Anti-Cancer Drugs 1997, 8, pp. 189–198

Messenger RNA expression of resistance proteins and related factors in human ovarian carcinoma cell lines resistant to doxorubicin, taxol and cisplatin

Ulrike Masanek, Gerd Stammler and Manfred Volm
German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.
Tel: (+49) 6221-423206; Fax: (+49) 6221-423225.

Doxorubicin- (OAW-dox, SK-OV-dox), taxol- (OAW-tax, SK-OV-tax) and cisplatin- (SK-OV-cis) resistant cells derived from the parental OAW-42 and SK-OV-3 cell lines were established. OAW-42 sublines showed high resistance, the SK-OV-3 sublines only low resistance. OAW-42 sublines showed a cross-resistance profile typical of multidrug resistance (MDR). The sublines of SK-OV-3 showed a cross-resistance profile different from the OAW-42 sublines. The mRNA expression of several resistance proteins and related factors was analyzed. An overexpression of P-glycoprotein 170 (P-170), glutathione-S-transferase-π (GST-π), thymidylate synthase (TS), glutathione peroxidase (GP) and c-*jun* was found in OAW-dox and OAW-tax cells. Additionally, OAW-tax cells expressed a higher mRNA level of protein kinase Cβ2. DNA analysis revealed a 2-fold gene amplification of P-170, whereas the genes for GST-π, TS and GP were not amplified. SK-OV-dox and SK-OV-tax cells showed a decreased level of histone 3 (H3) and TS mRNA. This shows that the sublines of OAW-42 developed resistance by co-expression of several resistance-related proteins and proto-oncogenes whereas the sublines of SK-OV-3 expressed resistance by decreased expression of the proliferation-dependent proteins H3 and TS.

Key words: Cell lines, co-expression, glutathione peroxidase, glutathione-S-transferase-π, ovarian carcinoma, P-glycoprotein, protein kinase C, proto-oncogenes, thymidylate synthase.

Introduction

Patients with ovarian carcinomas often respond initially to chemotherapy but the majority of these patients relapse and develop resistance to a wide range of drugs.[1] The factors leading to the development of resistance in ovarian carcinomas are still not clearly understood.

Some studies have shown that the phenotype of multidrug resistance (MDR) is caused by an overexpression of an 170 kDa plasma membrane glycoprotein (P-170) which functions as an ATP-dependent efflux pump for structurally different drugs.[2] The MDR-related protein (MRP), a distant member of the MDR family, has also been detected in multidrug resistant cell lines derived from several different tumor types.[3] Glutathione S-transferase-π (GST-π) and glutathione peroxidase (GP) may play an important role in protecting tissues from endogenous organic hydroperoxides produced during oxidative and chemical stress.[4,5] Overexpression of these proteins also results in drug resistance.[6] In addition, alterations in topoisomerase II have been identified in cell lines selected for resistance to etoposide, doxorubicin and teniposide.[7] It is also reported that thymidylate synthase (TS), a key enzyme for DNA synthesis, is involved in drug resistance.[8] Some of these proteins mentioned above are regulated by AP-1 binding domains responding to a heterodimer of *fos* and *jun* oncoproteins.[9] Several studies suggest additional roles for the protein product of c-*jun* and c-*fos* in the signal pathways associated with the acquisition of MDR.[10,11] Bushan *et al.*[12] demonstrated an elevation of the steady-state mRNA level of c-*fos* in multidrug resistant mouse sarcoma S180 and human KB cells. Funato *et al.*[13] were able to reverse cisplatin-resistance in human carcinomas by an anti-*fos* ribozyme. Increased c-*jun* levels were found in etoposide-resistant human leukemia cells.[10] The oncogenes c-*fos* and c-*jun* are regulated by a cascade involving protein kinase C (PKC).[14] Transfections with PKC genes and studies in which PKC modulators were used have suggested that the overexpression of PKC results in an increased level of resistance.[15,16] Additionally, the activation of the epidermal growth factor receptor (EGFR) stimulates protein kinases[17] and modulates the sensitivity of ovarian carcinoma cells to cisplatin.[18] The antimicrotubule agent taxol, which prevents the depolymerization of microtubules, has shown clinical

Correspondence to M Volm

antitumor activity against refractory cisplatin-resistant ovarian carcinomas.[19] Nevertheless, mechanisms of resistance to taxol have been described such as alteration in tubulin structure and changes in levels of tubulin.[20,21]

Often these complex regulatory mechanisms lead to multifactorial resistance which lower the positive outcome in human cancer. Up to now the regulatory mechanisms for ovarian cancer are not clear. Therefore, we established in the present study a panel of ovarian carcinoma sublines resistant to chemotherapeutic drugs generally used in the therapy against ovarian carcinomas and determined the cross-resistance phenotypes. In order to clarify the mechanisms of these phenotypes we investigated the mRNA expression of several proteins known to be involved in drug resistance.

Material and methods

The establishment of resistant human ovarian carcinoma cell lines

The human ovarian carcinoma cell lines OAW-42 and SK-OV-3 were derived from the Tumorbank of the German Cancer Research Center (Heidelberg). The parental OAW-42 and the established resistant cells were grown as monolayer cultures in a humidified CO_2 incubator at 37°C in α-MEM medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Immundiagnostik, Bensheim, Germany), 1% penicillin/streptomycin and 1% amphotericin (Biochrom). The parental SK-OV-3 and the corresponding resistant cells were grown in RPMI 1640 medium (Biochrom) with the same supplements as reported for OAW-42. Resistant cell lines were established by stepwise exposure to increasing concentrations of doxorubicin, cisplatin (both substances from Farmitalia Carlo Erba, Freiburg, Germany) and taxol (Bristol-Myers-Squibb, Munich, Germany) over 14 months. The development of resistance started with the concentrations of 0.1 μg/ml doxorubicin (dox) and increased stepwise to 19 μg/ml for OAW-dox and 7 μg/ml for SK-OV-dox. The taxol (tax)-treatment began with 0.03 μg/ml and ended with 30 μg/ml for OAW-tax and 6 μg/ml for SK-OV-tax. The initial concentrations for cisplatin (cis) were 2 μg/ml. The final concentrations were 8 μg/ml (OAW-cis) and 17 μg/ml (SK-OV-cis). The concentrations varied between the cell lines because of different primary sensitivity to the drugs. The experiments were carried out after pretreatment of the cells of at least 14 months.

