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Synergy of CD95 ligand and teniposide: No role of cleavable complex formation and enhanced CD95 expression [1]

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Abstract

Teniposide (VM26) enhanced the anti-glioma activity of the cytotoxic cytokine, CD95 ligand. Synergy was observed at concentrations of teniposide that were insufficient for cleavable DNA topoisomerase II complex formation. CD95 ligand did not modulate the formation or removal of such complexes after teniposide treatment. These processes were also unaffected by ectopic expression of *bcl*-2. Teniposide enhanced CD95 expression in a glioma cell line with wild-type p53 (LN-229) but not in two p53 mutant cell lines (T98G, LN-308). Forced expression of a transdominant negative p53 mutant prevented the teniposide induced augmentation of CD95 expression in LN-229 cells but did not prevent the synergy of CD95 ligand and teniposide. Teniposide did not alter CD95 ligand expression, and forced expression of CD95 did not modulate sensitivity to VM26. Thus, teniposide-induced DNA lesions and alterations in CD95 or CD95 ligand are not necessary for teniposide-induced sensitization of human malignant glioma cells to CD95-mediated apoptosis. © 1998 Published by Elsevier Science B.V.

Keywords: CD95; Teniposide; Topoisomerase; Glioma; Apoptosis

1. Introduction

CD95 (Fas/APO-1) ligand is a cytotoxic cytokine that induces apoptosis via activation of its receptor, CD95, a member of the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily. CD95-mediated apoptosis is a novel immunotherapeutic approach to human malignant glioma (Weller, 1996). Targeting CD95 appears to be feasible for the treatment of these inevitably lethal neoplasms because glioma cells do not resist CD95-mediated apoptosis (Weller et al., 1994) and since CD95 is not expressed by normal brain parenchymal cells (Leithäuser et al., 1993). The antiglioma activity of CD95 ligand is synergistically augmented by coexposure to CD95 ligand and various cancer chemotherapy drugs including the topoisomerase II inhibitor, teniposide (VM26) (Roth et al., 1997) and the topoisomerase I inhibitor, camptothecin (Weller et al., 1997b). Topoisomerase II plays a central role in DNA replication and DNA repair (Berger et al., 1996). Here, we examine the molecular mechanisms underlying the synergy of CD95 ligand and VM26 in three human malignant glioma cell lines, T98G, LN-229 and LN-308.

2. Materials and methods

2.1. Chemicals, cell lines and cell culture

VM26 was obtained from Bristol (Munich). All other chemicals were from Sigma (St. Louis, MO). [Methyl-^{3}H]-thymidine was from Amersham (specific activity: 20–40 Ci/mmol) (Braunschweig). T98G human glioma cells were obtained from ATCC (American Type Culture Collection). LN-229 and LN-308 human glioma cells were kindly provided by Dr. N. de Tribolet (Lausanne). These cell lines have been characterized in previous studies (Van Meir et al., 1994; Weller et al., 1994). Glioma cells engineered to express high levels of murine *bcl*-2 (T98G,

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LN-229) or human CD95 (LN-308) have been described (Weller et al., 1995a,b). For the present study, we also generated CD95 transfectants of the LN-229 cell line, using the BCMGS plasmid containing a human CD95 cDNA (Weller et al., 1995b). The generation of LN-229 cells engineered to express the murine temperature-sensitive p53 mutant val^{135} has been described (Trepel et al., 1996). Pooled transfected cells were always compared with neo (*bcl*-2, CD95) or hygro (p53) control cells which harbor the empty vector. Neuro2A murine neuroblastoma cells expressing murine CD95 ligand were generated and maintained as described (Rensing-Ehl et al., 1995) and served as the source for the CD95 ligand-containing supernatants used here.

2.2. Assessment of viability and apoptosis

Glioma cell proliferation was assessed by crystal violet staining. Apoptosis was measured by quantitative assessment of DNA fragmentation as described (Weller et al., 1994). Detached and adherent cells were harvested by centrifugation and trypsinization, respectively, pooled, and lysed for 10 min on ice in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 0.2% Triton X-100, pH = 7.5). Intact DNA was pelleted by centrifugation for 10 min at 13,000 rpm and 4°C. The supernatants containing fragmented DNA were transferred to new tubes. The pellets were disrupted by sonication and resuspended in lysis buffer. Disrupted pellets and supernatants were digested with RNase A (100 μg/ml) for 2 h at 37°C and the DNA content was separately determined by 1:10 dilution in 5 mM Tris–HCl/0.5 mM EDTA (pH = 7.6) containing 0.5 μg/ml ethidium bromide in a Millipore fluorimeter at 530 nm excitation and 620 nm emission wave lengths.

