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Report

Altered DNA-cleavage activity of topoisomerase II from WEHI-3B leukemia cells with specific resistance to ciprofloxacin

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In order to investigate the mechanisms of drug resistance arising in tumor cells, we investigated the capacity of fluoroquinolones to inhibit the *in vitro* growth of WEHI-3B monomyelocytic leukemia cells and then we established a variant of this line (currently maintained in the absence of drug). The line, named WEHI-3B/CPX, expresses a specific resistance to ciprofloxacin (CPX; resistance index=17.3 ± 2.2), and does not show cross-resistance with other fluoroquinolones, camptothecin and topoisomerase II inhibitors such as doxorubicin, etoposide and teniposide. Although a little decrease in intracellular accumulation of CPX is observed in WEHI-3B/CPX cells, these cells do not express MDR or LRP markers, and the resistance is not circumvented by verapamil. Purified nuclear extracts from WEHI-3B and WEHI-3B/CPX cells were tested for topoisomerase I catalytic activity and checking *in vitro* topoisomerase I sensitivity to CPX and camptothecin inhibition, but no difference was observed. As the treatment with CPX showed that the resistant cell line suffers a significantly lower number of breaks in the DNA molecule we also addressed our investigations to the topoisomerase II-dependent DNA cleavage that, in the resistant clone, was found dramatically less susceptible to be enhanced by CPX both in pre-strand and post-strand DNA passage conditions. WEHI-3B/CPX cells do not express any character of multidrug resistance and represent a rare case of specific drug resistance to CPX. The specific resistance to CPX observed in these cells is related to a functional decrease of topoisomerase II cleavage activity. It could be consequent to a decreased binding affinity of CPX for the topoisomerase II–DNA complex or to a decreased affinity or specificity of topoisomerase II for its DNA cleavage sites. [© 2001 Lippincott Williams & Wilkins.]

Key words: Ciprofloxacin, drug resistance, topoisomerases, WEHI-3B cells.

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Introduction

As is well known, multidrug resistance is a very important phenomenon which strongly reduces the efficacy of antineoplastic chemotherapy.[1–4] In order to study the mechanism able to produce drug resistance, many *in vitro* cell lines which express cross-resistance to a broad spectrum of drugs have been selected.[5] In our laboratory we demonstrated that murine myeloid progenitors (GM-CFU) and myelomonocytic leukemia cells WEHI-3B(D+) are sensitive to the inhibitory effect of fluoroquinolones (which are strong bactericidal agents having as target DNA gyrase, a prokaryotic type II topoisomerase). Although the mechanism of quinolones is not yet fully characterized, many reports indicated that these drugs also interfere with the functions of mammalian topoisomerases producing inhibition of their growth and proliferation.[6–8]

To investigate the mechanisms of inhibition of leukemia cell growth by fluoroquinolones we established, following exposure to increasing concentrations of the drug, a ciprofloxacin (CPX)-resistant line of murine myelomonocytic leukemia WEHI-3B(D+). This clone, named WEHI-3B/CPX, is very stable and studies on cross-resistance to other quinolones and to other topoisomerase inhibitors indicate that its resistance is not mediated by a MDR1 gene product.[9] This study compares the sensitivity to CPX of seven independent subclones derived from WEHI-3B/CPX and checks the parental line and the resistant clone for p110 protein expression (LRP),[10] DNA breakage and topoisomerase activity. Topoisomerase I preparations (from the parental line and the *in vitro* resistant variant) are tested for their sensitivity to increasing concentrations of CPX and camptothecin (CAM). The capacity of CPX to enhance the

topoisomerase II-dependent DNA cleavage activity was even investigated, finding that the enzyme from the resistant variant is 'resistant' to the CPX enhancement effect.

Materials and methods

Chemicals and reagents

CPX (348 FW) was purchased from Bayer (Wuppertal, Germany). Antineoplastic drugs doxorubicin (DOX, 580 FW) and CAM (348 FW) were purchased from Sigma (St Louis, MO). The drugs were dissolved in double-distilled autoclavated water (CPX) or DMSO (CAM) at a concentration of 10 000 mg/l, corresponding to 28.7 mM CPX and CAM, and 17.2 mM DOX. For testing the drugs activity on cell proliferation, working dilutions were performed in basal McCoy's medium (Seromed, Berlin, Germany). To test the *in vitro* activity on topoisomerases, the drug dilutions were performed in appropriate buffer as described below. Supercoiled plasmid pBR322 DNA and ATP were purchased from Sigma.

Cell growth and selection of resistant subclone

The selection of the CPX-resistant variant of the murine myelomonocytic leukemia WEHI-3B(D+)[11] was obtained by increasing the concentration of CPX in the culture medium according to a step-wise fashion previously described.[9] The resistant variant was subcultured in drug-free medium for more than 30 passages before testing. The stable resistant variant was subcultured by a limiting dilution technique, and seven subclones named A-1, B-10, E-5, E-9, G-4, G-11 and H-9 were isolated for testing their drug sensitivity. The parental strain, the resistant variant and the seven subclones were grown in 25 cm^2 plastic flasks (Nunc, Roskilde, Denmark) in McCoy's medium (Seromed) containing 5% FCS (Gibco, Grand Island, NY) in the absence of drug. The stability of the resistant phenotype was checked every 3 months by a MTT drug sensitivity test as described below.

In vitro drug sensitivity test

The drug sensitivity of the wild-type line (WEHI-3B), the CPX-resistant variant (WEHI-3B(D+)/CPX) and its subclones was determined by a MTT [3-(4,5 dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] colorimetric method on quadruplicate assays as previously described.[9]

Immunocytochemistry

The expression of p110 proteins in WEHI-3B(D+) and WEHI-3B(D+)/CPX cells was kindly checked by Dr A Izquierdo (Free University Hospital Amsterdam, The Netherlands) on cytocentrifuge preparations of the cell lines by using LRP-56 IgG2b monoclonal mouse antibodies. The preparation of antibodies and the immunocytochemistry procedure were performed as previously described.[10]

The expression of nuclear topoisomerase I has been verified on acetone-fixed cytospins of the cell lines using serum of a scleroderma patient (containing anti-topoisomerase I antibodies) diluted 1:2000.

