View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Drug Resistance Towards Etoposide and Cisplatin in Human Melanoma Cells is Associated with Drug-Dependent Apoptosis Deficiency

Heike Helmbach, Monika A. Kern, Evelyn Rossmann, Kristina Renz, Christine Kissel, Brigitte Gschwendt, and Dirk Schadendorf

Skin Cancer Unit at the German Cancer Research Center, University of Heidelberg, Germany

Anticancer drugs kill susceptible cells through induction of apoptosis. Alterations of apoptotic pathways in drug-resistant tumor cells leading to apoptosis deficiency might represent a potent mechanism conferring drug resistance. We have assessed the effect of etoposide and cisplatin on the apoptotic pathways of the drug-sensitive human melanoma cell line MeWo as well as its etoposide- and cisplatin-resistant sublines ($MeWo_{Eto01}$, $MeWo_{Eto1}$, and $MeWo_{Cis01}$, $MeWo_{Cis1}$). Etoposide and cisplatin induced apoptosis in drug-sensitive MeWo cells as indicated by dose-dependent (i) cytochrome c release, (ii) caspase activation, (iii) DNA fragmentation, and (iv) cleavage of poly(ADP-ribose)polymerase. In contrast, whereas low etoposide-resistant cells ($MeWo_{Eto01}$) demonstrated reduced but detectable apoptotic activities, highly etoposide-resistant cells ($MeWo_{Eto1}$) did not exhibit any of the apoptotic events observed in etoposide-induced cell death downstream of a strongly reduced cytochrome c release. Highly cisplatin-resistant cells ($MeWo_{Cis1}$), however, demonstrated a reduced caspase 9 activity and cytochrome c release but the extent of effector caspase activation as well as DNA fragmentation was comparable to that of sensitive MeWo cells at equitoxic concentrations. In addition, poly(ADP-ribose)polymerase cleavage was strongly reduced in highly cisplatin-resistant sublines. Taken together, sensitive and drug-resistant MeWo cells utilized different apoptotic pathways upon drug exposure in a drug-dependent fashion and apoptosis deficiency was strongly associated with the drug-resistant phenotype. ***Key words: caspase/cytochrome caspase antibodies/mitochondric. J Invest Dermatol 118:923–932, 2002***

Commonly used anticancer agents in the treatment of metastatic melanoma do not result in clinical meaningful responses in most patients (Garbe, 1993) suggesting a high drug resistance for unknown reasons. Dacarbazine remains the reference standard treatment for metastatic melanoma, but only a minority of patients obtain long-lasting responses (Middleton *et al*, 2000). Polychemotherapy regimes have been reported to produce various response rates. Cisplatin is frequently used in polychemotherapy regimens in melanoma, but partial response is observed only in approximately 10%. Treatment with etoposide is more common in lung cancer, leukemia, and testicular tumors and has been used in polychemotherapy regimens combined with cisplatin treating melanoma brain metastases but the response rates remain less than 13% (Fields *et al*, 1994; Planting *et al*, 1996).

Biochemical studies have not succeeded in identifying conclusively the basis of resistance in any type of cell selected with cisplatin and etoposide. In various cell types and using many approaches several mechanisms, such as DNA repair and DNA mismatch repair proteins, glutathione metabolism, multidrug-resistance-related proteins, or the p53 function, have been identified to be involved in sensitivity towards cisplatin (Niedner *et al*, 2001; Suzuki *et al*, 2001). Various mechanisms are discussed to cause etoposide resistance: topoisomerase II activity (Lage *et al*, 2000) and the regulation of death-receptor-mediated apoptotic pathways (Friesen *et al*, 1999).

Using an *in vitro* soft agar culture system to predict tumor cell sensitivity of freshly obtained metastatic melanoma samples, a high degree of resistance was found against all cytostatic drugs studied, suggesting the presence of intrinsic cellular mechanisms conferring drug resistance (Schadendorf *et al*, 1994). Various mechanisms have been described as mediating drug resistance in various types of cancer, but the cellular mechanisms conferring drug resistance in melanoma are only poorly understood (Schadendorf *et al*, 1994, 1995a, b, c; Helmbach *et al*, 2001). In recent years our group has performed an extensive search for underlying mechanisms. Drug resistance mediated by membrane transport proteins such as P-glycoprotein (Schadendorf *et al*, 1995a), multidrug-resistance-related protein, or lung resistance protein was analysed (Schadendorf *et al*, 1995c) and was found not to be associated with drug resistance in melanoma. Increased activity of glutathione S-transferase was demonstrated in gastrointestinal and ovarian tumors resistant towards alkylating agents (Zhang *et al*, 1998). Drug resistance in melanoma cells, however, was not associated with

Manuscript received August 28, 2001; revised January 29, 2002; accepted for publication February 19, 2002.

Reprint requests to: Dr. Dirk Schadendorf, Skin Cancer Unit (DKFZ), Universitäts-Hautklinik Mannheim, University Heidelberg, Theodor-Kutzer-Ufer 1, D-68135 Mannheim, Germany. Email: d.schadendorf@dkfz-heidelberg.de

Abbreviations: AFC, 7-amino-4-trifluoromethyl coumarin; DEVD, Asp-Glu-Val-Asp; IAP, inhibitor of apoptosis protein; IC, inhibitory concentration; LEHD, Leu-Glu-His-Asp; PARP, poly(ADP-ribose)polymerase.

0022-202X/02/$15.00 · Copyright © 2002 by The Society for Investigative Dermatology, Inc.

Table I Sensitivity of MeWo cells and the resistant sublines to cisplatin and etoposide at 72 h exposure times[a]

Drug	Cell line	IC_{75} (μg per ml)	IC_{85} (μg per ml)	IC_{95} (μg per ml)
Etoposide	MeWo	1 (20 ± 2.3%)	5 (14 ± 2.2%)	10 (7.2 ± 2.1%)
	$MeWo_{Eto01}$	5 (25.7 ± 2.4%)	20 (15.5 ± 2.2%)	40 (5.9 ± 1.4%)
	$MeWo_{Eto1}$	10 (27.2 ± 1.4%)	40 (16 ± 2.6%)	100 (8.0 ± 1.7%)
Cisplatin	MeWo	1 (24 ± 2.6%)	2 (14.2 ± 0.4%)	5 (3.7 ± 0.7%)
	$MeWo_{Cis01}$	2 (24.4 ± 5.5%)	5 (11.2 ± 1.3%)	10 (5.0 ± 1.7%)
	$MeWo_{Cis1}$	5 (24.7 ± 1.0%)	10 (15 ± 2%)	15 (7.0 ± 7.2%)

[a]Sensitivity of MeWo, etoposide-resistant sublines ($MeWo_{Eto01}$ and $MeWo_{Eto1}$), and cisplatin-resistant sublines ($MeWo_{Cis1}$) towards etoposide and cisplatin was assessed by incubating the cells with drugs over 72 h. Results are the mean of at least triplicate determinations. The IC_{75}, IC_{85}, and IC_{95} values represent drug concentrations (μg per ml) that inhibited cell growth by 75%, 85%, and 95%, respectively. The mean of cell survival (%) at the indicated drug concentration as well as the standard deviations are given in parentheses.

elevated GSH activity (Schadendorf *et al*, 1995b). On the basis of these analyses based on fresh tumor samples, it can be concluded that mechanisms commonly known to confer drug resistance in other tumors seem not to be involved in mediating drug resistance in melanoma. In order to study drug resistance in greater detail and to develop concepts for the *in vivo* situation, a panel of drug-resistant melanoma cell lines derived from human melanoma cell line MeWo were established and extensively studied (Kern *et al*, 1997; Helmbach *et al*, 2001)**.** MeWo and its drug-resistant sublines have already been extensively characterized with reference to some underlying mechanisms possibly conferring drug resistance (Kern *et al*, 1997; Lage *et al*, 1999, 2000; Nessling *et al*, 1999; Rünger *et al*, 2000; Sinha *et al*, 2000). Recent studies demonstrated an increased DNA repair in cisplatin- and fotemustine-resistant melanoma MeWo cells (Lage *et al*, 1999). Etoposide-resistant melanoma cells decreased DNA topoisomerase II activity, corresponding to an increasing degree of drug resistance (Lage *et al*, 2000), indicating that modulation of DNA topoisomerase II activity contributes to the phenotype of etoposide-resistant cells. As these observations did not explain the cross-resistance pattern in the panel of drug-resistant melanoma cells, however, other additional resistance mechanisms must be operating in these cells.

Recent publications demonstrated that chemotherapeutic agents exert their cytotoxicity by inducing apoptosis (Friesen *et al*, 1996; Fulda *et al*, 1998b; Houghton, 1999; Sellers and Fisher, 1999; Kaufmann and Earnshaw, 2000), leading to the attractive concept that acquired drug resistance may be the result of modulated apoptotic processes. Resistance to chemotherapy-induced apoptosis due to modulation of apoptotic pathways has recently been described in some tumors (Eliopoulos *et al*, 1995; Segal and Jacquemin, 1995; Los *et al*, 1997; Fulda *et al*, 1998a) and may also be responsible for drug resistance in melanoma (Jansen *et al*, 2000b; Raisova *et al*, 2000; Franco *et al*, 2001; Soengas *et al*, 2001). For various tumors etoposide and cisplatin were shown to induce apoptosis (Kuwahara *et al*, 1975; Seki *et al*, 1978; von Knethen *et al*, 1998; Hassan *et al*, 1999; Siitonen *et al*, 2000) and resistance toward these drugs has been associated with alteration of apoptotic processes (Henkels and Turchi, 1997; 1999; Breton *et al*, 1998; Minagawa *et al*, 1999). Furthermore, recent studies described how manipulation of apoptotic pathways on different levels could enhance the apoptotic potential of cancer therapies in tumor cells (Chmura *et al*, 1997; Wang *et al*, 1999; Jansen *et al*, 2000a).

