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# Induction of CD95 ligand and apoptosis by doxorubicin is modulated by the redox state in chemosensitive- and drug-resistant tumor cells

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## Abstract

**Induction of CD95 ligand (CD95-L) may contribute to drug-induced apoptosis in chemosensitive leukemias and solid tumors. Here we report that induction of CD95-L and apoptosis by doxorubicin in leukemic and neuroblastoma cells is regulated by the redox state and reactive oxygen species (ROS). Preincubation of chemosensitive cells with antioxidants such as N-acetyl-cysteine (NAC) or glutathione (GSH), significantly reduced doxorubicin-induced apoptosis, hyperexpression of ROS, loss of mitochondrial membrane potential ($\Delta\Psi_m$) and upregulation of CD95-L expression. Doxorubicin-resistant cells exhibited higher levels of GSH in comparison to chemosensitive cells and were deficient in hyperproduction of ROS, loss of $\Delta\Psi_m$ and upregulation of CD95-L in response to cytotoxic drugs. Downregulation of intracellular GSH concentrations reversed deficient drug-induced hyperproduction of ROS and CD95-L upregulation. In addition, overexpression of Bcl-$X_L$ in CEM cells blocked doxorubicin-triggered ROS and CD95-L expression. These findings suggest that induction of CD95-L by cytotoxic drugs is modulated by the cellular redox state and mitochondria derived ROS.**

**Keywords:** apoptosis; CD95 ligand; glutathione; doxorubicin; mitochondria; redox state

**Abbreviations:** AA, ascorbic acid; AICD, activation-induced cell death; BSO, L-buthionine-(S,R)-sulfoximine, CAT, catalase; CD95-L, CD95-ligand; CD95, CD95 receptor; CHX, cycloheximide; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; DOXO, doxorubicin; DOXOR, doxorubicin-resistant; $\Delta\Psi_m$, mitochondrial membrane potential; FSC, forward scatter; GSH, glutathione; HE, dihydroethidine; mBCl, monochlorobimane; MRP, multi-drug resistant protein; NAC, N-acetylcysteine; NGF, nerve growth factor; P-gp, p-glycoprotein; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PT, permeability transition; ROS, reactive oxygen species; SOD, superoxide dismutase; SSC, side scatter

## Introduction

Most anticancer drugs have been found to induce apoptosis in chemosensitive leukemias and solid tumors.[1,2] The CD95 receptor/CD95 ligand (CD95/CD95-L) system is a key signal pathway involved in regulation of apoptosis in several cell types.[3–7] The CD95 cell surface receptor (CD95) is a member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily of cell surface molecules which mediates apoptosis upon oligomerization.[8–13] CD95 cross-linking by agonistic antibodies or the natural ligand activates a signal cascade via FADD/MORT-1 and FLICE/MACH that directly leads to activation of ICE/Ced-3 proteases (caspases) which function as downstream effectors of cell death.[14] The CD95 ligand (CD95-L) is a member of the corresponding family of TNF-related cytokines, which is found in a soluble or membrane-bound form. CD95-L may cause autocrine suicide in sensitive $CD95^+$ T-cells and fracticide or paracrine death in neighboring T-cells or other target cells.[15–17]

Induction of CD95-L and upregulation of CD95 has been found after treatment of tumor cells with cytotoxic drugs.[18–25] Blocking of CD95/CD95-L interaction by antagonistic antibodies to the receptor downregulates drug-induced apoptosis.[18,21–23,25,26] Activation of the CD95 system has been observed in a variety of different tumor cell lines such as hepatoma,[23] neuroblastoma,[21] colon carcinoma[24] and medulloblastoma[22] and in *ex vivo* derived tumor cells[19] with different drugs, such as doxorubicin,[18,19,21] cisplatin,[21] etoposide,[21,25] teniposide,[25] cytarabine,[19] fluorouracil,[24] bleomycin[23] and methotrexate[18,19] although the relative contribution of CD95/CD95-L interaction is controversial.[27–33] Several recent reports have indicated that mitochondria play a major role in apoptosis including cell death induced by anticancer agents.[34–38] A crucial, common step in most forms of apoptosis involves a loss of mitochondrial membrane potential ($\Delta\Psi_m$). Disruption of $\Delta\Psi_m$ is mediated by opening of permeability transition (PT) pores resulting in hyperproduction of reactive oxygen species (ROS). Overexpression of ROS-scavenging enzymes and various antioxidants with ROS-scavenger properties were reported to block apoptosis induced by tumor necrosis factor (TNF), anticancer drugs such as doxorubicin,[39] $\gamma$-irradiation and activation-induced cell death (AICD).[40] Anti-apoptotic members of the Bcl-2 family have been shown to prevent apoptosis in response to a wide variety of apoptosis stimuli. Overexpression of Bcl-2 or Bcl-$X_L$ prevents mitochondrial permeability transition and hyperproduction of ROS by different apoptosis inducing stimuli.[34] Since some of these apoptosis inducers involve induction of CD95-L and activation of the CD95 system we investigated the role of the cellular redox state and ROS in drug-induced expression of CD95-L.

## Results

### Effects of NAC and GSH on doxorubicin-induced apoptosis

Reduced glutathione (GSH, a cystein-containing tripeptide: L-γ-glutamyl-L-cysteinyl-glycine), the most abundant intracellular thiol acts as a major antioxidant by protecting cells against damaging effects of free radicals and reactive oxygen species (ROS) and may protect cells from apoptotic cell death.[41–46] We therefore examined whether an increase in intracellular GSH would also affect drug-induced apoptosis in a chemosensitive leukemia cell line (CEM) and chemosensitive neuroblastoma cell line (SHEP). Since N-acetyl-cysteine (NAC) is known to increase intracellular GSH,[42] we examined whether pretreatment with NAC or GSH could prevent doxorubicin-induced apoptosis. The leukemia cell line CEM and the neuroblastoma cell line SHEP were pretreated with NAC or GSH for 2 h before doxorubicin-treatment (Figure 1A). After 30 h for CEM and 72 h for SHEP a significant reduction of doxorubicin-induced apoptosis was found in both cell lines pretreated with NAC or GSH in comparison to non pretreated cells. The inhibitory effect of NAC was mediated by upregulation of intracellular GSH, since coincubation of CEM cells with NAC and BSO, a specific inhibitor for GSH synthesis,[47] almost completely abolished the inhibitory effect of NAC (Figure 1B). We next investigated whether other non-thiol antioxidants with different ROS protecting effects such as catalase (CAT), superoxide dismutase (SOD) and ascorbic acid (AA) could also prevent doxorubicin-induced apoptosis. CEM cells were pretreated with catalase, superoxide dismutase or ascorbic acid 2 h before doxorubicin treatment. After 30 h a moderate protection against doxorubicin-mediated apoptosis was found (Figure 1C). These data demonstrate that only GSH could significantly protect cells from doxorubicin-induced apoptosis.

