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# A HUMAN SMALL CELL LUNG CARCINOMA CELL LINE, RESISTANT TO 4′-(9-ACRIDINYLAMINO)-METHANESULFON-*m*-ANISIDIDE AND CROSS-RESISTANT TO CAMPTOTHECIN WITH A HIGH LEVEL OF TOPOISOMERASE I

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**Abstract**—N417/AMSA cells, about 80-fold resistant to mAMSA [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide], were obtained by serial passages of the parental human small cell lung carcinoma NCI-N417 (N417/p) in stepwise drug concentrations. The N417/AMSA cells were found to be 114-, 100-, and 9-fold cross-resistant to the topoisomerase II (Topo II) inhibitors VM26, VP16 and Doxorubicin (DXR); they showed a 2-fold decrease in Topo II activity. Interestingly, N417/AMSA cells which exhibited a 3-fold increase in topoisomerase I (Topo I) activity were 28-fold cross-resistant to camptothecin (CPT), a specific inhibitor of Topo I. In order to investigate the cellular mechanisms leading to the development of resistance, the effects of mAMSA and CPT on parental and resistant cell lines were analysed by alkaline elution. A decrease in DNA single-strand breaks (DNA-SSB) was observed in N417/AMSA cells treated with mAMSA or CPT compared to parental cells. Similar differences were obtained in isolated nuclei, suggesting that no modification of mAMSA and CPT accumulation occurred in resistant cells. Topo I was purified from N417/p (Topo I/p) and N417/AMSA (Topo I/AMSA) cells in the exponential phase of growth, and the inhibitory effects of CPT on relaxation activities were determined. Topo I/AMSA was found to be about 7-fold less sensitive to CPT than Topo I/p, suggesting the possible involvement of a mutation outside the gene region sequenced (codons 420 to 642) or post-translational modifications of the Topo I enzyme. These data indicate that increased Topo I activity cannot be related to CPT resistance, and suggest that mAMSA can generate multiple cellular modifications which may be involved in resistance to various drugs.

*Key words:* drug resistance; DNA topoisomerases; mAMSA; camptothecin

Drug resistance is a major obstacle in cancer treatment. Resistance to drugs used as single agents is generally accompanied by the development of resistance to other drugs. This phenomenon is called multidrug resistance and is particularly relevant to antitumour drugs which inhibit Topo II†. Multidrug resistance to Topo II poisons is complex and involves several mechanisms such as overexpression of the 170-*p*-glycoprotein [1], increased activity of DNA repair [2] and detoxifying enzymes (e.g. GST$\pi$) [3, 4] and alterations in DNA Topo I and Topo II activity, sometimes associated with gene mutation [5–9].

DNA Topo I and Topo II are critical enzymes that regulate the topological conformation of DNA and participate in essential processes such as transcription, replication, and recombination, as well as chromosome condensation and segregation [10]. These enzymes catalyse the interconversion of topological states of DNA through a transient single-strand break for Topo I and a double-strand break for Topo II.

Two distinct Topo II called Topo II $\alpha$ and Topo II $\beta$ have been identified in mammalian cells [11]. The p170 kDa protein, encoded by the Topo II $\alpha$ gene localized on chromosome 17q21, and the p180 kDa isoform, encoded by the Topo II $\beta$ gene on chromosome 3p24 [12], are antigenically distinct, as established by immunoblotting and inhibition of catalytic activity with immune sera [13]. Recent studies have shown that the two Topo II isoforms are differentially expressed and regulated during the cell cycle [14–16].

Topo II has been shown to be the target of several anticancer drugs currently used in the treatment of

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† Abbreviations: CPT, camptothecin; DMSO, dimethyl sulfoxide; DTT, dithiotreitol; DNA-SSB, DNA single-strand breaks; DXR, Doxorubicin; HIFCS, heat-inactivated fetal calf serum; $IC_{50}$, inhibitory drug concentration leading to 50% of colony survival by soft agar clonogenic assays or growth inhibition by MTT assays; kDNA, kinetoplast DNA; mAMSA, 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonylfluoride; RPMI, Roswell Park Memorial Institute Medium; RRI, relative resistance index; SDS, sodium dodecyl sulfate; Topo I, topoisomerase I; Topo II, topoisomerase II; VM 26, 4′-dimethyl epipodophyllotoxin thenylidene-$\beta$-D-glucoside; VP16, 4-(4,6-*O*-ethylidene-$\beta$-D-glucopyranoside).

human cancer. This enzyme is inhibited by anticancer drugs including Adriamycin®, etoposide, and mAMSA [17] while Topo I is specifically inhibited by CPT and its derivatives [18].

The extent of DNA damage caused by Topo I and Topo II inhibitors is related to both the amount of Topo present and to the degree of interaction with the agent. The N417 cell line, resistant to mAMSA described in this study, exhibits a decrease in Topo II activity and an increase in Topo I activity, as observed in other cell lines resistant to Topo II inhibitors [5–7]. It has been postulated that a decrease in Topo II activity could be offset by an increase in Topo I. As CPT sensitivity is related to the level of Topo I [19–22], this drug may be more cytotoxic to cells exhibiting an elevated Topo I level, as observed in Calc18/AMSA cell line [6]. We therefore studied the effect of CPT on N417/AMSA in which a 2-fold decrease in Topo II activity and a 3-fold increase in Topo I were observed. Contrary to that initially predicted, the N417/AMSA cell line was found to be 28-fold resistant to CPT.