Analysis of mRNA

The culture medium was removed and the cells were rinsed twice with Hanks' solution (HBS) to remove floating dead cells, then the cells were harvested and washed twice in HBS. Total RNA was extracted from cells using 1 ml RNA-Clean (AGS, Heidelberg, Germany) and finally resolved in diethylpyrocarbonate water. Ten micrograms of RNA were size-fractionated by 1% agarose formaldehyde gel electrophoresis, transferred onto Qiabrane (Qiagen, Hilden, Germany) according to the manufacturer's instructions and fixed to the filters by UV irradiation. The membranes were prehybridized at 42°C for 2 h in prehybridization solution (5 × SSPE, 5 × Denhardt's, 0.5% SDS, 10% dextran sulfate, 20 μg/ml denatured salmon sperm DNA, 50% formamide) and hybridized overnight at 42°C in 5 ml of the same solution containing the ^{32}P-random-primed labeled probe. After two washes with 2 × SSPE, 0.1% SDS at room temperature for 5 min, 1 × SSPE, 0.1% SDS at 60°C for 15 min, radioactivity bound to the filters was detected by autoradiography. The probes of α-tubulin, β-tubulin, multidrug resistance-related protein (MRP), EGFR, glutathione S-transferase-π (GST-π), P-glycoprotein 170 (P-170), topoisomerase IIα (topo IIα) and β-actin were obtained by PCR amplification of specific DNA fragments with gene-specific primers of human cDNA (α-tubulin: 431–823,[22] β-tubulin: 461–750,[23] MRP: 988–1440,[24] EGFR: 397–810,[25] GST-π: 356–637,[26] P-170: 1832–2217,[27] topo IIα: 742–1040,[28] β-actin: 309–760[29]). A plasmid containing glutathione peroxidase (GP) cDNA was a gift from G Mullenbach (Chiron Research Laboratories, Emeryville, CA). The cDNA probe for thymidylate synthase (TS) was kindly provided by T. Seno and S. Kaneda (National Institute for Genetics, Mishima, Japan) and the histone 3 (H3) probe was a generous gift from NE Fusenig (German Cancer Research Center, Heidelberg, Germany). The probe of c-*jun* was offered by Dianova (Hamburg, Germany). A Hirschmann Elscript 400 densitometer was used for scanning the autoradiographs. The intensity of the hybridization signals of the investigated mRNA were normalized against β-actin.

Reverse-transcription (RT)-polymerase chain reaction (PCR) assay

Because c-*fos*, PKCα and PKCβ2 were not detectable by Northern hybridization we used PCR for the determination of the mRNA expression. A 50 ng

quantity of random hexamers was added to 3 μg RNA. The RNA was reverse transcribed following the instructions of the Super Script Preamplification System (Life Technologies, Karlsruhe, Germany). PCR was carried out with 100 ng cDNA, 100 μM of each dNTP, 0.3 μM of each primer, 1.25 U Taq polymerase (Boehringer, Mannheim, Germany), 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl and filled up to 50 μl with H_2O. A 2 min denaturation step at 94°C was followed by 30 cycles of primer annealing (55°C, 40 s), primer extension (72°C, 60 s) and denaturation (94°C, 40 s). The sequences of the primers were for c-*fos*, 5′-ACG CAG ACT ACG AGG CGT CA-3′ and 5′-CTT CAC AAC GCC AGC CCT GGA-3′;[30] for PKCα, 5′-CTA AAG GCT GAG GTT GCT GAT-3′ and 5′-GTC TTC AGA GGG ACT GAT GAC-3′;[31] for PKCβ2, 5′-ACG TGA TAT CAA AGA GCA TGC-3′ and 5′-TAA CAT CCA ATT ATC CAC AGA-3′;[32] and for glycerinaldehyde-3-phosphate dehydrogenase (GAPDH, as internal standard), 5′-GTC TTC ACC ACC ATG GAG AA-3′ and 5′-ATC CAC AGT CTT CTG GGT GG-3′.[33] Numerous negative controls were included in each set of PCR reactions to detect any possible contamination. The controls were constructed by using the cDNA synthesis mixture as described above without addition of reverse transcriptase. PCR products were separated in 1% agarose gels and transferred onto Qiabrane. For hybridization 5′-end labeled cDNA probes, constructed by PCR with the same primers as described above, were used. After densitometry the values of cDNA expression were normalized against the corresponding internal standard values.

Growth inhibition assay

Aliquots of 10^4 cells were seeded in 24-well plates (Becton Dickinson, Heidelberg, Germany) and after 2 days different concentrations of doxorubicin, cisplatin or taxol were added. For circumvention of resistance, cells were treated with doxorubicin or taxol plus verapamil (Stadapharm, Bad Vilbel, Germany). Seven days after application of the drug the cells were harvested and quantified using an electronic cell counter (Coulter, Krefeld, Germany). Cell samples from three independent wells per concentration were each counted three times.

DNA isolation and analysis

Total DNA was extracted from cells using standard protocols. The cells were resuspended in 1 volume digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8, 25 mM EDTA, pH 8, 0.5% SDS, 0.1 mg/ml proteinase K). For 3×10^7 cells 0.3 ml digestion buffer were used. The samples were incubated with shaking at 50°C overnight. DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After 10 min centrifugation at 1700 *g* the DNA was precipitated with 7.5 M ammonium acetate and 2 volumes of 100% ethanol. The DNA was sedimented at 1700 *g* for 2 min. The pellet was rinsed with 70% ethanol, vacuum dried, resuspended in TE buffer and frozen at −20°C until use. Ten microgram of DNA was restricted by endonucleases (*Hin*dIII or *Eco*RI; Amersham, Braunschweig, Germany) before size-fractionation by 1% agarose electrophoresis. Southern blotting and hybridization was carried out according to the manufacturer's instructions (Amersham). The prehybridization solution and the P-170 probe were the same as described for mRNA analysis. Radioactivity bound to the filter was detected by autoradiography.

The analysis of mRNA and growth inhibition were repeated and were reproducible.