The reaction was revealed by a peroxidase-labeled rabbit antiserum specific for human IgG (Dako, Glostrup, Denmark) diluted 1:100, followed by tyramide signal amplification (DuPont/NEN, Boston, MA) and development with diaminobenzidine (DAB; Sigma). Patient sera and peroxidase-labeled rabbit anti-human IgG were diluted in 0.05 M Tris-buffered saline (pH 7.2) containing 1% bovine serum albumin (fraction V; Sigma) and 0.001% Nonidet P-40 (Sigma).

Preparation of nuclear extracts

Nuclear extracts from exponentially growing cells were prepared according to the method suggested by Deffie *et al.*[12] with some modifications. Briefly, 120×10^6 cells, collected from a 72-h culture of 10^4 cells/ml, were washed in Nuclear Buffer at pH 6.5 (NB 6.5=100 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA-Na_2, 10% glycerol, 1 mM phenylmethylsulphonylfluoride (PMSF), 0.2 mM DTT and 10 μg/ml soybean trypsin inhibitor (STI)] and then resuspended in NB6.5 supplemented with 0.35% Triton X-100.

Cell suspension was vortexed for 1 min, maintained for 10 min at 4°C and then centrifuged at 1000 *g* for 10 min. The pellet was suspended in 1.5 ml Triton X-free NB adjusted at pH 7.5 (NB7.5) and nuclear protein were extracted by the addition of 5 M NaCl to a final concentration of 0.35 M. After incubation at 4°C for 40 min, nuclear debris were pelletted by centrifugation of 17 000 *g* for 10 min, and the supernatant (nuclear extract) was adjusted to a 50% final concentration of glycerol and stored at −20°C (used within 2 months).

The protein concentration of the preparation was determined by the method of Bradford.[13]

Isolation of topoisomerase I

A partial rapid purification of topoisomerase I has been performed by modifying the method suggested by Ishii

et al.[14] Briefly, the nuclear extracts were diluted 1:4 in NB 7.5 and applied to a heparin–Sepharose column (0.5 × 1.0 cm) (Pharmacia, Uppsala, Sweden) previously equilibrated with NB 7.5.

The column was washed with 4 ml of 0.4 M NaCl and the topoisomerase was eluted with a single step of 0.72 M NaCl. The eluted sample was concentrated 10 times by 50 000 molecular weight cut-off centricon filter (Millipore, Bedford, MA) and resuspended in NB7.5. After a further concentration 10 times, glycerol to 50% was added, and aliquots of topoisomerase preparation were aliquoted at −20°C and used within 1 month.

Immunostaining

A sample of nuclear extract prepared as described above was subjected to electrophoresis in 7.5% SDS–polyacrylamide gel by the method of Laemmli[15] and then transferred to nitrocellulose filters essentially as described by Towbin *et al.*[16] After incubation in 5% dry milk in PBS the blot was washed with PBS and incubated for 1 h at 37°C with serum obtained from a patient with scleroderma (diluted 1:1500 in 5% dry milk–PBS). After washing with PBS+Tween 0.3% the blot was incubated for 1 h at 37°C with an anti-human IgG peroxidase conjugate (Pierce, Rockford, MA) diluted 1:5000 in PBS–5% dry milk. After a further washing the blot was developed by an ECL technique (Amersham, Little Chalfont, UK) Western blotting detecting reagents and Hyperfilm ECL (Amersham). As positive control a sample of purified human topoisomerase I (TopoGEN, Columbus, OH) was subjected to electrophoresis and then immunoblotted as described above.

Topoisomerase I relaxation assay

The catalytic activity of topoisomerase I in nuclear extracts was measured by a DNA relaxation assay in the absence of ATP and Mg^{2+} by using supercoiled plasmid pBR322 DNA.[17]

Briefly, to 20 μl of reaction mixture containing 50 mM Tris–HCl, pH 7.5, 1 mM 2-mercaptoethanol, 165 mM KCl, 0.5 mM EDTA-Na_2 and 30 μg/ml BSA was added 10 μl of pBR322 DNA (150 ng) and 5 μl of nuclear extract (at 1:2 serial dilution) in NB 7.5 buffer, and the samples were incubated at 37°C for 10 min. The reaction was stopped by adding 10 μl of SDS 10%+250 mM EDTA+0.8 ng/ml proteinase K followed by incubation at 50°C for 30 min to digest the enzyme. The final products were mixed with loading solution (BBF 0.25%+60% sucrose) and subjected to electrophoresis in 1% agarose gel in 40 mM Tris–borate. Gel was stained with ethidium bromide and photographed (UV) through Kodak filters with Polaroid type 667 positive films. The amount of DNA was quantitated by scanning photographs with a densitometer. One unit of topoisomerase I activity is defined as the amount of extract which relaxes 50% of DNA under the above-described conditions. The specific activity of topoisomerase I in the extract was expressed as U/mg of protein content.

In vitro sensitivity of topoisomerase I to CPX and CAM

The sensitivity to CPX and CAM of topoisomerase I isolated from WEHI-3B and WEHI-3/CPX cells was evaluated by the pBR322DNA relaxing assay described above at a single dose of topoisomerase I extract corresponding to 5 U of enzyme. An aliquot of 5 μl of CPX (to a final concentration of 44.8–1436.8 μM) and CAM (from 9.12–287.3 μg/ml) was added to the suspension mixture before the reaction was started by the addition of the nuclear extract. Then the procedure was the same as described above for the topoisomerase I assay. The concentration of CPX and CAM able to give inhibition of 50% of the DNA relaxation (IC_{50}) was determined by densitometric analysis of the gels.

Topoisomerase II-mediated DNA cleavage

The assay was performed as described by Osheroff and Zechidrich.[18] Briefly, the reaction was started by adding 5 μl of nuclear extract (corresponding to 5 U) to a mixture of 10 μl of CPX (to final concentration of 43.1, 359.2 and 1436.8 μM for CPX) and 300 ng of pBR322DNA both in the absence (pre-strand DNA passage) and in the presence of 2 mM ATP (post-strand DNA passage). The reagents were prepared in a cleavage buffer (10 mM Tris, 50 mM NaCl, 50 mM KCl, 0.01 mM EDTA-Na_2, 5 mM $MgCl_2$ and 2.5% glycerol, pH 7.9) and the samples were incubated at 37°C for 10 min. Cleavage products were treated as described above for topoisomerase I assay and DNA cleavage was monitored by quantitating the conversion of supercoiled plasmid substrate to unit length linear molecules.