## MATERIALS AND METHODS

*Cell culture.* The small cell lung carcinoma cells NCI-N417 [23] (N417/p) were grown in RPMI 1640 supplemented with 10% HIFCS. Resistance to mAMSA was induced by stepwise increases of drug concentration: a resistance to 0.8 μM mAMSA (N417/AMSA) was obtained after 23 months of subculture corresponding to approximately 450 generations. The resistant cell line (N417/AMSA) was maintained in RPMI 1640 medium supplemented with 15% HIFCS in the presence of 0.8 μM mAMSA. All experiments were performed on resistant cells cultured in the absence of mAMSA for two passages.

*Cytotoxicity and cross-resistance studies.* The level of drug resistance was determined by clonogenic assay in soft agar as described by Hamburger and Salmon [24] and MTT assay using (3 - [4,5 - dimethylthiazol - 2 - yl] - 2,5 - diphenyltetrazolium bromide) [25].

Briefly, for clonogenic assay, exponential N417/p and N417/AMSA cells were treated by various mAMSA concentrations for 24 hr. The cells were washed twice in RPMI 1640 medium, centrifuged and then plated in 0.3% soft agar in culture medium at a concentration of $10^4$ cells per plate. All assays were carried out in triplicate in 35 mm Petri dishes. After agar solidification, the plates were incubated in a humidified atmosphere of 8% $CO_2$ in air at 37°. Colonies containing more than 50 cells were scored using an inverted phase microscope [26]. The RRI was estimated by the ratio of drug concentration giving rise to 50% of surviving colonies in resistant and sensitive cells, respectively.

For MTT assay, $8 \times 10^3$ exponentially growing cells in suspension in culture medium were seeded in each well (96-well microculture plates, Costar). After an incubation of 72 hr, drugs were added for 24 hr and then removed. After washing, cells were incubated for 72–84 hr in drug-free medium. Twenty microlitres of 5 mg/mL MTT was added and the plates were incubated at 37° for 4 hr. The plates were centrifuged, poured off and 100 μL of DMSO was added in each well. Absorbance was measured by densitometry (540 nm) and the percentage of viable cells estimated using a standard curve established for each cell line. The RRI was estimated by the ratio of $IC_{50}$ in resistant and sensitive cells, respectively.

*Topo I and Topo II activities and inhibition assays.* Topo I and II activities were measured in the nuclear extracts according to the method previously described [15]. Topo I activity was tested using the relaxation assay on supercoiled plasmid DNA pUC 18. One unit of Topo I activity is the quantity of extract required to relax 50% of pUC 18 DNA (150 ng) in 30 min at 37°. Topo II activity was tested by kDNA decatenation reaction. One unit of Topo II activity is the quantity of extract required to decatenate 50% of 250 ng kDNA in 30 min at 37°.

For CPT and mAMSA inhibition studies, Topo I and Topo II were partially purified from N417/AMSA and N417/p cells collected in the exponential phase of growth. All operations were performed at 4°. Nuclear extract, obtained as described by Lefevre *et al.* [6] was loaded on to a phosphocellulose column equilibrated with Tris–HCl 50 mM, pH 7.9, NaCl 0.2 M, EDTA 0.5 mM, DTT 0.5 mM, β-MSH 10 mM, aprotinine 4 μg/mL, PMSF 1 mM. The preparation was eluted with a 0.2–1 M potassium phosphate linear gradient containing 20% w/v glycerol, β-MSH 10 mM, PMSF 1 mM (buffer A). Topo I and Topo II active fractions eluated by 0.7 M and 0.35 M phosphate buffer A, respectively, were then loaded on to two separated hydroxyapatite columns equilibrated with 0.2 M potassium phosphate in previous buffer A. Active fractions of Topo were eluted stepwise with the same buffer A, containing 0.4–0.6 M and 0.45–1 M potassium phosphate for Topo II and Topo I, respectively, and further dialysed against conservation buffer (Tris–HCl 0.1 M, pH 7.9, glycerol 50%, β-MSH 10 mM, EDTA 1 M).

Topo I and Topo II inhibition studies were performed using 2 U of enzyme in the presence of various concentrations of CPT and mAMSA.

*CPT accumulation study.* Parental and resistant cells were harvested and $10^6$ cells were incubated in culture medium for 3 hr and then treated with 0.1, 1, 10 and 20 μM of CPT. Cells were incubated for 5, 20, 60 or 120 min with each drug concentration. Drug accumulation was stopped by addition of HCl to a final concentration of 0.01 N [27, 28]. Cells were washed quickly with ice-cold PBS containing HCl 0.01 N, resuspended in 500 μL of the same buffer and sonicated in ice (Sonicator, model W-10). CPT content in each sample was determined by spectrofluorometry using excitation and emission wavelengths of 368 and 435 nm, respectively.

