

Chronic lymphocytic leukemic cells exhibit apoptotic signaling via TRAIL-R1

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Abstract

Clinical trials have been initiated with Apo2L/TRAIL (Genentech) and agonistic mAbs to TRAIL receptors, -R1 and -R2 (Human Genome Sciences). The apoptosis-inducing ability of these mAbs and different TRAIL preparations, in the presence or absence of histone deacetylase inhibitors (HDACi), varied markedly against primary chronic lymphocytic leukaemia (CLL) cells and various tumor cell lines, demonstrating an unanticipated preferential apoptotic signaling via either TRAIL-R1 or -R2. Contrary to literature reports that TRAIL-induced apoptosis occurs primarily via signaling through TRAIL-R2, CLL cells, in the presence of HDACi, undergo predominantly TRAIL-R1-mediated apoptosis. Consequently, Apo2L/TRAIL, which signals primarily through TRAIL-R2, is virtually devoid of activity against CLL cells. To maximize therapeutic benefit, it is essential to ascertain whether a primary tumor signals via TRAIL-R1/-R2, prior to initiating therapy. Thus combination of an agonistic TRAIL-R1 Ab and an HDACi, such as the antic-convulsant sodium valproate, could be of value in treating CLL. *Cell Death and Differentiation* (2005) 12, 773–782.

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Abbreviations: CLL, chronic lymphocytic leukaemia; DISC, death-inducing signaling complex; HDACi, histone deacetylase inhibitor; HGS, Human Genome Sciences; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R1, TRAIL-receptor 1; TRAIL-R2, TRAIL-receptor 2

Introduction

TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is being developed as an antitumor agent as it

induces apoptosis in a wide range of tumor cell lines but not in most normal cells.^{1–4} TRAIL induces apoptosis by binding to two membrane-bound receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2), resulting in the recruitment of the adaptor molecule FADD/MORT1, followed by the recruitment and activation of caspase-8, into the death-inducing signaling complex (DISC).^{5–7} Active caspase-8, the apical caspase in death receptor-induced apoptosis, can then activate other caspases, such as caspase-3, which in turn cleave many cellular substrates resulting in the biochemical and morphological features characteristic of apoptosis.⁸

TRAIL is particularly promising for development as an anticancer agent as it appears to be nontoxic to normal tissues in contrast to other members of the TNF family, such as TNF or CD95, which cause systemic inflammatory syndrome and liver toxicity, respectively.^{9–11} Despite its early promise since its discovery in 1995, TRAIL is only now entering Phase I clinical trials partly due to reports of its potential liver toxicity.¹² Also, although many cancer cell lines show sensitivity to TRAIL, many primary cells from patients, such as those with chronic lymphocytic leukemia (CLL) and B-cell non-Hodgkin's lymphoma, are commonly resistant to TRAIL-mediated apoptosis.^{13–15} Although in many of these cells the mechanism(s) of TRAIL resistance is unclear, use of a combination of treatments with either chemotherapeutic agents or irradiation may sensitize tumor cells to TRAIL.^{9,11,16}

We have shown that resistance of CLL cells to TRAIL-induced apoptosis is upstream of caspase-8 activation at the level of the DISC.¹⁴ More recently we have shown that low concentrations of histone deacetylase inhibitors (HDACi) sensitize CLL cells to TRAIL-induced apoptosis by facilitating increased formation of the TRAIL DISC.¹⁷ We proposed that the combination of HDACi and TRAIL agonists could be of therapeutic benefit in CLL as well as other TRAIL-resistant hematological malignancies. The combination of TRAIL with HDACi may be particularly valuable as HDACi are reported to be relatively nontoxic.^{18,19}

In this study, we investigated the ability of different forms of TRAIL and agonistic Abs to induce apoptosis in a panel of cell lines and primary cells from patients with CLL in the presence or absence of HDACi. The different forms of TRAIL and Abs included those currently in Phase I and II clinical trials, namely Apo2L/TRAIL (Genentech),^{2,3} HGS-ETR1 and HGS-ETR2 (Human Genome Sciences),^{20–22} as well as TRAIL-His-LE (a recombinant TRAIL with low endotoxin) or TRAIL-His (recombinant TRAIL used in our previous study but not further purified to reduce endotoxin levels).^{14,17,23}

Results

HDACi sensitize CLL cells to TRAIL primarily via TRAIL-R1

Recently, we have shown that pretreatment of CLL cells with HDACi, such as depsipeptide or Trichostatin A, sensitizes resistant cells to TRAIL-induced apoptosis.¹⁷ In order to

exploit this observation clinically, we wished to identify which form of TRAIL and which HDACi would have optimal biological activity and could therefore be used in an initial clinical trial.

Peripheral blood lymphocytes from 28 patients with CLL at different clinical stages were studied. The clinical charac-

teristics of these patients are shown in Table 1. Cells from 10 of these patients have been characterized more extensively in terms of sensitivity to the different forms of TRAIL and are depicted in Figure 1a.

Spontaneous apoptosis in freshly isolated CLL cells from different patients varied significantly (3.4–46%, $n = 28$) after

