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# Cellular Resistance to the Antitumor DNA Topoisomerase II Inhibitor S16020-2: Importance of the *N*-[2(Dimethylamino)ethyl]carbamoyl Side Chain

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## ABSTRACT

The new olivacine derivative S16020-2 (NSC-659687) is a DNA topoisomerase II inhibitor endowed with a remarkable antitumor activity against various experimental tumors. In vitro physicochemical properties of this compound, in particular its interaction with DNA and DNA topoisomerase II, were very similar to those of ellipticine derivatives, except for a strictly ATP-dependent mechanism of cleavable complex induction. From the Chinese hamster lung fibroblast cell line DC-3F, a subline resistant to S16020-2, named DC-3F/S16, was selected by adding stepwise increasing concentrations of the drug to the cell growth medium. Whereas DC-3F/9-OH-E cells, a DC-3F subline resistant to 9-hydroxy-ellipticine, are cross-resistant to S16020-2, DC-3F/S16 cells are only very weakly cross-resistant to ellipticine derivatives, indicating that, despite their structural similarity, these compounds may differ in their mechanisms of action. Uptake and efflux rates of S16020-2 were identical in the resistant and the sensitive cells. Topoisomerase II$\alpha$ was expressed at the same level in both sensitive and resistant cells, whereas expression of the $\beta$-enzyme was approximately 50% lower in the resistant cells. Sequencing of both $\alpha$- and $\beta$-isoform cDNAs revealed a point mutation that converts Arg[486] to a Gly in the $\alpha$ cDNA, whereas the $\beta$ cDNA was not modified. This amino acid substitution in a highly conserved sequence of the enzyme appears to be responsible for the resistance to S16020-2. Comparative analysis of the properties of the ellipticine and S16020-2-resistant cells suggests that S16020-2, which is a DNA intercalator, might also interact with this enzyme amino acid sequence through its side chain.

DNA topoisomerase II inhibitors used in human cancer chemotherapy constitute a group of structurally unrelated compounds that share a common property, the capacity to induce a significant increase of the number of covalent enzyme-DNA complexes (cleavable complexes) present on the cell genome at a given time, thus triggering a cell response eventually leading to cell death by apoptosis (Liu, 1989; Froelich-Ammon and Osheroff, 1995). However, probably as a consequence of their structural diversity, DNA topoisomerase II inhibitors have been shown to be active in vitro through different mechanisms. It is generally admitted that these compounds are involved in the formation of a drug-enzyme-DNA ternary complex. Some drugs enter this complex by interacting predominantly with the protein (etoposide) or the DNA (ellipticine), but it is likely that most DNA-intercalating drugs bind to both the DNA, through intercalation of the chromophore at the enzyme-DNA interface, and the enzyme, through side chains, which are usually essential to their pharmacological activity. Some drugs [4'-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), etoposide] can inhibit the religation of the cleaved DNA, whereas others (ellipticine, genistein, quinolones) are presumed to accelerate the forward rate of complex formation (Froelich-Ammon and Osheroff, 1995).

The relatively simple structure of ellipticine has prompted chemists to design various structural modifications to synthesize compounds endowed with increased activity and specificity (Le Pecq et al., 1974; Auclair, 1987a). More than 70 molecules were thus obtained, but only one, 2-*N*-methyl-9-hydroxy-ellipticinium (NMHE), was finally used in human clinics with a moderate efficacy (Paoletti et al., 1980; Rouessé et al., 1993). Olivacine, a natural ellipticine isomer, differing by the shift of the methyl group from the 11 to the 1 position,

**ABBREVIATIONS:** *m*-AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide; 9-OH-E, 9-hydroxy-ellipticine; NMHE, 2-*N*-methyl-9-hydroxy-ellipticinium; MDR, multidrug resistance; SSB, single-strand break.

was not nearly as extensively studied, although it displayed a significant antitumor activity on various experimental tumors (Pierré et al., 1997).

Recently, a new series of 6*H*-pyrido[4,3*b*]carbazole derivatives, characterized by a basic *N*-dialkylaminoalkyl carboxamido side chain grafted onto an olivacine chromophore, was synthesized (Jasztold-Howorko et al., 1994). Some of these compounds displayed a remarkable activity against various experimental tumors. The most active of these compounds, S16020-2 (NSC-659687) (Fig. 1), has demonstrated a broad range of antitumor activity against a panel of murine and human tumor xenografts, being particularly active against the Lewis lung carcinoma and the human non-small cell lung carcinoma NCI-H460, two highly aggressive and chemoresistant models. In these models, NMHE was inactive and S16020-2 was at least as active as adriamycin taken as a reference in preclinical studies. Recently, two orthotopic models were used to confirm the potential activity of S16020-2 against experimental lung cancer. Murine Lewis lung carcinoma and human A549 tumor cells were grafted by the i.v route, the latter into SCID mice, to obtain lung metastases, which progressively invade the lungs resulting in the death of the animals. S16020-2 totally inhibited the growth of lung metastases and cured 89% of Lewis lung carcinoma-bearing mice and increased by 50% the survival of A549-bearing mice (Guilbaud et al., 1997). In addition, the drug retained its activity on several sublines expressing the multidrug resistance (MDR) phenotype (Guilbaud et al., 1996; Pierré et al., 1998). All these observations made S16020-2 a very promising antitumor agent. Because of its favorable pharmacokinetic characteristics and acceptable toxicity in different species (Pierré et al., 1997), S16020-2 is presently in clinical trials.

The molecular basis for the remarkable antitumor activity of S16020-2 is currently unknown. In vitro, S16020-2 displays strong similarities with NMHE (Le Mée et al., 1998). S16020-2 intercalates between adjacent DNA base pairs with an affinity comparable to that of NMHE. This new olivacine derivative is a specific topoisomerase II inhibitor that stimulates the cleavable complex formation in a concentration range close to that of NMHE and has no detectable effect on the DNA religation rate. However, S16020-2 differs markedly from any other topoisomerase II poison by its absolute ATP requirement to stimulate the enzyme-mediated DNA cleavage. In contrast with the results obtained in vitro, S16020-2 was at least 30-fold more cytotoxic than NMHE on the DC-3F cell line, and in these cells, the formation of the cleavable complexes was detected at concentrations 500-fold lower than with NMHE.

We now report the selection and properties of S16020-2-resistant cells derived from the Chinese hamster fibrosarcoma cell line DC-3F. Comparison of these cells with DC-3F/9-OH-E cells, a DC-3F variant resistant to 9-OH-E, led us to propose a new model for the mechanism of action of S16020-2.

## Materials and Methods

**Cell Lines and Culture Conditions.** The parental Chinese hamster lung fibroblast cell line DC-3F, the S16020-2-resistant subline DC-3F/S16, the 9-OH-E-resistant subline DC-3F/9-OH-E (Salles et al., 1982), clone 24 (Khélifa et al., 1999), and clone 11 cells (Dereuddre et al., 1997) were maintained as monolayer cultures in Eagle's minimal medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 50 $\mu$g/ml streptomycin (Dereuddre et al., 1997). The cells were incubated at 37°C in a humidified incubator with 5% $CO_2$ in air. The resistant cells DC-3F/S16 and DC-3F/9-OH-E were permanently grown in the presence of 100 nM S16020-2 and 0.6 $\mu$g/ml 9-OH-E, respectively. Before each experiment, the cells were grown for two or three passages in the absence of drug.

