

The XIAP inhibitor embelin sensitises malignant rhabdoid tumour cells to TRAIL treatment via enhanced activation of the extrinsic apoptotic pathway

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Abstract. Malignant rhabdoid tumour (MRT) is a rare, aggressive paediatric neoplasm, primarily diagnosed in those below the age of three. MRTs most commonly arise in the central nervous system and kidneys. A poor prognosis accompanies the MRT diagnosis, with a reported 2-year survival rate of 30%. Thus, there is an urgent need for the development of new therapies for this malignancy. Members of the inhibitor of apoptosis protein (IAP) family have previously been reported to be overexpressed in various cancers. As such, small molecule inhibitors of these family members have entered clinical trials. However, the role of IAPs in MRT has not been examined yet. The present study is the first report of the expression of a range of IAPs, including X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis protein 1 (cIAP1), cellular inhibitor of apoptosis protein 2 (cIAP2), livin and survivin in MRT cell lines. Furthermore, the results demonstrated the ability of the XIAP inhibitor, embelin, to sensitise MRT cell lines to TNF-related apoptosis-inducing ligand (TRAIL) treatment. The enhanced cell death detected upon cotreatment was dependent on caspase-8 and co-occurred with caspase-8 and caspase-3 cleavage, suggesting engagement of the extrinsic apoptotic pathway. Sensitisation to TRAIL was accompanied by livin cleavage, alongside downregulation of survivin and the caspase-8 inhibitor FLIP_L. In addition, knockdown of XIAP using siRNA enhanced TRAIL-mediated cell death, suggesting that this process may in part mediate sensitisation. In conclusion, the present results suggested that IAP inhibition may present a novel avenue for the treatment of MRT.

Introduction

Malignant rhabdoid tumour (MRT) is an aggressive paediatric neoplasm, primarily diagnosed in children under three (1,2). MRTs most frequently arise in the brain, where they are referred to as atypical/teratoid rhabdoid tumours (AT/RT), or the kidneys. Common to rhabdoid tumours is an inactivating mutation in the SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 (SMARCB1) gene, leading to loss of expression of SMARCB1, a component of the SWI/SNF chromatin remodelling complex (3,4). Unlike most cancers, MRTs are genomically stable, with SMARCB1 being the only commonly mutated gene identified (5-7). Currently, there is no standardised treatment protocol for MRT. Case reports in the literature, and numerous clinical trials are testament to this, with treatment regimens differing between institutions and often dependent upon tumour location. Treatment primarily consists of surgical resection of the tumour followed by aggressive chemotherapy, and if appropriate radiation therapy. Unfortunately, MRT is highly refractory to therapy, with retrospective reviews placing the 2-year event-free survival rate at $\leq 35\%$ (8,9). The most successful trial to date treated 20 AT/RT patients with surgical resection, intensive chemotherapy and radiation, resulting in a 2-year overall survival rate of 70% (10). However, the dangers of pursuing such an aggressive regimen in children was evident with several associated toxicities and one toxic death reported. Given the poor prognosis and lack of treatment options, there is an urgent need for new therapies for MRT.

TNF-related apoptosis-inducing ligand (TRAIL) is a proapoptotic cytokine expressed by many tissue types of immune cells. Unlike other members of the TNF superfamily, TRAIL exhibits selectivity towards cancer cells whilst sparing healthy cells (11). While chemotherapeutics and radiation therapy elicit apoptosis through the intrinsic or mitochondrial apoptotic pathway, TRAIL, activates the extrinsic apoptotic pathway (12). Binding of TRAIL to its cognate death receptors, death receptor 4 (DR4) or death receptor 5 (DR5), results in receptor oligomerisation and formation of a membrane associated death inducing signalling complex (DISC) consisting of the adaptor protein FAS-associated via death

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domain (FADD), caspases 8 and 10, and the caspase-8 analogue FLICE-inhibitory protein (FLIP). The primary result of DISC formation is caspase-8 activation. Once active, caspase-8 cleaves and activates caspases 3 and 7, thus inducing apoptosis. In addition, TRAIL can engage the intrinsic pathway, as caspase-8 may cleave the pro-apoptotic Bcl-2 protein Bid into its truncated form tBid, which mediates mitochondrial outer membrane permeabilization (MOMP). MOMP results in cytochrome c release which in turn facilitates caspase-9 activation thus potentiating apoptosis. MOMP and thus the intrinsic apoptotic pathway is regulated by the Bcl-2 family, which consists of pro and antiapoptotic members alongside regulatory proteins (13). In MRT, the antiapoptotic Bcl-2 protein myeloid leukaemia cell differentiation protein 1 (Mcl-1) has been demonstrated to be overexpressed (14). Unfortunately, clinical trials with TRAIL have yielded disappointing outcomes, with drug resistance being a primary issue (15-19). One approach to combat this, is the combination of TRAIL treatment with inhibition of negative regulators of the apoptotic process, such as FLIP-long form (FLIP_L), anti-apoptotic members of the Bcl-2 family and the inhibitor of apoptosis (IAP) family.

Of the IAP family, X-linked inhibitor of apoptosis (XIAP), cellular inhibitor of apoptosis protein (cIAP) 1, cIAP2, livin and survivin have been implicated in cancer progression and apoptosis inhibition, with their overexpression demonstrated in various malignancies, including acute myeloid leukaemia, neuroblastoma, hepatocellular carcinoma and renal cell carcinoma (20-28). To date, their role in MRT has not been examined. XIAP is the best characterised of these proteins and the only IAP which has been demonstrated to inhibit caspase activity directly (29-34). Specifically, XIAP has been demonstrated to bind and inhibit caspases 3, 7 and 9 (35,36). Embelin, the active constituent of the *Embelia ribes* shrub, is a small molecule which has been found to inhibit XIAP (37). Its tumour suppressive abilities have been demonstrated in leukaemia, prostate, breast, gastric and brain glioma cell lines (38-44). Its chemotherapeutic and radiation sensitising capacity has also been demonstrated in various cancers (45,46). Additionally, there are an increasing number of studies reporting that embelin can sensitise both cancer cell lines and xenograft models to TRAIL (47-54). To the best of our knowledge, there is only one published report on TRAIL treatment in MRT cell lines, which found 3 of 6 tested cell lines to be sensitive to TRAIL (55). However, no combination regimens were tested. Furthermore, there are currently no reports on the effects of embelin, either alone or in combination, in MRT.

The results reported in the present study demonstrated for the first time the expression of a range of IAPs in MRT cell lines. In addition, embelin was determined to act as a sensitising agent to TRAIL in otherwise resistant MRT cell lines. This enhanced apoptosis was mediated in part by XIAP inhibition. The current findings thus suggest potential novel therapeutic options for MRT.

