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Obatoclox enhances TRAIL-induced apoptosis in non-small-cell-lung-cancer cells by increasing TRAIL receptor expression, priming for mitochondrial apoptosis and reducing XIAP levels

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Abstract

Aim: Antitumor activity of TNF-related apoptosis-inducing ligand (TRAIL) can be inhibited by anti-apoptotic Bcl-2 family members. Here, we examined the potential of the BH-3 (bcl-2 homolog domain 3) mimetic obatoclox to sensitize non-small cell lung cancer (NSCLC) cells for TRAIL-induced apoptosis.

Methods: The sensitivity to obatoclox treatment in combination with TRAIL was measured with the tetrazolium-based 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. TRAIL-induced apoptosis was



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evaluated with flow cytometric analysis. Mitochondrial and cytosolic components were fractionated and western blotting was performed to determine the level of Cytochrome c, GAPDH, and Cox IV. Knockdowns were performed with small interfering RNA (siRNA). TRAIL receptor protein levels were assessed with fluorescence intensity index.

Results: Synergistic interactions were observed both in TRAIL-resistant (SW1573, A549) and TRAIL-sensitive (H460) cells, characterized by enhanced caspase-dependent apoptosis. Although concurrent treatment with obatoclast and TRAIL was sufficient for apoptosis activation in SW1573 cells, A549 cells required 48-h pre-incubation with obatoclast, during which TRAIL-R2 (TRAIL receptor 2) cell surface expression increased and X-linked inhibitor of apoptosis protein (XIAP) levels decreased. Interestingly, the knockdown of XIAP was sufficient to sensitize A549 cells for concurrent treatment.

Conclusion: Obatoclast is an effective sensitizer for TRAIL-induced apoptosis in NSCLC, although displaying cell type-specific effects.

Keywords: TRAIL, obatoclast, apoptosis, NSCLC, XIAP

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a disease with a poor prognosis and is the leading cause of cancer-related deaths worldwide^[1-3]. The 5-year survival in NSCLC and prognosis varies considerably between the stages; in stages I, II, IIIA, IIIB, and IV, the 5-year survival rate is about 68.4%, 45.1%, 26.2%, 17.3%, and 5.8%, respectively^[4,5]. Surgical resection is applicable mainly in stages I and II and may be considered in stage IIIA and can substantially improve the prognosis in combination with adjuvant or neoadjuvant treatment^[6]. Other treatment options are chemotherapy and radiotherapy, as well as combinations of these treatment modalities^[7-9]. Stereotactic ablative radiotherapy might even be non-inferior to surgery^[10,11]. Currently, patients in advanced stages have more options, such as tyrosine kinase-targeted therapy and immunotherapy^[8,12-14]. Many targeted therapies, e.g., Epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and immune checkpoint inhibitors, have been introduced to clinical practice, especially in adenocarcinoma^[7-9,15,16]. However, the molecular profile of some tumors is not suitable for any of these therapies, and most tumors acquire resistance. Therefore, the development of novel effective therapeutic agents beyond these treatment options is critical to reducing the mortality of NSCLC patients.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the tumor necrosis factor (TNF) superfamily, offers a promising anti-cancer strategy due to its selective induction of apoptosis in cancer cells while sparing normal cells^[17-20]. This unique capability has been validated in many experimental studies, distinguishing TRAIL from other therapeutic agents^[21,22]. However, several clinical trials have raised concerns about liver toxicity at high doses^[23]. TRAIL can bind to five different receptors: death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2); two presumed non-functional decoy receptors, DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4); and the soluble receptor osteoprotegerin (OPG)^[24,25]. The extracellular domain might be proteolytically cleaved from the cell surface, but the soluble form of TRAIL has a low ability to induce apoptosis compared to the membrane-bound form. Its release is possibly stimulated by cathepsin E^[24]. The binding of TRAIL to its death receptors TRAIL-R1 or TRAIL-R2 triggers the extrinsic apoptotic pathway. This process involves the assembly of the death-inducing signaling complex (DISC) through the recruitment of Fas-associated death domain (FADD) and procaspase-8, leading to subsequent activation of caspases 8 and 10, initiating apoptosis^[23]. Caspase 8 can either directly activate caspase 3 or induce apoptosis through the intrinsic apoptotic pathway via cleavage of the BH3 Interacting Domain Death Agonist (BID). The resulting truncated form of BID (tBID) activates Bcl-2 Antagonist Killer (Bak) and Bcl-2 Associated X protein (Bax), leading to pore formation in the mitochondrial membrane, resulting in the release of

apoptogenic SMAC/DIABLO and cytochrome c. In the cytosol cytochrome c, APAF-1 and procaspase 9 are assembled in the apoptosome, resulting in the activation of caspase 9 and subsequent caspase 3 cleavage and activation of other effector caspases -6 and -7, leading to irreversible apoptosis^[22,23,26,27]. TRAIL is still a promising anti-cancer therapeutic agent, but at least 50% of the NSCLC cell lines and approximately 60%-70% of tumor cell lines in general are TRAIL-resistant^[28,29]. Resistance to TRAIL-induced apoptosis in NSCLC can arise from various factors, including increased levels of decoy receptors like DcR2^[30-32] proteins such as Phosphoprotein Enriched in Diabetes (PED) and cellular FLICE-inhibitory protein (cFLIP)^[33,34], upregulation of clathrin-mediated endocytosis, or DR4/5 receptor internalization^[35], and microRNAs like miRNA-221 and miRNA-222^[36], ultimately reducing TRAIL efficacy. Furthermore, Bcl-2 family members have been implicated in TRAIL resistance - for example, overexpression of Bcl-2 has been shown to inhibit the activation of apoptosis^[35,37-39]. Bcl-2 family members are key regulators of mitochondrial apoptosis and have been classified into three distinct groups^[40-42]. The first group consists of pro-survival/ anti-apoptotic proteins: B-cell lymphoma-2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), Bcl-w, Myeloid Cell Leukemia-1 (Mcl-1), Bcl-B, and Bfl1. These proteins share a sequence homology consisting of 4 α -helical Bcl-2 homology (BH) domains, named BH1 to BH4. The pro-apoptotic proteins Bax and Bak lack the BH4 domain and are crucial for the permeabilization of the mitochondria, resulting in cytochrome c and SMAC/DIABLO release and ultimately apoptosis. The third group encompasses the “BH-3 only” pro-apoptotic proteins: BID, Bcl-2 Antagonist of Cell Death (BAD), Bcl-2 Interacting Killer (BIK), p53 Upregulated Modulator of Apoptosis (PUMA), NOXA, Bcl-2 Modifying Factor (BMF), and Harakiri (HRK). BH3-only proteins bind specifically to the hydrophobic groove of anti-apoptotic Bcl-2 family members to neutralize them and promote apoptosis. The cleavage of BID into tBID following TRAIL treatment can directly activate BAK and BAX via the BH3 domain, leading to mitochondrial outer membrane permeabilization (MOMP)^[23,43,44]. However, tBID can also interact with the BH3-binding groove of the anti-apoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, displacing BAK and/or BAX from these inhibitory complexes and facilitating apoptosis^[45-47]. The binding characteristics of tBID were used to synthesize small-molecule Bcl-2 inhibitors, such as ABT-737 and its oral variant ABT-263 (Navitoclax), ABT-199 (Venetoclax), and obatoclax, that bind to the hydrophobic groove of pro-survival Bcl-2 proteins in order to antagonize their function. ABT-737 and ABT-263 can bind and antagonize the function of Bcl-2 and Bcl-xL^[48-51]. However, overexpression of Mcl-1 was demonstrated to cause resistance to ABT-737^[52-55]. In this respect, obatoclax has the advantage that it also neutralizes Mcl-1 in addition to targeting Bcl-2 and Bcl-xL, thus evading Mcl-1-dependent resistance^[49,56,57]. Several clinical studies have been conducted to investigate obatoclax ([ClinicalTrials.gov](https://clinicaltrials.gov)). Obatoclax mesylate in combination with docetaxel in relapsed NSCLC (phase 1/2) showed minimal clinical response, with neutropenia as the most common adverse event^[58]. In a phase II trial in small-cell lung cancer (SCLC), the combination of obatoclax mesylate plus topotecan was disappointing^[59]. Similarly, a randomized phase II study of carboplatin and etoposide with or without obatoclax mesylate showed no clinical benefit in advanced-stage small cell lung cancer (SCLC)^[60].