**Drugs and Chemicals.** S16020-2 and S30761, the ellipticine analog of S16020-2 with the side chain grafted at position 1, were provided by Institut de Recherches Servier (Suresnes, France); NMHE was purchased from Pasteur-Mérieux (Lyon, France), doxorubicine (Adriablastine) from Farmitalia (Rueil Malmaison, France), vincristine (Oncovin) from Lilly France S.A. (St. Cloud, France), and actinomycine D (Lyovac Cosmegen) from MSD (Whitehouse Station, NJ). These drugs were dissolved in $H_2O$. 9-OH-E was kindly provided by Dr. E. Lescot (Institut Gustave Roussy, Villejuif, France) and dissolved in HCl $10^{-3}$ M. Etoposide and *m*-AMSA, obtained from Sigma Aldrich Chimie (Fallavier, France), were dissolved in dimethylsulfoxide. Stock solutions at 0.01 M were prepared for each drug. [$^3$H]S16020-2 (39 Ci/mmol) was synthesized by the CEA (Saclay, France).

**Population-Doubling Time.** DC-3F cells ($10^4$) and DC-3F/S16 cells ($5 \times 10^4$) were plated in 60-mm-diameter Petri dishes and incubated at 37°C. The cells were counted in duplicate every 12 h for 4 days. The data were analyzed by plotting the logarithm of the cell number as a function of time, and the population generation time was calculated from the exponential part of the growth curve.

**Tumorigenicity.** For determination of tumorigenicity, 1 or 2 $\times$ $10^6$ cells were injected s.c. into the left posterior flank of 6- to 8-week-old female *nu/nu* mice. The mice were examined biweekly for development of tumors. Cells were scored as tumorigenic if a palpable nodule appeared and grew progressively at the injection site within 10 weeks. Otherwise the mice were kept for at least 3 months after injection before being scored as nontumorigenic.

**Assay of Drug Sensitivity.** All of the experiments were carried out on exponentially growing cells. Two methods were used for determination of drug sensitivity: 1) The 72-h assay. The sensitivity of the DC-3F and DC-3F/S16 cells to the different drugs was determined by cell counting after 72-h drug exposure (Salles et al., 1982). Wells (16 mm) of a 24-well Falcon dish, containing 1 ml of minimal essential medium with graded drug concentrations, were inoculated with 1 ml of cell suspension containing either $10^4$ parental cells or $2 \times 10^4$ resistant cells. After 72 ($\pm$2) h of drug exposure, the number
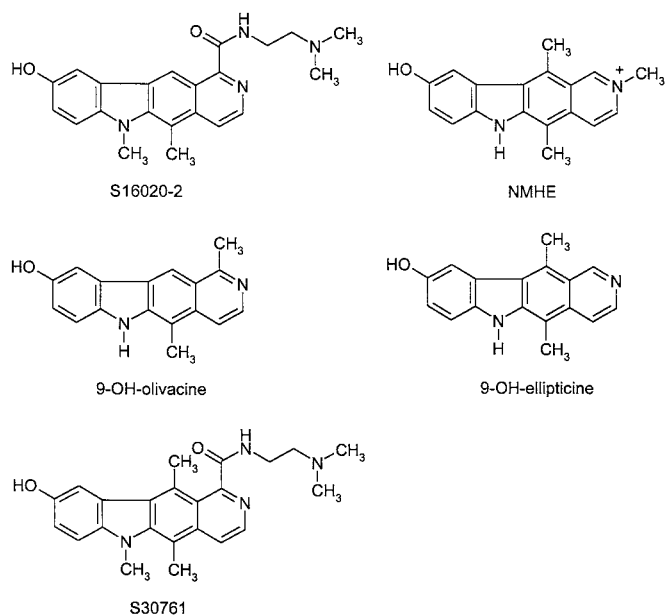


**Fig. 1.** Structures of olivacine and ellipticine derivatives.

of cells per well was determined with a model ZM Coulter Counter (Coultronic France, Margency, France). 2) The colony formation assay. This assay determines the cloning efficiency of the cell survival fraction after 3-h drug exposure. Parental cells or resistant cells (2 to $10^5$ or 4 to $10^5$, respectively) were grown into 60-mm-diameter Petri dishes (Falcon, Becton-Dickinson, Lincoln Park, NJ) for 18 h at 37°C before drug treatment. After 3 h of exposure to the drugs, the cells were trypsinized, and appropriate dilutions were made to seed 250 or 500 treated cells into 60-mm-diameter Petri dishes. Colonies were stained and counted 6 to 8 days later.

**Drug Uptake and Efflux Studies.** Cells were grown in 35-mm wells containing 4 ml of growth medium for 24 h to a cell density of $5 \times 10^5$ to $1 \times 10^6$ cells/well. The growth medium was then removed and replaced with 1 ml of fresh medium containing $^3$H-labeled S16020-2 at a concentration of 50 nM (specific activity adjusted to 2 Ci/mmol). At various times thereafter, the cells were washed three times with 1 ml of PBS at 4°C and lysed overnight with 1 ml of 2% Triton X-100 at room temperature. The cells lysates were withdrawn, and each well was washed with 1 ml of PBS. Each lysate and wash was collected in a scintillation vial and mixed with 10 ml of Ultima Gold (Packard Instruments Co., Meriden, CT) for liquid scintillation counting. Cell counting was carried out on duplicate samples grown in the same conditions with a model ZM Coulter Counter.

In efflux studies, cells were plated as described earlier and incubated for 30 min in growth medium containing 50 nM S16020-2. After the cells were washed three times with growth medium at 4°C, they were incubated at 37°C in 5 ml of drug-free medium. At the indicated times, the amount of drug that remained associated with the cells was determined as described above.

**cDNA Probes.** The SP1′ probe, obtained from a human cDNA library (Khélifa et al., 1994), selectively recognizes the 170-kDa form of the enzyme, whereas the PH1 probe, a *Pst*I/*Hin*dIII fragment isolated from the pBhamTOP2β plasmid, selectively detects the 180-kDa form (Dereuddre et al., 1997). The actin DNA probe was kindly provided by Dr. F. Dautry (Institut de Recherche Scientifique sur le Cancer, Villejuif, France). These probes were labeled by random priming with $\alpha$-$^{32}$P using the Multiprime labeling kit from Amersham (Buckinghamshire, UK).

**Anti-DNA Topoisomerase II Antibodies.** The rabbit polyclonal antibody designated A6 was previously described (Khélifa et al., 1994). This antibody raised against a 837-amino acid fragment of the human topoisomerase II$\alpha$, recognizes the $\alpha$- and $\beta$-enzymes from both human and hamster origins.

**Northern Blots.** RNA was extracted by the guanidine thiocyanate technique (Chirgwin et al., 1979). Polyadenylated RNAs were purified from total RNA using the mRNA purification kit from Pharmacia LKB Biotechnology (Saclay, France) and following the manufacturer's instructions. mRNAs (4 $\mu$g) were fractionated by electrophoresis in 1.2% (w/v) agarose gels containing 7% formaldehyde and transferred to a Hybond-N nylon membrane (RPN3050, Amersham, Buckinghamshire) in 150 mM ammonium acetate (Khélifa et al., 1994). With the PH1 probe, prehybridization was performed for 2 h at 42°C in 50% formamide, 5× SSC (SSC is 0.15 M NaCl, 15 mM sodium citrate), 50 mM phosphate buffer, pH 6.8, 5× Denhardt's solution, 1% SDS. Hybridization was then performed for 20 h in the same buffer, with 1× Denhardt's solution, containing the $^{32}$P-labeled cDNA probe. The membrane was washed twice at room temperature in 0.1% SDS, 2× SSPE (SSPE is 10 mM NaH$_2$PO$_4$, 0.13 M NaCl, 1 mM EDTA) and twice at 50°C for 30 min in 0.1% SDS/0.5× SSPE. With the SP1′ probe, prehybridization and hybridization were performed in the buffers described above containing 40% formamide. The second wash of the membrane was performed in 0.1% SDS, 1× SSPE.

