Inhibition of γ-secretase activity synergistically enhances tumour necrosis factor-related apoptosis-inducing ligand induced apoptosis in T-cell acute lymphoblastic leukemia cells via upregulation of death receptor 5

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Abstract. T-cell acute lymphoblastic leukemia (T-ALL) is a rare and aggressive hematopoietic malignancy prone to relapse and drug resistance. Half of all T-ALL patients exhibit mutations in Notch1, which leads to aberrant Notch1 associated signaling cascades. Notch1 activation is mediated by the γ-secretase cleavage of the Notch1 receptor into the active intracellular domain of Notch1 (NCID). Clinical trials of γ-secretase small molecule inhibitors (GSIs) as single agents for the treatment of T-ALL have been unsuccessful. The present study demonstrated, using immunofluorescence and western blotting, that blocking γ-secretase activity in T-ALL cells with *N*-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl] glycine-1,1-dimethylethyl ester (DAPT) downregulated NCID and upregulated the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5 (DR5). Upregulation of DR5 restored the sensitivity of T-ALL cells to TRAIL. Combination index revealed that the combined treatment of DAPT and TRAIL synergistically enhanced apoptosis compared with treatment with either drug alone. TRAIL combined with the clinically evaluated γ-secretase inhibitor 3-[(1r, 4s)-4-(4-chlorophenylsulfonyl)-4-(2, 5-difluorophenyl) cyclohexyl] propanoic acid (MK-0752) also significantly enhanced TRAIL-induced cell death compared with either drug alone. DAPT/TRAIL apoptotic synergy was dependent on the extrinsic apoptotic pathway and was associated with a decrease in BH3 interacting-domain death agonist and x-linked inhibitor of apoptosis. In conclusion, γ-secretase inhibition represents a potential therapeutic strategy to overcome TRAIL resistance for the treatment of T-ALL.

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Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; NCID, intracellular domain of Notch1; GSIs, γ-secretase inhibitors; DAPT, *N*-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl]glycine-1, 1-dimethylethyl ester; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; MK-0752, 3-[(1r, 4s)-4-(4-chlorophenylsulfonyl)-4-(2,5-difluorophenyl) cyclohexyl] propanoic acid

Key words: γ-secretase inhibitors, tumour necrosis-related apoptosis-inducing ligand, Notch1, T-cell acute lymphoblastic leukemia, synergy and death receptor 5

Introduction

T-acute lymphoblastic leukemia (T-ALL) is characterised by the infiltration of bone marrow with immature lymphoblasts that express T-cell immunophenotypic markers (1). T-ALL accounts for 10-15% of pediatric and 25% of adult ALL (2). Various genetic mutations have contributed to the pathogenesis of T-ALL. Constitutive activation of Notch1, attributed to various mutations, has been identified in over half of T-ALL patients (3,4). Notch1 is a transmembrane protein frequently associated with various oncogenic properties, including the promotion of cell survival, angiogenesis and resistance to current chemotherapies; therefore, it has attracted interest as a novel target for cancer therapy (5,6). γ-secretase is a membrane-embedded aspartyl protease responsible for the cleavage of several transmembrane proteins, including Notch and amyloid precursor protein (APP). The γ-secretase complex is required for effective Notch signaling (7). Small molecule γ-secretase inhibitors (GSIs) initially began clinical evaluation as a novel treatment for Alzheimer's disease, since they block the accumulation of APP (8). Subsequently, GSIs have been clinically evaluated as Notch targeting anti-cancer agents. Clinically evaluated GSIs include semagacestat (LY450139), RO4929097, avagacestat (BMS-708163), PF-03084014 and 3-[(1r, 4s)-4-(4-chloroph enylsulfonyl)-4-(2,5-difluorophenyl) cyclohexyl] propanoic acid (MK-0752) (data concerning these trials are available at clinicaltrials.gov.). Initial results from preclinical evaluations and clinical trials of GSIs have been disappointing, due to the lack of anti-tumour activity, toxicity and resistance (1,9,10). However, recent phase I clinical trials with the novel GSI PF-03084014, exhibited one complete response and several partial responses in advanced cancers (11); therefore, warranting continued clinical evaluation.

