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Murine Transgenic Cells Lacking DNA Topoisomerase IIβ Are Resistant to Acridines and Mitoxantrone: Analysis of Cytotoxicity and Cleavable Complex Formation

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ABSTRACT

Murine transgenic cell lines lacking DNA topoisomerase II (topo II)β have been used to assess the importance of topo IIβ as a drug target. Western blot analysis confirmed that the topo IIβ −/− cell lines did not contain topo IIβ protein. In addition, both the topo IIβ +/+ and topo IIβ −/− cell lines contained similar levels of topo IIα protein. The trapped in agarose DNA immunostaining assay (TARDIS) was used to detect topo IIα and β cleavable complexes in topo IIβ −/− and topo IIβ +/+ cells. These results show that both topo IIα and β are in vivo targets for etoposide, mitoxantrone, and amsacrine (mAMSA) in topo IIβ +/+ cells. As expected, only the α-isoform was targeted in topo IIβ −/− cells. Clonogenic assays comparing the survival of topo IIβ −/− and topo IIβ +/+ cells were carried out to establish whether the absence of topo IIβ caused drug resistance. Increased survival of topo IIβ −/− cells compared with topo IIβ +/+ cells was observed after treatment with amsacrine (mAMSA), methyl *N*-(4′-[9-acridinylamino]-2-methoxyphenyl) carbamate hydrochloride (AMCA), methyl *N*-(4′-[9-acridinylamino]-2-methoxyphenyl)carbamate hydrochloride (mAMCA), mitoxantrone, and etoposide. These studies showed that topo IIβ −/− cells were significantly more resistant to mAMSA, AMCA, mAMCA, and mitoxantrone, than topo IIβ +/+ cells, indicating that topo IIβ is an important target for the cytotoxic effects of these compounds.

Eukaryotic topoisomerase II (topo II) is an ATP-dependent nuclear enzyme that catalyzes changes in DNA topology. The reaction mechanism involves the passage of one DNA duplex through another by transiently cleaving a single DNA helix to create a DNA gate. During the cleavage reaction, a covalent enzyme-DNA intermediate is formed between a tyrosine residue of each topo II monomer and the 5′-phosphate group of the cleaved DNA. This covalent intermediate is known as a "cleavable complex" (Wang, 1996; Austin and Marsh, 1998). Topo II has been implicated in a number of cellular processes, including DNA replication, recombination, and chromatin organization (Earnshaw et al., 1985; Chen et al., 1996; Wang, 1996). In addition, it is an important cellular target for a number of currently available antineoplastic agents (Zwelling et al., 1987; Osheroff, 1989).

Mammals possess two isoforms of topo II, termed α (170 kDa) and β (180 kDa), which have been mapped to human chromosomes 17q21–22 and 3p24, respectively (Tan et al., 1992). They are highly homologous in the N-terminal three-quarters (78% identity) but show lower homology in the C-terminal quarter (34% identity) (Austin et al., 1993).

Topo IIα and β are differentially regulated during the cell cycle and are thought to carry out distinct cellular functions (Austin and Marsh, 1998, and references therein). Levels of topo IIα increase significantly during the S and G2/M phases of the cell cycle and subsequently decrease after mitosis (Heck et al., 1988; Prosperi et al., 1994). Topo IIα is therefore considered to be a specific marker for cellular proliferation, being required for chromosome condensation and segregation of intertwined daughter chromosomes (Heck and Earnshaw, 1986; Wang, 1996). In contrast, levels of topo IIβ fluctuate less throughout the cell cycle; however, they are still cell cycle dependent (Prosperi et al., 1994; Meyer et al., 1997). The cellular function of topo IIβ is as yet unknown.

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ABBREVIATIONS: topo, topoisomerase; mAMSA, amsacrine; AMCA, methyl *N*-(4′-[9-acridinylamino]-phenyl)carbamate hydrochloride; mAMCA, methyl *N*-(4′-[9-acridinylamino]-2-methoxyphenyl)carbamate hydrochloride; FITC, fluorescein isothiocyanate; TARDIS, trapped in agarose DNA immunostaining.

