

Enhanced etoposide sensitivity following adenovirus-mediated human topoisomerase II α gene transfer is independent of topoisomerase II β

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Summary The roles that the α and β isoforms of topoisomerase II (topo II) play in anticancer drug action were determined using MDA-VP etoposide-resistant human breast cancer cells and a newly constructed adenoviral vector containing the topo II α gene (Ad-topo II α). MDA-VP cells were more resistant to etoposide than to amsacrine and had more resistance to etoposide than did MDA-parental cells. MDA-VP cells also expressed lower topo II α RNA and protein levels than parental cells but had comparable topo II β levels. After infection with Ad-topo II α , topo II α , RNA and protein levels increased significantly, as did the cells' sensitivity to etoposide. In contrast, topo II β levels remained constant with little alteration in the cells' sensitivity to amsacrine. Band-depletion immunoblotting assays indicated that topo II α was depleted in etoposide-treated, Ad-topo II α -transduced MDA-VP cells but not in amsacrine-treated cells. Topo II β was depleted in amsacrine-treated, Ad-topo II α -MDA-VP cells, with little change in the topo II α levels. These results suggest that topo II α gene transfer does not alter topo II β expression and that enhanced sensitivity to etoposide is therefore secondary to change in topo II α levels. These studies support the theory that etoposide preferentially targets topo II α , while amsacrine targets topo II β . © 2001 Cancer Research Campaign <http://www.bjcancer.com>

Keywords: topoisomerase II α , topoisomerase II β , etoposide, amsacrine, drug targeting

DNA topoisomerase II (topo II) is a target for many anticancer drugs, including etoposide and amsacrine. These drugs stabilize the topo II-DNA cleavable complex, preventing religation of the DNA strand. Breaking of double-stranded DNA subsequently leads to cell death (Malonne and Atassi, 1997). Two distinct topo II isoforms have been identified. The topo II α gene, located on chromosome 17q21-22 encodes a 170-kDa enzyme, and the Topo II β gene located on chromosome 3p24, encodes a 180-kDa enzyme (Tsai-Pflugfeder et al, 1988; Jenkins et al, 1992). These two isoforms have different functions in DNA topography and the cell cycle (Austin and Marsh, 1998; Tan et al, 1992). However, both enzymes have been implicated in topo II-reactive drug action. Anticancer drug resistance has been attributed to alteration of topo II gene expression. Determining the importance of each isoform in anticancer drug action and resistance may create novel approaches to circumventing drug resistance and screening new isoform-specific drugs. Initial studies will require cell lines that either lack or express low levels of one of the topo II isoforms. We have previously shown that MDA-VP etoposide-resistant human breast cancer cells express low levels of topo II α compared MDA parental cells (Zhou et al, 1999). Here we show that MDA-VP and parental cells have comparable topo II β levels. MDA-VP cells therefore provide a useful model to study the role of each topo II isoform in drug sensitivity. The newly constructed adenovirus vector containing the human topo II α gene (Ad-topo II α) has made it possible to sensitize cells to topo II α -reactive drugs.

Our present study indicates that etoposide preferentially interacts with the topo II α isoform, while topo II β is the preferred target for amsacrine. These results confirm previously reported studies on the interaction between the two topo II isoforms and topo II-targeting drugs (Errington et al, 1999).

MATERIAL AND METHODS

Cell lines

MDA-MB-231 parental cells were obtained from American Type Culture Collection (Manassas, VA, USA). MDA-VP etoposide-resistant human breast cancer cells were initially derived and cloned from MDA-parental cells as described previously (Matsumoto et al, 1997). All cells were screened and found to be free of *Mycoplasma* (Gen-Probe Co., San Diego, CA, USA).

Infection of cells with Ad-topo II α virus

The Ad-topo II α virus was constructed and purified as described previously (Zhou et al, 1999). Cells were grown in logarithmic phase and were infected with Ad-topo II α at a multiplicity of infection of 100 pfu cell. Cells were harvested by standard methods after 48 h.