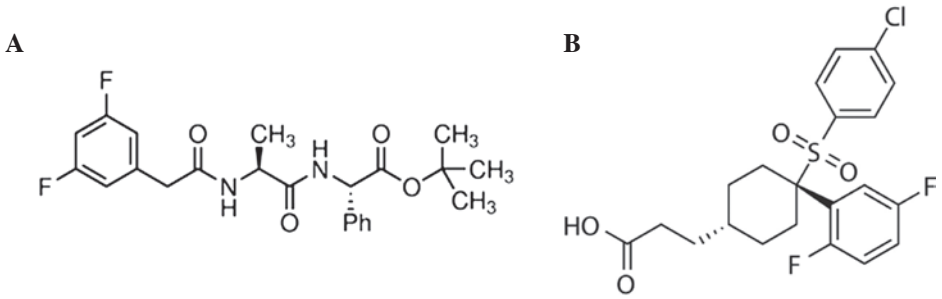

Figure 1. Chemical structure of the two γ-secretase inhibitors used in the present study: (A) N-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl] glycine-1, 1-dimethylethyl ester, also known at DAPT, and (B) 3-[(1r, 4s)-4-(4-chlorophenyl-sulfonyl)-4-(2,5-difluorophenyl) cyclohexyl] propanoic acid, also known as MK-0752.

Combination therapies may overcome problems associated with drug toxicity and resistance. The use of combination regimes have significantly improved the outcome of pediatric patients with T-ALL (12). The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-receptor system has attracted immense attention as a tumour-selective agent in terms of investment and clinical evaluation. The TRAIL death receptor (DR) 4 and DR5 are exclusively expressed on tumour cells (13). Ligands, including TRAIL, which bind to TRAIL receptors, may selectively activate the death signal in tumour cells (14). However as with numerous therapies, resistance has been identified for this type of therapy; endogenous low expression of DR4 and DR5 is responsible for inherent TRAIL resistance in T-ALL patients (15). Since, therapeutic agents that upregulate the expression of DR4 and DR5 may restore sensitivity to TRAIL-induced apoptosis, the present study investigated the potential of GSI and TRAIL combination treatment to synergistically induce apoptosis in T-ALL cells by restoring the DR-mediated pathway of apoptosis.

Materials and methods

Reagents. Reagents were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated, and tissue culture vessels were obtained from Greiner Bio-One GmbH (Frickenhausen, Germany). Recombinant human TRAIL (amino acid, 114-281; catalog no., 616374) and Caspase-8 Inhibitor z-IETD-FMK were purchased from Merck Millipore (Nottingham, UK). TRAIL was supplied in a buffer containing 500 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 2 mM KH_2PO_4, 1 mM dithiothreitol (DTT), ≤10% glycerol (1.2 mg/ml), and stored in aliquots at -70˚C. *N*-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl] glycine-1,1-dimethylethyl ester (DAPT; catalog no., D5942) was purchased from Sigma-Aldrich. MK-0752 (catalog no., S2660) was purchased from Selleckchem (Houston, TX, USA). Chemical structures of DAPT and MK-0752 are shown in Fig. 1. Caspase inhibitors and MK-0752 were dissolved in dimethyl sulfoxide (DMSO) and DAPT was dissolved in ethanol and stored at -20˚C. The final concentration of DMSO and ethanol did not exceed 0.1% (v/v) and 0.2% (v/v), respectively.