Topo II is an important cellular target for a number of currently available chemotherapy agents (Zwelling et al., 1987; Osheroff, 1989). These drugs inhibit the normal cellular function of topo II by stabilizing the usually transient "cleavable complex" that is formed during the catalytic cycle of the enzyme. Stabilized cleavable complexes are the primary lesions produced within the cell that initiate cell death. These are thought to be converted to permanent DNA breaks during DNA replication, causing various chromosomal aberrations (Pommier et al., 1985a; Chen et al., 1996; Suzuki et al., 1997).

Studies of drug-resistant cell lines have revealed a number of factors responsible for cellular resistance to topo II inhibitors. Classical multidrug resistance, caused by overexpression of genes encoding multidrug resistance protein 1 and multidrug resistance-associated protein leads to increased efflux of exogenous toxins, including topo II inhibitors, from the cell, thereby reducing their cytotoxicity (Long et al., 1991; Lorico et al., 1995). In addition, reduced levels of topo II and genetic mutations have been identified in various drug-resistant cell lines. Further investigations of sensitive and resistant cell lines have indicated that topo IIα and β may be differentially targeted by topo II agents (Mirski et al., 1993; Dereuddre et al., 1995).

Levels of topo IIα and β in neoplastic tissues can vary between tumor types (Giaccone et al., 1995). Cycling tumors contain higher levels of topo IIα than tumors with a low percentage of cycling cells, and slow-growing tumors contain significant levels of topo IIβ. It also has been reported that levels of topo IIβ are increased in human tumors compared with normal tissues (Turley et al., 1997). Furthermore, lymphomas and breast cancers have been shown to contain predominately more topo IIα than β, whereas seminomas contain equal levels of each isoform (Holden et al., 1992). Therefore, levels of topo IIα and β in cancerous tissues and the specificity of topo II inhibitors are important factors to consider when trying to make regimes for cancer chemotherapy more selective.

This paper investigates whether topo IIα and β are differentially targeted in vivo by several topo II inhibitors. Murine cell lines totally lacking topo IIβ have been used to assess the importance of topo IIα and β in the cytotoxic effects of etoposide, mitoxantrone, and three acridine derivatives, including amsacrine (mAMSA).

Materials and Methods

Construction of Murine Cell Lines

Targeted replacement of two exons of the murine *TOP2β* gene, one of which contains the codon for the active site tyrosyl residue of the enzyme, with a neomycin-resistance marker was carried out following standard protocols (W.L. and J.C. Wang, unpublished data). Heterozygous *TOP2β* +/− mice were intercrossed and stage 13.5 embryos genotyped. A TOP2β +/+ and a top2β −/− embryo were selected and used in the preparation of fibroblasts. Construction of immortalized fibroblasts, with simian virus 40 transformation, will be described elsewhere (L.L., W.L., and G.L., unpublished data).

Cell Culture

Murine topo IIβ −/− cell lines (mtop2β-5 and mtop2β-6) and a wild-type topo IIβ +/+ cell line (mTOP2β-4) were grown as monolayers at 37°C in a humidified atmosphere containing 5% CO_2. These were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (50 μg/ml)/streptomycin (50 μg/ml). Cell culture reagents were obtained from Gibco BRL, Paisley, UK. Cells were tested for *Mycoplasma* and were found to be free of contamination.

Whole-Cell Extracts

Whole-cell extracts were carried out as described by Mirski et al. (1993). Briefly, cells were seeded at ~2.5×10^5 cells/9-cm plate and left for 48 h to ensure the cells were growing exponentially. Cells were then scraped into 1 ml of ice-cold PBS and centrifuged at 1000*g* for 3 min. Cells were subsequently resuspended in four packed cell volumes of solution containing 0.25% SDS, 0.5 mg/ml deoxyribonuclease I, 0.25 mg/ml ribonuclease A, 10 mM MgCl, 50 mM Tris-HCl (pH 7.4) plus protease inhibitors (4 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin) and left on ice for 1 h. Proteins were then solublized by heating for 10 min at 68°C in 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.6 M Tris-HCl (pH7.5). Protein concentrations were subsequently estimated with the Bradford assay (Harlow and Lane, 1988).

Western Blotting

Proteins from whole-cell extracts of mTOP2β-4, mtop2β-5, and mtop2β-6 cells were electrophoresed (40 μg of protein/lane) on 5% SDS polyacrylamide gels and transferred to a nitrocellulose filter by standard protocols. For detection of topo IIα and β, blots were incubated with isoform specific antitopo II polyclonal antibodies, 18511(α) or 18513(β). They were used at 1:200 and 1:500 dilutions, respectively. The nitrocellulose filter was processed with the enhanced chemiluminescence detection kit (Amersham Corp., Amersham, UK) following the manufacturer's instructions.