Table 1 Summary of data from 28 patients with CLL

Patient number	Age / sex	Binet stage	Prior therapy	FAMP resistance	WCC $\times 10^9/l$	% IGHV homology	IGHV gene family	p53 mutation	% Spontaneous apoptosis	% Apoptosis: TRAIL plus HDACi
1	66/M	A	NIL	N/A	28	90	3–33		22.9	64.2
2	44/F	A(P)	NIL	N/A	126	88	4–34		41.2	80.8
3	71/M	C	FAMP FAMP + CAMPATH-1H	Yes	52	99	1–69		22.2	68.9
4	76/M	B	FAMP + cyclophosphamide (NR)	Yes	106	100	3–09	CODON 193 His → Leu	46.2	88.7
5	83/F	A(P)	Chlorambucil $\times 2$ courses	N/A	52	88	3–30		27.2	55.9
6	78/M	A(P)	Chlorambucil $\times 2$ courses	N/A	113	96	2–05		9.7	49.8
7	68/M	B	FAMP	N/A	332	100	4–59		32.1	63.2
8	57/M	C	NIL	N/A	156	97	3–53		43.7	85.8
9	66/M	C	Chlorambucil $\times 2$ courses	No	110	86	4–34		23.8	49.8
10	85/F	C	Chlorambucil FAMP	Yes	144	92	3–30	CODON 266 (Gly → Arg) CODON 267 (Arg → Trp)	25.5 37.1	73.1 58.2
11	69/M	A	NIL	N/A	50	96	1–69		5.8	38.6
12	62/F	A	NIL	N/A	128	88	3–64		8.1	18.6
13	74/M	A(P)	NIL	N/A	82	96	4–34		9.9	11.2
14	64/M	C	Chlorambucil FAMP FAMP + Cyclophosphamide CHOP FAMP + CAMPATH-1H	Yes	257	98	3–21		20.3	65.8
15	60/M	C	Chlorambucil CAMPATH-1H + FAMP	Yes	353	99	1–69	One allele deleted by FISH assay but no mutation detected	14.1	71.7
16	76/F	C	FAMP	No	87	99	4–39		22.9	76.7
17	82/F	A(P)	Chlorambucil	N/A	89	90	3–30		26.0	68.2
18	72/M	C	Chlorambucil FAMP FAMP + CAMPATH-1H	Yes	175	99	1–69		17.3	74.4
19	47/M	C	FAMP	No	250	99	1–02		19.7	64.4
20	84/M	C	Chlorambucil $\times 3$ courses	N/A	314	98	3–21		27.1	80.5
21	66/M	A(P)	NIL	N/A	119	100	3–09		19.6	64.4
22	81/M	C	Chlorambucil	N/A	159	97	3–11		16.1	65.9
23	71/M	C	FAMP	Yes	74	96	1–18		6.8	63.2
24	68/M	B	NIL	N/A	28	93	3–33		28.3	56.9
25	76/M	A	NIL	N/A	50	95	3–09		17.8	16.9
26	72/M	C	Primary Chlorambucil resistant	N/A	147	100	3–74		3.4	9.3
27	80/M	A(P)	NIL	N/A	144	100	3–23		18.1	46.0
28	55/M	C	Primary Chlorambucil resistant	N/A	280	94	3–33		22.2	52.7

A(P) denotes progressive Stage A CLL; FAMP denotes fludarabine; N/A denotes not applicable. Patients #1–12 correspond with the patient data shown in Figure 1a–d. The response to HDACi in all patients was measured with TRAIL-His (100 ng/ml). Patient #10 shows the sensitization data obtained both before and after acquiring a p53 mutation. We define $\geq 98\%$ homology of the IGHV gene to its germline sequence as unmutated

16 h culture *in vitro* (Table 1). No increase in the spontaneous level of apoptosis was observed in cells from these different patients exposed either to Apo2L/TRAIL (100–4000 ng/ml), TRAIL-His-LE (100 ng/ml) or TRAIL-His (100 ng/ml) alone (data not shown). Thus, as shown previously, primary CLL cells are totally resistant to TRAIL-induced apoptosis.

Firstly, we examined if depsipeptide could sensitize to different TRAIL preparations or was limited to a specific property of our own preparation of TRAIL (TRAIL-His). CLL cells were pretreated for 16 h with depsipeptide (10 nM) and then exposed to the different forms of TRAIL (Figure 1a). Pretreatment with depsipeptide at this concentration did not

increase the levels of spontaneous apoptosis but markedly sensitized cells from all 10 patients to subsequent exposure to either TRAIL-His-LE (100 ng/ml) or TRAIL-His (100 ng/ml) with the TRAIL-LE being slightly more potent in 5/10 patients (Figure 1a). Pretreatment with depsipeptide also sensitized CLL cells to Apo2L/TRAIL. However in all 10 patients, the sensitization to Apo2L/TRAIL was always less than that observed with the other forms of TRAIL even when Apo2L/TRAIL was used at 40-fold greater concentration than TRAIL-His-LE (Figure 1a). Although depsipeptide sensitized CLL cells to Apo2L/TRAIL, the dose response curve was relatively shallow and appeared to plateau at concentrations of Apo2L/TRAIL of 500 ng/ml with very little increase in sensitization occurring at higher concentrations of Apo2L/TRAIL (2000–4000 ng/ml) (Figure 1a).

Secondly, we assessed the activity of agonistic TRAIL receptor mAbs under the same conditions. Two agonistic human IgG1 mAbs, HGS-ETR1, a TRAIL-R1 mAb, and HGS-ETR2, a TRAIL-R2 mAb, have been developed for potential treatment of certain forms of human cancer and are currently in Phase I or II studies.^{20–22} When used alone, neither HGS-ETR1 (1 µg/ml) nor HGS-ETR2 (1 µg/ml) induced significant levels of apoptosis in freshly isolated CLL cells from different patients (*n* = 8) (Figure 1b). Pretreatment of CLL cells for 16 h with depsipeptide (10 nM) clearly sensitized CLL cells to apoptosis induced by HGS-ETR1, whereas little if any sensitization was observed with HGS-ETR2 (Figure 1b). The % apoptosis in the presence of depsipeptide either alone (26.9 ± 16 Mean \pm S.E.M., *n* = 8) or together with HGS-ETR1 or HGS-ETR2 were 56.7 ± 23 or 32.8 ± 17 , respectively (Mean \pm S.E.M., *n* = 8). These results strongly suggested that TRAIL-induced apoptosis in CLL cells occurred primarily by signaling through TRAIL-R1.