Cell culture. T-ALL Jurkat cells were obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The cells were cultured in RPMI-1640 containing GlutaMAX-1 supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37˚C under 5% CO_2. All cell culture materials were purchased from Gibco® (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Flow cytometric analysis of DNA content. T-ALL cells were treated with TRAIL (20, 40, 100 and 200 ng/ml), DAPT (0, 10, 20 and 50 μM) or MK-0752 (50 μM), or a combination of TRAIL and one GSI. The cells were also treated with 20 μM z-IETD-FMK in later experiments. Subsequent to 48 h, the cells were pelleted by centrifugation at 800 x g and fixed with ice-cold 70% ethanol with phosphate-buffered solution (PBS) overnight at -20˚C. Subsequently, the cells were treated with RNase A (0.5 mg/ml) and stained with propidium iodide (0.15 mg/ml). DNA content was measured using the BD Accuri C6 Flow Cytometer and analysed with associated software (BD Biosciences, Inc., Franklin Lakes, NJ, USA).

Western blotting. T-ALL cells were treated with 20 ng/ml TRAIL, 50 μM DAPT or a combination of the two. Following treatment, the cells were washed and re-suspended in PBS, lysed by addition of an equal volume of 2X Laemmli buffer [1X = 30 mM Tris base (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol] and briefly sonicated. Protein content was measured using Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). DTT was added at a final concentration of 50 mM following the BCA assay. Proteins were denatured by boiling at 100˚C for 5 min. Protein samples were resolved on 4-20% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) followed by transfer to Immobilon-P PVDF membranes (EMD Millipore, Billerica, MA, USA). The membranes were stained with 0.1% Ponceau S (w/v) in 5% acetic acid to ensure equal transfer. Only membranes exhibiting equal loading and even transfer were used for further analysis. The membranes were briefly washed in Tris-buffered saline (pH 7.7) and 0.05% Tween-20 (TBS-T) and blocked at room temperature in blocking buffer [5% (w/v) dried milk dissolved in TBS-T]. After 1 h, the membranes were probed with primary antibodies overnight at 4˚C. Rabbit monoclonal anti-cleaved Notch1 (Val1744; clone, D3B8; catalog no., 4147; 1:500), rabbit monoclonal anti-x-linked inhibitor of apoptosis (XIAP; clone, 3B6; catalog no., 2045; 1:1,000), rabbit polyclonal anti-BH3 interacting-domain death agonist (Bid; catalog no., 2002; 1:1,000) and mouse monoclonal anti-caspase-8 (clone, 1C12; catalog no., 9746; 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse

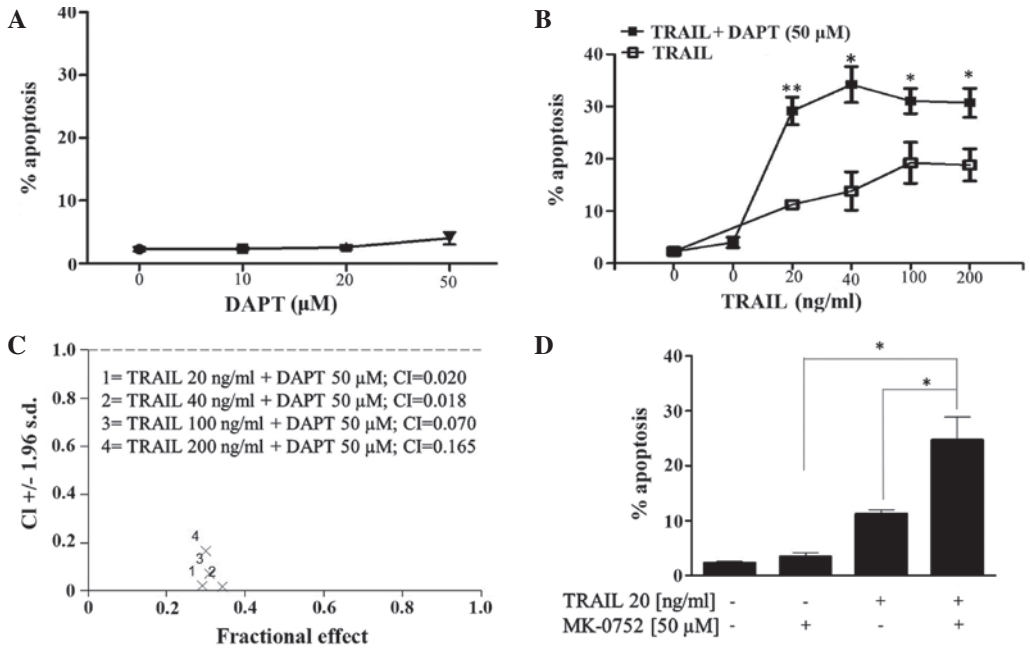

Figure 2. Inhibition of γ-secretase enhances TRAIL induced apoptosis in human T-cell acute lymphoblastic leukemia Jurkat cells. Jurkat cells were treated with (A) DAPT (0, 10, 20 and 50 μM), (B and C) TRAIL (20, 40, 100 and 200 ng/ml), DAPT (50 μM) or a combination of the two; and (D) TRAIL (20 ng/ml), MK-0752 (50 μM) or a combination of the two for 48 h. The first point on (B) refers to control vehicle-treated cells. The percentage of apoptosis (pre-G1 cell population) was detected by flow cytometric analysis of propidium iodide stained cells. Vehicle control for (A-C), 0.2% (v/v) ethanol; (D), 0.1% (v/v) dimethyl sulfoxide. Data shown are the mean ± standard error of the mean of ≥3 independent experiments. *P≤0.05, **P≤0.01. TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; DAPT, N-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester; MK-0752, 3-((1r, 4s)-4-(4-chlorophenyl sulfonyl)-4-(2,5-difluorophenyl) cyclohexyl) propanoic acid; CI, combination index.

monoclonal anti-caspase-3 (clone, AM1.4.1-1B; catalog no., AM39; 1:1,000) and anti-α-tubulin (clone, DM1A; catalog no., CP0; 1:2,500) antibodies were obtained from Merck Millipore. Subsequently, the membranes were washed three times in TBS-T and probed for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse (cat. no. W4021) or anti-rabbit (cat. no. W4011) secondary antibodies (1:1,000; Promega Corporation, Madison, WI, USA) secondary antibodies. The membranes were then exposed to Clarity™ ECL Western Blotting Substrate (Bio-Rad Laboratories Ltd.) for 2 min and images were detected using the Bio-Rad GelDoc XR system.

Flow cytometric analysis of DR5. T-ALL cells were treated with 50 μM DAPT. Following treatment, the cells were washed in ice cold PBS and re-suspended in PBS supplemented with 0.1% bovine serum albumin. DR5 cell surface expression was analysed using fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-TRAIL-R2 (clone, YM366; catalog no., MAB10418; Merck Millipore) for DR5 analysis. FITC conjugated IgG was used as an isotype control. The cells ($1x10^5$) were incubated on ice with 10 μg/ml antibody for 45 min, washed in ice-cold PBS, re-suspended in FACS buffer and analysed using the Accuri C6 Flow Cytometer. Values obtained for the isotype control were subtracted from the anti-TRAIL-R2 values and plotted accordingly.

Calculation of combination index (CI). Drug interactions were analysed using CalcuSyn 2.0 software (BIOSOFT, Cambridge, UK), as described by Bijnsdorp *et al* (16). CIs were extrapolated from drug cytotoxicity values derived from the quantification of the sub-G1 (apoptotic) population of cells. CalcuSyn software employs the Chou-Talalay method for drug combination, which is based on the median-effect equation, derived from the mass-action law principle (17). The resulting CI values depict synergy (<1), additive effect (=1) and antagonism (>1).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard error of the mean. Comparisons between specific groups within data sets were assessed using two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of GSI and TRAIL combinations in human T-ALL cells. The effect of the GSI DAPT alone and in combination with TRAIL in Jurkat T-ALL cells was determined by flow cytometric evaluation of the pre-G1 peak. The present results revealed that Jurkat cells are relatively resistant to DAPT up to concentrations of 50 μM (Fig. 2A). Combining DAPT with TRAIL (20-200 ng/ml) increased TRAIL-induced apoptosis in Jurkat cells (Fig. 2B). CI was used to determine synergism of DAPT and TRAIL combinations. Fig. 2C revealed that CI values ranged between 0.018 and 0.165, which is indicative of clear synergy. Furthermore, the second GSI MK-0752, which is currently under clinical evaluation for various cancer types (details at clinicaltrials.gov), also significantly enhanced TRAIL-induced apoptosis in T-ALL cells (Fig. 2D).

