Cancer Chemother Pharmacol (2001) 48: 1–8
DOI 10.1007/s002800100281

ORIGINAL ARTICLE

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Expression and integrity of DNA topoisomerase II isoforms does not explain generic drug resistance in malignant mesothelioma

Received: 14 September 2000 / Accepted: 5 February 2001 / Published online: 16 May 2001

Abstract *Purpose*: Malignant mesothelioma is a tumour that is highly resistant to a number of different chemotherapy agents, yet the mechanisms by which resistance occurs are poorly understood. The pattern of resistance is consistent with disruption of topoisomerase function or expression. Coupled with this, we have previously noted a common serological reaction to the β isoform of topoisomerase II, suggesting that it may be aberrantly expressed in patients with mesothelioma. *Methods*: We assessed the expression of topoisomerase II isoforms in sections of primary tumour. We tested a panel of five mesothelioma cell lines for sensitivity to the known topoisomerase-targeting drugs, doxorubicin and etoposide. We sequenced expressed segments of the topoisomerase genes from these cell lines that have previously been associated with drug resistance. We then investigated other potential resistance mechanisms. *Results*: We found that the β isoform of topoisomerase II was more frequently expressed in primary tumours. Only one of the five cell lines was highly resistant to etoposide and this cell line was found to have a point mutation in the gene for topoisomerase IIα. Protein levels of topoisomerase IIα and β did not correlate with sensitivity to either doxorubicin nor to etoposide. Semiquantitative analysis suggested that there was marked variation in the levels of mRNA expression of MRP, γ-GCS and MDR1. None of these findings could be associated with resistance to chemotherapy. *Conclusion*: We conclude that mutations in topoisomerase IIα can be associated with extreme resistance of mesothelioma to etoposide. The generic drug resistance of this tumour requires further investigation.

Keywords Mesothelioma · Topoisomerase II · Etoposide · Doxorubicin · Drug resistance

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Introduction

Malignant mesothelioma is a solid tumour of the pleura or peritoneum. It is generally regarded as resistant to treatment, including most chemotherapeutic agents and is uniformly fatal [9]. Thus, clinical studies of drug treatment have generally resulted in low response rates and no improvement in survival [25]. This intrinsic resistance is a significant barrier to the management of mesothelioma. Despite the increasing incidence of the disease, the mechanisms by which drug resistance occurs are little studied. Etoposide and doxorubicin are two chemotherapy agents that have activity in a number of solid tumours. They are important components of treatment regimens for breast, testicular and small-cell lung cancer. Studies of these drugs in mesothelioma have found little activity with response rates of 0–14% for doxorubicin [18, 33] and 4–7% with intravenous and oral etoposide [30]. These findings are consistent with those of studies demonstrating a high degree of resistance to these agents in mesothelioma cell lines in vitro [1].

Etoposide and doxorubicin are topoisomerase II inhibitors. The topoisomerases are a ubiquitous group of enzymes that are involved in the modification of DNA topology during replication and transcription. Topoisomerase II is present in humans as two isoforms, α and β, which appear to be independently regulated. The α form has a central role in cell division and appears to be essential for the viability of all cells, whereas viable cell lines which do not express the β form have been described [8]. These studies showed that topoisomerase II$\beta^{-/-}$ cells are more resistant to a number of drugs than $\beta^{+/+}$ cells, indicating that the β form is an important

target for these compounds. Quantitative and qualitative changes to both isoforms have been described in different studies [2, 7, 11, 26]. Etoposide and doxorubicin are also substrates for the drug efflux pumps multidrug resistance protein 1 (MDR1) and MDR-associated protein (MRP). Expression of these transmembrane proteins is associated with resistance to a number of different chemotherapy agents. A further mechanism of resistance to doxorubicin is by conjugation to glutathione, the production of which is regulated by the enzyme γ-glutamylcysteine synthetase (γ-GCS). Overexpression of γ-GCS has previously been found to correlate with doxorubicin sensitivity in mesothelioma cell lines [24].

Interestingly, we have recently shown that the majority ($>90\%$) of patients with mesothelioma have serum Ig antibodies to topoisomerase IIβ [29], suggesting that the β isoform is commonly either overexpressed or mutated in these patients. The current study was therefore established to define the level of expression of topoisomerase II in primary tumours and in five mesothelioma cell lines. We then went on to test if there was a relationship between the sensitivity of the cell lines to topoisomerase II poisons and the integrity of the topoisomerases or the expression of other drug resistance markers.