Clonogenic Assay

Murine topo IIβ +/+ (mTOP2-β4) and topo IIβ −/− (mtop2β-5 or mtop2β-6) cells were seeded (2.5×10^5/plate) into 9-cm plates. After 48 h, drug was added to exponentially growing cells at appropriate concentrations for 2 h. The clonogenic assay was carried out as described previously in Moses et al. (1988). Etoposide, mitoxantrone, and mAMSA were obtained from Sigma Chemical Co. (Poole, Dorset, UK). AMCA and mAMCA (analogs of mAMSA) were a gift from Dr. B. Baguley (University of Auckland School of Medicine, Private Bag 92019, Auckland, New Zealand).

Assay of Drug-Stabilized DNA Topo II-DNA Complexes

Preparation of Slides. The slide preparation method is described in detail by Willmore et al. (1998). Briefly, cells were seeded (3×10^4 cells/well) into six-well tissue culture plates. These were grown for ~48 h and drug was added to exponentially growing cells at appropriate concentrations. Microscope slides were precoated with agarose, and drug-treated or control (untreated) cells were immediately embedded in agarose and spread onto the slide. Slides were then placed in lysis buffer containing protease inhibitors for 30 min (after this stage slides could be stored at −20°C in PBS containing 10% glycerol), followed by 30 min in 1 M NaCl plus protease inhibitors. Slides were then washed three times in PBS (5 min/wash) and exposed to primary antisera for 1 to 2 h. Two antitopo II polyclonal antibodies, 18511(α) and 18513(β), were used. These antibodies were specific for the α- and β-isoforms of topo II, respectively. Both antibodies were used at a 1:50 dilution in PBS containing 0.1% v/v Tween 20 and 1% w/v BSA. Slides were washed three times in PBS containing 0.1% Tween 20 (PBST) and subsequently exposed for 1 to 2 h to a secondary antibody [anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody, $F(ab')_2$ fragment; Sigma] diluted in PBST containing 1% w/v BSA. Slides were washed three times in PBST followed by an overnight wash in PBS containing protease inhibitors, at 4°C.

Quantification of Cleavable Complexes. Slides were stained with Hoechst 33258 (10 μM in PBS; Sigma Chemical Co.) for 5 min

and cover slips were applied and secured. Images of blue (Hoechst-stained DNA) fluorescence and green (FITC-stained drug-stabilized topo II) immunofluorescence were then captured with an epifluorescence microscope attached to a cooled slow scan charge-coupled device camera. For each of eight randomly chosen fields of view, images of blue and green fluorescence were captured to give a total of ~100 cells/dose for each antibody.

Images were then analyzed to quantify the levels of Hoechst (blue) fluorescence and FITC (green) immunofluorescence with Imager 2 software (Astrocam, Cambridge, UK) based on Visilog 4 (Noesis, Paris, France). All images were corrected for stray light and camera background. Additionally, images were subjected to blue and green shade correction to compensate for variation in intensity of illumination and nonuniformities in light transmission (Willmore et al., 1998, and reference therein). Statistical analysis was carried out using GraphPad Prism software (Cherwell Scientific, Oxford, UK). Paired *t* tests, repeated measures one-way ANOVA (Tukey post test), and two-way ANOVA were the main forms of analysis used for statistical comparisons.

Results

Levels of Topo IIα and β. Western blot analysis confirmed that murine topo IIβ −/− cells (mtop2β-5 and mtop2β-6) did not contain any topo IIβ. Figure 1a illustrates levels of topo IIβ found in mTOP2β-4, mtop2β-5, and mtop2β-6 cells after the nitrocellulose filter was probed with 18513(β). A 180-kDa band was identified in mTOP2β-4 whole-cell extracts but was absent from the mtop2β-5 and mtop2β-6 whole-cell extracts, clearly illustrating that only mTOP2β-4 cells contain topo IIβ. Levels of topo IIα in mTOP2β-4, mtop2β-5, and mtop2β-6 cells also were investigated (Fig. 1b). Staining with 18511(α) identified a 170-kDa band of similar intensity in each whole-cell extract, indicating that they contained similar levels of topo IIα.