Cytostasis assay

A total of 5000 cells were seeded onto 96-well cell culture plates and allowed to adhere overnight. Cells were treated with different concentrations of etoposide or amsacrine (Sigma Co., St Louis, MO, USA). Their antiproliferative activity was determined by the

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3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue (MTT) assay as described previously (Fan et al, 1994).

Northern blot analysis

Total RNA was extracted with Trizol Reagent (Life Technologies, Inc., Grand Island, NY, USA). Then 20 µg of RNA was electrophoresed on a 1% formaldehyde/agarose gel and transferred to a Hybond-N⁺ membrane (Amersham Corp., Arlington Heights, IL, USA). Human topo II α gene probe ZII69 (Tsai-Pflugfeder et al, 1988), topo II β gene probe F12 (Austin et al, 1993; Herzog et al, 1998), and a GAPDH probe were used for hybridization. Probes were labelled using the Rediprime labelling system (Amersham).

Western blot analysis

The procedure involved 2 million cells, set up in a 100 mm dish, and treated as indicated. Cells were washed with cold phosphate-buffered saline (PBS) and lysed with buffer (50 mM Tris-HCl, pH 8.0, 425 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM β -mercaptoethanol) containing protease inhibitors. (Ganapathi et al, 1996) Then 50 µg protein was run on a 7.5% SDS/polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amersham). Human topo II α antibody (TopoGEN, Inc., Columbus, OH, USA), topo II β antibody (PharMingen Inc., San Diego, CA, USA), and β -actin antibody were used for protein detection with the ECL analysis system (Amersham).

Band-depletion immunoblotting assay

The band-depletion immunoblotting assay was performed as described previously (Zwelling et al, 1989). Cells were infected

Table 1 Comparison of resistance to etoposide or amsacrine between MDA-parent cells and MDA-VP cells.*

Cells	IC ₅₀ [†] for etoposide (M \pm SD) [‡]	IC ₅₀ for amsacrine (M \pm SD) [‡]
MDA-parental cells	3.0 \pm 0.2 μ M	5.6 \pm 0.3 μ M
MDA-VP cells	45.6 \pm 2.0 μ M	12.8 \pm 1.0 μ M
Relative resistance of MDA-VP cells[§]	15.0-fold	2.2-fold

*Cytostasis was measured by MTT assay as described in Material and methods. [†]IC₅₀ is the concentration that inhibits 50% of cell growth. [‡]M: mean value from at least 3 experiments, SD: standard deviation. [§]The relative resistance is calculated by dividing the IC₅₀ of MDA-VP by IC₅₀ of MDA-parent cells.

with Ad-topo II α or Ad- β -gal (control) for 48 h and then treated with 200 μ M etoposide or 100 μ M amsacrine at 37 °C for 1 h as indicated. Cell lysates were prepared in 2X Laemmli buffer by sonication for 30 s and boiled for 5 min. Proteins were resolved on a 7.5% SDS/polyacrylamide gel and immunoblotted using human topo II α , topo II β , and β -actin antibodies.

RESULTS

MDA-VP and parental cells were treated with various concentrations of etoposide or amsacrine. Table 1 shows that MDA-VP cells were 15-fold more resistant to etoposide than were MDA-parental cells. In contrast, MDA-VP cells were only 2.2-fold more resistant to amsacrine. To determine whether the differences in resistance were related to expression of the topo II isoforms in these cells, topo II α and topo II β RNA and protein levels were measured.

