TRAIL signals to apoptosis in CLL cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2.

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Running Title: Cross-linking facilitates signaling through TRAIL-R2

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Summary

TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, which is being developed as an anti-tumour agent due to its selective toxicity to tumour cells, induces apoptosis by binding to two membrane-bound receptors, TRAIL-R1 and TRAIL-R2. Clinical trials have been initiated with various preparations of TRAIL as well as agonistic mAbs to TRAIL-R1 and TRAIL-R2. Previously we reported that prior treatment of primary chronic lymphocytic leukaemia (CLL) cells with histone deacetylase inhibitors (HDACi) was required to sensitize CLL cells to TRAIL and using various receptor-selective TRAIL mutant ligands we demonstrated that CLL cells signalled to apoptosis primarily through TRAIL-R1. Some, but not all, agonistic TRAIL-receptor antibodies require cross-linking in order to induce apoptosis. We now demonstrate that CLL cells can signal to apoptosis through the TRAIL-R2 receptor, but only after cross-linking of the agonistic TRAIL-R2 antibodies, LBY135 and lexatumumab (HGS-ETR2). In contrast, signalling through TRAIL-R1 by receptor-selective ligands or certain agonistic antibodies, such as mapatumumab (HGS-ETR1) occurs in the absence of cross-linking. These results further highlight important differences in apoptotic signalling triggered through TRAIL-R1 and TRAIL-R2 in primary tumour cells. Such information is clearly important for the rational optimisation of TRAIL therapy in primary lymphoid malignancies, such as CLL. (200 words)

Keywords: TRAIL, apoptosis, chronic lymphocytic leukaemia (CLL), Cross-linking, TRAIL-R-antibodies.
Introduction

Chronic lymphocytic leukaemia (CLL) is often considered a disease of failed apoptosis. Apoptosis is a major form of cell death, used to remove unwanted or excess cells. Two major pathways of apoptosis have been described that ultimately result in the activation of a family of caspases that are responsible for the biochemical and morphological changes associated with apoptosis. The intrinsic pathway involves initial mitochondrial perturbation resulting from cellular stress or cytotoxic insults whereas the extrinsic pathway is triggered by activation of death receptors of the TNF family (Bratton, et al 2000, Sun, et al 1999). Ligation of death receptors, such as CD95 (Fas/Apo1), TNF receptor-1 (TNF-R1) and the TNF-related apoptosis-inducing ligand (TRAIL) receptors-1/-2 (TRAIL-R1/R2), by their cognate ligands or agonistic antibodies results in receptor aggregation and formation of a death-inducing signalling complex (DISC) resulting in activation of caspase-8, as the apical caspase (Ashkenazi and Dixit 1998, Peter and Krammer 2003). TRAIL is being developed as an anti-tumour agent as it induces apoptosis in a wide range of tumour cell lines but not in most normal cells and in contrast to other members of the TNF family, such as TNF or CD95L, does not appear to cause systemic inflammatory syndrome or liver toxicity, although there has been some controversy concerning its potential hepatotoxicity (Ashkenazi 2002, Hao, et al 2004, Koschny, et al 2007). TRAIL induces apoptosis by binding to two membrane-bound receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2) (Ashkenazi 2002). We and others have shown that primary cells, from patients with CLL and B-cell non-Hodgkin’s lymphoma, are universally resistant to TRAIL-induced apoptosis (MacFarlane, et al 2002, Snell, et al 1997). This resistance may be overcome by prior treatment with a histone deacetylase inhibitor (HDACi) that acts by facilitating increased formation of the TRAIL DISC (Inoue, et al 2004). Inhibition of HDAC1 and HDAC2 seems to be required for sensitization to TRAIL (Inoue, et al 2006). Based on these studies we proposed that the combination of HDACi and TRAIL agonists could be of therapeutic benefit in CLL as well as other TRAIL-resistant haematological malignancies. To ascertain which form of TRAIL may be optimal in such a combination, we utilized different forms of TRAIL, including soluble ligands selective for
TRAIL-R1 or TRAIL-R2, as well as mapatumumab (HGS-ETR1) and lexatumumab (HGS-ETR2) (Human Genome Sciences), selective agonistic antibodies for TRAIL-R1 and TRAIL-R2, respectively (Pukac, et al 2005). These two mAbs were derived from ScFv reagents that were selected for their ability to induce apoptosis via their cognate receptors and do not require cross-linking for efficacy. Both CLL cells and cell lines that signal to apoptosis predominantly through either TRAIL-R1 (Ramos cells) or TRAIL-R2 (Jurkat cells) (MacFarlane, et al 2005a, MacFarlane, et al 2005b) were analyzed for their sensitivity to these agents. As mapatumumab and the TRAIL-R1-selective ligands, but not lexatumumab and the TRAIL-R2-selective ligands, induced apoptosis in primary CLL cells after HDACi sensitization, we concluded that CLL cells signalled to apoptosis predominantly through TRAIL-R1 and not TRAIL-R2 despite expressing both receptors at the cell surface (MacFarlane, et al 2005a, MacFarlane, et al 2005b).