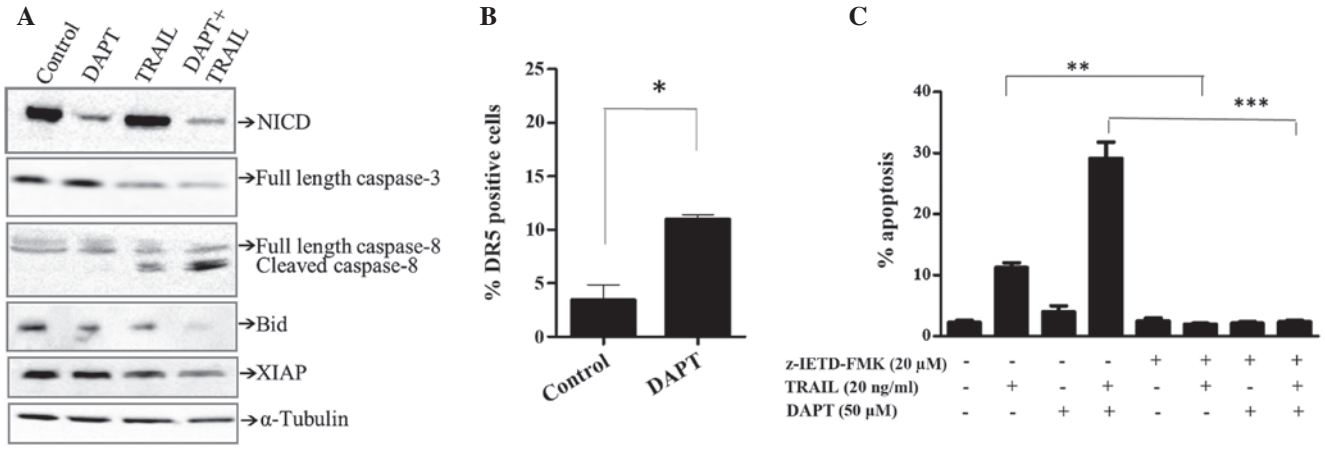

Figure 3. Downregulation of XIAP together with cleavage of caspase-8 and Bid are associated with DAPT/TRAIL apoptotic synergy. Jurkat cells were treated for 48 h in the presence of TRAIL (20 ng/ml), DAPT (50 μM) or a combination of the two. (A) Expression of various proteins was determined by western blotting. Equal loading was confirmed by α-tubulin. (B) Cell surface expression of DR5 was determined using flow cytometric analysis of cells stained with fluorescein isothiocyanate-conjugated anti-TRAIL-R2. (C) Jurkat cells were pre-treated for 1 h with the caspase-8 inhibitor z-IETD-FMK (20 μM) or control [0.1% (v/v) dimethyl sulfoxide] then treated with TRAIL, DAPT or a combination of the two. The percentage of apoptotic cells was detected by flow cytometric analysis of propidium iodide stained cells and quantification of the pre-G1 peak. Results are representative of ≥3 independent experiments. *P<0.05, **P≤0.01, ***P≤0.001. XIAP, x-linked inhibitor of apoptosis; Bid, BH3 interacting-domain death agonist; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; DAPT, N-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester; NCID, intracellular domain of Notch1; DR, death receptor.

Effects of GSI and TRAIL combinations on the expression of activated Notch and markers of apoptosis. Notch signaling is governed by the generation of the intracellular domain of Notch1 (NCID) by γ-secretase, which translocates to the nucleus and activates target genes. As expected, inhibiting γ-secretase activity with DAPT (50 μM) reduced the levels of NCID, as demonstrated by western blotting (Fig. 3A). TRAIL alone (20 ng/ml) did not alter the expression of NCID. However, DAPT-mediated inhibition of NCID formation was maintained in the presence of TRAIL. DAPT-mediated enhancement of TRAIL-induced apoptosis appeared to involve the extrinsic pathway, since activation of caspase-8 and cleavage of the caspase-8 substrate Bid was observed by western blotting (Fig. 3A). By contrast, the effects on caspase-3 were minimum (Fig. 3A). In addition, DAPT potentiated TRAIL-induced reduction of XIAP, which is a known inhibitor of the intrinsic and extrinsic apoptosis pathway (Fig. 3A).

