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Induction of apoptosis by chemotherapeutic drugs: the role of FADD in activation of caspase-8 and synergy with death receptor ligands in ovarian carcinoma cells

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Abstract

Although ovarian tumours initially respond to chemotherapy, they gradually acquire drug resistance. The aims of this study were to identify how chemotherapeutic drugs with diverse cellular targets activate apoptotic pathways and to investigate the mechanism by which exposure to a combination of drugs plus death receptor ligands can increase tumour cell kill. The results show that drugs with distinct cellular targets differentially up-regulate TRAIL and TNF as well CD95L, but do not require interaction of these ligands with their receptor partners to induce cell death. Factors that were critical in drug-induced apoptosis were activation of caspases, with caspase-8 being activated by diverse drugs in a FADD-independent manner. Certain drugs also demonstrated some dependence on FADD in the induction of cell death. Caspase-9 was activated more selectively by chemotherapeutic agents. Combining ligation of death receptors with exposure to drugs increased tumour cell kill in both drug resistant cell lines and primary ovarian carcinoma cells, even though these cells were not sensitive to death receptor ligation alone. CD95L was more consistent at combining with drugs than TRAIL or TNF. Investigation of the mechanism by which a combination of drugs plus CD95 ligation can increase cell death showed that caspase-8 was activated in cells exposed to a combination of cisplatin and anti-CD95, but not in cells exposed to either agent alone.

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Keywords: ovarian carcinoma; drug resistance; death receptors; apoptosis; adenovirus

Abbreviations: ALL, acute lymphoblastic leukaemia; FA, formaldehyde; FCS, foetal calf serum; NGS, normal goat serum; rhTNFα, recombinant human tumour necrosis factor alpha; TRAIL, TNF related apoptosis inducing ligand; CD95L, CD95 ligand; FLIP, FLICE inhibitory protein; FADD, Fas-associated death domain; GFP, green fluorescent protein; PI, propidium iodide; CHX, cyclohexamide; COX IV, cytochrome oxidase IV

Introduction

A major problem with the treatment of ovarian cancer is the development of drug resistance in tumour cells, with almost all ovarian tumours becoming refractory to the different drugs available within a few years. An understanding of how these drugs activate cell death pathways is important in order to be able to overcome drug resistance. The various drugs that are used in chemotherapy of ovarian cancer have distinct cellular targets which can be either DNA, RNA, topoisomerases I and II, microtubules or the plasma membrane, however the precise mechanism by which these drugs with different cellular targets activate cell death pathways remains unclear.

Initial studies revealed that cytotoxic drugs can activate CD95 signalling pathways via up-regulation of the receptor and/or ligand[1–5] and this mechanism may be an essential factor in the early phase of drug-induced cell death, depending on the cell type used.[6] Some cells, however, appear not to require CD95 receptor/ligand interaction for drugs to be effective,[7–11] although it has been demonstrated that chemotherapeutic drugs can induce CD95L-independent, FADD-mediated activation of the CD95 pathway, by inducing clustering of CD95 receptors.[12] There is also evidence that chemotherapeutic drugs can activate additional cell death pathways as well as those involving CD95 signalling. Thus, FLIP, which prevents the interaction of FADD with caspase-8, does not inhibit drug-induced apoptosis, although it does prevent CD95-dependent target cell lysis,[13] CD95 negative thymocytes from lpr/lpr mice retain their sensitivity to etoposide-induced apoptosis,[14] and CD95-negative erythroleukaemia cells transfected with CD95 show acquired sensitivity to the CD95 agonistic antibody, CH11, but not to drugs.[15] Whether the additional apoptotic pathways activated by drugs may involve other major death receptor signalling pathways i.e. TRAIL, TNF has not been fully investigated.

Drug-induced cell death may also involve mitochondrial regulated apoptosis. Thus, cytochrome *c* release has been seen in ovarian cancer cells and squamous carcinoma cells exposed to cisplatin and fluorouracil.[16,17] Release of cytochrome *c* can be regulated by members of the Bcl-2 family, particularly Bax which may interact directly with mitochondrial membranes. Evidence that drugs may act by altering Bax regulation comes from observations that several chemotherapeutic drugs, including cisplatin and topotecan can up-regulate p53 and Bax.[18–21] In addition, paclitaxel can induce phosphorylation of Bcl-2 in a variety

of cancer cell types, which in some model systems, reduces Bcl-2 heterodimerisation with Bax.[18,22]

Taken together, the above data demonstrate that chemotherapeutic drugs can activate different apoptotic pathways and, at present, it is not clear whether the pathway activated is dependent upon their distinct cellular targets. An understanding of this would help in choosing effective combinations of drugs to overcome drug resistance. Recent studies have indicated that death receptor signalling pathways can remain functional in drug resistant cells, since in a number of cell types, drug resistance can sometimes be overcome by combining exposure of cells to chemotherapeutic drugs with ligation of their surface death receptors.[23–28] The majority of this work has been carried out with established cell lines, therefore it is important to confirm whether these effects are also seen with primary human tumour cells. In addition the mechanism responsible has not been elucidated, since previous studies have indicated that the increase in cell death is not simply a result of drugs causing an increase in the number of death receptors available for ligands to interact with.[5,24]

The aims of the present study were to identify how chemotherapeutic drugs with distinct cellular targets activate apoptotic pathways and to investigate the mechanism by which exposure to a combination of drugs plus death receptor ligands can increase tumour cell kill. Our studies reveal that drugs with diverse targets induce differential up-regulation of TRAIL and TNF as well as CD95L, but do not require interaction of these ligands with their receptor partners to induce cell death. We also demonstrate that there is a critical role for caspases-8 and -3 activation in cell death induced by drugs with diverse cellular targets, however, caspase-9 appears to be activated more selectively. Activation of caspase-8 by drugs can occur when the function of FADD is inhibited. Finally, we show that combined treatment of drugs and death receptor ligation can increase tumour cell kill in primary ovarian carcinoma cells as well as cell lines, with caspase-8 being activated when drug resistant cells are exposed to a combination of cisplatin and CD95 ligation, but not in cells treated with either agent alone.

Results

Ovarian cell lines exhibit differential sensitivity to chemotherapeutic drugs with distinct cellular targets

Four ovarian carcinoma cell lines were exposed to a panel of drugs which have different cellular targets. Cisplatin forms crosslinks with DNA; fluorouracil inhibits DNA and RNA synthesis; topotecan prevents the breakage rejoining reaction of DNA-topoisomerase I; doxorubicin initiates a variety of potentially cytotoxic effects, including inhibition of topoisomerase II and intercalation into the DNA helix; paclitaxel and vincristine bind to tubulin, to stabilise or prevent the formation of microtubules. The four cell lines were assessed for their response to the panel of chemotherapeutic drugs by flow cytometric analysis of cell death (syto 16/propidium iodide uptake) and mitochondrial transmembrane potential ($DiOC_6(3)$ uptake). Measurement of cell death using the two fluorescent stains syto 16 and propidium iodide allows diagnosis of whether the mode of cell death is apoptosis or necrosis, whereas assessment of mitochondrial transmembrane potential reveals the extent of mitochondrial damage occurring when a cell dies.

The extent of cell death induced in each cell line 48 h following exposure to a single dose of drug is shown in Figure 1a. These doses were chosen from a range tested over a period of time as initiating up to 50% cell death in the drug sensitive A2780 cell line by 48 h (data not shown). Thus, A2780 cells exhibited between 35 and 50% cell death following exposure to drugs, the majority of which was apoptosis. A2780 cells which had been transfected with the plasmid pCΔj-bcl-2 and selected for stable over-expression of Bcl-2 (A27Bcl) demonstrated significantly reduced apoptosis levels following exposure to cisplatin or topotecan, but not to the remaining drugs. Selection of cells for resistance to cisplatin (A27CPR) conferred cross resistance to fluorouracil, topotecan and doxorubicin, but in contrast, A27CPR cells remained sensitive to paclitaxel and vincristine. An early passage ovarian carcinoma cell line, MG79, exhibited low levels of cell death at 48 h following treatment with all drugs and this probably represented a delay in the initiation of cell death as, by 96 h the level of cell death in MG79 cells was increased (data not shown).

The changes in mitochondrial transmembrane potential that occurred after A2780, A27Bcl, A27CPR and MG79 cells were exposed to the same drug concentrations are shown in Figure 1b. Loss of membrane potential correlated with the decrease seen in viability, with the exception that the presence of Bcl-2 did not inhibit the loss of mitochondrial membrane potential following topotecan treatment even though it had reduced cell death levels in cells treated with this drug.

These results reveal that in the four cell lines tested here, some degree of drug resistance was seen in three of them. The extent of this varied, with the presence of Bcl-2 only affording partial protection against cisplatin and topotecan, cisplatin resistant cells remaining refractory to exposure to fluorouracil, cisplatin, topotecan and doxorubicin and early passage MG79 cells exhibiting a reduced response to all drugs.