More recently we wished to extend these studies to two further TRAIL-R agonistic mAbs. Firstly, LBY135, is a potent agonistic antibody against TRAIL-R2, that exerts significant single agent activity in colon, breast and melanoma mouse xenograft models but in vitro requires cross-linking in order to induce apoptosis (Li 2007). Secondly, DR4-A, a mouse mAb that binds specifically to TRAIL-R1 but which in vitro requires cross-linking to induce apoptosis. We demonstrate that LBY135 and DR4-A show an absolute requirement for cross-linking in order to induce caspase-dependent apoptosis. Following cross-linking, LBY135 but not DR4-A induces apoptosis in Jurkat cells whereas both antibodies can induce apoptosis in Ramos cells. Our finding that cross-linking of LBY135 resulted in the induction of apoptosis in CLL cells from some, but not all, patients raised the possibility that signalling to apoptosis through TRAIL-R2 may be facilitated by oligomerisation of the receptor following cross-linking of the TRAIL-R2 agonistic antibody. Support for this hypothesis was provided by our finding that cross-linking also sensitised CLL cells to the agonistic mAb, lexatumumab. Our data highlight important differences in the ability to activate apoptosis through TRAIL-R1 and TRAIL-R2 in primary CLL cells.
Materials and Methods

Materials.

Media and serum were from Life Technologies, Inc. (Paisley, UK). LBY135, LBH 589 and DR4-A were all kindly provided by Novartis Pharma (East Hanover, NJ). LBY135 is a specific anti-TRAIL-R2 agonistic antibody, which has been generated by hybridoma technology and functional cell based screening (Li 2007). Mapatumumab (HGS-ETR1) and lexatumumab (HGS-ETR2) were kindly provided by Human Genome Sciences (Rockville, MD, USA). Human recombinant wild-type TRAIL, TRAIL-R1 (TRAIL.R1-5) and TRAIL-R2 (TRAIL.R2-6)-selective ligands were prepared as previously described (MacFarlane, et al 2005b). Low endotoxin TRAIL (TRAIL-LE) was from Alexis Corporation (Nottingham, UK), depsipeptide was kindly provided by Dr. E. Sausville (NCI, USA) and Trichostatin A was from Sigma (Poole, UK). Antibodies were sourced as follows: anti-acetylated tubulin mAb (clone 6-11B-1) was from Sigma (Poole, UK); anti-acetylated H3 (K9/14) and H4 (K5/8/12/16) polyclonal rabbit antibodies were from Upstate Biotechnology. The antibody to poly (ADP-ribose) polymerase (PARP) (clone C2-10) was from Alexis Corp. (San Diego, CA). F(ab’)_2 fragment goat anti-mouse IgG Fcγ fragment specific (used for DR4-A) and F(ab’)_2 fragment goat anti-human IgG Fcγ fragment specific (used for LBY135) cross-linking antibodies were from Jackson ImmunoResearch Laboratories, Inc (Baltimore, USA). Anti-caspase-3 and -8 antibodies were from previously described sources (Inoue, et al 2004, Sun, et al 1999).

Lymphocyte purification, cell lines and culture.

Blood samples were obtained from CLL patients with patient consent and local ethical committee approval. Samples were collected into lithium-Heparin tubes and CLL cells were purified as described previously (Inoue, et al 2004, MacFarlane, et al 2002). The clinical details of the patients are given in Table 1. Purified CLL cells were cultured in RPMI 1640 medium (4-5 × 10^6 cells ml^{-1}) at 37°C in an atmosphere of 5% CO₂ and incubated for 16 h with different HDACi, including depsipeptide (10 nM), Trichostatin A (0.25 μM) or LBH589 (1-50 nM). CLL cells were also pre-
treated with depsipeptide or LBH589 at the indicated concentrations for 16 h followed by treatment with different forms of TRAIL or agonistic mAbs to TRAIL-R1 or TRAIL-R2 for a further 4-6 h. Agonistic mAbs to TRAIL-R1 and TRAIL-R2 were used either alone or cross-linked with the appropriate isotype of F(ab')\textsubscript{2} fragment goat anti-IgG Fc\textsubscript{γ} fragment specific cross-linking antibodies (1:3 ratio of mAbs to cross-linking antibodies, pre-incubated at room temperature for 30 min).

Jurkat T cells (clone E6-1) obtained from ECACC (Wiltshire, UK) and Ramos cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 5% Glutamax. Ramos and Jurkat cells express predominantly TRAIL-R1 and TRAIL-R2, respectively, on their cell surface (MacFarlane, et al 2005a). Wild-type and TRAIL-R2/DR5-deficient BJAB cells, kindly provided by Dr. A. Thorburn (University of Colorado Health Sciences Center, Aurora, CO), were cultured in RPMI 1640 medium supplemented with 10% FBS and 5% Glutamax (Thomas, et al 2004). Where indicated, cells were pre-treated for 30 min with the caspase inhibitor, benzylxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD.fmk) (Enzyme Systems, CA, USA). Samples were either analyzed immediately for apoptosis or pelleted and stored at -80°C for subsequent Western blotting.

