Cell Death and Differentiation (2006) 13, 2160–2162

www.nature.com/cdd

Letter to the Editor

Upregulation of TRAIL-R2 is not involved in HDACi mediated sensitization to TRAIL-induced apoptosis

Cell Death and Differentiation (2006) **13,** 2160–2162. doi:10.1038/sj.cdd.4401977; published online 26 May 2006

Dear Editor,

TNF-related apoptosis inducing ligand (TRAIL) is selectively toxic to tumor cells.[1] TRAIL induces apoptosis by interacting with its two membrane-bound death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5/TRICK2), resulting in the recruitment of FADD/MORT1 and activation of caspase-8 in the death-inducing signaling complex (DISC).[1] Resistance to TRAIL is a major therapeutic problem, which can be overcome by pretreatment with chemotherapeutic agents, including proteasome inhibitors and histone deacetylase inhibitors (HDACi).[2] These combinations act by various proposed mechanisms including a decrease in c-FLIP levels, restoration of caspase-8 expression, increased cell surface expression of death receptors or inhibition of Akt.[3]

Many studies of HDACi-mediated sensitization to TRAIL have proposed that the primary mechanism of sensitization occurs through upregulation of TRAIL-R2.[4–10] Although most of these studies have claimed that this upregulation is responsible for the sensitization, no convincing mechanistic proof of this linkage was provided. Our recent finding that cycloheximide blocked TRAIL-R2 upregulation but not the HDACi-mediated sensitization to TRAIL, did not support this mechanism.[11] However, as nonspecific effects of the protein synthesis inhibitor cycloheximide may have complicated our interpretation, we wished to further investigate the role if any of HDACi-mediated TRAIL-R2 upregulation in the sensitization to TRAIL-induced apoptosis.

Some agents, such as bile acids, potentiate TRAIL-induced apoptosis through a JNK-dependent upregulation of TRAIL-R2.[12] In K562 cells, HDACi, such as depsipeptide, caused an upregulation of TRAIL-R2 together with a downregulation of TRAIL-R1 (Figure 1a) that was preceded by an early transient c-Jun and JNK phosphorylation (15–60 min) (data not shown). Exposure of K562 cells to SP600125 (10–20 μM), a JNK inhibitor,[13] totally inhibited the TRAIL-R2 upregulation but not the loss of TRAIL-R1 (Figure 1a) and the JNK phosphorylation (data not shown). Importantly the depsipeptide-mediated sensitization of TRAIL-induced apoptosis was largely unaffected by coexposure to SP600125 (Figure 1a), suggesting that neither JNK activation nor TRAIL-R2 upregulation was required for the HDACi-mediated sensitization to TRAIL-induced apoptosis in K562 cells.

Depsipeptide also induced upregulation of TRAIL-R2 in both freshly isolated primary chronic lymphocytic leukemic (CLL) cells from patients and in DU145, a human prostatic tumor cell line, and this upregulation was blocked by SP600125 (Figure 1b and c). Thus the upregulation of TRAIL-R2 in K562, CLL and DU145 cells appeared to be JNK dependent. Cells from some patients (4/11) were inherently sensitive to SP600125 alone and could not be used for this study, whereas cells from other patients (7/11) were not inherently sensitive and were used to examine the effect of SP600125 on HDACi-mediated sensitization. Depsipeptide sensitized both CLL and DU145 cells to TRAIL-induced apoptosis in the presence of SP600125 (Figure 1b and c). Thus in CLL, K562 and DU145 cells upregulation of TRAIL-R2 is clearly not required for HDACi-mediated sensitization to TRAIL-induced apoptosis.