However, previous investigations on the expression levels of pro-survival Bcl-2 family members in NSCLC patients revealed variable associations. In NSCLC, high levels of Bcl-2, Bcl-xL, and Mcl-1 were noted in 35%, 82%, and 56% of the patients, respectively^[61-64]. Elevated Bcl-xL or Mcl-1 corresponded to poor prognosis^[62,65,66], whereas increased expression of Bcl-2 surprisingly correlated with improved overall survival in NSCLC^[67-69]. On the other hand, in breast cancer, higher Bcl-2 expression correlated with resistance to therapy and poor survival, while in mantle cell lymphoma (together with low BAX), it was associated with poor prognosis^[70,71]. Therefore, we reevaluated these findings in our NSCLC model systems with low and high sensitivity to TRAIL. Although novel treatments using tyrosine kinase-targeting drugs and immunotherapy are effective for many patients, for a large group of NSCLC patients, no benefit was observed. For these patients, conventional combination chemotherapy remains the only option^[72-74]. One important resistance mechanism remains impaired apoptosis, which can be targeted specifically with

TRAIL^[39,75,76]. These patients may benefit from novel treatment options that can enhance the efficacy of TRAIL. In the present study, we explored the cytotoxic effects of obatoclax alone and combined with TRAIL in a panel of NSCLC cells sensitive and resistant to TRAIL. Underlying mechanisms of enhanced apoptosis activation were examined. The aim of this study is to investigate the potentially beneficial effect of TRAIL/obatoclax inhibition treatment on NSCLC and to understand the biological mechanisms underlying these effects.

METHODS

Cell culture

NSCLC H460 (NCI-H460; ATCC HTB-177), A549 (ATCC CRM-CLL-185) and SW1573 (SW-1573; ATCC CRL-2170) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The identity of the cell lines was verified by STR analysis. Cells were regularly tested for mycoplasma contamination and only used when proven to be mycoplasma-free. H460 and A549 cells were grown in RPMI-1640 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and SW1573 in Dulbecco's Modified Eagle's Medium (DMEM)(Gibco, Thermo Fisher Scientific, Waltham, MA, USA). The medium was supplemented with 10% fetal bovine serum (Greiner Bio-One, Frinckenhausen, Germany) and 100 units/mL penicillin/streptomycin (Lonza, Verviers, Belgium). Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂. Earlier, we extensively characterized these and other cell lines for their sensitivity to TRAIL; for the current investigations, we chose H460 as being very sensitive and SW1573 and A549 as being almost completely resistant to TRAIL^[25,77].

Antibodies and chemical compounds

Recombinant human TRAIL was produced non-commercially, as described earlier^[78]. Obatoclax was provided by Gemin X Pharmaceuticals (Quebec, Canada). The broad-spectrum caspase inhibitor zVAD-fmk was purchased from Promega, Madison, USA. For Western blotting, we followed the recommendations of manufacturers and the following primary antibodies were used: mouse monoclonal anti-caspase 8 (1C12, Cell Signaling Technology, Danvers, MA, USA, 1:1,000), rabbit anti-caspase 9 (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), anti-cleaved-caspase 9 (D330) (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), rabbit anti-caspase 3, rabbit anti-cleaved caspase 3 (Asp175) (Thermo Fisher Scientific, Waltham, MA, USA, 1:1,000), rabbit anti-PARP (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit anti-Bcl-2 (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit anti-Bcl-xL (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), rabbit anti-Mcl-1 (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit anti-cytochrome c (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit anti-COX IV (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit anti-Puma (Cell Signaling Technology, Danvers, MA, USA, 1:500), rabbit-anti-BID (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), rabbit anti-Bax (Cell Signaling Technology Danvers, USA), goat anti-Bak (G-23)(Santa Cruz Biotechnology, Heidelberg, Germany). 1:1,000), mouse anti-β-actin (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands, 1:10,000), rabbit anti-TRAIL receptor 2 (Cell Signaling Technology, Danvers, MA, USA, 1:1,000), mouse anti-NOXA (114C307) (Merck KGaA, Darmstadt, Germany, 1:500), mouse anti-XIAP clone 2F1 (MBL International, Woburn, USA, 1:1,000), goat anti-DR4 (C20) (Santa Cruz Biotechnology, Heidelberg, Germany, 1:5,000), and mouse anti-GAPDH (sc-32233) (Santa Cruz Biotechnology, Heidelberg, Germany, 1:10,000). Secondary antibodies used were goat-a-mouse-IRDye (1:10,000, 800CW;#926-32210 and 680;#926-32220, Westburg, The Netherlands) or goat-a-rabbit-IRDye (800CW;926-32211 and 680;#926-32221).

Growth inhibition assay

Cytotoxicity was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) assays as described previously^[79], using 96-well

plates. We used the MTT assay because it was validated by the National Cancer Institute (NCI) in 106 cell lines as appropriate for proliferation and drug testing^[80]. We subsequently validated the MTT and the sulforhodamine B (SRB) assays in 25 cell lines^[79] and also demonstrated an excellent correlation ($r = 1.0$) between MTT and SRB readings and with the cell count^[79,81]. Although we prefer the SRB protein assay for drug testing, we could not use this assay since TRAIL, being a protein, would interfere with the assay and underestimate drug sensitivity. All cell lines were plated at 5,000 cells/well in 100 μ L culture medium. After allowing the cells to be attached, drugs were added after 24 h in 100 μ L medium. For combination treatments, cells were exposed to increasing concentrations of obatoclax and a fixed concentration of TRAIL for 48 h; 50 ng/mL TRAIL for the TRAIL-resistant A549 and SW1573 cells and 1 ng/mL TRAIL for the TRAIL-sensitive H460 cells. Optical density was measured at 540 nm. IC₅₀ values were defined as the concentrations that correspond to a reduction of cell growth by 50% compared to untreated control cells and are given as means \pm SEM. To determine the interaction between obatoclax and TRAIL, the combination index (CI) was calculated (Calculusyn, Biosoft, Cambridge UK) as described previously^[82]. Per experiment, the CI values at fraction affected (FA) > 0.5 were averaged and used for the calculation of means between experiments. A CI < 0.9 indicates synergism and > 1.1 antagonism.

Chicken-embryo chorioallantoic membrane model for *in vivo* drug testing

For *in vivo* testing of the combination, we used the *ex ovo* chorioallantoic membrane (CAM) assay. Originally developed to study neovascularization and the effects of anti-angiogenic drugs^[83,84], the CAM of fertilized eggs serves as a respiratory organ. It fuses with the mesodermal layer of the chorion and undergoes rapid expansion from embryonic development day (EDD) 4 to EDD 10, completing the formation of its capillary network by EDD 14. This results in a richly vascularized structure. Notably, the chicken embryo does not develop immunocompetence until EDD 18. Therefore, according to European Union regulations, the CAM model is not classified as an animal experiment if used before EDD 18.

In order to perform TRAIL and obatoclax testing, we primarily used the optimized platform developed in our department as described by Li *et al.*^[85]. Fertilized White Leghorn chicken eggs were obtained from Het Anker B.V. (Ochten, The Netherlands) and were maintained horizontally at 38.5 °C and 55% humidity. On EDD 3, eggs were placed vertically and a small puncture hole was made on top and incubated for another 3 days, during which the CAM sank under the puncture hole. The hole was widened with a pointed tweezer to inspect the embryo and select eggs with a strong heartbeat and well-developed vessels for grafting SW1573 cells. A small puncture was made in a well-vascularized region at the center of the CAM to induce slight bleeding, into which the cells were grafted.