TRAIL activates apoptosis by binding to its relevant receptors, DR4 and DR5. Since DR5 is the predominant DR expressed in Jurkat cells, the effect of DAPT on DR5 was determined by the present study. As shown in Fig. 3B, DAPT (50 μM) significantly increased the expression of DR5 in Jurkat cells. Subsequently, the present study determined whether caspase-8 was essential for TRAIL/DAPT apoptotic synergy. The results obtained in Fig. 3C demonstrate that apoptosis induced by TRAIL alone and in combination with DAPT was completely inhibited by the caspase-8 inhibitor z-IETD-FMK, suggesting that caspase-dependent apoptosis was mediated by the extrinsic apoptotic pathway.

Discussion

Initial enthusiasm afforded by oncologists towards GSIs has dwindled, due to inherent tumour resistance to Notch-targeting therapies. Resistance to established and emerging chemotherapeutics, be it acquired or inherent, is an evolving paradigm posing a significant challenge to clinicians. Once the mechanism of resistance is established, rational therapeutic drug combinations may subsequently be designed and pre-clinically evaluated for potential therapeutic efficacy. T-ALL is a highly aggressive hematopoietic malignancy prone to relapse and resistance. Documented mechanisms of resistance include a low expression of DRs (DR4 and DR5) (15) and aberrations involving the T cell receptor, Notch1, HOXA cluster, tyrosine kinases, cyclin-dependent kinase inhibitor 2A locus and LIM domain only 2 proteins (2,18). The present study targeted two such resistance mechanisms, low expression of DR5 and Notch1, using two GSIs.

Various GSIs have been clinically evaluated as bespoke anti-cancer agents targeting the γ-secretase-generated Notch cleavage product NCID, which is associated with various malignancies (19). Initial clinical outcomes of GSI monotherapy with R04929097 fell short of clinical expectations (20,21). Combination studies incorporating GSIs with established anti-cancer treatments, including radiation; Temsirolimus, an inhibitor of mammalian target of rapamycin (20); capecitabine, a fluorouracil pro-drug (22); bicalutamide, an androgen antagonist; letrozole, a nonsteroidal aromatase inhibitor; temozolomide, an alkylating agent; vismodegib, an inhibitor of the hedgehog pathway and ABC transporters; tamoxifen, an antiestrogen; erlotinib hydrochloride, an inhibitor of epidermal growth factor receptor tyrosine kinase; gemcitabine hydrochloride, an antimetabolite (23); vinblastine and docetaxel, microtubule targeting agents; Cisplatin, a DNA targeting agent; and cediranib maleate, a vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor (24) (details of not referenced drugs are available from clinicaltrials.gov), have been evaluated or are currently undergoing evaluation for therapeutic efficacy. For certain trials with combination therapies, stable disease

was the best outcome (23-25), whilst other studies noted a partial clinical response (22). Pre-clinical research continues to aspire to identify suitable anti-cancer agents to combine with GSIs, with the aim of further improving clinical outcome.

As with GSIs, the TRAIL-DR ligand system primarily presents as a promising tumour selective anti-cancer strategy. However, TRAIL monotherapy has also succumbed to inevitable tumour resistance. A previous study revealed that there was therapeutic efficacy in combining GSI-I (Z-LLNle-CHO) with TRAIL in breast carcinoma-derived cells (26); Portanova *et al* (26) demonstrated that inhibition of Notch may cooperate with TRAIL to induce apoptosis in breast cancer cells.