We also wished to determine if CLL cells could be similarly sensitized to TRAIL by sodium valproate, as this is a widely used anticonvulsant drug with relatively little toxicity. Valproate also has activity as an HDACi^{24,25} and so could be used in a clinical trial in combination with either TRAIL or agonistic mAbs without the problems associated with using two novel

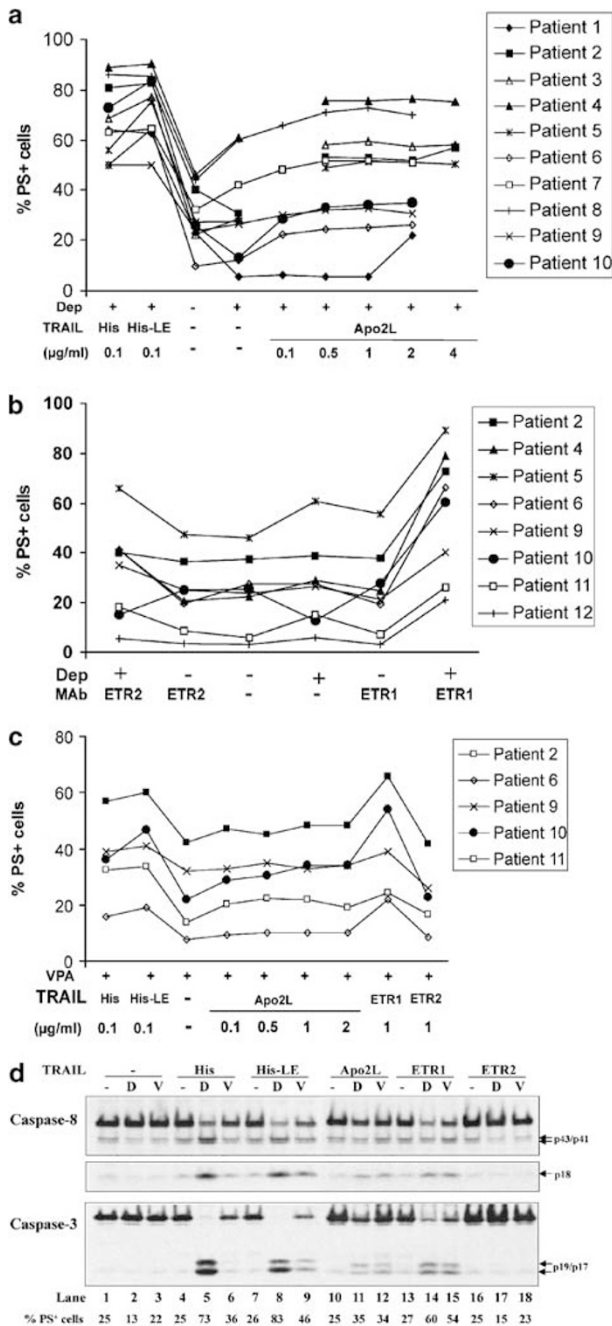


Figure 1 Potentiation of TRAIL-induced apoptosis in CLL cells by HDAC inhibitors occurs primarily by signaling through TRAIL-R1. **(a)** Freshly isolated and purified CLL cells from 10 individual patients, corresponding to the appropriate patient number in Table 1, were either incubated alone (Con) or preincubated for 16 h with depsipeptide (Dep) (10 nM). The cells were then treated for a further 4 h with TRAIL-His (100 ng/ml), TRAIL-His-LE (100 ng/ml) or Apo2L/TRAIL (Apo2L) (100–4000 ng/ml) and apoptosis assessed by measurement of phosphatidylserine (PS) externalization as described in Methods. Each line in Figure 1 (a–c) represents data from an individual patient. **(b)** CLL cells from different patients were preincubated for 16 h either alone or with depsipeptide (Dep) (10 nM) and then treated for a further 4 h with HGS-ETR1 (ETR1) (1 µg/ml) or HGS-ETR2 (ETR2) (1 µg/ml) and apoptosis measured by PS externalization. **(c)** CLL cells from different patients were preincubated for 16 h with sodium valproate (VPA) (2 mM) and then treated for a further 4 h with the different preparations of TRAIL or with the agonistic mAbs as indicated in (a) and (b) above. Apoptosis was measured by PS externalization. **(d)** Apoptosis was also assessed by measuring processing of caspase-8 and -3 in cells obtained from one patient (#10) treated with depsipeptide (D) or valproate (V) as described above in (a–c). Very similar data were obtained using cells, from another patient, exposed to all the different preparations. Similar results were also obtained when the effects of individual TRAIL preparations or Abs on cells from at least another five different patients were examined

agents in a clinical trial. Valproate (2 mM) alone induced little or no apoptosis in CLL cells (23.5 ± 12 , Mean \pm S.E.M., $n=5$) and sensitized CLL cells to TRAIL-induced apoptosis (Figure 1c). Interestingly, the magnitude of the valproate-mediated sensitization of CLL cells to HGS-ETR1 (41 ± 16 , Mean \pm S.E.M., $n=5$) was essentially identical to its sensitization to TRAIL-His-LE (40.1 ± 13 , Mean \pm S.E.M., $n=5$). Although valproate clearly sensitized CLL cells to HGS-ETR1, it caused no sensitization to HGS-ETR2 (23.1 ± 11 , Mean \pm S.E.M., $n=5$) (Figure 1c). These results support the suggestion that in CLL cells signaling through TRAIL-R1 is more important than TRAIL-R2 for cell death (Figure 1c).

To confirm that the depsipeptide and valproate-mediated sensitization was due to apoptosis, we examined the effects of the different treatments on the processing of caspase-8, the apical caspase in TRAIL-induced apoptosis,^{5-7,26} and caspase-3 the major effector caspase in many cells including CLL cells undergoing apoptosis. In untreated CLL cells, caspase-3 and caspase-8 were present as their unprocessed zymogens (Figure 1d lane 1). Exposure of CLL cells to depsipeptide (10 nM), valproate (2 mM), TRAIL-His (100 ng/ml), TRAIL-His-LE (100 ng/ml), Apo2L/TRAIL (2000 ng/ml), HGS-ETR1 (1 μ g/ml) or HGS-ETR2 (1 μ g/ml) alone neither induced apoptosis nor processing of caspase-3 or caspase-8 (Figure 1d lanes 2-4, 7, 10, 13 and 16). However in CLL cells, pretreated with either depsipeptide or valproate, processing of caspase-8 both to its p43 and p41 fragments and also its p18 catalytically active large subunit as well as processing of caspase-3 to its p19 and p17 forms was observed in CLL cells exposed to TRAIL-His, TRAIL-His-LE, Apo2L/TRAIL and HGS-ETR1 (Figure 1d lanes 5, 6, 8, 9, 11, 12, 14 and 15). The amount of caspase processing was generally commensurate with the induction of apoptosis induced by the different preparations of TRAIL or the mAbs; for example, no apoptosis or processing of caspases was observed with HGS-ETR2 (Figure 1d lanes 17 and 18). Depsipeptide was generally more effective than valproate at potentiating TRAIL-induced-apoptosis and processing of caspases. However, both depsipeptide (10 nM) and valproate (1-2 mM) inhibited HDACs in CLL cells, as assessed by an accumulation of acetylated H3 and H4 (data not shown), in agreement with our previous findings¹⁷ that both depsipeptide and Trichostatin A caused similar effects.