2.3. Determination of cleavable DNA topoisomerase II complexes

Cleavable DNA topoisomerase II complex formation was assessed as previously described (Rowe et al., 1986). Briefly, DNA of exponentially growing cells (10^5 cells/ml) was labeled with 2 μCi/ml [methyl-^{3}H]thymidine (specific activity: 20–40 Ci/mmol) over night. Cells were washed with phosphate-buffered saline (PBS) 3 times and trypsinized, and an aliquot was counted. The cells were adjusted to 100,000 cpm/ml and then incubated in 12 well plates for further 24 h. Subsequently, the cells were treated with different concentrations of VM26 for various time intervals, mostly 30 min, washed with PBS and lysed with 1 ml prewarmed (65°C) lysis solution (1.25% sodiumdodecylsulfate (SDS), 5 mM EDTA, pH 8.0, herring sperm DNA, 0.4 mg/ml). After breakage of chromosomal DNA by repeated passing of the lysates through an 22 gauge needle, the lysates were transferred to a reaction tube containing 250 μl 325 mM KCl, vortexed vigorously for 10 s, incubated for 10 min on ice and centrifuged for 10 min at 13,000 rpm/4°C. The pellets were resuspended in 1 ml washing solution (10 mM Tris/HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml herring sperm DNA) and kept at 65°C for 10 min. The suspensions were then cooled on ice for 10 min and recentrifuged. The pellets were washed once again and resuspended in 200 μl H_2O (65°C). After addition of 5 ml liquid scintillation cocktail, radioactivity was measured in a Wallac Liquid Scintillation Counter. For determination of the removal of cleavable complexes, the cells were washed with PBS after incubation with VM26, reincubated in fresh medium for the indicated times, and then prepared as described above.

2.4. Flow cytometry

CD95 expression was measured as described (Weller et al., 1995b). CD95 ligand expression was assessed accordingly, using anti-human CD95 ligand antibody (N-20) from Santa Cruz (rabbit polyclonal immunoglobulin G (IgG)) and rabbit IgG as an isotype-control. For assessment of CD95 ligand expression, the cells were permeabilized in 75% ice-cold ethanol in PBS for 10 min on ice prior to the labeling procedure.

2.5. Statistics

EC_{50} values were determined by linear regression analysis. Effects of simple treatments were compared by Student's *t*-test. Composite treatments were compared by analysis of variance (ANOVA).

3. Results

3.1. CD95 ligand fails to alter induction and removal of DNA lesions induced by VM26

Previous studies performed in our laboratory have shown that CD95 ligand-induced apoptosis of human malignant glioma cells is augmented by coexposure to VM26 in modified colony formation assays (Roth et al., 1997) (Table 1). Synergy of CD95 ligand and VM26 was observed at concentrations of VM26 of 0.1–1 μM. Lower concentrations were ineffective, and higher concentrations induced more than 50% growth inhibition when administered alone. In contrast, the acute cytotoxic effects of CD95 ligand were synergistically enhanced in LN-229 cells, but not in T98G or LN-308 cells, by VM26 at around 10 μM. Here we examined the molecular mechanisms underlying the synergy of CD95 ligand and VM26 in these cells. VM26 induced cleavable complexes of DNA and topoisomerase II in a concentration- and time-dependent manner (Fig. 1A, B). After prolonged exposure to VM26 (10 μM), an equilibrium of cleavable complex formation was achieved within 20 min. The concentrations required for significant induction of these DNA lesions

Table 1
Effects of coexposure to CD95 ligand and VM26, and p53 status, in human malignant glioma cell lines (Roth et al., 1997)