Distinct chemotherapeutic drugs initiate differential upregulation of the death receptor ligands TRAIL and TNFα, as well as CD95L, but are not dependent on ligation of their receptor partners to induce cell death

To determine whether drugs with distinct mechanisms of action can initiate up-regulation of TRAIL and TNF as well as CD95L, RNA levels were measured 16 h following exposure to drugs, prior to the onset of apoptosis. Semi-quantitative RT–PCR analysis was performed using ligand specific probes and a GAPDH probe as a loading control (Figure 2a).

The results demonstrate that certain chemotherapeutic agents can alter the regulation of TRAIL and TNF as well as CD95L. The extent of changes in RNA levels of these three death receptor ligands that occurred following drug treatment varied with individual drugs, with cisplatin being the most effective at up-regulating TNF, topotecan causing the greatest increase in TRAIL and doxorubicin initiating

the strongest up-regulation of CD95L. To determine whether the mechanism of action of any of the drugs used in this study was dependent on this up-regulation of death receptor ligands, experiments were carried out in which the interaction of CD95L, TRAIL or TNF with their receptor partners was blocked and the effect of this on drug-induced cell death assessed.

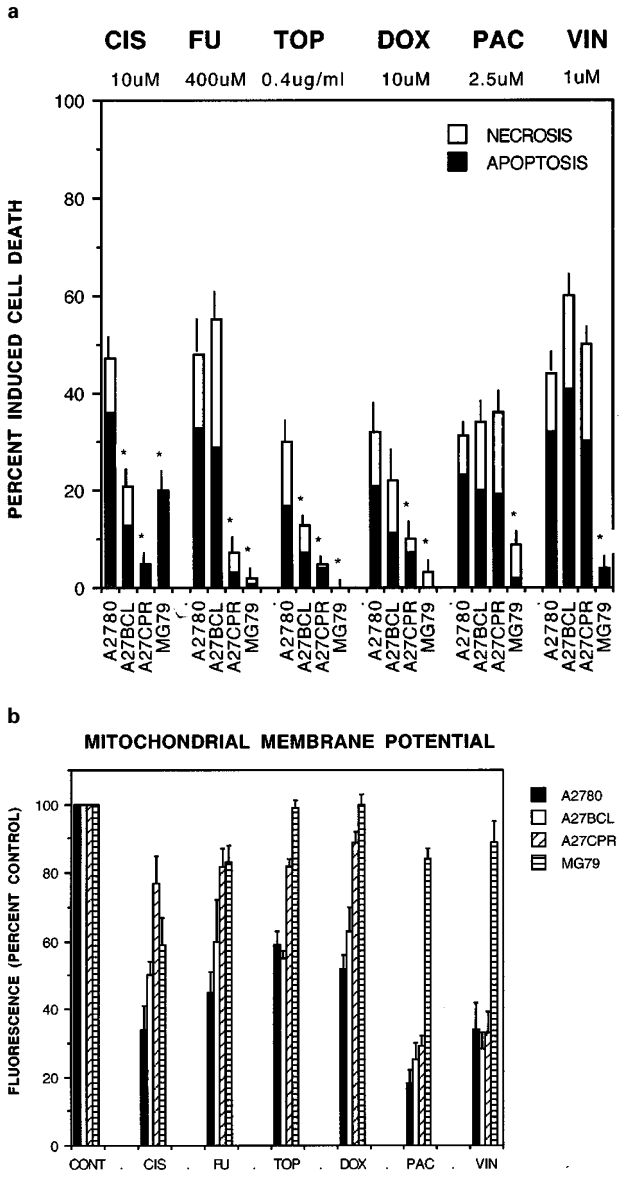


Figure 1 Analysis of the sensitivity of ovarian cancer cell lines to drugs with diverse cellular targets. (**a**) Parental (A2780) and isogenic Bcl-2 transfectants (A27Bcl) or cisplatin resistant (A27CPR) cells, along with early passage (MG79) cells were incubated with a single concentration of drugs, chosen from a range tested as initiating up to 50% cell death in A2780 cells by 48 h; cisplatin (cis), fluorouracil (fu), topotecan (top), doxorubicin (dox), paclitaxel (pac), vincristine (vin). Cell death was diagnosed as either apoptosis or necrosis after 48 h by assessing syto 16 and PI fluorescence by flow cytometry. Background cell death was subtracted from each cell line. Results are means of three separate experiments ± S.E. *$P<0.001$ compared to A2780 cells for each drug treatment. (**b**) A2780, A27Bcl, A27CPR and MG79 cells were exposed to drugs at the doses shown in (**a**). Mitochondrial transmembrane potential was measured 48 h after treatment by analysis of $DiOC_6(3)$ fluorescence by flow cytometry. The results are expressed as a percentage of the absorbance of untreated cells and are means of three separate experiments ± S.E.

A2780 cells were incubated with either the CD95 receptor/ligand blocking antibodies ZB4/Nok-1, TRAILR-Fc or a neutralising TNF antibody for 1 h prior to treatment with drugs (Figure 2b). The presence of either ZB4 or Nok-1 did not reduce drug induced cell death, although Nok-1 effectively inhibited CD95-induced apoptosis in A2780 and Jurkat T cells. Although ZB4 is typically used as a competitive inhibitor, we have seen that in some cell types, ligation of CD95 with ZB4 can initiate up to 20% apoptosis (data not shown), indicating that it may trigger a low level of signalling activity. This may explain the increase in cell death that occurred when cells were treated with drugs plus ZB4. There was no reduction in the extent of drug-induced cell death when TRAIL-R ligation was blocked and neutralisation of TNF did not inhibit apoptosis induced by any of the drugs. Therefore, our data indicate that although drugs with distinct cellular targets differentially up-regulate TRAIL and TNF as well as CD95L, drug-induced cell death is not dependent on the interaction of these ligands with their receptor partners in ovarian carcinoma cells.

The role of FADD in activation of caspase-8 by drugs

To further investigate how drugs activate apoptotic pathways, experiments were carried out to identify whether distinct chemotherapeutic agents show a common pattern of caspase activation. Activation of caspases-8, -9 and -3 was assessed 72 h following drug treatment by detection of cleaved, active caspases using Western blot analysis.

The p43, p41 or p18 active forms of caspase-8 could clearly be seen after treatment with all drugs (Figure 3a). As we had previously shown that ligation of death receptors was not essential for drug-induced cell death, it was important to determine whether drug-mediated activation of caspase-8 was dependent on FADD. This was particularly pertinent as previous studies have demonstrated that drugs can induce CD95L-independent, FADD-mediated activation of caspase-8, by inducing clustering of CD95 receptors.[12] Therefore, to test this hypothesis we examined drug-induced cell death, in cells which had been transfected with a FADD dominant-negative mutant (Figure 3b). Since A2780 cells and other ovarian carcinoma cells are difficult to transfect, we used a cervical carcinoma cell line (HeLa). This had the same degree of sensitivity as A2780 cells to the panel of drugs, showed a similar lack of requirement for CD95-ligation and also demonstrated caspase-8 activation following drug treatment.

The functionality of the FADD dominant-negative mutant was confirmed by comparing the level of cell death in parental cells and FADD dominant-negative transfectants following exposure to anti-CD95 antibody (1 μg/ml CH11) plus cyclohexamide (5 μg/ml). Viability of parental cells was reduced from 90 to 25% following 48 h treatment with cyclohexamide plus CH11, whereas viability of the transfectants was only reduced from 85 to 75%, indicating that the FADD dominant-negative mutant was effectively blocking the activity of FADD in these cells.