Apoptosis is a type of cell death involving characteristic morphologic and biochemical changes, which proceeds in part via aggregation and multimerization of upstream death effector molecules that sequentially activate a cascade of caspases (Rathmell and Thompson, 1999). There are currently two well characterized caspase-activating cascades that regulate apoptosis: one is initiated by a cell surface death receptor, e.g., TRAIL, FAS, and the other is triggered by changes in mitochondrial integrity (Scaffidi *et al*, 1998). In cells committed to apoptosis the latter is initiated by mitochondrial cytochrome c release, which binds to apoptotic protease activating factor 1 and thereby induces conformational changes of this apoptotic protein followed by recruitment of caspase 9 to the complex (Zou *et al*, 1997). The subsequent autocatalysis of caspase 9 is followed by activation of effector caspases (e.g., caspase 3, caspase 7) (Li *et al*, 1997; Zou *et al*, 1999) and induction of specific endonucleases resulting in DNA fragmentation (Liu *et al*, 1997). During apoptosis poly(ADP-ribose)polymerase (PARP), a nuclear protein involved in DNA repair (Le Rhun *et al*, 1998), is known to be one of the earliest substrates of apoptotic caspase 3 (Lazebnik *et al*, 1994; Tewari *et al*, 1995). A recently identified group of proteins regulating apoptosis is the group of chaperones, which is classified into several major groups: Hsp90, Hsp70, Hsp60, and the family of small Hsp. It was recognized that the Hsp may act proapoptotically as well as antiapoptotically by interacting with specific molecules, e.g., caspases, in the apoptotic pathway (Bukau and Horwich, 1998; Garrido *et al*, 1999; Mosser *et al*, 2000).

In this report, we have correlated apoptotic features of etoposide- and cisplatin-induced cell death in sensitive MeWo cells to differential expression and activation of apoptotic regulators in etoposide- and cisplatin-resistant cells. These data help us to understand the pathways by which etoposide and cisplatin induce cell death in human melanoma cells and suggest that these pathways are modulated in resistant melanoma cells. A more thorough understanding of the apoptosis deficiency involved in drug resistance would offer novel possibilities for modulating the clinical phenotype and consequently overcoming drug resistance in human melanoma.

MATERIALS AND METHODS

Cell culture The human melanoma cell line MeWo was derived from a lymph node metastasis of a patient with malignant melanoma at the Sloan Kettering Cancer Center (NY). Drug-resistant sublines exhibiting different levels of drug sensitivity were selected *in vitro* and characterized as described previously (Kern *et al*, 1997). Briefly, for selection of drug-resistant cell sublines, MeWo melanoma cells were continuously exposed to increasing concentrations of chemotherapeutic agents for at least 24 mo. The following drug-resistant MeWo cell sublines show different levels of resistance and have been maintained for up to 5 y in culture. The designation of the strains used is as follows: MeWo untreated (MeWo), MeWo resistant to etoposide 0.1 and 1 μg per ml ($MeWo_{Eto01}$, $MeWo_{Eto1}$), and MeWo resistant to cisplatin 0.1 and 1 μg per ml ($MeWo_{Cis01}$, $MeWo_{Cis1}$). Indices indicate drugs and drug doses used for selection of resistant cell lines. As positive controls in various experiments the human cervix carcinoma cell line HeLa, the T cell lymphoma cell line Jurkat, and the Burkitt lymphoma cell line SKW-6.4 were used.

Induction of cell death Twenty-four hours prior to chemotherapeutic drug treatment, cells were plated into tissue culture dishes. To induce apoptosis, cells were treated for 72 h at 37°C at equitoxic drug concentrations (IC_{75}, IC_{85}, IC_{95}) of cisplatin or etoposide. Seventy-two hours was chosen as an adequate time point after kinetic analysis revealing representative and reproducible apoptotic features. Cell survival was determined by counting cells after trypan-blue staining directly after removal of the drug. Inhibitory concentrations (IC) were calculated with

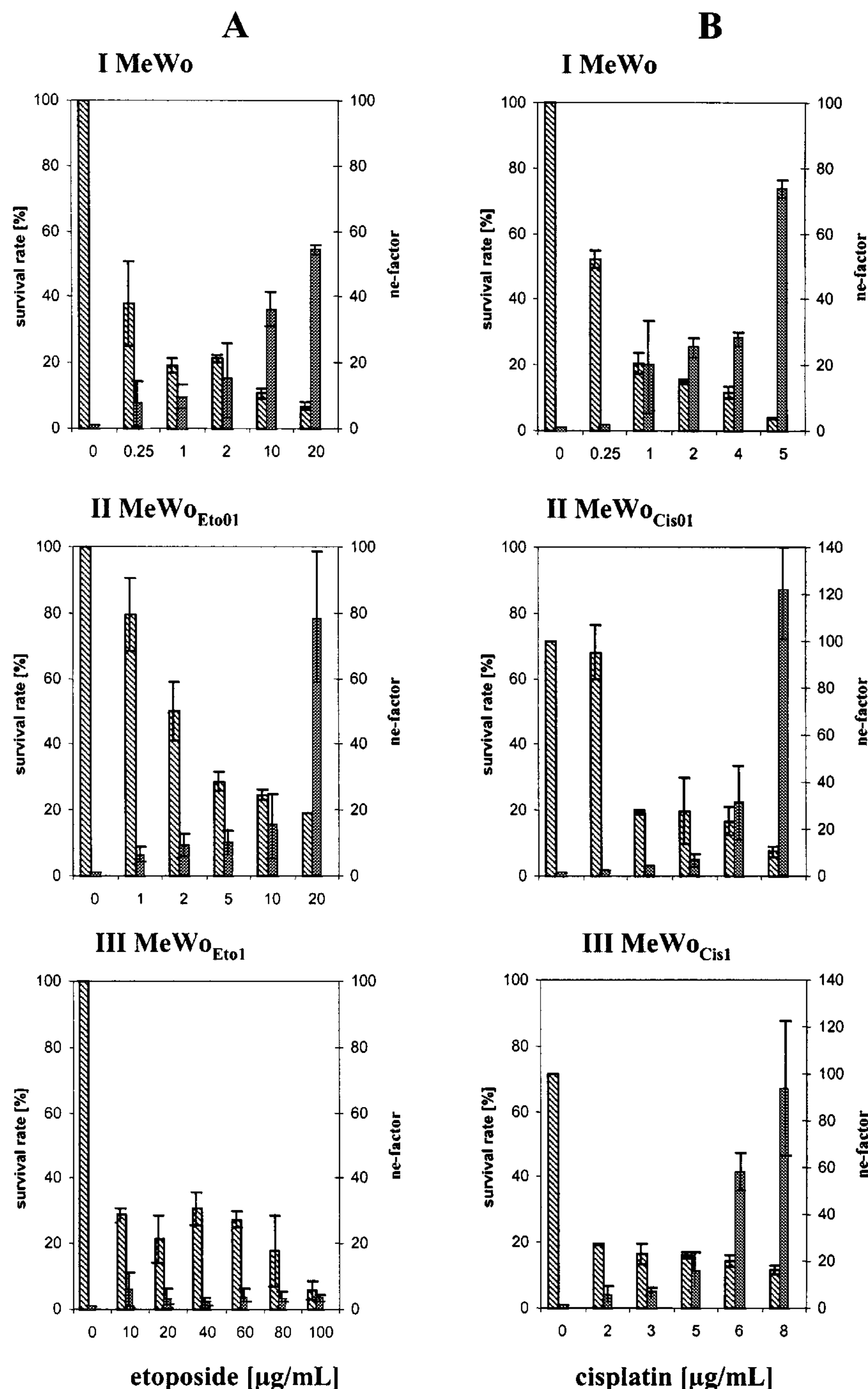

Figure 1. DNA fragmentation is induced at corresponding survival rates in sensitive and cisplatin-resistant cells but not in etoposide-resistant cells upon drug treatment. Cell extracts were prepared following continuous drug exposure for 72 h. DNA fragmentation is presented as nucleosomal enrichment factor (ne-factor) (*solid bars*) and is correlated to survival rate (*hatched bars*). Drug-sensitive MeWo cells were exposed to etoposide (*A*-I)and cisplatin (*B*-I); low- and high-level etoposide-resistant cells (*A*-II, *A*-III) were treated with etoposide; low- and high-level cisplatin-resistant cells with cisplatin (*B*-II, *B*-III). The data illustrate the results of an experimental setup that was repeated at least three times. Each bar represents the mean of experiments. The error bars indicate the observed range, and standard deviations are approximately 50% of these ranges.

reference to the untreated control and are shown in **Table I**. Sensitive and resistant cells were analyzed at their corresponding IC_{75}, IC_{85}, and IC_{95} dose of etoposide and cisplatin, respectively. Live adherent and nonadherent cells were collected and used for detection of DNA fragmentation and caspase activity as well as for RNA and protein isolation.

DNA fragmentation Histone-associated DNA fragments (mono-nucleosomes and oligonucleosomes) released from nuclear fractions of drug-treated cells were quantified using a Cell Death Detection ELISA (Roche, Penzberg, Germany) according to the manufacturer's instructions. Briefly, the sandwich-enzyme immunoassay uses mouse monoclonal antibodies directed against DNA and histones, respectively. A final peroxidase substrate reaction is measured as absorbance at 405 nm. Data were normalized against the control and interpreted as nucleosome enrichment factor (ne-factor). Results are presented as the mean of at least five experiments.