In the meantime, SW1573 cells were cultured to generate spheroids. For this purpose, SW1573 cells were resuspended in 20% Matrigel® Growth Factor Reduced Basement Membrane Matrix (Corning Life Sciences B.V., Amsterdam, The Netherlands, #354230) and 80% medium; 25 μ L of the suspension was placed on a Petri dish. The dish was closed and spheroids consisting of 1,500,000 cells were formed at the bottom of the droplet. On day 7, a small ring was placed on top of the CAM within the bleeding area, and the spheroids were applied inside the ring. The eggs were closed with adhesive tape and incubated. On EDD 9, embryo viability and tumor take were confirmed and eggs were randomized over treatment groups, control, TRAIL (at 50 ng/mL), 500 nM obatoclax, and the combination. Several eggs had to be eliminated, but 11-12 eggs could be selected for each group. Each day, 50 μ L of 0.9% NaCl was added to avoid dehydration. On day 17, the tumors were removed from the CAM and fixated. Pictures were captured and tumor volume was calculated using either a microscope or a ruler as follows: $\frac{4}{3} \times \pi \times a \times b^2$ as described earlier (Li *et al.*^[85]). Experiments were always terminated before day 18 to avoid hatching.

Flow cytometric analysis of cell death

Cell death measurements were performed as described previously^[82]. Briefly, 100,000 cells were seeded in a 6-well plate. After exposure to TRAIL, obatoclax, or the combination, cells were trypsinized, resuspended in the medium collected from the matching sample and centrifuged for 5 min at 1,200 rpm (Eppendorf 5702R Centrifuge, Eppendorf AG, Hamburg, German). Cells were washed with PBS and subsequently stained with propidium iodide-containing buffer (0.1 mg/mL propidium iodide, 0.1% RNase A) (Thermo Fisher Scientific, Waltham, MA, USA) in the dark on ice. DNA content was analyzed by a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) with the acquisition of 10,000 events. Cell death was determined by the sub-G1 peak.

Western blot analysis

Western blot analysis was performed as previously described^[77]. Briefly, cells were disrupted in lysis buffer (Cell Signalling Technology Inc, Danvers/Boston, MA, USA), and from each sample, 30–80 µg of protein was separated by 8%–12% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon™-FL PVDF, 0.45 µm). Membranes were blocked for 1 h at room temperature (RT) in InfraRedDye blocking buffer (Rockland Inc., Pennsylvania, USA) and incubated overnight at 4 °C with the primary antibodies. The membrane was then washed and incubated with the secondary antibody for 1 h at RT in the dark. Fluorescent proteins were detected using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE, USA) at 84 µm resolution, with 0 mm offset and high-quality settings, and quantified with Odyssey v 3.0. Software (LI-COR Biosciences, Lincoln, NE, USA). The outcome of the scans of the blots is provided in the [Supplementary Table 1](#).

Mitochondrial and cytosolic fractionation

Following TRAIL and/or obatoclax treatment, the release of cytochrome c was analyzed as described previously^[86]. Briefly, for each sample, approximately 20 million cells were harvested for lysis in homogenization buffer (0.25 M sucrose, 10 mM Hepes, pH 7.4, 1 mM EGTA) and subjected to 50 strokes in a 2-mL Dounce homogenizer at 4 °C. The homogenates were centrifuged at 1,000× g for 15 min at 4 °C to obtain a pellet containing nuclei and unbroken cells. The supernatant was centrifuged at 10,000× g for 15 min at 4 °C, resulting in cytosolic fraction (supernatant) and mitochondrial fraction (pellet). Mitochondrial and cytosolic fractions were subjected to western blot to determine Cytochrome c, GAPDH, and Cox IV levels.

RNA interference

For transient gene expression knockdown, cells were seeded at 2.5×10^5 per well in a 6-well tissue culture plate and allowed to settle overnight. siRNA duplexes were formed using Oligofectamine reagent according to the manufacturer's instructions (Invitrogen BV). The culture medium was replaced with OPTI-MEM®I (Invitrogen, Breda, The Netherlands) and cells were transfected with 100 nM small interfering RNA (siRNA) molecules targeting Bcl-xL: 5'-GGA AGA GAA CAG GAC UGA GGC C-dTdT-3' (sense) and 5'-GCC UCA GUC CUG UUC UCU UCC C-dTdT-3' (anti-sense) and for XIAP: 5'- GUG GUA GUC CUG UUU CAG C-dTdT-3' (sense) and 5'-GCU GAA ACA GGA CUA CCA CdTdT-3' (anti-sense) (Eurogentech, Seraing, Belgium)^[87]. As a control, siRNA (scrambled siRNA, Invitrogen) with no homology to the human genome was used. After 24 h, cells were reseeded for flow cytometric analysis.

Cell surface expression of TRAIL receptors

Cell surface expression levels of TRAIL receptors were determined as described previously^[88]. Briefly, cells were collected, washed, and incubated at room temperature (RT) for 15 min with 10 µg/mL antibodies against TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 (clone HS101, HS201, HS301, and HS402, respectively; Enzo Life Sciences, Inc., Farmingdale, USA). Mouse IgG1 (Dako, Glostrup, Denmark) was

used as an isotype-matched negative control. Following incubation, cells were washed and then incubated with PE-labeled rabbit anti-mouse secondary antibody (Dako) for 15 min at RT. After a final wash, fluorescence intensity was measured using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Relative fluorescence intensity (RFI) was calculated as follows: $RFI = \text{mean fluorescence intensity (MFI) of the TRAIL receptor} - \text{MFI of the IgG1 control}$. Basal expression levels were normalized to 100%.

Statistical analysis

All experiments were performed at least in triplicate. Data were analyzed using the student's *t*-test for unpaired data; If appropriate, a paired *t*-test was used. *P*-values < 0.05 were considered statistically significant and exact numbers are provided in the legends. If no specifications are given, there is no statistical difference. Statistical analysis was performed using GraphPad Prism (version 5.0. GraphPad Software, San Diego, CA, USA).

RESULTS

Bcl-2 family protein expression in NSCLC cells; Bcl-xL knockdown enhances TRAIL-induced apoptosis

First, we determined the basal expression levels of several anti- and pro-apoptotic Bcl-2 family proteins in TRAIL-sensitive H460 and TRAIL-resistant A549 and SW1573 cells by Western blot analysis [Figure 1A]. BID expression was low in SW1573 compared to A549 and H460 cells. H460 showed the highest expression level of Bak. The protein expression levels of Bax, Puma, Noxa, and Mcl-1 showed only small differences between all cell lines. Interestingly, high Bcl-2 expression was found in H460 cells, although Bcl-2 was undetectable in other NSCLC cell lines. The opposite was found for Bcl-xL, which was hardly expressed in H460 cells and clearly present in A549 and SW1573 cells. To determine whether high Bcl-xL levels are involved in TRAIL resistance, we used a specific siRNA to knock down Bcl-xL expression in A549 and SW1573 cells [Figure 1B]. TRAIL exposure (100 ng/mL for 24 h) significantly increased cell death from 7% up to 30% ($P < 0.005$) and from 6% to 27% ($P < 0.05$) for A549 and SW1573 cells, respectively. These results indicate that Bcl-xL is an important determinant for TRAIL resistance.

Synergistic effect of Obatoclax on TRAIL-induced apoptosis

Considering the high expression levels of Bcl-2, Bcl-xL, and Mcl-1 in NSCLC cell lines together with the TRAIL-sensitizing effect of Bcl-xL knockdown, we anticipated the therapeutic benefit of combined obatoclax and TRAIL treatment. For this purpose, cells were exposed to a range of obatoclax concentrations for 48 h with or without 1 ng/mL TRAIL for H460 cells, and 50 ng/mL TRAIL for A549 and SW1573 cells [Figure 2A]. Differential sensitivities to single-agent obatoclax were observed, with IC₅₀ values of 454 ± 9 , 113 ± 8 , and 93 ± 17 nM in H460, A549, and SW1573 cells, respectively. A high level of Bcl-xL expression correlated with enhanced obatoclax sensitivity (A549 and SW1573 cells). Combined TRAIL and obatoclax exposure clearly resulted in synergistic cell killing, as illustrated by combination indices (CIs) of 0.63 ± 0.1 , 0.73 ± 0.1 , and 0.04 ± 0.01 for H460, A549, and SW1573, respectively. CI values below 0.9 are indicative of synergistic interaction [Figure 2B]. Cell death measurements by determining subG₁ cell fractions showed that 48-h concurrent treatment with 0.5 μ M obatoclax and 50 ng/mL TRAIL resulted in strong apoptosis activation in SW1573 cells. This is in contrast to a lack of effect of concurrent treatment for 48 h with 2 μ M obatoclax and TRAIL in A549 cells [Figure 2C].