### Effects of NAC and GSH on doxorubicin-induced $\Delta\Psi_m$ reduction and ROS generation

Mitochondrial function involving loss of membrane potential by opening of PT pores and hyperproduction of ROS has been implicated in cell death induced by anticancer drugs.[34,35] Since antioxidants have been shown to inhibit doxorubicin-mediated death we analyzed the effects of antioxidants on doxorubicin-induced mitochondrial dysfunction. Incubation of chemosensitive CEM cells with doxorubicin lead to disruption of $\Delta\Psi_m$ and hyperproduction of ROS at early time points. Disruption of $\Delta\Psi_m$ and hyperproduction of ROS were significantly reduced when chemosensitive cell lines were pretreated with NAC or GSH before doxorubicin treatment (Figure 1D). These data suggest that NAC or GSH mediated inhibition of doxorubicin-induced apoptosis involves inhibition of disruption of $\Delta\Psi_m$ and generation of ROS from mitochondria.

### Effects of NAC and GSH on drug-induced induction of CD95-L

CD95-L is produced in chemosensitive leukemias and solid tumors in response to drug treatment[18–26] and deficient upregulation of CD95-L may contribute to drug-resistance of tumor cells.[19] We therefore asked whether upregulation of CD95-L by cytotoxic drugs would be modulated by intracellular GSH concentrations. Incubation of chemosensitive cell lines CEM and SHEP cells with doxorubicin after pretreatment with NAC or GSH completely blocked upregulation of CD95-L mRNA as shown by reverse transcriptase polymerase chain reaction (RT–PCR) (Figure 2A and B). In addition, no drug-induced upregulation of CD95-L protein was observed as shown by Western blot analysis (Figure 2A). These results are in agreement with data recently published showing that bleomycin-induced CD95-L mRNA involves ROS in hepatoma cells.[48] Furthermore, downregulation of CD95 expression was not found in chemosensitive cells after pretreatment with NAC or GSH at time points used in these experiments (data not shown). Defective activation of caspases by cytotoxic drugs after pretreatment with NAC or GSH was also reflected by the failure to cleave the prototype caspase substrate poly(ADP-ribose)polymerase (PARP).[49] PARP was processed to its characteristic 85 kDa fragment by doxorubicin in chemosensitive CEM and SHEP cells but not in chemosensitive cells pretreated with NAC or GSH before doxorubicin treatment (Figure 2C). To see whether differences in intracellular GSH concentrations would modulate chemosensitivity, we generated $CEM^{DOXOR}$ and $SHEP^{DOXOR}$, variants of CEM or SHEP cells, resistant to therapeutic concentrations of doxorubicin for over 24 months.[19] Both variants of resistant cells did not exhibit a multi drug-resistant (MDR) phenotype of drug-resistance since doxorubicin uptake was not altered in resistant cells as compared to sensitive parental cells.[19] Cytofluorometric analysis using mBCl (monochlorobimane)[44,45] for detection of intracellular GSH revealed higher GSH concentrations in doxorubicin-resistant cell lines compared to parental chemosensitive cell lines (Figure 3). These findings suggest that differences in intracellular GSH concentrations may modulate induction of CD95-L, full activation of the CD95 pathway and chemosensitivity.

### $\Delta\Psi_m$ reduction and ROS generation in doxorubicin-resistant cells

Since high GSH levels prevent cells from doxorubicin-induced loss of $\Delta\Psi_m$ and ROS generation and since doxorubicin-resistant cells show a higher concentration of GSH compared to chemosensitive cells, we asked whether disruption of $\Delta\Psi_m$ and hyperproduction of ROS was also reduced in doxorubicin-resistant cells. In contrast to parental CEM cells, loss of $\Delta\Psi_m$ and hyperproduction of ROS could not be detected in $CEM^{DOXOR}$ cells after drug treatment (Figure 4).

### Effect of Bcl-$X_L$ on activation of the CD95 system

Since Bcl-$X_L$ is located in mitochondria and prevents cells from doxorubicin-induced apoptosis and loss of $\Delta\Psi_m$ and ROS generation[34] (data not shown), we asked whether doxorubicin-induced CD95-L expression was modulated in Bcl-$X_L$ transfected CEM cells ($CEM^{Bcl\text{-}XL}$). In contrast to parental CEM cells, no CD95-L upregula-

tion was found in Bcl-$X_L$ transfected CEM cells after doxorubicin treatment (Figure 5). This suggests that ROS from mitochondria may be involved in doxorubicin-induced apoptosis.