Although widely used as a 'specific' JNK inhibitor, SP600125 inhibits other cellular targets at similar or greater potency including serum-and glucocorticoid-induced kinase, p70 ribosomal protein S6 kinase, cyclin-dependent protein kinase 2, casein kinase 1 and dual specificity, tyrosine phosphorylated protein kinase 1A.[14] SP600125 clearly prevented HDACi-mediated upregulation of TRAIL-R2 in a variety of cell types but had variable effects on TRAIL-R1 (Figure 1). Thus our data also support the suggestion that the regulation of TRAIL-R1 and -R2 is different and that activation of JNK or one of the other kinases may be involved in the upregulation of TRAIL-R2 but not in the down-regulation of TRAIL-R1.

In order to obtain direct genetic evidence that upregulation of TRAIL-R2 was not required for HDACi-mediated sensitization to TRAIL, DU145 cells were exposed to varying concentrations of TRAIL-R2 siRNA, which has previously been verified to decrease cell surface expression of TRAIL-R2 without affecting TRAIL-R1.[15] Depsipeptide (10 nM) caused an upregulation of TRAIL-R2 compared to control cells (Figure 1d compare lanes 1 and 2) and this upregulation was unaffected by transfection with a negative control RNAi oligonucleotide (Figure 1d compare lanes 2 and 3) but was decreased in a concentration dependent manner by TRAIL-R2 siRNA (Figure 1d lanes 4–6). Transfection of the siRNA for TRAIL-R2 had no effect on levels of TRAIL-R1 (Figure 1d top panel) confirming its specificity.[15] DU145 cells signal to apoptosis through both TRAIL-R1 and TRAIL-R2, as HDACi sensitized these cells to both TRAIL-R1 and -R2 agonistic Abs (unpublished data). To assess the role of TRAIL-R2 upregulation in sensitization to TRAIL, we examined the effects of TRAIL-R2 knockdown on HDACi-mediated sensitization to apoptosis induced by TRAIL and HGS-ETR2, a TRAIL-R2-specific agonistic Ab.[16] No apoptosis was observed in control cells following transfection of siRNA oligonucleotides either