	Synergy		p53 status
	acute cytotoxicity	growth inhibition	
T98G	−	+	mutation
LN-229	+	+	wild-type
LN-308	−	+	deletion

exceeded those sufficient to augment CD95-mediated apoptosis (Roth et al., 1997) more than 10-fold. Further, neither induction (Fig. 1C) nor removal (Fig. 1D) of DNA lesions was altered when T98G cells were coexposed to CD95 ligand and VM26 compared with VM26 treatment alone. Consistent with these data, quantitative DNA fluorometry failed to reveal enhanced induction of DNA fragmentation at 24 h by VM26 in the presence of CD95 ligand (data not shown). We have reported that glioma cells expressing high levels of murine *bcl*-2 protein are more resistant to VM26 than parental or neo-control cells (Roth et al., 1997). Interestingly, the enhanced resistance to VM26 afforded by ectopic *bcl*-2 expression was associated neither with decreased DNA lesion formation nor with enhanced complex removal after VM26 exposure (data not shown). Thus, formation of cleavable DNA topoisomerase II complexes may be necessary, but is not sufficient, for VM26-induced cytotoxicity, and is not required for augmentation of CD95-mediated apoptosis. Also, there is significant regulation of cell death down-stream in the killing pathway, as exemplified by the rescue from cell death afforded by *bcl*-2.