**Nuclear Extracts.** Nuclear extracts from DC-3F and DC-3F/S16 cells were prepared from 7 to $8 \times 10^7$ exponentially growing cells as previously described (Riou et al., 1991). Freshly prepared protease inhibitor phenylmethylsulfonyl fluoride (1 mM), benzamidine (1

mM), aprotinin (1 $\mu$g/ml), and soybean trypsin inhibitor (10 $\mu$g/ml) were added to all buffers. Protein concentration in the nuclear extracts was determined as previously described (Bradford, 1976). The extracts were used within 2 days.

**Western Blot.** Freshly prepared nuclear extracts (100 $\mu$g) were fractionated on 7.5% SDS-polyacrylamide gels. The proteins were transferred (4 mA/cm$^2$) to a nitrocellulose membrane (BioTrace NT, Gelman Sciences, East Hills, NY). Membranes were incubated with the polyclonal anti-topoisomerase II antibody A6. Bound antibody was visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence detection (Amersham, Buckinghamshire).

**Measurements of DNA Damage by Alkaline Elution.** The methodology of DNA alkaline elution has been previously described (Kohn et al., 1981). DNA single-strand breaks (SSBs) were assayed by DNA-denaturing alkaline elution carried out under deproteinizing conditions. Cellular DNA in exponentially growing DC-3F and DC-3F/S16 cells was radioactively labeled for 20 h at 37°C by adding to the growth medium either [2-$^{14}$C]thymidine (0.02 $\mu$Ci/ml, 56 mCi/mmol; Amersham, France) or [*methyl*-$^3$H]thymidine (0.1 $\mu$Ci/ml, 20 mCi/mmol; Amersham, France) diluted in $10^{-6}$ M unlabeled thymidine (internal standard cells). After the radioactive medium was removed, the cells were grown for at least 2 h in label-free medium before any experiment. [$^{14}$C]Thymidine-labeled cells were treated with the drug at increasing concentrations for 1 h. Drug treatments were terminated by removal of the drug-containing medium and resuspension of the cells in ice-cold Hanks' balanced solution containing 0.02% EDTA. $^{14}$C-labeled cells were mixed with untreated $^3$H-labeled cells (internal standard) that had been irradiated, on ice, with 1000 rad equivalents. The alkaline elution technique was the low-sensitive assay to detect SSB up to 3000 SSB rad equivalents (Kohn et al., 1981). The elution rate of [$^3$H]thymidine-labeled cells served to normalize the elution rate of DNA from cells labeled with [$^{14}$C]thymidine.

**Topoisomerase II-Mediated Cleavage Reactions.** pSP65 DNA (0.2 $\mu$g) was incubated with 5 $\mu$g of nuclear extracts from the different cell lines, in the absence or presence of drug, in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, 0.1 mM EDTA, 15 $\mu$g/ml BSA, 1 mM ATP for 15 min at 37°C. The cleavage reaction (15 $\mu$l) was terminated by the addition of SDS and proteinase K to final concentrations of 0.4% and 0.1 mg/ml, respectively, and the mixture was incubated for an additional 30 min at 50°C. After the addition of 4 $\mu$l of loading buffer (0.4% bromphenol blue, 0.4% xylene cyanol, 50 mM EDTA, 50% glycerol), the products of the reaction were fractionated by electrophoresis on 1% agarose gels for about 15 h (2.5 V/cm) in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) containing 0.5 $\mu$g/ml of ethidium bromide. DNA bands were visualized by transillumination with UV light and quantified by scanning the gel with a Bioprofil Scan (Vilbert-Lourmat, Marne-la-Vallée, France).

**Cloning of Topoisomerase II$\alpha$ and $\beta$ cDNAs from DC-3F/S16.** Polyadenylated RNAs were purified as described above. The cDNA libraries were constructed using the Zap cDNA synthesis kit from Stratagene (La Jolla, CA), following the manufacturer's instructions. cDNAs were cloned in the uni-ZAP XR vector and packaged in $\lambda$-phagemide as previously described (Dereuddre et al., 1995). Approximately $4 \times 10^5$ phages from each library were plated out and screened by plaque hybridization with the probes SP1′ or HP1. 9 plaques hybridizing with SP1′, and six plaques hybridizing with HP1 were picked up and purified by tertiary screening. cDNA fragments from single positive plaques were excised by digestion with *Xho*I and *Eco*RI. Six clones were found to contain inserts with sizes greater than 4.6 kb, the approximate size expected for a complete topoisomerase II$\alpha$ cDNA. Topoisomerase II$\beta$ cDNA contains two *Eco*RI-sensitive sites, and two clones presented the expected digestion pattern. pBluescript phagemids were then excised from the uni-ZAP vector and transfected in XL1-Blue bacteria, following the Stratagene protocol. Restriction analysis showed that these frag-

ments spanned the entire coding region of the topoisomerase IIα and β cDNAs, with approximately 95 and 160 bp upstream of the initiation codon, respectively.

**Sequencing of Topoisomerase IIα and β cDNAs.** DNA sequencing was performed and analyzed at ACT Gene-Euro Sequence Genes Services (Génopole, Evry, France), on an ABI 377 sequencer with an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit and AmpliTaq polymerase FS (Perkin-Elmer/Applied Biosystems division, Foster City, CA).

## Results

**Selection of S16020-2-Resistant Cells.** S16020-2-resistant DC-3F cells were selected by adding stepwise increasing drug concentrations to the cell growth medium. At each concentration (2, 5, 20, 50, and 100 nM), the cells were maintained until they recovered a normal growth pattern. After about 6 months of drug exposure in these conditions, a subline was established that was able to grow normally in the presence of S16020-2 at 100 nM with a doubling time (12 h) identical with that of the parental cells. From this subline, three independent clones were isolated that displayed identical properties, and one of them, designated DC-3F/S16, was chosen for further characterization.

Figure 2 shows the survival curves of DC-3F and DC-3F/S16 cells after treatment for 3 h with increasing concentrations of S16020-2. In these experimental conditions, the resistance level to S16020-2 of the DC-3F/S16 cells, calculated from the ratio of the $IC_{50}$ values shown in Table 1, was
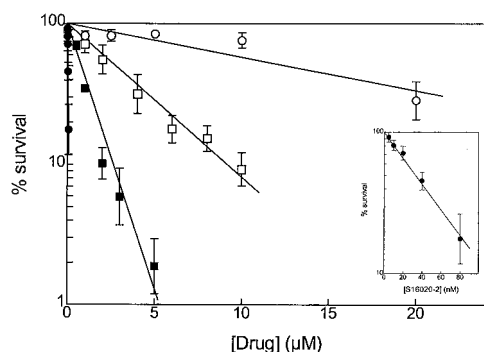


**Fig. 2.** Cytotoxicity of S16020-2 (●, ○) and NMHE (■, □) on DC-3F cells (filled symbols) and DC-3F/S16 cells (open symbols). Exponentially growing cells were treated with the drug at the indicated concentrations for 3 h. Cells were trypsinized, counted, and seeded, and after 6 to 7 days colonies were stained and counted as described under *Materials and Methods*. The inset shows an enlargement of the survival curve of DC-3F cells treated with S16020-2. Bar, S.D. of at least three independent determinations.

approximately 400-fold. This resistance level remained stable in cells grown in the absence of selecting agent for more than 4 months.

