progression until day 21, with tumors rapidly expanding thereafter until animals were humanely killed on day 35. In contrast, combined treatment with drozitumab and DOX caused significant inhibition of tumor growth and a substantial delay in tumor progression, which translated to a significant increase in survival. Histological examination of representative sections from the mammary tumors 48 h after treatment indicated that the combination of DOX and drozitumab induced apoptosis in a substantial proportion of the tumor mass, with intense TUNEL positive staining of tumor cells when compared to tumors in animals treated with either vehicle, drozitumab or DOX as single agents (Figure 7B). Since the tumor burden in the orthotopic site was remarkably reduced with survival benefit following combination treatment, these data suggest that patients with drozitumab-resistant tumors could benefit from drozitumab-based immunotherapy when used in combination with DOX.

Discussion

An important goal of anticancer strategies is the selective induction of apoptosis in cancer cells but not healthy cells. In the search for more effective treatments for cancer, combinations of agents with synergistic or additive activity are attractive because they enable the use of lower drug doses, which reduce toxic side-effects. Pro-apoptotic receptor agonists, including recombinant Apo2L/TRAIL or the agonistic antibody drozitumab, as well as chemotherapy induced cancer cell apoptosis through different but

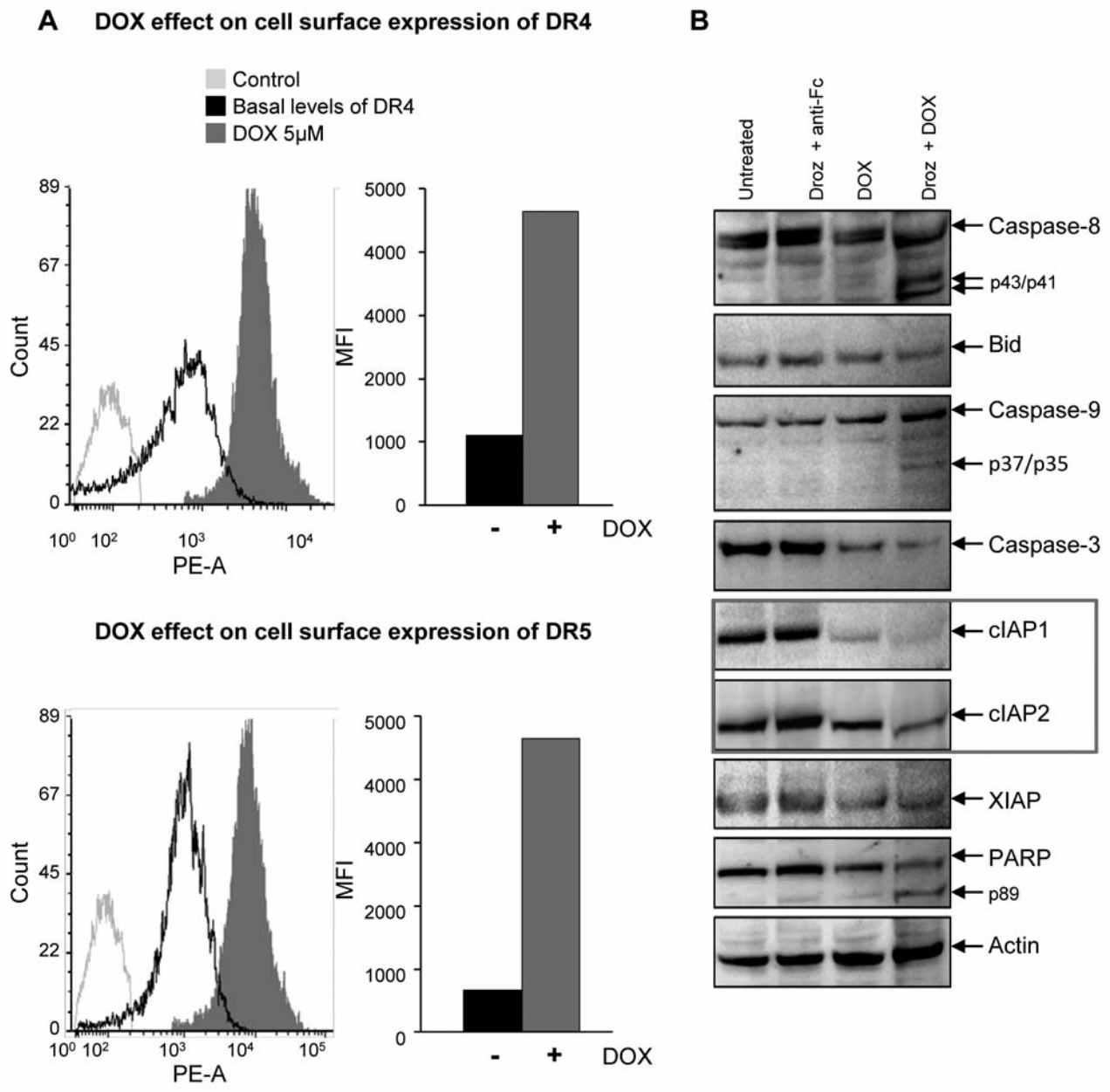