DNA breakage assay

The number of breaks induced in DNA by CPX treatment of WEHI-3B and WEHI-3B/CPX cells was determined by the fluorimetric assay of DNA rewinding (FADU) method as suggested by Kanter and Schwartz.[19] Briefly, both WEHI-3B and WEHI-3B/CPX

cells were pretreated for 1 h with increasing concentrations of CPX (28.7, 287.3 and 2873.6 μM). A set of controls did not receive drug but an equal quantity of distilled water. Drug-treated cultures and controls were centrifuged at 600 *g* for 10 min, resuspended to 10^6 cells/ml in ice cold PBS and distributed in nine aliquots of 1 ml. One set of three aliquots (group B) was tested for double-stranded DNA after 30 min denaturation at room temperature following the addition of 0.1 M NaOH. Another set (group A) was treated without incubation (non-denaturing condition) and the last set of three aliquots (group C) was sonicated for 5 s before denaturation in order to obtain complete DNA unwinding. Fluorescence (λ_{ex}=351 nm; λ_{em}=451 nm) was read on a Perkin-Elmer L50S. The residual double-stranded DNA (*F*) was calculated

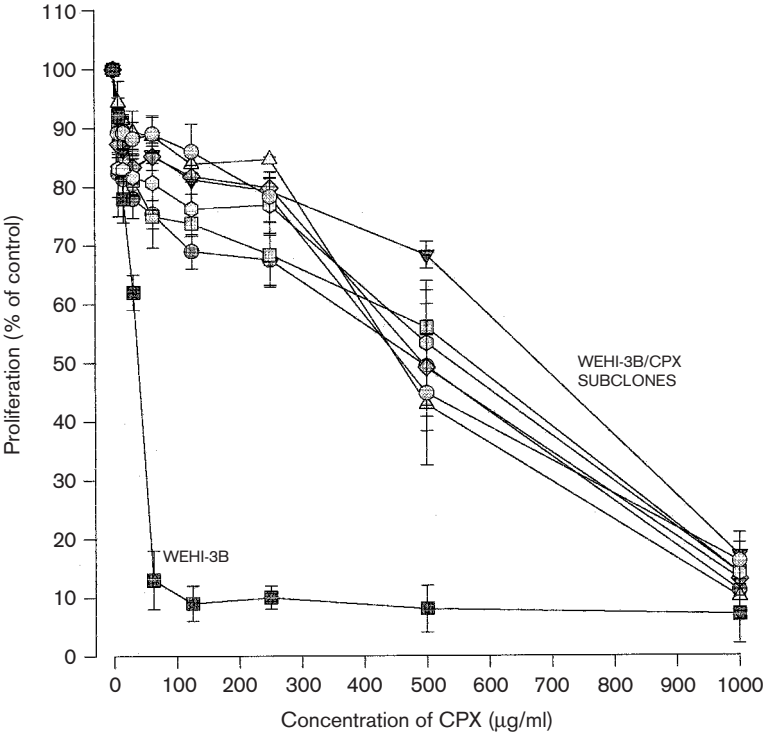

Figure 1. Sensitivity to CPX of WEHI-3B cells and seven subclones isolated from WEHI-3B/CPX variant. The graph reports the *in vitro* cell proliferation expressed as percentage of cell viability measured on control culture in the absence of drug. Each point represents the mean $\pm$ SE of three experiments performed in duplicate.

Table 1. Characteristics of WEHI-3B(D+)/CPX

Character studied	Result	Assay used
Resistance index[a] to CPX	17.3 $\pm$ 2.24	antiproliferation MTT test
Resistance index[a] to other quinolones (LMX, OFX, PFX and RFX)	1.45 $\pm$ 0.35	antiproliferation MTT test
Resistance index[a] to anti-topoisomerase I drugs (CPT)	1.88 $\pm$ 0.29	antiproliferation MTT test
Resistance to anti-topoisomerase II drugs (DOX, ETO and TNP)	1.1 $\pm$ 0.22	antiproliferation MTT test
P-170 expression	negative	Western blotting
LRP expression (p-110)	negative	immunocytochemistry
Resistance circumvention (by verapamil)	negative	antiproliferation MTT test
Intracellular accumulation of CPX	0.6-fold	fluorimetric assay
Growth kinetics	identical to parental line	antiproliferation MTT test
IL-3 production	identical to parental line	32DC13 bioassay

[a]Resistance index is calculated as the ratio between IC_{50} values. CPT, camptothecin; ETO, etoposide; CPX, ciprofloxacin; LMX, lomefloxacin; OFX, ofloxacin; TNP, teniposide; RFX, rufloxacin; PFX, pefloxacin.

according to the relationship $F=(B-C)/(A-C)$. A, B and C were the mean relative fluorescence intensity in groups A, B and C, respectively. Breaks per relative molecular mass of DNA ($n/10^9$) were determined by use of the Rydberg equation after determination of F.[20]

Statistical analysis

The IC_{50} values were determined by the Reed and Müench formula.[21]

Where required, the data were statistically analyzed by the Instat program (GraphPad, San Diego, CA) and the differences between the means were evaluated by a Tukey multiple comparison or by Student's t-test. Differences were considered significant at values of $p<0.05$ in a bidirectional test.[22]

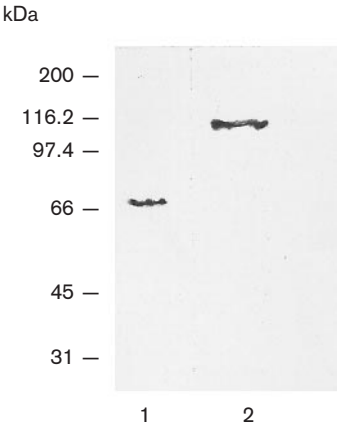

Figure 2. Immunoblot analysis of topoisomerase I protein. Lane 1, human standard preparation of Topoisomerase I from Topogen (positive control). Lane 2, partially purified nuclear extract from WEHI-3B cells. The migration positions of M_r protein markers (kDa) are indicated.