Materials and methods

Cell lines

Five human mesothelioma cell lines, established in our laboratory from the pleural fluid of patients with the disease, were used. These cell lines, designated JU77, LO68, NO36, ONE 58 and STY51, have been previously described [19]. None of the patients from whom the cell lines were derived had been exposed to chemotherapeutic agents. Two further cell lines were purchased from American Type Culture Collection (ATCC, Manassas, Va.) for use as controls: HT29 human colon adenocarcinoma (ATCC HTB38) and A549 human lung carcinoma (ATCC CCL185). These cell lines are part of the National Cancer Institute drug screening process and have well-described patterns of drug sensitivity. Cells were maintained in RPMI-1640 containing 5% fetal calf serum (Life Technologies, Mulgrave, Australia), 100 IU/ml penicillin (CSL, Perth, Australia), 50 µg/ml gentamicin (David Bull Laboratories, Melbourne, Australia), 5×10^{-5} *M* 2-mercaptoethanol and 20 m*M* HEPES buffer in a water-saturated atmosphere of 5% CO_2 in air at 37°C. This growth medium is referred to here as RF-5. All cells were checked at 3-monthly intervals for infection with *Mycoplasma* and remained negative to testing.

Chemotherapeutic agents

Doxorubicin and etoposide were obtained from Delta West (Perth, Australia). Both drugs were stored as advised by the manufacturer and dilutions of each drug were made on the day of use in RF-5 from these stocks.

Chemosensitivity assay

Drug sensitivities for each cell line were determined by use of the MTT assay modified from the method of Mossman [22]. Briefly, cells were seeded in 100 µl RF-5 at appropriate concentrations to maintain logarithmic growth for the duration of the assay. The cells were incubated for 24 h and then dilutions of drug were added to each well in triplicate to make a total volume of 200 µl. Plates were then incubated for 72 h, then 50 µl MTT (2 mg/ml; Sigma-Aldrich, Castle Hill, Australia) was added and a further 4-h incubation carried out. Plates were centrifuged at 2000 rpm for 5 min and the medium carefully aspirated from each well. Dimethyl sulphoxide (100 µl; Sigma-Aldrich) was added and each plate placed on a shaker for 30 min. Optical densities were determined using a SpectraMax 250 plate reader (Molecular Devices, Amersham Pharmacia Biotech, Castle Hill, Australia) at 570 nm. The IC_{50} was calculated as the concentration of drug that resulted in a 50% reduction in the optical density of treated cells compared to untreated cells. The standard deviation of triplicate values for each experiment was always less than 10%.

Polymerase chain reaction for MDR, MRP and γ-GCS

The expression of MDR1, MRP and γ-GCS were determined by polymerase chain reaction (PCR). Confluent cells were harvested, washed in PBS and lysed in RNAzol B (GeneWorks, Rundle Mall, Australia). RNA was extracted as recommended by the manufacturer and cDNA synthesized using Superscript reverse transcriptase (Life Technologies) according to the protocol supplied with the reagent.

Oligonucleotides were purchased from GeneWorks. The Genbank accession number and the position of the alignments relative to the canonical sequence are indicated in parentheses.

- γ-GCSf, GCTGCATCTCCCTTTTACCGAG (HUMGCSH, 932–954)
- γ-GCSr, TGGCAACTGTCATTAGTTCTCCAG (HUMGCSH, 1791–1814)
- MDR1f, CCCATCATTGCAATAGCAGG (HUMMDR1, 3020–3039)
- MDR1r, GTTCAAACTTCTGCTCCTGA (HUMMDR1, 3156–3175)
- MRPf, TGAAGGACTTCGTGTCAGCC (HUMMRPX, 4419–4438)
- MRPr, GTCCATGATGGTGTTGAGCC (HUMMRPX, 4655–4675)

Thermocycling conditions consisted of denaturation at 94°C for 3 min, followed by 30 cycles of 95°C for 45 s, 60°C for 45 s and 72°C for 2 min. DNA polymerization was catalysed using *Thermus aquaticus* polymerase (GeneWorks) in the manufacturer's buffer with the addition of 1.5 m*M* $MgCl_2$ and 1.0 µ*M* primer oligonucleotides. Concurrent PCR for the GAPDH transcript was performed to ensure the integrity of each of the cDNA samples.