Figure 4. *DOX treatment up-regulates DR4 and DR5 and decreases IAP levels in MDA-MB-231-TXSA-droz-R cells. A. Flow Cytometry analysis of cell surface expression of Apo2L/TRAIL death receptors in MDA-MB-231-TXSA-droz-R cells 24 h after DOX treatment at 5 μM. Graphs were obtained after staining with anti human TRAIL-R1 (DR4) and TRAIL-R2 (DR5) monoclonal antibodies as described in the Materials and Methods. DOX treatment dramatically increased the expression of DR4 and DR5 on the surface of the MDA-MB-231-TXSA-droz-R cell. B. Western blot analysis on MDA-MB-231-TXSA-droz-R cells following treatment with drozitumab alone at a concentration of 100 ng/ml or DOX alone at 5 μM or with the combination of drozitumab and DOX. The caspase-8, caspase-9 and poly(ADP-ribose) polymerase antibodies detect both full-length and processed forms of the antigen, whereas caspase-3 and Bid antibodies detect only the full-length antigens. The combination treatment led to activation of caspases -8, -3, -9 and also the apoptotic proteins BID and PARP. In addition there was dramatic processing of the IAP proteins, cIAP1 and cIAP2 following combination treatment of drozitumab and DOX.*

overlapping signaling pathways. Therefore, combination approaches may increase killing of tumor cells that resist death induction through a single pathway and may also reduce the probability of acquired resistance to each therapy.

Several lines of experimental evidence have revealed that some tumors exhibit or acquire resistance to pro-apoptotic receptor agonists. Although multiple mechanisms have been proposed, the basis for this resistance is not well-understood and