Results

Susceptibility of WEHI-3B and WEHI-3B/CPX cells to CPX

The study of sensitivity to CPX in seven subclones isolated from the resistant variant indicated a very similar kinetics (Figure 1). The resistance indices of these subclones ranged from 16 (clones E5 and G11) to 24 (clone H9) with a mean value of 18.9 ± 1.1, which is identical to the resistance index determined for WEHI-3B/CPX (17.3 ± 2.2) (Table 1).

No significant resistance of WEHI-3B/CPX was observed to other quinolones nor to the antitopoisomerases drug tested.

Extraction of topoisomerase I

Immunoblotting performed with anti-topoisomerase I antibodies obtained from a patient with scleroderma shows that topoisomerase I protein was present in partially purified preparations (Figure 2). The band detected (Figure 2, lane 2) was about 100 kDa, which is consistent with the reported molecular mass of topoisomerase I. No lower molecular weight species of topoisomerase I, as described by some authors, were detectable in our preparations whereas this type of molecule represents 100% of the standard preparation purchased from Topogen which bands to 66 Kda (positive control). This partially purified material had no endonuclease activity detectable by treating pBR322 DNA at 37°C for 4 h (data not reported).

Activity of topoisomerase I from WEHI-3B and WEHI-3B/CPX

The relaxing activities of topoisomerase I partially purified from WEHI-3B and WEHI-3B/CPX are com-

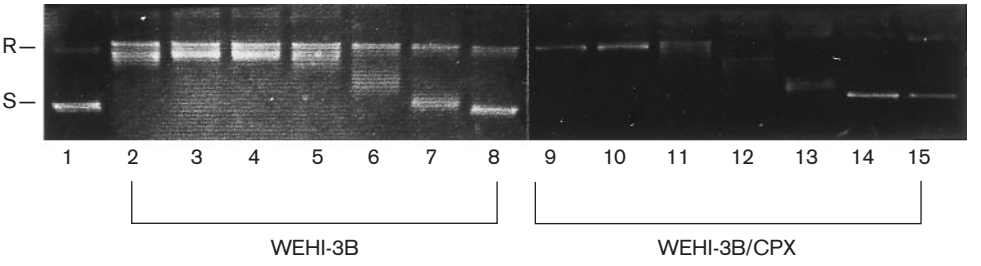

Figure 3. Catalytic activity of topoisomerase I from WEHI-3B and WEHI-3B/CPX cells (agarose gel run of pBR322). Lane 1, untreated pBR322 DNA (Control). Lanes 2–7, plasmid treated with 1.87, 3.75, 7.5, 15, 30 and 60 ng/ml of topoisomerase I from WEHI-3B cells. Lanes 9–13, plasmid treated with 1.87, 3.75, 7.5, 15, 30 and 60 ng/ml of topoisomerase I from WEHI-3B/CPX cells. R=relaxed DNA, S=supercoiled DNA.

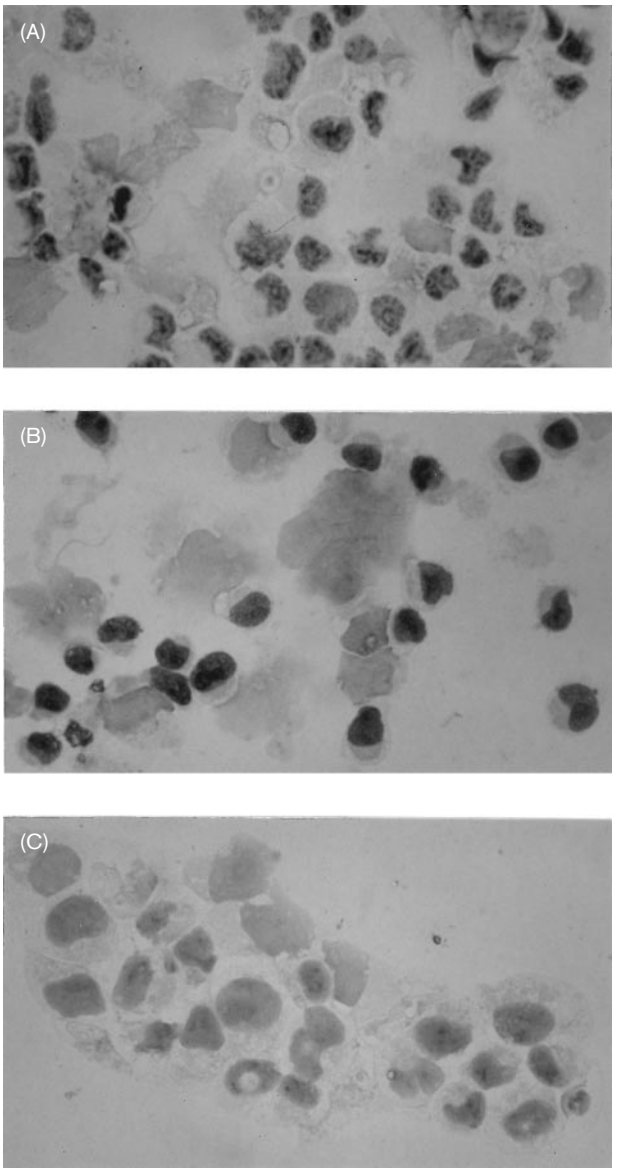

Figure 4. Immunoperoxidase staining of whole (A) WEHI-3B/CPX and (B) WEHI-3B cells treated with anti-topoisomerase I antibodies, and (C) negative control ($\times$ 100 magnification).

pared in Figure 3. It is evident that plasmid relaxation activities of topoisomerase I from WEHI-3B and WEHI-3B/CPX are quite similar.

The quantitation of the bands by densitometry was performed on gels from four independent experiments and expressed as the amount of relaxed plasmid DNA as a percentage of the total amount of DNA. The calculations confirmed similar levels of specific activity with the two topoisomerase I preparations: WEHI-3B = $8.5 \times 10^4 \pm 1.2 \times 10^4$ U/mg; WEHI-3B/CPX = $9.2 \times 10^4 \pm 0.98 \times 10^4$ U/mg. Even the statistical analysis did not indicate any significant difference between the relaxing activities of the two topoisomerase I preparations. These observations are consistent with the results of the immunocytochemistry studies on whole WEHI-3B or WEHI-3B/CPX cells which did not show any difference in the number of topoisomerase I-expressing cells (Figure 4).