Materials and methods

Cells and reagents. BT12 and BT16 cell lines are epithelial AT/RT cells isolated from a 2 month old female and a 2-year-old male, respectively. These cell lines were generously donated by Peter Houghton, St. Jude Children's Research Hospital

(Memphis, TN, USA). G401 cells (American Type Culture Collection, Manassas, VA, USA) are epithelial kidney MRT cells isolated from a 3 month old male. BT12 and BT16 cell lines were cultured in Gibco RPMI-1640 GlutaMAX (Biosciences, Dublin, Ireland). G401 cells were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM) GlutaMAX (Biosciences). All media used was supplemented with 10% (v/v) foetal bovine serum (FBS; Biosciences), 100 U/ml penicillin and 100 µg/ml streptomycin (Biosciences). Cultured cells were incubated at 37°C with 5% CO₂. Cells were screened for mycoplasma using PCR and were consistently found to be negative for infection. A 1 mg/ml stock of recombinant human TRAIL (Merck KGaA, Darmstadt, Germany) was prepared in sterile dH₂O and stored at -80°C. Dilutions were prepared fresh on the day of treatment. A 10 mM stock of embelin (Sigma-aldrich; Merck KGaA) was prepared in ethanol and stored at -20°C. Stocks (50 mM) of both the general caspase inhibitor (ZVAD-FMK; Calbiochem; Merck KGaA) and caspase-8 Inhibitor II [Z-IE(OMe)TD(OMe)-FMK; Calbiochem; Merck KGaA] were prepared in DMSO and ethanol respectively. Dilutions of both inhibitors were prepared fresh on the day of treatments in sterile dH₂O.

Cell viability assay. BT12, BT16 and G401 cell lines (200 µl) were seeded at densities of 10x10³, 10x10³ and 5x10³ cells/well, respectively, in 96-well plates. Cells were left overnight to adhere and then treated with a range of concentrations of TRAIL or embelin for 72 h. Viability was assessed via the alamar blue assay. Briefly, 20 µl of AlamarBlue (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA) was added to each well. The plate was incubated in the dark at 37°C for 5 h. Fluorescence was measured on a SpectraMax plate reader at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. All alamar blue assays were performed in triplicate.

Cell death analysis. For cell death analysis via propidium iodide (PI) staining, cells were collected by centrifugation and fixed in 70% ethanol overnight. The following day, 5 µl of FBS and 1 ml of PBS were added to each sample. Cells were collected by centrifugation and resuspended in BD FACSflow sheath fluid (BD Biosciences, San Jose, CA, USA) supplemented with 10 µg/ml of RNase A (Sigma-Aldrich; Merck KGaA) and 100 µg/ml of PI (Sigma-Aldrich; Merck KGaA). The samples were then incubated in the dark at 37°C for 30 min. Analysis was performed with a BD Accuri C6 flow cytometer using BD Accuri C6 software (BD Biosciences). Samples were first gated on vehicle controls. A total of 1x10⁵ cells from each sample were counted and results were visualised on histograms. PI was detected using a 585/40 bandpass filter. The sub G1/G0 population (<2n) was assigned as apoptotic cells.

For cell death analysis via Annexin V-fluorescein isothiocyanate (FITC) and PI staining, cells were collected by centrifugation and washed with 0.5 ml Annexin V binding buffer (5 mM HEPES, 70 mM NaCl, 1.25 mM CaCl₂ pH 7.4). The pellet from this wash was collected and stained with Annexin V-FITC (iQ Corporation, Groningen, Netherlands) for 30 min in the dark on ice. Cells were washed with 0.5 ml of Annexin V binding buffer and collected by centrifugation. The pellet was resuspended in 500 µl PI (0.5 µg/ml). The samples were then analysed immediately on BD Accuri C6

flow cytometer using BD Accuri C6 software. Samples were first gated on vehicle controls. These gates were then analysed on a FITC-Annexin V (535 nm; FL1 channel using a 530/30 bandpass filter) vs PI (488 nm; FL2 using a 585/40 bandpass filter) dot plot.

Gel electrophoresis and western blot analysis. For whole cell lysates, cell pellets were resuspended in ice-cold RIPA buffer (Sigma-Aldrich; Merck KGaA) supplemented with protease inhibitor cocktail II and III (Sigma-Aldrich; Merck KGaA) and complete ultra protease inhibitor (Roche Applied Science, Penzberg, Germany) and kept on ice for 30 min. Lysates were centrifuged at 10,000 x g for 10 min and the pellet was discarded. For the cytosolic lysates, the cell pellet was resuspended in 75 μ l subcellular fractionation buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCL, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, protease inhibitor cocktail) and kept agitated at 4°C for 30 min. The suspension was centrifuged at 720 x g for 5 min and the resultant supernatant was centrifuged further at 10,000 x g for 10 min. The final resultant supernatant was the cytosolic lysate. For all lysates, protein concentration was determined using the BCA assay (Pierce; Thermo Fisher Scientific, Inc.) and normalised. Cell lysates were boiled with Laemmli sample buffer supplemented with 50 μ M DTT for 10 min at 90°C. Equal concentrations of denatured samples were separated using polyacrylamide gel electrophoresis and transferred to an immobilion-P PVDF membrane (Merck KGaA). The membrane was blocked for 1 h in 5% non-fat dried milk and then incubated overnight at 4°C with the relevant primary antibody. The following primary antibodies were purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA): XIAP (1:2,500; cat no. 2045), cIAP1 (1:1,000; cat no. 7065), cIAP2 (1:1,000; cat no. 3130), livin (1:1,000; cat no. 5471), survivin (1:1,000; cat no. 2808), caspase-3 (1:1,000; cat no. 9662), cleaved caspase-3 (1:1,000; cat no. 9664), caspase-8 (1:1,000; cat no. 9746), cleaved caspase-8 (1:1,000; cat no. 9496), caspase-9 (1:1,000; cat no. 9508) Bid (1:1,000; cat no. 2002) and Mcl-1 (1:1,000; cat no. 4572). The following primary antibodies were also used: second mitochondria-derived activator of caspases (SMAC; 1:1,000; cat no. 612246; BD Biosciences), FLIP_L (1:2,000; cat no. MABC148; Merck KGaA) GAPDH (1:10,000; cat no. CB1001; Calbiochem), and α tubulin (1:1,000; cat no. CP0; Calbiochem). The following day the membrane was washed three times for 10 min each with TBST, probed with the relevant anti-rabbit (1:2,500; cat no. W402B; Promega Corporation, Madison, WI, U.S.A.) or anti-mouse (1:2,500; cat no. W401B; Promega Corporation) horseradish peroxidase (HRP)-conjugated secondary antibody, and then washed again as aforementioned. The membrane was soaked in enhanced chemiluminescence detection reagent (Merck KGaA). The western blot results were visualised using the BioRad Gel Doc™ XR+System with Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

XIAP knockdown using small interfering (si) RNA. On-targetplus human XIAP siRNA SMARTpool (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA) consisted of four siRNA sequences targeting XIAP, as follows: GUAGAU AGAUGGCAAUAUG, GAACUGGGCAGGUUGUAGA,

GAAAGAGAUUAGUACUGAA and GGACUCUACUACA CAGGUA. The negative control On-targetplus non-targeting pool (GE Healthcare Dharmacon, Inc.) consisted of four non-targeting siRNA sequences: UGGUUUACAUGUCGAC UAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAU GUUUUCUGA, and UGGUUUACAUGUUUCCUA. BT12 cells were seeded at 10x10⁴ cells/ml in antibiotic-free media in a 6-well plate. The following day, XIAP siRNA or non-targeting siRNA was transfected into cells using Lipofectamine (Thermo Fisher Scientific, Inc.) at a final concentration of 25 nM, as per manufacturer's instructions. Cell death was assessed 48 h post-treatment by flow cytometric analysis of PI-stained cells and subsequent quantification of the sub G1/G0 peak, as described above. Western blot analysis confirmed XIAP knockdown at 0 and 48 h of TRAIL treatment.