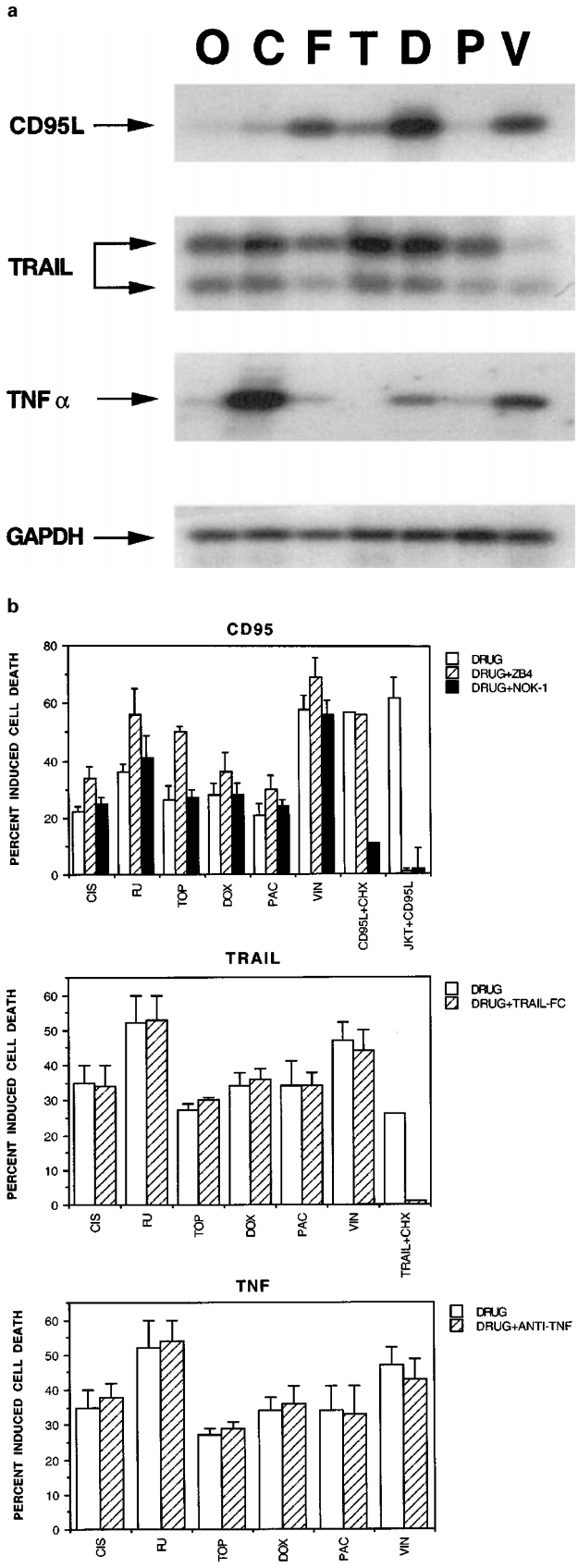


Figure 2 Analysis of whether drug-induced cell death is dependent on death receptor-mediated signalling pathways. (**a**) A2780 cells were treated with drugs at the doses shown in Figure 1, and RNA levels of the death receptor ligands CD95L, TRAIL and TNFα measured 16 h later. GAPDH RNA levels were included as a control. Results show Southern blots of cDNA prepared from total RNA, probed with specific [$^{32}P\gamma$]ATP labelled ligand probes. (**b**) A2780 cells were incubated with drugs in the presence or absence of the CD95 blocking antibody ZB4 (1 μg/ml), the CD95L blocking antibody NOK-1 (1 μg/ml), the TRAIL-R blocking molecule, TRAILR-Fc (500 ng/ml) or a TNFα neutralising antibody (500 ng/ml). As a control for the effectiveness of CD95 blocking, A2780 cells and Jurkat T cells were also exposed to the agonist CD95 antibody CH11 (500 ng/ml) (plus 5 μg/ml cyclohexamide in the case of A2780 cells). Cell death levels were measured after 48 h by flow cytometric analysis of syto16/PI stained cells. Results are means of three separate experiments $\pm$ S.E. Background cell death was subtracted

Figure 3c demonstrates that in both parental HeLa cells and HeLa cells transfected with a FADD dominant-negative mutant, the level of drug-induced cell death was unaffected by the presence of the CD95 blocking antibody Nok-1. A comparison of drug-induced cell death in parental *versus* FADD dominant negative transfectants revealed that the lack of FADD had no effect on cell death induced by either topotecan, doxorubicin or vincristine. However, cell death appeared to be reduced in cells lacking functional FADD following treatment with fluorouracil or paclitaxel, whereas cells exposed to cisplatin showed enhanced apoptosis when FADD functionality was inhibited. As these changes were relatively small ($<$20%) it was decided to investigate these differences in more detail by exposing cells to two concentrations of each drug and monitoring cell death levels over a period of time.

The results presented in Table 1 confirmed that Hela and Hela FADD dominant-negative cells exposed to topotecan, doxorubicin and vincristine had a similar response to both drug concentrations at each time point measured. However, cells in which FADD functionality had been inhibited showed a reduced response to both paclitaxel and fluorouracil at both concentrations by 48 or 72 h following treatment, with cell death levels being up to 20% less than in parental cells (Figure 3d). In contrast, cells lacking FADD showed an enhanced response to cisplatin which was evident at different drug concentrations and time points (Figure 3d).

Table 1 Time course of drug-induced cell death in parental or FADD dominant negative transfected Hela cells

	24 hours		48 hours		72 hours	
	Hela	**Hela FDN**	**Hela**	**Hela FDN**	**Hela**	**Hela FDN**
Control	5$\pm$1	6$\pm$1	5$\pm$1	6$\pm$1	5$\pm$1	7$\pm$1
Cis 4	10$\pm$4	12$\pm$1	26$\pm$1	42$\pm$1	43$\pm$1	67$\pm$1
8	33$\pm$2	53$\pm$2	81$\pm$1	92$\pm$1	95$\pm$1	97$\pm$1
Fu 400	10$\pm$1	9$\pm$1	45$\pm$2	36$\pm$1	76$\pm$3	62$\pm$1
800	10$\pm$1	9$\pm$1	54$\pm$1	49$\pm$2	77$\pm$2	66$\pm$2
Top 1	27$\pm$2	30$\pm$1	44$\pm$2	39$\pm$1	72$\pm$2	68$\pm$1
2	34$\pm$3	46$\pm$3	67$\pm$2	68$\pm$1	91$\pm$1	89$\pm$1
Dox 2.5	15$\pm$2	17$\pm$2	22$\pm$4	21$\pm$1	24$\pm$1	21$\pm$2
5	38$\pm$2	34$\pm$2	81$\pm$3	81$\pm$3	83$\pm$1	83$\pm$1
Pac 4	16$\pm$1	17$\pm$1	58$\pm$2	52$\pm$1	80$\pm$1	66$\pm$3
8	21$\pm$2	19$\pm$2	76$\pm$1	64$\pm$1	76$\pm$3	56$\pm$2
Vin 0.5	24$\pm$1	29$\pm$2	50$\pm$1	47$\pm$2	63$\pm$1	56$\pm$1
1	24$\pm$1	30$\pm$2	49$\pm$1	50$\pm$1	61$\pm$2	56$\pm$2

All drug concentrations are in μM, except topotecan which is in μg/ml

These experiments demonstrate that none of the chemotherapeutic agents used in this study are absolutely dependent on the presence of FADD to induce caspase-8 activation and cell death, as all drugs were able to kill cells transfected with a FADD dominant-negative mutant. Furthermore, the p43 active form of caspase-8 could clearly be seen in both parental and FADD dominant-negative mutants following drug treatment (Figure 3e). However, a detailed investigation revealed that the induction of cell death following exposure to paclitaxel and fluorouracil occurred via both FADD-dependent and FADD-independent mechanisms.

Selective activation of caspase-9 by drugs

Western blot analysis of caspase-9 showed that it appeared to be activated more selectively than caspase-8 in A2780 cells, following drug treatment, with the p37 product becoming clearly visible only after exposure to cisplatin and doxorubicin (Figure 4a). As caspase-9 activation is dependent on cytochrome *c* release from mitochondria, the extent of cytochrome *c* release was compared in cells treated with cisplatin (which had showed activation of caspase-9) with those exposed to paclitaxel or vincristine (which had showed no evidence of caspase-9 activation by Western blot). In A2780 cells treated with each of these drugs, cytochrome *c* was released from the mitochondria into the cytosol, as determined by Western blot analysis (Figure 4b). In contrast, a different mitochondrial protein, cytochrome oxidase IV (COX IV), which is not implicated in the apoptotic pathway, remained in the mitochondria in both control and drug treated cells (Figure 4b). These results suggested that although cytochrome *c* translocated from mitochondria into the cytosol following treatment with paclitaxel and vincristine, this did not result in activation of caspase-9. This observation tied in with our previous results demonstrating that Bcl-2, which can block mitochondrial-mediated apoptosis, was completely ineffective in blocking paclitaxel and vincristine-induced apoptosis (see Figure 1).

The relative role of caspases-8, -9 and -3 in drug-induced cell death

To further investigate the relative role of caspases-8 and -9 activation in drug-induced apoptosis, caspase inhibitors DEVD-FMK, IETD-FMK and LEHD-FMK were used to inhibit caspases-3, -8 and -9 like activity respectively. As these inhibitors are not 100% specific for these caspases, we initially tested their activity at 20 μM, 50 μM and 100 μM and found that 50 μM was the lowest effective dose (data not shown). At this dose we demonstrated that the inhibitors were acting in at least a partially specific manner, as IETD-FMK reduced CD95-induced apoptosis from 41 to 3% in Jurkat T cells, whereas LEHD-FMK only reduced cell death from 41 to 23% (data not shown).

Pre-incubation of cells with IETD-FMK for 1 h prior to drug treatment, reduced apoptosis levels by between 50 and 75% following treatment with all drugs, indicating that the caspase-8 activation seen by Western blot was essential for at least some of the cell death induced (Figure 5a). However, LEHD-FMK only significantly reduced apoptosis levels following treatment with cisplatin, fluorouracil or doxorubicin, strengthening the Western blot results that activation of caspase-9 was not part of the mechanism of cell death induced by paclitaxel or vincristine (Figure 5a). The observation that LEHD-FMK was equally effective as IETD-FMK in inhibiting apoptosis induced by cisplatin, ruled out the possibility that the differences in effectiveness of these inhibitors following apoptosis induced by topotecan, paclitaxel or vincristine was simply due to differences in their relative effectiveness. The presence of DEVD-FMK reduced apoptosis levels following treatment with all drugs indicating that activity of this downstream caspase is critical for drug-induced cell death (Figure 5a), and indeed the p17 active form of caspase-3 was seen on Western blot following treatment of A2780 cells with all drugs (Figure 5b).