**Cross-Resistance to Other Compounds.** With the colony formation assay, we first examined the sensitivity of DC-3F/S16 cells to ellipticine derivatives. Figure 2 and Table 1 show that NMHE and 9-OH-olivacine were approximately 30-fold less cytotoxic than S16020-2 on DC-3F cells. Surprisingly, DC-3F/S16 cells were only 3- to 4-fold cross-resistant to these drugs. We also tested the sensitivity to 9-OH-E, which was almost as cytotoxic as S16020-2 on DC-3F cells (Table 1). Again, the cross-resistance of DC-3F/S16 cells to this compound was very low (approximately 12-fold). However, DC-3F/S16 cells were approximately 90-fold cross-resistant to S30761, a 9-OH-E derivative carrying the same substitutions as S16020-2 (S16020-2 side chain grafted at position 1 and a methyl at position 6). Table 1 also shows that DC-3F/9-OH-E cells, an ellipticine-resistant variant of DC-3F cells (Salles et al., 1982), were highly cross-resistant to S16020-2. This unexpected pattern of cross-resistance to drugs, which belong to closely related chemical series and display very similar in vitro properties, suggested that different mechanisms of action might be involved in the toxicity of these compounds.

Cross-resistance of DC-3F/S16 cells to other antitumor agents was determined using an assay based on cell counting in control and drug-treated cultures after 72 h of drug exposure. As previously discussed (Charcosset et al., 1985), resistance levels to ellipticine derivatives measured by this assay are approximately 1 order of magnitude lower than those measured by the clonogenic assay. Table 2 shows that in these conditions the resistance of DC-3F/S16 cells to S16020-2 was approximately 36-fold, whereas the cross-resistance to 9-OH-E and NMHE was less than 10-fold, thus confirming the results of the clonogenic assay. Again, the presence of the side chain on S30761 was associated with an elevated cross-resistance (23-fold). A very low cross-resistance, 3- to 4-fold, was observed with genistein. In contrast, DC-3F/S16 cells were highly cross-resistant to other topoisomerase II inhibitors, such as *m*-AMSA and etoposide, and to a lesser extent to adriamycin. Finally, DC-3F/S16 cells are not cross-resistant to antitumor agents that are not in vivo topoisomerase II inhibitors such as camptothecin, vincristine, or actinomycin D. The lack of cross-resistance to the latter two compounds suggested that the MDR phenotype was not expressed in the DC-3F/S16 cells. Indeed, Northern blot analysis, with a probe that recognizes the transcripts from the three genes, *pgp1*, *2*, and *3* (Delaporte et al., 1991),

TABLE 1

Cross-resistance of DC-3F/S16 and DC-3F/9-OH-E cells to ellipticine derivatives

Drug toxicity was determined by the clonogenic assay after 3 h of drug exposure (see *Materials and Methods*).

| | DC-3F $IC_{50}$[a] | DC-3F/S16 | | DC-3F/9-OH-E | |
|---|---|---|---|---|---|
| | | $IC_{50}$[a] | R[b] | $IC_{50}$[a] | R[b] |
| | μM | μM | | μM | |
| S16020-2 | 0.030 ± 0.007 | 12.4 ± 5 | 413 | 12.5 ± 2.4 | 415 |
| S30761 | 0.045 ± 0.002 | 4.0 ± 0.3 | 90 | 5.7 ± 0.6 | 127 |
| 9-OH-olivacine | 0.11 ± 0.04 | 0.47 ± 0.19 | 4 | ND[c] | |
| NMHE | 0.80 ± 0.08 | 2.8 ± 0.03 | 3.5 | 110 ± 20 | 138 |
| 9-OH-E | 0.074 ± .004 | 0.93 ± 0.1 | 12 | 14.4 ± 1.3 | 194 |

[a] $IC_{50}$, drug concentration inhibiting cloning efficiency by 50% as measured after 3 h of exposure to the drug by the colony formation assay.
[b] R, resistance factor was calculated as the ratio of $IC_{50}$ values in DC-3F/S16:DC-3F or in DC-3F/9-OH-E:DC-3F.
[c] ND, not determined.

did not detect any increase in the expression of either one of these genes in the S16020-2-resistant cells (data not shown).

**Tumorigenicity of DC-3F/S16 Cells.** A decreased tumorigenicity is frequently observed in drug-resistant cell lines, including DC-3F/9-OH-E cells, the ellipticine-resistant variant of DC-3F cells (Remy et al., 1984). S16020-2-sensitive and -resistant cells were tested for their ability to form tumors after s.c. injection into nude mice. As previously reported (Remy et al., 1984), DC-3F cells were highly tumorigenic, with 100% tumor take within 1 week. Inoculation of $2 \times 10^6$ DC-3F/S16 cells provoked tumor take in approximately 70% of the animals within 10 days. After inoculation of $1 \times 10^6$ cells, the tumor take was reduced to 30% of the mice, whereas the latency was increased up to 1 month. These results show that development of resistance to S16020-2 was associated with a decreased oncogenic potential, although to a lesser extent than in DC-3F/9-OH-E cells, which completely lost their tumorigenicity.

**Cellular Accumulation and Efflux of S16020-2.** Cellular accumulation of S16020-2 by parental and resistant cells was measured by exposing cells to the $^3$H-labeled drug at $5 \times 10^{-8}$ M for the indicated times. Figure 3A shows that, after a rapid increase during the first 20 min, the drug uptake reached a plateau that remained stable for 3 h. This plateau was slightly higher (12%) for the resistant than for the sensitive cells. S16020-2 efflux kinetics were measured on the sensitive and resistant cells previously loaded for 30 min with the drug. The efflux kinetics (Fig. 3A) were identical for both cell lines: 50% of the drug was lost during the first 30 min, and the efflux progressively slowed down for the next 3.5 h. At that time, approximately 10% of the initial amount of drug was still present in each cell line.

Uptake of S16020-2 at different external concentrations was also determined in both cell lines. The cells were incubated for 30 min with the drug at concentrations ranging from $2 \times 10^{-8}$ M to $1.6 \times 10^{-7}$ M. In these conditions, the uptake of the drug increased linearly, and there was no difference between the sensitive and resistant cells (Fig. 3B).

**Stimulation of DNA Cleavage by DNA Topoisomerase II Poisons.** Stimulation of topoisomerase II-mediated DNA cleavage is a critical feature in the mechanism of action of many topoisomerase II inhibitors, and such a stimulation by S16020-2 was previously demonstrated in an in vitro assay using purified DNA topoisomerase II (Le Mée et al., 1998).

This experiment was repeated to compare nuclear extracts from the parental and resistant cell lines. Figure 4, A and B, shows that increasing concentrations of S16020-2 and NMHE stimulated the cleavable complex formation in the presence of nuclear extracts from DC-3F cells after a biphasic process, as previously described for other DNA intercalators (Pommier et al., 1986). At low concentrations, the amount of DNA cleavage increased with the drug concentration, whereas concentrations greater than 6 $\mu$M for S16020-2 and 8 $\mu$M for NMHE inhibited the cleavage. With the nuclear extracts from resistant cells, 75 and 45% reduced DNA cleavage was observed in the presence of S16020-2 and NMHE, respectively. Figure 4, C and D, also shows that DNA cleavage in the presence of nuclear extracts from the sensitive cells was stimulated by m-AMSA and etoposide in a concentration-dependent manner up to about 5 $\mu$M, and above that concentration the reaction tended to a plateau. With the nuclear extracts from DC-3F/S16 cells, the formation of the cleavable complex induced by m-AMSA and etoposide was decreased by 75 and 55%, respectively.