The current study extends the pre-clinical evaluation of TRAIL and GSIs as a possible therapy for the treatment of T-ALL. The present results revealed a clear apoptotic synergy in T-ALL cells when the GSI DAPT and TRAIL were administered as a combination, as opposed to treatment with either drug alone. A similar finding was observed when TRAIL was administered with the novel GSI MK-0752. TRAIL interacts with its receptors DR4 and DR5 and activates apoptosis via the extrinsic apoptosis pathway. Specifically, binding of TRAIL to DR4 or DR5 leads to the formation of the death-inducing complex and recruitment of the adaptor molecule Fas-associated protein with death domain and subsequent activation of caspase-8 (27). The present study reports that the GSI DAPT increases the levels of DR5 in Jurkat cells and re-sensitises the cells to TRAIL-induced apoptosis. These findings are in agreement with a previous study that revealed that GSI-I increased the levels of DR4 and DR5 in breast cancer MDA-MB-231 cells (26). By contrast to these findings, ectopic expression of NCID sensitised hepatocellular carcinoma cells to TRAIL-induced apoptosis by upregulating p53-dependent DR5 expression (28). However, a more recent study demonstrated that Notch4, but not Notch1, confers susceptibility to TRAIL-induced apoptosis in breast cancer cells (29). Taken together, these findings suggest that outcomes of TRAIL and Notch-based combination strategies may be dependent on the cell type and the Notch isoform expression profile, thus highlighting the requirement for additional pre-clinical studies in other malignancies.

TRAIL induces apoptosis by binding to DR4 and DR5 and initiating the extrinsic pathway via activation of caspase-8. In the present study, inhibition of caspase-8 using a selective inhibitor completely blocked TRAIL and DAPT/TRAIL-induced apoptosis; therefore confirming that DAPT enhances TRAIL-induced apoptosis by activation of the extrinsic apoptotic pathway. Caspase-8 initiates apoptosis by cleaving/activating caspase-3 or by cleaving Bid into truncated (t)Bid. Bid was identified as the preferred substrate of caspase-8 *in vitro* (30). It was previously reported that TRAIL induces the formation of an active caspase-8/tBid complex on the surface of the mitochondria, and thus activates the mitochondrial apoptotic pathway (31). In the present study, downregulation of Bid was more pronounced compared with caspase-3 during DAPT/TRAIL apoptotic synergy, suggesting a preference for Bid in the DAPT/TRAIL apoptotic pathway. A previous study has revealed that Bid is required for stage II cleavage of capase-3 (32). This suggests that Bid is the preferred substrate of caspase-8 and is required for the complete cleavage and activation of caspase-3. Bid was previously defined as the mediator of apoptotic synergy induced by TRAIL/etoposide combinations (33). Furthermore, ectopic expression of Bid sensitised prostate cancer cells to TRAIL-induced apoptosis (34). Taken together, these findings highlight a role for Bid in apoptosis promoted by TRAIL-based therapies. The anti-apoptotic protein XIAP inhibits mitochondrial amplification of the extrinsic pathway by binding to and inhibiting caspase-3, -7 and -9 (35). XIAP is inhibited by Smac or Bid dependent cleavage (32,36). Concomitant downregulation of XIAP during DAPT/TRAIL induced apoptosis, as observed in the present study, may be a Bid-dependent process. It is also possible that downregulation of XIAP may lead to the activation of caspase-3 at later time points, resulting in the subsequent amplification of the apoptotic signal by the intrinsic pathway.

In the present study, DAPT alone caused a clear decrease in NCID without inducing a significant amount of cell death. This finding is in agreement with other studies demonstrating that exposure of cells expressing Notch to GSI does not necessary result in cell death (37). The present study demonstrates that TRAIL maintains GSI-induced downregulation of NCID whilst synergistically inducing apoptosis. Therefore, it may be suggested that combining GSIs with other agents that trigger cell death independently of Notch whilst maintaining GSI-induced downregulation of Notch may prove to be a more successful method of treating Notch driven malignancies. In conclusion, the present study demonstrated that inhibition of γ-secretase presents a valid strategy to overcome TRAIL resistance in T-ALL cells.

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