*Alkaline elution assays.* The alkaline elution technique has previously been described in detail by Kohn *et al.* [29]. 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 24 hr, corresponding to approximately one and a half cell cycles. $^{14}$C-labelled cells ($2 \times 10^5$) were combined with $2 \times 10^5$ $^3$H-labelled internal standard cells irradiated from a $^{60}$Co source (300 rads) and layered on polycarbonate membrane

filter, lysed with 2 mL of 25 mM $Na_2$ EDTA, 2% SDS, pH 9.7, and deproteinized 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 [30]. Drug-induced break frequencies are expressed in rad equivalents.

The procedure for nuclei isolation has previously been described [31]. Cells were harvested 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*′-tetra-acetic acid (EGTA), 0.1 mM DTT, pH 6.4) and centrifuged at 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 for 30 min at 37° in the presence or absence of drug.

*DNA and RNA preparations.* DNA and total RNA were prepared from exponential growing cells using the guanidinium-isothiocyanate CsCl gradient technique [32]. Briefly, cells were ground in liquid nitrogen, then lysed in the guanidinium-isothiocyanate buffer. Lysate was layered on to 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) 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, spun down at the bottom of the centrifuge tube, was collected and precipitated by absolute alcohol. Denatured RNA samples (10 μg) were fractionated on a formaldehyde 1.2% agarose gel, transferred to a Hybond C extra filter and analysed by northern blot hybridization. Hybridizations were performed under stringent conditions with the appropriate human probes $^{32}$P-labelled by nick-translation (about $10^7$ cpm). Filters were exposed for various periods of time to autoradiographic films (Amersham).

*Probes.* The Topo I probe was the 0.7 kb *Eco*RI cDNA fragment of the human Topo I gene (clone pGEM45A) [33]; the Topo II α and Topo II β probes were the 1.5 kb *Eco*RI and the 1.5 kb *Eco*RI-*Pst*I cDNA fragments of the human Topo II α gene (clone SP1) and Topo II β gene (clone SP12), respectively (gift from Dr K. B. Tan) [12].

*Quantification of transcript levels.* The quantity of RNA loaded in each well was evaluated by scanning (Chromoscan 3, Joyce-Loebl) the 28S RNA species band, stained with ethidium bromide and UV photographed.

*Immunoblotting.* The Topo II and Topo I proteins were analysed by immunoblotting. Protein extracts were obtained from N417/p and N417/AMSA cells in the exponential phase of growth. Cells ($25 \times 10^6$) were lysed with 2% SDS in PBS plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 μg/mL leupeptin and 10 μg/mL soybean trypsin inhibitor; Sigma, St. Louis, MO, U.S.A.) at 68° for 5 min.

Total protein amount was estimated using Bio-rad protein assay. Protein (100 μg) was loaded on 7.5% SDS-polyacrylamide gel, electrophoresed following Laemmli conditions [34], and transferred on to nitrocellulose membrane as described above [6].

The membrane was soaked for 2 hr in PBS containing 5% milk (Régilait) and then incubated sequentially with Topo I or Topo II α-specific rabbit antibodies (gift from Dr J. Hwang, Institute of Molecular Biology, Taipei, Taiwan, R.O.C.) for 2 hr at room temperature. Bound immunoglobulins were detected by a peroxydase-conjugated anti-rabbit immunoglobulin using ECL detection system (Amersham).

*Topo I gene sequence.* cDNAs were obtained by reverse transcription of total RNAs from both N417/p and N417/AMSA cell lines using AMV reverse transcriptase (Appligene) and random hexamer primers. Topo I cDNA fragments were obtained by Polymerase Chain Reaction (Taq Polymerase, Promega) using two couples of primers (gift from Dr J.F. Riou, Rhône Poulenc Rorer, Vitry, France):

414
5′ GTT TCC TGG ACA GAG AAC AT 3′ sense
530
5′ AG GAA GTA CAA CTC TAC CAC 3′ antisense
509
5′ G GAG CAC ATC AAT CTA CAC 3′ sense
649
5′ GT TTG CAA GTT CAT CAT AG 3′ antisense

Amplification reactions were performed at 92° for DNA denaturation, 57° for primer annealing and 72° for extension (30 cycles). The sequences were determined by the Sanger dideoxy chain termination method using the same primers and Sequenase II kit (USB).

Table 1. Sensitivity of parental (N417/p) and resistant (N417/AMSA) cell lines to various anticancer agents

| Drugs | $IC_{50}$ (μM)* N417/p | N417/AMSA | RRI* |
|---|---|---|---|
| VM26 | 0.05 (± 0.01) | 5.7 (± 2.1) | 114 |
| VP16 | 3.1 (± 0.4) | 315 (± 49) | 100 |
| mAMSA† | 0.1 (± 0.05) | 8.2 (± 0.8) | 82 |
| CPT | 0.2 (± 0.015) | 5.6 (± 0.2) | 28 |
| DXR | 0.02 (± 0.01) | 0.18 (± 0.045) | 9 |

* $IC_{50}$ and RRI of parental and resistant cells were evaluated using MTT assays. $IC_{50}$ (± range) values represent the mean of two independent experiments. RRI is estimated by the ratio of $IC_{50}$ values in resistant and parental cells.