Trapping of DNA topoisomerase II on the cell genome by inhibitors can be observed in intact cells by the quantification of DNA single-strand breaks with the alkaline elution technique. Formation of DNA single-strand breaks was measured
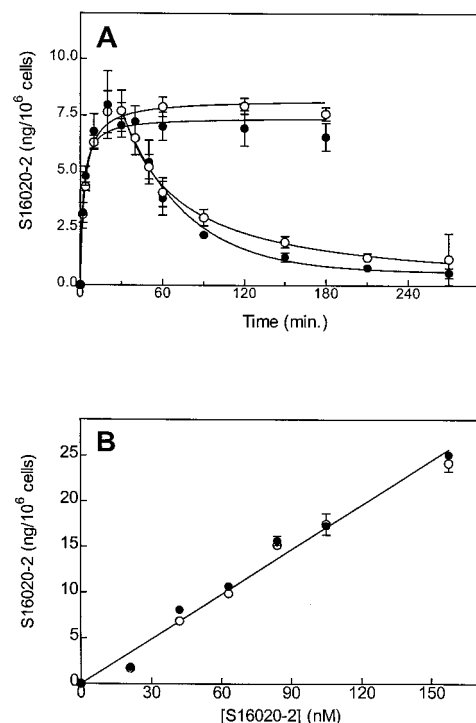
**Fig. 3.** Uptake and efflux kinetics of [$^3$H]S16020-2 by the parental (○) and S16020-2-resistant cells (○) at 37°C. A, cells were incubated with the drug for the indicated times. For efflux kinetics, cells, loaded with the drug for 30 min, were then incubated in drug-free medium for the indicated times. The amount of intracellular drug was determined as described under *Materials and Methods*. Values are means of two individual experiments, each done in triplicate. Bar, S.D. B, uptake of S16020-2 at different external concentrations by sensitive and resistant cells. The cells were incubated for 30 min with the drug at the indicated concentrations. The amount of intracellular drug was determined as in A.

TABLE 2

Cross-resistance of DC-3F/S16 to different antitumor agents

Drug toxicity was determined by the 72-h drug exposure assay (see *Materials and Methods*).

| | $IC_{50}$[a] | | $R$[b] |
|---|---|---|---|
| | DC-3F | DC-3F/S16 | |
| | *nM* | | |
| S16020-2 | 5 ± 1.8 | 180 ± 20 | 36 |
| S30761 | 7 ± 0.1 | 160 ± 10 | 23 |
| 9-OH-E | 32 ± 0.9 | 230 ± 40 | 7 |
| NMHE | 220 ± 30 | 840 ± 170 | 4 |
| Genistein | 6,600 ± 150 | 21,000 ± 5 | 3 |
| m-AMSA | 6 ± 0.4 | 1,960 ± 40 | 330 |
| Etoposide | 41 ± 4 | 4,800 ± 450 | 120 |
| Adriamycin | 5.3 ± 0.6 | 160 ± 6 | 30 |
| Vincristine | 2.5 ± 0.06 | 3.9 ± 0.1 | 1.6 |
| Actinomycin D | 0.39 ± 0.01 | 0.72 ± 0.05 | 1.8 |
| Camptothecin | 50 ± 1.6 | 67 ± 3.5 | 1.3 |

[a] $IC_{50}$, drug concentration inhibiting cell growth by 50% as measured by cell counting after 72 h of exposure to the drug (see *Materials and Methods*). Values are means ± S.D. of three to 15 determinations.
[b] R, resistance factor was calculated as the ratio of $IC_{50}$ values in DC-3F/S16:DC-3F.

after a 1-h exposure of DC-3F and DC-3F/S16 cells to the drugs. As previously shown (Le Mée et al., 1998), cleavable complex formation in the presence of S16020-2 was observed at concentrations about 500-fold lower than with NMHE (Fig. 5, A and B), which is markedly different from the in vitro experiments, in which conditions these compounds were active at very close concentrations. Formation of single-strand breaks was completely abolished in the DC-3F/S16 cells treated with S16020-2, *m*-AMSA, or etoposide (Fig. 5A, C and D). In contrast, a residual amount of complex formation, representing about 20% of the amount detected in the sensitive cells at 20 $\mu$M, was present in the resistant cells treated with NMHE (Fig. 5B). After a 3-h treatment, NMHE killed DC-3F cells at IC$_{50}$ concentrations of 0.5 to 1 $\mu$M. Since NMHE induced the complex formation with a much lower efficiency than the three other drugs, the technique was not sensitive enough to quantify the amount of single-strand breaks formed in sensitive and resistant cells at concentrations lower than 10 $\mu$M. However, this experiment suggested that the relative amount of cleavable complex formation induced by NMHE in DC-3F/S16 cells at concentrations close to the IC$_{50}$ might be sufficient to explain their low cross-resistance to this compound.

It should also be noted that the effects of these different drugs on the DC-3F/S16 cells are markedly different from their effects on DC-3F/9-OH-E cells: in the 9-OH-E-resistant cells, both in vitro and in vivo, ellipticine derivatives did not induce any detectable cleavable complex formation, whereas in the presence of *m*-AMSA and etoposide a 15 to 20% residual complex formation was observed (Pommier et al., 1986; Dereuddre et al., 1997). Although the mechanism underlying these differences is not presently understood, they are consistent with the other data indicating that different resistance mechanisms are involved in the resistance of DC-3F cells to S16020-2 and 9-OH-E.

**Analysis of DNA Topoisomerases II$\alpha$ and $\beta$ Expression in the DC-3F/S16 Cells.** The above results, showing the cross-resistance of the DC-3F/S16 cells to various topoisomerase II inhibitors and the alterations in the capacity of these compounds to induce the cleavable complex formation, suggested that quantitative and/or qualitative alterations of the DNA topoisomerase II activities might be involved in the resistance phenotype of DC-3F/S16 cells.

Expression of the topoisomerases II$\alpha$ and $\beta$ genes was first examined by Northern blot analysis. After fractionation by agarose gel electrophoresis, the mRNAs were successively hybridized with the SP1′ and HP1 probes, which specifically recognize the $\alpha$- and $\beta$-transcripts, respectively (Khélifa et al., 1994; Dereuddre et al., 1997). Figure 6B shows that expression of the $\alpha$-transcript was not modified in the DC-3F/S16 cells, whereas the $\beta$-transcript was 2- to 3-fold less abundant in the resistant as compared to the sensitive cells.

The amount of topoisomerase II$\alpha$ and $\beta$ present in the nuclear extracts from the DC-3F and the DC-3F/S16 cells was then analyzed by immunoblot using the A6 antibody. This antibody, raised against a 837-amino acid peptide from human topoisomerase II$\alpha$, recognizes both topoisomerase II isoforms (Khélifa et al., 1994). Figure 6A shows that the amount of isoform $\alpha$ was not modified in the DC-3F/S16 cells compared with the parental DC-3F cells, whereas the band corresponding to topoisomerase II$\beta$ was fuzzy and therefore difficult to quantify.