Results

Resistant human ovarian cell lines were established within 14 months by repeated treatment of the parental cell lines OAW-42 and SKOV-3 with taxol, doxorubicin and cisplatin. The detection of resistance and cross-resistance was determined by dose–response curves against these drugs. Figure 1 shows typical dose–response curves of OAW-42 cells pretreated by taxol (OAW-tax). In similar experiments the concentration producing 50% inhibition of growth (represented as IC_{50} values) was determined for each drug. Three experiments were repeated and each counted three times. The ratio between the IC_{50} of the parental cells and the IC_{50} of the resistant cells is the resistance factor. Table 1 summarizes the IC_{50} values and the resistance factors of OAW-42 and the resistant sublines. OAW-42 cell line developed high resistance against doxorubicin (OAW-dox) and taxol (OAW-tax) with an 93- and 83-fold resistance, respectively. Even after 14 months OAW-42 cells failed to develop resistance to cisplatin (data not shown). The investigation of the cross-resistance phenotype for OAW-dox reveals a 616-fold cross-resistance to taxol, 12-fold to cisplatin, 34-fold to colchicine and 118-fold to vincristine. OAW-tax cells were 93-fold cross-resistant to doxorubicin, 9-fold to colchicine, 75-fold to vincristine but showed no resistance to cisplatin. In contrast to the strong

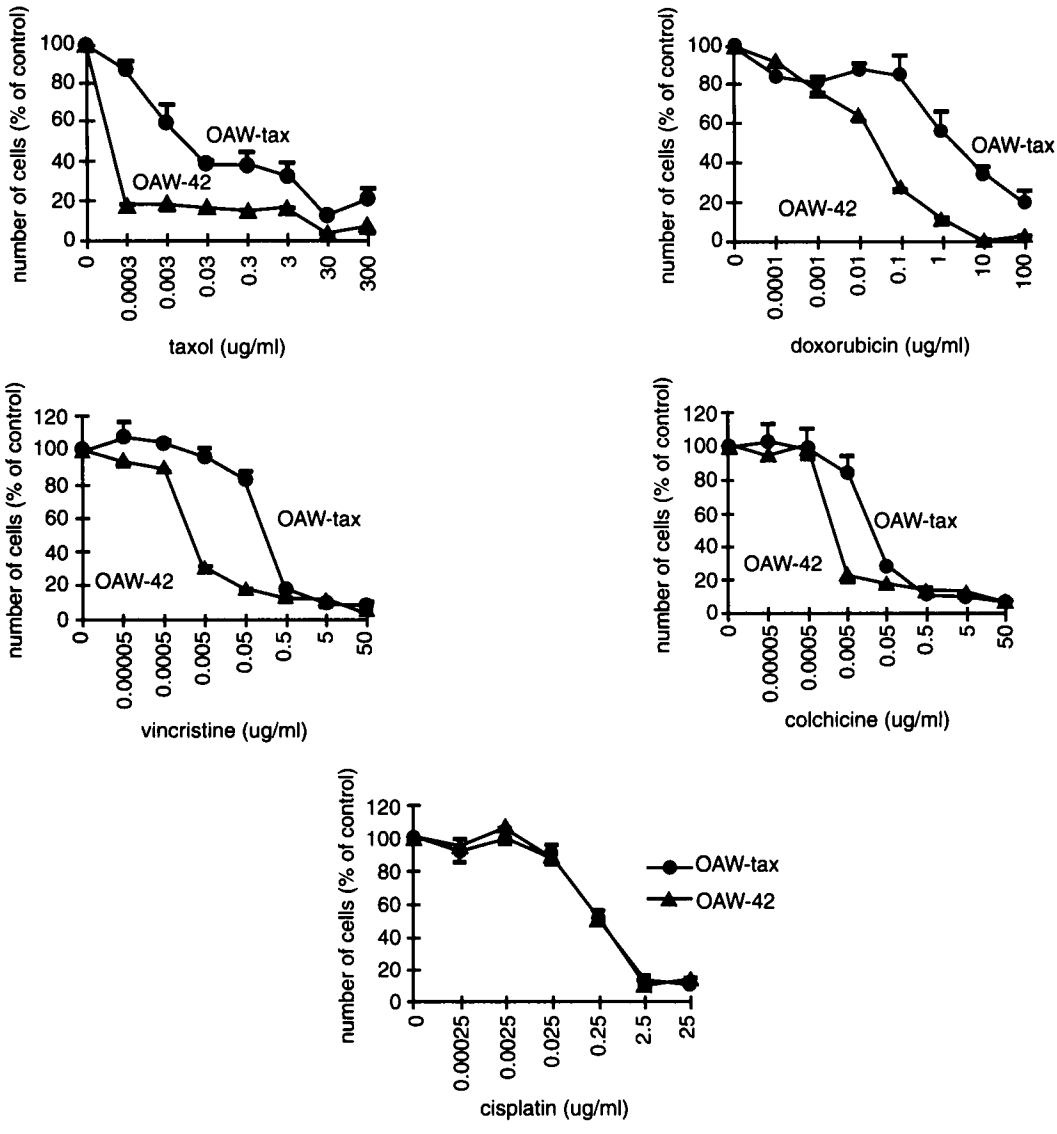

Figure 1. Detection of resistance and cross-resistance of OAW-tax (resistant) in comparison to the parental cells OAW-42 by growth inhibition assay. Dose–response curves were obtained by application of drugs. Abscissa, dose (ug/ml); ordinate, percent of the control; ▲, sensitive OAW-42 cells; ●, resistant OAW-tax. Values are the average of three independent measurements.

resistant sublines of OAW-42, the SK-OV-3 cell line developed only minor resistance to the drugs (Table 2). The doxorubicin-treated cells (SK-OV-dox) showed a resistance factor of 17. SK-OV-3 developed 12-fold resistance to taxol (SK-OV-tax) and a 6-fold resistance to cisplatin (SK-OV-cis). The cross-resistance phenotype is determined as follows. The cell line SK-OV-dox showed collateral sensitivity to taxol but 5-fold cross-resistance to cisplatin and showed no resistance to colchicine and vincristine. The cell line SK-OV-tax expressed a 6-fold resistance to cisplatin. However, they developed no resistance to doxorubicin, cholchicine and vincristine. The subline SK-OV-cis showed only a cross-resistance to doxorubicin (10-fold) and no resistance to cholchicine. It is worthy of note that this cell line was extremely sensitive to taxol and to vincristine. A comparison between the IC_{50} of OAW-42 (Table 1) and SKOV-3 (Table 2) shows that the sensitivity to each drug varied in both cell lines. A comparison of the sensitivity (IC_{50}) to doxorubicin and cisplatin of SK-OV-3 and OAW-42 cells revealed that they developed resistance to the drug to which they had primary lower sensitivity. The sensitivity to taxol in these cell lines showed reverse results. Thus, it is not possible to predict the development of the

Table 1. Inhibition (IC_{50}) of sensitive (OAW-42) and doxorubicin- (OAW-dox) and taxol- (OAW-tax) resistant cell lines by different drugs

Drugs	OAW-42	OAW-dox		OAW-tax	
	IC_{50}[a]	IC_{50}[a]	rf[b]	IC_{50}[a]	rf[b]
Doxorubicin	0.04	3.7	93	3.7	93
Taxol	1.8×10^{-4}	0.11	616	0.02	83
Cisplatin	0.11	1.38	12	0.11	0
Colchicine	3.42×10^{-3}	0.11	34	0.03	9.3
Vincristine	3.65×10^{-3}	0.43	118	0.28	75

[a]IC_{50} (inhibition concentration 50) in μg/ml.
[b]Resistance factor (ratio between IC_{50} of the sensitive cell lines and the resistant cell lines).

