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STUDY OF MOLECULAR MARKERS OF RESISTANCE TO *m*-AMSA IN A HUMAN BREAST CANCER CELL LINE

DECREASE OF TOPOISOMERASE II AND INCREASE OF BOTH TOPOISOMERASE I AND ACIDIC GLUTATHIONE *S* TRANSFERASE

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Abstract—Resistance to 0.8 μM 4′-(9-acridinylamino)methanesulphon-*m*-anisidide (*m*-AMSA) was induced by stepwise increases of drug concentration in the human tumor cell line CALc18 originating from a breast adenocarcinoma. The resistant cell line CALc18/AMSA exhibited a resistance index of 10 and a cross-resistance to other topoisomerase II inhibitors. A 3-fold decrease in the levels of topoisomerase II decatenating activity was found in CALc18/AMSA cells. By contrast, topoisomerase I activity was increased by about 3-fold in resistant cells. Interestingly this line was hypersensitive to camptothecin, a specific inhibitor of topoisomerase I. Restriction endonuclease patterns of the topoisomerase I and topoisomerase II loci were found to be identical in CALc18/AMSA and CALc18 with no evidence of gene amplification and rearrangements. Alkaline elution of *m*-AMSA-treated cells showed that DNA single strand breaks and DNA–protein crosslinks were decreased in CALc18/AMSA. The DNA lesions also obtained in *m*-AMSA-treated nuclei indicated that no drug uptake modification occurred in both cells. Moreover, the *in vitro m*-AMSA-induced DNA cleavage per unit of decatenating activity and the inhibitory effects of antitumoral drugs on decatenation were not found to be different with topoisomerase II from sensitive or resistant cells. However the specific cleavage induced by *m*-AMSA/per mg of crude protein from resistant cells was 2 to 3 times decreased. Multidrug resistance gene transcripts were not detected while levels of acidic glutathione *S* transferase mRNA were found to be 8 to 10-fold greater in resistant than in sensitive cell line with no amplification of the gene. In conclusion, the diminution of topoisomerase II activity and the increase of both topoisomerase I and acidic glutathione *S* transferase transcripts could contribute to the resistant phenotype of these breast cancer cells.

In breast cancer as well as in many other types of human cancers, drug resistance constitutes a major obstacle to patient recovery. Resistance process is complex, involving several mechanisms which likely differ with the tissue origin of tumour. Recent studies using cell model systems resistant to chemically unrelated drugs have led to the description of several mechanisms of action to explain the resistance phenomenon. A transmembrane P170 kDa glycoprotein encoded by multidrug resistance (MDR1)‡ gene was shown to control drug efflux and thus intracellular accumulation of drugs [1, 2]. MDR1 gene amplification and overexpression is correlated with the level of drug resistance expressed by multidrug resistance cell lines. MDR overexpression was also detected in a variety of normal human tissues and malignancies [3–5] and it was reported that drug treatment of primary tumour could play an important role in the selection of these types of resistant tumour cells [6, 7]. An isoenzyme of glutathione *S* transferase (GSTπ) belonging to a complex group of detoxifying enzymes was recently reported to be involved in the resistance process and suggested to act in concert with P-glycoprotein. Indeed this enzyme was found to be overexpressed in the breast cancer cell line MCF7 resistant to DXR (MCF7/DXR), a cell line also exhibiting an amplified and overexpressed MDR1 gene [8]. Other studies performed in tumour cell lines also appear to attribute to topoisomerases, a role in resistance. Topoisomerase I (Topo I) and topoisomerase II (Topo II) are essential enzymes involved in the interconversion of topological forms of single- and double-stranded DNA. Topo II was shown to be a major component of mitotic chromosome scaffold and nuclear matrix [9, 10] and was found to be implicated in cell proliferation [11–13]. Topo II was considered as a potent target of antineoplastic agents

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‡ Abbreviations: Topo I, topoisomerase I; Topo II, topoisomerase II; GSTπ, acidic glutathione *S* transferase; MDR1, multidrug resistance; kDNA, kinetoplast DNA; HIFCS, heat inactivated fetal calf serum; DMEM, Dulbecco's minimum essential medium; *m*-AMSA, 4′-(9-acridinylamino)-methanesulphon-*m*-anisidide; VM 26, 4′-demethyl epipodophyllotoxin thenylidene-β-D-glucoside; ACT-D, actinomycin D; CPT, camptothecin; DXR, doxorubicin; NHME, 2*N*-methyl-9-hydroxy-ellipticinium; VCR, vincristin; SDS, sodium dodecyl sulphate; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiotreitol; PMSF, phenylmethylsulphonylfluoride; RRI, relative resistance index; IC_{50}, inhibitory drug concentration leading to 50% colonies survival.

including anthracyclines, epipodophyllotoxins and amsacrine while Topo I was specifically inhibited with camptothecin analogs which are also antitumoral agents, but too toxic to be used in human cancer therapy [14–16]. Mammalian tumour cell lines expressing resistance to drugs that act on Topo I [17] or Topo II [18, 19] have been described and shown to be associated with decreased enzyme activities [20]. Recently alterations of Topo I and Topo II genes were observed in murine P388 leukemia cells resistant to camptothecin or amsacrine [21] suggesting that the modifications of topoisomerase activities were related to these alterations. Moreover of clinical relevance, Topo I was found to be increased in human colon cancers suggesting a role of the enzyme in the therapeutical activity of camptothecin [22].

To get a better insight to the mechanism of resistance we have established a human breast cancer cell line resistant to *m*-AMSA (4′-(9-acridinylamino)-methanesulphon-*m*-anisidide) (CALc18/AMSA) and studied several genes (MDR1, GSTπ, Topo I and Topo II) which could be involved in the resistance process.

MATERIALS AND METHODS

Cell culture

CAL18A cell line was recently established from a breast adenocarcinoma [23] and was kindly given by Dr Gioanni at its eighth passage. From subsequent passages, we isolated a cellular clone named CALc18 which exhibited the growth property of the pure cell type CAL18A. CALc18 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose), supplemented with 10% heat inactivated foetal calf serum (HIFCS), in a humidified atmosphere containing 5% CO_2 at 37°. The *m*-AMSA-resistant subline, CALc18/AMSA, was developed by continuous stepwise exposures of parental cells to *m*-AMSA. The highest concentration of *m*-AMSA permitting healthy growth of the bulk of the cells was used. Resistant cells were maintained in presence of 0.8 μM *m*-AMSA. Studies were performed with cells from their 38th passage. The doubling time of CALc18/AMSA was found to be 36 hr as compared to 18 hr for the parental cell line.