Cell Line Characteristics. The doubling time of mTOP2β 4, mtop2β-5, and mtop2β-6 cells was 16 to 20 h for all three cell lines with no obvious difference between them. In addition, all three cell lines grow in elongated fashion and spread over the tissue culture plate; however, mtop2β-6 cells appear to be slightly larger. Preliminary investigations suggest that there is no obvious difference in the cell cycle distribution of each cell line.

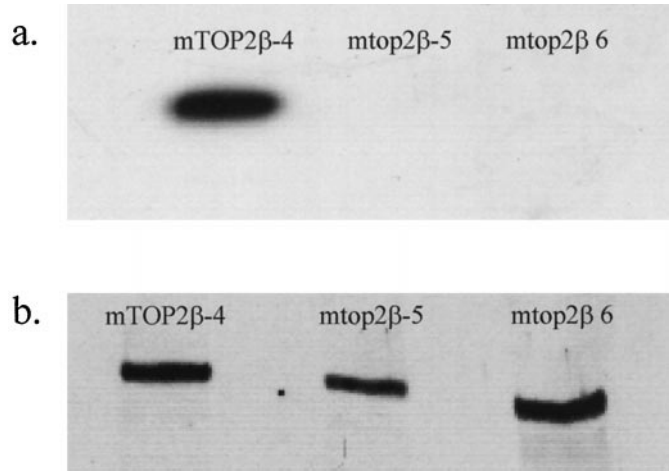

Fig. 1. Western blot analysis of levels of topo IIα and β in topo IIβ +/+ mTOP2β-4 cell and topo IIβ −/− cells (mtop2β-5 and mtop2β-6). Each lane was loaded with 40 μg of whole-cell extracts. These blots were probed with 18513(β) or 18511(α) isoform-specific antisera, shown in (a) and (b), respectively.

Sensitivity of Murine Topo IIβ +/+ and Murine Topo IIβ −/− Cells to Topo II Inhibitors. Clonogenic assays were carried out to examine the cytotoxicity of etoposide, mitoxantrone, and the three acridine derivatives mAMSA, AMCA, and mAMCA on mTOP2β-4, mtop2β-5, and mtop2β-6 cells to determine whether the absence of topo IIβ caused drug resistance. Initially, both mtop2β-5 and mtop2β-6 cells were used, however, both cell lines gave similar results so in subsequent experiments only mtop2β-5 cells were used.

Figure 2, a and b compare the survival of topo IIβ +/+ and topo IIβ −/− cells after exposure to a range of etoposide concentrations. IC_{50} values of mTOP2β-4, mtop2β-5, and mtop2β-6 cell lines were 1.7 ± 0.8, 2.1 ± 1.4, and 1.1 ± 1.0 μM, respectively. Student's *t* test comparing mTOP2β-4/mtop2β-5 and mTOP2β-4/mtop2β-6 IC_{50} values indicated that there was no significant difference in the sensitivity of mTOP2β-4, mtop2β-5, and mtop2β-6 cells to etoposide at the IC_{50}. This also was confirmed with two-way ANOVA. However, at high doses of etoposide (i.e., >10 μM) mtop2β-5 and mtop2β-6 cell survival was greater than observed for mTOP2β-4 cells, although this difference was not statistically significant (*t* test). For example, for cell line mtop2β-6 compared with mTOP2β-4, the *p* values at 20 and 50 μM were $p = .1285$ and $p = .1143$, respectively.

Figure 2c shows the survival of mTOP2β-4 and mtop2β-5 cells after exposure to a range of mitoxantrone concentrations. This cytotoxicity data indicate that mtop2β-5 cells were less sensitive than mTOP2β-4 cells at high concentrations. Statistical analysis (*t* test) comparing the IC_{50} values of mTOP2β-4 and mtop2β-5 cell lines (4.9 ± 4.0 and 5.2 ± 3.5 nM, respectively) indicated that both cell lines were equally sensitive to mitoxantrone, also confirmed with two-way ANOVA. However, the difference in mTOP2β-4 and mtop2β-5 cell survival at 50 nM was statistically significant ($p = .0449$; *t* test), indicating that topo IIβ −/− cells (mtop2β-5) were more resistant to mitoxantrone than wild-type topo IIβ +/+ cells (mTOP2β-4) at this concentration.