Table 2. Inhibition (IC_{50}) of sensitive (SK-OV-3) and doxorubicin- (SK-OV-dox), taxol- (SK-OV-tax) and cisplatin- (SK-OV-cis) resistant cell lines by different drugs

Drugs	SK-OV-3	SK-OV-dox		SK-OV-tax		SK-OV-cis	
	IC_{50}[a]	IC_{50}[a]	rf[b]	IC_{50}[a]	rf[b]	IC_{50}[a]	rf[b]
Doxorubicin	5.5×10^{-4}	9.55×10^{-3}	17	5×10^{-4}	0	5.5×10^{-3}	10
Taxol	2.19×10^{-3}	1.1×10^{-4}	−20	0.027	12	1.65×10^{-5}	−132
Cisplatin	2.05	10.4	5	12.6	6	12.62	6
Colchicine	2.7×10^{-3}	5×10^{-3}	1.7	4.3×10^{-3}	1.6	2.7×10^{-3}	0
Vincristine	1.2×10^{-3}	1.2×10^{-3}	0	5×10^{-4}	−2.4	3×10^{-5}	−40

[a]IC_{50} (inhibition concentration 50) in μg/ml.
[b]Resistance factor (ratio between IC_{50} of the sensitive cell lines and the resistant cell lines).

pattern of cross-resistance by comparing the drug-sensitivity of the parental cells.

In order to determine whether the known mechanisms of resistance to these drugs were operative in manifestation of the cross-resistance phenotype we used the sublines OAW-tax and OAW-dox for further investigations. We evaluated in the parental and the resistant cell lines the mRNA expression of the resistance-related proteins P-170, MRP, GST-π, topo IIα, TS, GP, α-tubulin, β-tubulin, the oncogene-protein c-*jun* and the proliferation marker H3 by Northern blotting. For the mRNA detection of the oncogene c-*fos* and the regulator proteins PKCα and PKCβ2 we used the more sensitive PCR method. As shown in Figure 2(a) the resistant OAW-tax and OAW-dox expressed a very high level of P-170 mRNA compared to the parental OAW-42. No obvious increase of MRP, which is also involved in MDR, was seen in resistant cell lines compared to parental cells (Figure 2f). The mRNA expression of MRP varied in the resistant and non-resistant cell lines. An elevation of the mRNA content was observed for GST-π (Figure 2b), GP (Figure 2c) and TS (Figure 2d) in OAW-tax and OAW-dox cells. Topo IIα was decreased in OAW-dox cells but showed the same level in OAW-tax cells compared to the parental cells (Figure 2e). The mRNA expression of α-tubulin (Figure 2g) and β-tubulin (Figure 2h) was not strongly altered, except for the decreased level of α-tubulin in OAW-dox cells. Additionally, we found that the mRNA expression of H3 seems to be lower in the resistant cells than in sensitive OAW-42 cells (Figure 2i) but this is not significant. Equal loading of mRNA was confirmed by β-actin hybridization (Figure 2k). The analysis of the mRNA expression of the proto-oncogenes c-*jun* (Figure 2j) and c-*fos* (Figure 3) showed that OAW-tax and OAW-dox cells have only an increased level of c-*jun*. This difference was only marginal. PKCα and PKCβ2 were not detectable in OAW-dox cells (Figure 3). OAW-tax cells showed an increased level of PKCβ2 (Figure 3), whereas no difference was visible for PKCα. These data suggests that both resistant cell lines developed severel protection mechanisms.

For further investigation of whether the elevated P-170 mRNA and the cross-resistance phenotype of OAW-tax cells and OAW-dox cells is involved in MDR we evaluated the influence of the P-170 blocker

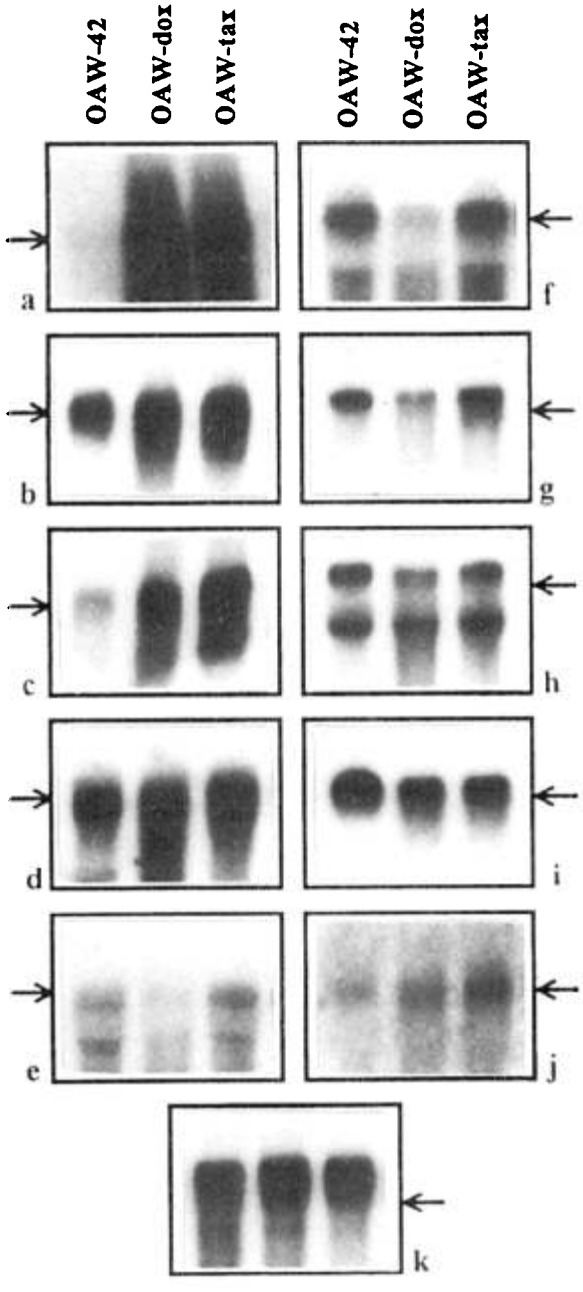

Figure 2. Northern blot analysis of OAW-42 (sensitive), OAW-dox and OAW-tax (both resistant). The membranes were hybridized with probes for P-170 (a), GST-π (b), GP (c), TS (d), topo IIα (e), MRP (f), α-tubulin (g), β-tubulin (h), H3 (i), c-*jun* (j) and β-actin as control (k).