Determination of synergism. TRAIL and embelin combination treatments were assessed for synergism based upon the apoptosis they elicited, both as single drugs and in combination with one another, as determined by flow cytometric analysis of PI-stained cells, as described above. The computer software program Compusyn (ComboSyn, Inc., New Jersey, U.S.A.) was used to study the interactions between the drugs. This program uses the Chou-Talalay method, based upon the median effect principle, to determine Combination Index (CI) values for each drug combination. CI values <1, =1 and >1 indicate synergistic, additive and antagonistic effects respectively.

Statistical analysis. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis of experimental data. Results are displayed as the mean \pm standard error of the mean. To determine statistical significance, Student's t-test was used for comparisons between two groups, while a one-way analysis of variance followed by Tukey's multiple comparison test was performed for comparisons of more than two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

MRT cell lines express a range of antiapoptotic IAP proteins. BT12, BT16 and G401 cell lines were firstly analysed for expression of XIAP, cIAP1, cIAP2, livin and survivin via western blotting (Fig. 1A). Densitometric analysis was then performed (Fig. 1B-G). All three cell lines were demonstrated to express a range of IAPs, with XIAP, cIAP1 and survivin universally expressed. Low levels of cIAP2 were detected in BT12 cells only. Livin exists as two splice variants; livin α and livin β . Both splice variants were detected in BT12 and G401 cells, while neither was detected in the BT16 cell line.

TRAIL and embelin exhibit limited effects on the viability of MRT cells. Yoshida *et al* (55) have previously reported a decreased viability of MRT cell lines upon TRAIL treatment. Given this, and combined with the XIAP expression demonstrated in Fig. 1, the viability of BT12, BT16 and G401 cell lines was assessed following treatment with TRAIL or the XIAP inhibitor, embelin, for 72 h via the alamarBlue assay (Fig. 2A). Viability dropped to only 60, 71 and 89% for G401, BT12 and BT16 cells, respectively, at 1,000 ng/ml

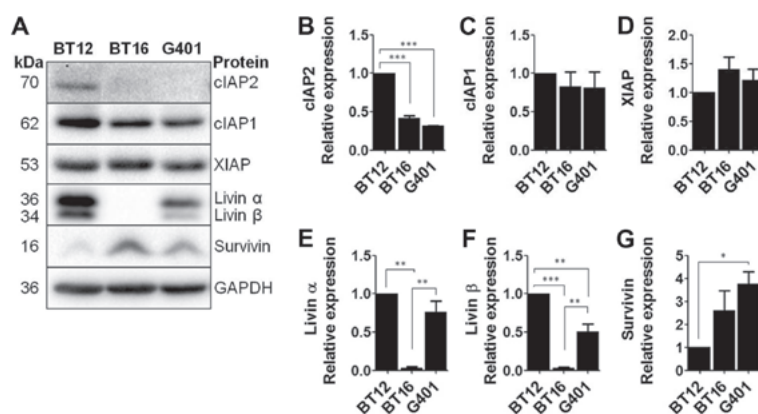


Figure 1. MRT cell lines express a range of IAP family members. (A) Expression levels of cIAP2, cIAP1, XIAP, Livin α , Livin β and survivin in malignant rhabdoid tumour cell lines, BT12, BT16 and G401, were detected by western blotting. GAPDH was used as a loading control. (B-G) Western blots were normalised to GAPDH and densitometric analysis was performed. Values are mean \pm standard error of the mean of at least three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 with comparisons indicated by brackets. MRT, malignant rhabdoid tumour; IAP, inhibitor of apoptosis protein; cIAP, cellular inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis.

TRAIL. Embelin reduced the viability of all three cell lines in a dose dependent manner. However, even at 50 μ M dose, the viability did not decrease below 33% for any cell line. BT12 and BT16 cells had similar IC_{50} values of 13.06 and 14.52 μ M, respectively, with viability plateauing approximately 44 and 37%, respectively. The IC_{50} value for the G401 cell line was considerably higher at 47.14 μ M.

Embelin synergistically enhances TRAIL-mediated cell death in MRT cell lines. Having shown the expression of XIAP in a range of MRT cell lines, and their resistance to TRAIL treatment, the present study next assessed if embelin could sensitise MRT cell lines to TRAIL. BT12, BT16 and G401 cell lines were treated with a range of concentrations of TRAIL, both alone and in combination with 10 μ M embelin, for 24, 48 and 72 h. This concentration of embelin is at the lowest end of the range (10–50 μ M) previously demonstrated to enhance susceptibility to TRAIL (47–54). It was selected for the present study, as lower doses are preferable in cancer therapeutics in order to minimise potential side effects. Cell death was assessed via PI staining of permeabilised cells and subsequent quantification of the sub G1/G0 peak. The computer program Compusyn was used to determine synergism. This program computes combination index (CI) values for each drug combination. CI values <1, =1 and >1 indicate synergistic, additive and antagonistic effects respectively, with lower CI values indicating greater synergism. In BT12 cells, treatment with TRAIL or embelin alone did not cause significant cell death compared with vehicle, at any concentration tested. Combination treatment resulted in a dose and time-dependent increase in cell death, which was significant and synergistic at all concentrations and at several timepoints (Fig. 2B; Table I). Treatment with TRAIL at 50 ng/ml in combination with 10 μ M embelin was determined to give the greatest synergism with a CI value at 0.169 (Table I). Annexin V-PI staining, was then employed to confirm the increased cell death upon combination treatment with concurring results obtained (Fig. 2C). Similarly, in BT16 cells, embelin synergistically enhanced TRAIL-mediated cell death across a range of timepoints and concentrations, with CI values <1 reported for all concentrations tested at 48 and 72 h

(Fig. 2B; Table I). In the G401 cell line, combination treatment did not result in significantly higher cell death compared with TRAIL treatment alone, at any concentration tested (Fig. 2B), possibly owing to differences between MRTs arising in the kidney and the brain.

Combination treatment induces apoptosis through the extrinsic apoptotic pathway. To determine the mode of cell death initiated upon combination treatment, BT12 cells were pretreated with an inhibitor of necroptosis, necrostatin 1 or the general caspase inhibitor, Z-VAD-FMK. Pretreatment with necrostatin 1 had no effect on cell death (Fig. 3A). By contrast, pretreatment with Z-VAD-FMK prevented cell death (Fig. 3B), indicating apoptosis was occurring. In the literature, TRAIL-induced cell death has mostly been attributed to activation of the extrinsic apoptotic pathway, resulting firstly in caspase-8 activation, followed by downstream activation of caspase-3, caspase-7 and in certain cell types caspase-9 (12). Pretreatment of BT12 cells with a specific caspase-8 inhibitor, Z-IETD-FMK, abrogated cell death (Fig. 3C), suggesting that the extrinsic apoptotic pathway was induced upon TRAIL and embelin treatment. Caspase inhibition was further confirmed via western blot analysis (Fig. 3D).