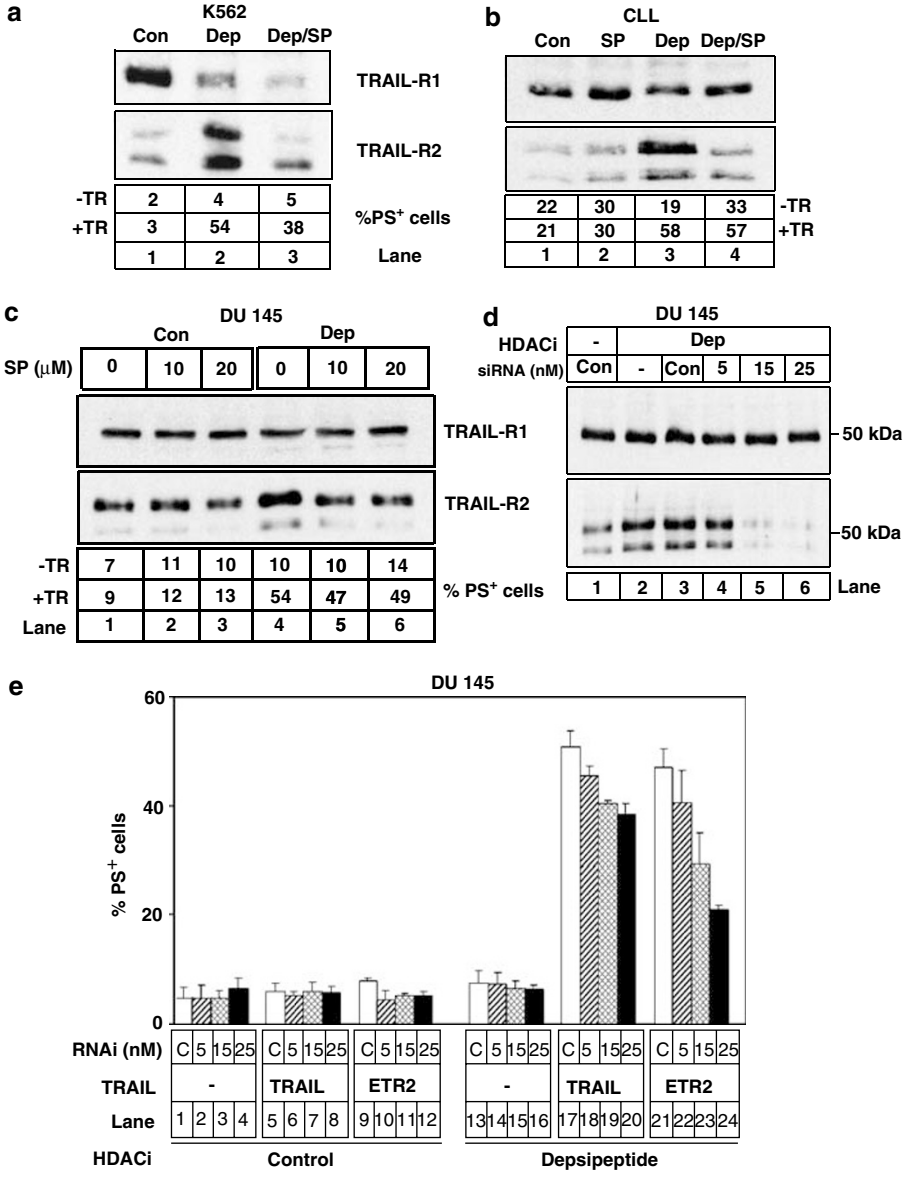

Figure 1 HDACi-mediated upregulation of TRAIL-R2 is not required for TRAIL-induced apoptosis. (**a**) K562 were incubated for 12 h either alone (Con) with depsipeptide either alone (Dep, 10 nM) or in the presence of SP600125 (20 μM) (Dep/SP). Following this incubation, either cell lysates were prepared and analyzed by Western blotting for TRAIL-R1 and -R2 or cells were incubated for a further 4 h in the presence of TRAIL (100 ng ml^{-1}) and apoptosis assessed by phosphatidylserine (PS) externalization.[11] The presence or absence of TRAIL is indicated by $+$TR or $-$TR, respectively. (**b**) CLL cells or (**c**) DU145 cells were incubated either alone or with depsipeptide (Dep) (10 nM) either for 16 h (CLL) or 8 h (DU145) and in the presence (20 μM in CLL) or absence of the indicated concentration of SP600125 (SP). Following incubation, cell lysates were analyzed by Western blotting for TRAIL-R1 and -R2 or cells were exposed for a further 4 h to TRAIL (100 ng ml^{-1}) and apoptosis assessed. Similar results were obtained with cells from seven individual CLL patients. (**d**) DU145 cells were seeded into six-well plates at 1.5×10^5 cells per well immediately prior to RNAi transfection with either a negative control siRNA (15 nM) (Con, **c**) or siRNAs for TRAIL-R2 (5–25 nM) and 10 μl Effectene (Qiagen). A negative control siRNA (#4611) and preannealed TRAIL-R2 siRNA oligonucleotides, with the sequence and validity described,[15] were from Ambion (Austin, TX, USA). After 40 h of transfection, the medium was changed and depsipeptide (Dep) (10 nM) added and incubated for a further 8 h and total TRAIL-R1 or TRAIL-R2 assessed.[11] (**e**) Following pretreatment with depsipeptide as described in (**d**), cells were exposed for a further 4 h to recombinant TRAIL (100 ng ml^{-1}) or the agonistic HGS-ETR2 antibody (0.5 μg ml^{-1}, Human Genome Sciences) and apoptosis assessed by phosphatidylserine (PS) externalization. Results are expressed as the Mean $\pm$ S.D. of three individual experiments

alone or in the presence of depsipeptide (Figure 1e lanes 1–4 or 13–16, respectively) or in the presence of TRAIL or HGS-ETR2 (Figure 1e lanes 5–8 and 9–12, respectively). Pretreatment with depsipeptide sensitized to both TRAIL- and HGS-ETR2-induced apoptosis in the presence of the control RNAi oligonucleotide (Figure 1e lanes 17 and 21). Knockdown of TRAIL-R2 caused only a modest decrease in TRAIL-induced apoptosis (Figure 1e lanes 19–20) despite the almost complete loss of TRAIL-R2 expression at the higher siRNA concentrations (Figure 1d lanes 5–6). As DU145 cells can signal through both TRAIL-R1 and TRAIL-R2, this modest loss was most probably due to the cells signaling through