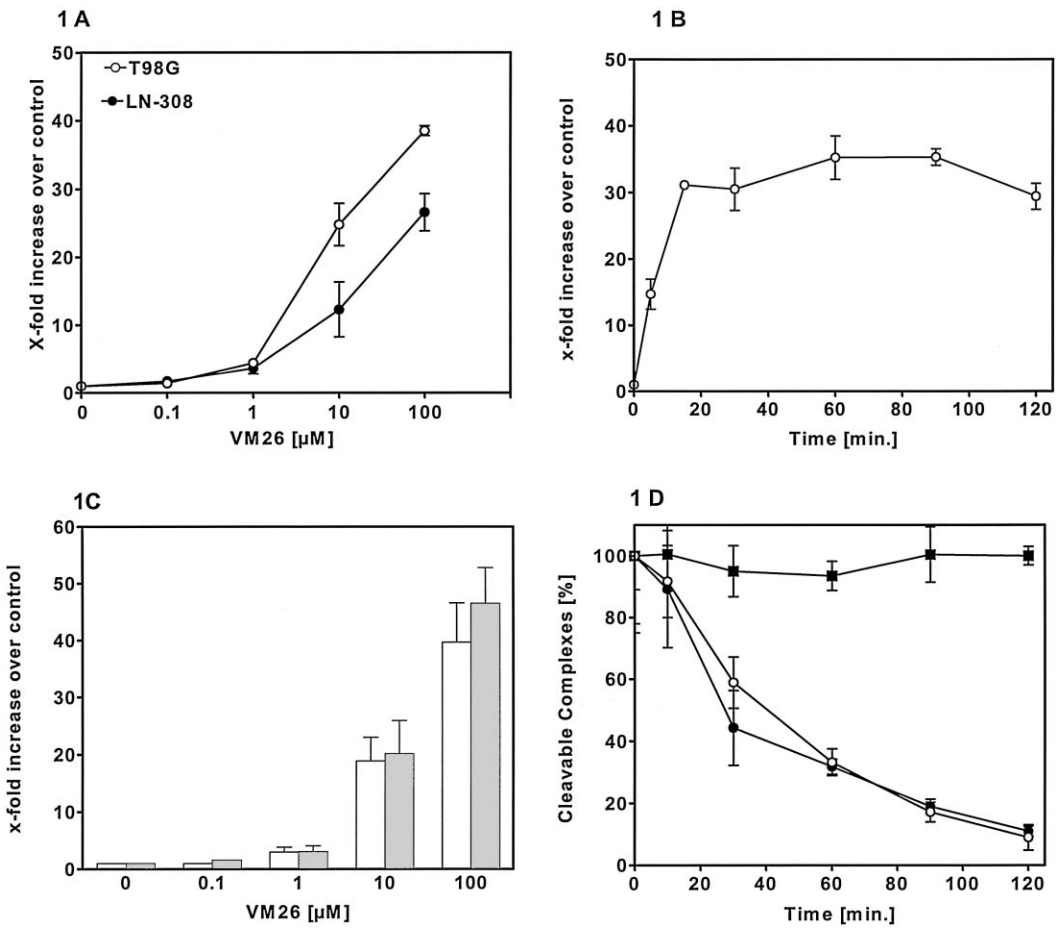

Fig. 1. Induction and removal of DNA lesions induced by VM26 are unaffected by CD95 ligand. (A) T98G (open circles) or LN-308 (closed circles) human glioma cells were exposed to increasing concentrations of VM26 for 30 min. Cleavable DNA/topoisomerase II complexes were measured by potassium chloride/SDS precipitation as described in Section 2. Data are expressed as X-fold increase over control (untreated cells). (B) T98G (open circles) cells were exposed to 10 μM VM26 for various times. DNA lesions were determined as in (A). (C) T98G cells were exposed to increasing concentrations of VM26 in the presence of neo control supernatant (open bars) or supernatant containing 50 U/ml CD95 ligand (closed bars) for 30 min. DNA lesions were measured as in (A) and (B). There was also no increase in complex formation in the presence of CD95 ligand when the incubation time was prolonged to 24 h (data not shown). (D) T98G cells were exposed to 10 μM VM26 for 30 min in the absence (open circles) or presence (closed circles) of CD95 ligand (50 U/ml). No change was observed when the cells were switched to medium containing VM 26 (closed squares). Removal of DNA lesions was monitored by determining levels of precipitable DNA/topoisomerase II complexes. Similar results were observed when the cells were exposed to CD95 ligand and VM26 for 24 h and complex removal was assessed thereafter (data not shown).

3.2. VM26-induced changes in CD95 expression are mediated by p53 but are not required for synergistic inhibition of glioma cell growth by CD95 ligand and VM26

Synergy of CD95 ligand and cancer chemotherapy drugs might involve drug-induced changes in the expression of CD95. Therefore, we examined the effects of VM26 on CD95 expression in LN-229, T98G and LN-308 cells. There was a significant increase in CD95 expression in LN-229 cells at 24 h, but not at 4 h, after exposure to VM26 at 0.5 and 10 μM (Fig. 2A–C). In contrast, no change in CD95 expression was seen in T98G or LN-308

