PERGAMON

European Journal of Cancer 38 (2002) 177–183

European Journal of Cancer

www.ejconline.com

Trail activity in human ovarian cancer cells: potentiation of the action of cytotoxic drugs

S. Vignati, A. Codegoni, F. Polato, M. Broggini*

Laboratory of Molecular Pharmacology, Department of Oncology, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy

Received 1 May 2001; received in revised form 30 July 2001; accepted 21 September 2001

Abstract

The ability of the TRAIL ligand to induce cell killing in three ovarian cancer cell lines was investigated using a glutathione-S-transferase (GST)-TRAIL fusion protein. One of the three lines was sensitive to TRAIL, which induced cell killing in a range of concentrations similar to those necessary to kill the TRAIL-sensitive leukaemic cell line Jurkat. The relative mRNA expression of the four TRAIL receptors did not explain the different sensitivities of the three ovarian cancer cell lines to TRAIL treatment. The TRAIL-sensitive IGROV-1 cell line expressed slightly lower levels of the anti-apoptotic protein FLIP than the two TRAIL-insensitive cell lines (A2780 and SKOV-3). Nevertheless, although TRAIL did not significantly reduce cell growth in the A2780 and SKOV-3 cells it did enhance the activity of paclitaxel and cisplatin (DDP), the two most widely used drugs for the treatment of ovarian cancer, increasing their ability to induce apoptosis. The use of TRAIL in combination with classical anticancer agents might thus boost the apoptotic response, improving the activity of DDP and paclitaxel in ovarian cancer.

Keywords: Apoptosis; Decoy receptors; Cisplatinum; Taxol (paclitaxel); Ovarian cancer

1. Introduction

Despite the introduction of new drugs for the treatment of ovarian cancer, the overall survival of patients suffering from this malignancy is far from satisfactory. One possible reason is that current treatments do not sufficiently induce apoptosis in cancer cells, a mechanism that is believed to be fundamental for the success of treatment. Research with ovarian cancer cells *in vitro* does in fact suggest that these cells show limited propensity for apoptosis compared with other cell types [1,2]. We recently demonstrated that forcing the drug-induced, p53-dependent activation of the proapoptotic gene *bax* strongly enhances the activity of paclitaxel, one of the most active drugs in the treatment of ovarian cancer [3], both *in vitro* and *in vivo* [4]. It therefore appears important to clarify whether ovarian cancer cells have impaired apoptotic pathways, either as a result of weak functioning of the apoptotic machinery, or amplification of survival factors and pathways.

The majority of ovarian cancer cell lines have an amplification of the *PIK3CA* gene [5] which can constitutively activate the survival factor akt, resulting in the inhibition of apoptosis. Another factor possibly involved in the lack of sustained drug-induced apoptosis is p53, which is mutated in ovarian cancer [6,7] and this could result in the reduced activation of p53-dependent proapoptotic genes such as *bax*, *PIGs*, *Fas* and others [8–11]. The possibility of combining anticancer agents and compounds that can reverse the 'anti-apoptotic phenotype' should ultimately increase the response of ovarian cancer cells to drug treatment.

The TRAIL ligand, a member of the tumour necrosis family (TNF) family, is one attractive candidate to be used in combination with anticancer agents. It is a potent inducer of apoptosis in various cancer cell lines [12–14]. It activates apoptosis through binding to different receptors, TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4; the first two can transduce the signal intracellularly, and the last two work as decoy receptors [15]. The presence and relative abundance of these

* Corresponding author. Tel.: +39-02-390-14472; fax: +39-02-354-6277.

E-mail address: broggini@marionegri.it (M. Broggini).

0959-8049/02/$ - see front matter
PII: S0959-8049(01)00345-8

receptors is an important factor in cell responses to TRAIL [16]. In addition—and probably most importantly—the expression level of the FLIP protein, which interacts with and inhibits TRAIL signalling, appears to be an important determinant of TRAIL activity [16–18]. Finally, TRAIL increases the cellular response to cytotoxic drugs in different cancer types [19–21], an important consideration that prompted us to study the possible synergism between TRAIL and anticancer drugs in ovarian cancer cells. We report the results obtained in ovarian cancer cells by combining TRAIL with cisplatin or paclitaxel, the two most widely used anticancer agents in this disease [3,22,23].