Combined drug and death receptor ligand treatment increases tumour cell kill in drug resistant ovarian cancer cell lines and primary cells

The three ovarian cancer cell lines that had acquired varying degrees of drug resistance (A27Bcl, A27CPR, MG79), along with primary ovarian tumour cells, were incubated with the death receptor agonists (anti-CD95 antibody CH11, rhTRAIL or rhTNFα), either alone or in combination with each of the six chemotherapeutic drugs previously tested. The single drug doses used in this experiment were chosen as causing approximately 50% cell death by 48 h in the drug sensitive, parental A2780 cells (see Figure 1). All cell lines were insensitive to ligation of death receptors alone with a maximum of 10% being induced by anti-CD95, TRAIL or TNF. However, when death receptors were ligated in combination with drug treatment, cell death levels were further increased by certain combinations and the effective combinations are shown in Figure 6a.

Combinations of cisplatin, topotecan or doxorubicin with CD95 or TNF ligation were the most effective at increasing cell death in A27Bcl cells, however none of the drugs used in these experiments were effective when combined with TRAIL ligation. Levels of cell death in A27CPR cells were increased by combinations of paclitaxel or vincristine with TRAIL and treatment of these cells with vincristine plus ligation of CD95 also increased tumour cell kill. Cells from the early passage MG79 cell line showed markedly enhanced cell death when CD95 or TRAIL-R were ligated following exposure to cisplatin or fluorouracil. Treatment of MG79 cells with topotecan also caused an increase in cell death following ligation of CD95, but not TRAIL-R. MG79 cells were not sensitised to TNF-mediated apoptosis by any of the drugs used in these experiments.

Similar experiments were performed on tumour cells isolated from ascitic fluid obtained from two patients who had become refractory to chemotherapy (OVBF, OVIN). The results show that when cells from these tumours were exposed to a combination of drugs and death receptor ligands, a further increase in cell death was seen following certain combinations, with the effective combinations being

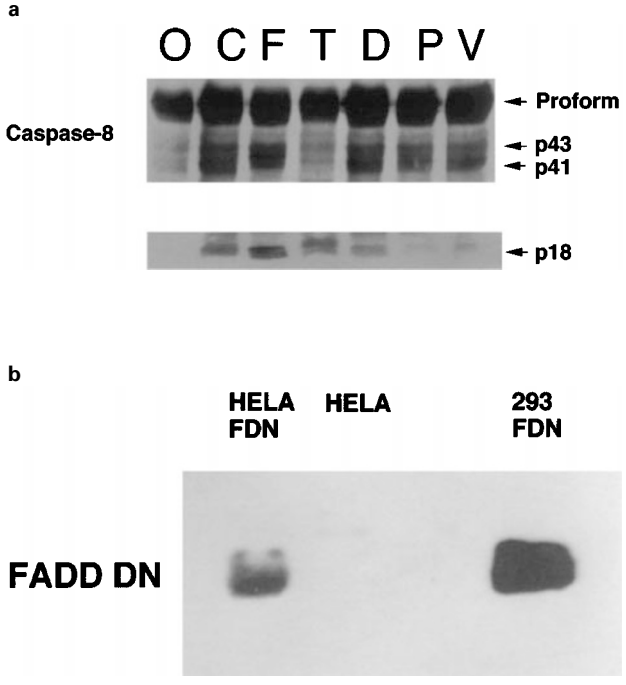
shown in Figure 6b. Interestingly, the optimum combination of drug and death receptor ligand needed to induce maximum apoptosis was different in each tumour. A combination of cisplatin or paclitaxel with anti-CD95 was most effective at increasing cell kill of OVBF cells, whereas an increase in cell death was seen following treatment with either fluorouracil, topotecan, doxorubicin, or paclitaxel and anti-CD95; fluorouracil, topotecan or doxorubicin and TRAIL; or topotecan and TNF in OVIN cells.

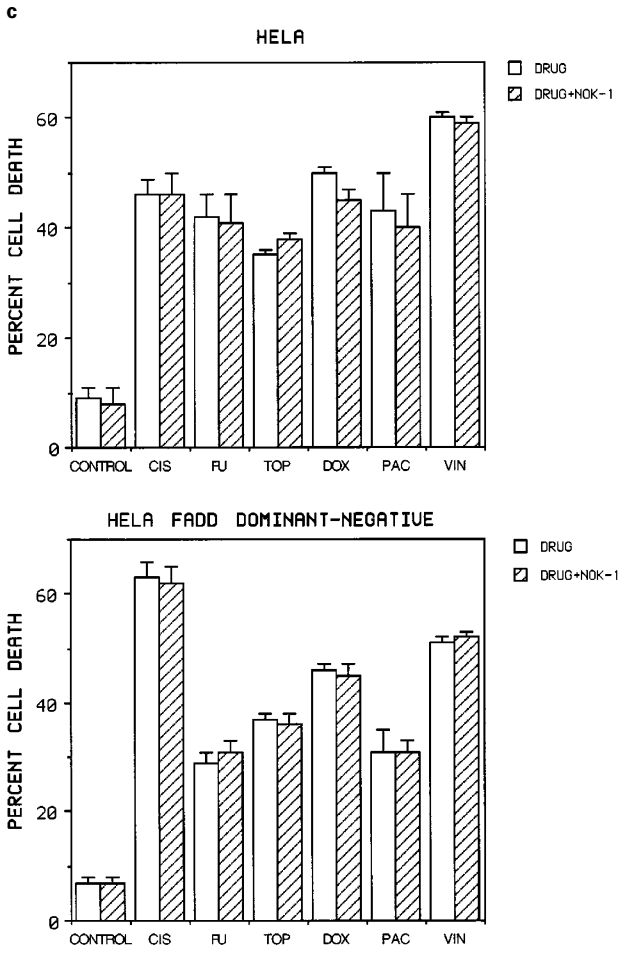
These results confirm that a combination of drugs and death receptor ligands can increase cell kill in drug resistant primary ovarian tumour cells as well as ovarian cancer cell lines *in vitro*. The results in both cases indicated that individual tumours are likely to have distinct responses to these agents and that ligation of CD95 rather than TRAIL or TNF receptor, was the most consistent at combining with drugs to give increased cell death in drug resistant cells.

CD95L delivered to ovarian cancer cells via an adenovirus vector can initiate enhanced cell death in drug resistant cells exposed to drugs

As CD95L cannot be given in soluble form *in vivo* because of its liver toxicity,[29] in order for it to be used in treatment it would have to be targeted specifically to tumour cells. Therefore, we wished to assess whether CD95L delivered to cancer cells via an adenovirus vector could also combine with drugs to give enhanced cell killing. A27Bcl cells were infected with a recombinant replication-defective adenovirus expressing either the CD95L gene (RAdFasL) or the GFP gene, exposed to chemotherapeutic drugs 48 h after infection and their metabolic status assessed after a further 48 h by MTT assay. A27Bcl cells, which had not been killed previously by exposure to soluble CD95 showed increased cell death, when infected with RAdFasL at 50 moi (Figure 7). This was a specific effect due to expression of CD95L, as a control virus carrying GFP did not kill A27Bcl cells. The addition of drugs to RAdFasL infected cells further enhanced cell death with the largest increases being seen following exposure to cisplatin, topotecan, doxorubicin and paclitaxel (Figure 7).

These results suggested that delivery of CD95L to cancer cells via a recombinant adenovirus may be an effective means of killing a proportion of the population even when these cells had not shown sensitivity to ligation of CD95 with soluble ligand. In addition, exposing virus infected cells to certain chemotherapeutic drugs may further enhance cell death.