However, pretreatment of A549 cells with 2 μ M obatoclax 48 h prior to the addition of TRAIL for 24 h resulted in a strong synergistic apoptotic response with up to 42% cell death [Figure 2D]. Exposure to lower concentrations of obatoclax prior to TRAIL treatment was not sufficient to induce synergistic levels of cell death in A549 cells (data not shown). Interestingly, pretreatment with obatoclax did not increase levels of apoptosis in SW1573 cells. These results indicate that different obatoclax and TRAIL treatment schedules

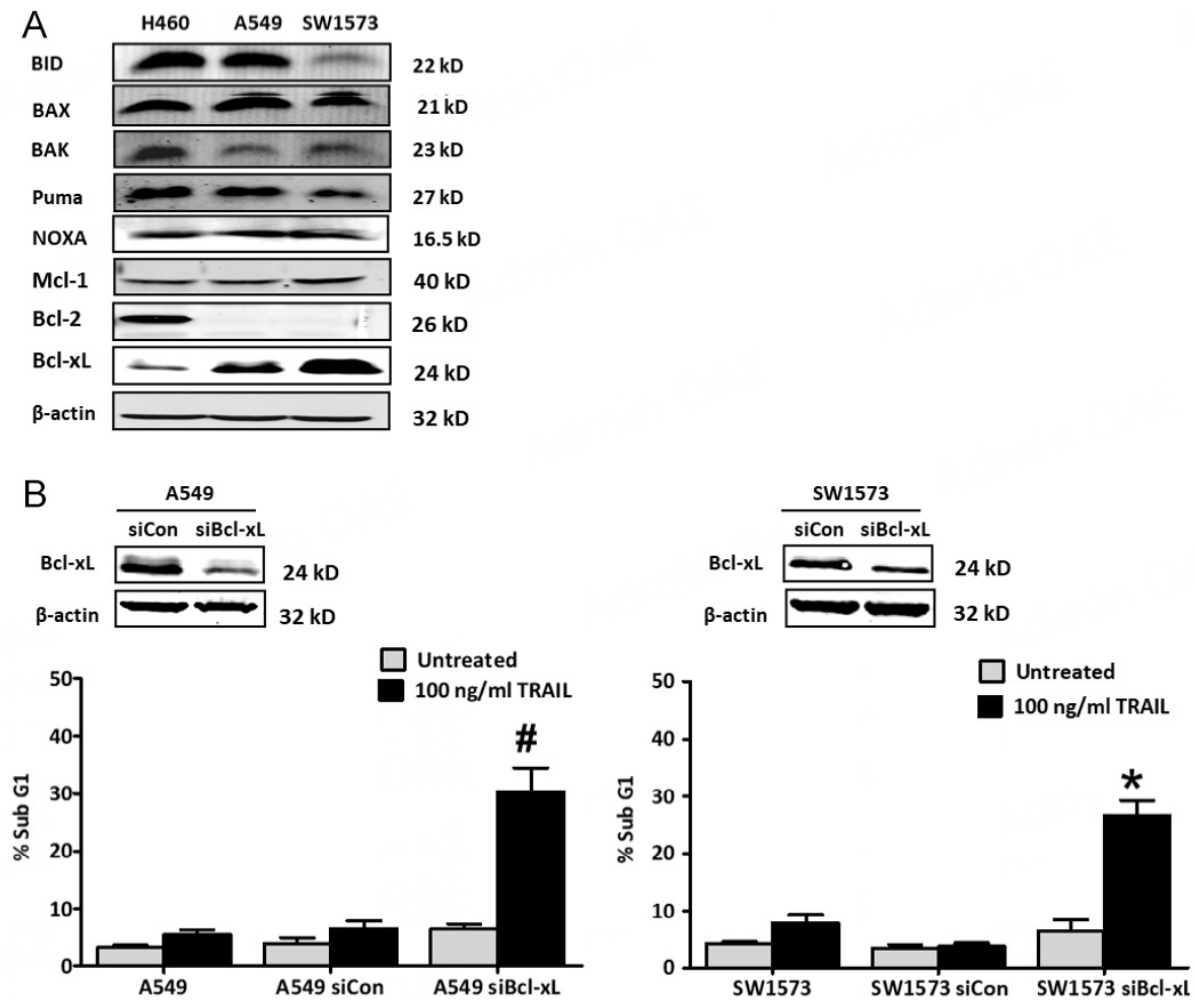


Figure 1. Bcl-2 family expression levels in A549 and SW1573 cells; silencing of Bcl-xL enhances TRAIL-induced cell death. (A) Expression levels of pro- and anti-apoptotic Bcl-2 family proteins as determined by Western blot analysis in H460, A549 and SW1573 cells. (B) Knockdown of Bcl-xL with a specific siRNA sensitizes for TRAIL-induced apoptosis in A549 and SW1573 cells. Values are means \pm SEM of at least three separate experiments. Statistics: (A) BID, SW1573 vs. H460 and A549, $P < 0.01$; BAX, A549 and SW1573 vs. H460, $P < 0.005$; Bcl-2: A549 and SW1573 vs. H460: $P < 0.001$; Bcl-xL, A549 and SW1573 vs. H460, $P < 0.01$; (B) [#] $P < 0.005$ and ^{*} $P < 0.05$, compared to untreated.

are required for optimal activity in the individual cell lines, suggesting different sensitization mechanisms. Regardless of this, in both cell lines, apoptosis induction by obatoclast and TRAIL was caspase-dependent, as the addition of caspase inhibitor zVAD-fmk clearly prevented TRAIL sensitization [Figure 2E].

Obatoclast enhances caspase cleavage and can increase TRAIL receptor expression

The onset of TRAIL-induced cell death is initiated by the activation of caspase 8. To investigate caspase cleavage patterns after exposure to obatoclast and/or TRAIL, we performed Western blot analysis [Figure 3A]. Concurrent treatment of SW1573 cells with 0.5 μ M obatoclast and 50 ng/mL TRAIL for 8 or 24 h clearly enhanced caspase 8 cleavage compared to cells exposed to TRAIL alone, accompanied by cleavage of caspases 9, 3 and PARP. Pre-incubation with 2 μ M obatoclast for 48 h followed by the addition of 50 ng/mL TRAIL for 8 or 24 h in A549 cells also resulted in enhanced cleavage of these caspases and PARP as indicated by the appearance of cleaved products and/or a reduction in the pro-forms. Increased caspase 8 activation is often related to a more effective assembly of the DISC that may involve TRAIL receptor