2. Materials and methods

2.1. Cell culture and treatment

Human ovarian cancer cell lines IGROV-1, A2780 and SKOV-3 and the human leukaemic cell line Jurkat were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS). Recombinant human glutathione-S-transferase (GST)-TRAIL (kindly supplied by Dr S. Lipkowitz, NCI, Bethesda, MD, USA) and GST were freshly prepared from bacterial cultures according to the published method in Ref. [19]. Cisplatin (cis-diamine-dichloro-platinum), DDP (Sigma, Milan, Italy) and paclitaxel (Bristol Myers Squibb, USA) were dissolved in medium immediately before use. Drug-induced cytotoxicity was evaluated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test: briefly, cells (2500/100 μl) were seeded in 96-well plates 72 h before treatment. Different concentrations of purified GST or GST-TRAIL were added and left for 72 h before the addition of 20 μl of MTT (6 mg/ml in saline). Cisplatin (DDP) treatment was for 1 h and paclitaxel treatment for 24 h. At the end of treatment, cells were washed and left in drug-free medium for 72 h before the addition of MTT. When used in combinations in TRAIL-resistant cells, GST and GST-TRAIL (25 ng) were added to the cells together with DDP and paclitaxel and, after washing, maintained in medium until the addition of MTT. At least three independent experiments, each consisting of six replicates were performed.

2.2. RNA extraction and polymerase chain reaction (PCR) analysis

Total RNA was extracted by the guanidium thiocyanate-caesium chloride method [24]. First-strand cDNA synthesis was done using 2 μg total RNA in 20 μl of total reverse transcription volume using a RNA PCR Core kit (Perkin-Elmer, Italy). To study the expression of death receptors TRAIL-R1, R2, and R3, 2 μl of the cDNAs were amplified in a total volume of 25 μl containing 2 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTPs) 2 μM primers, 1 μCi of α-[^{32}P] deoxycytidine-triphosphate (3000Ci/mmole, Amersham) and 0.5 U of Taq DNA polymerase. The sequences of primers used were:

TRAIL-R1 F, 5′-CGATGTGGTCAGAGCTGGTACAGC,

TRAIL-R1 R, 5′-GGACACGGCAGAGCCTGTGCCATC

TRAIL-R2 F, 5′-GGGAGCCGCTCATGAGGAAGTTGG

TRAIL-R2 R, 5′-GGCAAGTCTCTCTCCCAGCGTCTC

TRAIL-R3 F, 5′-GTTTGTTTGAAAGACTTCACTGTG

TRAIL-R3 R,5′-GCAGGCGTTTCTGTCTGTGGGAAC.

Amplification consisted of one cycle at 95 °C for 5 min, followed by 23 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, (for *TRAIL-R1* and *TRAIL-R2*) and 24 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s (for *TRAIL-R3*). Human *actin* was used as internal control for the reverse transcription and was amplified from the same cDNA. PCR products were separated on 7% polyacrylamide gel electrophoresis (PAGE), and the gels were dried and autoradiographed. Band intensities were quantitated using a Phosphorimager Storm 860.

To analyse the expression of *TRAIL-R4* and *FLIP*, cDNAs obtained as described above were amplified in a final volume of 25 μl containing 0.5 μM of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$,and 0.5U of Taq DNA polymerase (*β-actin* was used as control). The primer sequences for *TRAIL-R4* were 5′CTTTTCCGGCGGCGTTCATGTCCTTC and 5′GTTTGTTCCAGGCTGCTTCCCTTTGTAG and 5′AATTCAAGGCTCAGAAGCA and 5′GGCAGAAACTCTGCTGTTCC for *FLIP*.

The thermal cycle was: one cycle at 96, 60 and 72 °C for 1 min; 35 cycles of 96 °C for 30 s, 60 °C for 35 s and 72 °C for 1 min. PCR products were separated on 1.5% agarose gels and band intensities were evaluated using Image Master Total lab Software (Pharmacia).