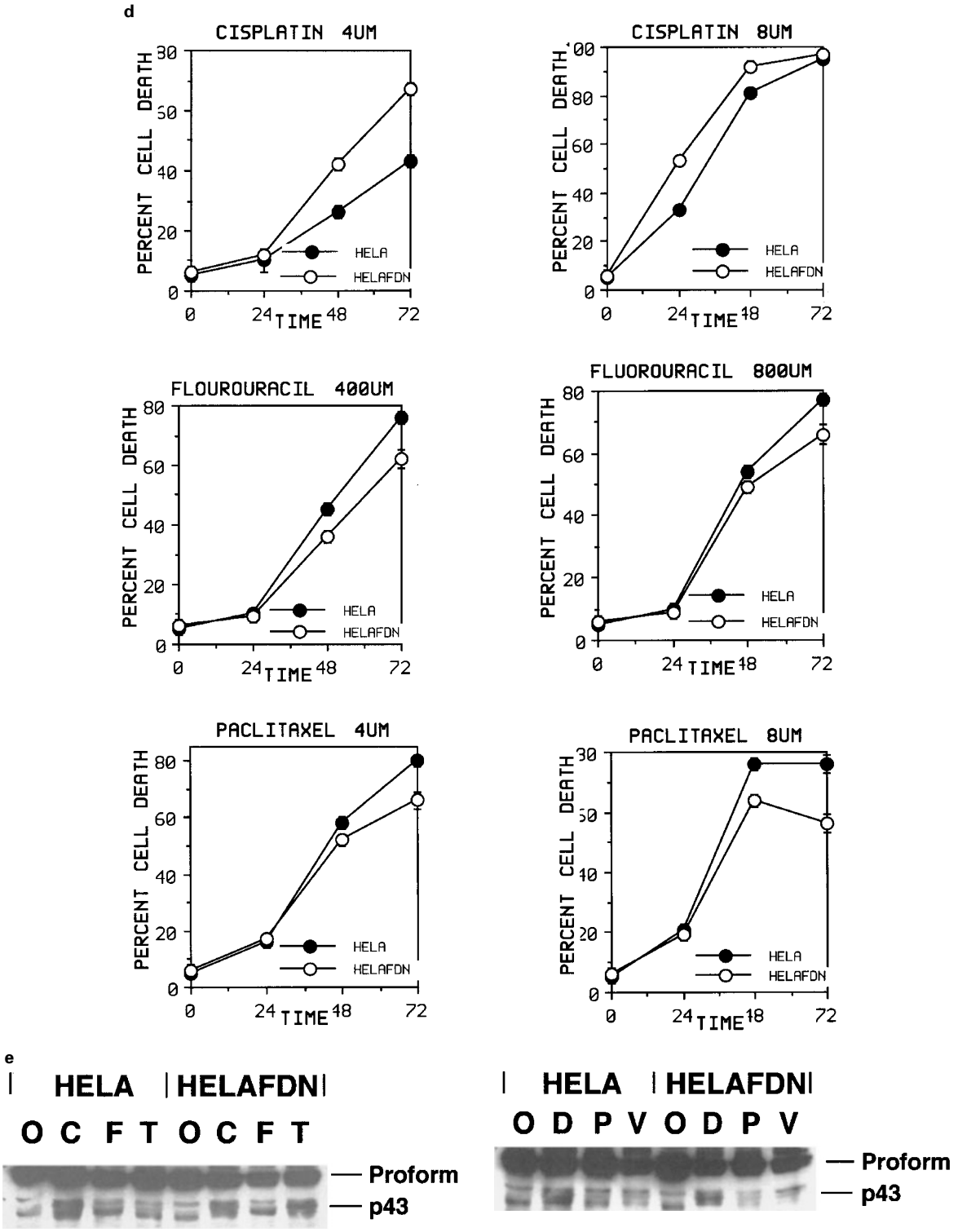


Figure 3 FADD-independent activation of caspase-8. (**a**) A2780 cells were exposed to drugs at the concentrations shown in Figure 1 and the presence of active caspase-8 assessed 72 h later by Western blot analysis. The antibody used was rabbit anti-caspase-8[41] (1/3000) which recognises both proform and active forms of the caspase. (**b**) Hela cervical carcinoma cells were stably transfected with a FADD dominant-negative mutant (Hela Fdn). The level of expression of dominant negative FADD was compared by Western blot with that expressed in 293 human epithelial kidney cells, transiently transfected with the dominant negative FADD gene. (**c**) HeLa and HeLa Fdn cells were treated with the same panel of drugs that A2780 cells had been exposed to, in the presence or absence of Nok-1 (1 μg/ml). Drug concentrations used were; cisplatin (8 μM), fluorouracil (800 μM), topotecan (1 μg/ml), doxorubicin (7.5 μM), paclitaxel (4 μM), vincristine (0.5 μM). Cell death levels were monitored 48 h later by flow cytometric analysis of syto 16 and PI fluorescence. (**d**) Hela and Hela Fdn cells were treated with two concentrations each of cisplatin, fluorouracil and paclitaxel. Cell death levels were measured at 24, 48 and 72 h after treatment by flow cytometric analysis of syto 16 and PI fluorescence. (**e**) The activation of caspase-8 in HeLa and HeLa Fdn cells following drug treatment, was measured by Western blot analysis as described for A2780 cells

Caspase-8 is activated in drug-resistant cells killed by a combination of cisplatin and anti-CD95, but not in cells exposed to either agent alone

The mechanism by which a combination of drugs and death receptor ligands can increase cell death in drug-resistant cells is not known. Previous studies have suggested that the increase in cell death seen when drugs are combined with death receptor ligands is not simply a result of drugs causing an increase in the number of death receptors available for ligand interaction.[5,24] In agreement with this we found that, although CD95 receptor levels were enhanced by exposure to some drugs in A27Bcl, A27CPR and MG79 cells, the degree of enhancement did not correlate with the increased sensitivity to CD95 ligation that we had seen in these cells when treated with drugs. For example, cisplatin, fluorouracil, topotecan and doxorubicin all initiated a similar increase in CD95 levels in A27 Bcl cells, however fluorouracil did not combine with CD95 ligation to enhance cell death (data not shown). In addition, the only drugs that up-regulated CD95 in A27CPR cells were topotecan and doxorubicin, but neither of these were effective at combining with CD95 ligation (data not shown).

As we had previously shown that in drug-sensitive cells, caspase-8 is activated by a wide variety of drugs, we wished to determine whether caspase-8 was being activated in drug-resistant cells treated with an effective combination of drug plus death receptor ligand. Our previous experiments had indicated that exposing A27Bcl cells to cisplatin whilst simultaneously ligating CD95 had increased cell death levels by approximately 30%, therefore caspase-8 activity was monitored by Western blot analysis in A27Bcl cells treated with either agent alone or both together (Figure 8). No activation of caspase-8 could be seen when A27Bcl cells were treated with either cisplatin or anti-CD95 alone, but following a combination of both, apoptosis levels increased by a further 27% above that seen following cisplatin treatment alone and activation of caspase-8 could clearly be seen by Western blot analysis.

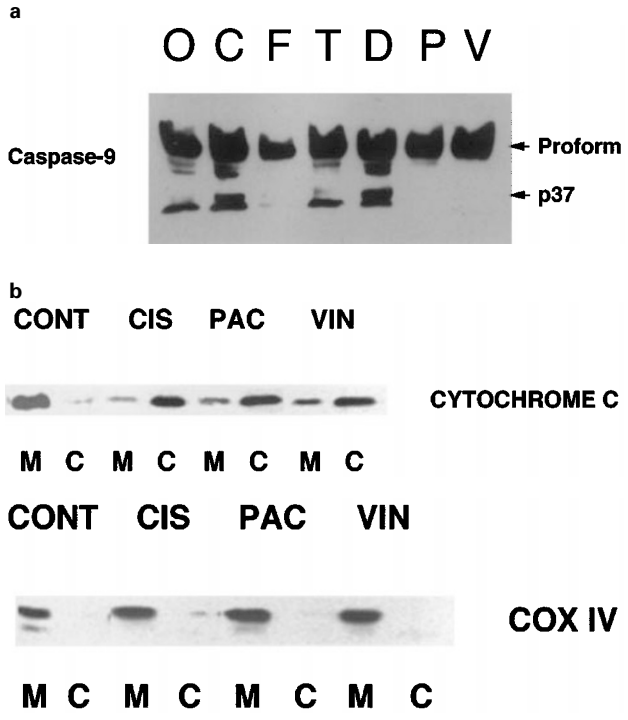


Figure 4 Selective activation of caspase-9 by drugs. (**a**) A2780 cells were exposed to drugs at the concentrations shown in Figure 1 and the presence of active caspase-9 assessed 72 h later by Western blot analysis. The antibody used was mouse anti-caspase-9 (Calbiochem, 1/100) which recognises both proform and active forms of the caspase. (**b**) The translocation of either cytochrome *c* or cytochrome oxidase IV from the mitochondria to the cytosol following exposure to either cisplatin, paclitaxel or vincristine was monitored by Western blot analysis. The concentrations of drugs used were those shown in Figure 1. M=mitochondria, C=cytosol. CONT=control, CIS=cisplatin, PAC=paclitaxel, VIN=vincristine

Discussion

In this study, we have investigated how chemotherapeutic drugs with distinct cellular targets activate apoptotic pathways in ovarian carcinoma cells, and have also identified a possible mechanism by which certain combinations of drugs and death receptor ligands can increase cell death in tumour cells resistant to either agent alone.

Our results demonstrated that in drug sensitive A2780 cells, a panel of drugs with diverse targets including DNA, RNA and microtubules, interact differently with death receptor signalling pathways to induce differential up-regulation of the death receptor ligands TRAIL and TNF as well as CD95L. Previous studies have shown that levels of CD95L can be enhanced by drugs, in particular doxorubicin,[1–3] and this was the most effective chemotherapeutic agent at increasing CD95L RNA levels in the ovarian carcinoma cells studied here. We have now shown that the other death receptor ligands TRAIL and TNF can also be regulated by drugs. An important point to clarify is whether the increase in death receptor ligands that can be initiated by certain chemotherapeutic agents is an essential part of their mechanism of action. Previous studies addressing this question with respect to CD95L have shown that in some cell types this is the case,[1–6] whereas in others it is not.[7–11] Our data reveal that ovarian carcinoma cells fall into the latter group and in addition, we showed that inhibiting the interaction of either TRAIL or TNF with their receptor partners had no effect on drug-induced apoptosis, suggesting that ligation of these receptors is also not part of the cell death mechanism induced by chemotherapeutic agents.