Western blot analysis Adherent and nonadherent cells were lysed in buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and small peptide inhibitors, 1% Triton X-100, and 10% glycerol). Protein content was determined using a peptide-binding dye Coomassie Brilliant Blue (Bio-Rad Laboratories, Muenchen, Germany) followed by photometric measurement at 595 nm. Lysates were mixed 4:1 with 5 × sample buffer [50% glycerine, 10% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 6.8), 25% β-mercaptoethanol, and 0.25 mg per ml bromophenol blue] and heated at 96°C for 3 min before loading. Equal amounts of protein were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 130 V and blotted at 100 V for 1 h to a Hybond nitrocellulose membrane (Amersham, Freiburg, Germany) in water-cooled transfer buffer [25 mM Tris–HCl, 0.19 M glycine, 20% methanol, 0.037% (wt/vol) SDS]. Membranes were blocked with 5% dry-milk in Tween–phosphate-buffered saline (PBS–0.05% Tween) for at least 1 h and were washed three times in Tween–

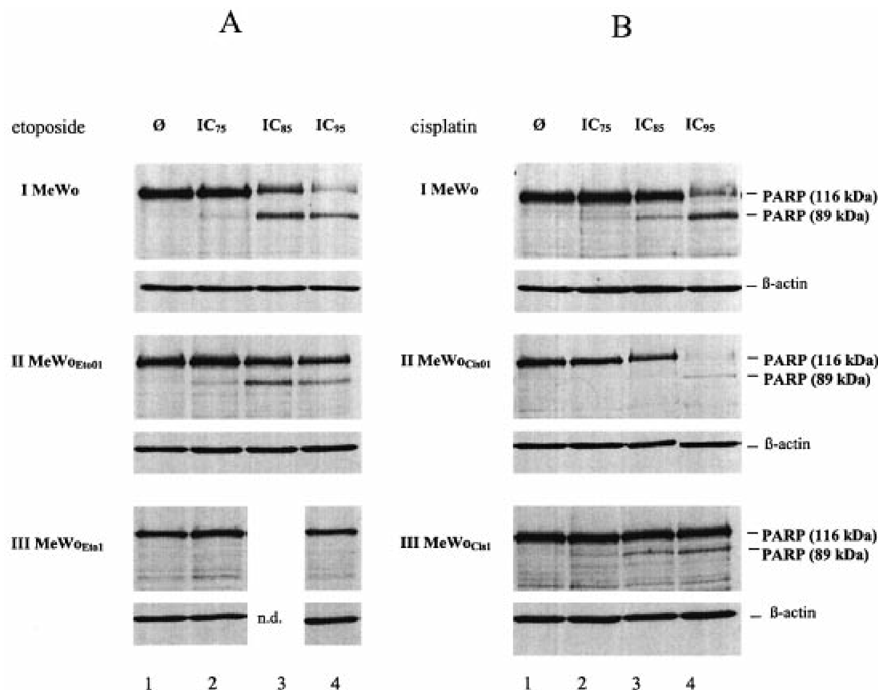

Figure 2. PARP cleavage upon drug treatment is detectable in drug-sensitive and cisplatin-resistant MeWo cells but not in etoposide-resistant cells. Expression of full-length PARP and PARP cleavage in drug-sensitive and drug-resistant MeWo cells detected by Western blot analysis. Polyclonal rabbit anti-PARP (1:10000 in Tween–PBS) and HRP-conjugated antirabbit antibody (1:5000 in Tween–PBS) were used. Full-length PARP and PARP cleavage fragments were detected in etoposide- and cisplatin-treated (*A*-I, *B*-I)sensitive MeWo cells, as well as in etoposide-treated $MeWo_{Eto01}$ and $MeWo_{Eto1}$ cells (*A*-II, *A*-III) and cisplatin-treated $MeWo_{Cis01}$ and $MeWo_{Cis1}$ cells (*B*-II, *B*-III). Untreated (*lane 1*) and treated (IC_{75}, IC_{85}, and IC_{95}) (*lanes 2–4,* respectively) cells were compared. The positions of full-length PARP (116 kDa) and its cleavage fragment (89 kDa) are presented on the right by arrows. Equal amounts of protein were confirmed by detection of β-actin, as denoted on the right. This blot illustrates one representative Western blot of at least three. (n.d., not determined.)

PBS. Membranes were incubated with the relevant primary antibody overnight at 4°C and subsequently washed three times in Tween–PBS. The corresponding horseradish peroxidase (HRP) conjugated secondary antibody was incubated for 1 h at room temperature followed by an additional washing with Tween–PBS. Proteins were visualized by enhanced chemoluminescence (Amersham), according to the manufacturer's recommendations. The membranes were recycled by incubation in stripping buffer [2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM β-mercaptoethanol] at 56°C for 30 min. To confirm equivalent loading and transfer of protein the blot was stained with the protein-binding dye Ponceau-S (Sigma, Deisenhofen, Germany). An additional hybridization with a 1:20,000 dilution of the monoclonal mouse anti-β-actin antibody followed by an incubation with a 1:10,000 diluted secondary HRP-conjugated antibody was performed. Mouse cytochrome c antibody and polyclonal caspase 3 antibody were purchased from Pharmingen (Heidelberg, Germany), and polyclonal caspase 7 antibody from Oncogen (Boston, MA). Rabbit anti-PARP was purchased from Roche (Mannheim, Germany) and the cytochrome-c-oxidase antibody was purchased from MoBiTec (Göttingen, Germany).

PARP Western blots have been evaluate by densitometry under consideration of the reduction of 116 kDa full-length PARP as well as the increase of the 89 kDa cleavage product.

RNase protection assay Cells (5×10^6) were plated in 182 cm^2 tissue culture flasks and cultured 24 h before drug treatment as indicated. Total RNA was isolated using the RNeasy Kit (Quiagen, Hilden, Germany) according to the manufacturer's recommendations. The presence of transcripts of caspase 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 was analyzed using the hApo-1c multiprobe template set (Pharmingen, Hamburg, Germany). Transcripts of members of the inhibitor of apoptosis protein (IAP) family (XIAP, survivin, c-IAP-1, c-IAP-2, NAIP, and TRPM-2) were analyzed using the hAPO-5c multiprobe template set (Pharmingen). In these template sets L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are included as internal controls. Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant Multi-Probe RNase Protection Assay System according to the manufacturer's instructions (Pharmingen). Finally, samples were analyzed by electrophoresis on denaturating polyacrylamide gel (5%) and quantified by autoradiography.

Leu-Glu-His-Asp 7-amino-4-trifluoromethyl coumarin (LEHD–AFC) cleavage assay For the specific quantitative *in vitro* determination of caspase 9 activity a fluorometric immunosorbent enzyme assay (R&D Systems, Wiesbaden, Germany) was used according to the manufacturer's recommendations. Briefly, 2×10^6 drug-treated or untreated cells were lysed in buffer. Protein contents were determined (see above) in order to use equal amounts. The lysates were incubated with the fluorogenic substrate LEHD–AFC (R&D Systems) at 37°C for at least 1 h. The fluorescence, directly proportional to enzymatic activity, was measured in a fluorescent microtiter plate reader every 30 min up to 3 h with a 409 nm excitation filter and a 500 nm emission filter. Data were normalized against the untreated control and interpreted as a factor of fold induction. Results are presented as one typical experiment of at least three.

Asp-Glu-Val-Asp (DEVD) AFC cleavage assay For the specific quantitative *in vitro* determination of caspase-3-like activity a fluorometric immunosorbent enzyme assay (Roche) was used according to the manufacturer's recommendations. Briefly, active caspase 3 was bound by a monoclonal mouse anti-caspase-3 antibody to a microtiter plate. After washing the fluorogenic substrate Ac-DEVD-AFC was added and cleavage rate was measured by fluorescent microplate reader after 1 h with a 409 nm excitation filter and a 500 nm emission filter. Data were normalized against the untreated control and interpreted as a factor of fold induction. They are presented as one typical experiment of at least three. The specificity of this assay was also confirmed using the specific caspase 3 inhibitor DEVD-CHO (Calbiochem, Gießen, Germany) (data not shown).

Preparation of cytosolic extracts for cytochrome c release For subcellular fractionation Apo Alert Cell Fractionation Kit (Clontech, Heidelberg, Germany) was used according to the manufacturer's recommendations. Briefly, untreated or treated cells (1×10^7) were washed in PBS and incubated on ice in 300 μl lysis buffer. Cells were lysed with strokes of a dounce homogenizer (Weathon) using a tight pestle and centrifuged ($900 \times g$ for 10 min, 4°C) to remove unbroken cells and nuclei. The mitochondria were pelleted by centrifugation ($15{,}000 \times g$ for 25 min, 4°C), resuspended in 80 μl lysis buffer, and stored at –20°C until use. The supernatant was ultracentrifuged at $100{,}000 \times g$ for 1 h (4°C). The resultant supernatant was designated as cytoplasmic fraction and stored at –20°C until use. Protein content was determined using a peptide-binding dye Coomassie Brilliant Blue (Bio-Rad Laboratories) followed by photometric measurement at 595 nm. Lysates were mixed 4:1 with 5 × sample buffer [50% glycerine, 10% SDS, 50 mM Tris (pH 6.8), 25% β-mercaptoethanol, and 0.25 mg per ml bromophenol blue] and heated at 96°C for 3 min before loading on a 12% SDS polyacrylamide gel. Lysates were analyzed by Western blot.