Cross-resistance studies

We have determined by clonogenic monolayer assay, the IC_{50} (drug concentration giving rise to 50% of surviving colonies) of various drugs DXR, VCR, NMHE, VM26, ACT-D and CPT. Briefly, lines were treated with drug for 30 min, before trypsinization. Cells (7×10^3) were further cultured in 60 mm Petri dishes for 7 days at 37°. Anchored colonies were fixed by methanol and stained with violet crystal. Colonies of more than 50 cells were scored under an inverted microscope. The percentage of surviving colonies was estimated and plotted as a function of *m*-AMSA concentration in order to determine the IC_{50} value. Results are the mean value of three independent determinations. The relative resistance index (RRI) was estimated by the ratio of IC_{50} in resistant and sensitive cells, respectively.

Tumorigenicity

Ten 8-week-old nude mice were given by subcutaneous route 10^7 cells in their flank. Tumour takes were followed in conventional conditions every week for 2 months.

Preparation of nuclear extracts and assessment of Topo II *and Topo* I *activities*

The method derives from that of Minford *et al.* [24]. Approximately 10^7 cells were scraped in nucleus buffer ($MgCl_2$ 5 mM; Tris–HCl 50 mM, pH 7.5; EDTA 0.5 mM, pH 8; DTT 0.5 mM; β-mercaptoethanol 10 mM; PMSF 1 mM), and centrifuged at 1500 rpm for 10 min, at 4° in a Beckman TJ 6 centrifuge. The cell pellets were resuspended in 0.75 mL nucleus buffer at 4°, and then mixed with an additional 0.25 mL nucleus buffer containing 3.5% Nonidet P40. The cell suspension was gently mixed for 10 min in ice and then centrifuged at 1500 rpm for 20 min at 4°. The nuclei pellets were resuspended in 0.5 mL nucleus buffer containing 0.33 M NaCl (final concentration). The salt extraction was performed by gentle stirring for 20 min in ice. The nuclei were then spun at 15,000 rpm for 20 min at 4°. Topo II activity of these extracts was immediately measured. First, serial dilutions of extracts were reacted with 100 ng of kDNA [25] for 30 min at 37° in 20 μL of decatenation buffer (Tris 20 mM pH 7.5; EDTA 0.5 mM; DTT 0.5 mM, BSA 20 μg/mL; KCl 165 mM; $MgCl_2$ 15 mM; ATP 0.5 mM). The reaction was stopped by 4 μL of 0.1% bromophenol blue, 0.1% SDS, 25% glycerol, and the reaction mixture was electrophoresed through a 2% agarose gel. Topo II decatenation activity was reported in units, one unit of Topo II being the quantity of nuclear extract which decatenates 0.1 μg of kDNA per nucleus or per mg of protein. The protein concentration of the nuclear extract was determined by the Biorad method. Then, the same dilutions of crude extracts were reacted with 200 ng of supercoiled pGFC 5A plasmid for 5 min at 37° in 20 μL of cleavage buffer (Tris 20 mM, pH 7.5; KCl 100 mM; $MgCl_2$ 15 mM; EDTA 0.5 mM; DTT 0.5 mM; BSA 20 μg/mL; ATP 0.5 mM; *m*-AMSA 10 μM). The reaction was stopped by 4 μL of 1% bromophenol blue, 0.1% SDS, 25% glycerol, 8 mg/mL proteinase K, and electrophoresed through a 1% agarose gel. Partially purified Topo II was also used in some experiments. Nuclear extracts were chromatographied on a phospho-cellulose column and eluted by 0.35 M phosphate buffer as previously described [26]. Topo I activity from the 1 M NaCl nuclear extract was determined by relaxation reaction. This reaction was performed with 100 ng of supercoiled plasmid pUC18 in a relaxation buffer containing 20 mM Tris pH 7.5, 0.5 mM DTT, 0.5 mM EDTA, 20 μg/mL BSA, 100 mM KCl. The reaction was stopped as described for Topo II assay and reaction mixture electrophoresed through a 1.2% agarose gel.

Immunoblotting

The Topo II proteins and P-170 kDa glycoprotein were analysed by immunoblotting. The Topo II proteins were obtained as described above. Three

volumes of cold acetone (−20°) were added to the samples for 30 min at −70°. The tubes were then centrifuged 10 min at 10,000 rpm, the acetone supernatants poured off and the tubes inverted to drain. The pellets were dissolved thoroughly in the solution described by Laemmli [27] and boiled for 5 min prior to electrophoresis in a 5–20% gradient SDS–polyacrylamide gel. Transfer of proteins from the gel to nitrocellulose membrane was performed in a Cera-Labo X-Blot cell at 0.8 mA/cm^2 for 1 hr. After transfer, the blot was soaked for 30 min in PBS containing 10% HIFCS and then incubated with Topo II-specific antiserum (a gift from Dr Liu) [28] (1/1000 dilution) in PBS 2% HIFCS for 1 hr at room temperature. Following incubation, the blot was washed four times for 10 min in PBS 2% HIFCS, 0.2% Triton X-100 and incubated with peroxydase labelled antibodies (1/200) in PBS 2% HIFCS for 1 hr at room temperature. The blot was washed four times as before and once in PBS alone. The peroxydase reaction was performed with DAB (3,3′-diaminobenzidine tetrahydrochloride) (5 mg/mL) and 0.03% H_2O_2 in Tris–HCl 0.1 M pH 7.6. Brown coloured bands appear immediately and the reaction was stopped with H_2O.

Membrane proteins were obtained from drug-sensitive (CALc18/p and MCF7/p) and -resistant (CALc18/AMSA and MCF7/DXR) cell lines and separated (50 μg per slot) by electrophoresis in a 7.5% SDS–polyacrylamide gel. Proteins were transferred to a polyvinylidine fluorure membrane (Millipore, St Quentin, France) and incubated with C219 mAb (Centocor, Cis-Oris, Saclay, France) as primary antibody, and, after washing, further incubated with ^{125}I-labelled sheep antimouse immunoglobin. P-170 kDa glycoprotein was then detected by autoradiography.

Isolation of cell nuclei for alkaline elution assays

The procedure for nuclei isolation has been previously described [29]. Cells were scraped in nucleus buffer at 4° (150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 mM DTT, pH 6.4) and centrifuged (2000 rpm for 10 min). Cells were rinsed once in nucleus buffer at 4°, spun down, and resuspended in 1/10 volume cold nucleus buffer. A 9/10 volume of the same buffer containing 0.3% Triton X-100 was added and the suspension was gently rotated for 10 min at 4°. Nuclei were centrifuged (2000 rpm for 10 min at 4°), resuspended in nucleus buffer and incubated at 37° in presence or absence of *m*-AMSA.

Alkaline elution assays

The alkaline elution technique has been previously described in detail by Kohn *et al.* [30].