**Quantification of Apoptosis and Western Blot Analysis.**

Apoptosis was quantified either by phosphatidylserine (PS) externalisation in the presence of propidium iodide or by loss of mitochondrial membrane potential (ΔΨ\textsubscript{m}) (Inoue, et al 2004, MacFarlane, et al 2002). Samples for Western blot analysis were prepared and caspases and cleaved PARP were detected as previously described (Inoue, et al 2004, MacFarlane, et al 2002). Extraction of histones for the detection of acetylated H3 and H4 were prepared as described (Inoue, et al 2004).
Results

**LBY135 but not DR4-A induces apoptosis in Jurkat cells but only when cross-linked.**

LBY135 (5-500 ng ml\(^{-1}\)) alone failed to induce apoptosis in Jurkat cells within 6 h, as assessed by externalisation of phosphatidylserine (PS) (Fig 1A). However in the presence of a cross-linking antibody, LBY135 (5-500 ng ml\(^{-1}\)) caused a concentration-dependent induction of apoptosis. These results demonstrated that cross-linking is absolutely required for LBY135 to induce apoptosis in Jurkat cells. On the other hand, DR4-A (5-500 ng ml\(^{-1}\)) did not induce apoptosis in Jurkat cells at 6 h even in the presence of a cross-linking antibody (Fig 1B). These results are generally in agreement with previous observations that Jurkat cells respond primarily through TRAIL-R2 (Kelley, et al 2005, MacFarlane, et al 2005a, MacFarlane, et al 2005b, Muhlenbeck, et al 2000).

LBY135, in the presence of a cross-linker, caused a time-dependent increase in PS externalisation together with a marked decrease in mitochondrial membrane potential (\(\Delta\Psi_m\)), both of which were almost completely abrogated by the broad spectrum caspase inhibitor z-VAD.fmK (10 \(\mu\)M) (Table 2). LBY135 induced a time-dependent processing of caspase-8, the apical caspase in the death-receptor pathway, to its p43 and p41 forms as well as its p18 catalytically active large subunit (Fig 1C, lane 3-4). This was accompanied by processing of caspase-3 to its p20 and p19/17 catalytically active large subunits, which was also catalytically active as poly(ADP-ribose) polymerase (PARP) was cleaved to its characteristic 85 kDa fragment (Fig 1C, lane 3-4). Pre-treatment with z-VAD.fmK (10 \(\mu\)M) completely blocked processing of caspase-8, caspase-3 and PARP cleavage (Fig 1C lane 5). No caspase processing or PARP cleavage was observed in the presence of either LBY135 or the cross-linker alone (Fig 1C lanes 6-7). Taken together these results demonstrated that LBY135, when cross-linked, induced a caspase-mediated apoptosis of Jurkat cells primarily through activation of TRAIL-R2.

**LBY135 and DR4-A induce apoptosis in Ramos cells.**
Previously we proposed that TRAIL induces apoptosis in Ramos cells primarily by signalling through TRAIL-R1 (MacFarlane, *et al* 2005a, MacFarlane, *et al* 2005b). Exposure of Ramos cells for 6 h to DR4-A (5-500 ng ml\(^{-1}\)), in the presence but not in the absence of a cross-linking antibody, caused a concentration-dependent induction of apoptosis (Fig 2A), indicating an absolute requirement for cross-linking in order for DR4-A to exert its pro-apoptotic activity *in vitro*.