The cytotoxic effects of mAMSA, AMCA, and mAMCA on mTOP2β-4 and mtop2β-5 cells are illustrated in Fig. 2, d–f, respectively. Each of these graphs follows a similar trend, the percentage of mtop2β-5 cells surviving is greater than mTOP2β-4 cells over the full range of drug concentrations used. IC_{50} values of mTOP2β-4 and mtop2β-5 cells were 0.06 ± 0.01 and 0.08 ± 0.03 μM (mAMSA), 0.33 ± 0.05 and 0.57 ± 0.18 μM (AMCA), and 0.34 ± 0.2 and 0.7 ± 0.3 μM (mAMCA), respectively. Two-way ANOVA was used to compare the survival of mTOP2β-4 and mtop2β-5 cells after treatment with mAMSA, AMCA, and mAMCA, and this test confirmed that mtop2β-5 cells were significantly more drug resistant than wild-type mTOP2β-4 cells ($p = .0014$, .0005, and .0034, respectively).

These data show that the sensitivity of topo IIβ −/− cells to etoposide was equivalent to that of the wild-type cells at lower drug concentrations. However, the topo IIβ −/− cells were significantly more resistant to mitoxantrone (at higher concentrations), mAMSA, AMCA, and mAMCA.

Quantification of Cleavable Complexes in mTOP2β-4, mtop2β-5, and mtop2β-6 Cells. The trapped in agarose DNA immunostaining (TARDIS) assay (Willmore et al., 1998) was used to quantify levels of drug-stabilized topo IIα and β cleavable complexes in mTOP2β-4, mtop2β-5, and mtop2β-6 cells. The assay involved embedding control

(i.e., untreated cells) or drug-treated cells in agarose on microscope slides. The cells were then lysed to disrupt the cellular membranes and remove soluble proteins. After this, salt extraction was used to remove nuclear proteins and any noncovalently bound topo II from the DNA matrix. Drug-stabilized topo IIα/β-DNA complexes remained and were detected by staining with isoform specific antisera, either 18511(α) or 18513(β), followed by an FITC-conjugated secondary antibody. Digital images of Hoechst (DNA) fluorescence and FITC immunofluorescence (drug-stabilized topo IIα/β cleavable complexes) were captured and levels of fluorescence were quantified.

Figures 3 to 5 show the results of TARDIS experiments to measure topo IIα and β cleavable complexes in mTOP2β-4, mtop2β-5, and mtop2β-6 cells after exposure to etoposide, mitoxantrone, and mAMSA, respectively. Figure 3 illustrates mean Hoechst fluorescence (a and c) and immunofluorescence (b and d) values from mTOP2β-4, mtop2β-5, and mtop2β-6 cells after a 2-h exposure to 0, 10, and 100 μM etoposide. As illustrated in Fig. 3, a and c, there was a slight increase in Hoechst fluorescence in mTOP2β-4, mtop2β-5, and mtop2β-6 cells with increasing concentrations of etoposide as reported previously for human leukemic CCRF-CEM cells (Willmore et al., 1998).

An etoposide dose-dependent increase in FITC immunofluorescence in mTOP2β-4, mtop2β-5, and mtop2β-6 cells after staining with 18511(α) is illustrated in Fig. 3b. The increase observed between 0 and 100 μM in each cell line was signif-