Thus following initial pretreatment with HDACi, the biological activities of different TRAIL preparations varied markedly in their ability to induce apoptosis in CLL cells with the potencies being TRAIL-His-LE \geq TRAIL-His \approx HGS-ETR1 $>$ Apo2L/TRAIL \gg HGS-ETR2. Taken together, these results strongly suggest that in CLL cells signaling to apoptosis occurs almost exclusively via TRAIL-R1 rather than TRAIL-R2.

Sensitization to TRAIL-induced apoptosis by HDACi is p53-independent

Some interindividual variation was observed in the depsipeptide-mediated sensitization of CLL cells to TRAIL-induced apoptosis (Table 1 and data not shown). The HDACi sensitization appeared to be p53-independent as it was

observed in CLL cells from patients with mutated p53 (Table 1). To confirm this, we used HCT116 cells in which the p53 gene is disrupted by homologous recombination.²⁷ Pretreatment with depsipeptide sensitized both parental and p53^{-/-} HCT116 cells to TRAIL-induced apoptosis to a similar extent (Figure 2), confirming that the ability of HDACi to sensitize cells to TRAIL is p53-independent.

Depsipeptide sensitization was effective at inducing apoptosis in patients with advanced CLL. Cells from all eight patients who were resistant to fludarabine or patients who harbored p53 mutations were sensitized to TRAIL by depsipeptide (Table 1), suggesting that this combination might be effective in this patient population, which overall has a median survival of about 12 months with conventional chemotherapeutic regimens.^{28,29} One valuable prognostic marker in CLL is the mutation rate within the immunoglobulin variable region (*IGHV*) gene sequences, with patients lacking mutations having a markedly inferior prognosis to those who do.^{30,31} Significantly, the combination of HDACi and TRAIL was equally effective in both subgroups of CLL (Table 1). Three patients (#13, 25 and 26) did not show enhanced apoptosis with TRAIL in the presence of HDACi. These patients were heterogeneous in terms of their clinical and *IGHV* mutational status. The mechanism by which these patients showed resistance to HDACi-mediated sensitization is unknown.

Depsipeptide sensitizes U937 and K562 cells to TRAIL-induced apoptosis

To examine if similar variations in the efficacy of different TRAIL preparations were also evident in other cell types, we examined the ability of depsipeptide to sensitize both the monoclastic leukemia cell line U937 and the Philadelphia chromosome-positive acute myeloid leukemia cell line K562 using the different TRAIL preparations.

Depsipeptide (10 nM), valproate (2.5 mM), TRAIL-His-LE (100 ng/ml), TRAIL (100 ng/ml), HGS-ETR1 (50-1000 ng/ml) or HGS-ETR2 (50-1000 ng/ml) alone did not induce significant levels of apoptosis in U937 cells as assessed by phosphatidylserine externalization (Figure 3a). Apo2L/TRAIL

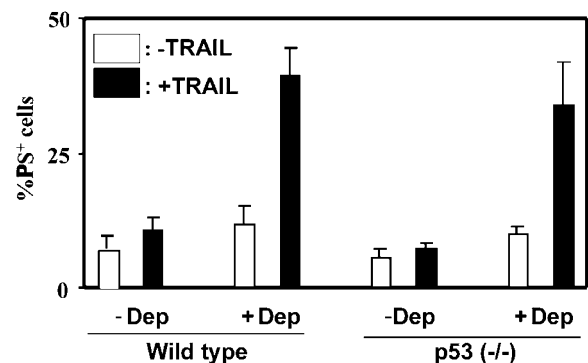


Figure 2 HDACi sensitization to TRAIL-induced apoptosis is p53-independent. Parental and p53^{-/-} HCT116 cells were incubated for 8 h with depsipeptide (10 nM) and then exposed to TRAIL-His (50 ng/ml) for a further 4 h. Apoptosis was then assessed by PS externalization as described in Methods. The results shown represent the Mean \pm S.E.M. of three separate determinations

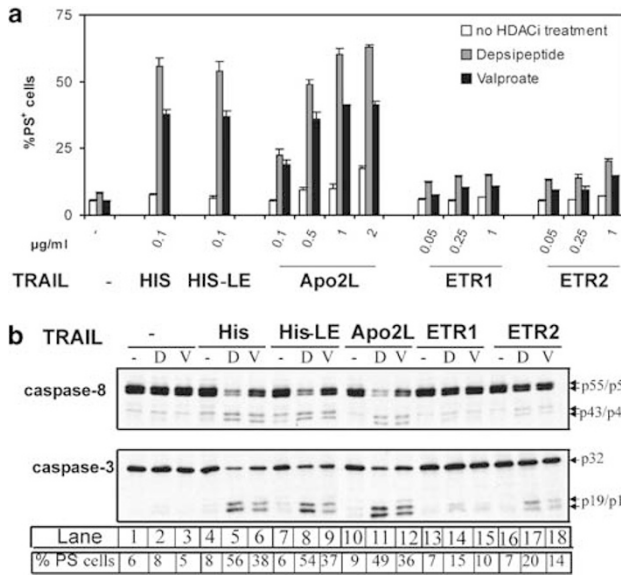


Figure 3 HDAC inhibitors sensitize U937 cells to TRAIL-induced apoptosis. U937 cells were incubated either alone or with depsipeptide (D) (10 nM) or sodium valproate (V) (2.5 mM) for 8 h and then exposed to TRAIL-His (His), TRAIL-His-LE (His-LE) or Apo2L/TRAIL (Apo2L) or to the agonistic mAbs as described in the legend to Figure 1. (a) Apoptosis was assessed by measurement of phosphatidylserine (PS) externalization. The results shown represent the Mean \pm S.E.M. of three separate determinations. (b) Apoptosis was also assessed by processing of caspase-8 and -3

(100 ng/ml) alone did not induce any apoptosis but higher concentrations (500–2000 ng/ml) caused a modest concentration-dependent induction of apoptosis (Figure 3a). Pretreatment with depsipeptide or valproate sensitized U937 cells to the different TRAIL preparations but had only very modest effects with the agonistic Abs (Figure 3a). The reasons for the modest potentiation of the Abs are unclear. Depsipeptide was more effective than valproate in sensitizing the cells ($P \leq 0.05$). The extent of sensitization of U937 cells varied being greatest with TRAIL-His-LE \approx TRAIL-His > Apo2L/TRAIL \gg HGS-ETR2 \approx HGS-ETR1, with TRAIL-His-LE and TRAIL-His being \sim 5-fold more potent than Apo2L/TRAIL.