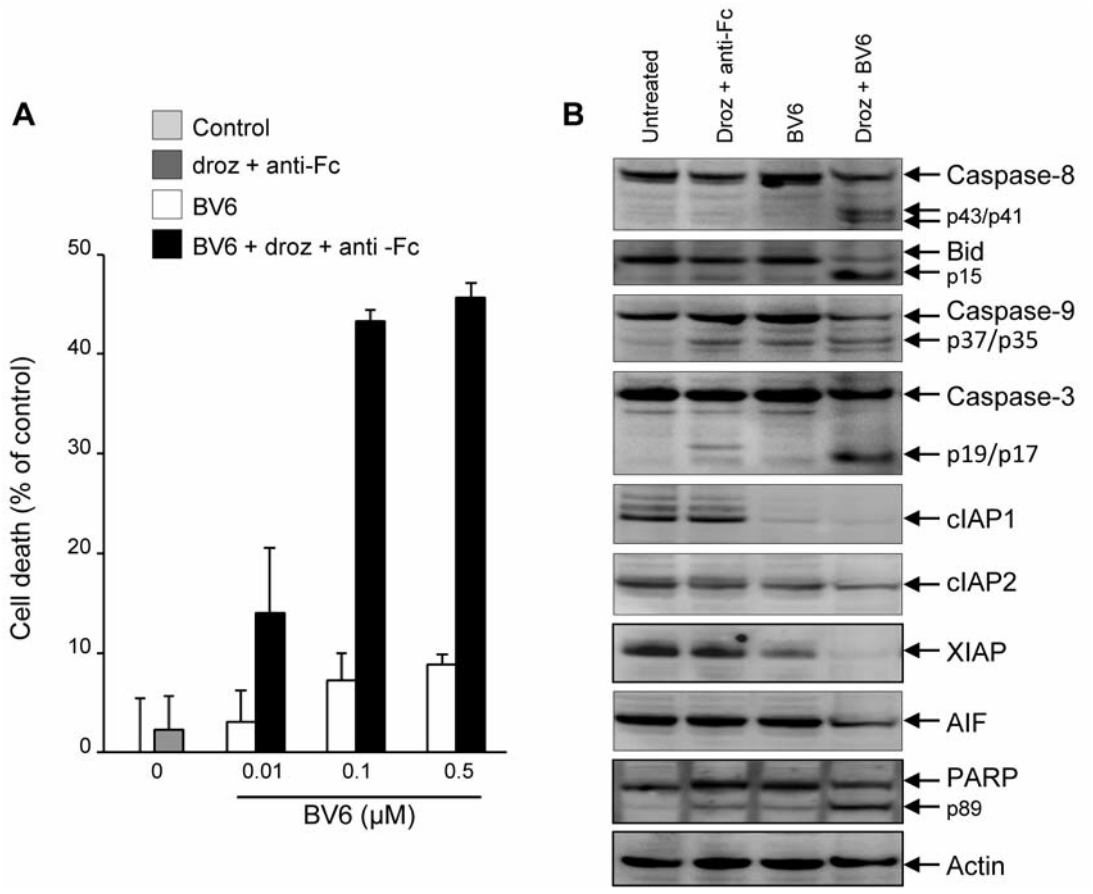

Figure 5. *BV6 re-sensitizes MDA-MB-231-TXSA-droz-R cells to drozitumab. A. MDA-MB-231-TXSA-droz-R cells were treated with increasing doses of BV6 (0-0.5μM) alone or in combination with drozitumab plus anti-human IgG Fc_γ at 100ng/ml and cell viability was assessed. BV6-alone had no effect on drozitumab-resistant MDA-MB-231-TXSA cells, whereas combination treatment of BV6 and drozitumab led to a significant increase of cell death in these cells. Data are presented as the mean±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells. B. Western blot analysis on MDA-MB-231-TXSA-droz-R cells following treatment with increasing concentrations of BV6 alone or in combination with drozitumab at 100 ng/ml. The combination treatment led to activation of caspases -8, -3, -9 and also the apoptotic proteins BID and PARP. In addition there was dramatic processing of the cIAP 1/2 proteins and also XIAP, following combination treatment of drozitumab and BV6.*

is under intense investigation by laboratories around the world, including ours (3-5, 7). Drozitumab resistance, whether intrinsic or acquired, like Apo2L/TRAIL resistance, is a major obstacle limiting anticancer efficacy. We have previously shown that not all breast cancer cell lines are sensitive to drozitumab-induced apoptosis and have now shown that prolonged exposure of sensitive breast cancer cells with drozitumab selects for a population of cells refractory to apoptosis induction by drozitumab. This type of resistance resembles drug resistance that develops in cancer patients following prolonged treatments with certain anticancer agents. Our aim was to evaluate the potential for the chemotherapeutic drug DOX to overcome resistance to drozitumab-based immunotherapy *in vitro* and to evaluate the anticancer efficacy of this combinatorial approach *in vivo* using an orthotopic model of breast cancer.

The breast cancer cell line MDA-MB-231-TXSA is extremely sensitive to drozitumab-induced apoptosis. However, we were able to generate a drozitumab-resistant subline with prolonged exposure of these cells with drozitumab. Development of resistance to drozitumab in the MDA-MB-231-TXSA cells could not be explained by major differences in Apo2L/TRAIL receptor expression between sensitive and resistant cells, indicating that other factors in the apoptotic pathways may be involved. While MDA-MB-231-TXSA-droz-R cells were resistant to drozitumab-induced apoptosis, these cells were not cross-resistant to a number of other clinically-relevant chemotherapeutic drugs, including doxorubicin, taxol, etoposide, cisplatin or the histone deacetylase inhibitor SAHA. Furthermore, ZVAD-fmk could not inhibit apoptosis induction by any of these

