The CD95/CD95 ligand system is not the major effector in anticancer drug-mediated apoptosis

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Abstract

Many anticancer drugs are able to induce apoptosis in tumor cells but the mechanisms underlying this phenomenon are poorly understood. Some authors reported that the p53 tumor suppressor gene may be responsible for drug-induced apoptosis; however, chemotherapy-induced apoptosis can also be observed in p53 negative cells. Recently, doxorubicin (DXR) was reported to induce CD95L expression to mediate apoptosis through the CD95/CD95L system. Thus, an impairment of such a system may be involved in drug resistance. We evaluated the *in vitro* antitumor activity of several cytotoxic drugs on two human p53-negative T-cell lymphoma cell lines, the HUT78-B1 CD95L-resistant cell line and the HUT78 parental CD95L-sensitive cell line. We demonstrated by Western blotting assay that DXR and etoposide (VP-16) were able to induce CD95L expression after 4 h of treatment. In contrast, they were unable to induce the expression of p53. DXR, at concentrations ranging from 0.001–1 $\mu\text{g/ml}$, and VP16, at concentrations ranging from 0.05–1 $\mu\text{g/ml}$, were equally cytotoxic and induced apoptosis in both cell lines as assessed by fluorescence microscopy and flow cytometry analyses. Although we observed a slightly reduced percentage of apoptotic cells in HUT78B1 when compared with the parental HUT78 cells after few hours of drug exposure, this difference was no longer evident at 48 or 72 h. Similarly, the exposure of HUT78 cells to a CD95-blocking antibody partially reduced early apoptosis (24 h) without affecting the long-term effects of the drugs including cytotoxicity. Furthermore, as observed with DXR and VP-16, both the CD95L-sensitive and the CD95L-resistant cell lines resulted equally sensitive to the cytotoxic effects of a number of different cytotoxic drugs (vincristine, camptothecin, 5-fluorouracil and methotrexate). The treatment with the

Caspase-3 tetrapeptide aldehyde inhibitor, Ac-DEVD-CHO, did not affect the DXR-induced apoptosis whereas it only modestly inhibited apoptosis and cytotoxicity of VP-16, while Z-VAD.FMK, a Caspase inhibitor that prevents the processing of Caspase-3 to its active form, was able to block DXR-induced apoptosis at 24 h but not at 48 h. Thus, our results do not confirm a crucial role for the CD95/CD95L system in drug-induced apoptosis and suggest the involvement of alternative p53-independent pathways at least in this experimental model system.

Keywords: apoptosis; anticancer drugs; CD95; CD95L; caspases

Abbreviations: CD95L, CD95 Ligand; DXR, Doxorubicin; VP-16, Etoposide; VCR, vincristine

Introduction

Programmed cell death or apoptosis is a genetically encoded process involved in the homeostasis of multicellular organisms and in carcinogenesis (Kerr *et al*, 1972; Williams and Smith, 1993; Martin and Green, 1995a; Williams, 1991). Several cytotoxic drugs employed in the chemotherapy of malignancies cause apoptosis in target cells (Fisher, 1994; Lennon *et al*, 1991; Collins *et al*, 1991; Walker *et al*, 1991), but the mechanisms by which these drugs induce apoptosis in neoplastic cells are not well understood at the molecular level. Several studies indicate that wild-type p53, a tumor suppressor gene which regulates both cell cycle and cell survival, plays a critical role in triggering cell death by apoptosis upon DNA damage caused by radio- and chemotherapy (Lowe *et al*, 1993a,b; Kinzler and Vogelstein, 1994). Recently, however, further evidence suggests that the p53 status does not necessarily correlate with chemo- or radio-resistance (Del Bufalo *et al*, 1996; Jung *et al*, 1992), and inactivation or mutation of p53 can even render cells more sensitive to cytotoxic agents (Fan *et al*, 1995; Xu *et al*, 1995). CD95 (also known as Fas or APO-1) is a cell surface protein belonging to the TNF receptor family which induces apoptosis upon interaction with agonistic antibodies or with the CD95 Ligand (CD95L). The CD95/CD95L system plays an important role in the immune response (Nagata, 1997; Krammer *et al*, 1994; Nagata and Golstein, 1995) and the expression of CD95L by tumor cells has been reported to be a possible determinant of tumor immunosurveillance and/or invasion *in vivo* (Strand *et al*, 1996; O'Connel *et al*, 1996; Hahne *et al*, 1996; Niehans *et al*, 1997; Saas *et al*, 1997). Crosslinking of CD95 results in rapid receptor aggregation and formation of the Death Inducing Signaling Complex (DISC) (Kischkel *et al*, 1995). This is composed of CD95, FADD or MORT-1 (Chinnaiyan *et al*, 1995; Boldin *et al*, 1995), a death domain-containing adaptor protein and Caspase 8 (FLICE/MACH protein) (Muzio *et al*, 1996; Boldin *et al*, 1996),