TRAIL-R1 following knockdown of TRAIL-R2. To overcome this problem, we also used HGS-ETR2. Following pretreatment with depsipeptide, siRNA for TRAIL-R2 caused a concentration-dependent reduction in HGS-ETR2-induced apoptosis (Figure 1e lanes 22–24). Most strikingly, sensitization to HGS-ETR2-induced apoptosis was still evident (Figure 1e lanes 23–24) in the complete inhibition of TRAIL-R2 upregulation and even in the presence of a decreased TRAIL-R2 expression compared to control cells (Figure 1d compare lanes 5 and 6 with lane 1).

Thus, our data on TRAIL-R2 knockdown and the experiments with SP600125 (Figure 1) demonstrate unequivocally that TRAIL-R2 upregulation is not required for HDACi-mediated sensitization to TRAIL-induced apoptosis. Our conclusions are in marked contrast to those studies claiming the importance of upregulation of TRAIL-R2 in HDACi-mediated sensitization to TRAIL.[4–10] In agreement with these studies, we observe an HDACi-mediated upregulation of TRAIL-R2. However, we show conclusively that this upregulation is not required for sensitization to TRAIL-induced apoptosis.

Our study has dissociated HDACi-mediated upregulation of TRAIL-R2 from sensitization to TRAIL-induced apoptosis. Similarly, upregulation of TRAIL-R2 was not critical for 5-fluorouracil-mediated sensitization of hepatoma cells to TRAIL.[17] Although upregulation of TRAIL-R2 by diverse agents, including proteasome inhibitors, bile acids, sulforaphane and methyl dihydroquercetin has been proposed to be important in the sensitization of different cell types to TRAIL-induced apoptosis, a mechanistic link has not been conclusively demonstrated.[12,18–20] However, two of these studies utilized siRNA to address the contribution of receptor upregulation to increased TRAIL sensitivity.[19,20] In the former study, knockdown of TRAIL-R2 resulted in a decrease of cell surface TRAIL-R2 expression below base line accompanied by a decreased ability of methyl dihydroquercetin to sensitize to TRAIL.[19] While this data may be compatible with a role for the upregulation of TRAIL-R2 in the sensitization, it required more definitive substantiation. In the latter study, siRNA for TRAIL-R2 in Hep3B cells also resulted in a knockdown of TRAIL-R2 expression below basal levels and as this receptor is involved in TRAIL-induced apoptosis in these cells, it is not unexpected that a loss of the sensitization to TRAIL was observed.[20] Furthermore, as the specificity of the TRAIL-R2 siRNA oligonucleotides was not demonstrated, it is difficult to agree with the conclusion that the upregulation of TRAIL-R2 was essential for the sensitization to TRAIL.[20] Thus, our present data highlight the necessity to re-examine the exact contribution, if any, of the upregulation of TRAIL-R2 to the reported increase in TRAIL sensitivity by a variety of diverse agents.

In summary, we clearly demonstrate that in several cell types upregulation of TRAIL-R2 is not required for HDACi-mediated sensitization of cells to TRAIL-induced apoptosis despite many claims to the contrary in the literature. Our results also suggest that it may be prudent to re-examine the claims that many chemotherapeutic agents sensitize cells to TRAIL-induced apoptosis by upregulation of TRAIL-R2.

Acknowledgements

We thank Drs Marion MacFarlane and Nick Harper for helpful discussions and advice on the siRNA. This work was supported by the Medical Research Council.

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