Furthermore DR4-A exerted its pro-apoptotic activity in Ramos but not in Jurkat cells consistent with our previous suggestions that these cells respond primarily by signalling through TRAIL-R1 and TRAIL-R2, respectively (MacFarlane, *et al* 2005a, MacFarlane, *et al* 2005b). LBY135, in the presence but not in the absence of a cross-linking antibody, caused a concentration-dependent induction of apoptosis in Ramos cells albeit to a lesser extent than DR4-A, with LBY135 (500 ng ml\(^{-1}\)) inducing a similar level of apoptosis as DR4-A (25 ng ml\(^{-1}\)) (compare Figs 2A and B). However some of the differing potencies of DR4-A and LBY135 may be due to different efficiencies of the specific cross-linking antibodies used. Both DR4-A (100 ng ml\(^{-1}\)) and LBY135 (100 ng ml\(^{-1}\)) induced a time-dependent induction of apoptosis in Ramos cells assessed by PS externalisation and changes in \(\Delta \Psi_m\), which were blocked by pre-treatment with z-VAD.fmk (10 \(\mu M\)) (Table 2). However, the cross-linked DR4-A induced a marked increase in PS externalisation and loss in \(\Delta \Psi_m\) at 3 h and further at 6 h (Table 2), while the cross-linked LBY135 induced these apoptotic changes at 6 h (Table 2). DR4-A and LBY135 alone, as well as the cross-linker alone, were completely inactive. DR4-A, in the presence of a cross-linking antibody, induced efficient time-dependent processing of caspase-8, caspase-3 and cleavage of PARP (Fig 2C lanes 3, 4 and 6). Within 3 h, caspase-8 was processed to its p43/41 forms and to its p18 catalytically active large subunit (Fig 2C lane 3). An uncharacterized p30 fragment was also detected, which may possibly represent the unprocessed large and small subunits of caspase-8 derived following cleavage of caspase-8 between its second N-terminal death effector domains and its large subunit. Caspase-3 was again processed to its p20 and p19/17 catalytically active large subunits (Fig 2C lanes 3 and 4). Z-VAD.fmk completely blocked the processing of caspase-8, caspase-3 and cleavage of PARP (Fig
2C lane 5). LBY135, in the presence of a cross-linking antibody, also induced processing of
caspases and cleavage of PARP (Fig 2C lanes 10-11) although to a lesser extent than DR4-A
commensurate with its lower efficiency in inducing apoptosis in these cells. Again, z-VAD.fmk
completely prevented the processing of caspase-8, caspase-3 and cleavage of PARP (Fig 2C lane
12). DR4-A and LBY135 alone as well as the cross-linker alone were completely inactive (Fig 2C
lanes 6, 7, 13 and 14). These results clearly indicate that in Ramos cells the cross-linked DR4-A is a
more potent apoptotic inducer than the cross-linked LBY135.

**LBY135 requires TRAIL-R2 to induce apoptosis in BJAB cells.**
The observation that LBY135 induced apoptosis in Ramos cells was unanticipated as we have
previously demonstrated that these cells signalled to apoptosis predominantly through TRAIL-R1,
although they express low levels of TRAIL-R2 on the cell surface (MacFarlane, *et al* 2005a,
MacFarlane, *et al* 2005b). The different sensitivity of Ramos cells to lexatumumab and LBY135
could be due to several possibilities including the different epitopes on TRAIL-R2 recognized by
these two mAbs, the requirement of the cross-linker for LBY135 or alternatively that cross-linked
LBY135 has some pro-apoptotic activity through TRAIL-R1 (DR4). To test the last hypothesis, we
tested the ability of LBY135 to induce apoptosis in BJAB cells lacking DR5 (TRAIL-R2) (Thomas,
*et al* 2004). In the presence of a cross-linking antibody, LBY135 (5-500 ng ml⁻¹) caused a
concentration-dependent induction of apoptosis in wild type but not in BJAB cells lacking TRAIL-
R2 (Fig 2D). These results demonstrated that the cross-linked LBY135 requires TRAIL-R2 for its
pro-apoptotic activity and exhibits no activity via TRAIL-R1.

**LBH589 sensitizes CLL cells to TRAIL-induced apoptosis.**
Our previous studies had shown that HDACi, such as depsipeptide and trichostatin A (TSA)
sensitize CLL cells to TRAIL-induced apoptosis (Inoue, *et al* 2004, Inoue, *et al* 2006, MacFarlane,
*et al* 2005a). We wished to examine if a novel HDACi, LBH589, (Qian, *et al* 2006) also sensitized
CLL cells to TRAIL. Firstly we ascertained at what concentration LBH589 would inhibit histone deacetylase activity in CLL cells. Using freshly isolated CLL cells from two individual patients (Table 1, Patients #3 and 9), depsipeptide (10 nM) caused an increase in acetylated H3 and H4 but not in acetylated tubulin (Fig 3A, lane 3) compatible with it being a class I HDACi (Inoue, et al 2006). TSA (0.25 µM) caused an increase in both acetylated H3 and H4, as well as an increase in acetylated tubulin (Fig 3A, lane 2) compatible with it being an inhibitor of class I and class II HDACs (Inoue, et al 2006). LBH589 (1-50 nM) caused a concentration-dependent acetylation of histones H3 and H4 as well as a concentration-dependent acetylation of tubulin (Fig 3A, lanes 4-9) supporting the previous suggestion that LBH589 is both a class I and class II HDACi (Qian, et al 2006). Next we examined the ability of LBH589 to sensitize primary CLL cells to TRAIL-induced apoptosis using depsipeptide as a positive control. Depsipeptide (10 nM) sensitized CLL cells to TRAIL-induced apoptosis (Fig 3B) in agreement with our previous findings (Inoue, et al 2004).

Pretreatment of CLL cells from a further eight different individuals for 16 h with LBH589 (1-50 nM) resulted in a concentration-dependent sensitization of CLL cells to TRAIL-induced apoptosis (Fig 3B). Furthermore, LBH589 appeared to be equipotent to depsipeptide in its ability to sensitize CLL cells to TRAIL (Fig 3B).