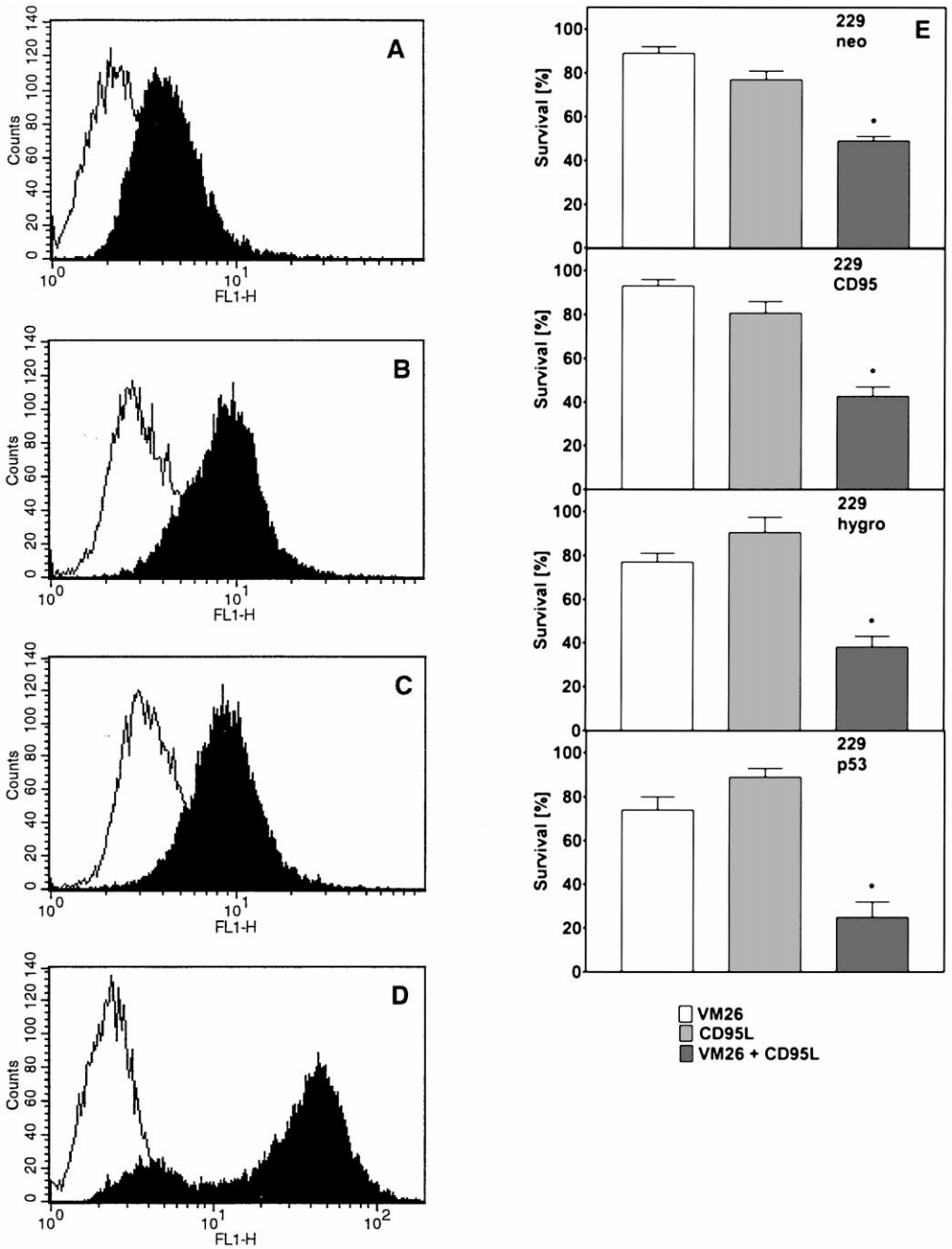

Fig. 2. No link between p53-dependent, VM26-induced augmentation of CD95 expression and the synergistic anti-glioma activity of VM26 and CD95 ligand. LN-229 cells were untreated (A) or exposed to VM26 at 0.5 μM (B) or 10 μM (C) for 24 h. CD95 expression was determined by flow cytometry as described in Section 2. SFI values were 1.83 (A), 2.40 (B) and 2.43 (C). No induction of CD95 expression was observed in T98G or LN-308 cells (data not shown). (D) LN-229 cells transfected with BCMGS-CD95 (Weller et al., 1995b) and CD95 expression analyzed by flow cytometry. The pool of transfected cells showed heterogenous CD95 expression, the SFI was in the range of 10–15. In Fig. 2A–D, black profiles indicate specific fluorescence for CD95, open profiles represent binding of an isotype control antibody. In Fig. 2E, 229 neo, 229-CD95, 229 hygro or 229-p53 cells were exposed for 16 h to VM26 (25 μM) (open bars) or CD95 ligand (25 U/ml (neo, CD95) and 8 U/ml (hygro, p53)) (light gray bars) or both (dark-gray bars). Survival was assessed by crystal violet staining. Data are expressed as means and S.E.M. ($n = 3$, $^{*}P < 0.05$).

cells (data not shown). Next we asked whether the synergy of VM26 and CD95 ligand in acute cytotoxicity assays with LN-229 cells is a result of VM26-induced augmentation of CD95 expression at the cell surface and whether this effect depends on wild-type p53 (Table 1). First, we generated LN-229 sublines engineered to express high levels of CD95 at the cell surface (Fig. 2D). These transfected LN-229 cells were not significantly more sensitive to CD95 ligand when administered alone than neo-control cells, confirming that an increase in CD95 expression beyond a critical threshold may not further enhance glioma cell sensitivity to CD95-mediated apoptosis in human malignant glioma cells (Weller et al., 1995b). Interestingly, these transfected cells, which were refractory to induction of CD95 expression by VM26 as a result of their high base-line CD95 expression, were nevertheless susceptible to synergistic cytotoxicity induced by coexposure to CD95 ligand and VM26, suggesting that the VM26-induced increase in CD95 expression is not required for synergy (Fig. 2E). Second, we took advantage of the temperature-sensitive p53 mutant val^{135} that acts as a transdominant negative mutant when expressed in mutant conformation at 38.5°C. Forced expression of p53 val^{135} in LN-229 cells in mutant conformation prevented the VM26-induced increase in CD95 expression, confirming that this effect is indeed mediated by wild-type p53. Yet, the synergy induced by coexposure to CD95 ligand and VM26 was unaffected in p53 val^{135}-transfected LN-229 cells (Fig. 2E).

3.3. VM26 fails to alter CD95 ligand expression in human malignant glioma cells

Drug-induced cytotoxicity of leukemia cells has recently been linked to the induction of CD95 ligand expression and CD95 ligand/CD95 interactions (Friesen et al., 1996). We found that exposure of T98G, LN-229 or LN-308 cells to 0.5 or 10 μM for 4 or 24 h did not result in altered CD95 ligand expression at the cell surface (data not shown). Base-line SFI levels for CD95 ligand expression were 7.37 for T98G, 8.9 for LN-229, and 8.7 for LN-308. That VM26 did not induce CD95 ligand expression was also consistent with the observation that CD95-transfected LN-229 were no more susceptible to VM26 than neo-control cells (Fig. 2E). Further, CD95-transfected LN-308 glioma cell clones (Weller et al., 1995b) did not differ from neo-control cells with regard to cleavable complex formation or cytotoxicity induced by VM26 (data not shown).