Factors that were found to be critical in drug-induced apoptosis were those acting at more distal points in cell death pathways i.e. activation of caspases. An investigation of whether drugs with different cellular targets activate distinct caspases demonstrated that a wide variety of drugs all activated caspase-8 and that this activation was required for drug-induced cell death. The mechanism by which caspase-8 is activated by drugs is not clear, as although both paclitaxel and fluorouracil activated FADD-dependent and -independent cell death pathways, the presence of FADD was not absolutely required for either cell death or caspase-8 activation by any of the drugs tested. This suggests that in this case, caspase-8 was not being activated by drugs inducing ligand-independent activation

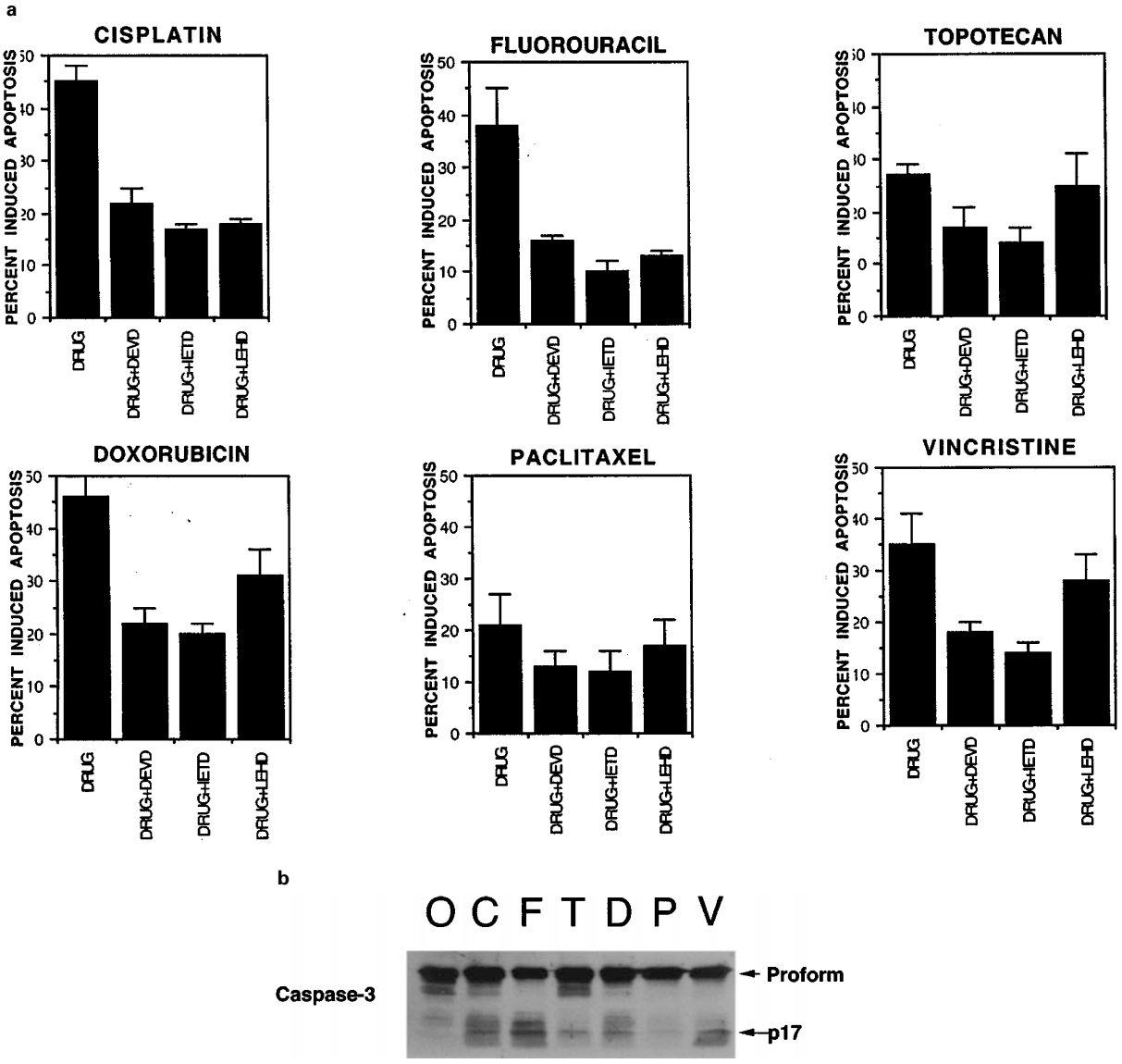


Figure 5 Effect of caspase inhibitors on drug-induced cell death. (**a**) A2780 cells were exposed to drugs at the concentrations shown in Figure 1, in the presence or abscence of caspase-3 like inhibitor (DEVD-FMK, 50 μM), caspase-8 like inhibitor (IETD-FMK, 50 μM) or caspase-9 like inhibitor (LEHD-FMK, 50 μM). These concentrations were chosen from a range of 20, 50 and 100 μM as being the lowest effective dose and acting in at least a partially specific manner. Cell death levels were analysed 48 h later by flow cytometric analysis of syto16/PI stained cells. Background cell death was subtracted. Results are means of three separate experiments $\pm$ S.E. (**b**) A2780 cells were exposed to drugs at the concentrations shown in Figure 1 and the presence of active caspase-3 assessed 72 h later by Western blot analysis. The antibody used was rabbit anti-caspase-3 (Pharmingen, diluted 1/5000) which recognises both proform and active forms of the caspase

of CD95 by initiating trimerisation of the receptor, as has been seen in other studies.[12] Therefore, our results along with previous studies showing that: (1) FLIP, which prevents the interaction of FADD with caspase-8, does not inhibit drug-induced apoptosis;[13] (2) CD95-negative thymocytes from lpr/lpr mice retain their sensitivity to etoposide[14] and (3) CD95-negative erythroleukaemia cells transfected with CD95 show acquired sensitivity to CD95 agonstic antibodies, but not to drugs,[15] all suggest that chemotherapeutic agents can initiate apoptosis independently of CD95 trimerisation.[30]

A possible mechanism for FADD-independent activation of caspase-8 is through a feedback loop via mitochondrial-regulated apoptosis, where caspase-9 activates caspase-3 which in turn can activate caspase-8.[42,43] In support of this, following cisplatin treatment, several factors suggested that mitochondrial-regulated apoptosis was essential for cell death. Thus, cisplatin-induced cell death was reduced in the presence of Bcl-2 (which can block mitochondrial-regulated apoptosis), and also by the caspase-9 inhibitor LEHD-FMK. In addition, cytochrome *c* was released from mitochondria following cisplatin treatment and activation of caspase-9 was seen by Western blot. As caspase-3 was also activated in these cells, it is possible that, following exposure to cisplatin, caspase-8 was activated downstream from mitochondria.