Embelin prevents the recovery of MRT cells from TRAIL treatment over time. BT12 and BT16 cells were treated with TRAIL and embelin, both alone and in combination, from 1 to 48 h. In both cell lines, TRAIL treatment resulted in an initial burst of cell death from 4 h (Fig. 4A and B). However, the cells recovered from TRAIL treatment with cell death decreasing steadily from 16 to 48 h in BT12 cells (Fig. 4A), and from 4 to 48 h in BT16 cells (Fig. 4B). By contrast, cell death increased upon combination treatment in a time-dependent manner in both cell lines. This suggests that embelin prevented the recovery of MRT cell lines from TRAIL treatment over time. Given the aforementioned findings that TRAIL and embelin-induced cell death was dependent on caspase-8 activation, the extrinsic apoptotic pathway was examined over a range of timepoints. Treatment with TRAIL alone resulted in a decrease in full length caspase-8 and generation of active

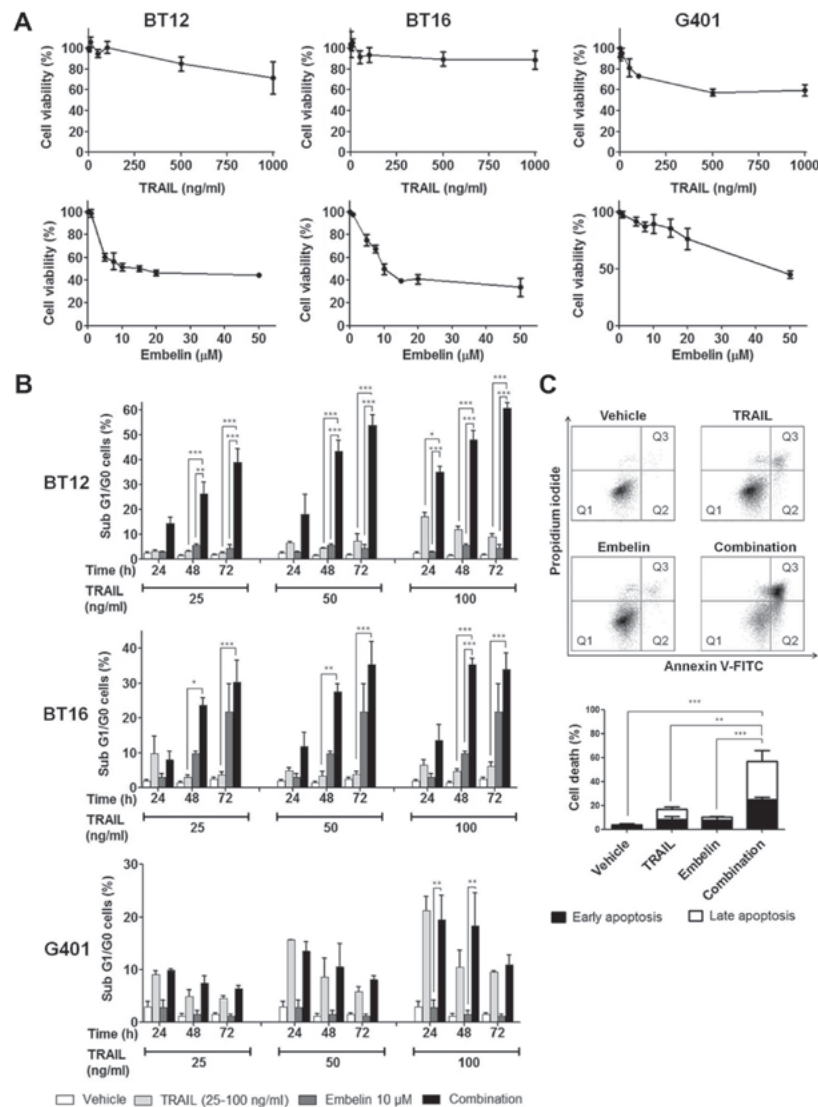


Figure 2. Embelin enhances TRAIL-mediated cell death in MRT cell lines. (A) MRT cell lines, BT12, BT16 and G401, were treated with a range of concentrations of TRAIL or embelin. Viability was assessed 72 h post-treatment via the alamar blue assay. (B) MRT cell lines, BT12, BT16 and G401, were treated with a range of concentrations of TRAIL alone or in combination with 10 μ M embelin. Cell death was assessed at 24, 48 and 72 h post-treatment via flow cytometric analysis of PI-stained cells and subsequent quantification of the sub G1/G0 peak. (C) BT12 cells were treated with 50 ng/ml TRAIL and 10 μ M embelin, both alone and in combination. Cell death was assessed 48 h post-treatment, via flow cytometric analysis of PI and Annexin-V FITC-stained cells. Cells which stained negative for PI and Annexin V are considered viable (Q1), cells which stained positive for Annexin V, but negative for PI were considered to be undergoing early apoptosis (Q2), and cells which stained positive for both PI and Annexin V were considered to be undergoing late apoptosis/necrosis (Q3), as shown in the representative plots. Values are mean \pm standard error of the mean of at least three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 with comparisons indicated by brackets. TRAIL, TNF-related apoptosis-inducing ligand; MRT, malignant rhabdoid tumour; PI, propidium iodide; FITC, fluorescein isothiocyanate.

caspase-8 from 4 h (Fig. 4D). However, in agreement with the cell death results, caspase-8 activation decreased from 4 to 48 h. By contrast, combination treatment resulted in enhanced caspase-8 cleavage compared with treatment with TRAIL alone from 4 h onwards (Fig. 4D). Bid, a target of caspase-8, was determined to be cleaved at 24 h in TRAIL-treated cells, with cleavage enhanced upon combination treatment (Fig. 4E). To determine if the generation of tBid was sufficient for MOMP, SMAC release into the cytosol was assessed from 1 to 24 h. SMAC is a pro-apoptotic protein usually resident within the mitochondria. However, upon MOMP, SMAC is released into the cytosol where it is free to carry out its proapoptotic functions by binding and inhibiting XIAP (33,56,57). Both TRAIL alone and combination

treatment resulted in SMAC release, with levels slightly enhanced at 24 h in combination-treated cells (Fig. 4F). Despite SMAC release into the cytosol, indicative of MOMP, caspase-9 cleavage fragments, which would be formed upon caspase-9 activation, were not detected using an antibody capable of detecting both full length and cleaved fragments (Fig. 4G). A further target of active caspase-8 is caspase-3. Cleavage of caspase-3 results in the generation of an inactive cleavage product of 19 kDa and active cleavage products of 17 and 15 kDa. Treatment with TRAIL alone resulted in generation of both active and inactive cleavage fragments from 4 h onwards (Fig. 4H). However, at later timepoints it was the inactive fragment which predominated. By contrast, combination treatment resulted in enhanced generation of

Table I. Synergism between TRAIL and embelin in BT12 and BT16 cells.