a member of the ICE-like family of cysteine proteases also known as Caspases (Alnemri *et al*, 1996), which play important roles in the execution stage of apoptosis (Martin and Green, 1995b; Kumar and Lavin, 1996). Moreover, CD95 crosslinking can also lead to the hydrolysis of sphingomyelin to form ceramide, which can then act as a second messenger to initiate multiple downstream pathways and several biological functions including apoptosis (Testi, 1996; Skowronski *et al*, 1996; Hannun, 1996). Recently, an increased expression of CD95L messenger RNA has been shown to occur in the lymphoblastic T cell CCRF-CEM leukemia exposed to doxorubicin or methotrexate (Friesen *et al*, 1996). Moreover, synthesis of ceramide, has been observed in murine leukemia cells treated with doxorubicin (Bose *et al*, 1995). These studies indicate that the CD95/CD95L system may be involved in apoptosis triggered by cytotoxic drugs. To understand the relationship between CD95 and drug-induced apoptosis, we have therefore studied the effects of doxorubicin (DXR) and etoposide (VP-16), two topoisomerase II inhibitors able to induce apoptosis in several tumor cell lines, in a T cell lymphoma clone resistant to CD95-mediated apoptosis (HUT78B1) as well as in its parental, CD95L-sensitive, counterpart (HUT78). HUT78B1 cells were selected by treatment of the parental HUT78 cell line with a CD95-specific monoclonal antibody, CH-11 and express both wild-type CD95 and a truncated form of the receptor lacking the intracellular death-signaling domain (Cascino *et al*, 1996).

Results

Effects of a CD95-agonistic antibody on HUT78 and HUT78B1 cells

HUT78 cells underwent apoptosis when exposed to increasing concentrations of the CD95-agonistic antibody, CH-11. Apoptosis was already observed after 4 h of treatment and reached a plateau at 24 h (90% of apoptotic cells). The CD95 IgG1 antibody, DX2, almost completely blocked the CH-11-induced apoptosis in HUT78 cells at 24 h (Figure 1) and to a similar extent at 48 and 72 h (data not shown). HUT78B1 cells were resistant to CH-11 treatment even at high concentra-

Figure 1 Percentage of apoptotic cells, evaluated by morphological analysis, after 24 h of exposure to the CD95-agonistic mAb CH11 in HUT78, HUT78B1, or HUT78 cells pretreated with the CD95-blocking Ab DX2. Data are the mean $\pm$ S.D. of four independent experiments. Flow cytometry analysis of apoptosis gave very similar results

tions, as previously reported (Cascino *et al*, 1996) (Figure 1).

Expression of p53 and bcl-2 protein in HUT78 and HUT78B1 cells

The expression of wild-type p53 and bcl-2 proteins was evaluated by Western blotting in HUT78 and HUT78B1 cells (Figure 2a and b). Similar levels of bcl-2 protein were observed in both cell lines while p53 protein was undetectable even upon treatment with DXR or VP-16 drugs for 12 or 24 h (Figure 2a).