Radioactive labelling of cells. Cellular DNA was labelled in exponentially growing cells by incubation with [^{14}C]thymidine (0.02 μCi/mL) or [^{3}H]thymidine (0.05 μCi/mL) for about one and a half cell cycles. ^{14}C-Labelled experimental cells (2×10^5) were combined with 2×10^5 ^{3}H-labelled internal standard cells.

DNA single strand breaks (SSB). Internal standard cells (2×10^5) have received 300 rad from a ^{60}Co source and were layered with 2×10^5 ^{14}C-labelled cells on polycarbonate membrane filter, lysed with 2 mL of 25 mM Na^2 EDTA, 2% SDS pH 9.7 and with 0.5 mg/mL proteinase K. Elution was performed with tetrapropylammonium hydroxide, EDTA, 0.1% SDS pH 12.2 using a peristaltic pump to control the flow rate at a speed of 0.03–0.04 mL/min (2 mL/hr). Fractions were collected at 3 hr intervals for 15 hr. Calculations were performed as previously described [31]. Drug induced break frequencies are expressed in rad equivalents.

DNA–protein crosslinks (DPC). DPC were measured by DNA denaturation (pH 12.2). Alkaline elution carried out under non deproteinizing conditions using protein absorbing filters (polyvinyl chloride). Both ^{14}C-labelled experimental cells and ^{3}H-labelled internal standard cells were γ-irradiated on ice and with 3000 rad just prior to elution. DPC frequencies, calculated as described by Ross *et al.* [32], were also expressed in rad equivalents.

DNA and RNA preparations

DNA and total RNA were prepared from exponential growing cells by the guanidinium isothiocyanate–CsCl gradient method [33]. Briefly, cells were ground in liquid nitrogen, then lysed in the guanidinium–isothiocyanate buffer. Lysate was layered onto a 5.7 M CsCl cushion and submitted to a 37,000 rpm centrifugation for 17 hr at 20° (SW55 Rotor Beckman Ultracentrifuge model L5). DNA was collected from the supernatant, dialysed and treated with proteinase K. After deproteinization by phenol–$CHCl_3$, DNA was precipitated by absolute ethanol. DNA preparations (5 μg per well) in solution in appropriate buffers, were incubated with various restriction endonucleases and the digest products analysed by Southern blot hybridization under stringent conditions using human DNA probes.

Total RNA was spun down at the bottom of the centrifuge tube and was collected. After treatment with DNase RNase-free (Sigma Chemical Co., St Louis, MO, U.S.A.), RNA was precipitated by absolute alcohol. Denatured RNA samples (10 μg/well) were fractionated on a formaldehyde 1.2% agarose gel and transferred to a Hybond C extrafilter and analysed by Northern blot hybridization. The quality of the RNA was verified by the integrity of the 28S and 18S ribosomal RNAs coloured by ethidium bromide. Hybridizations were performed in stringent conditions with the appropriate denatured human probes ^{32}P-labelled by nick-translation (about 10^7 cpm). Filters were exposed for various periods of times to Kodak XAR5 films.

Probes

The probes used were: the 1.8 kb *Eco*RI cDNA fragment of the human Topo II gene (clone Top2-ZII), [34]; the 0.7 kb *Eco*RI cDNA fragment of the human Topo I gene (clone $pGEM_4$5A) [35]; Topo I and Topo II probes were generously provided by Dr L. F. Liu (Johns Hopkins, Baltimore); the 1.38 kb *Eco*RI cDNA fragment of the human MDR1 gene (clone $pMDR_1$5A), a gift of Dr M. Gottesman (NCI, Bethesda) [36], the 0.725 kb *Eco*RI cDNA

Table 1. Cytotoxicity of anticancer drugs on human breast cell lines sensitive (CALc18) and resistant to *m*-AMSA (CALc18/AMSA)

Drugs	IC_{50} (μM) CALc18	IC_{50} (μM) CALc18/AMSA	Relative resistance index (RRI)
m-AMSA	1.5	15	10
DXR	0.22	1.8	8
VCR	0.09	0.08	0.9
NMHE	3	9	3
VM26	1.2	12	10
CPT	18	3	0.16
ACT-D	0.1	0.1	1

IC_{50} and resistance index of parental and resistant cell lines for anticancer drugs were evaluated using plating efficiency assay. IC_{50} values represent the mean of three independent experiments.

fragment of the human GSTπ gene, a gift of Dr K. Cowan (NCI, Bethesda) [37]; the 1.15 kb *Pst*I DNA fragment of the actin gene [38].

Quantitation of transcript levels

Quantitation of Topo II, Topo I and GSTπ transcript levels were performed by slot blot hybridization of total RNA prepared from parental and resistant cell lines. Serial 2-fold dilutions were deposited on nitrocellulose filter (Schleicher and Schuell, BA85). The mRNA levels were determined by densitometer scanning of the autoradiographic bands (Chromoscan 3, Joyce Loebl) comparatively to actin mRNA providing a control for the amount of RNA on the filters. MCF7 cell line resistant to Doxorubicin (MCF7/DXR) was used as a positive control for MDR1 mRNA and GSTπ mRNA as previously shown by Batist *et al.* [39] and Cowan *et al.* [40].

RESULTS

Characteristics of the resistant subline CALc18/AMSA

After 10 months of subculture, the subline resistant to 0.8 μM *m*-AMSA called CALc18/AMSA was obtained by treatment of the parental CALc18 cell line with increased concentrations of drug. CALc18/AMSA grew more slowly than CALc18 with doubling times of 36 and 18 hr, respectively. From the IC_{50} values of *m*-AMSA in CALc18 and CALc18/AMSA sublines, a relative resistance index (RRI) was found to be of 10 for resistant cells. As compared to CALc18, CALc18/AMSA cells did not show significant changes of morphology and contact inhibition. Cells were also treated with other antitumoral drugs (Table 1). CALc18/AMSA cells were found to be 10, 8 and 3-fold resistant to VM26, DXR and NMHE, respectively. By contrast CALc18/AMSA subline did not show any significant cross-resistance to ACT-D and VCR. Interestingly the resistant cells were 6-fold hypersensitive to CPT, a specific Topo I inhibitor.

Tumorigenicity

CALc18 and CALc18/AMSA cells (10^7) were injected into female nude mice by subcutaneous route [41]. Resistant as well as sensitive cells form growing cancers in all nude mice without latency period. However, tumour induced by CALc18/AMSA grows more slowly (Fig. 1). Histological examination showed that both tumours were moderately differentiated breast adenocarcinomas.