verapamil. As shown in Figure 4, we were able to modulate the resistance of OAW-tax and OAW-dox cells to a higher sensitivity. This suggests that the multidrug resistant phenotype was indeed realized.

Additionally, we analyzed OAW-tax and OAW-dox cells for amplification of the P-170, GST-π, GP and TS genes by Southern blot analysis and found a 2-

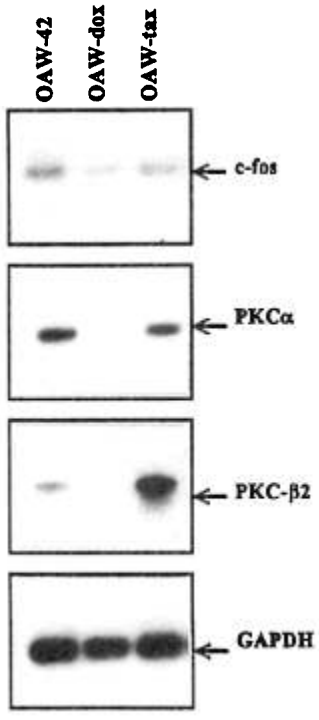

Figure 3. PCR-amplification of c-*fos*, PKCα, PKCβ2 and glycerin aldehyde phosphate dehydrogenase (GAPDH) as control in OAW-42, OAW-dox and OAW-tax cell lines.

fold amplification of the P-170 gene (Figure 5), whereas the genes of GP (Figure 6), GST-π and TS (data not shown) were not amplified.

The analysis of the resistant sublines derived from SK-OV-3 cells showed only a decreased level of H3 and TS mRNA (data not shown). The mRNA expression of the other resistant-related proteins showed no significant differences (data not shown). In order to confirm a possible involvement of the MDR phenotype in SK-OV-dox cells and SK-OV-tax cells we tried to circumvent resistance by verapamil and found no significant influence.

Discussion

In the present study we established taxol-, doxorubicin- and cisplatin-resistant human ovarian carcinoma sublines derived from the parental OAW-42 and SK-OV-3 cell lines. The sublines of OAW-42 cells developed high resistance, the SK-OV-3 sublines developed only low resistance. For the strong resistant OAW-dox cells and OAW-tax cells a typical cross-resistance profile was demonstrated which is explained by MDR (cross-resistance between doxorubicin, taxol, vincristine, colchicine). In contrast, the

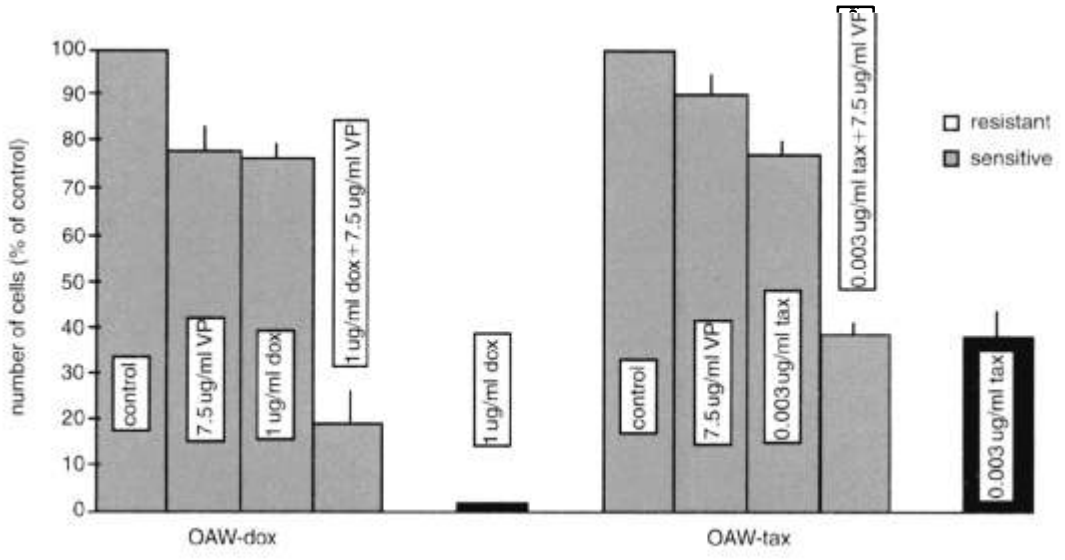

Figure 4. Reversal of resistance in OAW-dox and OAW-tax cells by verapamil. Effects on cell number after addition of doxorubicin (dox) (1 μg/ml) or doxorubicin (equal concentration) plus verapamil (VP) (7.5 μg/ml) to OAW-dox cells. OAW-tax cells were treated with taxol (tax) (0.003 μg/ml) or with taxol (equal concentration) plus verapamil (7.5 μg/ml).

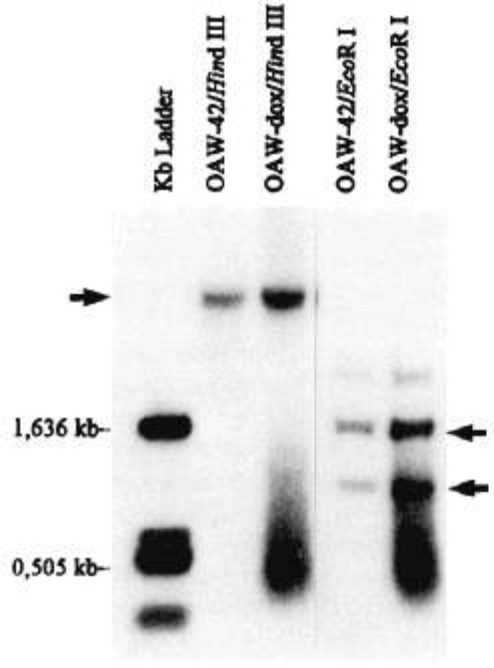

Figure 5. Southern blot analysis of *mdr-1* gene (coding for P-170) of OAW-42 and OAW-dox cells. DNA was restricted with *Hin*dIII and *Eco*RI. The arrows show the restriction fragments.