To confirm that the depsipeptide mediated-sensitization was due to apoptosis, we again examined the effects of the different treatments on caspase processing. In untreated U937 cells, caspase-3 and caspase-8 were present primarily as their unprocessed zymogens (Figure 3b lane 1). In U937 cells exposed to depsipeptide (10 nM) or valproate (2.5 mM) alone or to TRAIL-His (100 ng/ml), TRAIL-His-LE (100 ng/ml), Apo2L/TRAIL (500 ng/ml) and HGS-ETR1 (1 μ g/ml) alone, which did not induce any apoptosis, no processing of either caspase-3 or caspase-8 was observed (Figure 3b lanes 2–4, 7, 10, 13 and 16, respectively). In contrast, in cells pretreated with either depsipeptide or valproate, processing of both caspase-8 to its p43 and p41 fragments and caspase-3 to its p19 and p17 forms was observed in U937 cells exposed to TRAIL-His, TRAIL-His-LE and Apo2L/TRAIL (Figure 3b lanes 5, 6, 8, 9, 11 and 12), commensurate with their induction of apoptosis. Much smaller amounts of processed caspase-8 and caspase-3 were observed in cells pretreated with depsipeptide or valproate and subsequently exposed to

HGS-ETR1 or HGS-ETR2 (Figure 3b lanes 14, 15, 17 and 18), commensurate with the lower levels of apoptosis induced by the agonistic mAbs.

Depsipeptide (10 nM), valproate (2 mM), TRAIL-His (10–100 ng/ml), TRAIL-His-LE (10–100 ng/ml), Apo2L/TRAIL (10–500 ng/ml), HGS-ETR1 (50–1000 ng/ml) or HGS-ETR2 (50–1000 ng/ml) alone induced little or no apoptosis in K562 cells (Figure 4a). Pretreatment with depsipeptide or valproate sensitized K562 cells to the different TRAIL preparations except HGS-ETR1, with depsipeptide being somewhat more effective than valproate (Figure 4a). Marked differences in the efficacy of the different TRAIL preparations to induce apoptosis was noted compared to CLL cells with Apo2L/TRAIL > TRAIL-His-LE \approx TRAIL-His > HGS-ETR2 \gg HGS-ETR1. The greater potency of the Apo2L/TRAIL was most evident at low concentrations of the ligand (Figure 4a). In K562 cells, caspase-3 and caspase-8 were again processed commensurate with the induction of apoptosis induced by the different preparations of TRAIL (Figure 4b). Taken together, these data demonstrate that both depsipeptide and valproate can sensitize U937 and K562 cells to TRAIL-induced apoptosis.

Cell surface receptor expression in tumor cell lines

Differences in cell surface expression of TRAIL-R1 and -R2 receptors may partially explain the variation in activities of

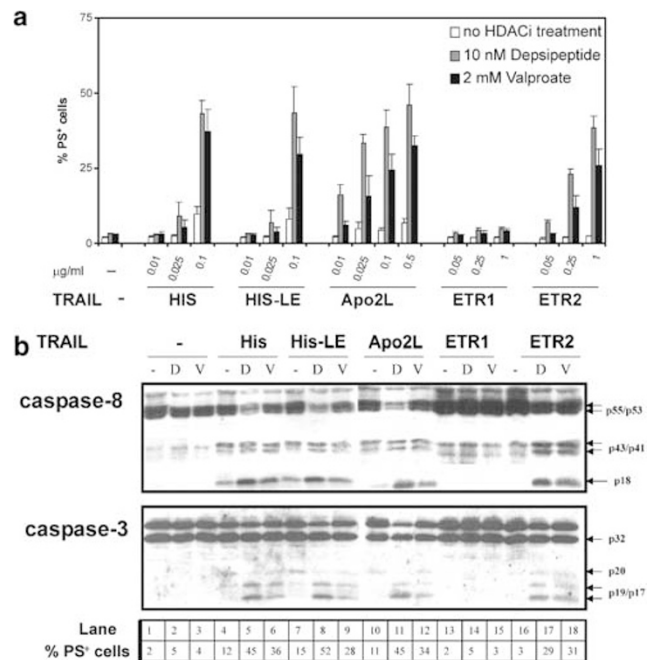


Figure 4 HDAC inhibitors sensitize K562 cells to TRAIL-induced apoptosis by signaling through TRAIL-R2. K562 cells were incubated either alone or with depsipeptide (D) (10 nM) or sodium valproate (V) (2 mM) for 12 h and then exposed to the indicated concentrations of TRAIL-His (His), TRAIL-His-LE (His-LE) or Apo2L/TRAIL (Apo2L) or to the agonistic mAbs as described in the legend to Figure 1. (a) Apoptosis was assessed by measurement of phosphatidylserine (PS) externalization. The results shown represent the Mean \pm S.E.M. of three separate determinations. (b) Apoptosis was also assessed by processing of caspase-8 and -3

different TRAIL preparations, although it has been shown that cell surface receptor expression alone does not account for the differential sensitivity of different cell lines to TRAIL.^{13,32,33} U937 cells display significant surface expression of TRAIL-R2 with little or no surface expression of TRAIL-R1 (Figure 5a). Following treatment of U937 cells with depsipeptide (10 nM) for 8 h, surface expression of TRAIL-R1 was unchanged whereas that of TRAIL-R2 increased slightly (Figure 5a). K562 cells displayed high and low levels of surface expression of TRAIL-R1 and TRAIL-R2, respectively (Figure 5a) but following treatment with depsipeptide, surface expression of TRAIL-R2 increased markedly whereas that of TRAIL-R1 decreased markedly (Figure 5a). Previously we have shown that CLL cells express low levels of both TRAIL-R1 and -R2 receptors but cell surface receptor expression alone does not correlate with susceptibility to apoptosis.^{14,17} Taken together, the data in CLL, K562 and U937 cells demonstrated that there was no clear relationship between cell surface expression of TRAIL-R1 or -R2 and sensitivity to TRAIL-induced apoptosis. Clearly other factors also influence the ability of the receptor to signal for apoptosis.^{13,17,32,33}