Immunostaining and confocal microscopy Cells were grown in 24-well plates on an object slide for 24 h prior to exposure to etoposide or cisplatin in a dose corresponding to the respective IC_{95}, as demonstrated in **Table I**. After 72 h continuous drug exposure cells were washed twice in PBS and fixed for 20 min at room temperature with 2% paraformaldehyde. Cells were blocked for 10 min with

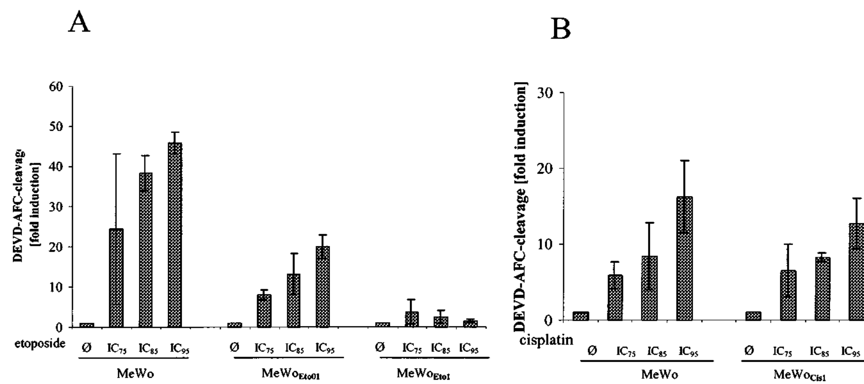

Figure 3. Effector caspases are not activated in drug-treated etoposide-resistant cells but in sensitive and cisplatin-resistant cells. Effector caspase activity (caspase-3-like activity) measured as DEVD–AFC cleavage in etoposide- and cisplatin-treated sensitive and resistant MeWo cells by a fluorogenic assay. Cell extracts were prepared following 72 h of continuous drug exposure at the respective IC_{75}, IC_{85}, and IC_{95} as described in *Materials* and *Methods*. (*A*) Etoposide-treated sensitive cells (MeWo) and etoposide-resistant cells ($MeWo_{Eto01}$ and $MeWo_{Eto1}$); (*B*)cisplatin-treated sensitive cells (MeWo) and cisplatin-resistant MeWo cells ($MeWo_{Cis1}$). The data were normalized against the respective untreated control (→) and are shown as *x*-fold induction. Each bar represents the mean of five experiments. The error bars indicate the observed range, and standard deviations are approximately 50% of these ranges.

PBS–fetal bovine serum before incubation with the anticytochrome antibody (1:200) (aAB6H2.B4, Pharmingen) for 1 h at room temperature revealed by a goat antimouse IgG fluorescein isothiocyanate (FITC) conjugate (1:200) (Southern Biotechnologies). Cells were counterstained with DAPI and covered with mounting medium. Slides were viewed with a Zeiss Axioplan confocal microscope. The images were transferred to Adobe Photoshop software for evaluation. At least three representative images were taken from each sample.

RESULTS

DNA fragmentation and PARP cleavage upon drug treatment is deficient in etoposide-resistant cells DNA fragmentation and PARP cleavage are considered as ultimate features of programmed cell death. Therefore, we analysed the response of drug-sensitive and drug-resistant MeWo cells to etoposide and cisplatin. The IC_{75}, IC_{85}, and IC_{95} values, representing drug concentrations necessary to kill 75%, 85%, or 95%, respectively, of the cells after 72 h of continuous drug treatment, were determined and are illustrated in **Table I**. In parental, drug-sensitive MeWo cells etoposide (**Fig 1*A*-I**) and cisplatin (**Fig 1*B*-I**) induced apoptotic cell death as indicated by decreasing survival rates (*hatched bars*), which were paralleled by increasing DNA fragmentation (*solid bars*). Low-level etoposide-resistant cells ($MeWo_{Eto01}$, **Fig 1*A*-II**) needed increased drug concentrations to reach comparable IC values; however, DNA fragmentation was just as high as in sensitive MeWo cells at the corresponding IC (**Figs 1*A*-I**, **1*A*-II**). In contrast, highly etoposide-resistant cells ($MeWo_{Eto1}$, **Fig 1*A*-III**) demonstrated a dramatically reduced DNA fragmentation at the corresponding IC compared to sensitive cells. These cells showed no increase in DNA fragmentation at any drug concentration tested. In contrast, cisplatin-resistant cells ($MeWo_{Cis01}$ and $MeWo_{Cis1}$, **Figs 1*B*-II, 1*B*-III**) did not demonstrate a deficient DNA fragmentation compared to sensitive MeWo cells at the corresponding IC.

In addition, cleavage of PARP was detected in sensitive MeWo cells in a dose-dependent manner upon exposure to etoposide and cisplatin, indicating apoptotic cell death (**Figs 2*A*-I**, **2*B*-I**, *lanes 2–4*). Densitometric analysis revealed an up to 16-fold reduction of the 116 kDa PARP and an up to 35-fold increase of the 89 kDa cleavage product in sensitive cells upon etoposide exposure. Similarly, the 89 kDa cleavage product increased up to 35-fold upon cisplatin exposure. In $MeWo_{Eto01}$ cells PARP cleavage was slightly reduced at IC_{85} and IC_{95} in response to etoposide compared to parental MeWo cells (**Fig 2*A*-II**), which was paralleled by the densitometric analysis. In contrast, high level resistant $MeWo_{Eto1}$ did not exhibit any 89 kDa PARP fragment (**Fig 2*A*-III**) and no change in 116 kDa and 89 kDa fragment levels were detectable by densitometry. In contrast, highly cisplatin-resistant cells ($MeWo_{Cis1}$) demonstrated a significant reduced PARP cleavage in response to cisplatin (**Fig 2*B*-III**) (up to 4.5-fold increase of the 89 kDa cleavage product at IC_{95} by densitometry), whereas $MeWo_{Cis01}$ cells still exhibited degradation of PARP (40-fold by densitometry at IC_{95}) (**Fig 2*B*-II**).

Effector caspase activation is deficient in etoposide-resistant cells As drug-induced DNA fragmentation and PARP cleavage at equitoxic concentrations in parental and resistant cells was altered, this observation prompted us to study the activation of effector caspases, e.g., caspase 3 and 7, in these cells by cleavage of fluorogenic substrates. The synthetic fluorogenic peptide Ac-DEVD-AFC was used to detect caspase-3-like activity (Garcia Calvo *et al*, 1999). Following 72 h treatment of sensitive MeWo cells with etoposide and cisplatin DEVD-AFC cleavage activity increased from ≈27-fold (IC_{75}) to ≈46-fold (IC_{95}) (**Fig 3*A***, *left panel*) and ≈4-fold (IC_{75}) to ≈16-fold (IC_{95}) (**Fig 3*B***, *left panel*), respectively. In comparison to parental MeWo cells, $MeWo_{Eto01}$ exhibited a strongly decreased DEVD–AFC cleavage. Nevertheless, a clear dose-dependent increase of DEVD–AFC cleavage activity from ≈7-fold (IC_{75}) to ≈18-fold (IC_{85}) and ≈22-fold (IC_{95}) was still demonstrable (**Fig 3*A***, *middle panel*). $MeWo_{Eto1}$ cells, however, demonstrated a dramatically restricted DEVD–AFC cleavage activity at IC_{75}. In addition, after exposure to maximum drug doses (up to IC_{95}) no significant DEVD–AFC cleavage was inducible in these cells (**Fig 3*A***, *right panel*). In comparison, in cisplatin-resistant $MeWo_{Cis1}$ cells DEVD–AFC cleavage activity increased dose-dependently in response to cisplatin (**Fig 3*B***, *right panel*). This cleavage activity was only slightly reduced compared to cisplatin-treated sensitive MeWo cells at the corresponding IC_{75}, IC_{85}, or IC_{95} (**Fig 3*B***, *left panel*).

Because of the differential cleavage activity of DEVD–AFC (**Fig 3**), a substrate thought to be preferred by caspase 3 and 7, we assessed the activation of these caspases by Western blot analysis (**Fig 4**). No differences in the expression of full-length caspase 3 and full-length caspase 7 in sensitive and resistant MeWo cells were observed (data not shown). Cleavage of caspase 3 to the 17 kDa fragment as well as cleavage of caspase 7 into the 20 kDa fragment were detected in sensitive MeWo cells upon etoposide (at IC_{85} and IC_{95}) (**Figs 4*A***, **4*C*-I**, *lanes 3–4*) and cisplatin exposure (IC_{95}) (**Figs 4*B***, **4*D*-I**, *lane 4*), indicating caspase 3 and caspase 7 activation in apoptotic cell death upon contact to both drugs.