Drug-induced apoptosis

DXR- and VP-16-induced apoptosis in HUT78 cells was concentration- and time-dependent (Figure 3a and b). About

Figure 2 Expression of p53 (a) and bcl-2 protein (b) in HUT78 and HUT78B1 cells. (a) As a control, the retinoblastoma cell line Y79 was incubated for 24 h in the presence (lane 1) or absence (lane 2) of camptothecin 0.5 μ M. HUT 78 cells (lanes 3–7) or HUT78B1 cells (lanes 8–12) were exposed to 0.9 μ g/ml of DXR for 12 (lanes 4 and 9) or 24 h (lanes 5 and 10) or to 1.5 μ g/ml of VP-16 for 12 (lanes 6 and 11) or 24 h (lanes 7 and 12). (b) Expression of bcl-2 in in HUT78 (lane 1), HUT78B1 (lane 2) and Y79 cells (lane 3)

Figure 3 Percentage of apoptotic cells, evaluated by morphological analysis, after 24 h (●), 48 h (■) or 72 h (▲) of exposure to DXR (a,c) or VP-16 (b,d) in HUT78 (a,b) and in HUT78B1 cells (c and d). Data are the mean $\pm$ S.D. of five independent experiments. Flow cytometry analysis of apoptosis gave very similar results

10% of the cells were apoptotic after 24 h of treatment with 0.6 $\mu\text{g/ml}$ of DXR or VP-16, and this percentage increased to 22% or 33% at 1.8 $\mu\text{g/ml}$ of DXR or 1.5 $\mu\text{g/ml}$ of VP-16, respectively. Finally DXR at 0.9 $\mu\text{g/ml}$ and VP-16 at 1.5 $\mu\text{g/ml}$ induced apoptosis almost in all cells at 72 h. Therefore, in spite of the absence of p53 and the presence of Bcl-2 proteins, HUT78 cells undergo apoptosis upon chemotherapeutic treatment. In the HUT78B1 CD95-resistant cells, a slightly lower percentage of apoptotic cells was observed at 24 h of drug exposure when compared to that of the wild-type HUT78 cells (Figure 3c and d). However, at 48 and 72 h the percentage of apoptotic cells in HUT78B1 was superimposable to that of HUT78 cells (Figure 3c and d).

Role of the CD95/CD95L system in drug-induced apoptosis in HUT78 and HUT8B1 cells

HUT78 cells express the CD95 receptor and are sensitive to apoptosis; thus we investigated the possible role of the CD95/

CD95L system in drug induced apoptosis. First the expression of CD95L in drug-treated HUT78 cells was evaluated by cytofluorimetric and Western blotting analyses. Since doubts have been raised about the specificity of some anti-CD95L antibodies COS-7 and 293T mock- or human CD95L- transiently transfected cells and NIH3T3 fibroblast transfected with the murine CD95L (Hughes and Crispe, 1995) were used along with the experimental samples. CD95L was detected in all transfectants as well as in the HUT78 cells treated with VP-16 and DXR by both cytofluorimetric (NOK-1 antibody) (Figure 4A and B) and Western blot (G247-4 and clone 33 antibodies) (Figure 4C and D) analyses but not in the mock transfected COS-7, 293T, NIH3T3 fibroblasts and in the untreated HUT78 cells (Figure 4). CD95L was induced at 4 h upon treatment with both VP-16 and DXR and slightly decreased at 8 h as detected by FACS analysis (Figure 4B), such decrease however was barely and only occasionally detectable on total cell lysates in Western blot (Figure 4D). Similar results were obtained with the CD95-

Figure 4 Expression of CD95L by cytofluorimetric and immunoblotting analyses. Surface CD95L expression detected with the NOK-1 or a isotype-matched control antibody by cytofluorimetric analysis (A, B) in mock or CD95L transfected 293T, COS-7, and NIH3T3 cells as indicated (A) and in HUT78 cells (B): untreated (a), treated with PMA (10 ng/ml and ionomycin (400 ng/ml) for 4 (b) and 8 h (c), treated with 1.5 $\mu\text{g/ml}$ of VP-16 for 4 (d) and 8 (e) h or with 0.5 $\mu\text{g/ml}$ of DXR for 4 (f) and 8 h (g). Immunoblotting analysis with the CD95L antibodies clone G247-4 and clone 33 as indicated (for details see Materials and Methods). (C) 293T, mock-transfected (lane 1); 293T, human CD95L-transfected (lane 2); COS-7 mock transfected (lane 3); COS-7 human CD95L-transfected (lane 4); NIH3T3 mock-transfected (lane 5); NIH3T3 murine CD95L-transfected (lane 6). (D): untreated HUT78 (lane 1), HUT78 treated with PMA and ionomycin for 4 (lane 2) and 8 h (lane 3), HUT78 treated with VP16 for 4 (lane 4) and 8 h (lane 5), HUT78 cells treated with DXR for 4 (lane 6) and 8 (lane 7) h