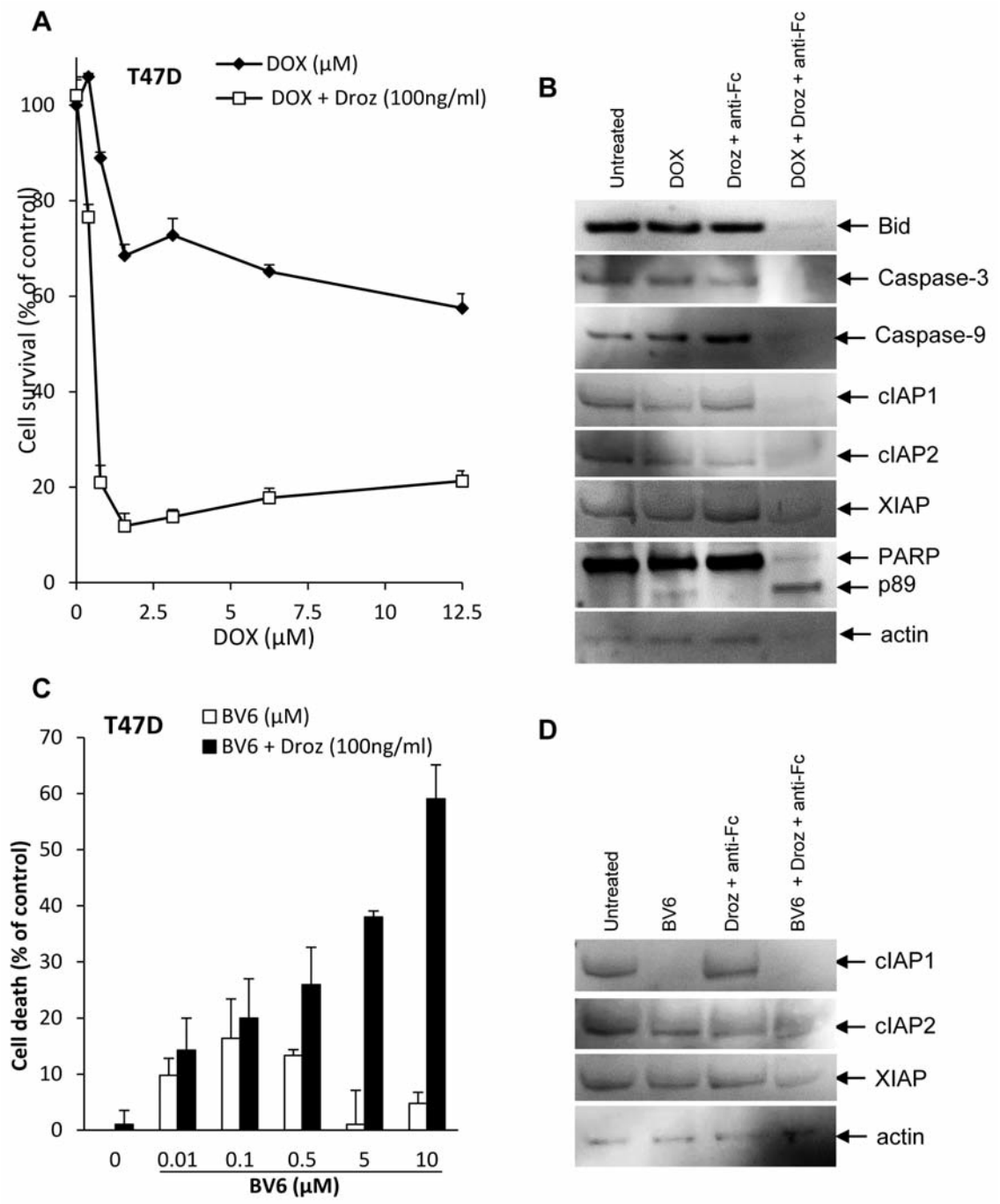

Figure 6. *Cooperation of drozitumab and DOX in apoptosis induction of T47-D breast cancer cells in vitro via inhibition of cIAPs. A. T47-D breast cancer cells were treated with increasing concentrations of DOX (0-12.5 μM) in combination with drozitumab plus anti-human IgG Fc$_\gamma$ at 100 ng/ml and cell viability was assessed. DOX-alone resulted in a 40% cell death only at the highest dose of 12.5 μM, whereas the combined treatment with drozitumab led to a significant loss in cell viability. B. Western blot analysis on T47-D cells following treatment with drozitumab alone at a concentration of 100 ng/ml or DOX-alone at 2 μM or with the combination of drozitumab and DOX. The combination treatment led to activation of caspases -3 and -9 and also the apoptotic proteins BID and PARP. In addition there was dramatic processing of the IAP proteins, cIAP1, and cIAP2 and XIAP following combination treatment of drozitumab and DOX. C. T47-D cells were treated with increasing doses of BV6 (0-10 μM) alone or in combination with drozitumab plus anti-human IgG Fcγ at 100 ng/ml and cell viability was assessed. BV6 alone had no significant effect on T47-D cells, whereas combination treatment of BV6 and drozitumab, led to a significant increase of cell death in these cells. Data are presented as the mean±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells. D. Western blot analysis on T47-D cells following treatment with BV6 alone (2.5μM) or in combination with drozitumab at 100 ng/ml. Cells were then lysed and protein was isolated at 24 h after treatment. The combination treatment of BV6 and drozitumab let to the dramatic processing of the cIAP 1/2 proteins and also XIAP.*