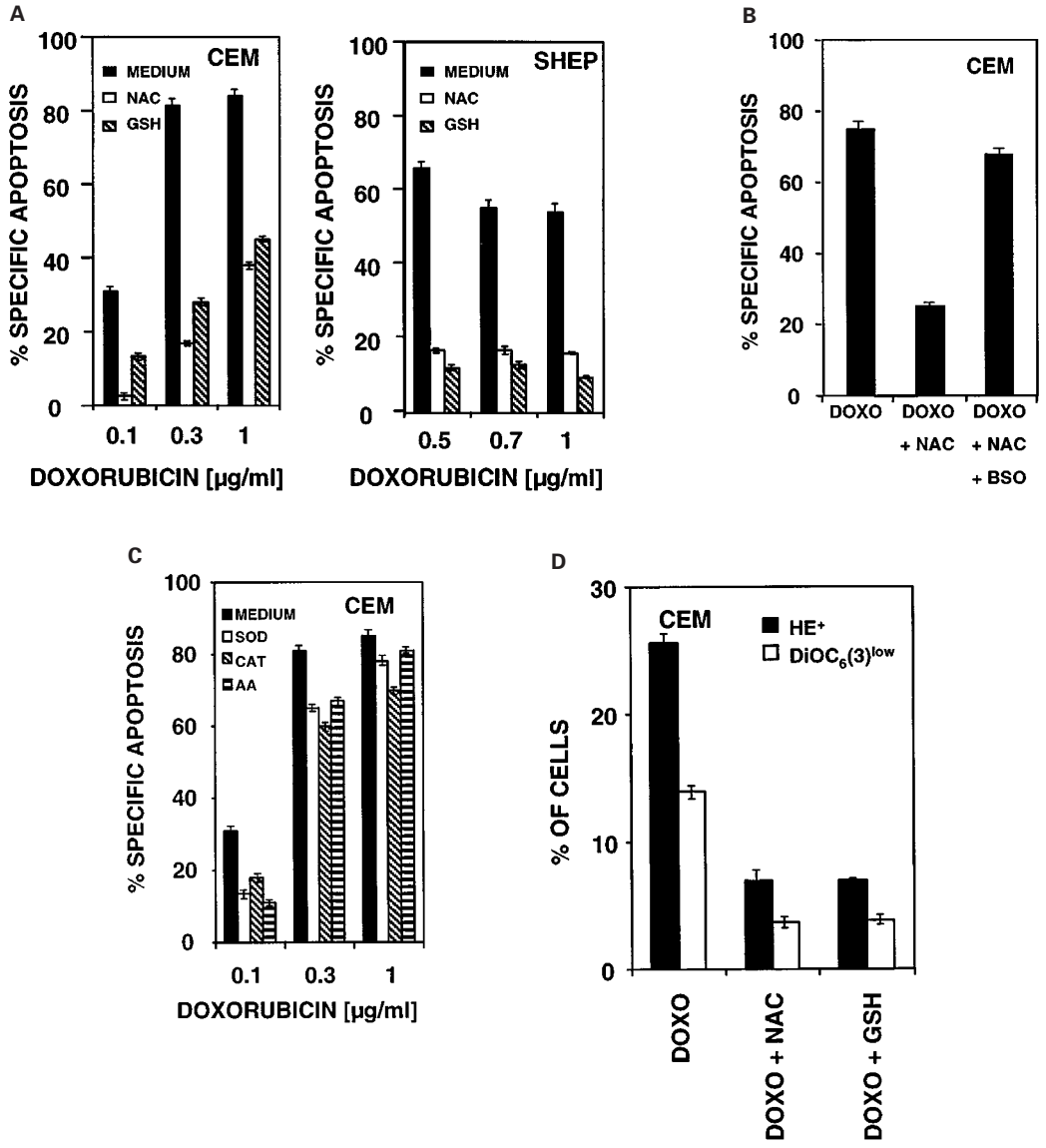


**Figure 1** Effects of antioxidants in doxorubicin-induced apoptosis. (**A**) Effects of NAC and GSH in doxorubicin-induced apoptosis in chemosensitive leukemia cells (CEM) and neuroblastoma cells (SHEP). CEM and SHEP cells were preincubated with MEDIUM (black bars), NAC (white bars) (1.5 mg/ml for CEM and 1.8 mg/ml for SHEP) or GSH (hachted bars) (1 mg/ml) in 96 well plates ($2 \times 10^4$ cells/well) for 2 h at 37°C by addition of doxorubicin in concentrations as indicated. After 30 h for CEM and after 72 h for SHEP cells percentage of apoptotic cells were determined by FSC/SSC in leukemia cells,[68] and by hypodiploid DNA in neuroblastoma cells as described.[67] Data are given as mean of triplicates with a standard deviation (SD) of less than 10%. Similar results were obtained in three independent experiments. Percentage of specific cell death was calculated as follows: 100 × (experimental dead cells (%) − spontaneous dead cells in medium (%)/100% − spontaneous dead cells in medium (%)). (**B**) Reduction of the inhibitory effect of NAC by BSO. CEM cells were preincubated with NAC (1.5 mg/ml) or NAC and BSO (1.5 mg/ml NAC and 30 µg/ml BSO) in 96 well plates ($2 \times 10^4$ cells/well) for 2 h at 37°C by addition of 0.3 µg/ml doxorubicin. After 30 h percentage of apoptotic cells were determined by FSC/SSC as described. Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments. Percentage of specific cell death was calculated as described in **A**. (**C**) Effects of catalase, superoxide dismutase and ascorbic acid in doxorubicin-induced apoptosis in chemosensitive leukemia cells (CEM). CEM cells were preincubated with MEDIUM, 100 µg/ml superoxide dismutase (SOD), 100 µg/ml catalase (CAT), and 0.1 µg/ml ascorbic acid (AA) in 96 well plates ($2 \times 10^4$ cells/well) for 2 h at 37°C by addition of doxorubicin at concentrations indicated. After 30 h percentage of apoptotic cells were determined by FSC/SSC. Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments. Percentage of specific cell death was calculated as described in **A**. (**D**) Effects of NAC or GSH in doxorubicin-induced apoptosis on ROS generation and $\Delta\Psi_m$ in chemosensitive leukemia cells (CEM). CEM cells were incubated with 0.3 µg/ml doxorubicin (DOXO), pretreated with 1.5 mg/ml NAC (DOXO+NAC) or 1 mg/ml GSH (DOXO+GSH) for 2 h by addition of 0.3 µg/ml doxorubicin at 37°C. After 12 h frequence of cells with enhanced ROS [$HE^+$] (black bars) and reduced $\Delta\Psi_m$ [$DiOC_6(3)^{low}$] (white bars) were measured as described.[34,38] Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments

## Reversal of doxorubicin-resistance and deficient activation of the CD95 system by downregulation of GSH

We next asked whether downregulation of intracellular GSH in resistant cell lines would reverse drug-resistance. Doxorubicin-resistant CEM ($CEM^{DOXOR}$) and doxorubicin-resistant SHEP ($SHEP^{DOXOR}$) cells were incubated for 24 h in the presence or absence of cycloheximide (CHX) which has been found to reduce intracellular GSH concentrations at non-toxic concentrations[42] in addition to inhibition of protein synthesis or L-buthionine-(S,R)-sulfoximine (BSO) which has been found to inhibit GSH synthesis.[42,47] In cytofluorometric analysis using mBCl to detect GSH,[44] downregulation of GSH concentrations was found in $CEM^{DOXOR}$ and $SHEP^{DOXOR}$ cells after treatment with highest non-toxic concentration of CHX or BSO (Figure 6A).