Cell line	Timepoint (h)	TRAIL (ng/ml)	Fa	CI value	Level of synergism
BT12	24	25	0.144	0.35798	+++ Synergism
		50	0.18	0.50820	+++ Synergism
		100	0.352	0.470532	+++ Synergism
	48	25	0.264	0.28718	++++ Strong Synergism
		50	0.434	0.16938	++++ Strong Synergism
		100	0.479	0.14939	++++ Strong Synergism
	72	25	0.39	0.43228	+++ Synergism
		50	0.538	0.33700	+++ Synergism
		100	0.607	0.31940	+++ Synergism
BT16	48	25	0.237	0.43825	+++ Synergism
		50	0.275	0.36781	+++ Synergism
		100	0.353	0.26640	++++ Strong Synergism
	72	25	0.3023	0.46910	+++ Synergism
		50	0.3527	0.35404	+++ Synergism
		100	0.3399	0.38200	+++ Synergism

MRT cells, BT12 and BT16, were treated with a range of concentrations of TRAIL with and without 10 μ M embelin. After the relevant treatment time, cells were harvested, fixed and stained with propidium iodide. Cells were analysed by flow cytometry and apoptotic cells were determined by quantification of the sub G1/G0 peaks. The mean of at least three independent experiments, each of which was done in triplicate, was entered into Compusyn. CI Values <1, =1 and >1 indicate synergistic, additive and antagonistic effects, respectively. TRAIL, TNF-related apoptosis-inducing ligand; CI, combination index.

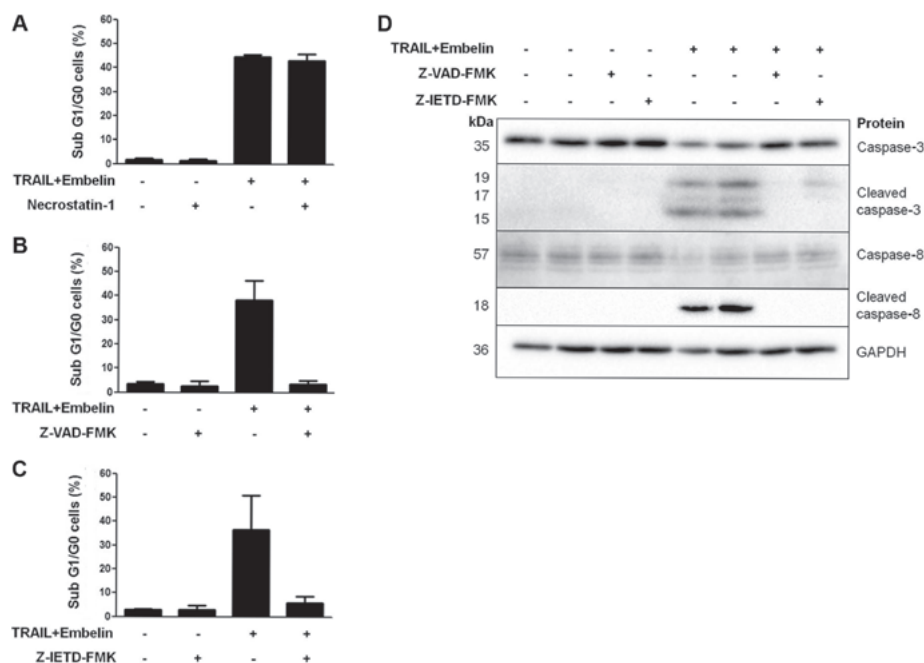


Figure 3. TRAIL and embelin induce cell death through the extrinsic apoptotic pathway in BT12 cells. (A) BT12 cells were pretreated with the receptor interacting threonine/serine kinase 1 inhibitor, necrostatin-1, (B) the general caspase inhibitor Z-VAD-FMK, or (C) the specific caspase-8 inhibitor, Z-IETD-FMK for 1 h. Cells were then treated with 50 ng/ml TRAIL in combination with 10 μ M embelin for 24 h and cell death was assessed via flow cytometric analysis of propidium iodide-stained cells and subsequent quantification of the sub G1/G0 peak. Values are mean \pm standard error of of two independent experiments, except for panel A where values are representative of three independent experiments. (D) Caspase inhibition was confirmed via western blotting. TRAIL, TNF-related apoptosis-inducing ligand.

active cleavage fragments over time, with active caspase-3 levels being higher compared with the TRAIL alone-treated cells from 4 h onwards (Fig. 4H). Caspase-3 activation was

also assessed in BT16 cells at 24 and 48 h. As per the BT12 cells, combination treatment resulted in enhanced generation of active caspase-3 over time (Fig. 4C).