**Cloning and Sequencing of DNA Topoisomerases II$\alpha$ and $\beta$ cDNAs from DC-3F/S16 Cells.** To further investigate the molecular basis of the reduced formation of DNA single-strand breaks in S16020-2-resistant cells, the complete cDNAs encoding topoisomerases II$\alpha$ and $\beta$ in DC-3F/S16 cells were cloned and sequenced. The sequence of the isoform $\beta$ cDNA was the same in the sensitive and resistant cells. In contrast, sequencing of the cDNA-encoding isoform $\alpha$
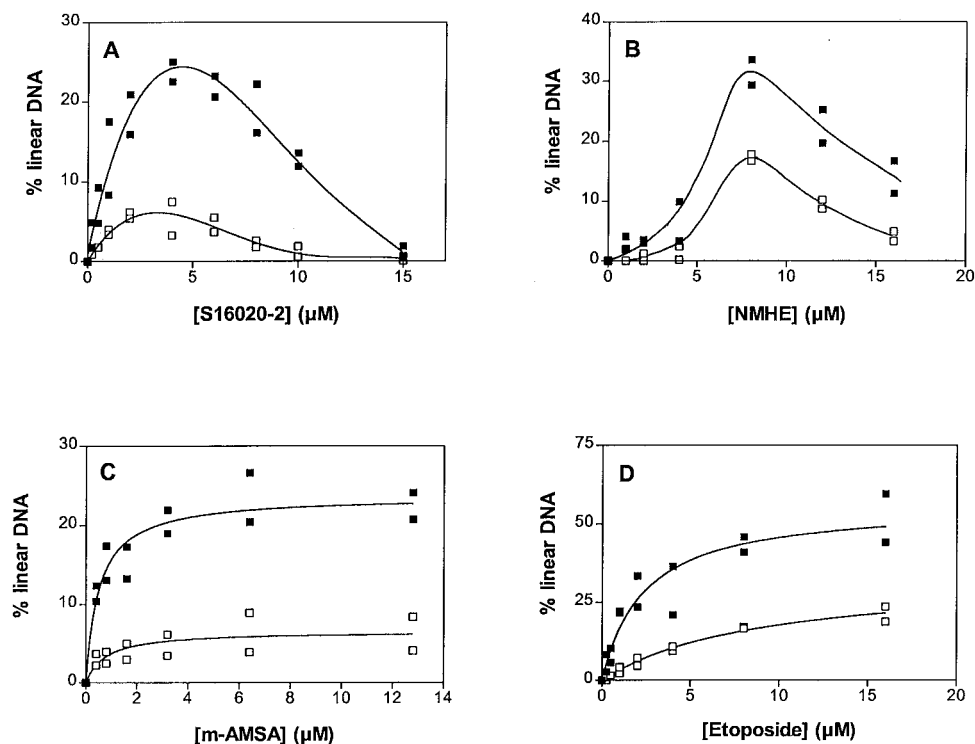


**Fig. 4.** Stimulation by S16020-2, NMHE, *m*-AMSA, and etoposide of DNA cleavage induced by nuclear extracts from DC-3F and DC-3F/S16. Nuclear extracts (5 $\mu$g) from parental (filled symbols) and resistant (open symbols) cells were incubated with 0.2 $\mu$g of pSP65 DNA in the presence of the indicated concentrations of S16020-2 (A), NMHE (B), *m*-AMSA (C), and etoposide (D). After agarose gel electrophoresis separation, the amount of linear DNA was quantified. The results of two independent experiments are shown.

revealed several differences compared with the sequence previously reported by Chan et al. (1993) for the sequence of the topoisomerase II$\alpha$ cDNA from a Chinese hamster ovary cell line. The open reading frame, also extending from nucleotide 1 to 4579, contained seven silent mutations, spread all over the coding region, and three additional mutations in the noncoding region (Table 3). Furthermore, a point mutation was identified at position 1456 that converts a codon AGA to a GGA, resulting in a missense mutation that changes Arg[486] to Gly (Table 3). This mutation was confirmed in three independent clones by sequencing of the corresponding region.

The presence of the silent mutations suggested an allele polymorphism in the parental DC-3F cells. PCR primers were designed to generate a 151-bp fragment containing the silent mutation at position 3933 that creates a *Bam*HI site and an *Acs*I site that would be common to both alleles. Therefore, it was possible to identify each allele in the DC-3F and DC-3F/S16 cells by its sensitivity to these enzymes. One 815-bp fragment was amplified from the genomic DNA with these primers, indicating the presence of intronic sequences in this region (Fig. 7, lanes 1 and 4). The fragment amplified from the DC-3F DNA was only partially digested by *Bam*HI (Fig. 7, lane 2). In contrast, the fragment amplified from the DC-3F/S16 DNA was completely digested by *Bam*HI, yielding two fragments of 716 and 99 bp (Fig. 7, lane 5). As expected, the DNAs from the two cell lines were completely digested by *Acs*I (Fig. 7, lanes 3 and 6). These results confirm that two different alleles are present in the parental cell line, one differing from the other by the presence of the silent mutations. The DC-3F/S16 cells only contain the allele with the silent mutations and the missense mutation.

**Role of DNA Topoisomerases II$\alpha$ and $\beta$ in the Resistance of DC-3F/9-OH-E Cells to S16020-2.** In the Chinese hamster lung cell line DC-3F/9-OH-E, made resistant to 9-OH-E, the amount of topoisomerase II$\alpha$ is 4- to 5-fold lower than in the parental DC-3F cell line. A mutation generating a stop codon in the topoisomerase II$\beta$ gene completely abol-

ishes the expression of this isoform in DC-3F/9-OH-E cells. To analyze the contribution of these quantitative alterations, the DC-3F/9-OH-E cells were transfected with a eukaryotic expression vector containing either the human $\alpha$-isoform or the hamster $\beta$-isoform (Dereuddre et al., 1997; Khélifa et al., 1999). The restoration of a normal topoisomerase II$\alpha$ or $\beta$ catalytic activity in the resistant cells had only very little effect on the toxicity of ellipticine derivatives. Because DC-3F/9-OH-E cells are highly cross-resistant to S16020-2, the sensitivity of $\alpha$- and $\beta$-transfected DC-3F/9-OH-E cells to S16020-2 was also determined. Figure 8 shows that the cytotoxicity of S16020-2 in DC-3F/9-OH-E cells transfected with either topoisomerase II$\alpha$ (clone 24)or $\beta$ (clone 11) cDNA, determined by colony formation assay, remained exactly the same as in the untransfected cells. In contrast, there was a partial reversion of sensitivity to *m*-AMSA in clone 24 ($\alpha$-enzyme) and an almost complete reversion in clone 11 ($\beta$-enzyme) (data not shown). Increased sensitivity of the transfected cells to these compounds was associated with an increased formation of the cleavable complex. In contrast, ellipticine derivatives remained unable to induce the cleavable complex formation in the $\alpha$- or $\beta$-transfected DC-3F/9-OH-E cells, which is consistent with the topoisomerase II-mediated mechanism of action of these compounds.

## Discussion

The data presented in this paper, together with our previous in vitro studies (Le Mée et al., 1998), indicate that: 1) DNA topoisomerases II are intracellular targets of S16020-2; 2) interaction of S16020-2 with these enzymes is strongly influenced by the *N*-[2(dimethylamino)ethyl]carbamoyl side chain; and 3) despite common properties between S16020-2 and other olivacine derivatives, in vivo induction of the cleavable complex in the presence of this compound appears to involve a peculiar mechanism, the al-
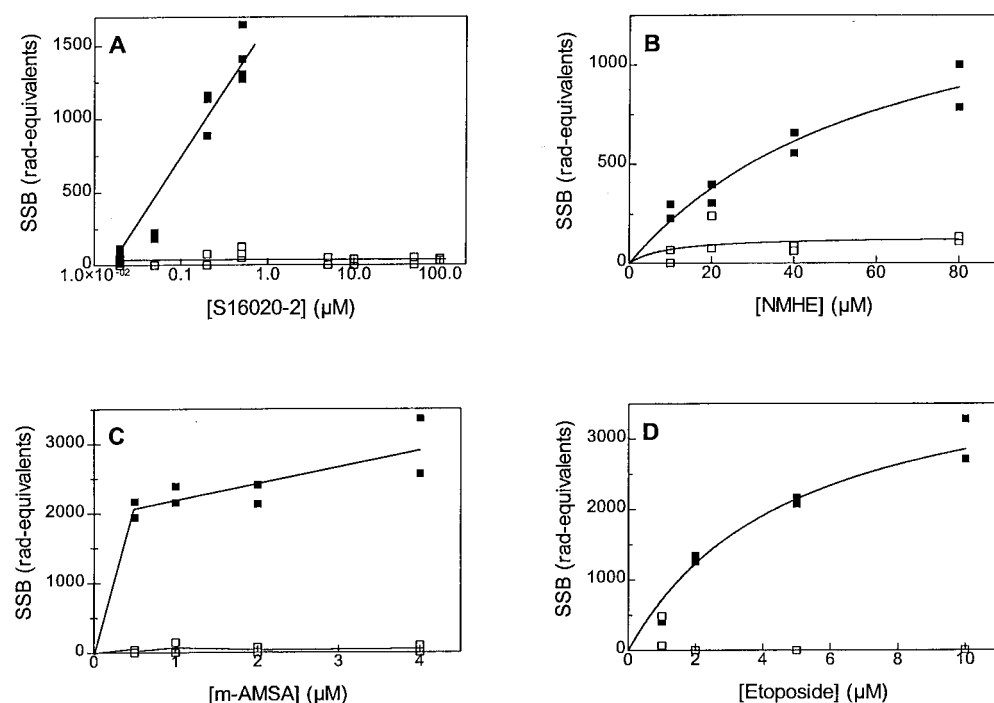