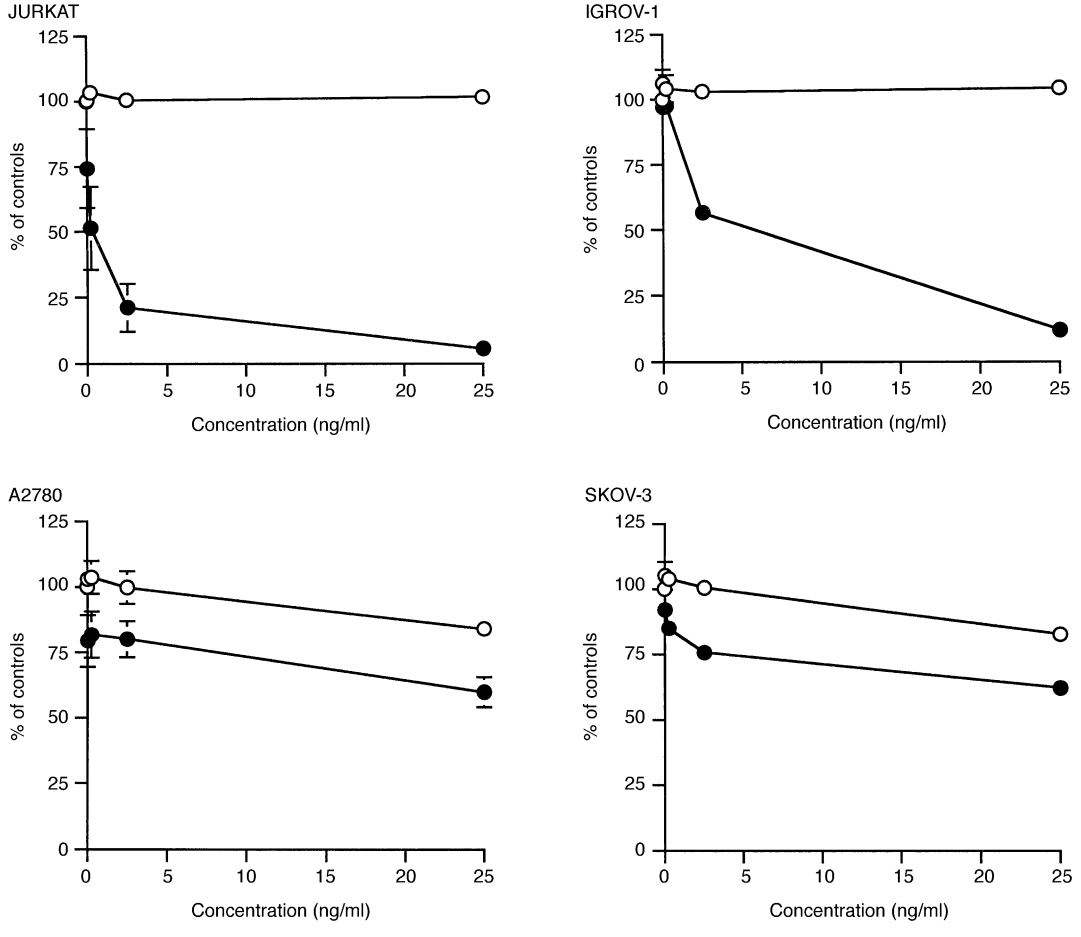
2.3. Evaluation of apoptosis

To evaluate apoptosis, cells were seeded on glass coverslips in 24-well plates and treated with the different drugs and combinations as for the evaluation of cytotoxicity. After 24 and 48 h, cells were fixed in 70% ethanol, washed and stained with 4′,6′-diamino-2-phenylindole (DAPI) and sulphorhodamine B [1]. The percentage of cells with apoptotic morphology was determined by two independent scientists in double blind analysis by counting different fields under a fluorescent microscope, each consisting of at least 100 cells.

3. Results

The three ovarian cancer cell lines showed different sensitivities to TRAIL (Fig. 1). IGROV-1 cells were killed by low concentrations of TRAIL (IC_{50} 2.7 ng/ml), in a range similar to that necessary to kill the TRAIL-sensitive leukaemic cell line Jurkat (IC50 0.42 ng/ml). By contrast, SKOV-3 and A2780 cells were resistant to TRAIL which in fact had no significant activity at concentrations 100–1000 times those active on IGROV-1. In all these experiments, recombinant GST, used as an internal control, had no noticeable effect.

Trying to correlate the different effects of TRAIL with the receptor status in the four cell lines, we used reverse transcriptase (RT)-PCR to quantitate the mRNA levels of *TRAIL-R1*, *TRAIL-R2*, *TRAIL-R3* and *TRAIL-R4* (Fig. 2, panels a and b). *TRAIL-R1* mRNA was detected in SKOV-3, A2780 and IGROV-1 cells with relatively high levels in SKOV-3 cells. The other transducing receptor, *TRAIL-R2*, was also measurable and its levels were lower in IGROV-1 cells than in A2780 and SKOV-3 cells. The decoy receptor *TRAIL-R3* was expressed at similar levels in SKOV-3 and A2780 cells, but lower amounts were seen in the IGROV-1 cells. Similarly, the levels of *TRAIL-R4* mRNA were lower in IGROV-1 cells than the A2780 and SKOV-3 cells. The TRAIL-sensitive leukaemic cell line JURKAT expressed the two signalling receptors *TRAIL-R1* and *TRAIL-R2*, but showed very low levels of the decoy receptors *TRAIL-R3* and particularly *TRAIL-R4*.