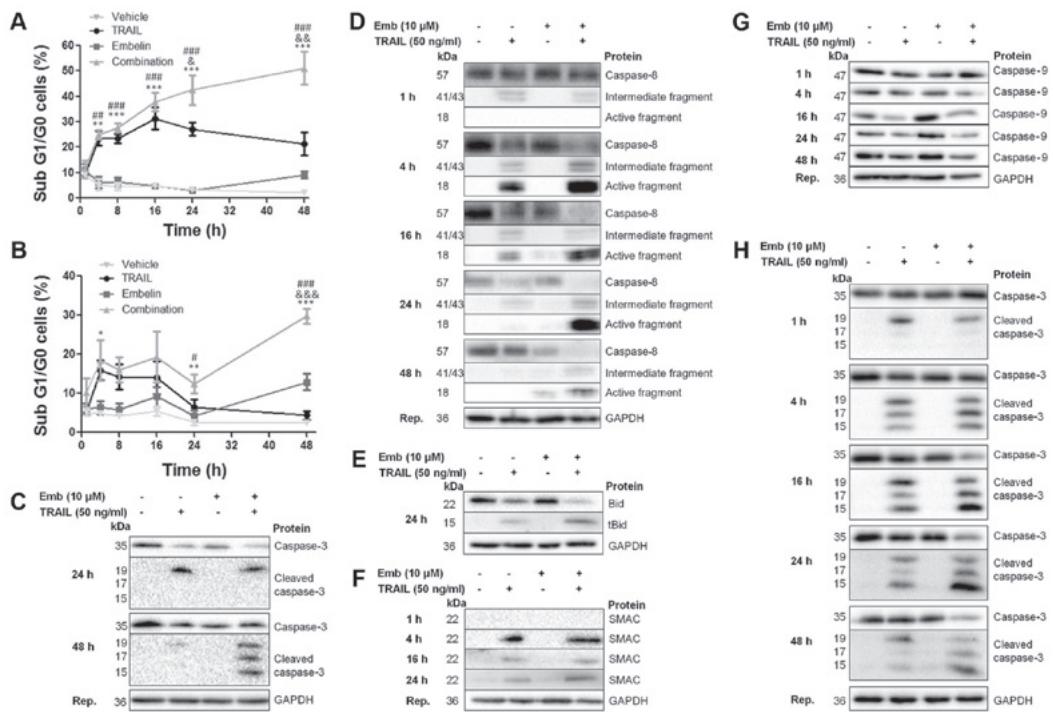


Figure 4. Embelin prevents the recovery of BT12 cells from TRAIL treatment with enhanced activation of the extrinsic apoptotic pathway over time. (A) BT12 and (B) BT16 cells were treated with 50 ng/ml TRAIL and 10 μ M embelin, both alone and in combination. Cell death was assessed at 1, 4, 8, 16, 24 and 48 h post-treatment via flow cytometric analysis of propidium iodide-stained cells and subsequent quantification of the sub G1/G0 peak. Values are mean \pm standard error of the mean of at least three independent experiments. * P <0.05, ** P <0.01 and *** P <0.001 vs vehicle; Δ P <0.05, $\Delta\Delta$ P <0.01 and $\Delta\Delta\Delta$ P <0.001 vs. TRAIL; and $\#$ P <0.05, $\#\#$ P <0.01 and $\#\#\#$ P <0.001 vs. embelin. (C) BT16 cells were treated with 50 ng/ml TRAIL and 10 μ M embelin, both alone and in combination. At 24 and 48 h post-treatment, expression of full length/cleaved caspase-3 was examined. (D) BT12 cells were treated with 50 ng/ml TRAIL and 10 μ M embelin, both alone and in combination. At 1, 8, 16, 24 and 48 h post-treatment, expression of full length/cleaved caspase-8, (G) caspase-9 and (H) full length/cleaved caspase-3 was assessed via western blotting. (E) Bid cleavage was examined at 24 h and (F) SMAC release into the cytosol at 1, 4, 16 and 24 h. Results are representative of at least three independent experiments, except those for Bid and tBid which are representative of two independent experiments. TRAIL, TNF-related apoptosis-inducing ligand; Bid, BH3 interacting domain death agonist; tBid, truncated Bid; SMAC, second mitochondria-derived activator of caspases.

Combination treatment results in decreased expression of antiapoptotic proteins. BT12 cells were treated with TRAIL and embelin, either alone or in combination, from 1 to 48 h and protein expression was analysed via western blotting. The expression levels of proteins which were implicated in sensitising the cells to apoptosis in the BT12 cells were then assessed in BT16 cells at 24 and 48 h. No differences in XIAP expression levels were observed following TRAIL or embelin treatment at any timepoint (Fig. 5A). Combination treatment resulted in slightly reduced expression at 24 and 48 h. XIAP expression in BT16 cells was similar (Fig. 6A). cIAP1 expression levels in BT12 cells were unaffected by any treatment at any timepoint (Fig. 5B). cIAP2 levels in BT12 cells were examined at 48 h only, due to technical limitations with the cIAP2 antibody, and were found to be slightly decreased following embelin or combination treatment (Fig. 5C). As aforementioned, livin exists as two splice variants, both of which can be cleaved by caspase-3, with the cleaved fragments suggested to be proapoptotic. In line with the cleaved caspase-3 data, treatment with TRAIL resulted in livin cleavage from 4 h in BT12 cells (Fig. 5D). However, by 48 h livin levels were comparable to vehicle, in accordance with the recovery of cells from treatment. By contrast, combination treatment resulted in increased livin cleavage compared with treatment with TRAIL alone from 4 h, with cleavage increasing in a time-dependent manner

(Fig. 5D). In BT12 cells, survivin levels were increased upon TRAIL treatment from 24 h (Fig. 5E). By contrast, treatment with embelin either alone or in combination with TRAIL resulted in reduced survivin expression from 16 h (Fig. 5E). Survivin levels in BT16 cells at 24 and 48 h were also decreased upon embelin or combination treatment (Fig. 6B). Expression levels of FLIP_L, an analog and inhibitor of caspase-8, were unaffected by TRAIL or embelin single treatment (Fig. 5F). However, combination treatment resulted in reduced FLIP_L expression from 16 h. By contrast, in BT16 cells, FLIP_L levels were unchanged (Fig. 6A). The anti-apoptotic Bcl-2 family member Mcl-1, has previously been demonstrated to be overexpressed in MRT. Mcl-1 levels were unaffected by TRAIL or embelin single treatment, but were found to be decreased from 4 h in combination-treated cells (Fig. 5G). Similarly, in BT16 cells, embelin as a single agent or in combination with TRAIL resulted in reduced Mcl-1 expression at 24 and 48 h (Fig. 6B). Densitometric analysis of the proteins examined in Figs. 5 and 6 have been included as supplemental material (Figs. S1 and S2).

Knockdown of XIAP enhances TRAIL-mediated apoptosis. As described above, TRAIL and embelin co-treatment did not result in downregulation of XIAP until 24 h in MRT cell lines, despite enhanced caspase activity from 4 h. However, the initial screening studies which identified embelin as an