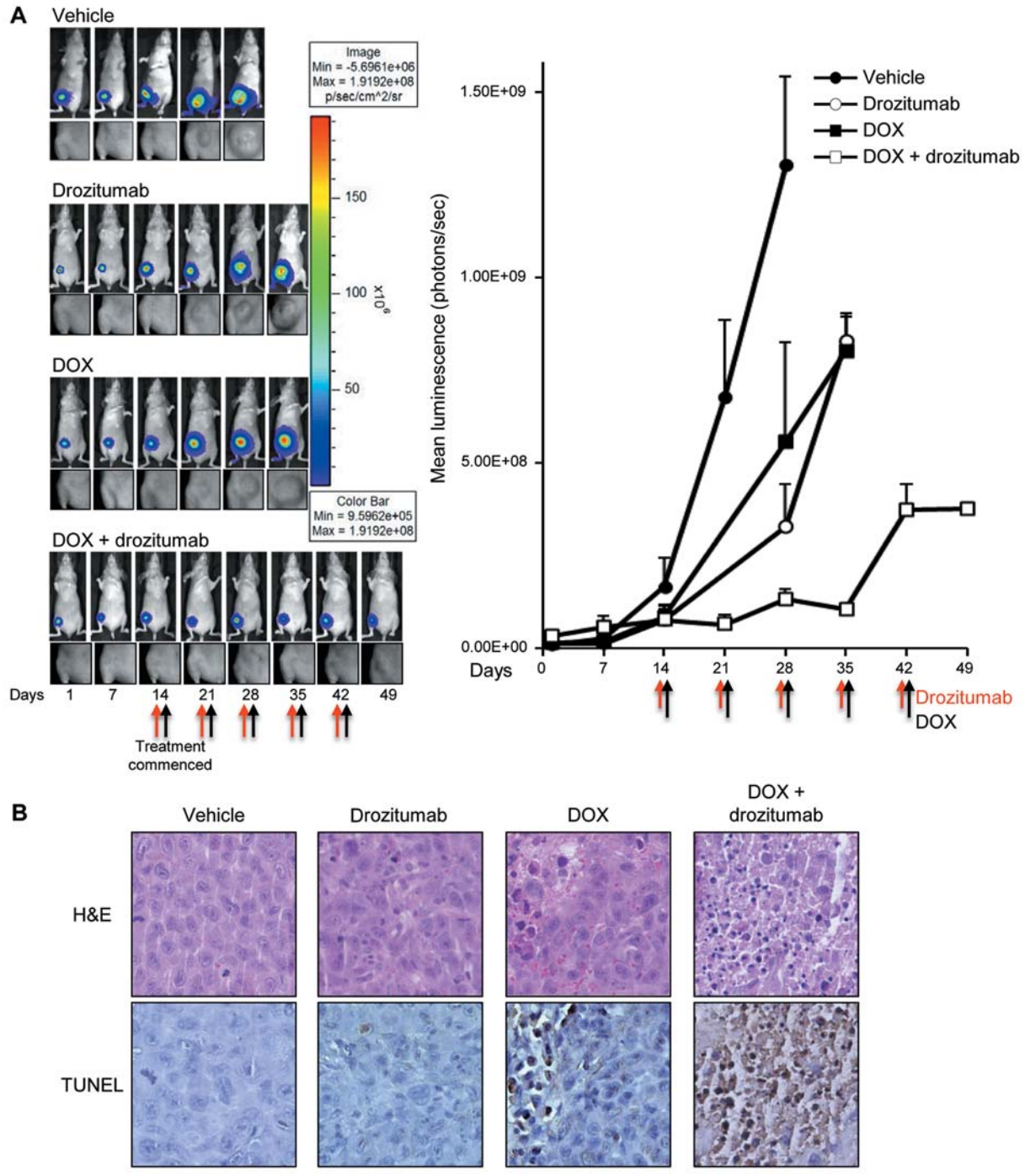

Figure 7. *Antitumor activity of drozitumab in combination with DOX against mammary tumors in vivo. Female nude mice were injected with 0.5×10^6 MDA-MB-231-TXSA-drozR-TGL cells into the mammary fat pad, as described in the Materials and Methods. Two weeks after cancer cell transplantation, mice were randomized into 4 groups of 5 mice per group and administered with vehicle (PBS) or drozitumab at 3mg/kg (in clear PBS i.p.) once weekly or single i.v. injection of DOX at 4mg/kg once weekly or a combination of DOX and drozitumab with a 4 hour gap between the two injections. A. Representative whole body BLI and photographs of the mammary tumors of a single animal from each group of mice during the course of the experiments are shown. All vehicle-treated animals were humanely killed on day 28 due to high tumor load. Animals that received treatment with a single agent also showed an increase in tumor growth and were humanely killed at day 35. In contrast, all mice receiving a combination treatment with drozitumab and DOX showed delayed or even inhibition of tumor growth, which was also concomitant with prolonged survival of the mice until the end of the experiment. B. Histological examination of representative sections from mammary tumors, prepared 48 h after treatment, indicate that drozitumab in combination with DOX induced apoptosis in a substantial proportion of the tumor mass, with intense TUNEL-positive staining of tumor cells when compared to vehicle-treated animals or with each agent alone.*