† $IC_{50}$ for mAMSA was also evaluated using clonogenic assays. RRI was found to be equal to 85.

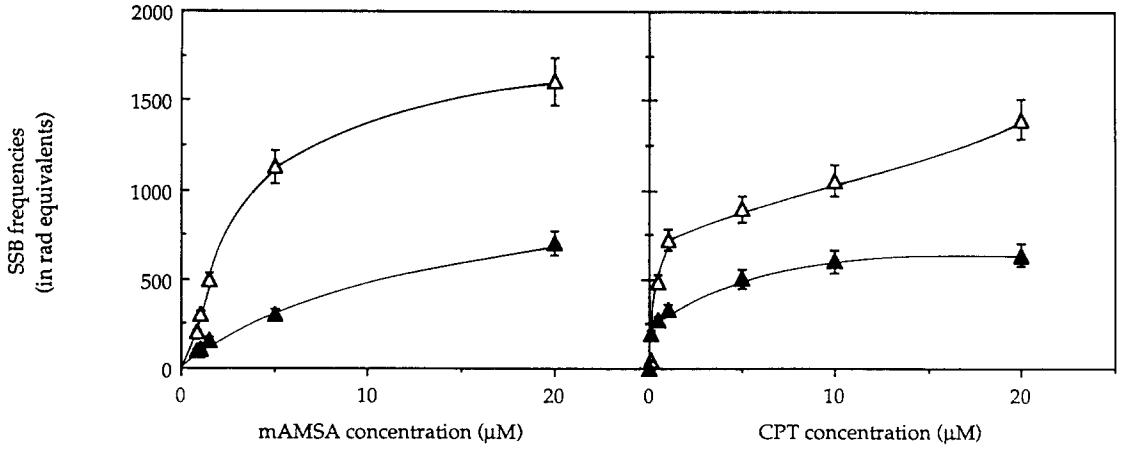


Fig. 1. DNA-SSB in N417/p (△) and N417/AMSA (▲) cells treated for 1 hr with various concentrations of mAMSA or CPT. DNA-SSB frequencies are expressed in rad equivalents ± SD; values represent the mean of at least three independent experiments.

## RESULTS

### *Characteristics of N417/AMSA subline*

The subline resistant to 0.8 μM mAMSA (N417/AMSA) was isolated by serial passages of the parental N417/p cell line in stepwise increasing concentrations of drug, after 23 months of subculture. N417/AMSA grew slightly more slowly than N417/p with doubling times of 18 hr and 16 hr, respectively. A morphological difference was rapidly detected in N417/p cells cultured in the presence of mAMSA when the cells became resistant to mAMSA. While parental cells grew as floating aggregates of refringent cells, N417/AMSA cells grew as a simple monolayer (data not shown). The cytotoxic effects of Topo II (mAMSA, VM26, VP16 and DXR) and Topo I (CPT) inhibitors were determined using an MTT assay for all the drugs (Table 1). Cytotoxicity studies of mAMSA were also performed using a clonogenic assay in soft agar, and similar data were obtained using both assays. N417/AMSA cells were found to be about 80-fold resistant to mAMSA and also highly cross-resistant to VM26 and VP16 (114- and 100-fold, respectively) but less resistant to DXR (9-fold). Interestingly, N417/AMSA cells were 28-fold resistant to CPT.

### *Alkaline elution assays*

The DNA-SSB produced by the stabilization of Topo II DNA cleavable complex by mAMSA were evaluated on N417/p and N417/AMSA cell lines by alkaline elution. In both cell lines, the frequency of DNA-SSB increased with mAMSA concentration (Fig. 1). When cells were treated for 1 hr with mAMSA, a significant decrease was observed in the frequency of DNA damage in N417/AMSA cells as compared to N417/p cells (Fig. 1, Table 2). Similar differences were found in isolated nuclei (Table 2), suggesting that differences in strand break induction were not related to altered drug uptake in mAMSA-resistant cells.

When cells were treated with CPT, DNA-SSB increased with drug concentration in both cell lines, but DNA damage was less severe in N417/AMSA cells than in N417/p cells. After 1 hr of treatment with 1 μM of CPT, a 2-fold decrease in the frequency of DNA damage was observed in N417/AMSA cells compared to that found in N417/p cells (Fig. 1, Table 2). To obtain a frequency in DNA-SSB damage equivalent to that induced by 600 rads, it was necessary to treat N417/AMSA cells with 30-fold more CPT (20 μM of CPT for N417/AMSA cells vs 0.7 μM of CPT for N417/p cells). The same differences were obtained for isolated nuclei (Table 2).