Apo2L/TRAIL induces apoptosis primarily via signaling through TRAIL-R2

Although the various TRAIL preparations were active, their activity was clearly dependent on the target cell line or pretreatment with an HDACi. TRAIL-His and TRAIL-His-LE were more potent than Apo2L/TRAIL in U937 cells (Figure 3a) and also in CLL cells (Figures 1a and c), which appear to signal almost exclusively through TRAIL-R1 (Figure 1b and c). However, in K562 cells following HDACi treatment, Apo2L/TRAIL was clearly the most potent particularly at low concentrations and in these cells HGS-ETR2 was much more active than HGS-ETR1 (Figure 4a). Taken together with the other data, this supports the hypothesis that Apo2L/TRAIL is active primarily by signaling through TRAIL-R2. This hypothesis is also consistent with TRAIL having a higher affinity for TRAIL-R2 than other receptors at physiological temperatures.³⁴ These results also suggested that Apo2L/TRAIL was relatively weak at inducing apoptosis via TRAIL-R1, whereas TRAIL-His and TRAIL-His-LE could induce apoptosis by signaling through either TRAIL-R1 or -R2.

To further test this hypothesis, we examined the sensitivity of a cell line that expresses primarily TRAIL-R1 and is sensitive to a TRAIL-R1 mAb, predicting that these cells should be relatively insensitive to Apo2L/TRAIL. Ramos cells expressed primarily cell surface TRAIL-R1 together with a low

surface expression of TRAIL-R2 (Figure 5a). Exposure of Ramos cells to HGS-ETR1 but not HGS-ETR2 showed a concentration-dependent induction of apoptosis (Figure 5b), suggesting that the TRAIL-R1 but not the TRAIL-R2 receptor was active. A dose response comparison of Ramos cells to the different forms of TRAIL showed that the cells were more

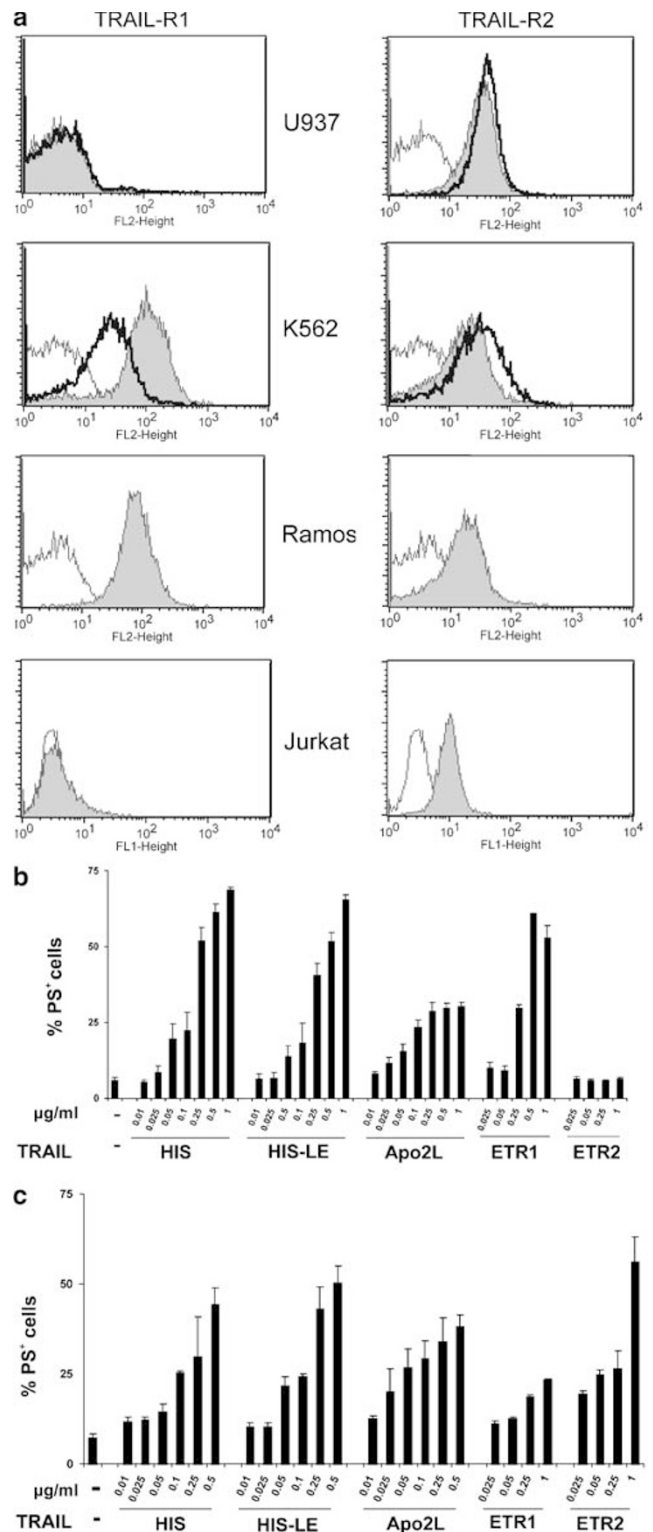


Figure 5 Apo2L/TRAIL induces apoptosis via TRAIL-R2. Cell surface expression levels of TRAIL-R1 and -R2 were measured in (a) U937, K562, Ramos and Jurkat cells. Cells were cultured either alone (gray shading) or in the presence of depsipeptide (10 nM) (dark black line) for 8 h and surface expression assessed by flow cytometry using mAbs to TRAIL-R1 and -R2. Cells labeled with control Ab alone were used to control for background fluorescence (pale line – no shading). (b) Ramos cells and (c) Jurkat cells were incubated for 4 or 8 h, respectively, with the indicated concentrations of TRAIL-His (His), TRAIL-His-LE (His-LE) or Apo2L/TRAIL (Apo2L) or to the agonistic mAbs and apoptosis assessed by phosphatidylserine (PS) externalization. Results are the Mean ± S.E.M. of at least three separate determinations