4. Discussion

Human malignant gliomas are refractory to current modes of treatment including cytoreductive surgery, radiotherapy, and chemotherapy. Current experimental approaches focus on immunotherapy and somatic gene therapy (Weller and Fontana, 1995). Recent experimental studies have evaluated immunochemotherapy based on coexposure of glioma cells to TNF-α and cytotoxic drugs including inhibitors of topoisomerase II (Kamikaseda et al., 1994; Morgavi et al., 1995). However, since glioma cells are largely resistant to TNF at relevant concentrations (Weller et al., 1994) and because TNF receptors are expressed almost ubiquitously in human tissues, CD95 targeting is probably much more promising therapeutically (Roth et al., 1997) because it can be targeted more selectively to glioma cells. The major risk of local CD95 activation for malignant glioma is systemic toxicity (Ogasawara et al., 1993). Combining CD95 ligand with chemotherapy could reduce the dose of CD95 ligand required for glioma therapy in vivo (Roth et al., 1997). Here, we examined the mechanism underlying the augmentation of CD95-mediated apoptosis in the presence of a topoisomerase II inhibitor, VM26. We found that the high nanomolar concentrations of VM26 sufficient for synergistic growth inhibition with CD95 ligand (Roth et al., 1997) were insufficient for the formation of cleavable DNA topoisomerase II complexes (Fig. 1A). Further, CD95 ligand did not enhance the formation of such complexes at higher VM26 concentrations, nor did it interfere with their removal after VM26 withdrawal (Fig. 1C, D). This suggests that VM26 has effects other than topoisomerase II inhibition detectable by cleavable complex formation. Inhibition of RNA and protein synthesis by VM26 at concentrations around 1 μM can be excluded as the mechanism augmenting the effects of CD95 ligand. Synergy based on inhibition of macromolecule synthesis, as exemplified by the effects of actinomycin D or cycloheximide on CD95-dependent apoptosis, is easily demonstrated in short-term cytotoxicity assays (Weller et al., 1994), whereas the synergy between VM26 and CD95 ligand in T98G and LN-308 cells becomes apparent only in modified colony formation assays (Roth et al., 1997). Interestingly, VM26-induced apoptosis can be *blocked* down-stream of DNA complex formation, as shown here and previously reported in the case of etoposide (Kamesaki et al., 1993), for *bcl*-2.

Recent studies suggested that the CD95/CD95 ligand signaling system might be directly involved in drug-induced apoptosis of leukemia and hepatoma cells (Friesen et al., 1996; Müller et al., 1997). We found that CD95 expression was enhanced by exposure to VM26 in a p53-dependent manner. This finding resembles the p53-dependent induction of CD95 in hepatoma cells (Müller et al., 1997). However, using gene transfer approaches, we demonstrated that (i) ectopic expression of neither CD95 — to nullify a significant increase in CD95 expression induced by VM26 — nor of a transdominant negative p53 mutant — to block p53-dependent augmentation of CD95 expression — altered the sensitization to CD95-mediated apoptosis induced by VM26 in LN-229 cells in acute cytotoxicity assays (Fig. 2). Further, modulation of the

expression of CD95 or CD95 ligand appeared to play no role in the synergistic activity of VM26 and CD95 ligand in either cell line in modified colony formation assays. That glioma cells coexpress CD95 and CD95 ligand without committing fratricide or suicide is puzzling but has been confirmed independently (Saas et al., 1997; Weller et al., 1997a). In regard to the regulation of drug sensitivity, glioma cells differ significantly from leukemia or hepatoma cells (Friesen et al., 1996; Müller et al., 1997) with regard to the role of the CD95/CD95 ligand system. The mechanisms underlying potentiation of CD95-mediated apoptosis by cytotoxic drugs such as VM26 thus remain obscure at present. We hypothesize that such drugs might act further down-stream to facilitate signaling of the death pathway triggered by CD95 ligation, e.g., at the level of caspase activation or ceramide generation, both of which are thought to be essential steps in CD95 ligand-induced apoptosis.

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