In vitro sensitivity of topoisomerase I to CPX and CAM

The topoisomerase I preparations from parental WEHI-3B cells and the resistant variant WEHI-3B/CPX have also been tested for their susceptibility to inhibition by CPX and CAM (Figure 5).

The catalytic activity of topoisomerase I from WEHI-3B and WEHI-3B/CPX cell extracts in the presence of CPX shows a similar curve of inhibition. A complete inhibition of plasmid relaxation resulted from 1436.8 μM of CPX in both the lines. Densitometric quantification of plasmid bands of gels in four independent experiments indicated that the concentrations of CPX required to produce 50% inhibition of topoisomerase I activity (IC_{50}) were 302.9 ± 24.5 μM for WEHI-3B and 344.3 ± 41.6 μM for WEHI-3B/CPX. Topoisomerase I from the two cell lines also shows a very similar sensitivity to CAM, with an IC_{50} value of 50.4 ± 7.8 for WEHI-3B and 49.4 ± 1.5 for WEHI-3B/CPX (see Table 2). The statistical analysis confirmed the absence of difference between the two topoisomerases I in their sensitivity towards CPX and CAM. Also DOX (that exerts its antitopoisomerase I activity by acting as intercalator) gave the same results on the two cell lines with IC_{50} values of 0.39 μM for WEHI-3B and 0.53 μM for WEHI-3B/CPX (gel runs not reported).

DNA breakage

To evaluate the relative capacity of CPX to cause DNA breakage in WEHI-3B and WEHI-3B/CPX cells, a FADU assay which gives the same information as the alkaline elution technique was used.

In WEHI-3B cells the *F* values, which represent the fraction of DNA in the duplex form after alkaline unwinding, is higher ($F=0.72 \pm 0.05$) than in WEHI-3B/CPX ($F=0.6 \pm 0.06$), but this difference is not statistically significant ($p=0.09$). The slope of linear regression due to CPX treatment is significant ($p < 0.03$) in both the lines and correlation coefficients are comparable ($R=-0.91$ for WEHI-3B and $R=-0.89$ for WEHI-3B/CPX). The determination of the number of breaks present in DNA (Figure 6B) and the relative increase

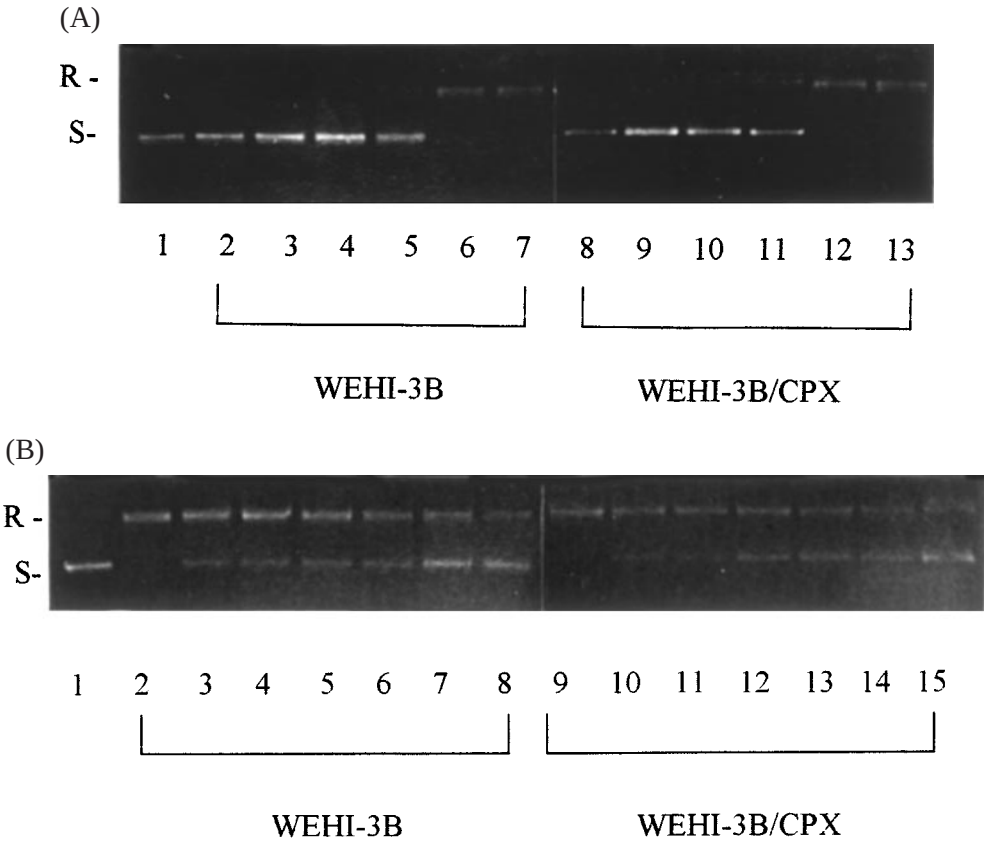

Figure 5. *In vitro* sensitivity of topoisomerase I from WEHI-3B and WEHI-3B/CPX cells to CPX (A) and CAM (B). Lane 1, untreated pBR322 DNA (negative control). Lanes 2 and 9, plasmid treated with 4 U of topoisomerase I in the absence of drug (positive control). Lanes 3–8, plasmid treated with topoisomerase I from WEHI-3B in the presence of drug. Lanes 10–15, plasmid treated with topoisomerase I from WEHI-3B/CPX in the presence of drug. CPX concentrations (A): 44.8 μM (lanes 3 and 10), 89.8 μM (4 and 11), 179.5 μM (5 and 12), 359.2 μM (6 and 13), 718 μM (7 and 14), and 1436.8 μM (8 and 15). CAM concentrations (B): 9.12 μM (lanes 3 and 10), 17.9 μM (4 and 11), 35.9 μM (5 and 12), 71.8 μM (6 and 13), 143.6 μM (7 and 14) and 287.3 μM (8 and 15).