sensitive to TRAIL-His, TRAIL-His-LE and HGS-ETR1 than to Apo2L/TRAIL. Exposure of the Ramos cells for a further 4 h to Apo2L/TRAIL (1000 ng/ml) resulted in only a small increase of apoptosis from 28 to 39%. We also examined the sensitivity of Jurkat T cells that express surface TRAIL-R2 with no detectable surface expression of TRAIL-R1 (Figure 5a). As expected, these cells were more susceptible to HGS-ETR2 than HGS-ETR1 (Figure 5c). At low concentrations of the ligands, Apo2L/TRAIL appeared somewhat more potent than TRAIL-His and TRAIL-His-LE (Figure 5c). Taken together, these results were compatible with the suggestion that Apo2L/TRAIL signals primarily through TRAIL-R2, whereas TRAIL-His and TRAIL-His-LE can signal through both TRAIL-R1 and -R2, possibly with a preference for TRAIL-R1.

Discussion

Many hematological malignancies, including CLL, remain incurable with current therapy. Overall survival has not improved significantly despite the introduction of new therapeutic agents and the prognosis for certain subgroups of patients, particularly for those with either clinical resistance to fludarabine or mutations of p53, is measured in months.^{28,29} New therapeutic approaches are urgently needed for CLL and related B-cell malignancies.

Significantly, different preparations of TRAIL varied markedly in their ability to induce apoptosis *in vitro* and they acted preferentially via either TRAIL-R1 or -R2 (Figures 1, 3–5). The molecular basis for this selective activation via the two receptors is not known, but together with the data using the receptor-specific mAbs implicates independent regulation of signaling via homotrimeric TRAIL-R1 and -R2 receptors. In addition, sensitivity to the various reagents could not be predicted on the basis of receptor level expression alone. In CLL cells signaling to apoptosis occurred predominantly, if not exclusively, via TRAIL-R1 (Figure 1) despite surface expression of TRAIL-R2.^{14,17} Taken together, our data strongly suggest that it may be essential to test individual tumor types functionally with TRAIL rather than simply on the presence or absence of cell surface TRAIL receptor expression.

We propose that an HDACi in combination with TRAIL or an appropriate mAb signaling predominantly through TRAIL-R1 may be effective therapy for CLL, including patients refractory to fludarabine or with p53 mutations. The possible clinical use of an HDACi has not been extensively investigated in CLL. Sodium valproate acts as an HDACi in CLL cells as evidenced by an increase in acetylated H3 and H4 (data not shown). Doses of sodium valproate, resulting in serum concentrations of 346–693 μ M have been used in patients with myelodysplasia without serious toxicities.³⁵ In preliminary studies, valproate (0.75 mM) potentiated TRAIL-induced apoptosis in CLL cells of some but not all patients. In the present study, valproate (2 mM) was used to ensure a response in all patients but prior to entering a clinical trial, the lowest concentration of valproate that sensitizes CLL cells to TRAIL could be ascertained on an individual basis. HDAC inhibition is a simple means of overcoming TRAIL resistance and may operate in solid malignancies as well as hematological diseases, although the molecular mechanism underlying this

phenomenon remains unclear.^{18,19,36} Based on our current data, we propose that valproate in combination with the agonistic Ab, HGS-ETR1, or alternatively a form of TRAIL that signals predominantly through TRAIL-R1, could be used to obtain proof of principle for the concept of the therapeutic benefit of such combinations. Interestingly, HGS-ETR1 alone was also devoid of activity in CLL cells and activity was only manifested in combination with an HDACi.

Although most studies suggest that TRAIL-R2 is the primary TRAIL receptor leading to cell death,^{9,11,37,38} few studies have used primary tumor cells from patients; the great majority having utilized cell lines. TRAIL had a modest activity in killing primary precursor B-cell acute lymphoblastic leukemic (ALL) cells but no correlation was seen with the levels of receptor expression.¹³ Primary multiple myeloma cells are also killed by TRAIL but the receptor involved is not known.³⁹ Upregulation of TRAIL-R2 was found in several human tumors including liver, breast, and 5/8 lymphomas examined, although changes in TRAIL-R1 were not assessed.³⁸ In this study, the tumoricidal effect of an agonistic TRAIL-R2 Ab was observed without any hepatocyte toxicity.³⁸ It is presently unclear whether our finding that primary CLL cells signal through TRAIL-R1 is restricted to malignant cells of hematopoietic origin or may be a more general feature of primary tumor cells.

Another interesting aspect of the requirement to signal through TRAIL-R1 relates to the potential effects on liver toxicity. The potential of TRAIL to induce liver toxicity was first proposed following its induction of apoptosis in normal primary human hepatocytes.¹² This caused particular concern due to the known liver toxicity of CD95.⁴⁰ Although the toxicity was ascribed to the use of nonoptimized TRAIL preparations, which contain poor zinc coordination and a less ordered conformation,^{11,41} it may also have been due to enhanced oligomerization of TRAIL caused by dithiothreitol present in the purification procedure.⁴² In the present study, we used two forms of His-TRAIL. Analysis of one of these forms, TRAIL-His-LE, showed that it was >99% pure and did not contain dimers or trimers by SDS-PAGE analysis (Alexis Corp.). There are conflicting reports on the presence of TRAIL receptors in human liver. Both TRAIL-R1 and -R2 were proposed to be on the cell surface by virtue of their susceptibility to agonistic mAbs.⁴³ However, another study proposed an absence of cell surface TRAIL-R2 by virtue of its insensitivity to an agonistic mAb.³⁸ Most recently it has been shown that freshly isolated human hepatocytes express no TRAIL-R1 and very small amounts of TRAIL-R2 on the cell surface.⁴⁴ In this elegant study, TRAIL was not hepatotoxic in severe combined immunodeficient/Alb-*uPA* chimeric mice containing human hepatocytes.⁴⁴ Thus, the proposed combination of an HDACi and an agonistic TRAIL-R1 Ab should not exacerbate any potential liver toxicity.