resistant HUT78B1 cells (data not shown). Next we evaluated the susceptibility to drug-induced apoptosis in HUT78 cells treated with a CD95-blocking antibody (DX2) and we compared the results to that obtained in HUT78B1. Likewise to that observed in HUT78B1, at 24 h of drug exposure a slightly lower percentage of apoptotic cells in the DX2-treated HUT78 than in the HUT78 cells was observed. (Figure 5a and b). However, at 48 (Figure 5c and d) and at 72 h (Figure 5e and f) a similar percentage of apoptotic cells in HUT78, HUT78 exposed to DX2 and in HUT78B1, was detected.

Drug-induced cytotoxicity

To test whether the CD95/CD95L system may influence antitumoral effects other than apoptosis we evaluated the cytotoxicity of DXR and VP-16 in HUT78 and in HUT78B1 cells by the trypan blue dye exclusion test. Both drugs produced similar cytotoxic effects in the two cell lines (Figure 6). In addition, the treatment with the DX2 antibody did not affect the cytotoxicity of DXR or VP-16 in HUT78 cells (data not shown). Moreover the cytotoxic effects of other

chemotherapeutic drugs, which differ in structure and mechanism of action from DXR and VP-16, were tested in HUT78 and HUT78B1 cells (Table 1). No significant differences in cytotoxicity were detected in the sensitive and in the resistant cell lines.

Role of Caspases in drug-induced apoptosis

Caspase-3 (CPP32, Yama, apopain) is activated through a protease cascade in response to several apoptotic stimuli, including CD95 crosslinking, and cleaves specific intracellular substrates during apoptosis (Martin and Green, 1995b; Kumar and Lavin, 1996). To understand whether Caspase-3 activation was directly involved in drug-mediated apoptosis, we studied the effects of DXR and VP-16 in HUT78 cells treated with the caspase 3 inhibitor Ac-DEVD-CHO (Acetyl Asp-Glu-Val-Asp aldehyde). While Ac-DEVD-CHO was able to inhibit, almost completely, the CH-11-induced apoptosis (86%, data not shown), it had no effect on apoptosis induced by DXR and only a partial inhibitory effect on apoptosis and cytotoxicity was observed in HUT78 cells exposed to Ac-DEVD-CHO and VP-16 (Figure 7). The

Figure 5 Comparison between the percentages of apoptosis, evaluated by morphological analysis, induced by DXR or VP-16 in HUT78, HUT78B1, or HUT78 cells exposed to the blocking anti-CD95 Ab DX2; (a) after 24 h of exposure to DXR; (b) after 24 h of exposure to VP-16; (c) after 48 h of exposure to DXR; (d) after 48 h exposure to VP-16; (e) after 72 h of exposure to DXR; (f) after 72 h exposure to VP-16. Data are the mean $\pm$ S.D. of five independent experiments. Flow cytometry analysis of apoptosis gave very similar results

Figure 6 Cytotoxicity of DXR and VP-16 in HUT78 (■) or HUT78B1 (▲) cells after 72 h of exposure to the agents. Cytotoxicity was evaluated by the trypan blue dye exclusion test. Data are the mean $\pm$ S.D. of five independent experiments

Caspase inhibitor Z-VAD.FMK (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone) (Slee *et al*, 1996) markedly inhibited DXR-induced apoptosis at 24 hours but not at 48 h and failed to inhibit apoptosis at 48 h when VP-16 was used at 5 μ g/ml (Figure 7). Z-VAD.FMK was however able to block CH-11-induced apoptosis both at 24 and 48 h (95%, data not shown).