Investigation of potential apoptotic activity of LBY135 and DR4-A in CLL cells

We then investigated cells from a further 11 CLL patients to determine their sensitivity to TRAIL, DR4-A and LBY135. Neither TRAIL, DR4-A nor LBY135 alone induced apoptosis in cells from any individual unless the cells had been pre-treated with an HDACi. No activity of DR4-A or LBY135 was observed in cells in the absence of a cross-linker. Cells from one patient were resistant to all the treatments and cells from two other patients had a high level of spontaneous apoptosis (≥ 40 % within 24 h) so leaving 8 CLL cases for analysis. CLL cells from these 8 patients all responded to the combination of HDACi and TRAIL but appeared to comprise 3 subsets depending on their response to DR4-A or LBY135. Cells from 3 patients (Table 1, Patients #1, 2 and 7) were
sensitized to TRAIL but were not sensitized to DR4-A or LBY135, with a representative example given in Fig 4A. Cells from a further 3 patients (Table 1, Patients #3, 4 and 6) showed a sensitization ranking as follows: TRAIL > DR4-A > LBY135 with a representative example shown in Fig 4B. Cells from the remaining 2 patients (Table 1, Patients #5 and 8) showed a sensitization ranking as follows: TRAIL > LBY135 > DR4-A with a representative example given in Fig 4C. To confirm that this induction of cell death by LBY135 was due to apoptosis, we also examined the processing of caspases in CLL cells from one patient (#8), which were sensitive to LBY135 in the presence of a cross-linker (Fig 4D). Induction of apoptosis assessed by PS externalisation was accompanied by processing of caspase-8 to its p43/41 fragments as well its p18 catalytically active large subunit together with processing of caspase-3 to its catalytically active p19/17 large subunit (Fig 4D lane 8).

**Cross-linking of lexatumumab enables CLL cells to signal to apoptosis**

The above results indicated that under some circumstances CLL cells may possibly signal through TRAIL-R2 as well as TRAIL-R1. To further investigate this possibility, we examined the ability of lexatumumab (HGS-ETR2), a fully human agonistic TRAIL-R2 antibody, to signal to apoptosis in CLL cells. Previously we had shown that lexatumumab did not signal to apoptosis in CLL cells even when the cells had been pretreated with an HDACi (MacFarlane, et al 2005a, MacFarlane, et al 2005b). In these studies we also compared the effects of soluble forms of TRAIL (100 ng ml⁻¹), TRAIL.R1-5, a TRAIL-R1-selective ligand and TRAIL.R2-6, a TRAIL-R2-selective ligand (MacFarlane, et al 2005b). We have now examined cells from a further 9 patients to determine their sensitivity to lexatumumab in the presence and absence of a cross-linker. None of the preparations of TRAIL induced apoptosis in CLL cells unless the cells were pre-treated with depsipeptide, an HDACi. Cells from one patient (Table 1 #15) were completely resistant to all treatments whereas cells from the other 8 patients were all sensitized following pre-treatment with depsipeptide, albeit to differing extents (Fig 5). Some inter-individual variation was observed with cells from different patients but some important common themes emerged. Following depsipeptide pre-treatment,
TRAIL and TRAIL.R1-5, but not TRAIL.R2-6, induced significant levels of apoptosis above the controls in CLL cells (Fig 5). Mapatumumab (ETR1) induced similar levels of apoptosis irrespective of whether or not it was cross-linked (Fig 5). Importantly cross-linking of lexatumumab (ETR2) resulted in an enhancement of apoptosis above lexatumumab alone in cells from 7/8 patients (Fig 5). Some inter-individual variation was also observed in that depsipeptide alone (10 nM) induced apoptosis in cells from some patients possibly because of the slightly longer times of incubation compared to those used in our earlier studies (Inoue, et al 2004, MacFarlane, et al 2005a). Thus, CLL cells can signal to apoptosis via TRAIL-R2 but only following cross-linking of agonistic antibodies to TRAIL-R2.
Discussion

Novel therapies are urgently required for the treatment of CLL. Although TRAIL has been reported to be selectively toxic to many tumour compared to normal cells (Ashkenazi 2002, Koschny, et al 2007), we observed that primary cells from patients with CLL were completely resistant to TRAIL, even to concentrations as high as 2000 ng ml\(^{-1}\) (MacFarlane, et al 2002). Similarly we and others have shown that in addition to CLL, primary cells from patients with other haematological malignancies including B-cell acute lymphoblastic leukaemia, B-cell non-Hodgkin’s lymphoma and mantle cell lymphoma are also resistant to TRAIL (Clodi, et al 2000, MacFarlane, et al 2005b, Snell, et al 1997). Subsequently we demonstrated that primary CLL and mantle cell lymphoma cells could be sensitised to TRAIL by prior exposure to a HDACi (Inoue, et al 2004, MacFarlane, et al 2005b). In this study, we have shown that another HDACi, LBH589, also sensitises CLL cells to TRAIL-induced apoptosis (Fig 3). Nanomolar concentrations of HDACi, such as LBH589, are sufficient to sensitise CLL cells to TRAIL-induced apoptosis whereas much higher concentrations (~1000-fold) of these HDACi alone, such as LBH589, are required to induce apoptosis in CLL cells (Inoue, et al 2007).