To see whether a downregulation of GSH concentrations could reverse doxorubicin-resistance, $CEM^{DOXOR}$ and $SHEP^{DOXOR}$ cells were incubated in the presence or absence of the highest non-toxic dose of CHX in addition to doxorubicin. After 24 h for $CEM^{DOXOR}$ cells and after 72 h for $SHEP^{DOXOR}$ cells sensitivity to doxorubicin-induced apoptosis was measured by FSC/SSC analysis (Figure 6B). These time points were chosen according to the different kinetic of drug-induced apoptosis in both cell lines. In doxorubicin-resistant cells, induction of apoptosis was only found in the presence of CHX. Similar results were observed in $CEM^{DOXOR}$ cells pretreated with BSO for 12 h before doxorubicin treatment (Figure 6B). Furthermore, addition of high non-toxic doses of GSH reversed the effect of BSO or CHX (data not shown). These data suggest that downregulation of intracellular GSH using CHX or BSO might reverse doxorubicin-resistance.

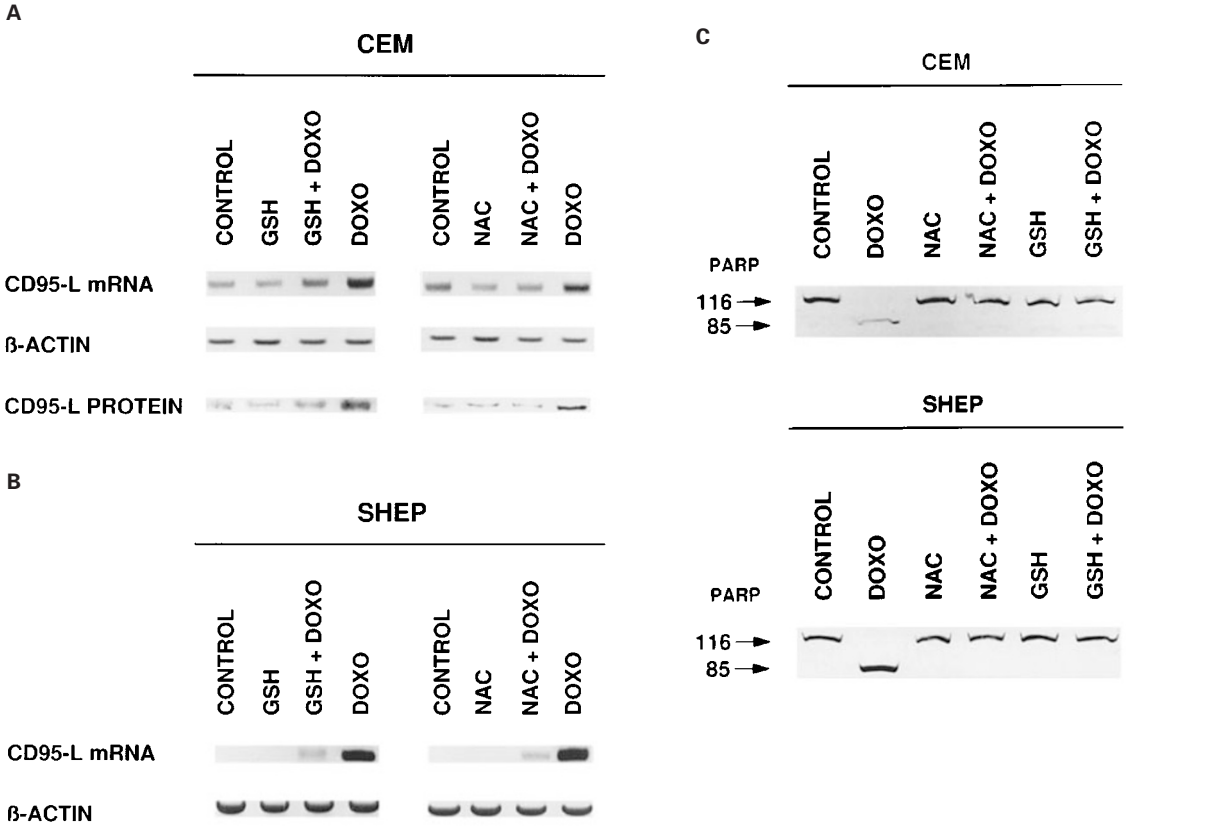


**Figure 2** Inhibition of doxorubicin-induced CD95-L expression and PARP cleavage in chemosensitive leukemia cells (CEM) and neuoblastoma cells (SHEP) by NAC and GSH. (**A**) Inhibition of CD95-L mRNA and CD95-L protein expression in chemosensitive leukemia cells (CEM) by GSH and NAC. CEM cells were incubated with medium (CONTROL), incubated with 1 mg/ml GSH (GSH) or 1.5 mg/ml NAC (NAC), preincubated with 1 mg/ml GSH or 1.5 mg/ml NAC for 2 h at 37°C before addition of 0.3 $\mu$g/ml doxorubicin (GSH+DOXO, NAC+DOXO) or incubated with 0.3 $\mu$g/ml doxorubicin (DOXO) at 37°C. After 2 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integrity. After 8 h CD95-L protein was detected by Western blot. Protein loading was controlled by Ponceau Red staining. (**B**) Inhibition of CD95-L mRNA and CD95-L protein expression in chemosensitive neuroblatoma cells (SHEP) by NAC and GSH. SHEP cells were incubated with medium (CONTROL), incubated with 1 mg/ml GSH (GSH) or 1.8 mg/ml NAC (NAC), preincubated with 1 mg/ml GSH or 1.8 mg/ml NAC for 2 h at 37°C before addition of 0.5 $\mu$g/ml doxorubicin (GSH+DOXO, NAC+DOXO), or incubated with 0.5 $\mu$g/ml doxorubicin (DOXO) at 37°C. After 24 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integrity. (**C**) Inhibiton of doxorubicin-induced cleavage of the prototype caspase substrate PARP (poly(ADP-ribose)polymerase) in chemosensitive leukemia cells (CEM) and neuroblastoma cells (SHEP) by NAC and GSH. CEM cells were incubated with medium (CONTROL), incubated with 0.3 $\mu$g/ml doxorubicin (DOXO), incubated with 1.5 mg/ml NAC (NAC) or 1 mg/ml GSH (GSH), or preincubated with 1.5 mg/ml NAC or 1 mg/ml GSH for 2 h at 37°C before addition of 0.3 $\mu$g/ml doxorubicin (NAC+DOXO, GSH+DOXO) for 30 h. SHEP cells were incubated with medium (CONTROL), incubated with 0.5 $\mu$g/ml doxorubicin (DOXO), incubated with 1.8 mg/ml NAC (NAC) or 1 mg/ml GSH (GSH), or preincubated with 1.8 mg/ml NAC or 1 mg/ml GSH for 2 h at 37°C before addition of 0.5 $\mu$g/ml doxorubicin (NAC+DOXO, GSH+DOXO) for 72 h. PARP protein was detected by Western blot. Protein loading was controlled by Ponceau Red staining