The results of the cytotoxicity study and alkaline elution assays are consistent. They show that N417/

Table 2. DNA-SSB frequencies induced by mAMSA or CPT treatment in parental (N417/p) and resistant (N417/AMSA) cells and in isolated nuclei

| | mAMSA | | | CPT | | |
|---|---|---|---|---|---|---|
| | N417/p | N417/AMSA | R | N417/p | N417/AMSA | R |
| Whole cells | 467 ± 37 | 109 ± 10 | 4.3 | 700 ± 52 | 300 ± 22 | 2.3 |
| Nuclei | 397 ± 62 | 117 ± 22 | 3.4 | 541 ± 21 | 270 ± 33 | 2.0 |

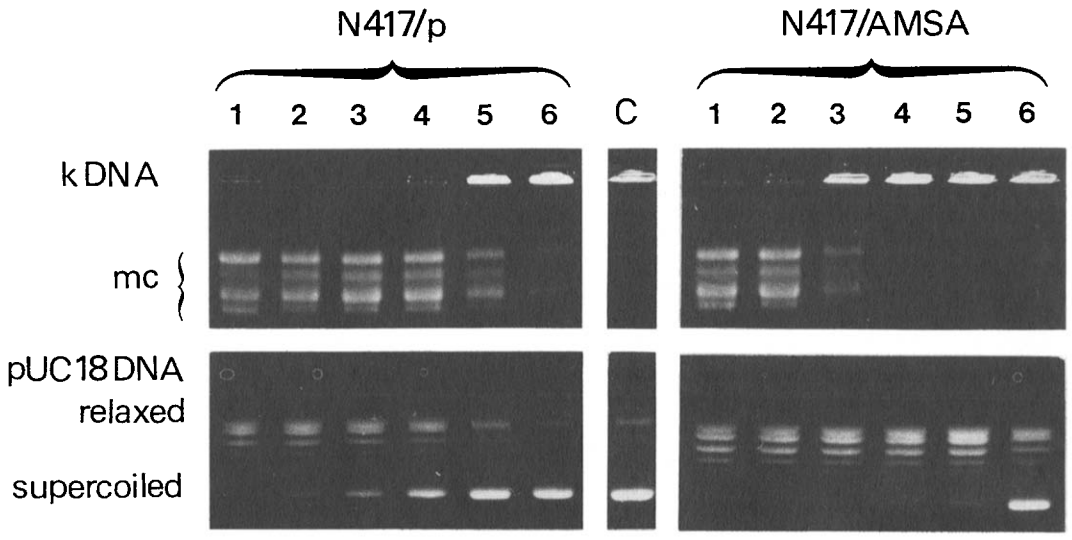


Fig. 2. Topo II and Topo I activities in parental and resistant cells. Topo II decatenation activity: 0.33 M NaCl nuclear extracts were prepared from N417/p and N417/AMSA cells in exponential phase of growth and Topo II activities determined using kDNA decatenation assay. C: *Control* kDNA (250 ng); 1: *Control* kDNA plus 1 μL (0.5 μg of protein) of N417/p or N417/AMSA cells; 2–6: *Control* kDNA plus serial dilutions (0.25, 0.125, 0.062, 0.031, 0.0015 μg) of Topo II extract (mc, free minicercles). Topo I relaxation activity: 0.01 M NaCl nuclear extracts were prepared from N417/p and N417/AMSA cells in exponential phase of growth and Topo I activities determined using pUC 18 DNA relaxation assay. C: *Control* pUC 18 DNA; 1: *Control* pUC 18 DNA plus 1 μL (0.1 μg of protein) of nuclear extract from parental or resistant cells; 2–6: *Control* pUC 18 DNA plus serial dilutions (0.05, 0.025, 0.0125, 0.0062, 0.0031) of Topo I extract.

AMSA cells are cross-resistant to CPT (Table 1) and exhibit a decrease in the CPT-stabilized cleavable complex (Fig. 1, Table 2), suggesting that an alteration occurs at the level of Topo I and Topo II.

*Cellular CPT content*

In order to determine whether the decrease of CPT sensitivity in N417/AMSA cells was due to a lower intracellular CPT accumulation, we measured the CPT content in N417/p and N417/AMSA cells by spectrofluorometric assays. Intracellular CPT content was similar in both parental and resistant cells for the different drug concentrations and incubation times (data not shown). These results are not in favour of a CPT uptake modification in N417/AMSA cells.

In vitro *activity of Topo II and Topo I and inhibition studies with mAMSA and CPT*

Topo II activity of nuclear extracts obtained from N417/p and N417/AMSA cells was determined using the kDNA decatenation assay. A 2.5-fold decrease in the level of Topo II activity was found in N417/AMSA cells compared to that of N417/p cells (Fig. 2). The inhibition of Topo II activity by mAMSA was studied on partially purified Topo II (see Materials and Methods). No difference was found between Topo II/AMSA and Topo II/p in sensitivity to mAMSA (data not shown).

Topo I ATP-independent relaxation activity present in nuclear extracts from N417/p and N417/AMSA cells was determined using the pUC 18 DNA relaxation assay. Topo I relaxation activity was found to be approximately 3-fold higher in nuclear extracts from N417/AMSA cells than in extracts from N417/p cells (Fig. 2). Topo I was partially purified (see Materials and Methods) and the inhibition of relaxation activity by CPT was determined using enzyme dilutions adjusted according to equal activity (2 units). Topo I/p was found to be roughly 7-fold more sensitive to the inhibitory effect of CPT than was Topo I/AMSA. Indeed, inhibition of 50% of the relaxation activity of Topo I/p was obtained with 11.25 μg/mL of CPT vs 82.5 μg/mL for Topo I/AMSA (Fig. 3).