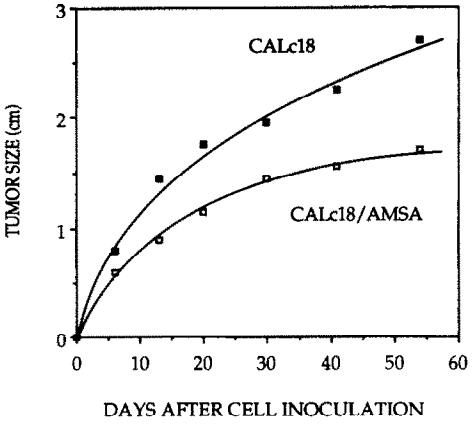

Fig. 1. Tumorigenicity assay. 10^7 cells from parental CALc18 and resistant CALc18/AMSA cells were injected in nude mice (each value corresponds to the mean size of 10 tumours obtained with both cell lines).

Decrease of DNA single strand breaks (SSB) and DNA–protein crosslinks (DPC) in CALc18/AMSA

SSB and DPC frequencies produced by *m*-AMSA in CALc18 and CALc18/AMSA cell lines were compared using alkaline elution technique. In both lines, SSB and DPC formation increased with drug concentration and the frequency of SSB and DPC is significantly higher in sensitive than in resistant cells (Fig. 2). As an example, at 1.5 μM of *m*-AMSA, CALc18/AMSA showed a decrease of DNA lesions of about 3-fold as compared to CALc18. Interestingly the breakage frequencies in both lines were nearly the same when they were treated at their respective IC_{50} value. About 10 times more

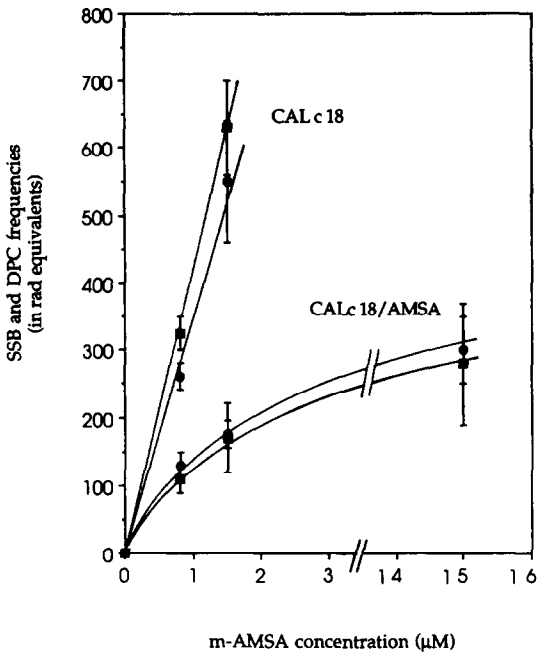

Fig. 2. DNA single strand break (SSB) and DNA-protein crosslink (DPC) frequencies in parental breast cancer cell line (CALc18) and resistant subline (CALc18/AMSA). SSB (■) and DPC (●) frequencies were measured after exposure to different concentrations of *m*-AMSA for 0.5 hr. Values represent the mean of at least two independent experiments.

drug were needed to produce the same cleavage frequencies in CALc18/AMSA than in CALc18. In addition the ratio SSB/DPC is nearly equal to 1 in both resistant and sensitive cell lines, as already described for *m*-AMSA-induced Topo II cleavage [42]. These data suggest a modification of Topo II activity.

To test the hypothesis that a lack of drug accessibility to the nucleus could be involved, *m*-AMSA-induced DNA breakage activity was measured in isolated nuclei. SSB frequencies were found to be similar in isolated nuclei and in whole cells of each line (Table 2). Moreover, as previously found in whole cells, *m*-AMSA induced DNA damage which is 2.5-times lower in isolated nuclei from CALc18/AMSA than in those from CALc18. These results indicate that the drug uptake is not modified in resistant cells.

Decrease of Topo II *and increase of Topo* I *in CALc18/AMSA*

Topo II activity measured using kDNA decatenation assay, was found to be more than 2-fold lower in resistant cells than in sensitive cells (Table 3). The stimulation of the *in vitro* Topo II–DNA cleavage reaction by *m*-AMSA was determined in crude extracts from both cells. When *m*-AMSA (10 μM) was added to the reaction mixture, the circular DNA molecules were linearized (DNA form III) as shown by electrophoresis in agarose gels [43] (data not shown). The relative proportion of linear DNA band was evaluated by densitometric scanning of negative UV photograph (Chromoscan 3, Joyce Loebl). Data showed that, for the same concentration of proteins of the crude extract, the percentage of linear DNA band was about 2.5-fold lower when assay was performed with Topo II from CALc18/AMSA (Fig. 3).

Immunoblotting assay of Topo II performed with specific antibodies [28] showed no difference in the size of Topo II protein from sensitive and resistant cells but exhibited a 2–3-fold reduction of the amount of protein in resistant cells (Fig. 4).

The inhibition by antitumoural drugs (*m*-AMSA, VM26, VP16, DXR and NMHE) of the decatenating activity was studied with the purified Topo II. The assays were performed using enzyme amounts which fully decatenate kDNA namely 0.1 and 0.28 μg of Topo II proteins purified from CALc18 and CALc18/AMSA, respectively. Inhibition of enzyme activities were obtained with the same concentrations of drug (Table 4).

Total RNAs were prepared from the two lines harvested in exponential phase of growth and analysed by Northern blot hybridization using sequentially human Topo II [34] and Topo I [35] probes. The amounts of total RNA loaded in wells for electrophoresis are equivalent as shown by the intensity of 28S and 18S ribosomal RNA coloured with ethidium bromide and by the actin transcript signal (Fig. 5A). Transcript bands with expected

Table 2. DNA single strand break (SSB) induced by *m*-AMSA in human breast cancer cell line sensitive (CALc18) and resistant to *m*-AMSA (CALc18/AMSA)

	SSB frequencies in rad equivalents (±SD)*		
	Sensitive cells	Resistant cells	R
Whole cells	402 ± 93	188 ± 12	2.1
Isolated nuclei	342 ± 43	141 ± 70	2.4

SSB frequencies expressed in rad equivalents were measured on whole cells and isolated nuclei from parental and resistant cells following exposure to 1 μM *m*-AMSA for 30 min. Each value represents the mean of two independent experiments. R is the ratio of frequencies obtained in sensitive cells over those obtained in resistant cells.
*SD, standard deviation.