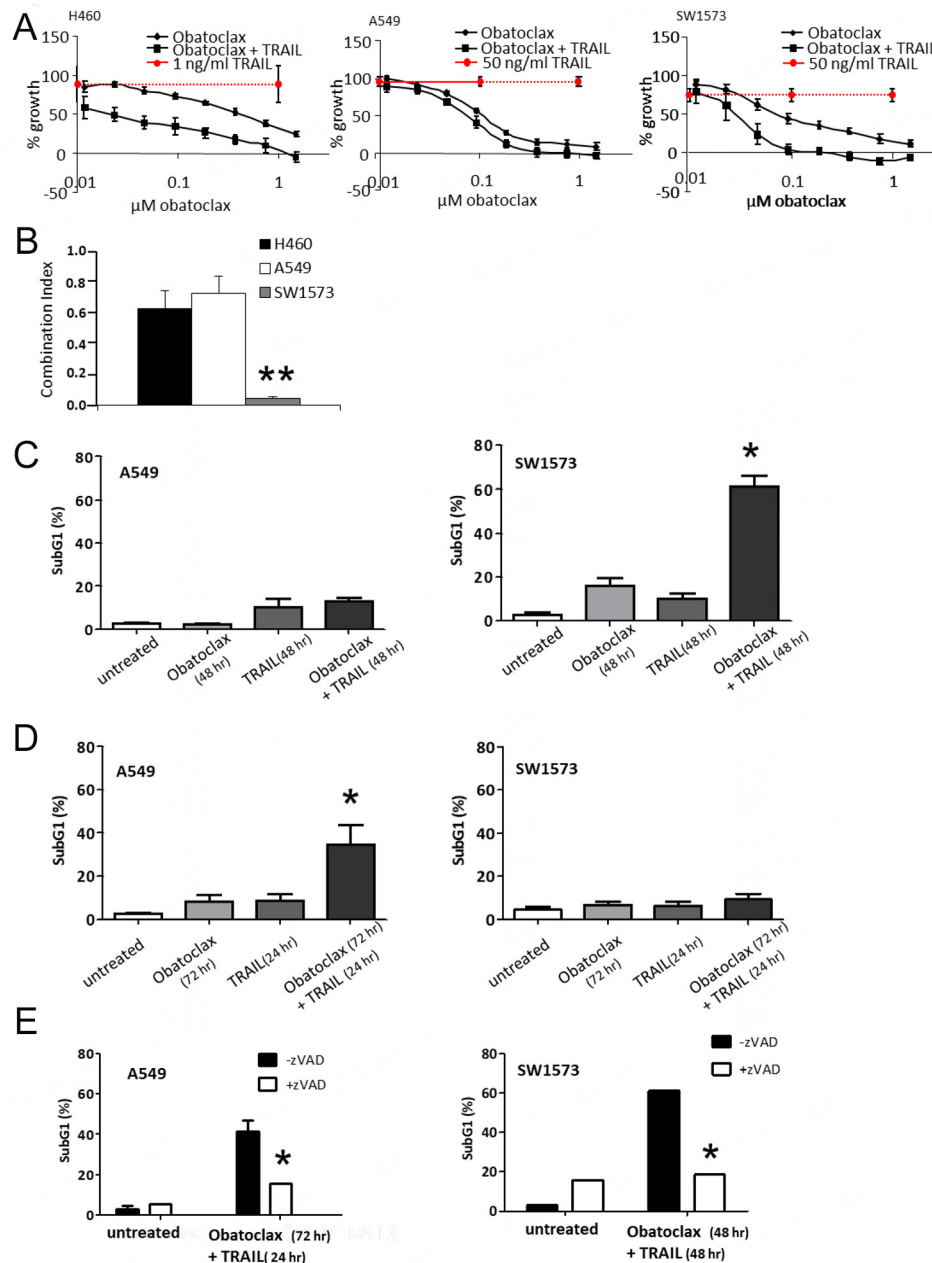


Figure 2. Synergistic interactions between obatoclox and TRAIL require different treatment schedules. (A) Dose-response curves for obatoclox, TRAIL, or the combination following 48-h treatment of H460, A549, and SW1573 cells. (B) Synergistic activity was calculated and represented by the Combination Index (CI); $\text{CI} < 0.9$ = synergy, $\text{CI} < 0.3$ = strong synergy. (C) Cell death measurement after 48-h concurrent treatment with 2 μM obatoclox, 50 ng/mL TRAIL, or both for A549 and 0.5 μM obatoclox, 50 ng/mL TRAIL, or the combination for SW1573. (D) Measurement of cell death after 48-h obatoclox treatment followed by the addition of TRAIL for 24 h: 2 μM obatoclox, 50 ng/mL TRAIL, or the combination, for A549; and 0.5 μM obatoclox, 50 ng/mL TRAIL, or the combination for SW1573. (E) Measurement of cell death after incubation with caspase inhibitor zVAD using pre-incubation or concurrent treatment for A549 and SW1573 cells, respectively. Values are means \pm SEM of at least three separate experiments. Statistics: (B) $^{**}P < 0.0001$, SW1573 compared to H460 and A549; (C and D) $^{*}P < 0.01$, higher sub-G1 for the combination of obatoclox with TRAIL compared to single agents; (E): $P < 0.01$, lower for zVAD compared to no zVAD.

upregulation. To explore this possibility, A549 and SW1573 cells were exposed to 2 and 0.5 μM obatoclox, respectively, for different time periods. Western blot analysis revealed enhanced expression of TRAIL-R1 and TRAIL-R2 in A549 cells after 48- and 24-h treatment, respectively. Enhanced TRAIL-R1 and TRAIL-R2

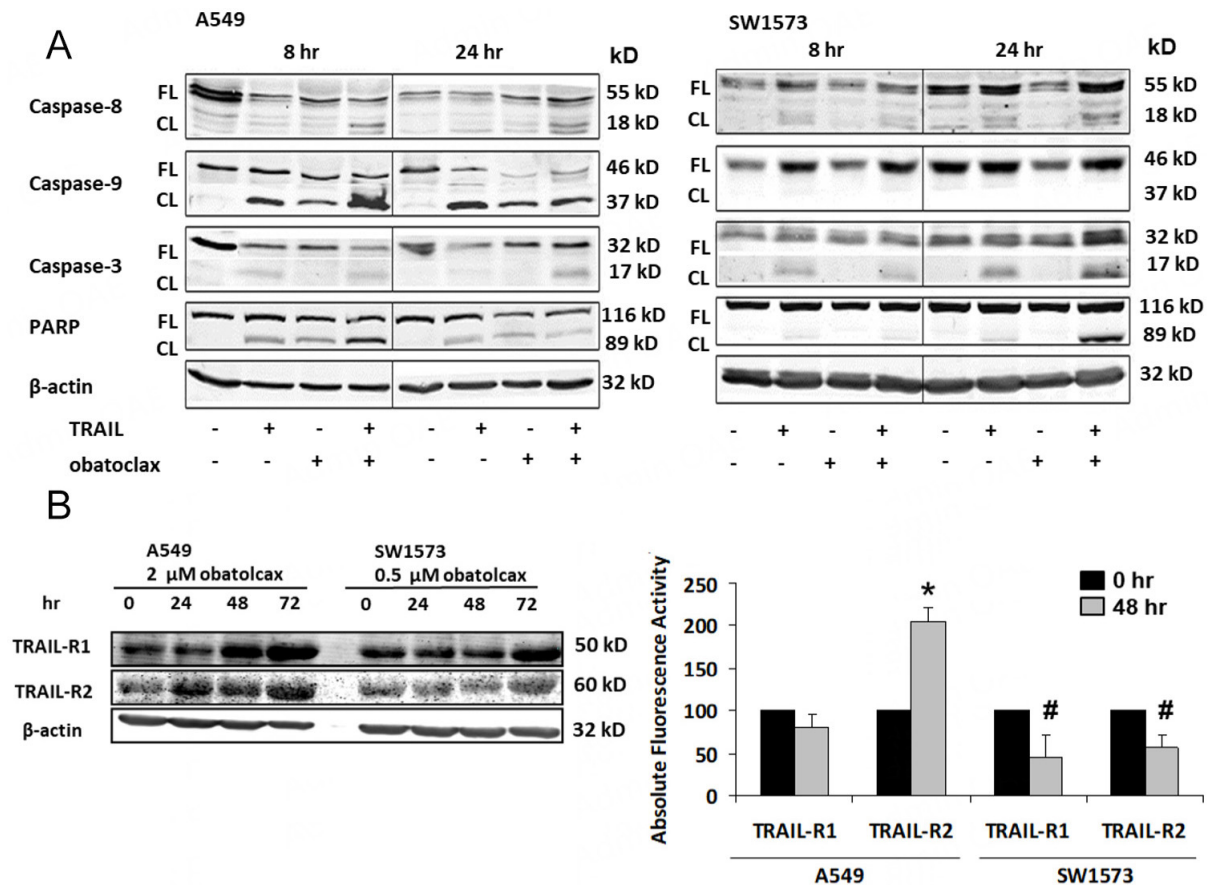


Figure 3. Obatoclox treatment enhances TRAIL-induced caspase cleavage and increases TRAIL receptor expression in a cell-dependent way. (A) Caspase- and PARP cleavage were determined by Western blot analysis after exposure to obatoclox (2 μ M for A549 and 0.5 μ M for SW1573), TRAIL (both cell lines 50 ng/mL), or the combination using pre-incubation or concurrent protocols for A549 and SW1573 cells, respectively. (B) Cell surface expression of TRAIL-R1 and TRAIL-R2 were determined by FACS analysis after 48-h exposure to 0.5 or 2 μ M for A549 and SW1573 cells, respectively. Western blots show a representative result from at least three separate experiments. Values are means \pm SEM of at least three separate experiments. Statistics: * $P < 0.01$, 48 h > 0 h; # $P < 0.05$, 48 h < 0 h.

was also seen in SW1573, although mainly after 72-h incubation. Next, cell surface expression of the TRAIL receptors was determined by FACS analysis following 48-h obatoclox treatment. A strong significant ($P < 0.01$) increase in TRAIL-R2 was observed after 48-h exposure to 2 μ M obatoclox in A549 cells [Figure 3B]. In SW1573, such an increase was not found, indicating differential responses to obatoclox in A549 and SW1573 cells.