Table 1 Cytotoxicity of anticancer drugs in HUT78 and HUT78B1 after 72 h of exposure

Drugs	HUT78	HUT78B1	RI*
	IC ₅₀ (ng/ml)	IC ₅₀ (ng/ml)	
DXR	48.0	57.0	1.2
VCR	5.3	9.2	1.7
VP-16	270.0	290.0	1.1
Camptothecin	4.9	5.6	1.1
Methotrexate	27.3	25.0	0.9
5-fluorouracil	650.5	260.2	0.4

RI: Resistance Index

Discussion

To examine the role of the CD95/CD95L system in the drug sensitivity/resistance of tumor cells, we studied the effects of DXR and VP-16, as well as of other agents, on the T cell lymphoma line HUT78 and on the CD95-resistant derived clone HUT78B1. HUT78 cells express CD95 and undergo apoptosis when treated with CH-11 agonistic antibody; moreover, apoptosis can be blocked by the addition of the DX2 antibody at 24 h (Figure 1) and to a similar extent at 48 and 72 h (data not shown). HUT78B1 cells express both a wild-type and a truncated receptor lacking the intracellular death domain and are completely resistant to the CH-11 antibody. Furthermore, we studied the expression of p53 in HUT78 and B1 clone, employing a monoclonal antibody specific for a sequence at the NH₂-terminus of the protein (aa 18–30). Wild-type p53 protein in both cell lines even upon treatment with DXR and VP-16 was not detectable by Western blot analysis. DXR and VP-16 induce apoptosis in HUT78 and HUT78B1 cells in a dose- and time-dependent manner. The DX2 antibody was able to interfere with DXR-

Figure 7 Effect of the Caspase inhibitors Ac-DEVD-CHO and Z-VAD.FMK on DXR- and VP16-induced apoptosis and cytotoxicity in HUT78 cells. Cells were exposed to 200 μ M of Ac-DEVD-CHO or 50 μ M of Z-VAD.FMK and after 90 min DXR or VP16 were added. (a) DXR induced apoptosis in HUT78 cells in the presence or absence of Ac-DEVD-CHO or Z-VAD.FMK; evaluation after 24 and 48 h; (b) VP16 induced apoptosis in HUT78 cells in the presence or absence of caspase inhibitors; evaluation after 24 and 48 h. (c) DXR induced cytotoxicity in HUT78 cells in the presence or absence of caspase inhibitors; evaluation after 24 and 48 h; (d) VP16 induced cytotoxicity in HUT78 cells in presence or absence of caspase inhibitors; evaluation after 24 and 48 h

and VP16-induced apoptosis in HUT78 cells only at 24 h, while it did not influence the percentage of apoptotic cells at 48 or 72 h of treatment. In other words, the addition of the CD95-blocking monoclonal antibody caused only a modest slow down of the apoptotic effects of DXR and VP-16 in HUT78. However, this was not due to the lack of CD95L, in fact a clear increase in CD95L expression shortly after the application of DXR or VP-16 was observed in HUT78 cells as assessed by cytofluorimetric and Western blot analyses (Figure 4). These results suggest that the CD95/CD95L system might play some role only in the early stages of drug treatment while it does not appear to be involved in the apoptosis observed after longer periods (48 or 72 h) of exposure to DXR and VP-16. This conclusion is further supported by the experiments with the HUT78B1 clone. In fact, while a lower percentage of apoptotic cells was observed at 24 h of treatment with DXR or VP-16 in HUT78B1 cells in comparison with the parental cell line, this difference was no longer evident at 48 or 72 h of treatment.

Moreover, the CD95/CD95L system did not influence the cytotoxic effects of other antineoplastic drugs, such as camptothecin, vincristine, 5-fluorouracil and methotrexate in this experimental system.

Remarkably the two cell lines do not express p53, thus this protein is not involved in the apoptotic process. It is tempting to speculate that new p53 independent mechanisms are responsible for the drug-induced apoptosis in this model system.