Topo II α mRNA levels were lower in MDA-VP cells than in MDA-parental cells (Figure 1A). Densitometric analysis showed only 20% topo II α gene expression in the etoposide-resistant MDA-VP cells compared to the MDA-parent cells (Figure 1B,

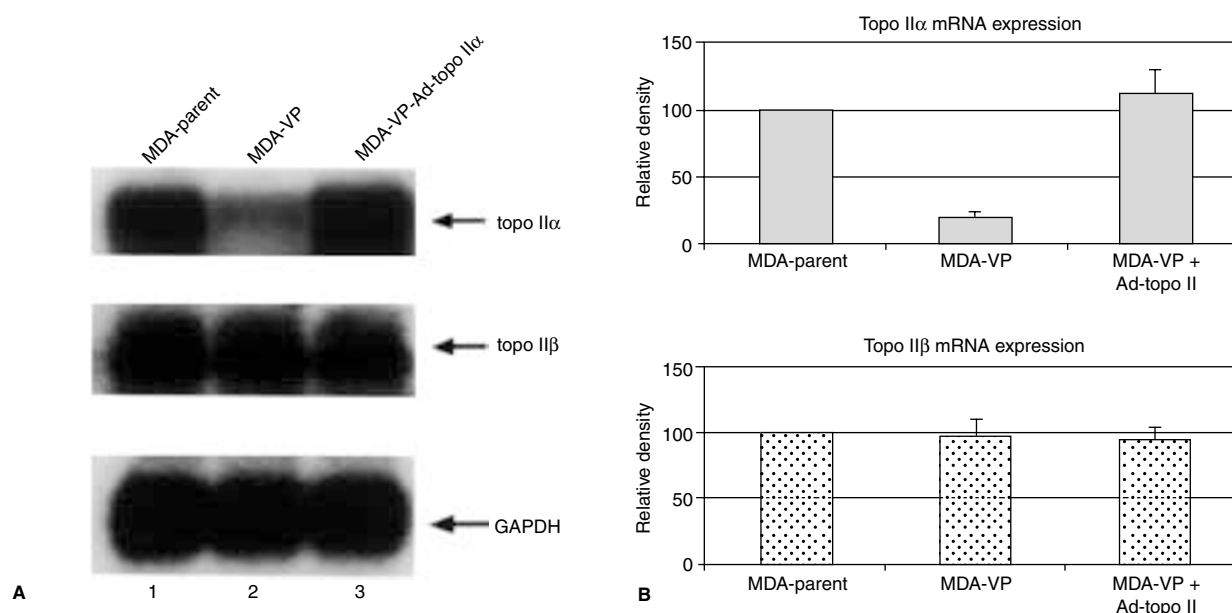


Figure 1 (A) Expression of topo II α and topo II β RNA in MDA-parental cells, MDA-VP cells, and MDA-VP cells infected with Ad-topo II α . Total RNA was extracted from MDA-parental cells (lane 1), MDA-VP cells, (lane 2) and MDA-VP cells infected with Ad-topo II α at 100 pfu/cell (lane 3). Northern blot analysis was performed using topo II α , topo II β and GAPDH probes. (B) Topo II α and β mRNA expression was quantified using densitometric analysis. The relative density at each point was calculated by dividing that value by the density in MDA-parent cells and adjusted by GAPDH loading control. The columns represent the mean from 3 independent experiments; the bars represent the standard deviation

upper panel). Topo II β mRNA levels were not significantly different in the two cell types (Figure 1B, lower panel). Topo II α protein levels were also much lower in MDA-VP cells than in MDA-parental cells, (Figure 2A and 2B, upper panel) while topo II β protein levels were *not* significantly different in the 2 cell types (Figure 2B, lower panel).

To further explore the relationship between drug resistance and isoform expression, MDA-VP cells were infected with the Ad-topo II α virus, then the topo II RNA and protein levels, as well as drug sensitivity, were quantified. Topo II α mRNA levels were elevated after infection; however, topo II β levels were not significantly altered (Figure 1). Topo II α protein levels also increased in MDA-VP cells after Ad-topo II α infection, but topo II β protein levels remained constant (Figure 2). The sensitivity of MDA-VP cells to etoposide increased 4.5-fold after infection with Ad-topo II α . The IC₅₀ of MDA-VP cells infected with Ad-topo II α went from 45.6 μ M to 10.1 μ M. By contrast, the sensitivity to amsacrine only increased 1.3-fold following infection with Ad-topo II α (Table 2). Therefore, the increased sensitivity of cells to etoposide following topo II α gene transfer correlated with increased topo II α levels but not with topo II β levels.