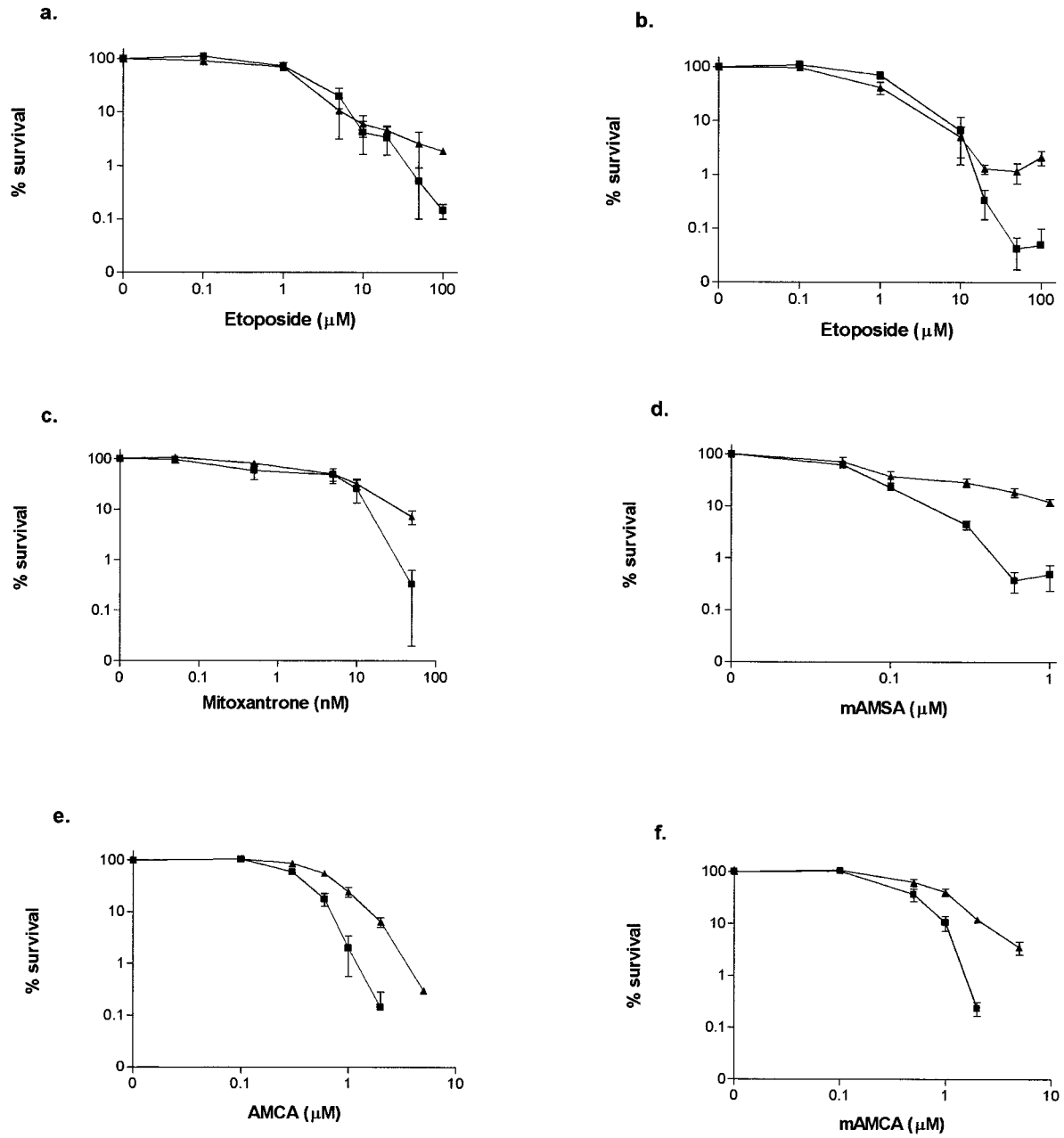

Fig. 2. Survival curves wild-type mTOP2β-4 cells (■) and topo IIβ −/− mtop2β-5 cells [▲, in (a) and (c–f)] or mtop2β-6 cells [▲ in (b)] cells after exposure to five different topo II inhibitors. Each plot is derived from at least three independent experiments. Each data point shows the mean of at least two to four values ± S.E. a and b, percentage of survival of mTOP2β-4/mtop2β-5 and mTOP2β-4/mtop2β-6 cells after a 2-h exposure to etoposide. c–f, survival curves comparing the survival of mTOP2β-4 and mtop2β-5 cells after a 2-h exposure to mitoxantrone, mAMSA, AMCA, and mAMCA, respectively.

icant (all p values $< .01$). These results confirmed that etoposide-topo IIα-stabilized cleavable complexes were formed in all three cell lines. Figure 3d shows the levels of FITC immunofluorescence from mTOP2β-4, mtop2β-5, and mtop2β-6 cells after probing with 18513(β). As illustrated, etoposide induces a clear dose-dependent increase in FITC immunofluorescence in mTOP2β-4 cells that was highly significant at 100 μM ($p < .001$). In contrast, the topo IIβ −/− cells (mtop2β-5 and mtop2β-6) showed no clear increase in FITC immunofluorescence with increasing concentrations of etoposide.