IC50 values (ng/ml)

	GST-Trail	GST
JURKAT	0.42	>100
IGROV-1	2.7	>100
SKOV-3	>100	>100
A2780	>100	>100

Fig. 1. Cytotoxicity of glutathione-S-transferase (GST)-TRAIL (●) and GST (○) in Jurkat, IGROV-1, A2780 and SKOV-3 cells. Each value is the mean±standard deviation (S.D.) of three different experiments each consisting of six replicates. The concentrations inhibiting the growth by 50% (IC_{50}) are listed at the bottom of the figure.

We also tested the mRNA levels for the inhibitor *FLIP*. This inhibitor was present at lower levels in the Jurkat cells, whereas the TRAIL-sensitive ovarian cell line IGROV-1 had only a slightly lower level than the TRAIL-insensitive cell lines A2780 and SKOV-3. In the two latter lines, we checked whether TRAIL, which alone was uneffective, could increase the sensitivity to DDP and paclitaxel, two drugs that are widely used for the therapy of ovarian cancer.

In the SKOV-3 cells, 25 ng of TRAIL, which alone did not reduce cell number, boosted the sensitivity to DDP (Fig. 3). The dose of 50 μM DDP alone caused 33% growth inhibition, which became 70% when combined with TRAIL. Co-treatment with GST did not increase the inhibition by DDP alone (35%). This effect was also observed at lower DDP concentrations (25 μM DDP caused 13% inhibition, while 25 μM DDP plus 25 ng of TRAIL caused 42% inhibition).

Similarly, the activity of paclitaxel was increased by co-treatment with TRAIL. Fig. 4 reports the results using 25 ng of TRAIL in combination with 10 or 100 nM paclitaxel. At the latter concentration, the addition of TRAIL increased the activity of paclitaxel from 55 to 83%.

Using the same treatment conditions, TRAIL also boosted DDP and paclitaxel activity in the A2780 cells (Fig. 5a and b). For example, 50 μM DDP resulted in 54% inhibition of growth which became 73% when given in combination with TRAIL. Again, GST alone did not change the activity of DDP. The addition of TRAIL to 1 nM paclitaxel reduced the surviving cells from 20 to 10%.

To test whether the ability of TRAIL to boost the cytotoxic activity of paxlitaxel and DDP was due to an increase in the number of apoptotic cells, we calculated the percentage of apoptotic cells in the same treatment conditions as for cytotoxicity. Fig. 6 reports the results with SKOV-3 cells. GST-TRAIL and GST alone did not increase the basal level of apoptosis. DDP alone did not induce significant apoptosis which was clearly observable with paclitaxel. In combination, GST-TRAIL but not GST raised the number of apoptotic cells, the increase being greater for the combination with DDP. Similar results were obtained in A2780 cells (data not shown).