Table 2. Comparison between the IC_{50} values determined for CPX, CAM and DOX towards topoisomerase I *in vitro* activity

Drug	WEHI-3B (μM)	WEHI-3B/CPX (μM)	Ratio
CPX	302.9±24.5	344.3±41.6	0.88
CAM	50.4±7.8	49.4±1.5	1.02
DOX	0.67±0.07	0.91±0.1	1.36

after CPX treatment clearly evidenced that CPX treatment produces a linear increase of breaks in the two cell lines, but at the highest CPX concentrations (2873 μM) the number of breaks induced in WEHI-3B (13.2) is more than 2-fold higher than that induced in WEHI-3B/CPX-resistant cells (5.9).

Effect of CPX on topoisomerase II-mediated DNA cleavage

The upper gel in Figure 7 shows the pre-strand passage DNA cleavage/religation equilibrium (absence of ATP). As shown by the increased linear DNA, CPX enhanced the capacity of topoisomerase II from WEHI-3B cells to modulate the formation of double-strand breaks in DNA. Even 1436.8 μM CPX does not enhance the pre-strand cleavage activity of topoisomerase II extracted from WEHI-3B/CPX, whereas 359.2 μM of CPX enhances significantly the cleavage activity of topoisomerase II extracted from WEHI-3B cells. A very similar pattern of enhancement was observed in experiments performed in the presence of ATP (post-strand passage DNA cleavage condition) (lower gel of Figure 7).

Discussion

As previously reported[9] and summarized in Table 1, the resistant cell line WEHI-3B/CPX is a genetically stable variant expressing a high degree of resistance to CPX. It does not show any cross-resistance with other quinolones (LMX, OFX, PFX and RFX), with CAM or with topoisomerase II inhibitors (DOX, ETO and TEN). The lower accumulation of CPX observed in WEHI-3B/CPX cells does not by itself explain the resistance, but it could have a role in producing resistance in synergism with some other mechanism. However, the resistance is not circumvented by verapamil, and these cells do not express p170 glycoprotein and even p110 glycoprotein that regulates LRP mechanism often present as a secondary mechanism of resistance in MDR-negative cells.

The analysis of seven subclones derived from the resistant line (Figure 1) indicates a high homogeneity of cell subpopulations: the IC_{50} values in the seven clones ranged from 1275 to 1949.3 μM with a mean of 1500.9 ± 90.5 μM, whereas the IC_{50} determined on wild-type sensitive cells was 82.0 ± 6.3 μM. The high genetic stability of this resistant variant has been confirmed by its high resistance index of 18.9 after 6 years of growth in the absence of CPX. All these characteristics, taken together, clearly demonstrate that the mechanism of resistance of WEHI-3B/CPX is specifically directed to CPX and it is absolutely not a MDR mechanism. The cytotoxicity exerted by CPX and other quinolones on eukaryotic cells has been described as a consequence of their capacity to inhibit the catalytic activity of topoisomerase I and II.[6–9,23] So, to explain the resistance observed we investigated, in a first step, both a possible difference in the catalytic activity of topoisomerase I of the two cell lines and a different sensitivity of topoisomerase I to inhibitory activity of CPX and CAM which act by trapping cleavage complexes.[24] As reported in Results (see Figure 3), the catalytic activity of topoisomerase I proved to be very similar in the two cell lines (specific activity of $8.5 \times 10^4 \pm 1.2 \times 10^4$ U/mg for WEHI-3B and $9.2 \times 10^4 \pm 0.98 \times 10^4$ U/mg for WEHI-3B/CPX) and was relatively higher than the specific activity observed in solid tumors.[25] Even the expression of topoisomerase I, checked by a immunocytochemistry technique, confirmed that the two cell types are comparable (Figure 4).

The studies on topoisomerase I sensitivity to CPX (Figure 5A) showed that the IC_{50} value determined in WEHI-3B (302.9 ± 24.4 μM) does not differ from that of the enzyme extracted from the resistant variant (344.3 ± 41.6 μM) and that these values are similar to the data reported by other authors[4,20] who studied the effect on CPX to topoisomerase I of calf thymus. A similar sensitivity of the topoisomerase I from the two lines has also been confirmed by using CAM (Figure

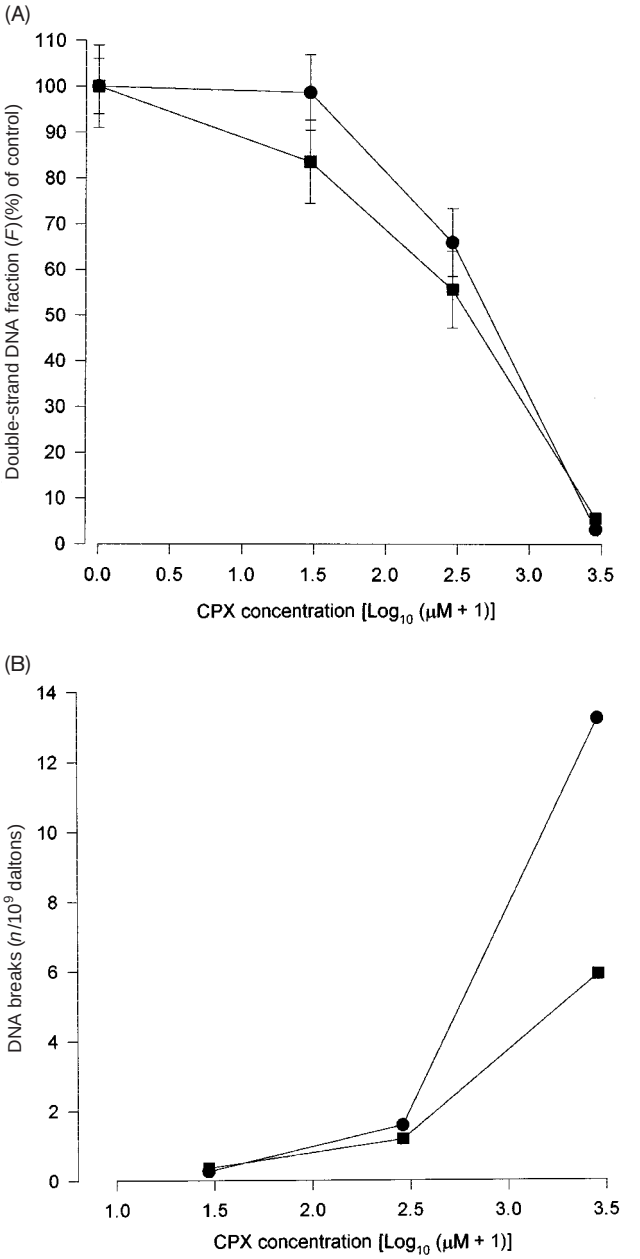

Figure 6. DNA breakage in WEHI-3B (●) and WEHI-3B/CPX (■) cells treated by CPX. Kinetics of DNA damage induced into cells by CPX treatment was evaluated by FADU. (A) Fraction of DNA in the duplex form (*F*) mean from three experiments) is reported as percentage calculated on the *F* values determined in untreated cells (controls) considered as 100% (*F*=0.72+0.04 for WEHI-3B and *F*=0.6+0.06 for WEHI-3B/CPX). (B) Breaks per relative molecular mass of DNA ($n/10^9$) determined by the Rydberg equation (see Materials and methods).