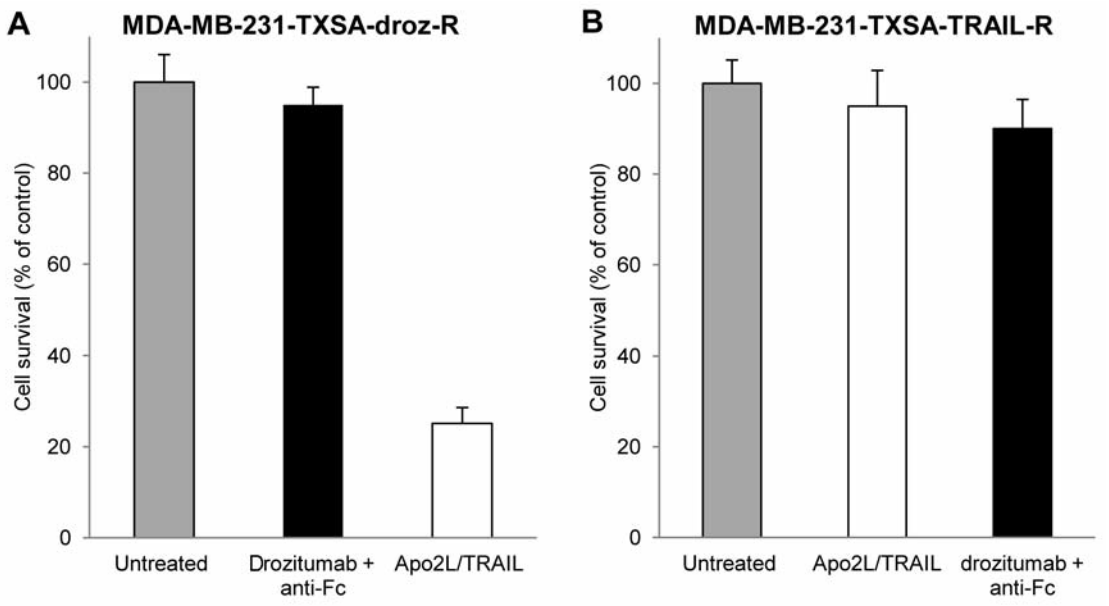

Figure 8. *Effects of drozitumab and Apo2L/TRAIL on MDA-MB-231-TXSA-droz-R and MDA-MB-231-TXSA-TRAIL-R cells. Drozitumab-resistant (MDA-MB-231-TXSA-droz-R) and Apo2L/TRAIL-resistant (MDA-MB-231-TXSA-TRAIL-R) breast cancer cells were treated with drozitumab plus anti-human IgG Fc_γ (100 ng/ml) or Apo2L/TRAIL (100 ng/ml) and cell viability was assessed. A. MDA-MB-231-TXSA-droz-R were refractory to drozitumab-induced apoptosis showing maintenance of 95% cell viability. However treatment of these cells with Apo2L/TRAIL resulted in 75% loss of cell viability. B. MDA-MB-231-TXSA-TRAIL-R cells demonstrated strong resistant to both drozitumab and Apo2L/TRAIL. Data are presented as the mean±SEM of triplicate wells from a representative experiment, repeated at least three times and are expressed as a percentage of the number of control cells.*

stimuli (data not shown). These data suggest that, while drozitumab-induced apoptosis mediated by DR5-signaling was impaired, apoptosis induction *via* the intrinsic signalling pathway was intact. These data are consistent with our previously published work (20) in which we have shown that prolonged exposure of the same cell line to recombinant Apo2L/TRAIL protein leads to acquired resistance to Apo2L/TRAIL-induced apoptosis, while apoptosis induction by these other activators of the intrinsic pathway was not affected. Detailed examination of the drozitumab-resistant cells however, demonstrated that these cells express elevated levels of IAP proteins, particularly cIAP2, compared to the sensitive cells. Alterations in cIAPs levels have been associated with the development of tumors and resistance to chemotherapy and IAP antagonists are in development as anticancer agents (11, 14, 15). Our results are in line with various preclinical and clinical studies that have demonstrated IAP proteins to be expressed at very high levels in human malignancies (2, 10, 14, 15, 19, 23). Mechanistically, Varfolomeev et al. have previously shown that down-regulation of IAP expression using siRNA sensitised other cancer cell lines to drozitumab in a similar manner to what we have shown here with doxorubicin and the IAP antagonist BV6. Taken together, these data provide additional evidence that drozitumab resistance in some cell lines is mediated, at least in part, by high levels of IAPs (21). This raises the possibility that IAP proteins act as important modulators of the Apo2L/TRAIL-based apoptotic signalling pathways and may play a significant role in causing resistance to Apo2L/TRAIL-based agents in cancer.

In our attempts to reverse the resistance of the MDA-MB-231-TXSA-droz-R cells to drozitumab, we tested these cells for their sensitivity to the chemotherapeutic agent DOX. DOX, when used alone, showed limited cytotoxic effect on the MDA-MB-231-TXSA-droz-R cells. However, when combined with drozitumab, DOX cooperated synergistically to induce apoptosis, when compared to the effect of each agent alone. This effect was associated with a significant degradation of cIAP1 and cIAP2 proteins, an effect that was more profound when combined with drozitumab treatment. To assess if IAP proteins play a role in determining the resistance of MDA-MB-231-TXSA-droz-R cells to drozitumab, we used a pan-IAP-antagonist, BV6, to inhibit their action in these cells. Co-administration of drozitumab and BV6 resulted in a synergistic increase in cell death when compared to drugs used alone. These data suggest that IAP proteins play a significant role in inhibiting drozitumab-induced apoptosis.