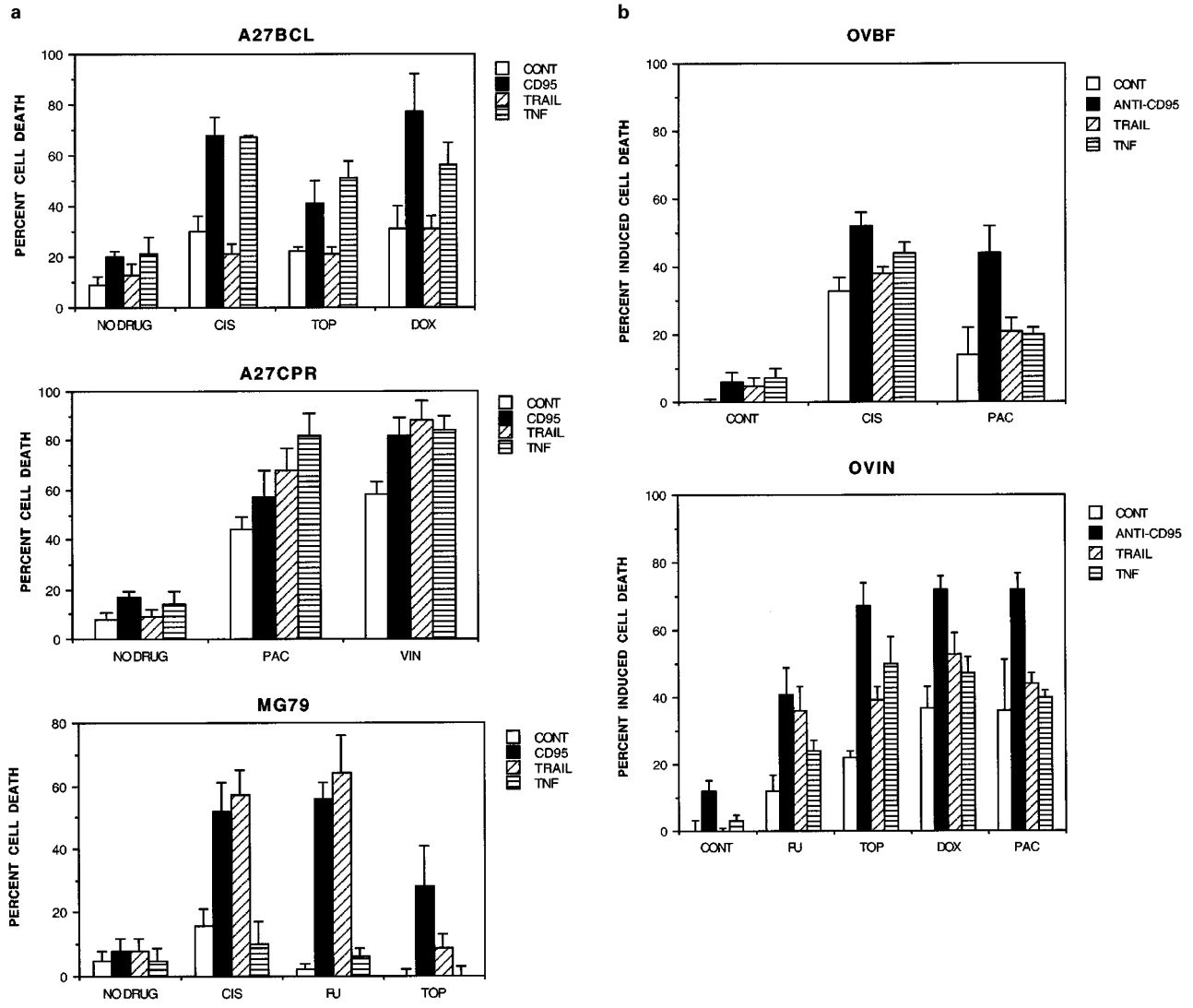


Figure 6 Combination treatment of drug resistant cell lines with drugs plus death receptor ligation. (**a**) The three cell lines that had exhibited varying degrees of drug resistance (A27Bcl, A27CPR and MG79) were incubated with 20 ng/ml anti-CD95 (CH11), 50 ng/ml rhTRAIL or 50 ng/ml rhTNFα, either alone or immediately after cells had been treated with each of the six chemotherapeutic drugs used in this study. After a further 48 h, cells were harvested and analyzed for cell death levels by analyzing syto16/PI fluorescence. Only effective combinations are shown. Results are means of three experiments $\pm$ S.E. (**b**) Primary cells from two different ovarian tumours which had become refractory to chemotherapy (OVBF, OVIN), were exposed to 20 ng/ml rhCD95L, 50 ng/ml rhTRAIL or 50 ng/ml rhTNFα either alone or immediately after cells had been treated with each of the six chemotherapeutic drugs. After a further 48 h, cells were harvested and analyzed for cell death levels by analyzing syto16/PI fluorescence. Only effective combinations are shown. Background cell death was subtracted. Results are means of three experiments $\pm$ S.E.

In contrast to cisplatin, several results suggested that the microtubule disrupting drugs paclitaxel and vincristine, did not appear to be acting via mitochondria in the ovarian carcinoma cells used in this study. Thus, (1) Bcl-2 was completely ineffective at blocking paclitaxel or vincristine-induced apoptosis; (2) The presence of LEHD-FMK did not significantly reduce apoptosis levels in cells exposed to paclitaxel or vincristine and (3) The active form of caspase-9 was not seen on Western blot in cells treated with paclitaxel or vincristine, even though cytochrome *c* was released from mitochondria. However, activation of caspase-8 was clearly seen by Western blot in cells treated with paclitaxel and vincristine and the caspase-8 inhibitor, IETD-FMK, effectively blocked apoptosis in these cells. Therefore, the mechanism by which paclitaxel and vincristine activate caspase-8 remains to be elucidated.

Our experiments investigating whether it is possible to reverse drug resistance by combination treatment of drugs and death receptor ligation, utilised cell lines with varying degrees of drug resistance and also primary ovarian tumour cells. Drug resistance mediated by Bcl-2 afforded partial protection only against cisplatin and topotecan in these ovarian carcinoma cells, which correlates with previous studies demonstrating that the presence of Bcl-2 reduces cisplatin-induced cell death in ovarian and bladder cancer cells,[31,32] but does not impart protection against paclitaxel,

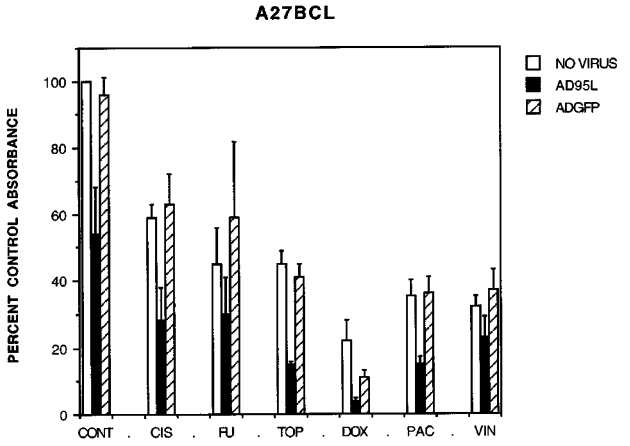


Figure 7 Combination treatment of drug resistant cells with drugs and infection with adenovirus expressing CD95L. A27Bcl cells were infected with adenovirus expressing either CD95L (AD95L) or GFP (ADGFP) at 50 moi for 2 h. Forty-eight hours after infection cells were exposed to chemotherapeutic drugs at concentrations shown in Figure 1. After a further 48 h cell viability was analyzed by MTT assay. Results are expressed as a percentage of the absorbance of untreated cells and are the means of three separate experiments ± S.E.

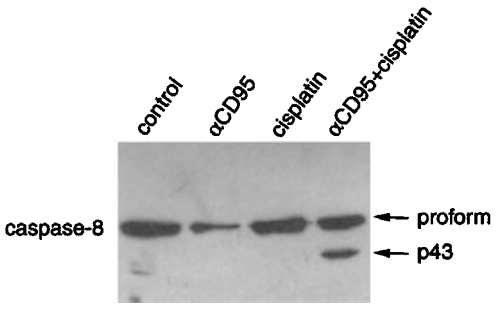


Figure 8 Activation of caspase-8 in cells exposed to both cisplatin and anti-CD95. A27 Bcl cells were exposed to either 10 μM cisplatin, 20 ng/ml anti-CD95 (CH11) or to both agents simultaneously. After 48 h Western blot analysis was performed to look for the presence of the active, cleaved form of caspase-8. The antibody used was rabbit anti-caspase-8[41] (1/18,000), which recognises both the proform and active forms of the caspase (note that in Figure 3, this same antibody was diluted 1/3000)

which inhibits the anti-apoptotic activity of Bcl-2 by inducing its phosphorylation.[18,33] Cells acquiring resistance to cisplatin, also developed strong resistance to fluorouracil, topotecan and doxorubicin, but not to the microtubule disruptors paclitaxel and vincristine and early passage MG79 cells[34] demonstrated a delayed response to all drugs used in this study. In addition to the established cell lines, we also analysed the response of primary cells taken from two different ovarian tumours, which had become refractory to chemotherapy. Although drug resistant cells were not sensitive to death receptor ligation alone we found that tumour cell kill could be increased by combination treatment of drugs and death receptor ligands. The optimum combination to induce maximum apoptosis in tumour cells varied between individual cell lines and tumours, however ligation of CD95 rather than TRAIL-R or TNF-R1 was the most consistent at combining with drugs to give increased cell death in drug resistant cells.

As CD95L cannot be given in soluble form *in vivo* because of its liver toxicity,[29] it would have to be targeted specifically to tumour cells if it was to be used *in vivo*, therefore we assessed whether CD95L delivered to cancer cells via an adenovirus vector could also enhance chemotherapy-mediated cell killing. Importantly we found that infection with the adenovirus containing CD95L (but not GFP) alone, induced cell death in a proportion of drug resistant cells that had not been sensitive to soluble rhCD95L. This may be due to membrane bound CD95L being more effective at trimerising the CD95 receptor than soluble CD95L and supports other studies highlighting differential effects of membrane-bound *versus* soluble CD95L.[35,36] These results suggest that targeting CD95L to cancer cells via an adenoviral vector may be an effective means of killing a proportion of the population. Combining infection of cells with the CD95L virus, with exposure to chemotherapeutic drugs further enhanced cell death suggesting that death receptor ligands delivered in this way will be similarly effective as soluble ligands at combining with drugs to promote cytotoxicity in drug resistant cells.

The reason for the increase in cell death seen when drugs are combined with death receptor ligands is not known, since it is not simply a result of drugs causing an increase in the number of death receptors available for ligands to interact with.[5,24,our data] Our results showed that when A27Bcl cells were killed using a combination of cisplatin and CD95 ligation activation of caspase-8 was clearly seen, although there was no detectable activation of caspase-8 following administration of either agent alone. Our experiments with drug sensitive ovarian cancer cells had shown that caspase-8 can be activated by a wide variety of drugs with different cellular targets during drug-induced cell death. Therefore, we hypothesise that in drug resistant cells administration of either drugs or death receptor ligands alone may activate a small amount of caspase-8, but not enough to form sufficient oligomers to activate downstream caspases. However, when the two are added together, oligomerisation of caspase-8 may be able to increase above the threshold needed to trigger the apoptotic pathway.