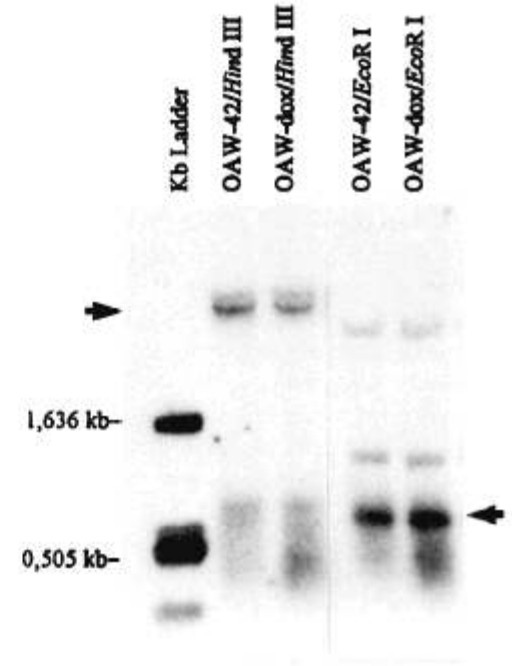

Figure 6. Southern blot analysis of GP of OAW-42 and OAW-dox cells. DNA was restricted with *Hin*dIII and *Eco*RI. The arrows show the restriction fragments.

lower resistant cells derived from SK-OV-3 showed a cross-resistance profile different to the strong resistant OAW-dox and OAW-tax sublines. The low resistant SK-OV-dox cells developed a cross-resistance to cisplatin and a collateral sensitivity to taxol. SK-OV-tax cells showed a resistance to cisplatin as well, but neither of the resistant sublines revealed a resistance to drugs involved in the MDR phenotype.

In order to discover the mechanisms responsible

for the drug resistance, the mRNA expression of several resistance proteins and resistance-related proteins was investigated. In the strong resistant OAW-dox and OAW-tax sublines an overexpression of P-170, GST-π, TS, GP and c-*jun* were found at the mRNA level. Additionally, OAW-tax cells expressed a higher level of PKCβ. This is in agreement with earlier studies in which we demonstrated significant correlations between doxorubicin resistance *in vitro* and the overexpression of P-170, GST-π and TS in human lung tumors.[34,35] The typical multidrug resistance phenotype is mediated by an overexpression of the ATP-dependent efflux pump P-170. Several studies showed an overexpression of P-170 in cell lines resistant to doxorubicin[35,36,43] or taxol[37] which showed a comparable cross-resistance phenotype. In order to prove the significance of P-170 for the resistance in these cell lines we treated the cells with the calcium canal blocker verapamil. In fact, the resistance was modulated nearly to the level of the sensitive cells. In contrast MRP, which has been reported to be involved in MDR,[3] was not important for this resistance.

In OAW-tax and OAW-dox sublines the MDR phenotype was associated with an overexpression of several other resistance-related proteins. In addition to the elevated expression of P-170, we found a co-expression of GST-π and GP. A corresponding co-expression was obtained by Chao *et al.*[38] who found in human colon carcinoma cells resistant to doxorubicin an overexpression of P-170 mRNA and an increased GST-π activity. Other groups confirmed the involvement of elevated GP and GST-π activity in the development of doxorubicin resistance.[39,40] This may be due to quinone-containing drugs like doxorubicin, which are easily reduced and, therefore, develop hydrogen or lipid peroxides. GST and GP are able to protect cells by their glutathione-dependent peroxidase activity against oxygen radical toxicity.[4] Elevated GST-π and GP expression is additionally involved in cisplatin resistance.[41] This may be the reason why cross-resistance to cisplatin in OAW-dox cells was found. In contrast to OAW-dox cells, the taxol-resistant cell line (OAW-tax) showed no cisplatin resistance although they expressed an enhanced level of GST-π and GP mRNA.

Our study demonstrated an elevated mRNA level of TS both in OAW-dox and OAW-tax cells. Normally TS catalyzes the synthesis of dTMP and is therefore involved in DNA replication. Chu *et al.*[42] showed that exposure of human breast and colon cancer cells to doxorubicin led to an increased expression of TS. It might be possible that DNA destruction induced by the intercalation of doxorubicin with the DNA results in an enhanced level of TS. Further investigations are necessary to explain the higher expression of TS in OAW-tax cells.

Furthermore, we analyzed topo IIα and found a significant decreased mRNA level of topo IIα in OAW-dox cells and an elevated level of P-170 mRNA. This is in correlation with data of renal cell carcinomas in which we found inter-relationships between increased expression of P-170 and GST-π, and down-regulation of topo II.[43] Because topo II is a proliferation-dependent enzyme,[44] we suggest that the decreased topo IIα level was associated with the lower proliferation activity in OAW-dox cells. Non-proliferating cells have been shown to be more resistant to cytotoxic agents than proliferating cells. This is supported by earlier experiments indicating that tumors with a low rate of proliferation often show no response to chemotherapy.[45,46] In contrast, the topo IIα level was unaltered in OAW-tax cells.

A second mechanism of taxol-resistance which differs from the typical multidrug resistance phenotype has been described. This kind of resistance is often associated with mutations or altered expression of α-tubulin or β-tubulin.[47,48] We therefore investigated the mRNA expression of α-tubulin and β-tubulin but could not find differences between sensitive and resistant cells (OAW-dox, OAW-tax). We observed a reduced level of the proliferation marker H3 in OAW-dox and OAW-tax cells. This was in agreement with the low proliferation rate found in these resistant cell lines.