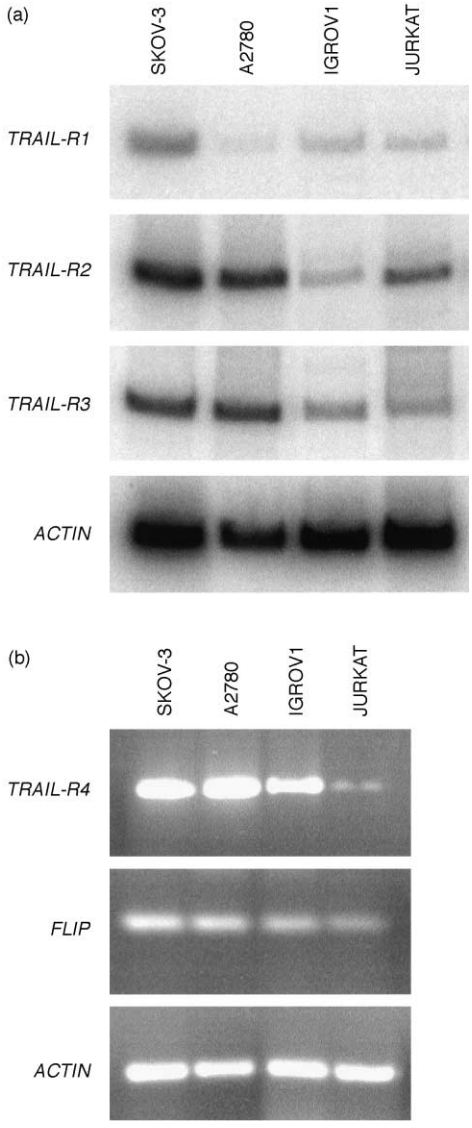


Fig. 2. mRNA expression of the four TRAIL receptors (*TRAIL-R1*, *TRAIL-R2*, *TRAIL-R3*, *TRAIL-R4*) and *FLIP* in Jurkat, IGROV-1, A2780 and SKOV-3 cells, evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) (panels a and b).

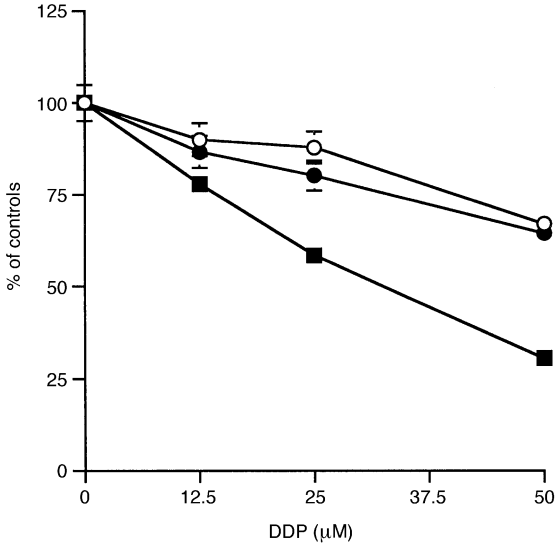


Fig. 3. Concentration-dependent activity of DDP alone (●) or with 25 ng of glutathione-S-transferase (GST) (○) or 25 ng of GST-TRAIL (■) in SKOV-3 cells. Each value is the mean±standard deviation (S.D.) of at least three experiments, each consisting of six replicates.

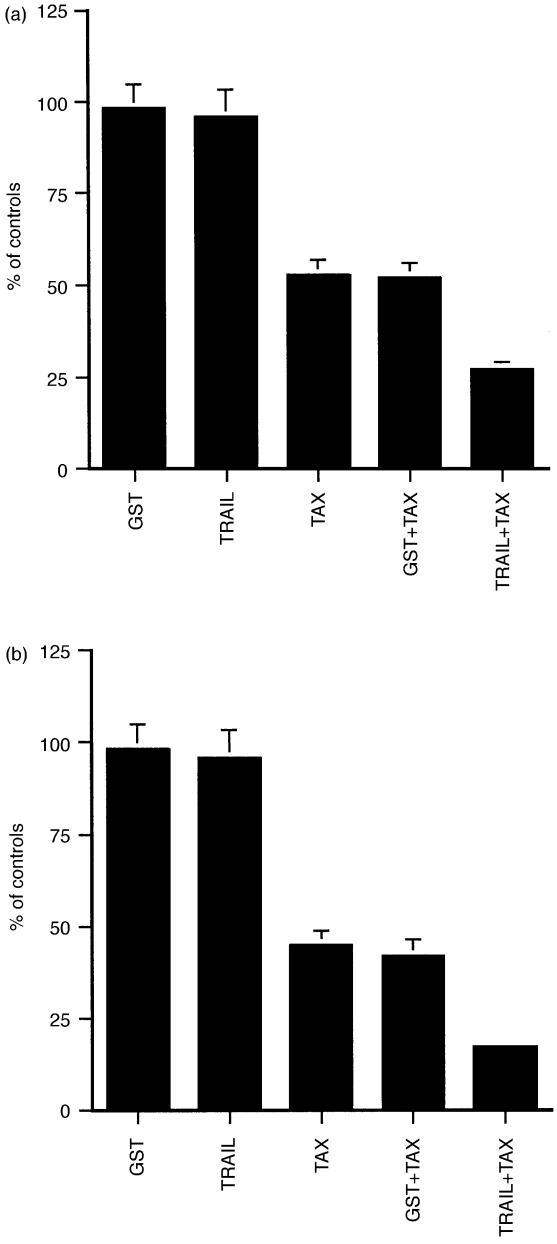


Fig. 4. Inhibition of SKOV-3 cell growth by 10 nM (panel a) or 100 nM (panel b) paclitaxel alone or in combination with glutathione-S-transferase (GST) or GST-TRAIL. Each value is the mean±standard deviation (S.D.) of at least three experiments, each consisting of six replicates.