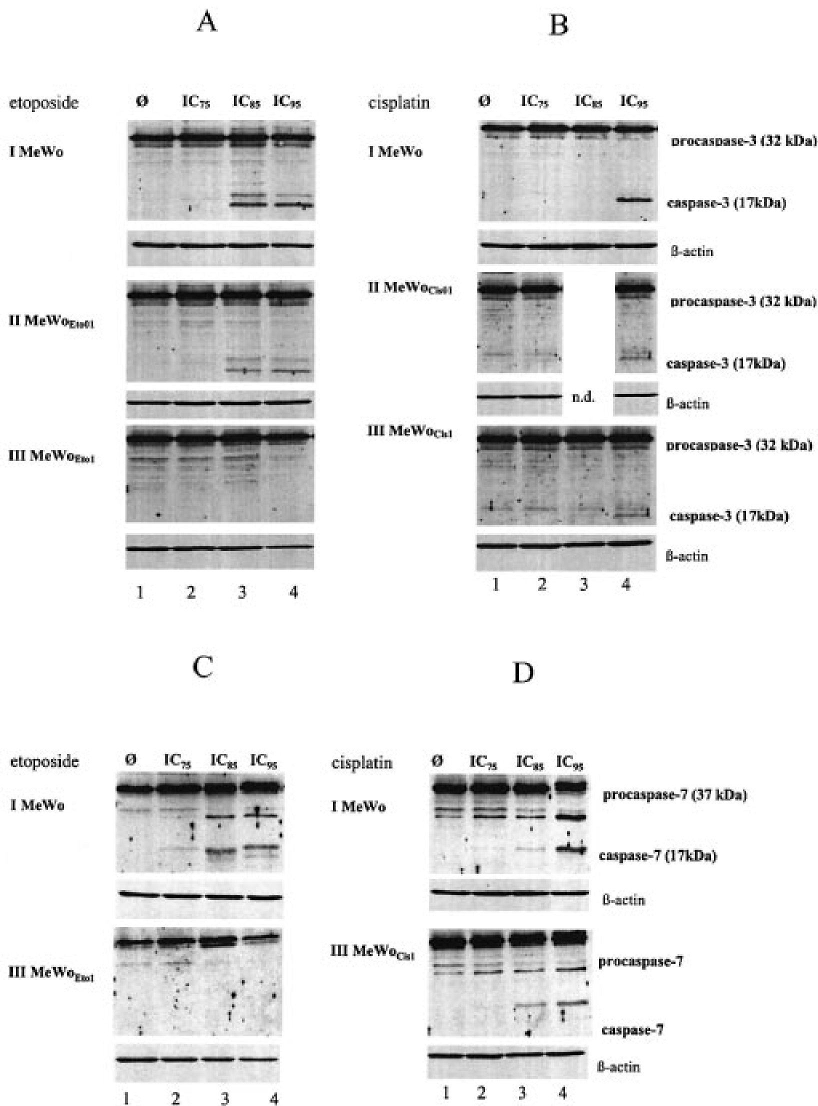

Figure 4. Different activation of caspase 3 and caspase 7 in sensitive and resistant MeWo cells. Western blot analysis was performed in drug-sensitive and drug-resistant MeWo cells using the polyclonal rabbit anticaspase 3 (*A*, *B*) (1:2000 in Tween–PBS) and HRP-conjugated antirabbit antibody (1:5000 in Tween–PBS) as well as the polyclonal mouse anticaspase 7 (*C*, *D*) (1:1000 in Tween–PBS) and HRP-conjugated antimouse antibody (1:5000 in Tween–PBS). Expression was detected in etoposide- and cisplatin-treated sensitive MeWo cells (*A–D*/-I), as well as in etoposide-treated $MeWo_{Eto01}$ and $MeWo_{Eto1}$ cells (*A*; *C*-II, *C*-III) and cisplatin-treated $MeWo_{Cis01}$ and $MeWo_{Cis1}$ cells (*B*; *D*-II, *D*-III). Untreated (*lane 1*) and treated (IC_{75}, IC_{85}, and IC_{95}) (*lanes 2–4,* respectively) cells were compared. The positions of full-length caspase and its cleavage fragment are denoted on the right. Equal amounts of protein were confirmed by detection of β-actin as denoted by an arrow on the right. This blot demonstrates one representative Western blot of at least three. (n.d., not determined.)

Consistent with the DEVD–AFC cleavage activity (**Fig 3*A***), caspase 3 activation, detected by Western blot, in $MeWo_{Eto01}$ cells was significantly reduced at IC_{85} and IC_{95} in response to etoposide (**Fig 4*A*-II**, *lanes 3*, *4*). Highly resistant $MeWo_{Eto1}$ cells did not exhibit cleavage fragments (**Figs 4*A*, 4*C*-III**). In comparison, low cisplatin-resistant cells, $MeWo_{Cis01}$, demonstrated a significantly reduced caspase 3 activation in response to cisplatin (**Fig 4*B*-II**, *lane 4*). In $MeWo_{Cis1}$ cells proteolytic products of caspase 3 and 7 could be detected weakly upon cisplatin exposure at IC_{95} (**Figs 4*B*, 4*D*-III**, *lane 4*) in comparison to sensitive cells.

Expression of caspases and inhibitory molecules is not different in sensitive and resistant cells Activation of caspases during apoptosis proceeds by stepwise processing of caspases by other proteases (Martin and Green, 1995); however, the detailed sequence in this process remains obscure. Our initial results demonstrated deficiencies in caspase activation, where various caspases themselves as well as caspase inhibitors might be responsible. Expression of caspases was determined on the mRNA level. Using an RNase Protection Assay, transcripts of all caspases investigated, except caspase 10, were present (**Fig 5*A***). On the mRNA level none of these caspases was differentially expressed, either in drug-resistant (*lanes 6–9*) or in untreated or drug-treated sensitive MeWo cells (*lanes 1–5*). Additional Western blot analysis of total cellular protein lysates confirmed that expression levels of caspase 3, 7, and 9 were not significantly different in sensitive and resistant cell lines (data not shown). Expression of IAP family members known to inhibit caspases was determined on the mRNA level using an RNase Protection Assay (**Fig 5*B***). All cells studied expressed a comparable pattern of mRNA species encoding XIAP, survivin, c-IAP-1, and TRPM-2. NAIP and c-IAP-2 specific mRNAs were not detectable (**Fig 5*B***). Additional Western blot analysis confirmed that protein levels of cIAP-1, cIAP-2, X-IAP, and survivin did not differ between drug-sensitive and drug-resistant cells (data not shown).

Cytochrome c release is strongly reduced in etoposide-resistant cells Release of cytochrome c from mitochondria into the cytoplasm has been implicated as an important step in apoptosis mediated by cytostatic drugs (Liu *et al*, 1996; Bossy-Wetzel *et al*, 1998). The extent of cytochrome c translocation from mitochondria into the cytoplasm in drug-sensitive and drug-resistant cells after drug exposure was assessed by Western blotting and immunocytologic staining. Release of cytochrome c in drug-exposed sensitive MeWo cells (**Figs 6*A*-II, *A*-IV**) was demonstrated by a diffuse homogeneous staining pattern in contrast to the untreated control (**Fig 6*A*-I**), with a granulated staining pattern representing a mitochondrial localization of cytochrome c. This translocation of cytochrome c into the cytosol was also induced in cisplatin-resistant cells exposed to cisplatin (**Fig 6*A*-III**). Upon etoposide treatment, however, $MeWo_{Eto1}$ cells exhibited the characteristic granulated FITC staining beside a slight diffuse FITC staining (**Fig 6*A*-V**), suggesting strongly reduced cytochrome c translocation.

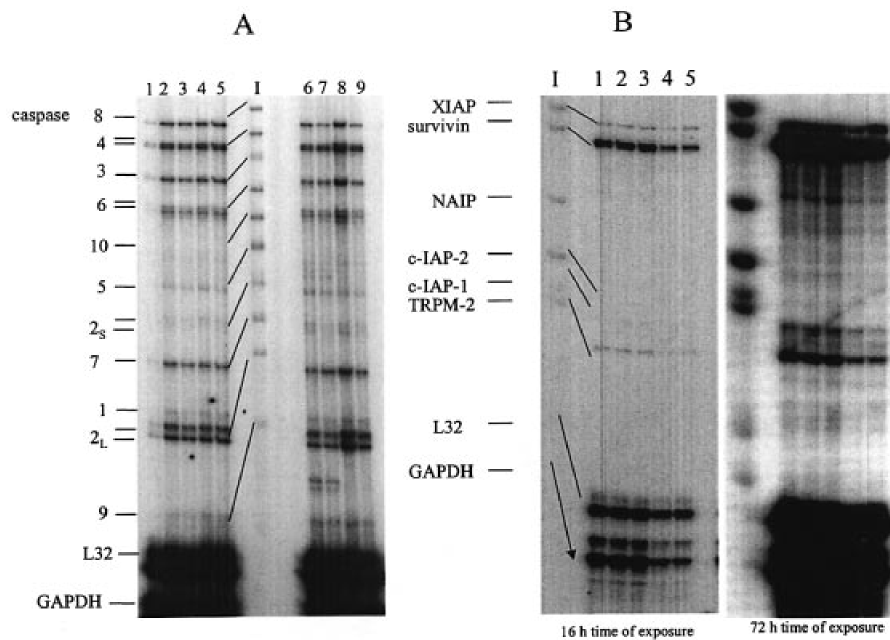

Figure 5. mRNA of several caspases and members of the IAP family is equally expressed in sensitive and resistant MeWo cells. For detection of mRNA expression of several (*A*) caspases and (*B*) members of the IAP family RNase protection assay was performed. (*A*) Cells were treated with cisplatin or etoposide for 20 h. RNA was analyzed from untreated sensitive MeWo cells (*lane 1*), sensitive cells treated with cisplatin 0.1 μg per ml and 1 μg per ml (*lanes 2, 3*), sensitive cells treated with etoposide 0.1 μg per ml and 1 μg per ml (*lanes 4, 5*), $MeWo_{Cis01}$ (*lane 6*) and $MeWo_{Cis1}$ (*lane 7*), and $MeWo_{Eto01}$ (*lane 8*) and $MeWo_{Eto1}$ (*lane 9*). Weak signals detected in RNA from MeWo (*lane 1*) are due to reduced RNA loading. (*B*) Cells were treated with cisplatin or etoposide for 20 h. RNA was analyzed from untreated sensitive MeWo cells (*lane 1*), sensitive MeWo cells treated with cisplatin 1 μg per ml (*lane 2*) and etoposide 1 μg per ml (*lane 3*), $MeWo_{Cis1}$ (*lane 4*), and $MeWo_{Eto1}$ (*lane 5*). The undigested probe (I) was used to establish a standard curve for identifying specific RNase-protected bands in the samples. The respective transcripts are denoted on the left. Equal loading was checked by analyzing the housekeeping genes L32 and GAPDH. RNA loading from cisplatin- and etoposide-resistant cells was reduced in *lanes 4 and 5*. Results were confirmed by one additional RNase protection assay.