PCR for topoisomerase isoforms

PCR was performed using cDNA prepared as above. Oligonucleotides were synthesized with additional restriction endonuclease sites at the 5′ end to facilitate cloning of the products. The Genbank accession number and the position of the alignments relative to the canonical sequence are indicated in parentheses.

- Topoisomerase IIAf, AAGGAATTCCAAGAGCTTTGGATCAAC (HUMTOP2, 1190–1208)
- Topoisomerase IIAr, TCCGGATCCTTTACAATGGGAGTGATA (HUMTOP2, 1754–1774)
- Topoisomerase IIBf, TTCGAATTCGAAAACATGACTCTGCAG (HSTOPIIB, 1183–1200)
- Topoisomerase IIBr, TCCGGATCCCCTGCTTATTTTTGCTTG (HSTOPIIB, 1786–1804)

These primers were designed to amplify the region most commonly described to contain mutations associated with resistance to topoisomerase II poisons [26]. For thermocycling the conditions were essentially as described above with the exception that for topoisomerase IIα the $MgCl_2$ concentration was 1.5 m*M* and for topoisomerase IIβ 2.5 m*M*.

The PCR products were extracted from agarose gels using a QIA Quick Spin Gel Extraction kit (Qiagen, Clifton Hill, Australia) as recommended by the manufacturer. The products were cloned into the pBluescript vector (Stratagene, La Jolla, Calif.) using compatible overhangs generated by restriction enzyme digestion. Plasmids containing the appropriate inserts were selected for further purification and sequencing reactions were carried out using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). The sequence was acquired using an Applied Biosystems 373A with a BigDye filter wheel upgrade.

Western blotting

Equal numbers of cells were lysed in 2× sodium dodecyl sulphate (SDS) gel loading buffer (50 m*M* Tris-HCl, 2% SDS, 0.1% bromophenol blue, 50% glycerol, 2-mercaptoethanol) and loaded onto 7.5% polyacrylamide gels. The gels were run at 100 V for approximately 1 h and the separated proteins were transferred to nitrocellulose membranes. The membranes were exposed to primary antibody at 4°C overnight. For topoisomerase IIα the antibody was used at a final concentration of 2.5 μg/ml (Calbiochem-Novabiochem, Croydon, Australia). For topoisomerase IIβ, a 1/100 dilution of a culture supernatant from clone 3H10 was used (a gift from Dr Kikuchi, Nagoya, Japan). 3H10 is an IgG2a monoclonal which recognizes residues 1583–1601 of the human topoisomerase IIβ sequence. The membranes were then exposed to secondary antibody conjugated to horseradish peroxidase and the specific bands visualized using the ECL protocol (Amersham Pharmacia Biotech) and subsequent exposure to XAR-5 Kodak film. Analysis of films was performed using ImageQuant software.

Immunohistochemistry

Tumour samples embedded in paraffin were sectioned at 5 μm and mounted on Superfrost slides. Slides were dewaxed in xylene and exposed to ethanol/3% hydrogen peroxide to remove any endogenous peroxidases. The slides were then blocked for nonspecific binding by incubation with Tris-buffered saline (TBS) containing 20% horse serum. Primary antibody (as described for the Western blot) was applied to the sections at 4°C overnight. Biotinylated secondary antibody and then streptavidin peroxidase were applied sequentially to the section, each for 45 min at room temperature. The slides were developed using filtered diaminobenzidine (DAB) solution (6 mg DAB/10 ml TBS plus 10 μl hydrogen peroxide) and then exposed to $CuSO_4$ solution (2 g $CuSO_4$ plus 3.2 g NaCl in 400 ml distilled water). At least two different areas of each tumour sample were examined and the percentage of positively stained cells in a single high power field was calculated for each area. Only cells that were clearly positive, in which the nucleus was overstained brown, were counted. An overall percentage of positively stained cells for the tumour was then determined and the sample assigned to one of seven groups as detailed in Table 1.

Table 1 Grading of immunohistochemistry samples

Percentage of positively stained cells	Grade
< 1	1
1–4.9	2
5–9.9	3
10–24.9	4
25–49.9	5
50–74.9	6
75–90	7
> 90	8

Results

Topoisomerase IIα and β expression in primary tumours

Immunohistochemistry was performed on tissue samples taken from 18 unselected patients with mesothelioma. Histological examination confirmed the diagnosis in all of these samples. Salient characteristics of the patients are shown in Table 2. The age of this patient group ranged from 49 to 82 years with a mean age of 64.8 years. The survival ranged from 1 to 18 months with a median survival of 10 months. The group included two women among the 18 patients. The median survival of patients who were treated in the cytokine trials was not different from that of other patients at 10.5 months. This was despite this group having a younger mean age (59 years) than those who were not in trials (67 years).