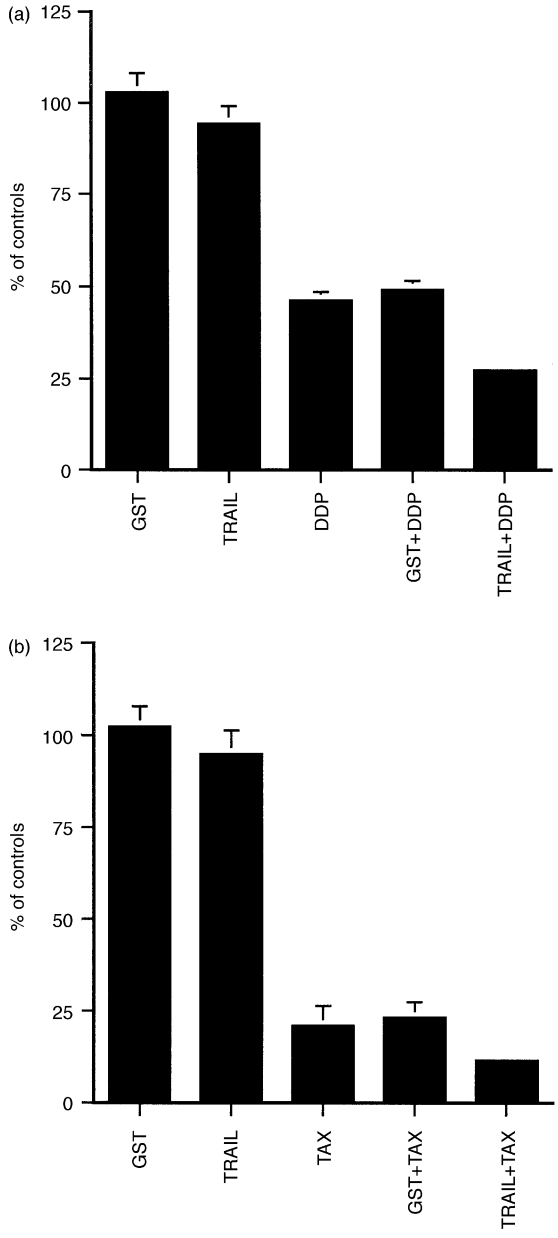


Fig. 5. Inhibition of growth in A2780 cells. Panel a: 50 μM DDP alone or in combination with glutathione-S-transferase (GST) or GST-TRAIL. Panel b: 1 nM paclitaxel alone or in combination with GST or GST-TRAIL. Each value represents the mean±standard deviation (S.D.) of two independent experiments each consisting of six replicates.

4. Discussions

The TRAIL ligand has been reported to induce apoptosis in different cellular systems *in vitro* and *in vivo*, although only a small percentage of cell lines shows sensitivity to this ligand [10,18,25–27]. We used a GST-TRAIL fusion protein reported to induce apoptosis in TRAIL-sensitive breast cancer cells [19]. One of three ovarian cancer cell lines examined here did show sensitivity to TRAIL, which was active at concentrations similar to those necessary to kill the TRAIL-sensitive leukaemic cell line Jurkat. The two other lines did not respond even to high concentrations of TRAIL.

The relative expression of the four TRAIL receptors might partly explain the cell sensitivity to TRAIL [16,28]. This may be true for the leukaemic cell line Jurkat, which expresses the two TRAIL-transducing receptors TRAIL-R1 and TRAIL-R2, but has very low levels of the decoy receptors TRAIL-R3 and TRAIL-R4, but we could not find any clear difference between