To examine if the synergistic activity of drozitumab and DOX seen *in vitro* could be translated into *in vivo* efficacy, MDA-MB-231-TXSA-droz-R cells were transplanted into the mammary gland of female athymic mice and allowed to

establish for 2 weeks. Animals treated with vehicle developed aggressive, rapidly growing mammary tumors. When animals were administered drozitumab or DOX as single agents, tumor growth was delayed by approximately one week. In contrast, drozitumab cooperated with DOX to mediate a significant inhibition of tumor growth and a substantial delay in tumor progression, which translated to a significant increase in survival.

The present study describes a number of novel findings that are distinct from those previously published. Firstly, while we and others have previously published on the development and characterisation of acquired resistance to recombinant Apo2L/TRAIL, this is the first study to demonstrate that resistance to the drozitumab antibody can also be acquired by continuous and prolonged exposure of cancer cells (MDA-MB-231-TXSA) to drozitumab converting this highly sensitive line to a relatively resistant one. Secondly, we used this isogenic drozitumab resistant line to evaluate the potential of chemotherapy to reverse such resistance *in vitro* and *in vivo* using an orthotopic breast cancer mouse model. Thirdly, we provided some mechanistic evidence showing that IAPs, at least in some instances, likely contribute to the acquired resistance of tumor cells to drozitumab-induced apoptosis and that modulation of IAP levels by chemotherapy or small IAP antagonists can reverse such resistance. It must be emphasized that acquired resistance to the Apo2L/TRAIL ligand is mediated by impairment of both DR4 and DR5 signalling, while acquired resistance to drozitumab is mediated by impairment of DR5 signalling. This is supported by our data showing that MDA-MB-231-TXSA-TRAIL-R cells, which have acquired resistance to Apo2L/TRAIL after their prolonged exposure to the ligand (20) are also cross-resistant to drozitumab-induced apoptosis. In contrast, the isogenic drozitumab-resistant cells developed in this study retain their sensitivity to Apo2L/TRAIL, presumably *via* functional DR4 signaling (Figure 8). Therefore, the mechanisms of acquired Apo2L/TRAIL resistance appear to be distinct from those of acquired drozitumab resistance. Understanding the basis of the resistance of tumor cells to drozitumab will undoubtedly provide new treatment strategies for the clinical application of drozitumab in cancer therapy. For example, cancer patients who develop resistance to drozitumab following their prolonged treatment with the antibody may benefit by switching over to Apo2L/TRAIL therapy or continue with drozitumab therapy in combination with chemotherapy.

Defects in apoptosis signalling is a hallmark of cancer development, progression and metastasis and multiple factors involved in both the extrinsic and intrinsic apoptotic signalling pathways that impair PARP–mediated apoptosis have been described by many investigators including work from this laboratory (7-9, 20). IAP proteins represent one line of defence against apoptosis induction of cancer cells by inhibiting caspase activity, essential for tumor survival and maintenance of therapeutic resistance. IAP proteins are expressed at elevated levels in many tumor types including breast cancer and, as such, make IAP proteins attractive targets for anticancer therapy.

In conclusion, this study indicates that prolonged exposure to drozitumab can result in the development of drozitumab resistance while retaining sensitivity to Apo2L/TRAIL and that acquired resistance to drozitumab can be reversed by chemotherapy. Mechanistically, the data presented in this study suggests that IAPs contribute to drozitumab resistance and that degradation of IAPs with chemotherapy or IAP antagonists, such as BV6, in some cancer cells, in which the only defect may be IAP deregulation, can reverse such resistance and sensitise cancer cells to drozitumab-induced apoptosis. The identification of specific biomarkers for detecting acquired or innate resistance to these agents is of great importance and, when this is achieved, new combinatorial approaches can be developed to improve the survival of cancer patients. Taken together, these results suggest that combination of drozitumab with agents that modulate IAP levels, including the currently developed IAP antagonists, may be a useful strategy to target tumor cell resistance in cancer patients and improve survival.

Conflicts of Interest

No potential conflicts of interest are disclosed.

Acknowledgements

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