Ki67 staining was used as an independent measure of cellular proliferation within the samples. Only 14 of the biopsies were stained for Ki67 due to insufficient clinical material being available in the remainder. In general, the numbers of cells that stained positive for topoisomerase IIα and Ki67 in any one specimen were low, with 16/18 and 14/14, respectively, having less than 10% of cells positive (Table 2). With topoisomerase IIβ, positive cell staining was much more common with 15/17 specimens having more than 25% positive and 9/17 having more than 50% positive (Fig. 1, Table 2). There was some correlation between staining for topoisomerase IIα and Ki67. Specimens that were grade 2 for topoisomerase IIα (ten) were all also either grade 1 (four) or grade 2 (six) for Ki67. All three specimens that were grade 3 for topoisomerase IIα were also grade 3 for Ki67. There was no correlation between staining for topoisomerase IIβ and either topoisomerase IIα or Ki67 and no correlation was found between survival and any of the immunohistochemistry data. Overall topoisomerase IIβ was highly expressed in the mesothelioma specimens and topoisomerase IIα had low levels of expression consistent with proliferation as determined by Ki67 staining.

Chemosensitivity of cell lines

The five mesothelioma cell lines showed only minor variations in their sensitivity to doxorubicin. There was a threefold difference between the least sensitive cell line (NO36, 0.69 m*M*) and the most sensitive cell line (STY51, 0.22 m*M*; Fig. 2). In contrast, the maximum difference in sensitivity to etoposide was greater than 30-fold and with this drug the positions were reversed. The least sensitive cell line was STY51 (24.1 m*M*) and the most sensitive was NO36 (0.75 m*M*). In general, the cells were more sensitive to doxorubicin on a molar basis but there was no correlation between sensitivities to the two drugs.

Table 2 Summary of patients' features and immunohistochemistry. Immunohistochemistry grades are as defined in Table 1. (*GM-CSF* granulocyte-macrophage colony stimulating factor given as intralesional/intrapleural infusion [6], *IFNα* interferon alpha given as intralesional infusion [3], *VV-IL2* vaccinia IL2 gene therapy construct given intralesionally [23])

Patient (sex)	Age at diagnosis (years)	Survival from diagnosis (months)	Asbestos exposure	Treatment	Topo IIα	Ki67	Topo IIβ
1 (M)	54	3	Yes	Nil	2	1	5
2 (M)	50	12	Yes	GM-CSF	3	3	7
3 (M)	74	5	Yes	Nil	2	2	
4 (M)[a,b]	70	Unknown	Probable	Nil	4	1	6
5 (M)	62	15	Yes	Nil	2	2	6
6 (F)[c]	55	7	Yes	GM-CSF	3	3	5
7 (M)	49	18	No	Nil	2	1	5
8 (M)[b]	74	Unknown	No	Unknown	2	2	8
9 (M)[d]	80	4	Possible	Nil	5		6
10 (M)[c]	60	10	Yes	IFNα	2		5
11 (M)	56	5	Yes	Nil	2		7
12 (F)	69	11	No	Nil	2		6
13 (M)	55	6	Yes	GM-CSF	2	2	5
14 (M)	73	11	Yes	IFNα	2	2	3
15 (M)	73	10	Yes	Nil	2	1	6
16 (M)	61	11	Yes	VV-IL2	2	1	5
17 (M)[e]	82	1	Yes	Nil	2	2	8
18 (M)	69	16	Yes	Nil	3	3	4

[a]Could not recall any specific asbestos exposure but had worked in shipyards in England in the 1960s
[b]Lost to follow-up
[c]Known metastatic disease at the time of death (the presence of metastases is uncommon in mesothelioma): patient no. 6 had pancreatic and kidney metastases, patient no. 10 had liver and bone marrow metastases
[d]Had casual exposure to asbestos during work as a carpenter but was not aware of any high level exposure
[e]Short survival due to the diagnosis being made during operation for a chronic empyema. The patient succumbed to postoperative complications