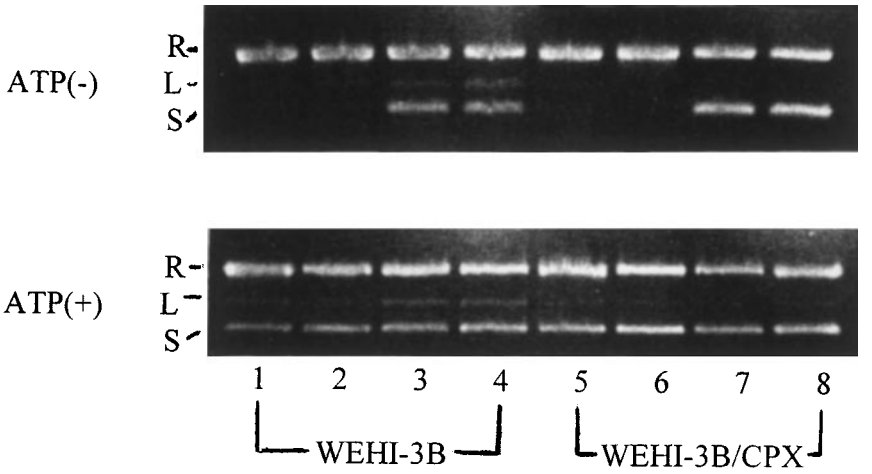

Figure 7. Effect of CPX on topoisomerase II-mediated DNA cleavage evaluated in the presence (ATP+) and in the absence (ATP−) of ATP (see Materials and methods). Lanes 1 and 5, pBR322 DNA treated with nuclear extract in the absence of CPX (controls). Lanes 2, 3, 4, 6, 7 and 8, pBR322 DNA treated with nuclear extract in the presence of CPX at 43.1 μM (lanes 2 and 6), 359.2 μg/ml (lanes 3 and 7) and 1436.8 μM (lanes 4 and 8). R=relaxed DNA, L=linear DNA, S=supercoiled DNA.

5B) that is a non-binder inhibitor of topoisomerase I. These data do not explain the high resistance index expressed by WEHI-3B/CPX cells to CPX in the antiproliferative assay, but allow us to exclude that the resistance may be due to some important alteration of topoisomerase I–DNA complex stabilization by the drug.

As reported by Rubin *et al.*[26] and Oomori *et al.*,[27] studies on two myeloid cell lines (one of them resistant to 9-nitro-20(*S*)camptothecin) indicated that, although the lines had similar levels of topoisomerase I catalytic activity, the topoisomerase I from the resistant cell clone was about 10-fold more resistant to the *in vitro* drug inhibition in comparison with the parental sensitive line. In general, the resistance to CAM described is characterized by a reduced CAM accumulation, a reduced topoisomerase I content[28] or by a functional alteration of topoisomerase I which results into a decreasing production of protein-linked DNA breaks. This last mechanism could also involve a poly-ADP-Ribose polymerase (PARP) because its inhibition by 3-aminobenzamide potentiates CAM toxicity in the resistant line.[29] In WEHI-3B/CPX cells it seems that topoisomerase I is not involved in the resistance to CPX and as this drug (differently to CAM) is also able to inhibit topoisomerase II, we addressed the second step of our study to check the role of this enzyme in our model.

Using a FADU technique we observed that treatment with CPX in the DNA of the resistant cells produced a significantly lower number of breaks in comparison with the parental sensitive cell line (Figure 6B). This observation correlates well with results from the study on enhancement of topoisomerase II cleavage activity by CPX and CAM. Cleavage activity of topoisomerase II from wild-type cells is much more susceptible to be enhanced by CPX than topoisomerase II from WEHI-3B/CPX cells. As shown in Figure 7, CPX increases topoisomerase II cleavage from WEHI-3B cells at 359.2 μM, whereas the drug was ineffective on cleavage activity of topoisomerase II from WEHI-3B/CPX cells up to the maximum tested concentration of 1436.8 μM.

It is demonstrated that quinolones do not affect topoisomerase II religation activity[30,31] and that their cytotoxicity towards mammalian cells correlates with the ability to enhance topoisomerase II-mediated DNA cleavage. Therefore the ability of CPX to shift the DNA cleavage/religation equilibrium of the enzyme toward the cleavage event, observed both before and after its double-strand DNA passage (as shown in Figure 7), may be considered an important step in producing cytotoxicity. These data, taken together, strongly support the hypothesis that the resistance to CPX expressed by the WEHI-3B/CPX subclone is due to an altered sensitivity to CPX of cleavage activity of topoisomerase II.

Conclusions

(a) The CPX-resistant clone of WEHI-3B/CPX cells does not express any multidrug resistance character and therefore this line represent a unique case of specific drug resistance towards CPX. The lower CPX phenomenon accumulation

observed does not explain by itself the resistance, but could take part in this phenomenon.

(b) The mechanism of resistance does not correlate with alteration of topoisomerase I catalytic activity and not even with a decreased sensitivity of topoisomerase I to CPX.

(c) The observation that topoisomerase II from WEHI-3B/CPX resistant cells is refractory to the enhancement effect produced by CPX on DNA cleavage activity correlates well with the lower efficiency of CPX to introduce breaks into DNA molecules of WEHI-3B/CPX cells if compared with WEHI-3B.