The drugs might act at a different step of the apoptotic process, such as at the level of the activation of the Caspase cascade. Interestingly, Ibrado *et al.* have shown that Ara-C, mitoxantrone and VP-16 induce activation of Caspase 3, which results in the cleavage of poly-(ADP-ribose) polymerase (PARP) and apoptosis (Ibrado *et al.*, 1996). However, the precise mechanism by which these drugs cause activation of Caspase-3 is still not clear. In order to understand the role of Caspase-3 in drug-induced apoptosis and cytotoxicity in our model system we treated HUT78 cells with DXR and VP-16 in the presence of the specific Caspase-3-like inhibitor Ac-DEVD-CHO. Although Ac-DEVD-CHO treatment almost completely blocked CH-11-induced apoptosis, non univocal results were observed with the antineoplastic drugs. In fact, whereas DXR-induced apoptosis was not affected, Ac-DEVD-CHO induced a partial inhibition of apoptosis and cytotoxicity induced by VP-16. These results are in agreement with recent reports indicating that the activation of Caspase-3 or other ICE-like cysteine proteases may not be a rate-limiting step in the chemotherapy-induced apoptotic process. In fact, cells transfected by CrmA, a cowpox protein which inhibits the apoptotic proteases, became resistant to CD95-mediated apoptosis but remained sensitive to the apoptotic effects of chemotherapy (Decaudin *et al.*, 1997). However, we observed that Z-VAD.FMK, a Caspase inhibitor acting upstream to Caspase-3 activation, was able to markedly inhibit DXR and VP-16 induced apoptosis. This inhibition was evident only in the first 24 h of treatment but not after 48 h. When a moderately cytotoxic concentration of etoposide was used, the apoptotic inhibitory activity of Z-VAD.FMK was evident also at 48 h (data not shown). Z-

VAD.FMK was able to block completely CH-11-induced apoptosis at 24 and 48 h. The complete inhibition of DXR-induced apoptosis by Z-VAD.FMK but not by Ac-DEVD-CHO observed after 24 h support the hypothesis that Caspases different from Caspase-3 may be involved in the early phases of DXR-induced apoptosis in this experimental model.

In conclusion, we think that the mechanisms underlying drug-induced apoptosis may involve several different molecular determinants which can be different depending on the experimental cellular model and the drug employed. Further studies may lead to the identification of new molecular targets for chemotherapeutic drugs.

Materials and Methods

Cell culture

HUT78 and HUT78B1 cells were grown in RPMI 1640 (Gibco Grand Island, NY, USA) containing 10% FCS (Gibco) supplemented with 100 U/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), and 2 mM L-glutamine (Sigma Chemical Co, St Louis, MO, USA) in a 5% CO₂ atmosphere at 37°C. Monkey COS-7 cells (American Type Culture Collection, Rockville, MD; CRL 1651) murine fibroblast, 293T cells (kindly provided by Dr. R. Beyaert, Laboratory for Molecular Biology, University of Gent, Belgium) were grown in DMEM containing 10% FCS. NIH3T3 transfected cells (kindly provided by Dr. N. Crispe, Yale University School of Medicine, New Haven, USA) were grown in presence of 0.5 mg/ml of G418-supplemented medium.

Chemicals and antibodies

DXR, VP-16, camptothecin, vincristine, methotrexate and 5-fluorouracil were from Sigma Chemical Co. The Caspase inhibitors Ac-DEVD-CHO and Z-VAD.FMK were purchased from Alexis Biochemicals (Laufelfingen, Switzerland). The following antibodies were used: the CD95 monoclonal antibody, DX2, kindly provided by Dr. R. Testi (Department of Experimental Medicine and Biochemical Sciences, Tor Vergata University, Rome, Italy); the CD95-agonistic antibody, CH-11, Upstate Biotechnology (Lake Placid, NY, USA); the CD95L antibodies: clone NOK-1, (Lot. N. M021271) PharMingen (San Diego, CA, USA), clone G247-4 (Lot N. M021163) PharMingen and clone 33, (Lot N. 2) Transduction Laboratories (Lexington, KY, USA); the p53, clone DO1, and the Bcl2 monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the Fluorescein-linked or horseradish peroxidase (HPR)-coupled sheep anti-mouse Ig from Amersham (Bucks, UK) and the Alkaline Phosphatase (AP)-linked anti-mouse IgG from Promega Corporation (Madison, USA).