Figure 4, a and d show Hoechst fluorescence for mTOP2β-4, mtop2β-5, and mtop2β-6 cells after treatment with mitoxantrone. mTOP2β-4 cells showed higher levels of Hoechst staining than mtop2β-5 or mtop2β-6 cells in control and drug-treated cells. Also, a slight increase in Hoechst fluorescence was observed between untreated and drug-treated cells, as seen with etoposide (Willmore et al., 1998).

A mitoxantrone dose-dependent increase in FITC immunofluorescence in mTOP2β-4, mtop2β-5, and mtop2β-6 cells was observed after staining with 18511(α) (Fig. 4, b and c). The difference between 0 to 1 and 0 to 10 μM was significant for all cell lines (all p values $< .05$). There was no further increase in 18511(α) immunofluorescence between 1 and 10 μM mitoxantrone in mTOP2β-4 and mtop2β-5 cells (Fig. 4b); in fact, a slight decrease was observed. On further investigation, it was determined that if concentrations were raised to 100 μM there was a further decrease in immunofluorescence, to approximately 2.5×10^4 fluorescence units (data not shown). In addition, Fig. 4b demonstrates that at 10 μM mitoxantrone mTOP2β-4 cells had significantly higher levels of 18511(α) immunofluorescence than mtop2β-5 cells ($p = .0317$). In contrast, the plateau in immunofluorescence and the reduction in 18511(α) immunofluorescence in topo IIβ −/− cells was not observed in mtop2β-6 cells (Fig. 4c).

Figure 4, e and f illustrate the levels of FITC immunofluorescence from mTOP2β-4, mtop2β-5, and mtop2β-6 cells after staining with 18513(β). There was a dose-dependent increase that was significant from 0 to 1 and 0 to 10 μM mitoxantrone (both p values $< .05$) in mTOP2β-4 cells. Levels of immunofluorescence plateau between 1 and 10 μM (Fig. 4e). In contrast, there was no increase in immunofluorescence in topo IIβ −/− (mtop2β-5 and mtop2β-6) cells, as expected.

Figure 5 illustrates Hoechst and FITC immunofluorescence after treatment with mAMSA. Figure 5, a and c, show that mTOP2β-4 cells generally have higher levels of Hoechst staining than mtop2β-5 and mtop2β-6 cells. In addition, levels of Hoechst fluorescence, in each cell line, increase in drug-treated cells compared with untreated controls.

The mean immunofluorescence values from mTOP2β-4, mtop2β-5, and mtop2β-6 cells after staining with 18511(α) illustrates a dose-dependent increase for each cell line (Fig. 5b). Furthermore, Fig. 5d shows a dose-dependent increase in 18513(β) immunofluorescence in mTOP2β-4 cells between 0.5, 5, and 50 μM mAMSA with a slight decrease at 100 μM.