A band-depletion immunoblotting assay was performed with topo II α and topo II β antibodies to analyze the interaction of the 2 isoforms with etoposide and amsacrine. Topo II α band depletion was seen following treatment with etoposide, while little change was seen following amsacrine treatment (Figure 3, lanes 2,3, upper panel). In contrast, topo II β was more depleted in cells treated with amsacrine than in cells treated with etoposide (Figure 3, lanes 2,3, middle panel). Densitometric analysis indicated that etoposide induced 70% depletion of topo II α protein and only 10% depletion of topo II β protein (Figure 3, lane 2). Conversely, amsacrine induced only a 10% reduction of topo II α protein, but a 60% reduction of topo II β protein (Figure 3, lane 3). After infection of MDA-VP cells with Ad-topo II α , topo II α protein levels

Table 2 Enhancement of sensitivity to etoposide or amsacrine following infection of MDA-VP cells with Ad-topo II α *

Cells	IC ₅₀ for etoposide (M \pm SD)	IC ₅₀ for amsacrine (M \pm SD)
MDA-VP cells	45.6 \pm 2.0 μ M	12.8 \pm 1.0 μ M
MDA-VP cells infected with Ad-topo II α	10.1 \pm 0.5 μ M	9.3 \pm 0.4 μ M
Sensitivity enhancement[†]	4.5-fold	1.3-fold

*MDA-VP cells were infected with Ad-topo II α (100 pfu/cell) for 48 h, then treated with different concentrations of etoposide or amsacrine. [†]Sensitivity enhancement is calculated by dividing the IC₅₀ of MDA-VP by IC₅₀ of MDA-VP with Ad-topo II α .

were once again significantly increased (Figure 3, lane 4, upper panel) with relatively no change in topo II β protein levels (Figure 3, lane 4, middle panel). Neither topo II α protein levels nor topo II β protein levels were significantly altered following infection with Ad- β -gal (Figure 3, lane 7). The band-depletion pattern in MDA-VP cells following infection with Ad-topo II α (Figure 3, lanes 5, 6) and Ad- β -gal (Figure 3, lanes 8, 9) was the same as that seen in MDA-VP control cells (Figure 3, lanes 2,3). Etoposide treatment induced a significant reduction in topo II α , with little change in topo II β . By contrast, treatment with amsacrine did not affect the increased topo II α protein levels in the MDA-VP-Ad-topo II α cells.

DISCUSSION

Topo II, a nuclear enzyme involved in a number of important cellular processes, is the target for several anticancer drugs. The specific roles of topo II α and topo II β isoforms in the action of these topo II-targeting drugs are still poorly understood. Our data provide further evidence that topo II α is the main target for etopo-