Western blot results presented in **Fig 6**(***B***, ***C***) confirmed minimal levels of cytochrome c (15 kDa) only in the cytoplasm of extracts prepared from untreated cells (**Figs 6*B***, **C**, *lane 5*). After 72 h etoposide treatment of sensitive MeWo cells a clear dose-dependent increase of cytoplasmic cytochrome c was detectable (**Fig 6*B*-I**, *lanes 6–8*). Similar results were obtained after cisplatin exposure of sensitive MeWo cells (**Fig 6*C*-I**, *lanes 6–8*). In contrast, in $MeWo_{Eto1}$ cells a strongly reduced release of cytochrome c into the cytoplasm (**Fig 6*B*-III**, *lanes 5–8*) was observed. Instead, there was a significant upregulation of mitochondrial cytochrome c in drug-exposed $MeWo_{Eto1}$ cells (**Fig 6*B*-III**, *lane 4*). We also performed similar experiments exposing etoposide-resistant cells to teniposide, another topoisomerase II inhibitor, with identical results (data not shown). $MeWo_{Eto01}$ cells demonstrated a detectable but reduced cytochrome c release (**Fig 6*B*-II**). $MeWo_{Cis01}$ cells exhibited a significant cytochrome c release comparable to sensitive cells (**Fig 6*C*-II**). In comparison, highly cisplatin-resistant cells ($MeWo_{Cis1}$) showed a reduced cytochrome c release into the cytoplasm at high drug concentrations (IC_{85}, IC_{95}) (**Fig 6*C*-III**, *lanes 7, 8*) by Western blot analysis.

LEHD–AFC cleavage activity As sensitive and resistant MeWo cells showed different levels of cytochrome c release, effector caspase activation, PARP cleavage, and DNA fragmentation at comparable survival rates, we consequently analyzed caspase 9 activity by LEHD–AFC cleavage. Caspase 9 is an initiator caspase promoting apoptotic cell death initiated by cytochrome c and apoptotic protease activating factor 1. Various studies have shown that caspase 9 is activated in response to cytochrome c due to clustering of caspase 9 by apoptotic protease activating factor 1 (Li *et al*, 1997; Pan *et al*, 1998). A preferred substrate of caspase 9 *in vitro* is LEHD, which was used to assess caspase 9 activity in drug-sensitive and resistant MeWo cell lines. Peptide cleavage was measured in extracts prepared from cells after 72 h of drug treatment and was normalized against the untreated control. After etoposide exposure of sensitive MeWo cells LEHD–AFC cleavage activity increased from ≈4-fold (IC_{75}) to ≈17-fold (IC_{95}) dose-dependently (**Fig 7*A***, *left panel*). In comparison, etoposide exposure of $MeWo_{Eto01}$ cells resulted in reduced LEHD–AFC cleavage activity. Nevertheless, this activity still increased dose-dependently from ≈2-fold (IC_{75}) up to ≈8-fold (IC_{95}) (**Fig 7*A***, *middle panel*). LEHD-specific peptide cleavage activity could not be detected in extracts prepared from $MeWo_{Eto1}$ cells exposed to etoposide at any concentrations tested (**Fig 7*A***, *right panel*). In sensitive MeWo cells cisplatin induced a dose-dependent LEHD–AFC cleavage activity starting at an ≈2-fold induction at IC_{75} up to an ≈12-fold induction at IC_{95} (**Fig 7*B***, *left panel*). In $MeWo_{Cis1}$ cells exposed to cisplatin low caspase 9 activity was measured, which was maximally ≈2.5-fold induced and did not increase dose-dependently (**Fig 7*B***, *right panel*), whereas low-resistant $MeWo_{Cis01}$ cells demonstrated an ≈7-fold increase of LEHD–AFC cleavage at IC_{85} (**Fig 7*B***, *middle panel*).

DISCUSSION

Many cytostatic drugs are well known to act through apoptotic mechanisms. In order to investigate mechanisms conferring drug resistance towards etoposide and cisplatin, human melanoma MeWo cells and their resistant variants were analyzed. Apoptotic cell death in drug-sensitive MeWo cells upon exposure to cisplatin and etoposide was associated with a dose-dependent DNA fragmentation and PARP cleavage. Furthermore, cell death was accompanied by mitochondrial release of cytochrome c into the cytoplasm, the induction of LEHD–AFC cleavage activity suggesting the activation of the initiator caspase 9, DEVD–AFC cleavage activity, and proteolytic degradation of the effector

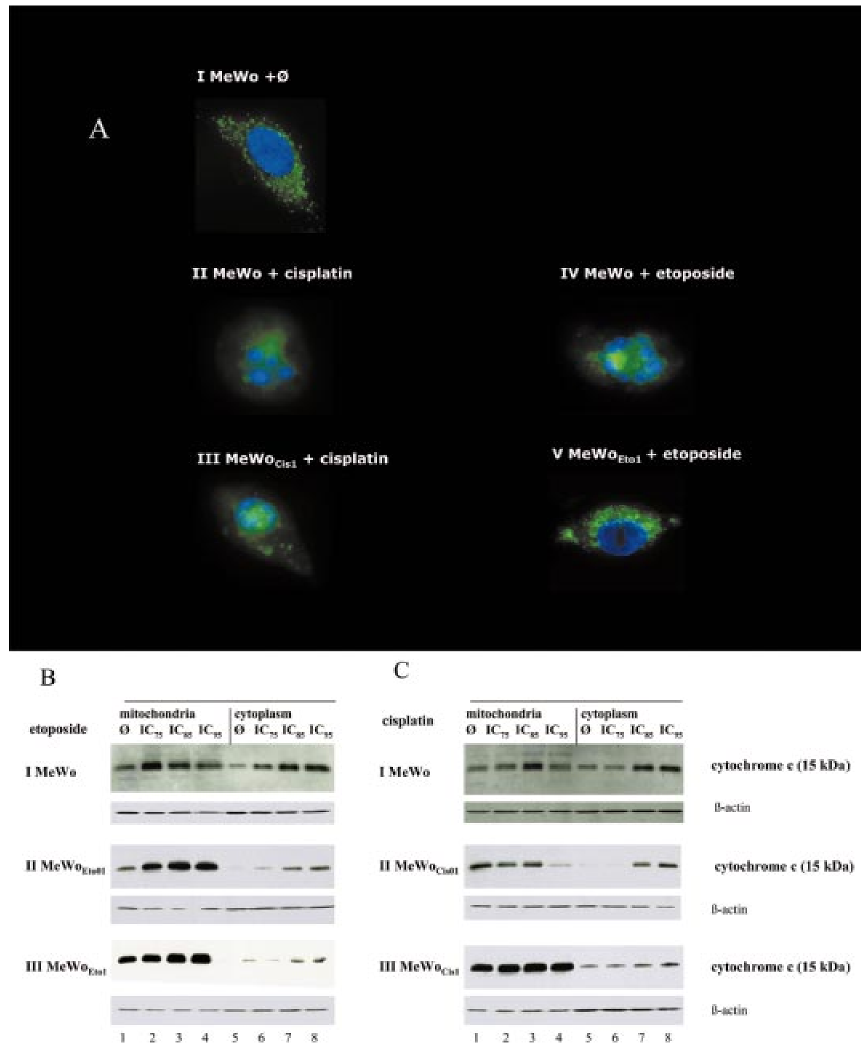

Figure 6. Cytochrome c release in etoposide-resistant MeWo cells is strongly reduced. Release of cytochrome c was investigated by immunocytologic staining (*A*) as well as Western blot analysis (*B*, *C*). (*A*)Untreated nonapoptotic cells are characteristically featured by punctuated FITC/cytochrome c (green) staining and the intact nucleus (DAPI/blue) (*A*-I)as seen in the untreated MeWo control. Sensitive and resistant cells were treated with cisplatin and etoposide, respectively, for 72 h in a concentration that represents the IC_{95} (**Table I**). Cisplatin-treated sensitive MeWo cells (*A*-II) as well as $MeWoCis_1$ (*A*-III) demonstrated nucleus fragmentation and a diffuse homogeneous FITC staining representing release of cytochrome c into the cytosol. This appearance was detectable also in sensitive cells treated with etoposide (*A*-IV). The staining of drug-exposed highly etoposide-resistant cells is shown in (*A*-V)where the granulated cytochrome c-FITC staining and a slight diffuse homogeneous FITC background as well as the unfragmented nucleus are seen. (*B*)Treated and untreated cells were collected and subcellular fractions were separated as described in *Materials* and *Methods*. Mitochondrial (*lanes 1–4*) and cytoplasmic fractions (*lanes 5–8*) were separated by SDS-PAGE. Western blots were probed with a 1:1000 dilution of a mouse anticytochrome c monoclonal antibody. Sensitive MeWo cells and etoposide-resistant cells, $MeWo_{Eto01}$ and $MeWo_{Eto1}$ (*B*-I, *B*-II, *B*-III) cells untreated (*lanes 1*, *5*) and treated with etoposide at IC_{85}, IC_{95}, and IC_{95} (*B*-I, *lanes 2–4*, *6–8*), were analyzed. The same experiment was performed with sensitive MeWo cells and cisplatin-resistant cells, $MeWo_{Cis01}$ and $MeWo_{Cis1}$, exposed to cisplatin (*C*).The position of cytochrome c (15 kDa) is marked on the right. Equal amounts of protein were confirmed by detection of β-actin, as denoted by an arrow. The purity of the subcellular fractions was controlled by additional Western blot analysis with a monoclonal antibody detecting cytochrome-c-oxidase, which is strictly localized in the mitochondrial membrane (data not shown).

caspases, caspase 3 and caspase 7, determined by Western blot analysis. Cytochrome c release was strongly reduced in etoposide-resistant cells. Therefore, this work provides additional evidence that cisplatin and etoposide resistance is associated with an altered apoptotic pathway specific for the drug employed. These data further demonstrate that etoposide- and cisplatin-induced apoptosis in drug-sensitive MeWo cells is mediated by mitochondrial pathways as has been described for various cytostatic drugs in other tumor entities previously (Hirsch *et al*, 1997; Zhao *et al*, 1998; Henkels and Turchi, 1999). Taken together the results implicate a marked apoptosis deficiency in highly etoposide-resistant cells, although caspase-independent apoptosis as well as programmed cell death without DNA fragmentation have been described.