**Fig. 5.** DNA SSBs induced by S16020-2 (A), NMHE (B), *m*-AMSA (C), and etoposide (D) in DC-3F and DC-3F/S16 cells. DC-3F (filled symbols) and DC-3F/S16 cells (open symbols) were exposed to the indicated drug concentrations for 1 h at 37°C. The drug was then removed by washing the cells twice with Hanks' balanced salt solution at 0°C. Cells were scraped, and DNA SSBs were measured by DNA-denaturing alkaline elution. Note that abscissa in A is logarithmic.

teration of which would account for the properties of DC-3F/S16 cells.

S16020-2 induces cleavable complex formation in vivo at concentrations that are approximately 500-fold lower than NMHE, whereas, in in vitro experiments using purified enzyme, both drugs displayed similar properties. We first hypothesized that this difference might result from a higher intracellular accumulation and/or a peculiar intracellular localization of the molecule, because of the relative hydropho-

bicity of its side chain (Auclair et al., 1987b). Because S16020-2 is not fluorescent, its intracellular localization will be difficult to analyze. Comparison of uptake and efflux kinetics of S16020-2 with those of NMHE (Charcosset et al., 1983; Delaporte et al., 1988) showed that S16020-2 uptake rate was faster than that of NMHE, whereas efflux rates were identical for both drugs. Determination of drug accumulation showed that, for an equal external concentration, the amount of intracellular S16020-2 was approximately 4-fold higher than that of NMHE. However, because S16020-2 is 30-fold more cytotoxic than NMHE, its estimated intracellular amount at equitoxic concentrations was approximately 6-fold lower than that of NMHE (40 and 250 $\mu$M, respectively). Therefore, a higher intracellular accumulation cannot account for the in vivo activity of S16020-2. This indicates that different mechanisms are involved in the toxicity of S16020-2 and NMHE, a conclusion also strongly sup-
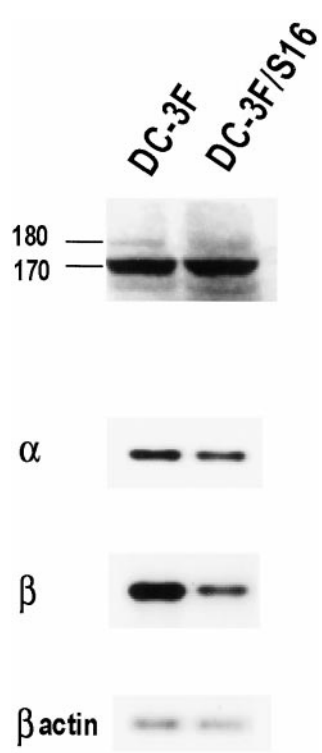


**Fig. 6.** Expression of topoisomerase II$\alpha$ and $\beta$ in DC-3F/S16 cells. A, immunoblot analysis. Nuclear proteins (100 $\mu$g) extracted by 0.35 M NaCl from S16020-2-sensitive and -resistant cells were separated on SDS-polyacrylamide gel, transferred to nylon membranes, and probed with the A6 antibody. B, Northern blot analysis. Topoisomerase II transcripts were fractionated by agarose gel electrophoresis (1.2%) and transferred to a nylon membrane. The RNAs were sequentially hybridized with the SP1′ ($\alpha$), HP1 ($\beta$), and $\beta$-actin probes.

TABLE 3

Topoisomerase II$\alpha$ allele polymorphism in DC-3F/S16

The accession number to the complete sequence of the DC-3F/S16 topoisomerase II$\alpha$ cDNA in the EMBL/EBI database is Y16595.

| Nucleotide No. | Nucleotide Change | Amino Acid Change |
|---|---|---|
| Silent mutations in the *Bam*HI-sensitive allele | | |
| 87 | T → C | Ile$^{29}$ → Ile$^{29}$ |
| 342 | A → G | Leu$^{114}$ → Leu$^{114}$ |
| 2877 | T → C | Tyr$^{959}$ → Tyr$^{959}$ |
| 3072 | C → T | Leu$^{1024}$ → Leu$^{1024}$ |
| 3630 | C → T | His$^{1210}$ → His$^{1210}$ |
| 3933 | C → T | Asp$^{1311}$ → Asp$^{1311}$ |
| 4314 | A → G | Ser$^{1438}$ → Ser$^{1438}$ |
| 5114 | G → T | Noncoding region |
| 5346 | A → G | Noncoding region |
| 5408 | G → A | Noncoding region |
| Missense mutation in DC-3F/S16 | | |
| 1456 | A → G | Arg$^{486}$ → Gly$^{486}$ |
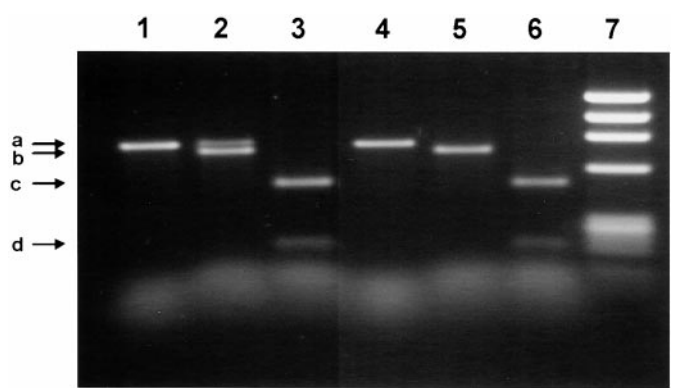


**Fig. 7.** Topoisomerase II$\alpha$ allele polymorphism. From the genomic DNA, 815-bp fragments, containing the *Bam*HI site associated with the mutation at position 3933 in the cDNA of the mutated allele, were amplified with appropriate primers. After the sample was digested with the restriction enzymes *Bam*HI and *Acs*I, the products were analyzed by agarose gel electrophoresis. Lanes 1 and 4, undigested fragments, amplified from DC-3F and DC-3F/S16 cells; lanes 2 and 3, *Bam*HI and *Acs*I fragments from DC-3F cells; lanes 5 and 6, *Bam*HI and *Acs*I fragments from DC-2F/S16 cells; lane 7, size markers. The sizes of the fragments, labeled a to d, are 815, 716, 486, and 230 bp, respectively. A 99-bp fragment, generated by either *Bam*HI or *Acs*I digestion, was not visible in these experimental conditions.
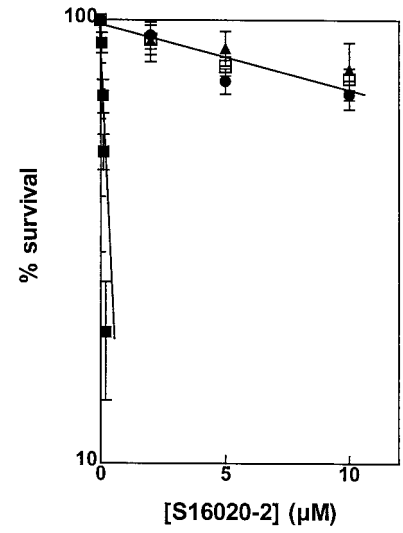


**Fig. 8.** Cytotoxicity of S16020-2 in DC-3F (■), DC-3F/9-OH-E (□), clone 11 (●), and clone 24 (▲) cells. Experimental details are as in Fig. 2.

ported by analysis of cross-resistance patterns in DC-3F/9-OH-E and DC-3F/S16 cells.