Obatoclox enhances mitochondrial cytochrome c release upon TRAIL exposure

Obatoclox binds to the BH-3 groove present in Bcl-2, Bcl-xL, and Mcl-1, thereby preventing the binding of pro-apoptotic Bcl-2 family proteins, allowing them to initiate MOMP. We examined whether obatoclox and TRAIL exposure may result in altered expression of these anti-apoptotic proteins. A clear downregulation of Mcl-1 was observed only in A549 cells after pretreatment with 2 μ M obatoclox for 48 h prior to the addition of TRAIL for 24 h [Figure 4A]. To further determine the involvement of the mitochondrial pathway, the release of cytochrome c from the mitochondria was examined in concurrently treated SW1573 cells and obatoclox-pretreated A549 cells [Figure 4B]. Combined exposure clearly triggered enhanced release of cytochrome c in SW1573 and, to a lesser extent, in A549 cells. This indicates that, as expected, obatoclox stimulates the intrinsic apoptotic pathway.

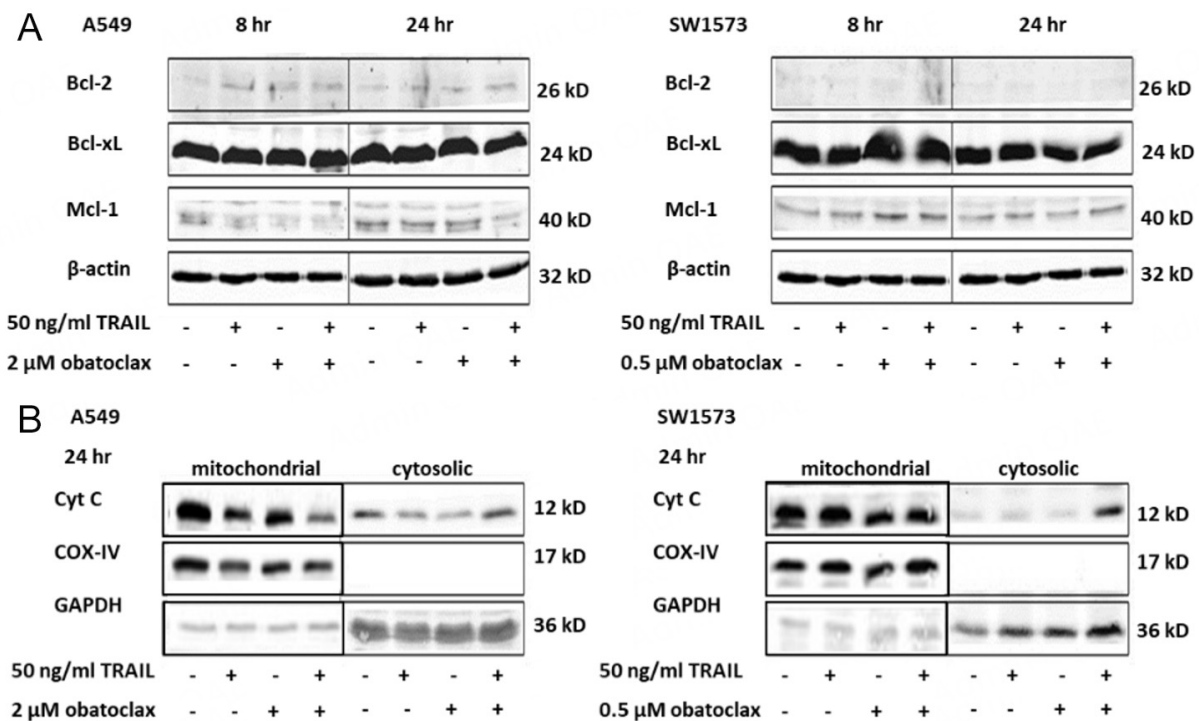


Figure 4. Obatoclox facilitates activation of the mitochondrial pathway by TRAIL. (A) Western blot analysis showing Bcl-2, Bcl-xL, and Mcl-1 protein expression levels after exposure to obatoclox, TRAIL, or the combination. SW1573 cells were concurrently exposed to 0.5 μM obatoclox, 50 ng/mL TRAIL, or the combination for 8 or 24 h. A549 cells were incubated with 2 μM obatoclox, followed by the addition of 50 ng/mL TRAIL for 8 or 24 h. (B) Treated and untreated cells were fractionated in cytoplasmic and mitochondrial lysates and cytochrome c levels were determined by Western blotting. Cox-IV and GAPDH were used as controls for the mitochondria and cytosolic fraction, respectively, showing that the mitochondrial fraction is not contaminated with cytosolic proteins, and that the cytosolic fraction does not contain mitochondrial proteins. SW1573 cells were concurrently exposed to 0.5 μM obatoclox, 50 ng/mL TRAIL, or the combination for 24 h. A549 cells were incubated with 2 μM obatoclox, followed by the addition of 50 ng/mL TRAIL for 24 h. Western blots show one representative result from at least three separate experiments. Cyt c: cytochrome c.

Obatoclox reduces XIAP expression

The aforementioned results indicate that the TRAIL-sensitizing effect of obatoclox involves the stimulation of mitochondrial apoptosis and increased expression of TRAIL receptors, leading to overall enhanced caspase activation. The caspase inhibitor XIAP has previously been implicated in TRAIL resistance in tumor cells^[87,89,90]. We observed high XIAP expression in A549 cells and explored whether XIAP contributes to the sensitivity of these cells to obatoclox and TRAIL [Figure 5A]. Surprisingly, exposure to obatoclox resulted in a time-dependent reduction in XIAP levels in both SW1573 and A549 cells [Figure 5A]. Based on these findings, we hypothesized that one of the effects of obatoclox pretreatment in A549 cells is to reduce the XIAP level sufficiently for TRAIL sensitization. To obtain further evidence for this notion, XIAP expression was silenced by transfection with a specific siRNA [Figure 5B] and the efficacy of combinations of TRAIL and obatoclox was evaluated. Reduced XIAP expression in A549 and SW1573 did not affect sensitivity to either obatoclox or TRAIL alone compared to siRNA controls. However, concurrent treatment with TRAIL and obatoclox was more effective in XIAP knockdown SW1573 cells than in control cells (increased from 48% to 55%, $P < 0.05$), and even more pronounced in A549 cells (increased from 14% to 24%, $P < 0.001$). These findings support our hypothesis that the reduction of XIAP levels by obatoclox contributes to its ability to sensitize cells to TRAIL. Moreover, they help explain why pretreatment of A549 cells is necessary to achieve effective TRAIL sensitization.