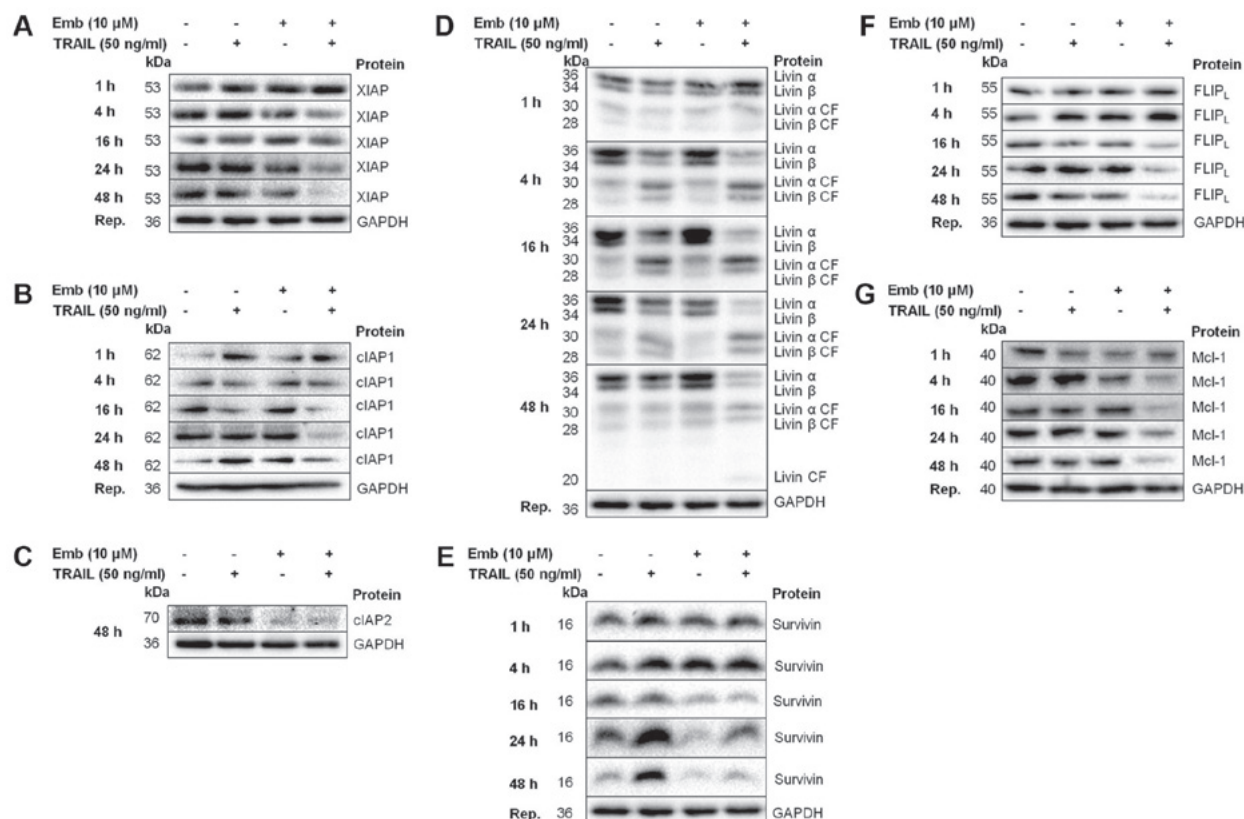


Figure 5. Decreased expression of antiapoptotic proteins, including early downregulation of survivin, FLIP_L and Mcl-1 accompanies combination treatment in BT12 cells. BT12 cells were treated with 50 ng/ml TRAIL and 10 μM embelin, both alone and in combination. At 1, 8, 16, 24 and 48 h post-treatment, expression of (A) XIAP, (B) cIAP1, (C) cIAP2 (examined only at 48 h), (D) livin, (E) survivin, (F) FLIP_L and (G) Mcl-1 was assessed via western blotting. Results are representative of at least three independent experiments. FLIP_L, FLICE-inhibitory protein long form; Mcl-1, myeloid leukaemia cell differentiation protein 1; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis; cIAP, cellular inhibitor of apoptosis protein.

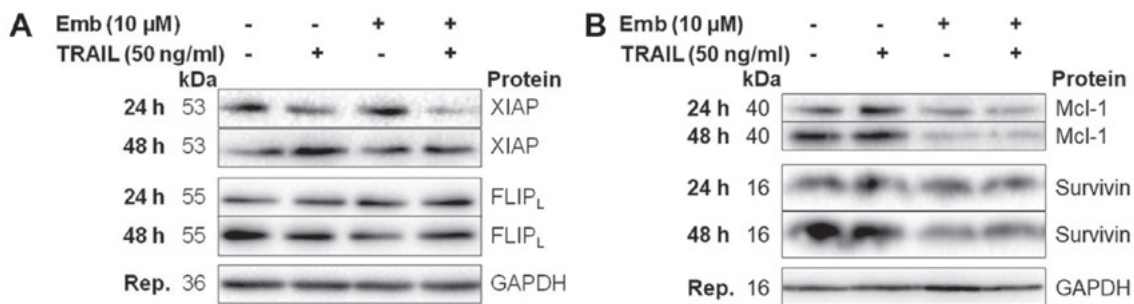


Figure 6. Embelin prevents the recovery of BT16 cells from TRAIL treatment with enhanced apoptosis induction, and decreased survivin and Mcl-1 expression over time. BT16 cells were treated with 50 ng/ml TRAIL and 10 μM embelin, both alone and in combination. At 24 and 48 h post-treatment expression of (A) XIAP and FLIP_L, and (B) survivin and Mcl-1 was assessed by western blotting. Results are representative of at least three independent experiments. TRAIL, TNF-related apoptosis-inducing ligand; Mcl-1, myeloid leukaemia cell differentiation protein 1; XIAP, X-linked inhibitor of apoptosis; FLIP_L, FLICE-inhibitory protein long form.

inhibitor of XIAP did not suggest degradation was necessary for inhibition (37). Given this and combined with previous reports of enhanced susceptibility to TRAIL upon XIAP knockdown, we next assessed if XIAP inhibition could account for the sensitisation of MRT cell lines to TRAIL upon embelin co-treatment. BT12 cells were treated with TRAIL in combination with XIAP siRNA or non-targeting siRNA and XIAP knockdown was confirmed via western blotting (Fig. 7A). While significant knockdown was achieved, low levels of XIAP expression were still detected (Fig. 7A). Despite

this, cells treated with XIAP siRNA exhibited significantly enhanced TRAIL-mediated cell death, in contrast to cells treated with non-targeting siRNA (Fig. 7B). It should be noted that this enhanced cell death was not as pronounced as with the embelin cotreatment (Figs. 2B and 4A). While the incomplete knockdown of XIAP may account for this, it is likely that XIAP inhibition via embelin only partially mediates the enhanced cell death. A complex multifactorial mechanism of cell death is supported by the reduced survivin, FLIP and Mcl-1 expression demonstrated upon cotreatment.

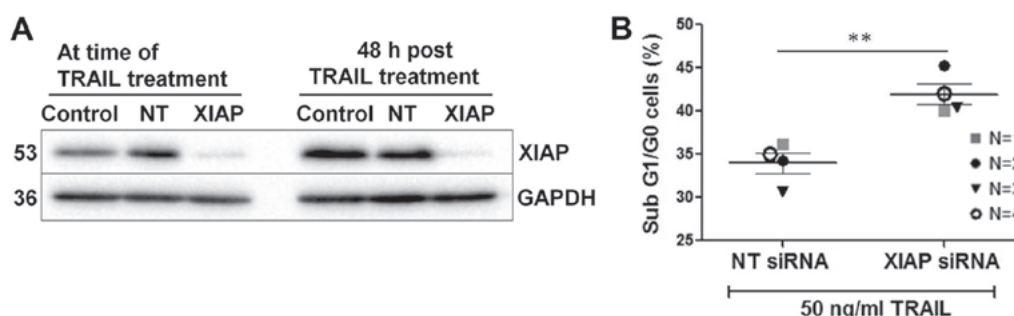


Figure 7. XIAP knockdown partially sensitises MRT cells to TRAIL treatment. BT12 cells were pretreated for 24 h with NT siRNA or XIAP siRNA. Cells were then treated with 50 ng/ml TRAIL for 48 h. (A) XIAP knockdown was confirmed by western blotting. (B) Cell death as assessed in the control and knockdown cells by flow cytometric analysis of propidium iodide staining and subsequent quantification of the sub G1/G0 peak. Values are mean \pm standard error of the mean of four independent experiments. ** $P < 0.01$. XIAP, X-linked inhibitor of apoptosis; MRT, malignant rhabdoid tumour; TRAIL, TNF-related apoptosis-inducing ligand; si, small interfering; NT, non-targeting.

Discussion

MRT is a highly aggressive paediatric tumour, for which there is no standardised treatment. Its diagnosis carries with it a poor prognosis, and given its early age of onset, aggressive treatment is hindered by associated toxicities. As such, there is an urgent need for the development of safe effective therapies. TRAIL has demonstrated efficacy in many cancer cell lines and in *in vivo* models as a cancer therapeutic with limited side effects (11). However, clinical trials with TRAIL have yielded disappointing outcomes, with resistance being a primary issue (15-19). In recent years, reports have demonstrated enhanced susceptibility of cancer cells to TRAIL upon cotreatment with the XIAP inhibitor embelin (47-54). The present study, therefore, assessed the efficacy of TRAIL and embelin cotreatment in MRT cell lines.