In addition to increased GSH levels and deficient reduction of $\Delta\Psi_m$ and ROS generation, $CEM^{DOXOR}$ and $SHEP^{DOXOR}$ failed to upregulate CD95-L expression after doxorubicin-treatment (Figure 7).[19] Since downregulation of intracellular GSH levels and hyperproduction of ROS restored doxorubicin sensitivity in doxorubicin-resistant cells we asked whether downregulation of GSH with hyperproduction of ROS would also restore upregulation of CD95-L following drug treatment. After incubation of doxorubicin-resistant cell lines ($CEM^{DOXOR}$, $SHEP^{DOXOR}$) with doxorubicin alone or in the presence of highest non-toxic dose of CHX or BSO, CD95-L mRNA expression was analyzed by RT–PCR. Upregulation of CD95-L mRNA was only observed in doxorubicin-resistant cells lines pretreated with CHX or BSO (Figure 7). Production of CD95-L protein in doxorubicin-resistant cells treated with CHX was also confirmed by Western blot (data not shown). Activation of caspases downstream of CD95-triggering system by doxorubicin after treatment with the highest non-toxic dose of CHX was also indicated by PARP cleavage (data not shown). Taken together, these results demonstrate that induction of CD95-L and subsequent activation of downstream apoptosis effector pathways by doxorubicin is controlled by intracellular GSH levels and ROS which may be produced in mitochondria. Downregulation of high GSH levels in drug-resistant cells and hyperproduction of ROS may reverse the defect in drug-induced activation of CD95.

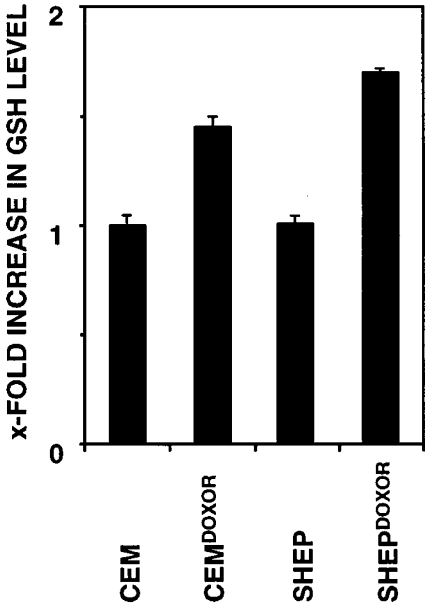


**Figure 3** Analysis of intracellular GSH content of chemosensitive parental and doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP). For determination of intracellular GSH chemosensitive parental and doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP) were labeled with mBCl and analyzed by cytofluorometry. Data of doxorubicin-resistant cells are shown as $\times$-fold increase in GSH levels comparison to chemosensitive parental cell lines. Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments

## Discussion

Sensitivity and resistance of tumor cells for cytotoxic drugs seems to depend on activation of apoptosis pathways which may involve death receptor pathways such as the CD95 system. Treatment with cytotoxic drugs induces CD95 and CD95-L expression and may trigger autocrine or paracrine death.[18–25] Deficiences in this system may lead to resistance

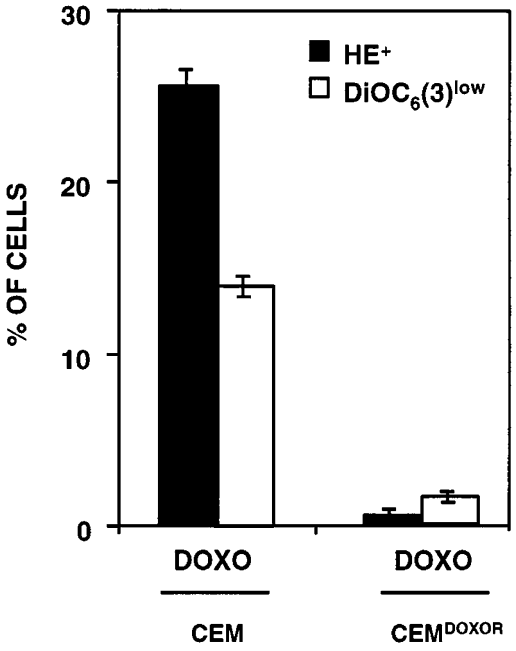


**Figure 4** Effects of doxorubicin-induced apoptosis on ROS generation and $\Delta\Psi_m$ in chemosensitive and doxorubicin-resistant (DOXOR) leukemia cells (CEM). Chemosensitive and doxorubicin-resistant (DOXOR) leukemia cells (CEM) were incubated with 0.3 $\mu$g/ml doxorubicin at 37°C. After 12 h frequence of cells with enhanced ROS [$HE^+$] (black bars) and reduced $\Delta\Psi_m$ [$DiOC_6(3)^{low}$] (white bars) were measured as described.[34,36,38] Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments

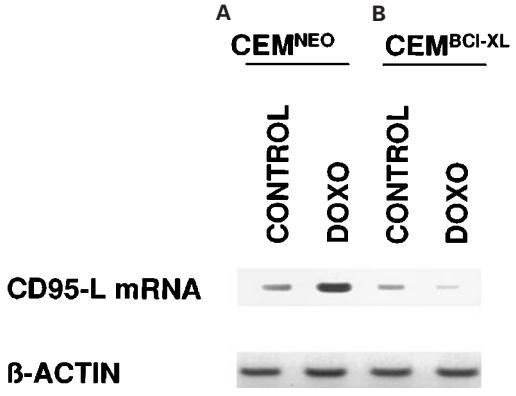


**Figure 5** Inhibition of doxorubicin-induced CD95-L expression in Bcl-$X_L$ transfected cells. (**A**) $CEM^{NEO}$ and (**B**) $CEM^{Bcl\text{-}XL}$ were incubated with 0.3 $\mu$g/ml doxorubicin (DOXO) at 37°C. After 6 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integrity

towards chemotherapy independent of multi-drug resistant protein (MRP), p-glycoprotein (P-gp, MDR-1) or p53 function.[19] Since cytotoxic drugs such as doxorubicin have been shown to generate ROS and may perturb mitochondrial function,[34,35] alterations of the redox state may provide increased levels of CD95-L and activate the CD95/CD95-L