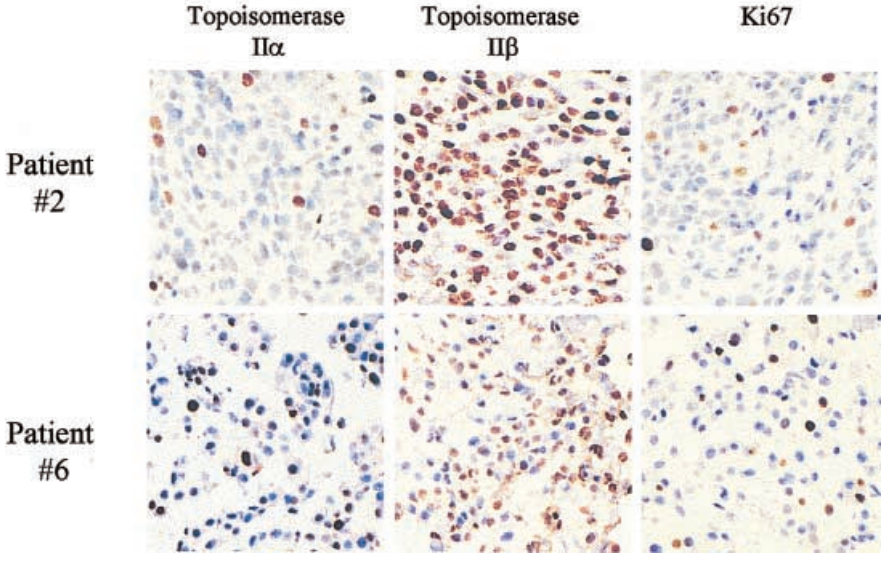

Fig. 1 Photomicrographs of stained sections from tumour biopsies from two randomly selected patients with mesothelioma. Cells that demonstrate positive staining for each antibody are brown. The grade of staining in each of these sections is provided in Table 2 (all ×100)

Topoisomerase II expression by the cell lines

The expression of topoisomerase II protein was quantitated for each cell line by Western blotting (Fig. 3, Table 3). The values in Table 3 were obtained from two independent blots that were performed on different occasions. There were marked differences in the levels of topoisomerase IIα expressed between cell lines. NO36 expressed approximately 20-fold more topoisomerase IIα than JU77. The expression of topoisomerase IIα correlated with sensitivity to doxorubicin ($r^2 = 0.86$) but not with sensitivity to etoposide. There was much less variation (2.5-fold) in the expression of topoisomerase IIβ (Fig. 2, Table 3) but there was a weak inverse correlation between topoisomerase IIβ levels and resistance to doxorubicin ($r^2 = 0.52$).

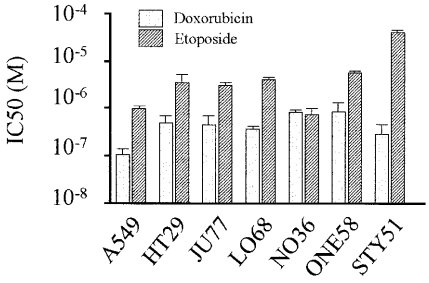

Fig. 2 IC_{50} of topoisomerase II poisons against five mesothelioma cell lines and two control cell lines (A549 and HT29) as determined by the MTT assay. Note that the *y*-axis is a log scale. Values are the means ± SE from three experiments

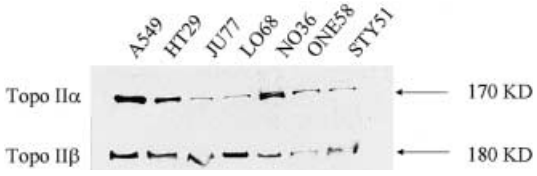

Fig. 3 Expression of topoisomerase IIα and topoisomerase IIβ in a representative Western blot. Gels were stained with Coomassie blue to confirm even loading. Quantitation of data, collated from three independent blots is presented in Table 3

Table 3 Expression of topoisomerase isoforms and drug resistance markers in various mesothelioma cell lines

	JU77	LO68	NO36	ONE58	STY51
Topoisomerase IIα	5	12	100	34	14
Topoisomerase IIβ	86	100	74	40	71
MDR1	100	20	48	5	0
MRP	100	95	92	97	8
γ-GCS	46	100	44	44	1

Relative expression of topoisomerase IIα and topoisomerase IIβ protein was determined by western blotting. Relative expression of MDR1, MRP and γ-GCS mRNA were determined by semi-quantitative RT-PCR (see Figs. 4–6). In each series, the cell line with the highest level of expression was arbitrarily assigned the value of 100. Data are means from at least two independent experiments