The present study is the first to report the expression levels of antiapoptotic IAP family members in a panel of MRT cell lines. Further studies are underway into IAP expression in rhabdoid tumour patient samples (data not shown). XIAP, cIAP1, cIAP2, livin and survivin have frequently been reported to be overexpressed in a broad range of tumour types, including acute myeloid leukaemia, renal cell carcinoma, neuroblastoma and hepatocellular carcinoma (20-28). To this end, SMAC mimetics, inhibitors of XIAP, cIAP1 and cIAP2, have been developed, with several of them currently in clinical trials. The paediatric preclinical testing program (PPTP) has previously assessed treatment with the SMAC mimetic L6161 in a panel of cancer cells, including two MRT cell lines (BT12 and CHLA-266 cells), with IC_{50} values $>10 \mu M$ obtained for both (58). Despite the lack of response to L6161 as a single agent in MRT reported by the PPTP, it would be interesting to evaluate its effects in combination with other cancer therapeutics, considering previous reports on its sensitising ability (59-62). In addition, YM155 is an agent which suppresses survivin gene promotion and is currently in clinical trials as a possible chemotherapeutic sensitising agent (63,64). Given the demonstrated IAP expression reported in the present study, there is a rationale for the preclinical evaluation of a broad range of IAP targeting agents, such as SMAC mimetics, YM155 and the XIAP inhibitor embelin in MRT, both alone and in combination with various cancer therapeutics.

The current study examined the efficacy of embelin as a single agent in a panel of MRT cell lines. Embelin was found to elicit a partial decrease in viability at concentrations up to $50 \mu M$ with IC_{50} values of 13.06, 14.55 and $47.14 \mu M$ obtained for BT12, BT16 and G401 cells, respectively, upon 72 h treatment. While this is the first report on embelin treatment in MRT cell lines, its anticancer effects have previously been demonstrated in numerous other malignancies. IC_{50} values of 65, 28, 56 and $60 \mu M$ have been reported for HCT-116 (colon carcinoma), MCF-7 (breast cancer), MIAPaCa-2 (pancreas ductal carcinoma) and PC-3 (prostate cancer) cells, respectively, upon 48 h treatment (65). IC_{50} values $<6 \mu M$ upon 72 h treatment have been reported in MCF-7 and MDA-MB-231 breast cancer cells (40). This places MRT within the mid-range of response to embelin as a single agent. However, the modest decrease in viability of MRT cells in response to embelin suggests that its clinical potential may lie in its use as a combinatorial rather than a single agent.

To date, the only report on the treatment of MRT with TRAIL was by Yoshida *et al* (55), which reported sensitivity in 3 out of 6 tested cell lines upon 18 h treatment. The present study has reported on a further 3 cell lines, with resistance detected in all 3 upon 72 h treatment. Notably, when tested between 1 and 48 h, MRT cells initially responded to TRAIL treatment, but recovery was observed after 16 h. This was confirmed by cell death and caspase analysis across a broad range of timepoints. The reasons for this recovery are not entirely obvious. It is possible that the surviving population increased proliferation upon TRAIL treatment mediating recovery. In support of this, previous reports have demonstrated nuclear factor (NF)- κB and extracellular signal-regulated kinase (ERK) activation upon TRAIL stimulation (66-70). However, engagement of these pathways was not detected at a range of early and later timepoints in the present study (data not shown). Additionally, it is also possible that the cells became resistant to TRAIL over time. In line with this, studies by Flusberg *et al* (71) demonstrated that MCF10A and HCT116 cancer cells treated with TRAIL developed reversible resistance (71). While their surviving cells exhibited enhanced NF- κB signalling, this was found to be dispensable for resistance. The experimental design of the current study was clearly different to that described by Flusberg *et al*, which investigated exclusively the development of resistance in a survivor population. Yet, both

suggest that cancer cells can develop resistance to TRAIL treatment independently of enhanced proliferative signalling.

Of note, the recovery of cells from TRAIL was inhibited by embelin co-treatment which resulted in synergistically enhanced cell death and caspase activity in BT12 and BT16 cells. The present results are supported by the literature where embelin has been reported to enhance TRAIL-mediated cell death in acute myeloid leukaemia, malignant glioma, pancreatic, inflammatory breast, and non-small cell lung cancer cell lines (47-54). Previous reports have differed in their proposed mechanism with changes in expression of anti-apoptotic proteins FLIP, XIAP, survivin and Bcl-2 family members, alongside altered regulation of the NF- κ B and ERK pathways among the factors implicated. The mechanism may thus be concentration or cell type-specific. In MRT, at the doses used, enhanced cell death was accompanied by reduced expression of Mcl-1 from 4 h and reduced FLIP_L and survivin levels from 16 h. No changes in XIAP expression were noted until relatively late timepoints, by which time caspase-3 activity was already enhanced. While this may suggest that the enhanced cell death observed did not rely upon embelin-mediated XIAP inhibition, it should be noted that the original screening studies which identified embelin as an inhibitor of XIAP did not suggest protein degradation to be necessary for inhibition (37). In the present study, XIAP knockdown, via siRNA, significantly enhanced TRAIL-mediated cell death. This was further supported by analysis of the cleaved caspase-3 fragments, with the inactive fragment predominating in TRAIL-treated cells in contrast to further processing evident upon combination treatment. This is in line with results previously reported, where enhanced SMAC release resulted in increased XIAP inhibition and completed caspase-3 processing (72,73). In addition, livin cleavage, likely mediated by caspase-3 was observed in correlation with increased caspase activity. Livin has been demonstrated to bind SMAC and is suggested to inhibit its XIAP inhibitory activity (74). Livin cleavage may thus facilitate enhanced SMAC inhibition of XIAP. Furthermore, the cleaved livin fragments generated have previously been demonstrated to be proapoptotic (75,76). The present results suggest that the enhanced cell death observed upon embelin cotreatment is likely multifactorial, with reduced expression of the antiapoptotic proteins survivin, FLIP_L and Mcl-1 being combined with livin cleavage and XIAP inhibition, to most likely account for the synergism observed.

In conclusion, the current study has highlighted the antiapoptotic IAP family members as possible targets for the treatment of MRT and suggests that further preclinical evaluation of their inhibition is warranted. Furthermore, the present results suggest that the XIAP inhibitor embelin may be combined with TRAIL as a potential novel treatment strategy for MRT, an aggressive malignancy desperately in need of new therapeutic options.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Author's contributions

DMZ and MJO'S decided upon this line of study. Experiments were planned and designed by DMZ and RC. Experiments were executed by RC, with KS and LE performing initial optimisation experiments and repeating a number of experiments. RC prepared and labelled all figures, executed experiments for Figs. 2B-7 independently and performed repeats for Figs. 1 and 2A. KS performed a number of replicates for Fig. 2A and optimisation experiments for Fig. 2B. LE performed optimisation experiments for Figs. 1 and 2, and a number of replicates for Fig. 1A. The manuscript was written by RC and reviewed by DMZ and MJO'S. DMZ and MJO'S reviewed all figures for publication. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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