*Topo I and Topo II transcript levels*

We analysed Topo II $\alpha$ and $\beta$, and Topo I mRNA in the N417/p and N417/AMSA cell lines using the northern blot technique. Transcripts of Topo II $\alpha$, Topo II $\beta$ and Topo I with expected sizes (6.2, 6.2 and 4.0 kb) were observed. In N417/AMSA cells, the Topo II $\alpha$ transcript level was significantly lower (2-fold) than that of parental cells (Fig. 4), while Topo II $\beta$ transcripts could not be detected (Fig. 4). By contrast, the Topo I transcript level had increased by approximately 3-fold (Fig. 4).

*Topo I and Topo II protein levels*

We studied Topo I and Topo II $\alpha$ proteins in parental and resistant cells by the western blot technique using specific antibodies. An immunoreactive band corresponding to Topo I (100 kDa) and Topo II $\alpha$ (170 kDa) was observed in both cell

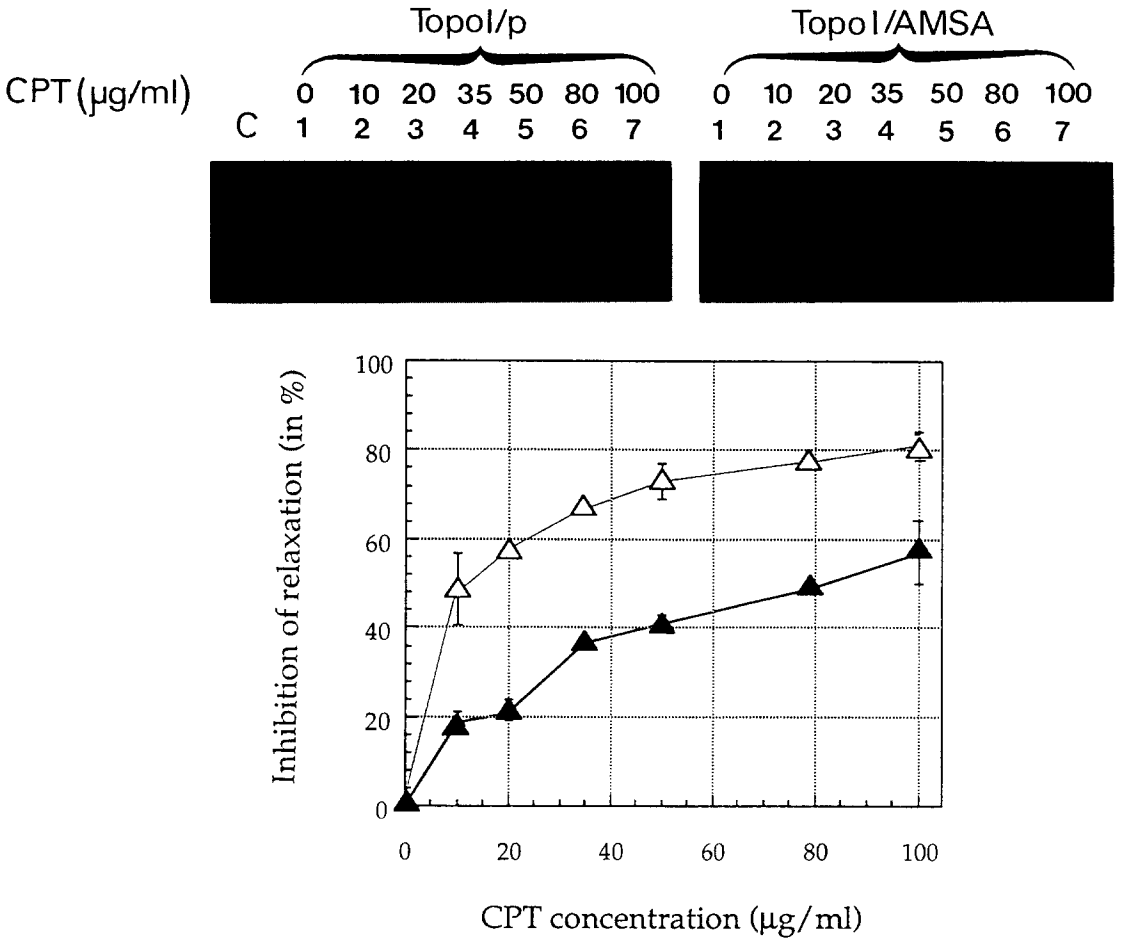


Fig. 3. Inhibitory effect of CPT on the relaxation activity of Topo I partially purified from parental (Topo I/p) (△) and resistant (Topo I/AMSA) (▲) cells. Agarose gel electrophoresis (upper panel). C: Control pUC 18 DNA (150 ng); lanes 1–7: plus 2 units of partially purified Topo I with various concentrations of CPT. Gel was stained with ethidium bromide. The negative UV photographs were scanned using a soft laser densitometer (Chromoscan 3 Joyce-Loebl). Diagrams were traced with values expressed in percentage of relaxed DNA, considering that 100% of relaxed pUC 18 DNA was observed in the absence of CPT (lanes 1). Inhibition of 50% of relaxation was obtained with 82.5 μg/mL and 11.25 μg/mL of CPT for Topo I/AMSA and Topo I/p, respectively.

lines. In N417/AMSA cells, the Topo II α protein level decreased 3-fold (Fig. 5), whereas the Topo I protein level increased 2-fold compared to that of parental cells (Fig. 5). These results are consistent with northern blot analysis.