According to the co-expression of several resistance mechanisms in OAW-dox and OAW-tax we investigated regulation mechanisms responsible for this co-expression. It is supposed that the expression of P-170, GST-π, TS and topo II is regulated at the transcriptional level by an AP-1 binding domain responding to homo- or heterodimeres of c-Fos and c-Jun oncoproteins.[9] The regulation of c-Fos and c-Jun involves phosphorylation by members of PKC.[14] Several investigators have confirmed an involvement of these regulatory proteins in drug resistance. Bhushan *et al.*[12] selected human and murine cells by increasing concentrations to doxorubicin and showed an elevation in the mRNA level of c-*fos*. Yu *et al.*[16] demonstrated in *mdr-1* transfected MCF-7 breast carcinomas an increased resistance to doxorubicin and vinblastine when these cells were additionally transfected with PKCα. We therefore investigated the expression of c-*jun*, c-*fos*, PKCα and PKCβ2. We found an increased mRNA level of c-*jun* in OAW-dox and OAW-tax cells but not an increased level of c-*fos*. Interestingly, OAW-dox cells

showed no expression of PKCα and PKCβ2, whereas in OAW-tax cells the expression of PKCβ2 was significantly elevated. The results show that there might be an influence of c-*jun* on the co-regulation of the described resistance proteins in OAW-dox and OAW-tax cells. These results are in concordance with data from other authors. Slapak *et al.*[49] reported that selection of cells with doxorubicin results in an overexpression of c-*jun* and *mdr-1*. Ibrado *et al.*[50] demonstrated that co-treatment with PKC stimulator phorbol 12,13-dibutyrate significantly reduced taxol-induced DNA fragmentation and apoptosis. It might be possible that higher mRNA expression of PKCβ2 in OAW-tax cells leads to resistance by reducing the cell damage.

To test if gene amplification is responsible for elevated mRNA expression of P-170, GST-π, TS and GP, we analyzed OAW-tax and OAW-dox cells, and found a 2-fold amplification of the P-170 gene in OAW-dox cells, whereas the other genes were not amplified in either of the cell lines. Chao *et al.*[51] also found gene amplification of P-170 in doxorubicin-resistant human colon carcinoma cell lines.

In contrast to the strong resistant OAW-dox and OAW-tax cells we found only decreased mRNA levels of TS and H3 and a minor proliferation rate in the low resistant SK-OV-tax and SK-OV-dox in comparison to the sensitive SK-OV-3 cells (data not shown).

Conclusion

We demonstrated that the strong resistant sublines of OAW-42 developed resistance by co-expression of several resistance-related proteins (P-170, GST-π, GP and TS) and by overexpression of c-*jun*. In contrast, the sublines of SK-OV-3 developed resistance by decreased expression of the proliferation-dependent proteins H3 and TS. Therefore, we suggest that the resistant sublines developed different mechanisms to protect them against exposure to different drugs.

References

1. Einhorn N. Treatment of ovarian cancer: the state of the art. *Med Oncol Tumor Pharmacother* 1993; **10**: 139–42.
2. Bradley G, Juranca PF, Ling V. Mechanism of multidrug resistance. *Biochim Biophys Acta* 1988, **948**: 87–128.
3. Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SP, Deeley RG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 1994; **54**: 357–61.
4. Doroshow JH, Akman S, Chu F-F, Esworthy S. Role of the glutathione–glutathione peroxidase cycle in the cytotoxicity of the anticancer quinones. *Pharmacol Ther* 1990; **47**: 359–70.
5. Dusre L, Mimnaugh EG, Myers CE, Sinha BK. Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. *Cancer Res* 1989; **49**: 511–5.
6. Akman SA, Forrest G, Chu FF, Esworthy RS, Doroshow JH. Antioxidant and xenobiotic-metabolizing enzyme gene expression in doxorubicin-resistant MCF-7 breast cancer cells. *Cancer Res* 1990; **50**: 1397–402.
7. Hofmann GA, Mattern MR. Topoisomerase II in multiple drug resistance. *Cytotechnology* 1993; **12**: 137–54.
8. Scanlon KJ, Kashani-Sabet M. Elevated expression of thymidylate synthase cycle genes in cisplatin-resistant human ovarian carcinoma A2780 cells. *Proc Natl Acad Sci. USA* 1988; **85**: 650–3.
9. Scanlon KJ, Kashani-Sabet M, Tone T, Funato T. Cisplatin resistance in human cancers. *Pharmacol Ther* 1991; **52**: 385–406.
10. Ritke MK, Bergoltz VV, Allan WP, Yalowich JC. Increased c-*jun*/AP-1 levels in etoposide-resistant human leukemia K562 cells. *Biochem Pharmacol* 1994; **48**: 525–33.
11. Yamazaki H, Schneider E, Meyers CE, Sinha BK. Oncogene overexpression and *de novo* drug-resistance in human prostate cancer cells. *Biochim Biophys Acta* 1994; **1226**: 89–96.
12. Bhushan A, Abramson R, Chiu J-F, Tritton TR. Expression of c-*fos* in human and murine multidrug-resistant cells. *Mol Pharmacol* 1992; **42**: 69–74.
13. Funato T, Yoshida E, Jiao L, Tone T, Kashani-Sabet M, Scanlon KJ. The utility of an anti-*fos* ribozyme in reversing cisplatin resistance in human carcinomas. *Adv Enzyme Regul* 1992; **32**: 195–209.
14. Angel P, Karin M. The role of JUN, FOS and AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1991; **1072**: 129–57.
15. Chaudhary PM, Roninson IB. Activation of MDR1 (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncol Res* 1992; **4**: 281-90.
16. Yu G, Ahmad S, Aquino A, Fairchild CR. Transfection with protein kinase Cα confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein. *Cancer Commun* 1991; **3**: 181–9.
17. Carpenter G, Zendegui JG. Epidermal growth factor, its receptor, and related proteins. *Exp Cell Res* 1986; **164**: 1–10.
18. Christen RD, Hom DK, Porter DC, *et al.* Epidermal growth factor receptor regulates the in vitro sensitivity of human ovarian carcinoma cells to cisplatin. *J Clin Invest* 1990; **86**: 1632–40.
19. Rowinsky EK, Onetto N, Canetta RM, Arbuck SG. Taxol: the first of the taxanes, an important new class of antitumor agents. *Semin Oncol* 1992; **19**: 646–62.
20. Van der Zee AGJ, Hollema HH, De Bruijn HWA, *et al.* Cell biological markers of drug resistance in ovarian carcinoma. *Gynecol Oncol* 1995; **58**: 165–78.
21. Minotti AM, Barlow SB, Cabral F. Resistance to antimitotic drugs in Chinese hamster ovary cells correlates with changes in the level of polymerized tubulin. *J Biol Chem* 1991; **266**: 3987–94.