Table 3. Topo II decatenating and Topo I relaxing activities from human breast cancer cell line sensitive (CALc18) and resistant to *m*-AMSA (CALc18/AMSA)

Cell lines	Topo II activity (decatenation) Units/nucleus (±SD)*	Topo II activity (decatenation) Units/mg of protein (±SD)	Topo I activity (relaxation) Units/nucleus
CALc18	$9.6\ (\pm 0.1) \times 10^{-4}$	$14.7\ (\pm 3.9) \times 10^{3}$	16.0 ± 10^{-3}
CALc18/AMSA	$4.4\ (\pm 0.6) \times 10^{-4}$	$5.5\ (\pm 0.3) \times 10^{3}$	29.3×10^{-3}

Values represent the mean of at least three independent experiments for Topo II assay and two experiments for Topo I assay. *SD, standard deviation.

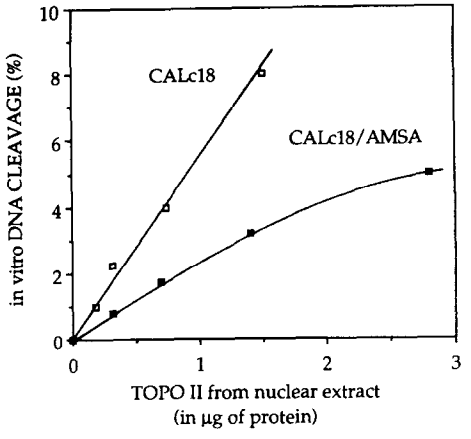

Fig. 3. *In vitro* Topo II DNA cleavage stimulated by *m*-AMSA. Topo II was extracted from human breast cancer cell lines sensitive (CALc18) and resistant to *m*-AMSA (CALc18/AMSA). Electrophoresis in 1.2% agarose gel was performed on circular DNA of form I, after incubation with *m*-AMSA (10 µM) and serial dilutions of 0.33 M NaCl crude extract containing Topo II activity. Circular DNA was cleaved in linear DNA form III. The percentage of linear DNA band was evaluated by scanning (Chromoscan 3, Joyce Loebl) the electrophoretic bands coloured with ethidium bromide.

Table 4. Inhibitory effects of antitumoural drugs on the decatenating activity of Topo II purified from CALc18 and CALc18/AMSA

Drug	Drug concentration (µM) Topo II from CALc18	Drug concentration (µM) Topo II from CALc18/AMSA
m-AMSA	50	50
VM 26	50	50
VP 16	100	100
DXR	5	5
NMHE	5	5

Enzyme assays were performed using the minimal amount of purified Topo II which fully decatenates 100 ng of kDNA in the absence of drug (0.1 and 0.28 µg for Topo II from CALc18 and CALc18/AMSA, respectively). The drug concentrations entirely inhibit the decatenation reaction.

sizes of 6.1 and 4.0 kb for Topo II and Topo I, respectively, were observed in both cell lines. Thus, Topo II and Topo I mRNAs were not grossly altered in the CALc18/AMSA cell line. However the Topo II transcript band was found significantly less intense and the Topo I transcript band was found significantly more intense in CALc18/AMSA as compared to parental cells. Quantitation of the transcript levels

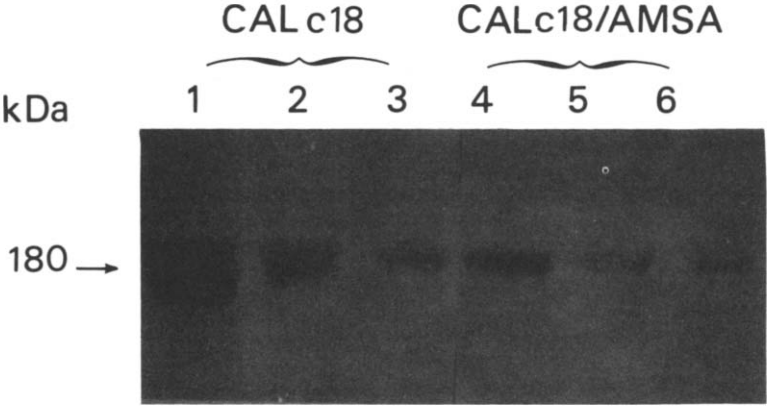

Fig. 4. Western blot analysis. Sensitive and resistant nuclear extracts were prepared as described in Materials and Methods. Serial dilutions of proteins were electrophoresed in a 5–20% gradient SDS–polyacrylamide gel. After transfer to nitrocellulose, Topo II was detected with specific antiserum. 1, 2, 3: 100, 75 and 50 µg of total proteins from CALc18 crude extract, corresponding to 1400, 1050 and 700 units respectively. 4, 5, 6: 100, 75 and 50 µg of total proteins from CALc18/AMSA crude extract, corresponding to 510, 380 and 255 units respectively. Topo II activity was measured by decatenation assay before electrophoresis.