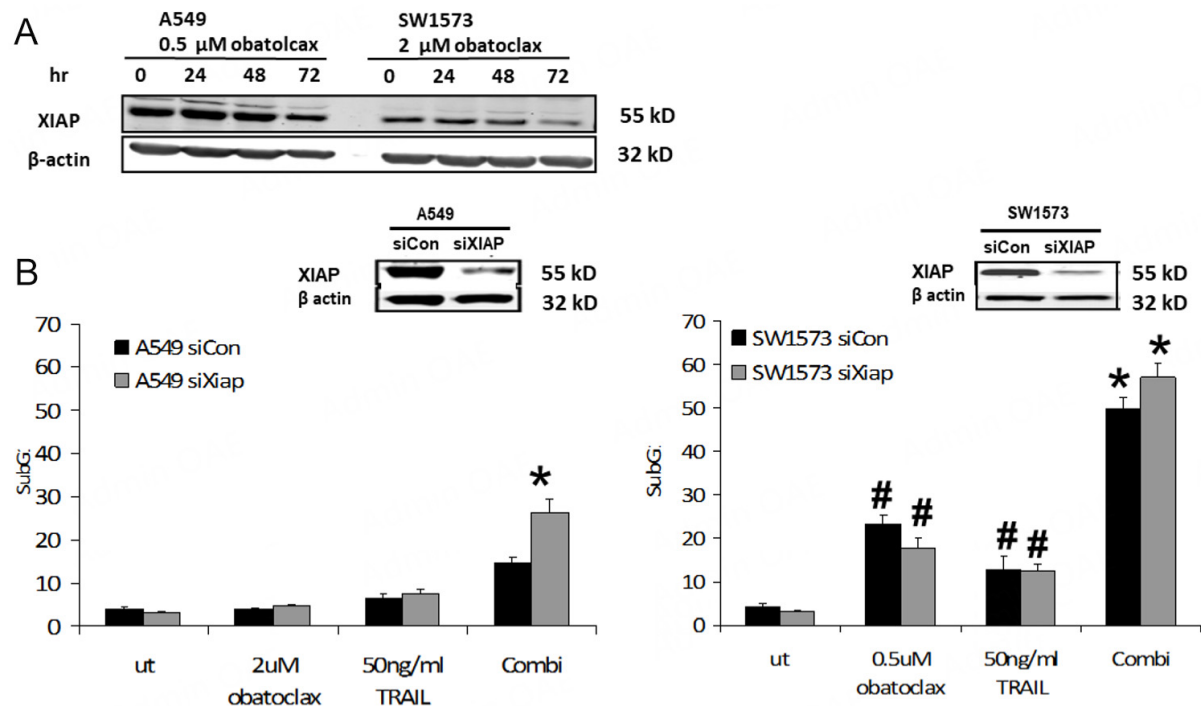


Figure 5. XIAP knockdown enhances obatoclast-mediated TRAIL-induced apoptosis. (A) XIAP expression levels were determined by Western blot analysis after incubation with 0.5 μM or 2 μM for A549 and SW1573 cells, respectively, at the indicated times. (B) XIAP-silenced A549 and SW1573 cells were exposed to concurrent treatment with 50 ng/mL TRAIL and 0.5 and 2 μM obatoclast for SW1573 and A549, respectively. Western blots show one representative result from at least three separate experiments. Values are means ± SEM of at least three separate experiments. Statistics, compared to ut (untreated), #*P* < 0.01; **P* < 0.001.

Obatoclast and TRAIL synergistically inhibit *in vivo* tumor growth in the CAM assay

To validate the synergistic interaction of TRAIL and obatoclast, we used the CAM assay. SW1573 cells were selected for this experiment because they are resistant to TRAIL and reproducibly formed tumors in the CAM model. This *in vivo* model features a well-defined tumor microenvironment characterized by neo-vascularization and immune competent cells, and it does not require the use of an animal-specific form of TRAIL. As expected, SW1573 cells remained resistant to TRAIL in this *in vivo* setting [Figure 6], and obatoclast also failed to exert any antitumor effect. The growth of the control and the treated tumors was similar. In contrast, the combination treatment resulted in a marked and statistically significant reduction in tumor growth of approximately 50%, consistent with our *in vitro* findings.

DISCUSSION

Anti-apoptotic Bcl-2 family members are well-established inhibitors of the TRAIL apoptotic pathway. For example, Bcl-xL is one of the crucial proteins involved in acquired resistance to TRAIL-induced apoptosis in TRAIL-R4- knockdown cells^[91]. Accordingly, siRNA-mediated knockdown of Bcl-xL in our panel of NSCLC cell lines sensitized the cells to TRAIL-induced apoptosis. This finding prompted us to examine the possible synergistic effects of combining the Bcl-2-targeting agent obatoclast with recombinant TRAIL in NSCLC cells. We observed a synergistic interaction between TRAIL and obatoclast in both TRAIL-sensitive (H460) and -resistant (A549 and SW1573) NSCLC cells. This synergism was dependent on caspase activation, as evidenced by Western blot analysis, and was further supported by functional experiments showing that the addition of the pan-caspase inhibitor zVAD suppressed cell death. Interestingly, optimal TRAIL sensitization required different obatoclast exposure schedules depending on the cell line. In SW1573 cells, simultaneous treatment with 0.5 μM obatoclast and 50 ng/mL TRAIL was sufficient, whereas in A549

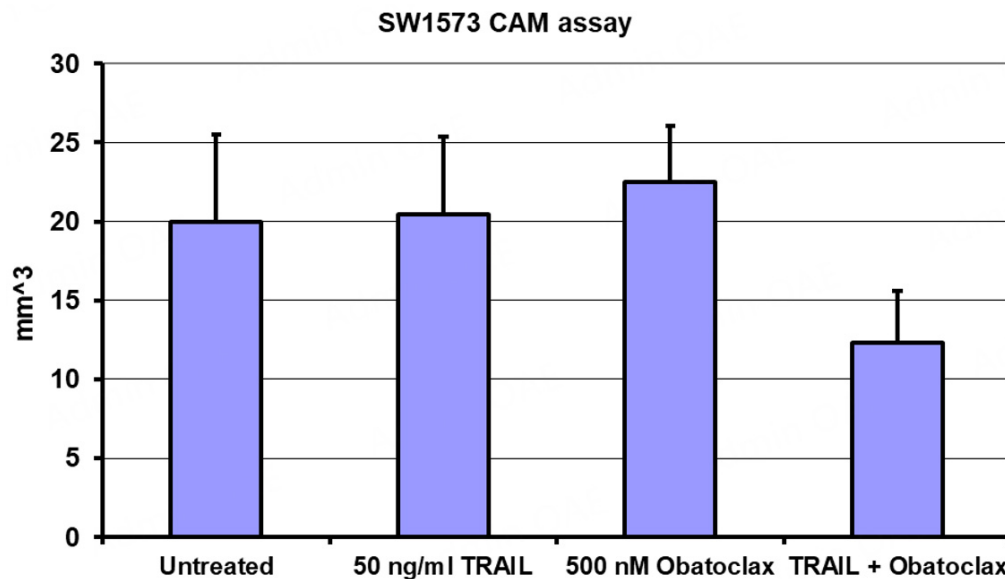


Figure 6. Antitumor effect of the combination of TRAIL and obatoclox in the *in vivo* CAM model. SW1573 tumors were treated in the CAM model from EDD 13 to EDD 17 with TRAIL at 500 ng/mL, 500 nM obatoclox, or the combination. Values are means \pm SEM of 11-12 eggs. Tumor size in the TRAIL-obatoclox group was significantly reduced compared to the control and single-agent treatment groups ($P < 0.001$ for all comparisons; Student's *t*-test for unpaired samples).

cells, pre-incubation with 2 μ M obatoclox for 48 h prior to TRAIL exposure was more effective at inducing cell death. Notably, the same concentrations also produced a significant antitumor effect *in vivo* in SW1573 tumors. Pretreatment with obatoclox in combination with TRAIL has previously been reported to be effective in other cancer cell types^[92-94]. Based on these findings, we further explored the schedule dependency and underlying mechanisms of this combination therapy in NSCLC.