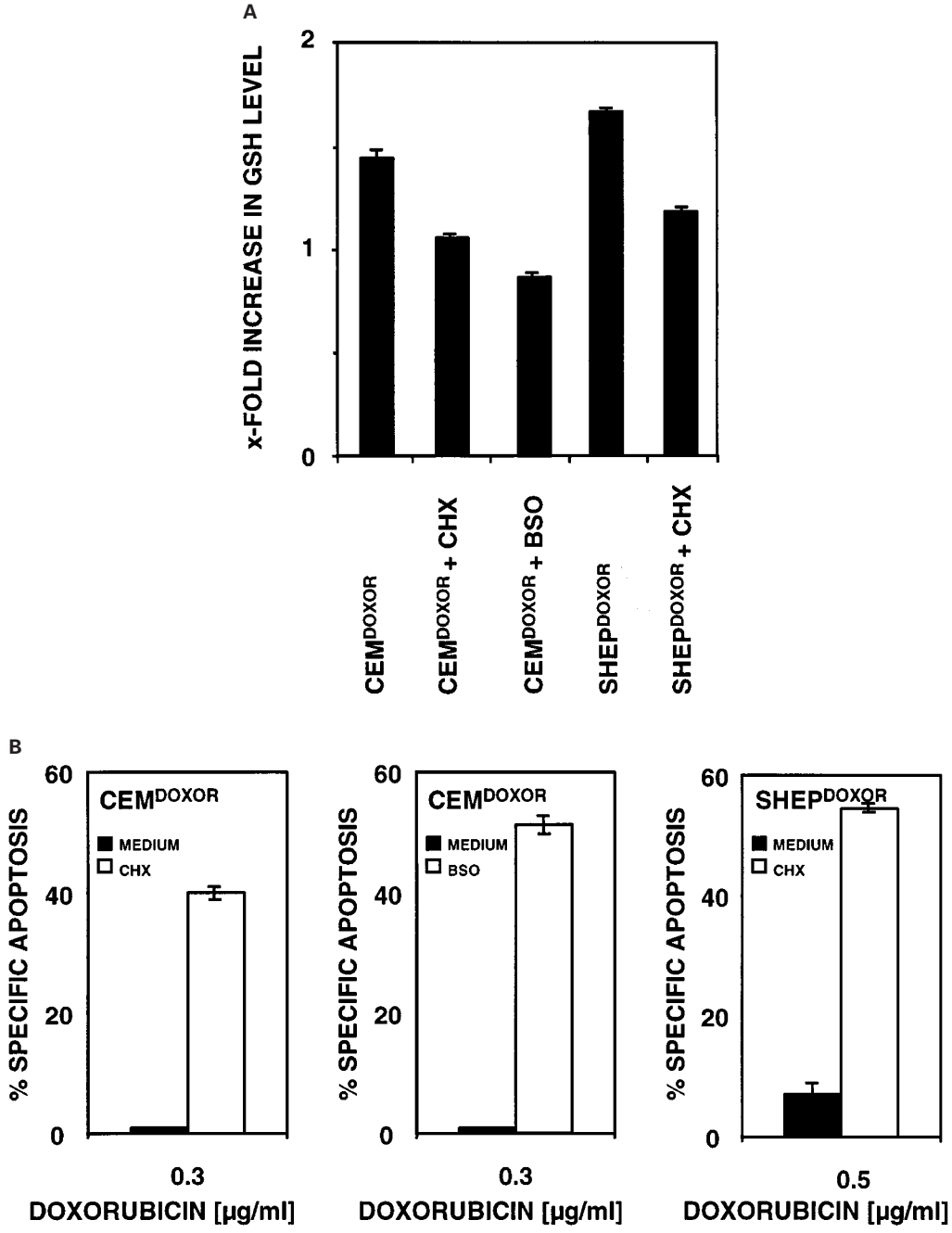


**Figure 6** (**A**) The effect of CHX on intracellular GSH concentration in doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP). Doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP) were incubated in the presence or absence of CHX (1 $\mu$g/ml CHX for $CEM^{DOXOR}$ or 5 $\mu$g/ml CHX for $SHEP^{DOXOR}$) or BSO (100 $\mu$g/ml) for 24 h. Intracellular GSH concentrations were determined with mBCl and analyzed by cytofluorometry. Data of resistant cells and cells treated with CHX or BSO are shown as $\times$-fold increase in GSH levels comparison to chemosensitive parental cell lines CEM or SHEP. Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments. (**B**) The effect of CHX or BSO in doxorubicin-induced apoptosis in doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP). Doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP) were incubated in 96 well plates ($2 \times 10^4$ cells/well) with MEDIUM (black bars), CHX (1 $\mu$g/ml CHX for $CEM^{DOXOR}$ or 5 $\mu$g/ml CHX for $SHEP^{DOXOR}$) (white bars) or preincubated for 8 h with 100 $\mu$g/ml BSO (white bars) by addition of doxorubicin at 37°C at concentrations indicated. After 24 h for $CEM^{DOXOR}$ and after 72 h for $SHEP^{DOXOR}$ the percentage of apoptotic cells was measured by FSC/SSC in leukemia cells[67] and by hypodiploid DNA in neuroblastoma cells as described.[68] Data are given as mean of triplicates with a SD of less than 10%. Similar results were obtained in three independent experiments. Percentage of specific cell death was calculated as described in Figure 1A