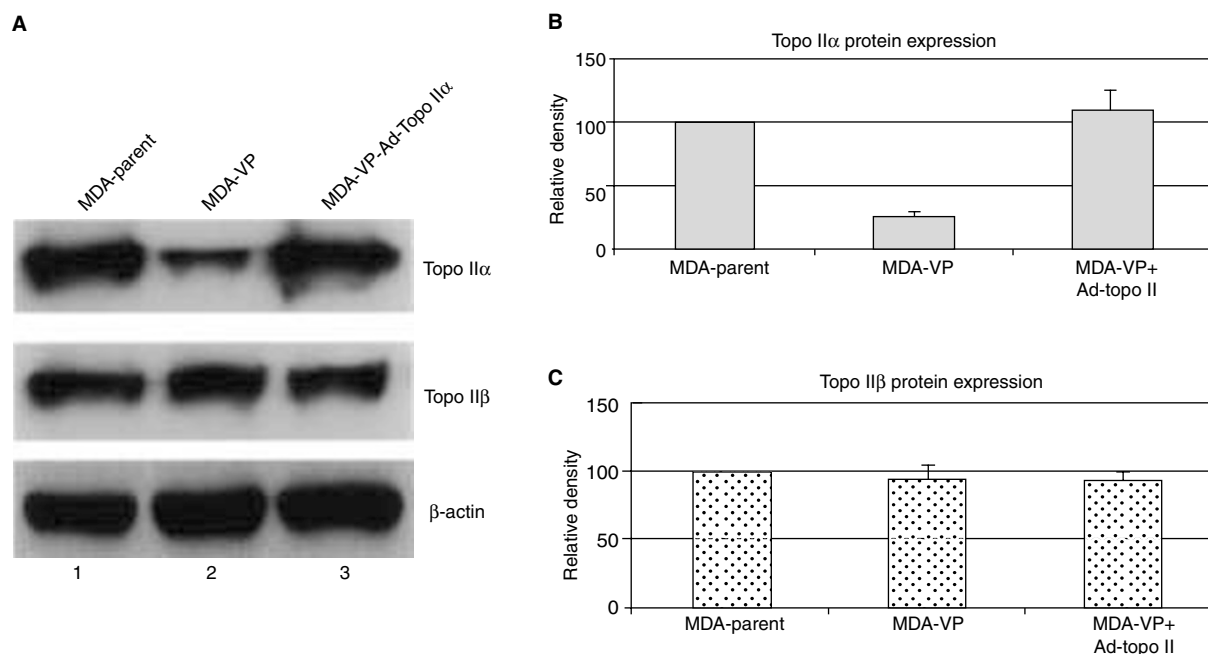


Figure 2 (A) Comparison of protein levels in MDA-parental cells, MDA-VP cells, and MDA-VP cells infected with Ad-topo II α . Protein was extracted from MDA-parental cells (lane 1), MDA-VP cells (lane 2), and MDA-VP cells infected with Ad-topo II α (lane 3). Western blot analysis was performed using anti-human topo II α , topo II β , and β -actin antibodies. (B) Topo II α and β protein expression was quantified using densitometric analysis. The relative density was calculated compared with MDA-parental cells and adjusted by β -actin loading control. The columns represent the mean from 3 independent experiments; the bars represent the standard deviation

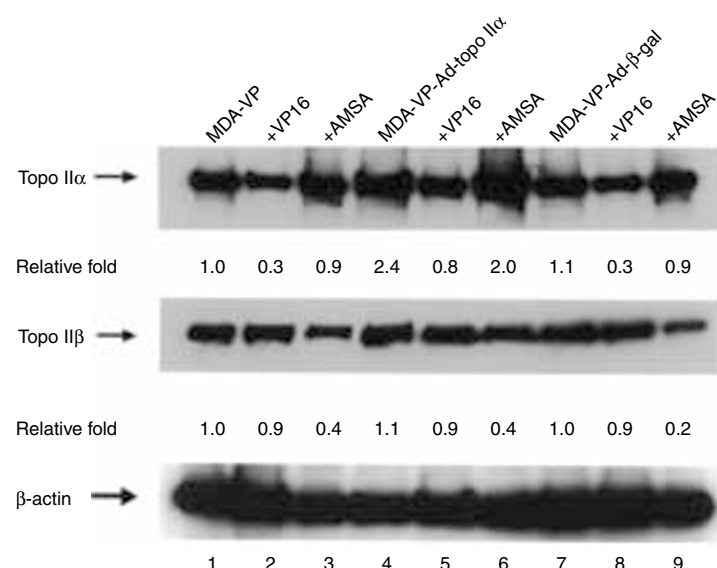


Figure 3 Band-depletion immunoblotting assay using topo II α and topo II β antibodies. MDA-VP cells were treated with PBS buffer as a control (MDA-VP), with 200 μ M etoposide (VP16), or with 100 μ M amsacrine (AMSA) for 1 h at 37°C. Identical treatment was performed on MDA-VP cells 48 h after infection with Ad-topo II α (lanes 4–6) or Ad- β -gal (lanes 7–9). Topo II protein isoforms were extracted and quantified using a band-depletion immunoblotting assay with human topo II α , topo II β , and β -actin antibodies. The relative fold was calculated using densitometric analysis from 3 independent experiments. MDA-VP cells were designated as 1.0 and calculations were adjusted according to the β -actin protein loading control