In studies to assess which of the many available forms of TRAIL may be most appropriate for a clinical study in CLL, we discovered that soluble TRAIL appeared to signal to apoptosis primarily by activation of TRAIL-R1 (MacFarlane, et al 2005a, MacFarlane, et al 2005b). The present studies were undertaken partly to confirm and extend our previous work to two novel agonistic Abs, LBY135 and DR4-A, which activate their respective receptors only upon cross-linking. Surprisingly, our new data demonstrate that cross-linking of agonistic Abs to TRAIL-2, such as LBY135 or lexatumumab can enhance apoptosis in cells, which otherwise signal predominantly through TRAIL-R1, such as CLL or Ramos cells (Figs. 2, 4 and 5). By contrast, no cross-linking of mapatumumab was required for it to signal to apoptosis in CLL or Ramos cells (Fig 5) (MacFarlane, et al 2005a, MacFarlane, et al 2005b). These results confirm and extend our previous observations
where we proposed that TRAIL signals predominantly through TRAIL-R1 and not through TRAIL-R2 in CLL and Ramos cells (MacFarlane, et al 2005a, MacFarlane, et al 2005b). In these initial studies, exposure to lexatumumab in the absence of a cross-linker resulted in the induction of apoptosis in Jurkat but not in Ramos or CLL cells. Based on these observations, together with data using receptor-selective TRAIL mutant ligands, we proposed that Jurkat cells signalled to apoptosis primarily through TRAIL-R2 whereas CLL and Ramos cells signalled to apoptosis primarily through TRAIL-R1 (MacFarlane, et al 2005a, MacFarlane, et al 2005b). However, it is now apparent that cross-linking of agonistic antibodies particularly mAbs to TRAIL-R2, such as LBY135 and lexatumumab, enables these antibodies to signal to apoptosis in CLL cells. These results indicate that signalling through TRAIL-R1 and TRAIL-R2 is clearly different and suggests that at least in some cell types, such as CLL and Ramos cells, increased oligomerisation of TRAIL-R2 may be required in order to facilitate induction of apoptosis by agonistic TRAIL-R2 antibodies. Our data are compatible with previous studies using cells in which the receptors TRAIL-R1 and -R2 were over-expressed, which showed that TRAIL-R1 was sensitive to both apoptosis and NF-κB/JNK induction following exposure to cross-linked or non-cross-linked soluble TRAIL, whereas TRAIL-R2 only responded to cross-linked forms of TRAIL (Muhlenbeck, et al 2000). These results suggested distinct cross-linking requirements of TRAIL-R1 and -R2 for apoptosis and NF-κB induction and for activation of JNK. Furthermore, in support of these conclusions, this group also demonstrated that TRAIL-R1 was activated by both soluble and membrane-bound forms of the ligand, whereas TRAIL-R2 was only activated by membrane-bound TRAIL or an epitope-tagged form of soluble TRAIL cross-linked with a mAb (Wajant, et al 2001).

In the present and our previous studies, we have shown that in response to wild type TRAIL, receptor-selective TRAIL mutants and non-cross linked agonistic antibodies, CLL cells signal predominantly through TRAIL-R1 and not through TRAIL-R2. However, we now show that following cross-linking of certain agonistic TRAIL-R2 antibodies, CLL cells can also signal
through TRAIL-R2. *In vitro* cross-linking can be induced by various reagents including anti-immunoglobulin antibodies or by chemical cross-linkers, whereas *in vivo* effector molecules or cells responsible for cross-linking are restricted to Fc receptors present on most immune effector cells or the complement component C1q (Chuntharapai, *et al* 2001, Motoki, *et al* 2005). Such endogenous cross-linkers may vary in different individuals either due to immunosuppressive therapy or polymorphisms of FcR, therefore in terms of therapy it may be preferable to use an agent that signals via TRAIL receptors but does not require such cross-linking (Cartron, *et al* 2002, Kono, *et al* 2002, Motoki, *et al* 2005). Taken together our data suggest that therapies that target TRAIL-R2 and that rely on efficient cross-linking *in vivo* may not be optimal as the reliance on activation through Fc receptors may well be comprised in patients with CLL, who are also often receiving concomitant therapy with immunosuppressive agents. In conclusion, we propose that it may still be preferable to treat patients with CLL with a histone deacetylase inhibitor followed by a receptor-selective TRAIL mutant ligand specific for TRAIL-R1 or an agonistic antibody to TRAIL-R1 that does not require cross-linking in order to induce apoptosis.
Figure Legends

Fig 1. LBY135 but not DR4-A induces apoptosis in Jurkat cells.
Jurkat cells were incubated for 6 h with the indicated concentration of (A) LBY135 or (B) DR4-A alone or in the presence of a cross-linking antibody and apoptosis was assessed by PS externalisation. Results are expressed as the Mean ± SE of at least four independent experiments. (C) Jurkat cells were incubated for the indicated times with LBY135 (100 ng ml\(^{-1}\)) either alone or in the presence of a cross-linking antibody. Where indicated cells were pretreated for 30 min with z-VAD.fmk (10 \(\mu\)M) and then incubated with LBY135 in the presence of a cross-linking antibody. Cell lysates were prepared at the indicated times and analyzed by Western blotting for the processing of caspase-8, caspase-3 and PARP cleavage.