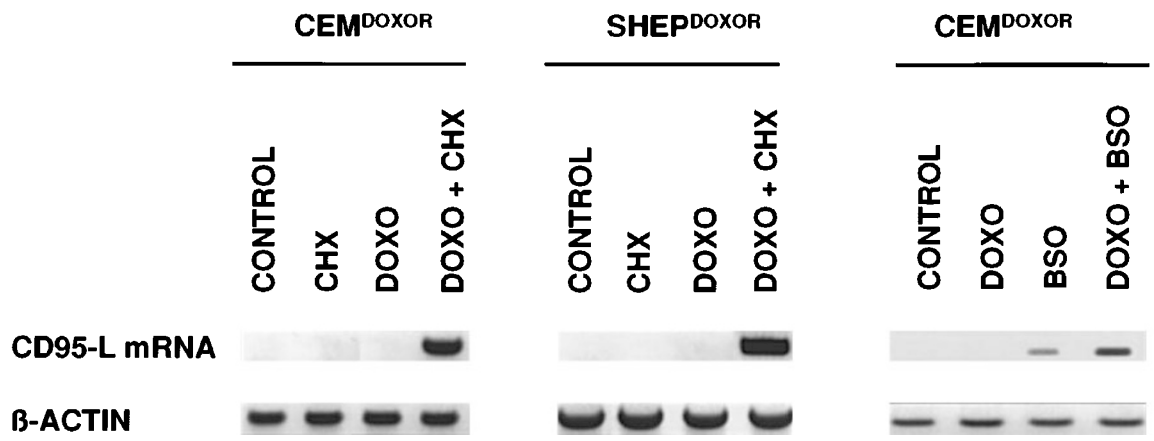


**Figure 7** Induction of CD95-L expression in doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuoblastoma cells (SHEP) by CHX or BSO. Induction of CD95-L mRNA in doxorubicin-resistant (DOXOR) leukemia cells (CEM) and neuroblastoma cells (SHEP). $CEM^{DOXOR}$ cells were incubated with medium (CONTROL), 1 $\mu$g/ml CHX (CHX) or 100 $\mu$g/ml BSO (BSO), 0.3 $\mu$g/ml doxorubicin (DOXO) or 1 $\mu$g/ml CHX by addition of 0.3 $\mu$g/ml doxorubicin (DOXO+CHX) at 37°C. After 4 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integritiy. $SHEP^{DOXOR}$ cells were incubated with medium (CONTROL), 5 $\mu$g/ml CHX (CHX), 0.5 $\mu$g/ml doxorubicin (DOXO) or 5 $\mu$g/ml CHX by addition of 0.5 $\mu$g/ml doxorubicin (DOXO+CHX). After 24 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integritiy. $CEM^{DOXOR}$ cells were incubated with medium (CONTROL), preincubated for 8 h with 100 $\mu$g/ml BSO (BSO), 0.3 $\mu$g/ml doxorubicin (DOXO) or preincubated for 8 h with 100 $\mu$g/ml BSO by addition of 0.3 $\mu$g/ml doxorubicin (DOXO+BSO) at 37°C. After 4 h CD95-L mRNA expression was determined by RT–PCR. Human $\beta$-actin served as control for equal loading and RNA integritiy

system following drug treatment. Here we report that activation of the CD95 system by doxorubicin is regulated by the redox state and ROS dependent on intracellular GSH. Since most of the molecular oxygen is consumed in the electron transport chain, mitochondria presumably are the major source of ROS. NAC, which increases intracellular GSH concentrations[42] (data not shown) or GSH *per se* significantly reduced doxorubicin-induced apoptosis in chemosensitive CEM leukemia cells and SHEP neuroblastoma cells. However, protection from drug-induced cell death was less pronounced upon prolonged incubation ($>96$ h) (data not shown). In addition, preincubation with NAC or GSH before doxorubicin treatment significantly reduced loss of $\Delta\Psi_m$ and hyperproduction of ROS and inhibited doxorubicin-induced upregulation of CD95-L expression and cleavage of PARP. In contrast, non-thiol antioxidants such as catalase, which metabolizes hydrogen peroxide in cytoplasma or superoxide dismutase, which metabolizes superoxide radicals in mitochondria and cytoplasma or ascorbic acid exhibited only a moderate protection against doxorubicin-induced apoptosis and did not confer a significant protection against ROS hyperproduction and upregulation of CD95-L (Figure 1C) (data not shown). Furthermore, CD95-L expression and cleavage of PARP were completely blocked in doxorubicin-resistant cells which exhibit higher GSH levels compared to chemosensitive cells. In doxorubicin-resistant cells no significant disruption of $\Delta\Psi_m$ and hyperproduction of ROS were found in response to doxorubicin treatment. Downregulation of GSH levels by the highest non-toxic dose of CHX or BSO, a specific inhibitor for GSH synthesis,[47] restored deficient doxorubicin-induced apoptosis and deficient upregulation of CD95-L by cytotoxic drugs in doxorubicin-resistant cells. This effect of BSO and CHX could be overcome by addition of high doses of GSH (data not shown). In addition, in Bcl-$X_L$ transfected CEM cells, doxorubicin-induced hyperproduction of ROS and loss of $\Delta\Psi_m$ was significantly reduced and activation of doxorubicin-mediated CD95-L ligand upregulation was blocked.

ROS are physiological metabolites, which may be generated from mitochondria.[50] When ROS are produced in excessive amounts in mitochondria, nucleic acids, proteins, and lipids are extensively modified by oxidation.[50] To counteract the harmful consequence of ROS generation mitochondria are equipped with MnSOD and a redox cycle using reduced GSH and GSH peroxidase.[50] GSH is the most abundant intracellular thiol that functions in reduction of disulfide linkages of proteins and acts as an antioxidant.[41–46] Hydrogen peroxide generated within the electron transport chain can undergo several fates: conversion to hydroxyl radicals with the participation of transition metals in the Haber-Weiss and Fenton reaction, reduction to water catalyzed by GSH peroxidase, with the required participation of reduced GSH, or diffusion out of mitochondria, where it may be metabolized by the action of catalase. Since mitochondria do not contain catalase, GSH in the mitochondrial matrix is the only defense available to cope with the potential toxic effect of hydrogen peroxide produced endogeneously in the electron transport chain.[51] ROS produced in a controlled fashion are involved in several biological processes.[52] ROS may act as signal transducers and represent a cellular control mechanism for gene regulation. Thus, the redox environment within the cell may play a regulatory role in controlling activation and DNA binding of several transcription factors such as NF-$\kappa$B, which requires mitochondrial electron transport.[52–54] Generation of ROS has been suggested as a main mechanism of anthracycline cytotoxicity. Mitochondria rapidly metabolize anthracyclines to anthracycline semiquinone radicals and oxygen radicals.[55–58] Anthracyclines such as doxorubicin have been found to activate NF-$\kappa$B

possibly through generation of oxygen radicals.[53,59] Recently, the promotor of CD95-L,[60] whose expression is induced following doxorubicin treatment, has been found to contain an NF-$\kappa$B binding site.[25] Furthermore, it was found, that CD95-L mRNA induction involves ROS in hepatoma cells treated with bleomycin.[48]

GSH has also been found to modulate T-cell apoptosis e.g. during HIV infection.[61] Seropositive individuals display GSH deficiencies in the plasma and peripheral blood lymphocytes and enhanced susceptibility to apoptotic stimuli early during the course of infection. NAC, which acts both as the precursor of glutathione and as an antioxidant by itself inhibits apoptosis in T-cells from patients.[62] T-cells from HIV-infected individuals also exhibit a significant disruption of $\Delta\Psi_m$ compared to normal T-cells,[63,64] (also Friesen and Debatin, unpublished results). Since activation of the CD95 system, with increased levels of CD95-L has been implicated in accelerated T-cell apoptosis during HIV infection this may suggest that increased CD95-L expression is mediated by the altered redox state of T-cells in these patients. NAC, which upregulates intracellular GSH levels may prevent depletion of HIV-infected lymphocytes.[62] In contrast to HIV increased intracellular GSH levels, which prevent mitochondrial dysfunction, may prevent drug-resistant cells from drug-induced apoptosis and upregulation of CD95-L by cytotoxic drugs.