The resistance to CPX observed in WEHI-3B/CPX cells is consistent with a specific 'resistance' of topoisomerase II to be enhanced by CPX and this is the first model described in the literature. Further experiments will be required to investigate if this functional alteration is dependent on changes of topoisomerase II specificity for topoisomerase II cleavage sites or on a decreased binding affinity of CPX to the topoisomerase II–DNA complex because the alterations of topoisomerase II (at-MDR) described until now are all associated with multidrug resistance.[32]

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References

1. Bellami WT, Dalton WS. Multidrug resistance in the laboratory and clinic. *Adv Clin Chem* 1994; **31**: 1–61.
2. McKenna MS, Padna RA. Multidrug resistance in leukemia. *Br J Haematol* 1997; **96**: 659–74.
3. Lehnert M. Clinical multidrug resistance in cancer: a multifactorial problem. *Eur J Cancer* 1996; **32A**: 912–20.
4. Parchment R, Pessina A. Topoisomerase I inhibitors and drug resistance. *Cytotechnology* 1998; **27**: 149–64.
5. Clynes M. Cellular models for multiple drug resistance in cancer. *In Vitro Cell Dev Biol* 1993; **29A**: 171–9.
6. Hussy P, Maas G, Tümmler B, Grosse F, Schomburg U. Effect of 4 quinolones and novobiocin on calf thymus DNA polimerase α primase complex, topoisomerase I and II and growth of mammalian lymphoblast. *Antimicrob Agents Chemother* 1986; **29**: 1073–8.
7. Pessina A, Neri MG, Muschiato A, Mineo E, Cocuzza G. Effects of fluoroquinolones on the *in vitro* proliferation of myeloid precursor cells. *J Antimicrob Chemother* 1989; **24**: 203–8.
8. Hoshino K, Sato K, Akahane K, Yoshida A, Hayakawa I, Sato M, Une T, Osada Y. Significance of the methyl group on the oxazine ring of ofloxacin derivatives in the inhibition of bacterial and mammalian type II topoisomerases. *Antimicrob Agents Chemother* 1991; **35**: 309–12.
9. Pessina A, Mineo E, Gribaldo L *et al*. Susceptibility of leukemia cell lines to quinolones and induction of resistance to ciprofloxacin in WEHI-3B (D^+) leukemia cells. *Cancer J* 1993; **6**: 291–7.
10. Scheper RJ, Broxterman HJ, Scheffer GL, *et al*. Overexpression of a M_r 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. *Cancer Res* 1993; **53**: 1475–9.
11. Warner LN, Moore MAS, Metcalf D. A transplantable myelomonocytic leukemia in BALB/c mice: cytology, caryotype and neuroaminidase content. *J Natl Cancer Inst* 1969; **43**: 963–82.
12. Deffie AM, Batra JK, Goldenberg GJ. Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and resistant P388 leukemia cell lines. *Cancer Res* 1989; **49**: 58–62.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; **72**: 248–54.
14. Ishii K, Hasegawa T, Fujisawa K, Ando T. Rapid purification and characterization of DNA topoisomerase I from cultured mouse mammary carcinoma FM3A cells. *J Biol Chem* 1983; **258**: 12728–32.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; **227**: 680–2.
16. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350–4.
17. Drake H, Hofmann GA, Mong SM, *et al*. *In vitro* and intracellular inhibition of topoisomerase II by the antitumor agent merbarone. *Cancer Res* 1989; **49**: 2578–83.
18. Osheroff N, Zechiedrich EL, Gale KC. Catalytic function of DNA–topoisomerase II. *BioEssays* 1991; **13**: 269–75.
19. Kanter PN, Schwartz HS. A fluorescence enhancement assay for cellular DNA damage. *Mol Pharmacol* 1982; **22**: 145–51.
20. Rydberg B. The role of strand separation in alkali of DNA of irradiated mammalian cells. *Radiat Res* 1975; **61**: 274–84.
21. Reed LJ, Müench HA. A simple method of estimating fifty per cent endpoints. *Am J Hyg* 1938; **27**: 493–97.
22. Snedecor GW. *Statistical methods*. Ames, IA: Iowa University Press 1952.
23. Moreau NJ, Robaux H, Baron L, Tabary X. Inhibitory effects of quinolones on pro- and eucaryotic DNA topoisomerases I and II. *Antimicrob Agents Chemother* 1990; **34**: 1955–60.
24. Pommier Y, Pourquier P, Fan Y, Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* 1998; **1400**: 83–106.
25. McLeod HL, Douglas F, Oates M, *et al*. Topoisomerase I and II activity in human breast, cervix, lung and colon cancer. *Int J Cancer* 1994; **59**: 607–11.

26. Rubin E, Pantazis P, Bharti A, Toppmeyer D, Giovannella B, Kufe D. Identification of a mutant human topoisomerase I with intact catalytic activity and resistance to 9-nitro camptothecin. *J Biol Chem* 1994; **269**: 2433–9.
27. Oomori Y, Yasue T, Aoyama H, Hirai K, Suzue S, Yokota T. Effects of Fleroxacin on HeLa cell functions and topoisomerase II. *J Antimicrob Chemother* 1988; **22D**: 91–7.
28. Eng WK, McCabe FL, Tan KB, *et al*. Development of a stable camptothecin-resistant subline of P388 leukemia with reduced topoisomerase I content. *Mol Pharmacol* 1990; **38**: 471–80.
29. Beidler D, Chang J-Y, Zhou B, Cheng Y. Camptothecin resistance involving steps subsequent to the formation of protein-linked DNA breaks in human camptothecin-resistant KB cell lines. *Cancer Res* 1996; **56**: 345–53.
30. Elsea SH, McGuirk PR, Gootz TD, Moynihan M, Osheroff N. Drug features that contribute to the activity of quinolones against mammalian topoisomerase II and cultured cells: correlation between enhancement of enzyme-mediated DNA cleavage *in vitro* and cytotoxic potential. *Antimicrob Agents Chemother* 1993; **37**: 2179–86.
31. Robinson MJ, Martin BA, Gootz TD, *et al*. Effects of quinolone derivates on eukaryotic topoisomerase II. *J Biol Chem* 1991; **266**: 14585–92.
32. Beck WT, Danks MK, Wolverton JS, *et al*. Resistance of mammalian tumor cells to inhibitors of DNA topoisomerase II. *Adv Pharmacol* 1994; **293**: 145–69.

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