Materials and Methods

Cell culture

The human ovarian carcinoma cell line A2780 and its cisplatin resistant subclone, A27CPR,[37] were kindly provided by Dr J Plumb (University of Glasgow, Glasgow, UK). The A2780 line was transfected with the plasmid bCΔj-bcl-2[38] using the calcium phosphate technique and stable transfectants (A27Bcl) resistant to the selective marker geneticin (Gibco, Uxbridge, UK) were obtained.[31] Jurkat T cells were kindly provided by Professor A Rickinson (University of Birmingham, Birmingham, UK). MG79 ovarian tumour cells were derived from continuous culture of cells isolated from ascites fluid obtained from a patient with epithelial adenocarcinoma of the ovary.[34] HeLa cervical

carcinoma cells were transfected with the FADD dominant negative gene (kindly provided by Dr V Dixit), cloned into pCDNA3.hygro (Invitrogen), using the calcium phosphate technique and stable transfectants (HeLaFdn) resistant to the selective marker Hygromycin (Gibco) were obtained. A2780 lines, HeLa lines and Jurkat T cells were maintained in RPMI (Gibco) and MG79 cells in DMEM (Gibco), both supplemented with 10% FCS (Flow Laboratories, Irvine, UK) and 2 mM glutamine (Gibco) and kept at 37°C in a 5% CO_2 atmosphere. Primary ovarian cells were isolated from ascites fluid by two rounds of centrifugation through lymphoprep gradients[34] and cultured in RPMI plus 10%FCS and 2 mM glutamine. After 5 days, any non adherent cells were removed by washing.

Flow cytometric analysis of cell viability

Cells were trypsinised and plated out at a density of 2×10^5/ml in 48-well plates and allowed to attach overnight. Following treatment with either cisplatin, fluorouracil, topotecan, doxorubicin, paclitaxel or vincristine and/or anti-CD95 (CH11, Coulter Immunotech, Luton, UK), rhCD95L (Alexis Corporation, San Diego, USA), rhTRAIL (Alexis Corporation, San Diego, USA), rhTNF (R&D Systems, Abingdon, UK), cells were trypsinised and resuspended in 0.5 ml saline (pre-warmed to 37°C). Syto 16 (Molecular Probes Europe, Leiden, The Netherlands) was added at a concentration of 25 nm (A27 lines, MG79 cells and primary tumour cells) or 250 nm (Jurkat T cells) and incubated with the cells at room temperature for 1 h, at which time 5 μg/ml propidium iodide was added. Samples were analyzed immediately on a Coulter EPICS XL flow cytometer. A two-dimensional dot plot was generated of syto 16 fluorescence *versus* propidium iodide fluorescence. Syto 16 is only taken up by viable cells and propidium iodide only enters cells whose membranes have become permeabilised, therefore this technique distinguishes between viable cells (syto 16 +ve, propidium iodide −ve), apoptotic cells (syto 16 −ve, propidium iodide −ve) and necrotic cells (syto 16 −ve, propidium iodide +ve).[39] Data for 10 000 cells was collected for each sample and, prior to data collection cell debris was excluded by setting a gate on a forward *versus* side scatter two dimensional dot plot.

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential was analyzed by assessment of uptake of the dye $DiOC_6(3)$. Cells were plated out at a concentration of 2×10^5/ml into 24-well plates and allowed to attach for 24 h prior to drug treatment. Forty-eight hours following exposure to drugs, cells were trypsinised and resuspended in 1 ml fresh growth medium (pre-warmed to 37°C). $DiOC_6(3)$ was added to a final concentration of 20 nM and the cells were incubated at 37°C for 15 min before being analyzed immediately for fluorescence by flow cytometry.

Analysis of CD95L, TRAIL and TNFα RNA levels

Preparation of RNA: Cells were plated out at a density of 2×10^5/ml in 10 ml wells and allowed to attach overnight prior to drug treatment. Twenty-four hours following treatment cells were trypsinised, washed once with PBS and pelleted. After 5 min treatment with RNAzol, chloroform was added to one tenth volume, samples were vortexed for 15 min then incubated on ice for 3 min. Samples were pelleted by centrifugation at 13 000 r.p.m. at 4°C. The upper, acqueous layer was added to isopropanol and stored at −20°C for 30 min. Samples were pelleted by centrifugation at 13 000 r.p.m. at 4°C, washed once with 70% ethanol and resuspended in DEPC. After incubating on ice for 1 h, RNA samples were stored at −70°C.

Preparation of cDNA: RNA samples were treated with DNase for 20 min at RT prior to addition of EDTA and incubation at 65°C for 5 min. OligodT was added and samples incubated at 70°C for 10 min. cDNA was generated by PCR at 42°C for 1 h, followed by 70°C for 10 min following which the cDNA samples were incubated with RNase for 20 min at 37°C.

Southern blot analysis of DNA: A PCR reaction was carried out using 1 μl cDNA with primers specific for either GAPDH, CD95L, TRAIL or TNF as previously described.[40] Initial experiments were performed to define the linearity curve for analysis of gene amplification. PCR products were run on a 1.5% agarose gel at 100 V for 1 h. The gels were soaked in denaturing solution followed by neutralising solution for 30 min each, before being washed once with distilled water. Gels were blotted overnight with Hybond, after which the Hybond filters were fixed in 0.4 M NaOH for 20 min, then washed twice with SSC prior to pre-hybridisation at 42°C for 2 h. Hybridisation with specific [$^{32}P\gamma$]-ATP labelled probes was carried out at 42°C overnight, following which the Hybond filters were washed three times with SSC+0.1% SDS.

Western blot analysis

Cells were harvested, washed once in ice cold PBS, pelleted by centrifugation and pellets lysed for 20 min on ice. Samples were pelleted at 13 000 r.p.m. at 4°C and lysates decanted and stored at −70°C. Aliquots containing 100 μg protein were subjected to either 12% SDS–PAGE (p53, caspase-8, FADD), 10% SDS–PAGE (caspases-9, and -3) or 15% SDS–PAGE (cytochrome *c*, COX IV). The fractionated proteins were transferred to nitrocellulose membranes that were incubated with either rabbit anti-caspase-8[41] (a kind gift from Dr G Cohen, University of Leicester, UK, diluted 1/3000), mouse anti-caspase-9 (Calbiochem, diluted 1/100), rabbit anti-caspase-3 (Pharmingen, diluted 1/5000), mouse anti-cytochrome *c* (Pharmingen, diuted 1/500), mouse anti-COX IV (Molecular Probes, diluted 1/250) or mouse anti-FADD (Transduction Laboratories). Secondary antibodies were either goat anti-mouse HRP (Sigma, diluted 1/1000) or goat anti-rabbit HRP (Sigma, diluted 1/7500). Target proteins were visualised using ECL reagent (Amersham International).

Measurement of cytochrome *c* release from mitochondria

Cells were washed twice in ice-cold PBS and then resuspended at 5×10^7/ml in ice cold lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor).[44] The cell suspension was homogenised with 25 strokes of a Dounce homogeniser and centrifuged at $700 \times g$ for 7 min at 4°C. The pellet was discarded and the supernatent centrifuged at $20\,000 \times g$ for 15 min. The resulting pellet containing the mitochondria was resuspended in lysis buffer and the supernatent containing the cytosol retained in a fresh tube. The relative purity of the fractions was ascertained by Western blot using an anti-COX IV antibody as a marker for mitochondria.

Adenoviral infection of cells

Adenovirus vectors: RAdFasL virus was generated by homologous recombination between the Ad5-based *dl309* plasmid pJM17 (kind gift from F Graham) and an adenovirus transfer vector pMC3, into which

FasL cDNA was cloned under the control of the cytomegalovirus immediate early promoter (CMV-IE). Virus rescue was performed in the AdE1a and SV40 large T-transformed human renal epithelial cell line 293T by co-transfecting pMC3-FasL and pJM17. Following homologous recombination between the plasmids, plaques of recombinant adenovirus were visible from day 6 post-transfection. Following several rounds of plaque purification on 911 cells, the presence of recombinant virus was verified by RT – PCR in infected target cells, using primers for FasL in RNA samples from RAdFasL-infected cells.

Infection of cells with adenovirus: Cells were plated out at a density of 5×10^4/ml in 96-well plates and allowed to attach overnight, after which cells were exposed to 50 moi RAdFasL or a control virus carrying green fluorescent protein (GFP). After a 2 h exposure period, virus was removed and cells replenished with complete medium.

MTT assay

Cells were plated out at a density of 5×10^4/ml in 96-well plates and allowed to attach overnight prior to treatment. Following treatment, 20 μl MTT (3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) in PBS were added to each well and incubated for 4 h at 37°C and the formazan crystals formed were dissolved in DMSO. The absorbance was recorded at 550 nm.

Acknowledgements

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