Topoisomerase II cDNA sequencing

An expressed segment from each of the genes was selected for further study based on previous reports of mutational hot spots associated with drug resistance. Essentially, we examined residues 384–579 of the 1531 residues of topoisomerase IIα and residues 395–601 of the 1621 residues of topoisomerase IIβ. Each segment was amplified from cDNA by PCR, cloned into a plasmid vector and at least three independent inserts were sequenced in an attempt to identify both alleles. Differences from the canonical sequences were only recorded if their presence was confirmed in an independent sequencing run. STY51 exhibited a point mutation in topoisomerase IIα at nucleotide 1559 which would be expected to result in the substitution of threonine for

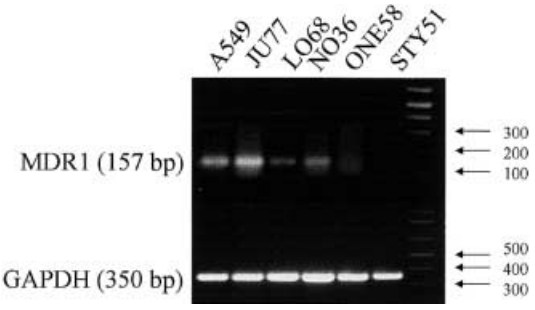

Fig. 4 RT-PCR for MDR1 showing GAPDH controls. The MDR1 amplicon includes positions 3020 to 3175. The complementarity determining sequence is included by positions 425–4265. Quantitation of collated data is presented in Table 3

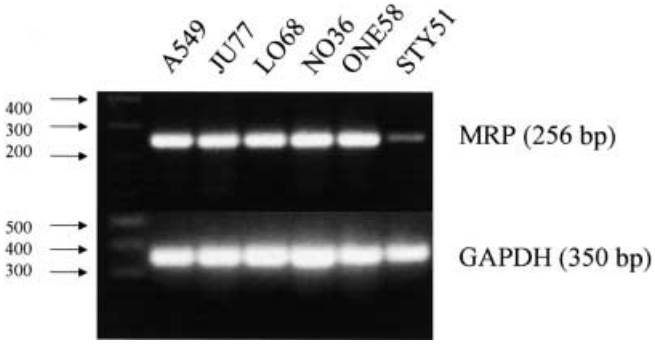

Fig. 5 RT-PCR for MRP showing GAPDH controls. The MRP amplicon includes positions 4418 to 4675. The complementarity determining sequence is included by positions 197–4789. Quantitation of collated data is presented in Table 3

arginine at position 508 of the translated protein (N508 T). This mutation occurred in two inserts and the wild-type sequence was recorded in a third isolate, suggesting that the mutation was monoallelic and that both alleles were expressed by this cell line.

Several mutations were found in the expressed segment of topoisomerase IIβ. When compared to the canonical sequence, three point mutations were found in LO68 one of which would be expected to be silent (S411P, L532L and T556P). Two identical mutations were found in NO36 (L532L, T556P). The silent mutation recurred and an independent change was observed in ONE58 (L532L, S563P). The common variations at residues 532 and 556 are therefore likely to represent single nucleotide polymorphisms. Thus, only ONE58 and LO68 carry mutations in this segment of their topoisomerase IIβ gene.

Expression of MDR1, MRP and γ-GCS

Three of the five mesothelioma cell lines expressed levels of MDR1 similar to the control cell line, A549. ONE58 and STY51 showed only weak expression (Fig. 4, Table 3). All of the mesothelioma cell lines expressed MRP,

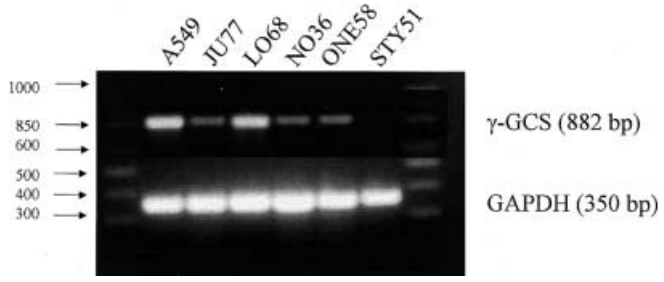

Fig. 6 RT-PCR for γ-GCS showing GAPDH controls. The γ-GCS amplicon includes positions 932 to 1814. The complementarity determining sequence is included by positions 93–2006. Quantitation of collated data is presented in Table 3

albeit at slightly lower levels for STY51 (Fig. 5, Table 3). All of the mesothelioma cell lines expressed γ-GCS, but there was a greater than 100-fold difference in the level of expression of γ-GCS between the highest (LO68) and the lowest expressing cell line (STY51) (Fig. 6, Table 3).