Transient transfection

A cDNA coding for a full length CD95L receptor was obtained by RT-PCR using the synthetic oligonucleotides: GR142 GGGGGTACCATGCAATTA and GR115 GCGGATCCTTAGAGCTTATATAAGCCGAAA and then cloned into the *KpnI* and *BamHI* sites of the pcDNA3 eukaryotic expression vector (Invitrogen Corp., San Diego, CA). The integrity of the insert was confirmed by sequencing analysis. COS-7 or 293T cells respectively by the DEAE-dextran and the Calcium-phosphate methods were transfected with pcDNA3 or with pcDNA3CD95L plasmids. After 48 h, the transfected cells were analyzed by Flow cytometry and immunoblotting.

Cytotoxicity assays

To evaluate the number of live and dead cells, the cells were treated with trypan blue and counted on a hemocytometer. Cells which showed trypan blue uptake were interpreted as non viable. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 ml of complete medium and treated with different concentrations of each drug. After 72 h of incubation, the number of viable cells was determined and expressed as percent of the number of viable control cells. Resistance index (R.I.) for each drug was calculated from the ratio: IC50 in HUT78B1/IC50 in HUT78.

Flow cytometry analysis

The cells were washed once in ice-cold PBS and resuspended at 1×10^6 /ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μ g/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40 (Sigma). After 30 min of incubation in this solution, the samples were filtered through nylon cloth, 40 mm mesh, and their fluorescence was analyzed as single-parameter frequency histograms using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G₀-G₁ peak after labeling with propidium iodide (Darziynkiewick *et al*, 1992). For CD95L expression analysis cells were incubated at 4°C for 30 min with the NOK-1 (5 μ g/ml) or an isotype matched control antibody, washed and treated with FITC-conjugated sheep anti-mouse Ig. Relative fluorescent intensities of individual cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson).

Morphological evaluation of apoptosis

The morphological effects of the drugs were studied by fluorescent microscopy after labeling with acridine orange and ethidium bromide (Duke and Cohen, 1992). Cells (2×10^5) were centrifuged (300 g) and the pellet was resuspended in 25 μ l of the dye mixture. Thereafter 10 μ l of the cell suspension was placed on a microscope slide, covered with a 22 mm² coverslip and examined in oil immersion with a 100 $\times$ objective using a fluorescent microscope. Live cells were identified by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies.

Western blotting analysis of p53 and bcl-2

Cells were lysed for 30 min at 4°C with a solution (15 μ l/10⁶ cells) consisting of 1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS in phosphate buffer saline, pH 7.4, containing inhibitors of proteases (25 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium ortovanadate, 10 mM NaF, 25 μ g/ml leupeptin, 0.2 mM sodium pyrophosphate) and sonicated three times for 10 s. Equivalent amounts of protein (40 μ g) of the supernatants, obtained after centrifugation at 15000 g for 20 min at 4°C, were resolved in SDS-polyacrylamide gel electrophoresis (10% acrylamide). After electroblotting on nitrocellulose filters, proteins were immunoreacted for 2 h with p53 monoclonal antibody, DO1, (dilution 1 : 1000), or bcl-2 monoclonal antibody (dilution 1 : 500) followed by a 30-min incubation with anti-mouse IgG AP-linked antibody. Visualization was performed using nitroblue tetrazolium and bromochloro-indoyl-phosphate.

Western blotting analysis of CD95L

Cells were lysed in 50 mM Tris pH 7.6, 150 mM NaCl, 0.5% NP-40, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM PMSF and an equal amount of proteins (30 μ g) for each sample was loaded on a 12% SDS-PAGE under reducing conditions and transferred to nitrocellulose. CD95L was detected with the CD95L antibodies clone 33 (0.3 μ g/ml) or clone G247-4 (2 μ g/ml) using HRPO-coupled sheep anti-mouse Ig and the enhanced chemoluminescence (ECL) system (Amersham).

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