This concept of apoptosis deficiency is supported by the observed close association between the gradual loss of apoptotic features and the increasing level of etoposide resistance at equitoxic drug concentrations used for exposure. An altered drug–target interaction cannot be completely ruled out, however, as in highly etoposide resistant cells ($MeWo_{Eto1}$) a decrease of topoisomerase activity was observed previously (Lage *et al*, 2000), which itself might be in part responsible for drug resistance. Although etoposide-resistant cells exhibit a marked cross-resistance towards cisplatin (Kern *et al*, 1997), cisplatin exposure of etoposide-resistant cells can reactivate certain apoptotic characteristics, such as DNA fragmentation and caspase activation (data not shown).

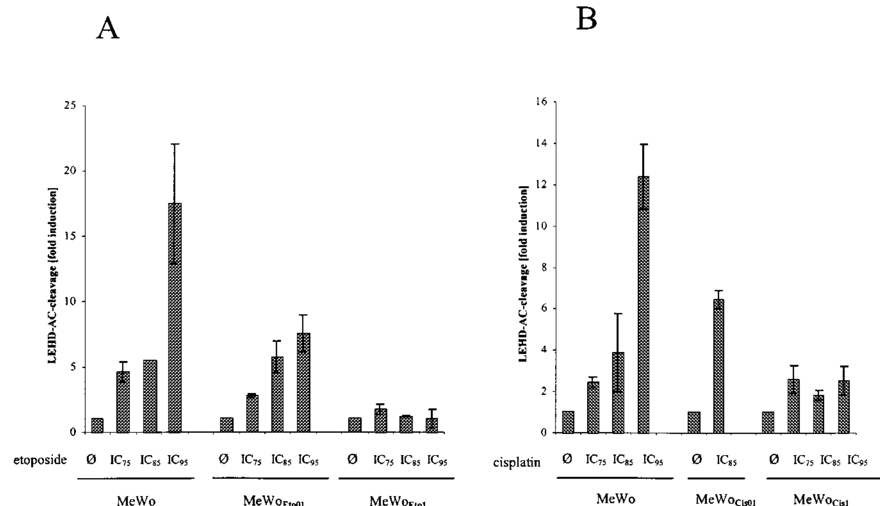

Figure 7. LEHD cleavage (caspase 9 activity) in etoposide- and cisplatin-treated resistant MeWo cells is strongly reduced. Cell extracts were prepared following 72 h continuous drug exposure at the respective IC_{75}, IC_{85}, and IC_{95} as described in *Materials* and *Methods*. (*A*)Etoposide-treated sensitive cells (MeWo) and etoposide-resistant cells ($MeWo_{Eto01}$ and $MeWo_{Eto1}$); (*B*) cisplatin-treated sensitive cells (MeWo) and cisplatin-resistant cells ($MeWo_{Cis1}$). The data were normalized against the respective untreated control (→) and are shown as *x*-fold induction. Each bar represents the mean of five experiments. The error bars indicate the observed range, and standard deviations are approximately 50% of these ranges.

Taken together, chemotherapeutic drugs, such as etoposide and cisplatin, induced apoptotic cell death in MeWo cells via involvement of mitochondria. Etoposide- and cisplatin-resistant sublines derived from MeWo followed different pathways leading to cell death compared to the sensitive parental cell line. Cell death in etoposide-resistant MeWo cells was characterized by an absence of typical hallmarks of apoptosis, implicating an apoptosis deficiency as the possible origin of the drug resistance observed. Cisplatin-resistant cells showed another dramatic modulation of their apoptotic pathway compared to the sensitive parental as well as to the etoposide-resistant cell line. Apoptosis in these cells was less dependent on cytochrome c release and caspase 9 activation and associated with reduced PARP cleavage. This constellation suggests a modulation of the apoptotic pathway on the mitochondrial level, where cytochrome c release and caspase 9 activation is reduced. High expression of Hsp, namely Hsp70 and Hsp27, in breast, endometrial, or gastric cancer has been associated with metastasis, poor prognosis, and resistance to chemotherapy (Maehara *et al*, 2000; Munster *et al*, 2001; Wataba *et al*, 2001). In apoptosis induced by the anticancer agent etoposide caspase 9, but not cytochrome c release, is inhibited by Hsp27. Thus, Hsp27 may exert its effect on the apoptosome (Garrido *et al*, 1999). This might be a relevant mechanism in etoposide-resistant cells as we detected here a remanent cytochrome c release but no activation of caspase 9; this should be proved in future experiments.

In a recent report (Henkels and Turchi, 1999) cisplatin-induced apoptosis in cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines was shown to proceed via caspase-3-independent and caspase-3-dependent pathways, respectively. In that report an alteration of the apoptotic pathway present in sensitive cells was associated with the cisplatin-resistant phenotype. Other studies reported defects in the ability to activate apoptotic processes in a cisplatin-resistant leukemia cell line, which correlated to a lack of endonuclease activity and DNA fragmentation (Segal and Jacquemin, 1995). In cisplatin-resistant human squamous carcinoma cells apoptosis deficiency was associated with Bcl-x_L dependent abrogation of cytochrome c release (Kojima *et al*, 1998). These differences emphasize that apoptotic pathways might be dependent not only on the apoptotic stimulus but also on the cell system.

The differences observed in cisplatin- and etoposide-resistant cells highlights an important aspect with respect to the transduction of the cellular signals sensing DNA damage induced by different anticancer drugs. In any case, it will be of interest to determine the effect of other apoptotic stimuli on the apoptotic pathway in resistant MeWo cells. These studies will show whether specific apoptotic defects associated with specific drug resistance also confer resistance to other drugs and if these defects can be bypassed or corrected.

Furthermore, alternative mechanisms leading to drug resistance such as alterations in DNA mismatch repair (Lage *et al*, 1999; Rünger *et al*, 2000) and DNA topoisomerase II activity (Lage *et al*, 2000) were associated with the acquired etoposide-resistant phenotype exhibited by $MeWo_{Eto1}$ cells. These data demonstrated a constitutive decreasing activity of topoisomerase IIβ with increasing etoposide resistance of MeWo cells. Furthermore, Lage *et al* (1999) showed a deficiency in DNA mismatch repair (MMR) by decreased nuclear MMR protein expression levels (MSH-1, MSH-6) in fotemustine- and cisplatin-resistant MeWo cells. Rünger *et al* (2000) demonstrated an increased DNA repair in the cisplatin-resistant $MeWo_{Cis}$ cells and fotemustine-resistant cells as well. DNA repair might therefore be an essential part in conferring resistance towards alkylating drugs.

The drug-resistant MeWo cell line represents a unique system to study underlying drug resistance mechanisms. Work on cell lines has the advantage of reproducibility; however, whether data obtained are representative needs to be proven. Nevertheless, the results obtained might lead to new concepts and might stimulate further research on fresh tumor samples and other melanoma cell lines. Differences in drug-induced apoptosis dependent on various cytostatic drugs have not been analyzed before. Our data provide further support for the involvement of apoptosis deficiency in drug resistance of melanoma and may help towards an understanding of etoposide- and cisplatin-induced apoptosis and mechanisms conferring chemoresistance towards etoposide and cisplatin in human melanoma. Moreover, the results support the concept that modulation of apoptotic pathways in human MeWo cells is drug dependent and highly associated with the drug-resistant phenotype.

This work was supported by a grant of Deutsche Forschungsgemeinschaft (DFG, Scha 422/7–2) and Forschungsfond Klinikum Mannheim.

REFERENCES

Bossy-Wetzel E, Newmeyer DD, Green DR: Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *Embo J* 17:37–49, 1998

Breton C, Story MD, Meyn RE: Bcl-2 expression correlates with apoptosis induction but not loss of clonogenic survival in small cell lung cancer cell lines treated with etoposide. *Anticancer Drugs* 9:751–757, 1998

Bukau B, Horwich AL: The Hsp70 and Hsp60 chaperone machines. *Cell* 92:351–366, 1998

Chmura SJ, Mauceri HJ, Advani S, *et al*: Decreasing the apoptotic threshold of tumor cells through protein kinase C inhibition and sphingomyelinase activation increases tumor killing by ionizing radiation. *Cancer Res* 57:4340–4347, 1997

Eliopoulos AG, Kerr DJ, Herod J, Hodgkins L, Krajewski S, Reed JC, Young LS: The control of apoptosis and drug resistance in ovarian cancer: influence of p53 and Bcl-2. *Oncogene* 11:1217–1228, 1995

Fields KK, Zorsky PE, Hiemenz JW, Kronish LE, Elfenbein GJ: Ifosfamide, carboplatin, and etoposide: a new regime with a broad spectrum of activity. *J Clin Oncol* 12:544–552, 1994