As expected, obatoclox enhanced mitochondrial apoptosis in NSCLC cells. However, additional apoptosis-sensitizing effects were observed. Obatoclox appeared to increase the cell surface expression of particularly TRAIL-R2 in A549 cells, which is likely responsible for the associated enhanced caspase 8 activation cleavage that we observed [Figure 3B]. Previously, obatoclox-induced inhibition of the reported TRAIL-R2 transcriptional repressor protein Yin-Yang 1 (YY1) was related to enhanced TRAIL sensitivity^[93]. Moreover, the BH3 mimetic ABT-737 also enhanced the expression of TRAIL-R2, whereas FADD, cFLIP, and caspase 8 expression remained unaffected^[95]. In contrast, we did not observe obatoclox-dependent enhanced TRAIL-R2 levels in SW1573 cells, and even detected a reduction in expression, which may contribute to the failure of obatoclox pretreatment (48 h) to enhance TRAIL-induced apoptosis in these cells. Since TRAIL-R2 upregulation leads to enhanced DISC formation and subsequent caspase activation, the lower baseline levels of caspases [Figure 1] might suggest existing resistance mechanisms in this cell line. Since baseline levels of anti-apoptotic proteins are comparable between cell lines [Figure 1], the underlying cause might be rather a defect in TRAIL receptor signaling, or alternative cell death pathways might be overactivated in this cell line. Future studies should address these issues to provide a broader understanding of this therapeutic modality and to identify potential resistance mechanisms at an early stage, thereby helping to prevent treatment failure. Furthermore, examination of XIAP expression indicated a high basal level in A549 cells that was reduced by obatoclox exposure [Figure 5]. We found that part of the obatoclox-sensitizing effect was mediated by a decrease in XIAP, as siRNA-mediated silencing of XIAP in A549 cells was sufficient for cells to respond to concurrent obatoclox/ TRAIL treatment. Thus, the downregulation of XIAP expression appears to be an additional effect of obatoclox in NSCLC cells. This is in line with a previous report showing

that obatoclast reduced XIAP expression levels by suppressing NF- κ B activity through an unknown mechanism in B-Non-Hodgkin Lymphoma Ramos cells, resulting in enhanced TRAIL sensitivity^[93]. In chronic lymphocytic leukemia, both the XIAP overexpression in bone marrow and the increased Bcl-2 expression in peripheral blood were associated with a venetoclax-resistant phenotype. The increased expression of CD86 might explain why CLL cells avoid venetoclax-mediated apoptosis^[91,96-98]. It was also shown that XIAP overexpression-mediated ABT-263 resistance can be reduced by the use of metformin in combination with ABT-263^[99]. Moreover, targeting XIAP was sufficient for bypassing TRAIL resistance as a result of Bcl-2 overexpression in pancreatic cancer cells^[100]. Together, this suggests that obatoclast and other Bcl-2 inhibitors-mediated downregulation of XIAP are important mechanisms in TRAIL sensitization.

Martinez-Paniagua *et al.* found that inhibition of NF- κ B by obatoclast, in addition to dissociation of Bak from Mcl-1, was involved in the synergistic interaction of TRAIL and obatoclast in B-Non Hodgkin Lymphoma cell line Ramos^[93]. Inhibition of NF- κ B resulted in reduced expression of several NF- κ B-regulated gene products, including Bcl-xL, Mcl-1, and XIAP. We also observed a reduction in Mcl-1 expression in A549 cells after 48-h pretreatment with obatoclast followed by the addition of TRAIL for 24 h. However, our data, as well as other studies, did not show any alterations in the expression patterns of Bcl-2, Bcl-xL, and Mcl-1 upon obatoclast treatment alone^[92,94]. Interestingly, the treatment of leukemia with combined RBM10 and a Bcl-2 inhibitor (venetoclax) led to alternative splicing and inactivation of XIAP, as well as the downregulation of Bcl-2A1 (anti-apoptotic protein inhibiting the response to venetoclax), improving the final outcome^[101]. Moreover, combined inhibition of Bcl-2 (venetoclax) and XIAP (embelin) in acute myeloid leukemia cells enhanced the therapeutic effect^[102].

The specificity of obatoclast as an agent targeting the Bcl-2 family is under debate. According to Vogler *et al.*^[103], obatoclast induces cell death by inflicting severe mitochondrial damage, leading to non-specific cytochrome c release and caspase cleavage independent of Bak, Bax, and caspase 9^[103]. Therefore, other mechanisms by which obatoclast can modulate cell death should be taken into account, such as autophagy and cell cycle regulation, as reported by others^[92,94,104]. Brem *et al.* described that obatoclast as a BH-3 mimetic might also affect p53 by inhibiting Bcl-2, which influences cell cycle regulation^[104]. Moreover, induction of apoptosis, autophagy, and necroptosis has been observed in MLL-AF4 ALL cell lines upon obatoclast exposure^[105]. These findings illustrate the wide-ranging effects of obatoclast.

A derivative of obatoclast, SC-2001, has been described to be more potent as an inducer of cell death compared to obatoclast in hepatocellular carcinoma cells^[106]. Since 56% of lung cancer patients show high levels of Mcl-1, obatoclast or its derivatives are expected to circumvent the Mcl-1-dependent resistance that was reported for ABT-737^[64]. Currently, many inhibitors of Bcl-2 are in clinical use or are planned to be tested, including venetoclax, oblimersen sodium, AT-101, APG-2575, S-55746, BGB-111417, AZD-0466, AMG-176, AMG-397, S-64315, PRT-1419, and AZD-5991, mainly in hematological malignancies^[107,108].

The main limitation of the study was the use of only two TRAIL-resistant cell lines and one sensitive cell line. The resistant SW1573 cell line was able to establish tumors in the CAM assay and exhibited complete resistance *in vivo*. However, simultaneous treatment with obatoclast, which was ineffective when used alone, reversed this resistance. We focused on mechanistic aspects by using siRNA to downregulate XIAP and Bcl-xL, but we did not investigate gain-of-function effects, as one of the cell lines already exhibited high XIAP expression.

These results indicate a promising area of study, especially in light of previously identified major factors influencing TRAIL sensitivity. These factors include alterations in Bcl-2 family members (BID, Bcl-2, Mcl-1;

the impact on the intrinsic apoptotic pathway), relocalization of the TRAIL-R1 receptor within lipid rafts (probably activating the extrinsic apoptotic pathway), and immune system involvement through increased activation of caspases 1 and 4^[25]. Taken together, these results might help explain why major clinical trials targeting TRAIL have failed and suggest possible strategies to overcome resistance to TRAIL-based inhibitors.

Preclinical research has shown that obatoclax triggers non-apoptotic cell death. Clinically, obatoclax's side effects included transient central nervous system (CNS) adverse events such as confusion, ataxia, somnolence, and euphoric mood^[109]. Specifically, in a phase 1/2 trial for NSCLC, common side effects were neutropenia, febrile neutropenia, and dyspnea, mostly classified as grade 3/4 adverse events^[58]. Moreover, obatoclax had limited response rates that were not superior to chemotherapy alone in NSCLC^[58]. Due to these factors, a business decision was made to discontinue its clinical development^[110]. However, with an optimized dosing regimen, obatoclax (or its analogs) could potentially achieve clinical success. Our paper provides evidence of a synergistic effect that could be explored as a new treatment strategy for obatoclax or related compounds. This approach might also shift the mode of cell death from non-apoptotic^[111] to TRAIL-mediated apoptosis.

Overall, we observed a synergistic interaction between TRAIL and obatoclax in NSCLC cells. Obatoclax appears to exert additional mechanistic effects beyond those previously anticipated. The combined use of TRAIL and obatoclax may represent a promising strategy worthy of further clinical studies.

DECLARATIONS

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Authors' contributions

Study conception and design: de Wilt LHAM, Peters GJ, Kruyt FAE

Data collection, draft manuscript preparation: de Wilt LHAM, Sobocki BK

analysis and interpretation of results: de Wilt LHAM, Sobocki BK, Snoek BC, Kruyt FAE, Peters GJ

Editing, rewriting, supervision: Peters GJ, Jansen G, de Jong S, Kruyt FAE

Funding: de Jong S, Peters GJ, Kruyt FAE

All authors reviewed the results and approved the final version of the manuscript.

Availability of data and materials

All data are provided in this paper. Some data are provided in [Supplementary Material](#).

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Conflicts of interest

Peters GJ is an Editorial Board member of *Journal of Cancer Metastasis and Treatment*. Peters GJ was not involved in any part of the editorial process, including reviewer selection, manuscript handling, or decision making, while the other authors have declared that they have no conflicts of interest.

Ethical approval and consent to participate

The chicken embryo does not become immunocompetent before EDD (embryonic development day) 18. According to regulations in the European Union, the CAM (chorioallantoic membrane) model is not classified as animal experiments if used before EDD 18. There is no ethics involved.

Consent for publication

Not applicable.

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