**Analysis of Cross-Resistance Patterns.** DC-3F/9-OH-E cells, selected up to the highest possible level of resistance to 9-OH-E, display a complex phenotype (Larsen and Jacquemin-Sablon, 1985). Among other traits, these cells are highly cross-resistant to all topoisomerase II inhibitors, including S16020-2. This resistance involves a 4- to 5-fold decreased expression of a mutated topoisomerase II$\alpha$ and the complete loss of the $\beta$-enzyme (Khélifa et al., 1994). Restoration of a normal $\alpha$- or $\beta$-activity in DC-3F/9-OH-E cells, by transfection of the corresponding cDNAs, had similar effects on their drug sensitivity: formation of cleavable complexes and sensitivity to etoposide and $m$-AMSA were partially restored, whereas resistance to ellipticine derivatives, genistein, and S16020-2 was unchanged, these compounds remaining unable to induce the cleavable complex formation (Dereuddre et al., 1997; Khélifa et al., 1999). We interpreted these data as indicating that: 1) topoisomerase II alterations in DC-3F/9-OH-E cells were associated with cross-resistance to all topoisomerase II inhibitors; and 2) resistance to drugs that do not inhibit the religation step (ellipticines and genistein) involves a peculiar mechanism not presently understood. Because S16020-2 belongs to this latter group (Le Mée et al., 1998), the same mechanism should account for the resistance of DC-3F/9-OH-E cells to this compound.

The cross-resistance pattern of DC-3F/S16 cells was very different: despite an elevated resistance to S16020-2, these cells are poorly cross-resistant to other compounds, such as NMHE, 9-OH-E, and genistein, but highly cross-resistant to the religation inhibitors etoposide and $m$-AMSA. Ellipticine derivatives and S16020-2 are poor substrates of the Pgp protein (Delaporte et al., 1988; Pierré et al., 1998). Absence of any detectable alteration of S16020-2 cellular accumulation or efflux in DC-3F/S16 cells and lack of cross-resistance of these cells to drugs sensitive to the MDR, MRP protein, and lung resistance protein phenotypes indicate that none of these mechanisms is involved in the resistance to S16020-2 or in the cross-resistance of DC-3F/S16 cells to other drugs. Both in vivo and in vitro, the resistance to DNA topoisomerase II inhibitors in DC-3F/S16 cells is associated with a decreased formation of the cleavable complex. In vivo, the alkaline elution technique did not detect any cleavable complex in resistant cells treated with S16020-2, etoposide, and $m$-AMSA. In contrast, in presence of 20 $\mu$M NMHE, the amount of residual complex in DC-3F/S16 cells was 20 to 25% of that detected in sensitive cells. The sensitivity of the technique does not allow an accurate determination of the complex formation at concentrations close to the $IC_{50}$ value for NMHE in DC-3F/S16 cells (0.5–1 $\mu$M). However, it is likely that, at cytotoxic concentrations of NMHE in DC-3F/S16 cells, the formation of cleavable complexes should be sufficient to explain their low cross-resistance to this compound.

**Importance of the $N$-[2(Dimethylamino)ethyl]carbamoyl Side Chain.** Alterations in drug capacity to increase the cleavable complex formation indicated that resistance of DC-3F/S16 cells to topoisomerase II inhibitors should be related to quantitative and/or qualitative alterations of either topoisomerase II$\alpha$ or $\beta$ or both. Northern and immunoblot analyses did not reveal any change in the expression of topoisomerase II$\alpha$. The amount of topoisomerase

II$\beta$ transcripts was approximately 50% lower in resistant than in sensitive cells, whereas the aspect of the band in Western blot was more fuzzy and therefore difficult to quantify. Because previous studies indicated that $m$-AMSA may target primarily the $\beta$-enzyme (Dereuddre et al., 1997; Herzog et al., 1998), transcriptional and/or eventual post-translational alterations of this isoform might account at least partially for the high cross-resistance of DC-3F/S16 cells to this compound. The only genetic modification identified in S16020-2-resistant cells is a point mutation converting Arg[486] to a Gly in the topoisomerase II$\alpha$ cDNA. Arg[486] is part of a sequence of eight amino acids -Pro-Leu-Arg-Gly-Lys-(Leu/Ile/Met)-Leu-Asn- present in all eukaryotic and bacterial topoisomerases II. In HL60 cells (Lee et al., 1992) and in patients with small cell lung cancer (Kubo et al., 1996), replacement of Arg[487] (corresponding to hamster Arg[486]) by a Lys has been associated with the acquisition of the resistance to $m$-AMSA and etoposide, whereas the same substitution had no effect in yeast (Wasserman and Wang, 1994). In the B subunit of *Escherichia coli* DNA gyrase, a mutation of Lys[447] (position corresponding to Arg[486] in the hamster enzyme) to glutamic acid confers resistance to nalidixic acid (Yamagishi et al., 1986). As previously suggested (Lee et al., 1992), the high degree of conservation and the drug resistance associated with the majority of the mutations of Arg[486] suggest that the -Pro-Leu-(Arg/Lys)-Gly-Lys-(Leu/Ile/Met)-Leu-Asn- sequence should play an important part in the topoisomerase II catalytic cycle and be involved in the formation of the ternary complex with certain drugs. Molecules like S16020-2 or $m$-AMSA would bind to DNA by intercalation and to the enzyme through interaction of their side chains with the -Pro-Leu-(Arg/Lys)-Gly-Lys-(Leu/Ile/Met)-Leu-Asn- sequence. This latter interaction would be essential to their pharmacological activity because either a mutation in the target sequence or a chemical modification of the drug side chain resulted in a decreased activity. DC-3F/S16 cells display a very low cross-resistance to drugs, like olivacine, 9-OH-E, or NMHE, which do not carry the appropriate side chain to interact with the target sequence and, therefore, are not sensitive to the Arg[486] mutation. We then predicted that DC-3F/S16 cells should be cross-resistant to S30761 (Fig. 1), an ellipticine derivative that carries the same side chain as S16020-2 and is almost as cytotoxic. Tables 1 and 2 show that this was indeed the case.

**Mechanism of Cleavable Complex Induction.** Biochemical and structural studies have led to a model that describes topoisomerase II as an ATP-modulated clamp with two molecular gates at opposite ends (Berger et al., 1996). The enzyme catalytic cycle is driven by a series of conformational changes triggered by cofactor binding. Because S16020-2 induces topoisomerase II-mediated DNA cleavage only in the presence of ATP, we hypothesize that it would only bind to the enzyme-DNA complex when it is in the ATP-bound conformation. In this conformation of the complex, the eight-amino acid target sequence would take a position proximal to the catalytic site, thus allowing S16020-2 to interact with both the enzyme and the DNA. The cleavage-religation equilibrium would then be shifted to the cleaved form, possibly through a deformation of the DNA molecule. Such a reaction would increase the number of cleavable complexes on the genome at a given time and would then account for the drug toxicity. After the T fragment

passage, the religation of the G fragment requires that the enzyme recovers a conformation similar to that before ATP binding, a transition that might involve the hydrolysis of one ATP molecule (Harkins and Lindsley, 1998; Harkins et al., 1998). This conformation change would provoke the release of the drug then unable to interfere with the religation step. The strict ATP dependence of S16020-2 would be a property dependent on the side chain. However, a similar mechanism could be extended to other compounds that do not inhibit the religation step, except that they might enter the enzyme-DNA complex before the ATP binding.

## Acknowledgments

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