Franco AV, Zhang XD, Van Berkel E, *et al*: The role of NF-kappaB in TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of melanoma cells. *J Immunol* 166:5337–5345, 2001
Friesen C, Herr I, Krammer PH, Debatin KM: Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat Med* 2:574–577, 1996
Friesen C, Fulda S, Debatin KM: Cytotoxic drugs and the CD95 pathway. *Leukemia* 13:1854–1858, 1999
Fulda S, Los M, Friesen C, Debatin KM: Chemosensitivity of solid tumor cells *in vitro* is related to activation of the CD95 system. *Int J Cancer* 76:105–114, 1998a
Fulda S, Susin SA, Kroemer G, Debatin KM: Molecular ordering of apoptosis induced by anticancer drugs in neuroblastoma cells. *Cancer Res* 58:4453–4460, 1998b
Garbe C: Chemotherapy and chemoimmunotherapy in disseminated malignant melanoma. *Melanoma Res* 3:291–299, 1993
Garcia Calvo M, Peterson EP, Rasper DM, Vaillancourt JP, Zamboni R, Nicholson DW, Thornberry NA: Purification and catalytic properties of human caspase family members. *Cell Death Differ* 6:362–369, 1999
Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, Solary E: HSP27 inhibits cytochrome c-dependent activation of procaspase-9. *Faseb J* 13:2061–2070, 1999
Hassan Z, Fadeel B, Zhivotovsky B, Hellstrom Lindberg E: Two pathways of apoptosis induced with all-*trans* retinoic acid and etoposide in the myeloid cell line P39. *Exp Hematol* 27:1322–1329, 1999
Helmbach H, Rossmann E, Kern MA, Schadendorf D: Drug-resistance in human melanoma. *Int J Cancer* 93:617–622, 2001
Henkels KM, Turchi JJ: Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cell lines. *Cancer Res* 57:4488–4492, 1997
Henkels KM, Turchi JJ: Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. *Cancer Res* 59:3077–3083, 1999
Hirsch T, Marchetti P, Susin SA, *et al*: The apoptosis–necrosis paradox. Apoptogenic proteases activated after mitochondrial permeability transition determine the mode of cell death. *Oncogene* 15:1573–1581, 1997
Houghton JA: Apoptosis and drug response. *Curr Opin Oncol* 11:475–481, 1999
Jansen B, Wacheck V, Heere-Ress E, *et al*: Chemosensitisation of malignant melanoma by BCL2 antisense therapy. *Lancet* 356:1728–1733, 2000a
Jansen B, Wacheck V, Heere-Ress E, *et al*: Bcl-2 antisense plus dacarbacine therapy for malignant melanoma. *Proceedings of the Am. Assoc. Cancer Res. Conference Programmed Cell Death Regul. A95*, 2000b
Kaufmann SH, Earnshaw WC: Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 256:42–49, 2000
Kern MA, Helmbach H, Artuc M, Karmann D, Jurgovsky K, Schadendorf D: Human melanoma cell lines selected *in vitro* displaying various levels of drug resistance against cisplatin, fotemustine, vindesine or etoposide: modulation of proto-oncogene expression. *Anticancer Res* 17:4359–4370, 1997
von Knethen A, Lotero A, Brune B: Etoposide and cisplatin induced apoptosis in activated RAW 264.7 macrophages is attenuated by cAMP-induced gene expression. *Oncogene* 17:387–394, 1998
Kojima H, Endo K, Moriyama H, *et al*: Abrogation of mitochondrial cytochrome c release and caspase-3 activation in acquired multidrug resistance. *J Biol Chem* 273:16647–16650, 1998
Kuwahara D, Tsutsumi K, Kobayashi T, Hasunuma T, Nishioka K: Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. *Cancer Lett* 148:65–71, 1975
Lage H, Christmann M, Kern MA, Dietel M, Pick M, Kaina B, Schadendorf D: Expression of DNA repair proteins hMSH2, hMSH6, hMLH1, O6-methylguanine-DNA methyltransferase and N-methylpurine-DNA glycosylase in melanoma cells with acquired drug resistance. *Int J Cancer* 80:744–750, 1999
Lage H, Helmbach H, Dietel M, Schadendorf D: Modulation of DNA topoisomerase II activity and expression in melanoma cells with acquired drug resistance. *Br J Cancer* 82:488–491, 2000
Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC: Cleavage of poly(ADP-ribose)polymerase by a proteinase with properties like ICE. *Nature* 371:346–347, 1994
Le Rhun Y, Kirkland JB, Shah GM: Cellular responses to DNA damage in the absence of poly(ADP-ribose)polymerase. *Biochem Biophys Res Commun* 245:1–10, 1998
Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147–157, 1996
Liu X, Zou H, Slaughter C, Wang X: DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 89:175–184, 1997
Los M, Herr I, Friesen C, Fulda S, Schulze Osthoff K, Debatin KM: Cross-resistance of CD95- and drug-induced apoptosis as a consequence of deficient activation of caspases (ICE/Ced-3 proteases). *Blood* 90:3118–3129, 1997
Maehara Y, Oki E, Abe T, Tokunaga E, Shibahara K, Kakeji Y, Sugimachi K: Overexpression of the heat shock protein HSP70 family and p53 protein and prognosis for patients with gastric cancer. *Oncology* 58:144–151, 2000
Martin SJ, Green DR: Protease activation during apoptosis: death by a thousand cuts?. *Cell* 82:349–352, 1995
Middleton MR, Lorigan P, Owen J, Ashcroft L, Lee SM, Harper P, Thatcher N: A randomized phase III study comparing dacarbazine, BCNU, cisplatin and tamoxifen with dacarbazine and interferon in advanced melanoma. *Br J Cancer* 82:1158–1162, 2000
Minagawa Y, Kigawa J, Itamochi H, Kanamori Y, Shimada M, Takahashi M, Terakawa N: Cisplatin-resistant HeLa cells are resistant to apoptosis via p53-dependent and -independent pathways. *Jpn J Cancer Res* 90:1373–1379, 1999
Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B: The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol Cell Biol* 20:7146–7159, 2000
Munster PN, Basso A, Solit D, Norton L, Rosen N: Modulation of Hsp90 function by ansamycins sensitizes breast cancer cells to chemotherapy-induced apoptosis in an RB- and schedule-dependent manner. See: E. A. Sausville, Combining cytotoxics and 17-allylamino, 17-demethoxygeldanamycin: sequence and tumor biology matters, *Clin Cancer Res*, 7:2155–2158, 2001. *Clin Cancer Res* 7:2228–2236, 2001
Nessling M, Kern MA, Schadendorf D, Lichter P: Association of genomic imbalances with resistance to therapeutic drugs in human melanoma cell lines. *Cytogenet Cell Genet* 87:286–290, 1999
Niedner H, Christen R, Lin X, Kondo A, Howell SB: Identification of genes that mediate sensitivity to cispaltin. *Mol Pharmacol* 60:1153–1160, 2001
Pan G, K OR, Dixit VM, Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 273:5841–5845, 1998
Planting AS, van der Burg ME, Goey SH, *et al:* Phase II study of a short course of weekly high-dose cisplatin combined with long-term etoposide in metastatic melanoma. *Eur J Cancer* 32A:2026–2028, 1996
Raisova M, Bektas M, Wieder T, Daniel P, Eberle J, Orfanos CE, Geilen CC: Resistance to CD95/Fas-induced and ceramide-mediated apoptosis of human melanoma cells is caused by a defective mitochondrial cytochrome c release. *FEBS Lett* 473:27–32, 2000
Rathmell JC, Thompson CB: The central effectors of cell death in the immune system. *Annu Rev Immunol* 17:781–828, 1999
Rünger TM, Emmert S, Schadendorf D, Diem C, Epe B, Hellfritsch D: Alterations of DNA repair in melanoma cell lines resistant to cisplatin, fotemustine, or etoposide. *J Invest Dermatol* 114:34–39, 2000
Scaffidi C, Fulda S, Srinivasan A, *et al*: Two CD95 (APO-1/Fas) signaling pathways. *Embo J* 17:1675–1687, 1998
Schadendorf D, Worm M, Algermissen B, Kohlmus CM, Czarnetzki BM: Chemosensitivity testing of human malignant melanoma. A retrospective analysis of clinical response and *in vitro* drug sensitivity. *Cancer* 73:103–108, 1994
Schadendorf D, Herfordt R, Czarnetzki BM: P-glycoprotein expression in primary and metastatic malignant melanoma. *Br J Dermatol* 132:551–555, 1995a
Schadendorf D, Jurgovsky K, Kohlmus CM, Czarnetzki BM: Glutathione and related enzymes in tumor progression and metastases of human melanoma. *J Invest Dermatol* 105:109–112, 1995b
Schadendorf D, Makki A, Stahr C, *et al*: Membrane transport proteins associated with drug resistance expressed in human melanoma. *Am J Pathol* 147:1545–1552, 1995c
Segal BE, Jacquemin SA: Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process. *Exp Cell Res* 218:201–212, 1995
Seki K, Yoshikawa H, Shiiki K, Hamada Y, Akamatsu N, Tasaka K: Cisplatin (CDDP) specifically induces apoptosis via sequential activation of caspase-8-3 and -6 in osteosarcoma. *Cancer Chemother Pharmacol* 45:199–206, 1978
Sellers WR, Fisher DE: Apoptosis and cancer drug targeting. *J Clin Invest* 104:1655–1661, 1999
Siitonen T, Mantymaa P, Saily M, Savolainen ER, Koistinen P: Etoposide-induced apoptosis is not associated with the Fas pathway in acute myeloblastic leukemia cells. *Leukemia Res* 24:281–288, 2000
Sinha P, Kohl S, Fischer J, *et al*: Identification of novel proteins associated with the development of chemoresistance in malignant melanoma using two-dimensional electrophoresis. *Electrophoresis* 21:3048–3057, 2000
Soengas MS, Polsky CPD, Mora J, *et al*: Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 409:207–211, 2001
Suzuki T, Nishio K, Tanabe S: The MRP family and anticancer drug metabolism. *Curr Drug Metab* 2:367–377, 2001
Tewari MLT, Quan OR, Desnoyers KS, *et al:* Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose)polymerase. *Cell* 81:801–809, 1995
Wang CY, Cusack JC Jr, Liu R, Baldwin AS Jr: Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. *Nat Med* 5:412–417, 1999
Wataba K, Saito T, Fukunaka K, Ashihara K, Nishimura M, Kudo R: Over-expression of heat shock proteins in carcinogenic endometrium. *Int J Cancer* 91:448–456, 2001
Zhang K, Mack P, Wong KP: Glutathione-related mechanisms in cellular resistance to anticancer drugs. *Int J Oncol* 12:871–882, 1998
Zhao EG, Song Q, Cross S, Misko I, Lees Miller SP, Lavin MF: Resistance to etoposide-induced apoptosis in a Burkitt's lymphoma cell line. *Int J Cancer* 77:755–762, 1998
Zou H, Henzel WJ, Liu X, Lutschg A, Wang X: Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3 [see comments]. *Cell* 90:405–413, 1997
Zou H, Li Y, Liu X, Wang X: An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 274:11549–11556, 1999