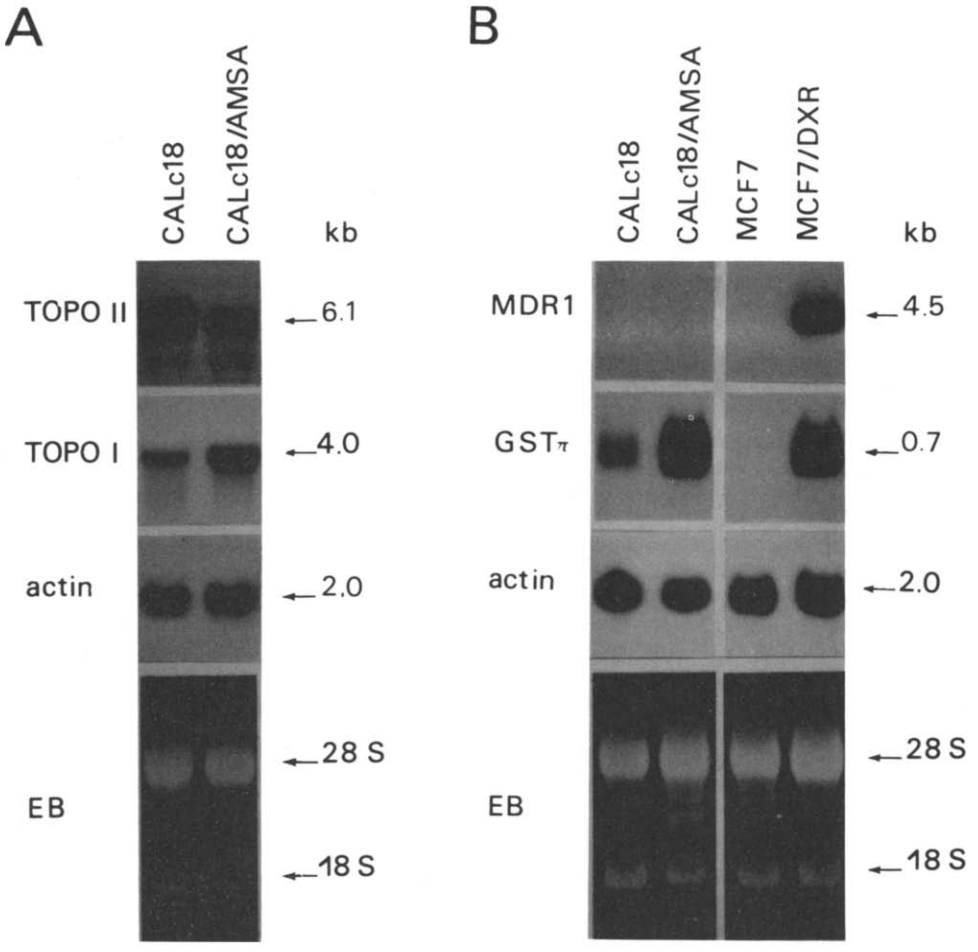

Fig. 5. Northern blot analysis. Total RNAs (10 μg per well) were prepared from cell lines in exponential phase of growth. Hybridizations were performed on two separated blots using sequentially Topo II, Topo I and actin probes (A) and MDR1, GSTπ and actin probes (B), exposure time to Kodak XAR5 film, 48 hr; filters were also exposed to films for long exposure times (15 days) for the detection of eventual low levels of MDR1 mRNA (data not shown). The transferred blots after the agarose gels were coloured with ethidium bromide (EB) are shown (lower panel).

by slot blot hybridization showed that Topo II mRNA level was about 3-fold lower and Topo I mRNA level was found 3-fold greater in the resistant cell line than in the parental cell line (Fig. 6). The Topo I relaxation activity (without ATP and Mg^{2+}) as determined in 1 M NaCl nuclear extracts showed that the specific activity per nucleus was about 2-fold greater in CALc18/AMSA than in CALc18 (Table 3).

Genomic DNAs digested with several restriction enzymes (*Bam*H1, *Eco*RI, *Hind*III, *Xba*I, *Pst*I, *Pvu*II, *Hpa*II, *Msp*I, *Xmn*) were analysed by Southern blot. The expected cleavage patterns were obtained for Topo II (Fig. 7) and Topo I loci (data not shown) revealing no difference between CALc18 and CALc18/AMSA. DNAs from both cell lines were incubated using *Hpa*II, an enzyme which does not cleave methylated cytosines. When probed with Topo I and Topo II cDNAs, a band of high molecular weight was observed with about the same intensity in sensitive as well as in resistant cells suggesting that both Topo I and Topo II genes were equally hypermethylated in these cells (Fig. 8). *Msp*I, which recognizes the same base sequences as *Hpa*II, but which cuts methylated cytosine, provides a complete cleavage pattern (Fig. 8).

No expression of multidrug resistance (MDR1) gene but overexpression of glutathione S *transferase (GSTπ) gene in CALc18/AMSA*

The two genes MDR1 and GSTπ were recently isolated and their products shown to be important markers of drug resistance in experimental cell system as well as in fresh human cancers [1, 2, 6, 7]. Therefore, transcription of these genes was analysed in our breast cancer cell lines. The expected 4.5 kb MDR1 transcript band was not detected in both lines (Fig. 5B). P-170 kDa glycoprotein was also checked by immunoblotting. No signal was obtained neither with CALc18 nor CALc18/AMSA while it was elevated in MCF7/DXR used as positive control (data not shown). In contrast, the GSTπ gene

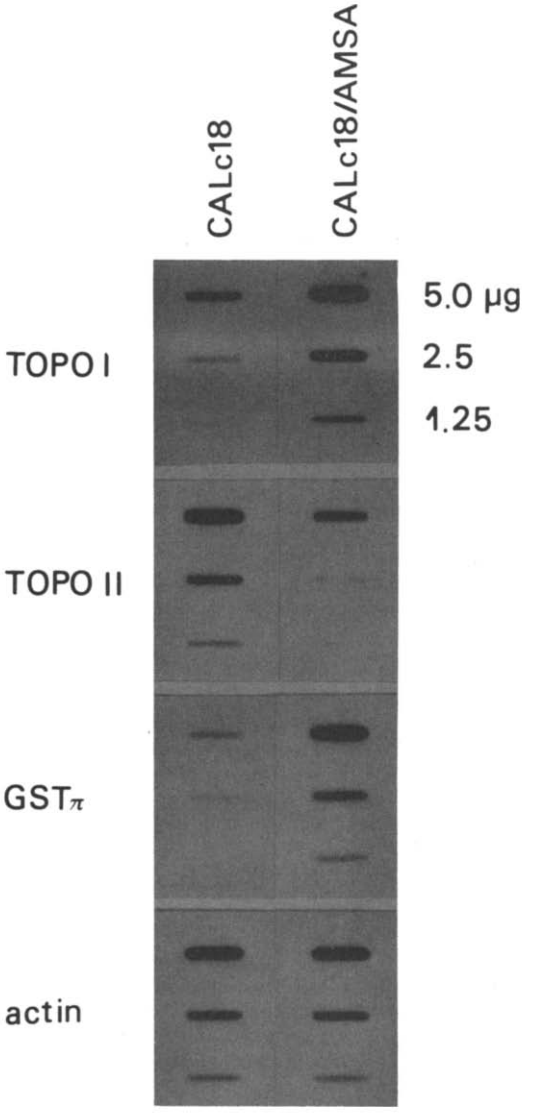

Fig. 6. Slot blot analysis. Total RNAs after 2-fold serial dilutions (5, 2.5 and 1.25 µg per slot) were analysed for Topo I, Topo II and GSTπ transcript levels.

transcript band of 0.7 kb was observed in sensitive and resistant cell lines (Fig. 5B). However, the GSTπ mRNA level detected in CALc18/AMSA was found to be 8–10-fold greater than that detected in sensitive line (Fig. 6). Positive controls for MDR1 and GSTπ gene transcripts were performed using MCF7/DXR, these transcripts were not observed in the parental MCF7 cell line [39].

Cellular DNA preparations were incubated with *Hind*III, *Xba*I, *Hpa*II, *Msp*I, *Eco*RI, *Bam*HI, *Xmn*, *Pst*I and *Pvu*II enzymes and analysed by Southern blot hybridization. No amplification of the GSTπ gene was observed in CALc18/AMSA and the restriction cleavage patterns obtained with the various enzymes were not found to be different in resistant and sensitive cells (data not shown). When DNAs were analysed after incubation with *Hpa*II enzyme, the degree of hypermethylation of GSTπ gene was not different in both lines and was lower than that observed with Topo I and Topo II genes (Fig. 8).