22. Cowan NJ, Dobner PR, Fuchs EU, Clereland DW. Expression of human alpha-tubulin genes: interspecies conservation of 3′ untranslated regions. *Mol Cell Biol* 1983; **3**: 1738–45.
23. Hall JL, Dudley L, Dobner PR, Lewis SA, Cowan NJ. Identification of two human beta-tubulin isotypes. *Mol Cell Biol* 1983; **3**: 854–62.
24. Cole SP, Bhardwaj G, Gerlach JH, *et al.* Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; **258**: 1650–4.
25. Merlino GT, Ishii S, Whang-Peng J, *et al.* Structure and localisation of genes encoding aberrant and normal epidermal growth factor receptor RNAs from A431 human carcinoma cells. *Mol Cell Biol* 1985; **5**: 1722–34.
26. Kano T, Sakai M, Muramatsu M. Structure and expression of a human class pi glutathione S-transferase messenger RNA. *Cancer Res* 1987; **47**: 5626–30.
27. Chen CJ, Chin JE, Ueda K, *et al.* Internal duplication and homology with bacterial transport proteins in the *mdr*1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 1986; **47**: 381–9.
28. Tsai-Pflugfelder M, Liu LFF, Liu AA, *et al.* Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome 17q21-22. *Proc Natl Sci USA* 1988; **85**: 7177–81.
29. Ponte P, Ng SY, Engel J, Gunning P, Kedes L. Evolutionary conservation in the untranslated regions of actin mRNA: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res* 1984; **12**: 1687–96.
30. Van Straaten F, Mueller R, Curran T, Van Beveren C, Verma JM. Complete nucleotide sequence of a human c-oncogene: deduced amino acid sequence of the human c-*fos* protein. *Proc Natl Acad Sci USA* 1983; **80**: 3183–7.
31. Finkenzeller G, Marme D, Hug H. Sequence of humen protein kinase C alpha. Nucleic Acids Res 1990; **18**: 2183–3.
32. Kubo K, Ohno S, Suzuki K. Primary structures of human protein kinase C beta I and beta II differ only in their C-terminal sequences. *FEBS Lett* 1987; **223**: 138–42.
33. Tokunaga K, Nakamura Y, Sakata K, *et al.* Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res* 1987; **47**: 5616–9.
34. Volm M, Mattern J, Samsel B. Overexpression of P-glycoprotein and glutathione S-transferase π in resistant non-small cell lung carcinoma of smokers. *Br J Cancer* 1989; **64**: 700–4.
35. Volm M, Mattern J. Elevated expression of thymidylate synthase in doxorubicin resistant human non-small cell lung carcinomas. *Anticancer Res* 1992; **12**: 2293–6.
36. Volm M, Efferth T, Günther A, Lathan B. Detection of murine S180 cells expressing a multidrug resistance phenotype using different *in vitro* test systems and monoclonal antibodys. *Arzneim-Forsch/Drug Res* 1987; **37**: 862–7.
37. Dumontet C, Duran GE, Steger A, Beketic-Oreskovic L, Sikic BI. Resistance mechanismus in human sarcoma mutants derived by single-step exposure to Paclitaxel (Taxol). *Cancer Res* 1996; **56**: 1091–7.
38. Chao CC-K, Huang Y-T, Ma CM, Chou W-Y, Lin-Chao S. Overexpression of glutathione S-transferase and elevation of thiol pools in a multidrug resistant human colon cancer cell line. *Mol Pharmacol* 1992; **41**: 69–75.
39. Samuels BL, Murray JL, Cohen MB, *et al.* Increased glutathione peroxidase activity in a human sarcoma cell line with inherent doxorubicin resistance. *Cancer Res* 1991; **51**: 521–7.
40. Benchekroun MN, Pourquier P, Schott B, Robert J. Doxorubicin-induced lipid peroxidation and glutathione peroxidase activity in tumor cell lines selected for resistance to doxorubicin. *Eur J Biochem* 1993; **211**: 141–6.
41. Wang Y, Teicher BA, Shea TC, *et al.* Cross-resistance and glutathione S-transferase-π levels among four human melanoma cell lines selected for alkylating agent resistance. *Cancer Res* 1989; **49**: 6185–92.
42. Chu E, Drake JC, Koeller DM, *et al.* Induction of thymidylate synthase associated with multidrug resistance in human breast and colon cancer cell lines. *Mol Pharmacol* 1991; **39**: 136–43.
43. Volm M, Kästel M, Mattern J, Efferth T. Expression of resistance factors (P-glycoprotein, glutathione S-transferase π, and topoisomerase II) and their interrelationship to proto-oncogene products in renal cell carcinomas. *Cancer* 1993; **71**: 3981–7.
44. Sullivan DM, Glisson BS, Hodges PK, Smallwood-Kentro S, Ross WE. Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry* 1986; **25**: 2248–56.
45. Volm M, Koomägi R, Mattern J. Prognostic significance of proliferating cell nuclear antigen (PCNA) in adenocarcinoma of the lung. *Int J Oncol* 1995; **6**: 359–62.
46. Stammler G, Sauerbrey A, Volm M. Messenger RNA expression of resistance factors and their correlation to the proliferative activity in childhood acute lymphoblasic leukemia. *Cancer Letters* 1995; **89**: 129–35.
47. Ohta S, Nishio K, Kubota N, *et al.* Characterization of a taxol-resistant human small-cell lung cancer cell line. *Jpn J Cancer Res* 1994; **85**: 290–7.
48. Ohta S, Nishio K, Kubo S, *et al.* Characterization of a vindesine-resistant human small-cell lung cancer cell line. *Br J Cancer* 1993; **68**: 74–9.
49. Slapac CA, Rubin EH, Kufe DW. Constitutive c-*jun* overexpression in doxorubicin and vincristine selected, multidrug-resistant U937 cells. *Proc Am Ass Cancer Res* 1993; **34**: A1874.
50. Ibrado AM, Ponnathpur V, Reed J, *et al.* Protein kinase C and tyrosine kinase activities affect taxol induced apoptosis in human leukemic cells. *Proc Am Ass Cancer Res* 1994; **35**: A1868.
51. Chao CK, Ma CM, Cheng P-W, Lin-Chao S. Increased *mdr* gene expression and decreased drug accumulation in a human colonic cancer cell line resistant to hydrophobic drugs. *Biochem Biophys Res Commun* 1990; **172**: 842–9.

(Received 12 November 1996; accepted 26 November 1996)