Bcl-2 located in the outer mitochondrial membrane has been shown to act in an antioxidant pathway and inhibit apoptosis by suppressing the ROS function in mitochondria and antagonizing mitochondrial dysfunction.[34,65] We found that in Bcl-$X_L$ transfected CEM cells doxorubicin-induced apoptosis, hyperproduction of ROS and loss of $\Delta\Psi_m$ are significantly reduced in comparison to the parental cell line and activation of doxorubicin-induced CD95-L ligand expression is inhibited. This may suggest that Bcl-$X_L$ blocks doxorubicin-induced CD95-L expression by decreasing the release of ROS from mitochondria.

Taken together, our data suggest that induction of CD95-L expression in response to treatment with cytotoxic drugs depends on hyperexpression of ROS, which may be produced in mitochondria and which may be modulated by intracellular GSH. Thus, mitochondrial dysfunction, may not only be involved in the execution phase of apoptosis, it may also act as an initial amplifier system that contributes to drug-mediated death. Alteration of the cellular redox state e.g. by downregulation of GSH, provides a possible mean to restore deficient activation of the CD95 system by cytotoxic drugs.

## Materials and Methods

### Cell lines and culture conditions

Leukemia cells (CEM) and neuroblastoma cells (SHEP) were grown in RPMI 1640 (GIBCO BRL Eggenstein, Germany) containing 10% fetal calf serum (Conco, Wiesbaden, Germany), 10 mM HEPES, pH 7.3 (Biochrom, Berlin, Germany), 100 U/ml penicillin (GIBCO), 100 $\mu$g/ml streptomycin (GIBCO) and 2 mM L-glutamine (Biochrom). $CEM^{DOXOR}$, a variant of CEM resistant towards doxorubicin up to 1 $\mu$g/ml was generated by continuous culture in doxorubicin for more than 24 months.[19] For experiments $CEM^{DOXOR}$ cells were washed and cultured for 2 weeks in the absence of doxorubicin. Likewise $SHEP^{DOXOR}$ cells were generated by the same conditions as described above. $CEM^{NEO}$ and $CEM^{Bcl\text{-}XL}$ were generated as described.[66] All cell lines were mycoplasma free.

### Drugs and reagents

Doxorubicin (Farmitalia, Milano, Italy) was freshly dissolved in sterile distilled water prior to each experiment to ensure constant quality of the preparations.

Reduced glutathione (GSH), N-acetyl-cysteine (NAC), L-buthionine-(S,R)-sulfoximine (BSO), catalase (CAT), superoxide dismutase (SOD), ascobic acid (AA) (Sigma, Deisenhofen, Germany) and cycloheximide (CHX) (Aldrich, Steinheim, Germany) was freshly dissolved in sterile destilled water prior to each experiment to ensure constant quality of the preparations.

### Induction of apoptosis

For quantitative determination of apoptosis, cells were lysed in Nicoletti-buffer (0.1% sodium citrate plus 0.1% Triton X-100 containing propidium iodide 50 $\mu$g/ml) as described.[67] Propidium iodide stained nuclei[67] or forward scatter/side scatter (FSC/SSC) profile of cells[68] were analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany).

### Cytofluorometric analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$) and reactive oxygen species (ROS) generation

To measure $\Delta\Psi_m$ and ROS generation, cells ($5\times10^5$/ml) were incubated with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$(3), 460 ng/ml; FL-1) (Moleculare Probes, Inc., Eugene, OR, USA) for $\Delta\Psi_m$ and dihydroethidine (HE, 126 ng/ml, FL-3) (Moleculare Probes) for ROS generation for 12 min at 37°C in the dark followed by analysis on a flow cytometer (FACScan).

### Cytofluorometric analysis of non-oxidized glutathione

Intracellular GSH (mainly non-oxidized glutathione) was measured by staining the cells with 40 $\mu$M monochlorobimane (mBCl) (Moleculare Probes) for 10 min at room temperature in the dark before measuring fluorescence on a FACS Vantage (Becton Dickinson) (excitation, wavelength 325 nm; emission, wavelength 450 nm).

### RT–PCR for CD95-L mRNA

Total RNA was prepared using the Qiagen RNA kit (Qiagen, Hilden, Germany). RNA was converted to cDNA by reverse transcription and amplified by polymerase chain reaction (PCR) in a thermocycler (Stratagene, Heidelberg, Germany) using the Gene Amplification kit (Perkin Elmer, Branchburg, NJ, USA) following the manufacturer's instructions. Primers used for amplification of CD95-L fragment were according to the sequence of human CD95-L.[69]

Expression of $\beta$-actin (MWG-Biotech, Ebersberg, Germany) was used as a standard for RNA integritiy and equal gel loading. PCR-reaction products were run at 60 V for 2 h on a 1.5% agarose gel stained with ethidiumbromid and visualized by UV illumination.

### Western blot analysis

Proteins were extracted from cells lysed for 30 min at 4°C in PBS with 0.5% Triton X (Serva, Heidelberg, Germany) and 1 mM PMSF (Sigma) followed by high-speed centrifugation. Membrane proteins were eluated by buffer containing 0.1 M glycin/HCl, pH 3.0 in PBS and lower Tris, pH 8.8. Protein concentration was assayed using bicinchoninic acid (Pierce, Rockford, USA). 100 $\mu$g protein per lane was separated by 12% SDS – PAGE and electroblotted to nitrocellulose (Amersham, Braunschweig, Germany). Equal protein loading was controlled by Ponceau red staining of membranes. After blocking for 1 h in PBS supplemented with 2% BSA (Sigma) and 0.1% Tween 20 (Sigma), immunodetection of PARP and CD95-L was done using rabbit anti-PARP polyclonal antibody (Enzyme Systems Products, Dublin, CA, USA), rabbit anti-CD95-L polyclonal antibody IgG (Santa Cruz Biotechnology, Santa Cruz, California) and horseradish peroxidase-conjugated goat anti-rabbit IgG. Enhanced chemiluminescence system (ECL) (Amersham) was used for detection.

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