side, while topo II β is the preferred target for amsacrine in MDA-VP cells. The etoposide sensitivity and resistance are more related to topo II α gene expression than to topo II β expression. MDA-VP cells expressed lower levels of topo II α RNA and protein than MDA parental cells. In contrast, topo II β RNA and protein levels were relatively the same in both cell types. MDA-VP cells are more resistant to etoposide than to amsacrine and this correlates to the topo II α and topo II β protein levels. Correlation between mRNA topo II levels and cell kill are not always universal. The level of drug-stabilized cleavable complex formation is the most important factor (Koo et al, 1999). Our previous studies show that etoposide-induced topo II α -DNA cleavable complex formation is also significantly lower in MDA-VP cells than in parental cells, supporting the hypothesis that low levels of topo II α account for the etoposide resistance of these cells. Drug uptake and participation of P-glycoprotein or the multiple drug-resistant associated protein do not play a role in resistance of MDA-VP cells (Asano et al 1996).

Transfer of the human topo II α gene into MDA-VP cells using an adenoviral vector increased topo II α protein levels without an appreciable change in topo II β protein levels. The topo II α protein produced following transduction was sensitive to etoposide but not to amsacrine. Etoposide-induced cytotoxicity was enhanced 4.5-fold in cells transduced with topo II α , whereas amsacrine-induced cytotoxicity did not change significantly. These results indicate that topo II α gene transfer does not alter topo II β expression and that the enhanced sensitivity to etoposide is secondary to the change in topo II α .

The involvement of topo II β in amsacrine sensitivity is also supported by others. Herzog et al (1998) have shown that topo II β mRNA levels in HL60/AMSA amsacrine-resistant human leukaemia cells are only 10% of those in HL-60 parental cells and that topo II β protein is not detectable in HL60/AMSA cells. However, these cells are sensitive to etoposide. Withoff et al (1996) have additionally demonstrated that amsacrine resistance in

GLCA/AM3y cells, a subline of the human small cell lung carcinoma cell line, is linked to a major decrease in topo II β protein. Dereuddre et al were able to increase the sensitivity of a Chinese hamster lung cell line to amsacrine by transfection with the topo II β gene (Dereuddre et al, 1997).

Topo II β have different tissue distribution. High levels of topo II α expression have been seen in aggressive proliferating tumours, whereas topo II β appears to be expressed ubiquitously in quiescent cells (Turley et al, 1997). Topo II α is essential for survival of eucaryotic cells (Wang, 1996), while topo II β does not appear to be essential for either proliferation or survival (Yang et al, 2000; Herzog et al, 1998). Such findings may help explain the greater clinical utility of etoposide versus amsacrine. Each topo II isoform appears to carry out a different cellular function and plays a different role in drug resistance. It is important to understand how tumour cell sensitivity may be influenced by differential expression of these two isoforms.

In summary, our data indicate that topo II α gene transfer does not affect topo II β expression, and the ability to circumvent etoposide resistance using topo II α gene transfer is secondary to enhanced production of the drug-sensitive protein. These data substantiate the hypothesis that etoposide preferentially targets topo II α , while amsacrine targets topo II β . In addition we have shown that we can successfully manipulate topo II α gene expression in cells without the problem of feedback inhibition previously experienced by us and other laboratories (Asano et al, 1996). We attributed this to our use of the strong cytomegalovirus promoter in our adenoviral vector construct. This vector can be manipulated by making mutations in specific parts of the gene and thus provide a valuable tool with which to investigate the biology of human topo II α expression and function.

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