Discussion

The elucidation of the mechanisms by which drug-resistant tumours such as mesothelioma are able to avoid damage by chemotherapeutic agents is an important step towards maximizing the potential of currently available drugs. Although drugs that target topoisomerase II, such as those used in this study, have proved to be useful in the management of a wide array of cancers they have little activity against mesothelioma. Doxorubicin has generated responses in up to 15% of patients whereas etoposide is effective in less than 7% of patients [30]. In the current study, five mesothelioma cell lines were examined for a number of different factors that are known to be associated with resistance to these drugs.

Firstly, we focused on the topoisomerases. Sequence analysis was carried out on segments of topoisomerase IIα and β genes that have previously been demonstrated to contain mutations in drug-resistant cell lines [26]. One cell line in this study (STY51) showed extreme resistance to etoposide; it had a point mutation in topoisomerase IIα encoding a change from asparagine to threonine at amino acid 508. This mutation has not previously been described, but it is possible that the change in the enzyme is sufficient to alter the sensitivity of the cell line to etoposide. It is also possible that there are further mutations in areas of topoisomerase IIα and β outside the area we examined. Protein expression of topoisomerase IIα was not different in this cell line from two of the other more sensitive cell lines but this cell line did demonstrate lower functional topoisomerase II activity in decatenation assays (unpublished observations). This is consistent with the mutation being associated with a change in function rather than a change in level of expression.

A total of seven differences from the canonical sequence were found in topoisomerase IIβ in three different mesothelioma cell lines. Two of these (L532L and T556P) recurred in different cell lines and are therefore most likely to be polymorphisms. In the cell lines with unique mutations, one (ONE58, S563P) was relatively resistant to etoposide, the other (STY51, N508 T) was highly resistant to etoposide. The third unique mutation (LO68, S411P) did not relate to a difference in sensitivity to either drug. While few mutations have been found in these cell lines it must be borne in mind that only a small portion of the gene was examined and that these cell lines are chemonaive. Previous studies of resistance have usually been in cells that have been exposed to increasing concentrations of a single agent over a long period. Such selection pressures are more likely to induce specific mutations. Other less well defined mechanisms of alterations to topoisomerase II have been proposed which reduce the action of topoisomerase II poisons. A reduction in phosphorylation of topoisomerase II has been described in a leukaemia cell line resistant to etoposide. This cell line did not contain mutations of topoisomerase II [28].

Mutations that have previously been described in drug resistance to topoisomerase II poisons range from single base pair changes [5, 26] to gross rearrangements of the gene [21]. The majority of these studies have been in cell lines that had been made drug resistant by progressive exposure to a chemotherapy agent. For example, Kubo et al. found mutations in topoisomerase IIα in 1 of 13 patients with small-cell lung cancer that had been treated with etoposide [17]. The patients from whom the mesothelioma cell lines in the current study were derived had not been treated with chemotherapy. So this study was seeking spontaneous mutations associated with drug resistance that might offer a selective growth advantage to the cells.

The mesothelioma cell lines expressed varying levels of both topoisomerase IIα and β by Western blotting. NO36, which showed the highest expression of topoisomerase IIα, was the most sensitive to etoposide but, overall, topoisomerase IIα protein expression had the best correlation with resistance to doxorubicin. This finding is the opposite of what would be expected if the levels of topoisomerase IIα determined the sensitivity of the cell to doxorubicin. The inverse correlation between topoisomerase IIβ levels and resistance to doxorubicin, while weak, is consistent with this isoform being the target for this agent. The lack of correlation between expression of either topoisomerase II isoform and sensitivity to etoposide is surprising, given that etoposide is a more "pure" topoisomerase II poison. The finding does have precedent in the literature. A positive correlation between sensitivity to doxorubicin and topoisomerase IIα expression, coupled with an inverse correlation with topoisomerase IIβ has been reported for lymphoblastic leukaemia [2]. In that study also no correlation with etoposide sensitivity and either topoisomerase isoform was found.