Fig 2. LBY135 and DR4-A induce apoptosis in Ramos cells.
Ramos cells were incubated for 6 h with the indicated concentration of (A) DR4-A or (B) LBY135 alone or in the presence of a cross-linking antibody and apoptosis assessed by PS externalisation. Results are expressed as the Mean ± SE of at least four independent experiments. (C) Ramos cells were incubated for the indicated times with either DR4-A (100 ng ml\(^{-1}\)) or LBY135 (100 ng ml\(^{-1}\)) either alone or in the presence of a cross-linker. Where indicated cells were pre-treated for 30 min with z-VAD.fmk (10 \(\mu\)M) and then incubated with cross-linked DR4-A (100 ng ml\(^{-1}\)). Cell lysates were prepared at the indicated times and analyzed by Western blotting for the processing of caspase-8, caspase-3 and PARP cleavage. (D) Wild type (wt) and DR5 null (TRAIL-R2\(^{-/-}\)) BJAB cells were incubated for 6 h with the indicated concentration of LBY135 in the presence or absence of a cross-linker. Apoptosis was assessed by PS externalisation. Results are expressed as the Mean ± SE of at least three independent experiments.

Fig 3. LBH589 sensitizes CLL cells to TRAIL-induced apoptosis.
(A) CLL cells from two patients were incubated for 16 h either alone with TSA (0.25 μM), depsipeptide (Dep, 10 nM) or the indicated concentration of LBH589 (LBH). Cells were harvested and cell lysates analyzed for inhibition of HDAC activity by accumulation of acetylated tubulin (Ac-tubulin) and acetylated histone H3 or H4 (Ac-H3 or Ac-H4). (B) CLL cells from eight patients were pre-treated with depsipeptide (Dep, 10 nM) or LBH589 (1-50 nM) for 16 h then treated for further 4 h with TRAIL (100 ng ml⁻¹) and apoptosis assessed by PS externalisation. Results are expressed as the Mean ± SE of eight different patients.

**Fig 4. Marked inter-individual variation in response of CLL cells to DR4-A and LBY135.**
CLL cells were pre-treated with depsipeptide (Dep, 10 nM) or LBH589 (10 nM) for 16 h then treated for a further 4 h (except for individual #2 where cells were treated for 6 h) with TRAIL (100 ng ml⁻¹) or cross-linked DR4-A or cross-linked LBY135 and apoptosis assessed by PS externalisation. (A) Cells from patient #2 responded to TRAIL but did not respond to cross-linked DR4-A or LBY135. Cells from patients # 1 and 7 behaved similarly. (B) Cells from patient #3 responded to TRAIL and DR4-A to a greater extent than to LBY135. Cells from patients # 4 and 6 behaved similarly. (C) Cells from patient #5 responded to TRAIL and LBY135 to a greater extent than to cross-linked DR4-A. Cells from patient # 8 behaved similarly. (D) Cells from patient #8 were incubated for 16 h with depsipeptide (10 nM) followed by incubation for a further 4 h with TRAIL (100 ng ml⁻¹), cross-linked DR4-A (5 μg ml⁻¹) or cross-linked LBY135 (5 μg ml⁻¹) and then cell lysates prepared and analyzed by Western blotting for the processing of caspase-8 and caspase-3. Apoptosis assessed by PS externalisation is also shown for cells from this patient.

**Fig 5. CLL cells can signal through TRAIL-R2 following cross-linking of agonistic antibodies.**
CLL cells were pre-treated with depsipeptide (Dep, 10 nM) for 16 h then treated for further 6 h with low endotoxin TRAIL (TRAIL-LE, 100 ng ml⁻¹), the TRAIL-R1-selective ligand (R1-5, 500 ng ml⁻¹) or the TRAIL-R2 selective ligand (R2-6, 500 ng ml⁻¹), Mapatumumab (ETR1)(1 μg ml⁻¹) either
alone or cross-linked or lexatumumab (ETR2) (1 μg ml⁻¹) either alone or cross-linked. Results shown are from cells from 7 patients as indicated. The dark black line indicates the Mean value for the % apoptotic cells induced by the specific treatment. Cells from patient #2 (Table 1) did not respond to the cross-linking and are omitted from this analysis. Cross-linking alone did not induce any apoptosis in the presence or absence of depsipeptide.
Table 1: Clinical data of patients in current study