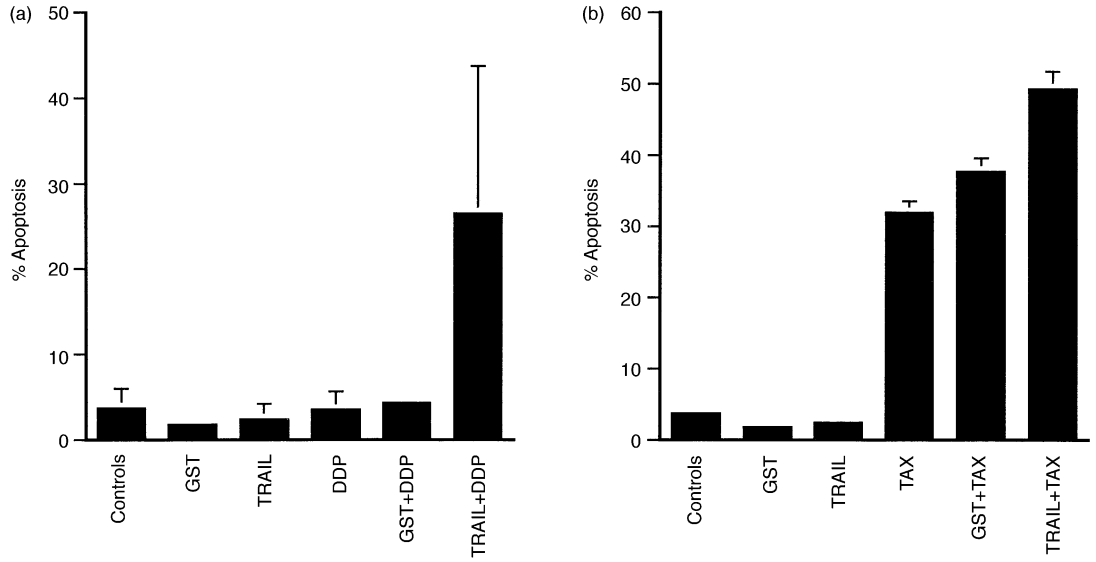


Fig. 6. Induction of apoptosis in SKOV-3 cells by DDP (50 μm) (panel a) or paclitaxel (10 nM) (panel b) alone or in combination with glutathione-S-transferase (GST) or GST-TRAIL. Values represent the mean ± standard deviation (S.D.) of at least four fields in which at least 100 cells were counted.

the TRAIL-sensitive and the two TRAIL-insensitive ovarian cancer lines. These data are in agreement with reports regarding other non-haematological cell systems where the receptor status did not correlate with the sensitivity to TRAIL [25]. Although the small number of cell lines examined here does not allow any firm conclusion for ovarian cancer cells, it seems that the levels of the apoptosis inhibitory protein FLIP may indeed influence cell sensitivity to TRAIL. This would be in agreement with previous reports in cell lines of different origin [16,17].

Cell lines not responding to TRAIL *in vitro* were reported to be more prone to apoptosis when TRAIL was combined with anticancer agents, particularly with doxorubicin and topoisomerase I inhibitors [19–21]. We extended these studies by combining TRAIL with the two most widely used drugs for the treatment of ovarian cancer, paclitaxel and DDP [22,23] in the two TRAIL-insensitive ovarian cancer cell lines.

Concentrations of TRAIL that were completely inactive on their own, boosted the activity of both paclitaxel and DDP in the A2780 and in SKOV-3 cell lines. This correlates well with the increased ability of these two drugs when combined with TRAIL to induce apoptosis. The two cell lines express a wt-p53 and no p53, respectively, suggesting, in agreement with what has been reported in breast cancer cell lines [19], that TRAIL increases the sensitivity of paclitaxel and DDP by a p53-independent mechanism. In addition, the effects observed here are specific for TRAIL since GST alone did not affect the activity of either paclitaxel or DDP.

We previously reported that paclitaxel and DDP treatment of different ovarian cancer cell lines resulted in a relatively limited induction of apoptosis [1,2]. However, by forcing the expression of the proapoptotic gene *bax* after paclitaxel treatment of the ovarian cancer cell line A2780, there was a strong increase in paclitaxel activity and induction of apoptosis through caspase activation [4], clearly suggesting that the apoptotic machinery involving activation of caspases is functional in this cell line. This fits well with the proposed mechanism by which TRAIL increases the apoptosis induced by doxorubicin which would be an easier induction of caspase activation [10,19].

The finding that TRAIL can boost the activity of paclitaxel and DDP in ovarian cancer cell lines could have important therapeutic implications for the treatment of ovarian cancer.

Acknowledgements

We are particularly indebted to Dr Stan Lipkowitz (NCI, Bethesda, MD) for the generous supply of GST-TRAIL. The generous contribution of the Italian Association for Cancer Research (AIRC) and of the Italian Ministry of Health is gratefully acknowledged. Sara Vignati is a recipient of a "Famiglie Belloni e Guglielmetti" fellowship.