Both of the drugs examined are susceptible to the action of MDR1 and this efflux pump has been shown to be present in the majority of mesotheliomas by immu-

nohistochemistry [27] but not in cell lines [12, 24]. Two of the cell lines express low levels of MDR1. These two were no more sensitive to doxorubicin and etoposide than the others, making it unlikely that MDR1 is the major determinant of sensitivity in these cells.

Resistance to doxorubicin in mesothelioma cells has been found to be related to coordinated overexpression of MRP and γ-GCS [24]. Conjugation of doxorubicin to glutathione is a necessary prerequisite for export of the drug through the MRP pump. All of the cell lines in the current study expressed MRP and γ-GCS. The lowest expresser for both was STY51 and this was indeed the most sensitive to doxorubicin. Overall, however, the relative expression of γ-GCS did not correlate with sensitivity to doxorubicin. This might be explained, at least in part, by differential expression of glutathione-S-transferase, another component of the glutathione conjugation pathway. This enzyme has been shown to be both commonly expressed in mesothelioma [32] and to be associated with resistance to a related drug, epirubicin [15]. Future work will address this possibility.

The level of expression of MRP in the mesothelioma cell lines was similar to that of the control cell line A549. The high level of expression of MRP in this and a similar study [24] suggests that this pump may play a role in the general resistance of mesothelioma to chemotherapy. The most notable difference in the IC_{50} values for the mesothelioma cell lines treated with topoisomerase II poisons was that of STY51 and etoposide. While STY51 was the most sensitive of the mesothelioma cell lines to the action of doxorubicin, it was eight times more resistant to etoposide than the mesothelioma cell line with the next highest IC_{50}. STY51 does not express MDR1 and it shows the lowest expression of both MRP and γ-GCS of the mesothelioma cell lines. It does show increased levels of p53 protein but the level of expression is similar to that of STY51 and NO36 and less than that of ONE58 [4]. None of these factors explains the relative resistance of this cell line to etoposide. This resistance appears to be specific for etoposide. When tested against a range of chemotherapy agents, STY51 is actually one of the more sensitive mesothelioma cell lines [20]. Therefore, one might expect that the resistance of the cell line to etoposide could be best explained by some difference in the drug target, topoisomerase II. Any other nonspecific resistance feature not examined in this study, such as an alteration in apoptotic factors, would most likely result in more generalized resistance to chemotherapy. Decreased uptake of etoposide has been described in non-small-cell lung cancers that were relatively resistant to this agent [14] and this could also potentially be a contributing factor.

The immunohistochemistry demonstrated that topoisomerase IIβ was expressed in the majority of mesothelioma tumour cells. In contrast, only small numbers of cells stained positively for topoisomerase IIα. These findings are in general agreement with other published studies. Positive staining has been noted in more than 90% of cells in breast cancer [31] and in more than 50% of cells in a variety of other tumours [34]. It can be inferred from the high frequency of expression of topoisomerase IIβ that this is not the limiting factor in determining the resistance of mesothelioma to topoisomerase II poisons. The overexpression of this isoform may be associated with a humoral immune response in these patients.

The lower levels of positive staining for topoisomerase IIα and the similarities with Ki67 staining have been demonstrated in other tumours (reviewed in reference 10). For lung cancers, only 5/27 non-small-cell tumours had positive staining for topoisomerase IIα in more than 5% of cells. More rapidly growing small-cell lung cancers had more than 5% topoisomerase IIα-positive cells in 28 of 29 specimens [16]. Interestingly, of ten small-cell cancers in this study that were treated with etoposide, seven had decreased levels of topoisomerase IIα when rebiopsied following treatment. In another study, the level of topoisomerase IIα expression by immunohistochemistry in breast cancer has been found not to correlate with response to epirubicin, a drug related to doxorubicin [13].

In summary, topoisomerase IIα and topoisomerase IIβ were both expressed in biopsies of mesothelioma. In general, topoisomerase IIβ was expressed more commonly than topoisomerase IIα. The frequency of cells staining with topoisomerase IIα strongly correlated with a marker of cellular proliferation, Ki67. We found a correlation between expression of topoisomerase IIα and resistance to doxorubicin and a weak correlation between expression of topoisomerase IIβ and sensitivity to doxorubicin. The sensitivity of mesothelioma cells to etoposide did not correlate with the expression of either isoform of topoisomerase II. Only two mesothelioma cell lines expressed mutations in a critical region of topoisomerase IIα. One of these mutations (N508 T) may be associated with extreme resistance to etoposide.

Acknowledgement B.R.M. was supported by the National Health and Medical Research Council of Australia.

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