*Topo I nucleotide sequencing*

Several point mutations have been reported in tumour cells resistant to CPT [35–37]. Nucleotide sequencing of the 666 bp Topo I cDNA fragments, comprised between codons 420 and 642, was performed. Both N417/p and N417/AMSA cell lines had the same nucleotide sequence pattern. When compared to the sequence obtained by D'Arpa *et al.* [38] for human normal cells, we identified a silent mutation at codon 591 ($ACG^{Thr} \rightarrow ACA^{Thr}$), also observed in other human cell lines (data not shown), suggesting the presence of a polymorphic site.

## DISCUSSION

In this study we describe the establishment of a new mAMSA resistant cell line and its characterization. The cells obtained by progressive adaptation to increased concentrations of mAMSA were 83-fold resistant to this drug. This cell line was cross-resistant to many anticancer drugs including inhibitors of Topo II (VP16, VM26, ADR) and CPT, a specific inhibitor of Topo I.

Quantitative and qualitative changes in the Topo II enzyme have already been reported to account for the resistance process in cell lines resistant to Topo II inhibitors. Most cell lines resistant to mAMSA or other Topo II inhibitors exhibit a decrease in Topo II gene expression, reduced total Topo II activity and drug-induced cleavage [5–9, 39–43]. Moreover, yeast cells overexpressing the Topo

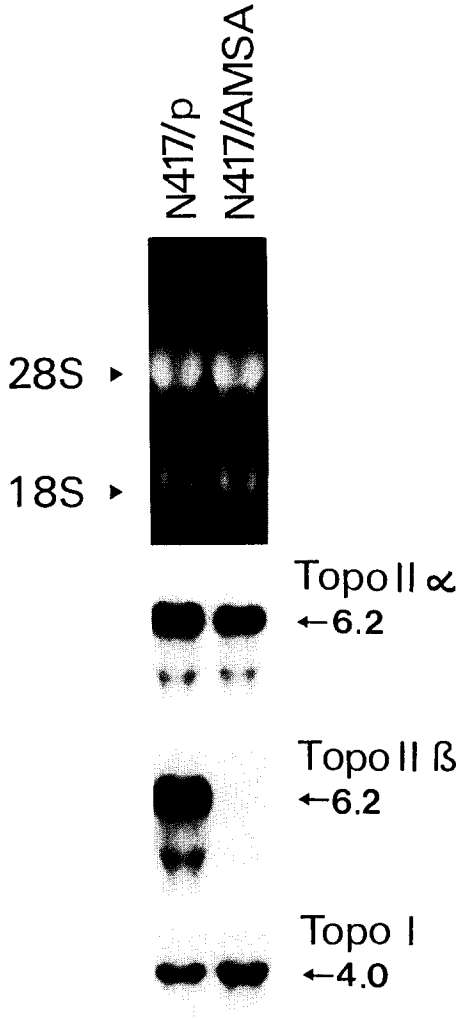


Fig. 4. Northern blot analysis. Total RNAs (10 $\mu$g per lane) were prepared from cell lines in exponential phase of growth. Hybridizations were performed using Topo II $\alpha$, Topo II $\beta$ and Topo I probes. Filters were exposed to X-ray films for various lengths of time. The transferred blots after the agarose gels were stained with ethidium bromide and are shown in the upper panel.

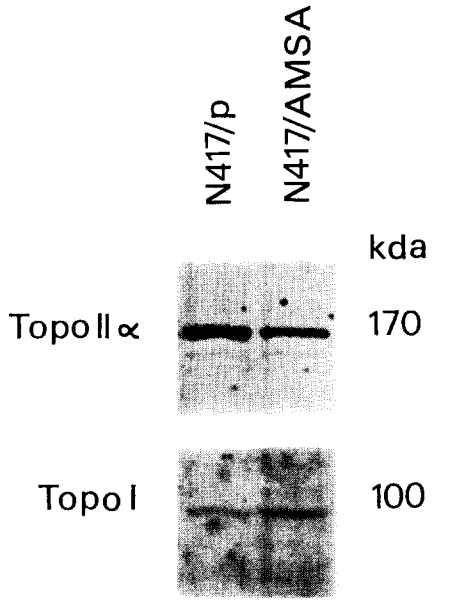


Fig. 5. Western blot analysis of Topo I and Topo II $\alpha$ in N417/p and N417/AMSA cells using specific antibodies (see Materials and Methods).