DISCUSSION

We have established a resistant breast cancer cell line (CALc18/AMSA) by progressive adaptation of parental cells to increasing concentrations of *m*-AMSA which could mimic a situation found in the chemical treatment of breast cancers. After 40 passages, cells were resistant to 0.8 µM of *m*-AMSA and the resistant phenotype was found to be stable. CALc18/AMSA presented a multiple drug resistance phenotype (Table 1) as already described in other cell lines [44–47]. The cross-resistance was observed with DXR, NMHE or VM26, compounds known to act on Topo II. The absence of cross resistance to the *Vinca* alkaloid derivative VCR, apparently indicates that MDR1 gene is not involved in the mechanism of resistance of CALc18/AMSA cells, as assessed by the absence of MDR1 gene expression in these resistant cells.

Several cell lines showing a high index of resistance to the Topo II inhibitors were obtained by drug adaptation [19, 45, 46, 48–50] or mutagenesis [51]. In addition, VM26, ellipticines or DXR were shown to develop resistance according to mechanisms of action other than Topo II inhibition. In some cases, resistance to these drugs is multifactorial since alteration of Topo II, MDR1, GSTπ or cytochrome P450 gene expression could be observed in the same resistant cells [40, 52, 53]. By contrast and in agreement with the present study, *m*-AMSA has never been reported to induce MDR1 gene expression [54] or to be cross-resistant to cell lines exhibiting the MDR1 phenotype [55]. These results are in agreement with recent data obtained in other resistant cell lines [56]. Furthermore it was recently reported that two distinct phases were apparent during the *m*-AMSA resistance development in human myeloid leukaemia cell line [57]. A low-degree of resistance (5–7-fold) was developed during the first 14 months followed by a rapid acquisition of high level of resistance (100-fold). Our resistant cell line could be at a first step of low resistance to *m*-AMSA.

The lack of MDR involvement in the resistance phenotype of CALc18/AMSA and its cross resistance to Topo II inhibitors led us to examine alteration of the Topo II functions. A concording 2–3-fold decrease of enzymatic activities, mRNA and protein levels were observed in resistant cells as compared to the parental cells. This quantitative decrease could be related to a lower activity of the gene.

It has also been reported that reduced Topo II levels were related to the natural resistance of various cell lines to epipodophillotoxins [58, 59]. In another study it has been suggested that low levels of Topo II found in leukemic and normal lymphocytes from patients contribute to Adriamycin® resistance [60].

The resistance phenotype was associated with a decrease of tumoral cell proliferation rate when cultured *in vitro* or injected to nude mice. Since Topo II is an essential enzyme for replication and cell division its decrease in CALc18/AMSA could

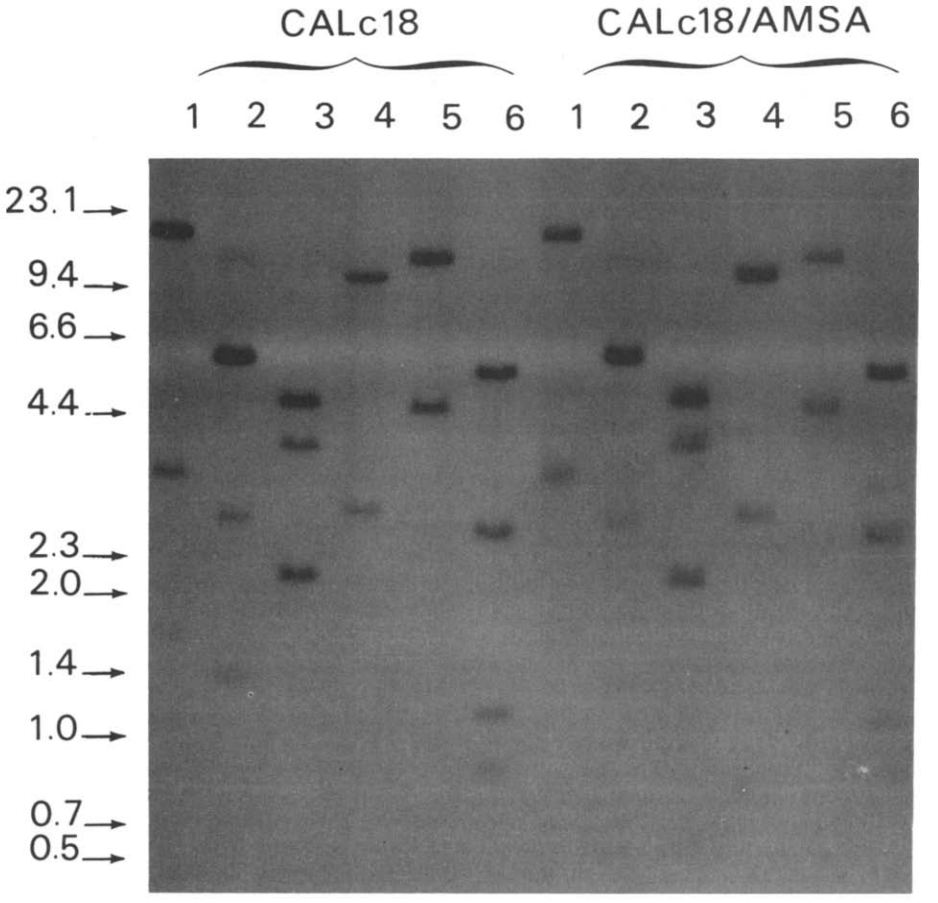

Fig. 7. Analysis of Topo II gene by Southern blot. Genomic DNAs (5 μg per well) from CALc18 and CALc18/AMSA were digested by: (1) *Eco*R1, (2) *Xba*1, (3) *Pst*1, (4) *Pvu*II, (5) *Hind*III, (6) *Msp*I, and hybridized using Topo II probe. Size markers in kb (*Hind*III-digested λ phage DNA and *Hae*III-digested *Trypanosoma cruzi* minicircles).