Taken together our results strongly suggest that subtle alterations in different forms of TRAIL can alter their propensity to activate different TRAIL receptors, thus markedly altering their biological activities. The presence of the His-tag or some other change occurring during the purification of both TRAIL-His and TRAIL-His-LE has possibly resulted in a subtle conformational change enabling the ligand to activate more selectively TRAIL-R1. In this regard it is interesting to consider a recent study,³⁷ which showed that mutation of

certain residues in Apo2L/TRAIL resulted in selective binding to either TRAIL-R1 or -R2. As the TRAIL-R2, but not the TRAIL-R1-selective variants, induced apoptosis in selected cell lines, the authors concluded that signaling through TRAIL-R2 rather than -R1 may be more important in TRAIL-induced apoptosis in cancer cells expressing both receptors.³⁷ However, our results suggest that this conclusion is dependent at least in part on the cell lines used. Nevertheless, subtle conformational changes of the TRAIL ligand clearly affect its ability to interact selectively with different receptors.³⁷ In support of their findings, we clearly show that Apo2L/TRAIL is more active than other forms of TRAIL in cells that signal primarily through TRAIL-R2, namely K562 cells (Figure 4a) and to a lesser extent in Jurkat cells (Figure 5c). However, Apo2L/TRAIL is virtually devoid of activity in cells that signal primarily through TRAIL-R1, namely CLL and Ramos cells (Figures 1 and 5). As Apo2L/TRAIL (Genentech) has just entered Phase I clinical trials, our data highlight the importance of assessing the selectivity of different primary tumors to signaling via TRAIL-R1 or -R2 prior to commencement of treatment. Prior knowledge of whether a tumor is activated via TRAIL-R1 or -R2 to induce apoptosis before initiating therapy will facilitate the choice of an appropriate preparation of TRAIL, so maximizing the therapeutic ratio by averting any possible toxicity due to the use of therapeutically inactive TRAIL preparations.

In summary, our data indicate an unanticipated preferential apoptotic signaling via either TRAIL-R1 or -R2 in tumor cells. Thus the rational clinical use of TRAIL-targeted therapy will require prior *in vitro* testing to assess the sensitivities of different tumors to TRAIL-R1 or -R2.

Materials and Methods

Lymphocyte purification, cell lines and culture

Blood samples were obtained from CLL patients, staged according to the Binet system, during routine diagnosis at the Leicester Royal Infirmary with patient consent and local ethical committee approval. Blood samples were analyzed while patients were no longer receiving any chemotherapy. CLL cells were purified as previously described^{14,17} resulting in mean average purity of >95% CD19⁺ CD5⁺ CLL cells. The cells were resuspended in RPMI 1640 medium (5×10^6 cells/ml) at 37°C in an atmosphere of 5% CO₂ and incubated with different preparations of TRAIL or agonistic mAbs to TRAIL-R1 or TRAIL-R2. Cells were also incubated with desipeptide (10 nM) or sodium valproate (1–2.5 mM) for the indicated times (8–16 h) prior to treatment with TRAIL. U937, K562 and Jurkat T cells (clone E6-1) were cultured as previously described.¹⁷ Ramos, an Epstein–Barr virus-negative Burkitt's lymphoma cell line, was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Parental and p53^{-/-} HCT116 cells (provided by Dr. B Vogelstein) were maintained in McCoy's 5A medium supplemented with 10% FBS and Glutamax. Samples were either analyzed immediately for apoptosis or pelleted and stored at -80°C for subsequent Western blotting.

Materials

Media and serum were from Life Technologies, Inc. (Paisley, UK). TRAIL-His-LE (a recombinant TRAIL with low endotoxin – Lot L11447), which was

>99% pure and did not contain dimers or trimers by SDS-PAGE analysis, was obtained from Alexis Corporation (Nottingham, UK). A similar form of human recombinant TRAIL-His was prepared as previously described (but not further purified to reduce endotoxin levels).¹⁴ Apo2L/TRAIL (Genentech), and HGS-ETR1 and HGS-ETR2 were kindly provided by Genentech (South San Francisco, CA, USA) and Human Genome Sciences (Rockville, MD, USA), respectively. Phycoerythrin (PE)-conjugated mouse anti-DR4 and anti-DR5 mAb for flow cytometric detection of cell surface expression of TRAIL-R1 and -R2, respectively, were from eBiosciences Inc. (San Diego, CA, USA). Desipeptide was kindly provided by Dr. E Sausville (NCI, USA) and sodium valproate was from Calbiochem (Nottingham, UK). Caspase antibodies were from sources previously described.⁴⁵ Other reagents were from Sigma (Poole, UK).

Quantification of apoptosis and Western blot analysis

Apoptosis was quantified by phosphatidylserine (PS) externalization in the presence of propidium iodide as previously described.¹⁴ Samples for Western blot analysis were prepared and the processing of caspase-3 and -8 was detected as previously described.⁴⁶

Assessment of cell surface receptor expression

Cells were resuspended in blocking buffer (10% normal goat serum in PBS) and incubated for 30 min on ice to block nonspecific binding, then incubated with anti-TRAIL receptor antibodies (diluted 1:25) or an isotype-matched control antibody for 1 h on ice, washed three times with PBS and then analyzed as previously described.^{14,17}

p53 analysis

DNA was extracted from peripheral blood lymphocytes using the DNeasy tissue kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. PCR was performed using 100 ng of DNA, 2 µM of each primer and 12.5 µl of Extensor readymix (Abgene, Surrey, UK). Cycling parameters were denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 15 s), annealing (60°C for 30 s), extension (68°C for 30 s) and a final extension at 72°C for 10 min in a Techne Touchgene gradient thermal cycler. Primers were generated as described for exons 5–9⁴⁷ and exon 4.⁴⁸

IGHV analysis

IGHV analysis was performed using genomic DNA as a template as previously described using the BIOMED2 primers.⁴⁹ When more than one rearranged IGHV allele was detected, the PCR products were gel eluted and purified using the Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Homology of the rearranged IGHV sequences with the germline IGHV sequences was determined using IgBlast at the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) and aligned using CLUSTALW (<http://www.ebi.ac.uk/clustalw/>).

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