II gene are hypersensitive to mAMSA [44]. We studied the Topo II mRNA levels and the catalytic activity of the partially purified enzymes Topo II/p and Topo II/AMSA. We found a 2-fold decrease in total Topo II catalytic activity in the N417/AMSA cell line associated with a decrease in the mRNA level. This decrease is the result of modifications in both Topo II $\alpha$ and Topo II $\beta$ gene expression. Indeed, a 2-fold decrease in Topo II $\alpha$ gene expression and the total disappearance of Topo II $\beta$ gene expression in N417/AMSA cells were observed using northern blot analysis. The Topo II $\alpha$ protein analyses were in agreement with these results.

mAMSA-induced DNA-SSB were found to have greatly decreased numbers of resistant cells. No modification in mAMSA accessibility to nuclei occurred in resistant cells since the ratio of DNA-SSB was the same in whole cells and isolated nuclei.

A decrease in the Topo II level seems to be sufficient for mAMSA resistance [6, 9, 43]. However, it has been shown that most cell lines resistant to Topo II inhibitors contain a Topo II enzyme resistant to drugs [28, 39–42]. Topo II/AMSA and Topo II/p were partially purified from exponentially growing cells and sensitivity to mAMSA was determined using an inhibition of decatenation test and *in vitro* cleavage experiments. No modification was found (data not shown). However, the implication of the Topo II $\beta$ enzyme in cell sensitivity to drugs may not be ruled out, since this enzyme is known to be less stable than Topo II $\alpha$ and may undergo degradation in partially purified extracts. Drake *et al.* [13] showed that Topo II $\alpha$ and $\beta$ isoforms differed in their sensitivity to drugs. The Topo II $\beta$ isoenzyme might indeed be more sensitive to mAMSA than Topo II $\alpha$, and its selective loss would be sufficient to confer resistance to cells. Using northern blot analyses and a specific probe for Topo II $\beta$, we found that the 6.2 kb transcript had disappeared completely in the resistant cell line when compared to parental cells.

Topo I and II are key enzymes in the preservation of DNA topology. In this study as in others, mAMSA [5, 6, 28] and etoposide [7] resistant cell lines increased both Topo I activity and mRNA level, thereby offsetting the decrease in the Topo II level as shown in yeast [45, 46]. In the Calc18/AMSA cell line [6] an increase in Topo I has been related to a CPT-hypersensitive phenotype, a Topo I-specific inhibitor. This cell line, which exhibits a 3-fold increase in Topo I activity and strong DNA drug-induced cleavage, was found to be over 6-fold

hypersensitive to CPT. Indeed, CPT sensitivity seems to be related to the level of Topo I since CPT resistant cell lines exhibit a decrease in the level of Topo I [19–21, 46], and baby hamster kidney cells, stably transfected by wild-type Topo I gene, are hypersensitive to CPT [38].

In contrast to results already published, and in spite of the increase in Topo I activity and the mRNA level, our data clearly indicate that the N417/AMSA resistant cell line is cross-resistant to CPT. A decrease in single-strand breaks was found by alkaline elution in N417/AMSA cell line treated with various concentrations of CPT compared to that found in N417/p cells. No difference was detected when these experiments were performed on isolated nuclei. Moreover, using spectrofluorometric assays, we showed that there was no difference in cellular CPT contents between parental and resistant cells. This result is not consistent with a model of drug resistance involving an alteration of drug accumulation, and suggests that the resistance process is localized at nuclear level.

The N417/AMSA cells were cloned at the 23rd passage. Resistance parameters as well as topoisomerase content were further investigated in three independent clones. The three clones exhibited similar resistance indices to mAMSA (range 72 to 107) and CPT (range 22 to 32) compared to those obtained for uncloned cells (Table 1). A 2-fold decrease in Topo II activity and mRNA level and a 3- to 4-fold increase in Topo I activity were observed in these clones.

Cellular drug resistance may be due to a variety of mechanisms that may be simultaneously at work in some resistant cell lines. CPT resistance has been shown to be related to reduced Topo I activity [19, 20, 46], or reduced enzyme sensitivity to CPT, whether associated with gene alteration or not [35–37, 47–49]. We have thus evaluated the inhibitory effect of CPT on a partially purified Topo I. The enzyme extracted from N417/AMSA cells (Topo I/AMSA) was found to be approximately 7-fold less sensitive to CPT than Topo I/p. The N417/AMSA cell line is the first to exhibit an increase in Topo I activity associated with a CPT resistant phenotype. In several CPT-resistant cell lines a decrease in Topo I sensitivity to CPT has previously been associated with Topo I gene rearrangement, hypermethylation [5, 20, 21] or point mutations [35–37]. These gene alterations were investigated in N417/AMSA and N417/p cells by studying the restriction enzyme pattern of the entire Topo I locus and the nucleotide sequence of the Topo I gene region in which point mutations have been described. No modification was found in the restriction pattern, nor any sign of mutation. However, compared to the published normal sequence [38], a polymorphic site was found at codon 591 ($ACG^{thr} \rightarrow ACA^{thr}$) in both N417/p and N417/AMSA cell lines, as well as in other human cell lines (data not shown).

In conclusion, the N417/AMSA cell line provides an interesting model to study the mechanisms of resistance to the Topo I and Topo II inhibitors. Phosphorylation [50–56] and polyADPribosylation [57, 58], which have been reported to change Topo I and Topo II activity, could be among these mechanisms.

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