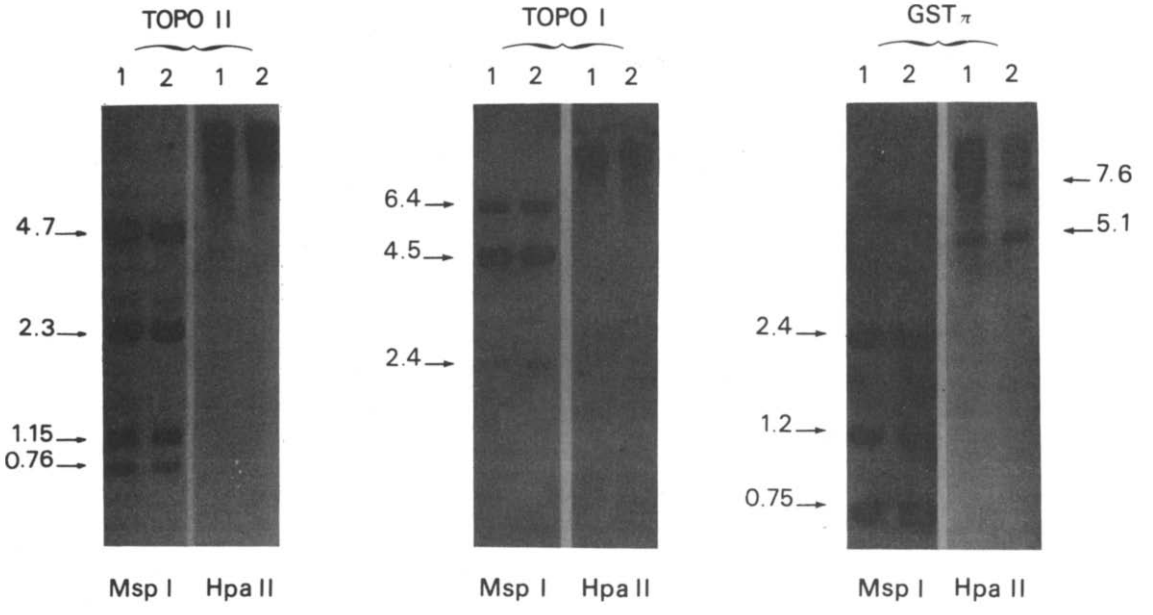

Fig. 8. Analysis of Topo I, Topo II and GSTπ genes by Southern blot. Genomic DNAs (5 μg per well) from CALc18 (lane 1) and CALc18/AMSA (lane 2) were digested with *Msp*I and *Hpa*II and hybridized using sequentially Topo I, Topo II and GSTπ probes.

be related to the diminution of cell proliferation rate. It is possible that *m*-AMSA has selected cells with a lower growth rate [61].

The mechanism of Topo II inhibition by *m*-AMSA is mediated by the stabilization of the enzyme by the drug under a cleavable complex with DNA [62, 63]. We found that in *m*-AMSA-treated sensitive and resistant cells, SSB and DPC frequencies increased proportionally to drug concentration but with a lower rate in CALc18/AMSA. For the same cleavage frequencies, the parental and resistant cells exhibited the same cytotoxicity. About 600 rad equivalents led to 50% of dead cells in the two cell lines. These results are observed when the resistant cells are treated with 10 times more drug than the sensitive cells in good agreement with the resistance index of 10 obtained with *m*-AMSA. This suggests that the ability of the drug to form cleavage complex may explain the mechanism of resistance of the CALc18/AMSA cells. In addition, no modification of the drug accessibility to nuclei occurs in resistant cells, since the ratio SSB in whole cells over SSB in isolated nuclei is the same in sensitive and resistant cells (Table 2). This does not rule out a modification of the plasma membrane permeation for cross resistant drugs. A 2–3-fold increase of SSB frequencies were also observed in nuclei from CALc18/AMSA cells. These results strongly suggest that a modification of the *m*-AMSA-induced Topo II cleavage activity *in vivo* is involved in the mechanism of CALc18/AMSA cell resistance since a 2–3-fold lack of interaction between the drug and the enzyme may lead to a decreased antitumoral activity.

To determine whether qualitative modification of the enzyme occurs in CALc18/AMSA, we have analysed *in vitro* the ability of the extracted enzyme to generate *m*-AMSA-induced DNA cleavage. Our results show that the specific cleavage activity induced by *m*-AMSA per mg of Topo II protein from resistant cells is 2–3 times decreased. Such decrease corresponds to the lower specific Topo II catalytic activity (per mg of protein) measured by the decatenation reaction. These results indicate that *m*-AMSA-induced cleavage is identical in sensitive and resistant cells relatively to Topo II activity. It can be concluded that Topo II from CALc18/AMSA does not exhibit any qualitative modification. Moreover, the inhibition by antitumoral drugs of the purified Topo II decatenation activity was observed with the same drug concentrations (Table 4). These results are different from those of Zwelling *et al.* [50] who found that Topo II from HL60/AMSA is less sensitive to *m*-AMSA as Topo II from parental HL60 line.

Southern blot analysis of genomic DNA from CALc18 and CALc18/AMSA did not reveal any differences in the Topo II restriction cleavage patterns (Fig. 7). Moreover the degree of hypermethylation of Topo II gene was not found to be higher in resistant than in sensitive cells as shown after incubation of the genomic DNAs with *Hpa*II enzyme which does not cleave methylated cytosines in contrast to *Msp*I enzyme which cuts them (Fig. 8). Our data are different from those showing that, in cells resistant to *m*-AMSA, the HL60 line [50] and the murine P388 leukemia [21], Topo II gene exhibits an altered pattern with certain restriction enzymes and a higher dose of hypermethylation.

We have shown a significant increase of Topo I enzyme activity and gene transcript level in concert with a diminution of Topo II in CALc18/AMSA. The Topo I increase was related to a hypersensitivity of CALc18/AMSA cells to camptothecin, a specific Topo I inhibitory drug (Table 1). A moderate increase of Topo I activity was described in cell lines resistant to etoposide [56] or *m*-AMSA [21]. In our resistant cells, the decrease of Topo II activity could be partially compensated by an increase of Topo I activity as shown in yeast for certain function [64, 65]. No evidence of Topo I gene rearrangement could be detected and the degree of Topo I hypermethylation was found to be similar in CALc18 and CALc18/AMSA. These results differ from those of Tan *et al.* [21]. The clinical relevance of increased Topo I level in resistant cell may have a clinical incidence depending on the drug used as this was recently reported in human colon adenocarcinomas [22]. Surprisingly no cross resistance was observed with the Topo II inhibitory drug ACT-D which was recently shown to also inhibit Topo I [66, 67]. The lack of cross resistance would result from the balance between Topo I and Topo II levels, so that ACT-D should have the same toxic effect in both cell lines.

It is well established that estrogen receptor (ER)-positive breast cancers are of better prognosis than ER-negative cancers. The interesting observation that MCF7/DXR cell line became ER-negative and GSTπ-positive prompted Moscow *et al.* [68] to analyse these two genes in breast cancers. Data showed an inverse correlation between the expression of both genes suggesting a clinical interest to test GSTπ in breast cancers. In the present study, we show that CALc18/AMSA cells which were ER-negative exhibited a 8–10-fold increase of GSTπ transcript level without amplification and rearrangement of the gene. The GSTπ gene was moderately methylated as compared to Topo I and Topo II genes which disclosed a high degree of methylation. These data are in favour of a role of GSTπ gene in the resistant process as this was recently shown in other cell models resistant to alkylating agents [69]. In conclusion our data show that CALc18/AMSA cell line provides a suitable model to further study of resistance parameters but also to study the genes involved in breast cancer cell growth and tumorigenesis.

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