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sex</th>
<th>Age</th>
<th>Stage</th>
<th>WCC</th>
<th>Cytogenetics</th>
<th>IGVH status</th>
<th>Rx</th>
<th>F Res</th>
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<td>18</td>
<td>11q and 13q del</td>
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<td>0</td>
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CLL was staged by the Binet classification. WCC: White cell count x10^9/L at diagnosis; Age at diagnosis; F res: Fludarabine resistance; IGHV: Immunoglobulin Heavy Chain, U: Unmutated, M: Mutated; nd: not done, N/A: not applicable, t: translocation, del: deletion; Rx number of different chemotherapy/antibody regimes.
Table 2. Differential sensitivity of cells to cross-linked LBY135 and DR4A.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cross-linker</th>
<th>Jurkat</th>
<th>Ramos</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LBY 135</td>
<td>LBY 135</td>
<td>DR4-A</td>
</tr>
<tr>
<td></td>
<td>% PS</td>
<td>% ΔΨm</td>
<td>% PS</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>3.2 ± 1.0</td>
<td>90 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3.6 ± 0.1</td>
<td>93 ± 0.6</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>3.4 ± 0.5</td>
<td>93 ± 0.3</td>
</tr>
<tr>
<td></td>
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<td>2.6 ± 0.2</td>
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</tr>
<tr>
<td>3</td>
<td>-</td>
<td>3.9 ± 0.6</td>
<td>92 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25 ± 8.4</td>
<td>76 ± 4</td>
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<tr>
<td>6</td>
<td>-</td>
<td>4.9 ± 0.5</td>
<td>91 ± 0.8</td>
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<tr>
<td></td>
<td>+</td>
<td>54.3 ± 11</td>
<td>47 ± 3</td>
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<tr>
<td></td>
<td>z-VAD.fmk</td>
<td>+</td>
<td>3.2 ± 0.5</td>
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</table>

Jurkat and Ramos cells were exposed to either LBY135 or DR4-A for 0-6 h in the presence or absence of a cross-linker as indicated. Apoptosis was then assessed either by PS externalisation or loss in mitochondrial membrane potential (ΔΨm) as described in Materials and Methods. Some cells were also incubated for 6 h in the presence of the indicated cross linked antibody and the broad spectrum caspase inhibitor, z-VAD.fmk (10 μM) prior to assessment of apoptosis. The results represent the Mean ± SE of at least 3 separate experiments.
References


**Acknowledgements.**

This work was supported in part by a grant from Novartis. We thank Dr. Andrew Thorburn (University of Colorado Health Sciences Center, Aurora, CO, USA) for the TRAIL-R2/DR5-deficient BJAB cells.
Figure 1

A

% PS+ cells

- Crosslinker

+ Crosslinker

LBY135 (ng/ml)

B

% PS+ cells

- Crosslinker

+ Crosslinker

DR4-A (ng/ml)

C

<table>
<thead>
<tr>
<th>z-VAD -fmk</th>
<th>Crosslinker</th>
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<tbody>
<tr>
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<tr>
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Caspase-8

p43/p41

p18

Caspase-3

p20

p19/p17

PARP

p85

LANE

1  2  3  4  5  6  7
Figure 2

A. Ramos cells

B. Ramos cells

C. Ramos cells

D. wt BJAB cells

TRAIRL-R2-/- BJAB cells
Figure 3

A

Patient #3

<table>
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<th>TSA</th>
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Lane

1 2 3 4 5 6 7 8 9

Ac-H3

Ac-H4

Tub

Ac-Tub

Patient #9

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</table>

Lane

1 2 3 4 5 6 7 8 9

B

CLL cells

% PS cells

- TRAIL

+ TRAIL

Con Dep 1 2.5 5 10 25 50

LBH589 (nM)
Figure 4

A. Patient #2

B. Patient #3

C. Patient #5

D. Patient #8

<table>
<thead>
<tr>
<th></th>
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<th>TRAIL</th>
<th>Fab' m</th>
<th>DR4-A</th>
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<th>Fab' h</th>
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<td>0.5</td>
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<td>5</td>
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<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>TRAIL</th>
<th>Fab' m</th>
<th>DR4-A</th>
<th>DR4-A + Fab' m</th>
<th>Fab' h</th>
<th>LBY135</th>
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<tbody>
<tr>
<td>µg/ml</td>
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<td>0.1</td>
<td>5</td>
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<td>1</td>
<td>5</td>
<td>0.5</td>
<td>1</td>
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</tbody>
</table>

| TRAIL (0.1 µg/ml) | - | + | - | + | - | + | - | + |
| Cr-DR4-A (5 µg/ml) | - | + | - | + | - | + | - | + |
| Cr-LBY135 (5 µg/ml) | - | + | - | + | - | + | - | + |

% PS+ cells

Caspase-8

p43/p41

Caspase-3

p18

p19/p17

% PS+ cells
Figure 5

- △ Patient #1
- ◇ Patient #4
- ★ Patient #5
- ▼ Patient #10
- ▪ Patient #11
- ■ Patient #12
- ○ Patient #13

+ Depsipeptide