References

1. Debernardis D, Sire EG, De Feudis P, *et al.* p53 status does not affect sensitivity of human ovarian cancer cell lines to paclitaxel. *Cancer Res* 1997, **57**, 870–874.
2. De Feudis P, Debernardis D, Beccaglia P, *et al.* DDP-induced cytotoxicity is not influenced by p53 in nine human ovarian cancer cell lines with different p53 status. *Br J Cancer* 1997, **76**, 474–479.
3. Rowinsky EK, Donehower RC. Paclitaxel (taxol). *N Engl J Med* 1995, **332**, 1004–1014.

4. De Feudis P, Vignati S, Rossi C, *et al*. Driving p53 response to Bax activation greatly enhances sensitivity to taxol by inducing massive apoptosis. *Neoplasia* 2000, **2**, 202–207.
5. Shayesteh L, Lu Y, Kuo WL, *et al*. PIK3CA is implicated as an oncogene in ovarian cancer. *Nature Genet* 1999, **21**, 99–102.
6. Harris CC. The 1995 Walter Hubert Lecture—molecular epidemiology of human cancer: insights from the mutational analysis of the p53 tumour-suppressor gene. *Br J Cancer* 1996, **73**, 261–269.
7. Hollstein M, Soussi T, Thomas G, von Brevern MC, Bartsch H. P53 gene alterations in human tumors: perspectives for cancer control. Recent.Results. *Cancer Res* 1997, **143**, 369–389.
8. Ko LJ, Prives C. p53: puzzle and paradigm. *Genes Dev* 1996, **56**, 2649–2654.
9. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000, **408**, 307–310.
10. Grotzer MA, Eggert A, Zuzak TJ, *et al*. Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. *Oncogene* 2000, **19**, 4604–4610.
11. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev* 1996, **10**, 2438–2451.
12. Kastan M. On the TRAIL from p53 to apoptosis? *Nature Genet* 1997, **17**, 130–131.
13. Ahmad M, Shi Y. TRAIL-induced apoptosis of thyroid cancer cells: potential for therapeutic intervention. *Oncogene* 2000, **19**, 3363–3371.
14. Herr I, Posovszky C, Di Marzio LD, Cifone MG, Boehler T, Debatin KM. Autoamplification of apoptosis following ligation of CD95-L, TRAIL and TNF-alpha. *Oncogene* 2000, **19**, 4255–4262.
15. French LE, Tschopp J. The TRAIL to selective tumor death. *Nature Med* 1999, **5**, 146–147.
16. Kim K, Fisher MJ, Xu SQ, el-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* 2000, **6**, 335–346.
17. Irmler M, Thome M, Hahne M, *et al*. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997, **388**, 190–195.
18. Walczak H, Miller RE, Ariail K, *et al*. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nature Med* 1999, **5**, 157–163.
19. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* 1999, **59**, 734–741.
20. Gibson SB, Oyer R, Spalding AC, Anderson SM, Johnson GL. Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. *Mol Cell Biol* 2000, **20**, 205–212.
21. Gliniak B, Le T. Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity in vivo is enhanced by the chemotherapeutic agent CPT-11. *Cancer Res* 1999, **59**, 6153–6158.
22. Thigpen JT. Chemotherapy for advanced ovarian cancer: overview of randomized trials. *Semin Oncol* 2000, **27**, 11–16.
23. Partridge EE, Barnes MN. Eputhelial ovarian cancer: prevention, diagnosis, and treatment. *CA Cancer J Clin* 1999, **49**, 297–320.
24. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989.
25. Zhang XD, Franco A, Myers K, Gray C, Nguyen T, Hersey P. Relation of TNF-related apoptosis-inducing ligand (TRAIL) receptor and FLICE-inhibitory protein expression to TRAIL-induced apoptosis of melanoma. *Cancer Res* 1999, **59**, 2747–2753.
26. Kim CH, Gupta S. Expression of TRAIL (Apo2L), DR4 (TRAIL receptor 1), DR5 (TRAIL receptor 2) and TRID (TRAIL receptor 3) genes in multidrug resistant human acute myeloid leukemia cell lines that overexpress MDR 1 (HL60/Tax) or MRP (HL60/AR). *Int J Oncol* 2000, **16**, 1137–1139.
27. Kagawa S, He C, Gu J, *et al*. Antitumor activity and bystander effects of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. *Cancer Res* 2001, **61**, 3330–3338.
28. Sheikh MS, Huang Y, Fernandez-Salas EA, *et al*. The anti-apoptotic decoy receptor TRID/TRAIL-R3 is a p53-regulated DNA damage-inducible gene that is overexpressed in primary tumors of the gastrointestinal tract. *Oncogene* 1999, **18**, 4153–4159.