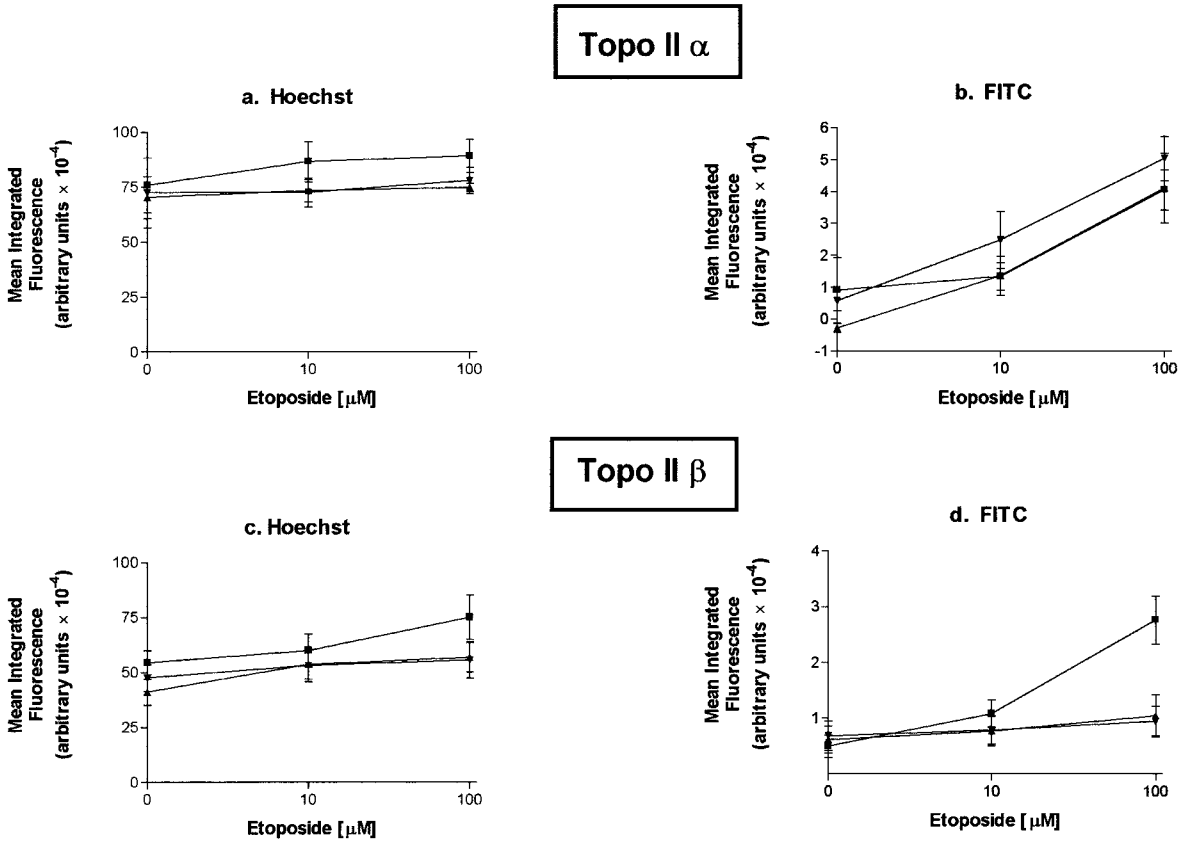

Fig. 3. TARDIS analysis of cells treated with etoposide for 2 h. Slides were prepared and processed as described in *Materials and Methods*. Plots show means ± S.E. of mean fluorescence values from three to eight independent experiments. a and c, Hoechst fluorescence; b and d, FITC immunofluorescence. a and b, slides stained for topo IIα with antisera 18511(α). c and d, slides stained for topo IIβ with antisera 18513(β). ■, mTOP2β-4; ▲, mtop2β-5; ▼, mtop2β-6.

The increase is highly significant from 0 to 100 μM (p = .0094; t test). In contrast, there is no significant increase in 18513(β) FITC immunofluorescence in mtop2β-5 and mtop2β-6 cells.

Discussion

Homozygous topo IIβ −/− cells (mtop2β-5 and mtop2β-6) and wild-type control cells (mTOP2β-4) have been exploited to assess the importance of topo IIα and β in the cytotoxic effects of five topo II inhibitors. Our results show that in mTOP2β-4 cells, both topo IIα- and β-stabilized cleavable complexes are formed after treatment with etoposide, mitoxantrone, and mAMSA. In addition, they suggest that topo IIβ is important for the cytotoxic action of the acridine derivatives mAMSA, AMCA, and mAMCA and the anthraquinone mitoxantrone.

Quantification of etoposide-induced topo IIα- and β-cleavable complexes in mTOP2β-4 cells indicated that both isoforms are in vivo targets for etoposide. These studies confirm data obtained from earlier investigations on CCRF-CEM human leukemic cells (with the same assay) that showed that both isoforms formed etoposide-stabilized cleavable complexes in vivo (Willmore et al., 1998). In vitro data (Austin et al., 1995; Perrin et al., 1998) and yeast complementation studies (Meczes et al., 1997) also have implicated both topo IIα and β as possible targets for etoposide. In contrast, cytotoxicity data presented herein suggest that topo IIβ is probably not the major cytotoxic target for etoposide. This is in agreement with previous data, which have implicated topo IIα as the main target of etoposide. For example, mutations and down-regulation of topo IIα, in vivo, leads to increased resistance (Feldoff et al., 1994; Mirski and Cole, 1995; Son et al., 1998).

At high concentrations of mitoxantrone and mAMSA, levels of 18511(α) and 18513(β) immunofluorescence reach a plateau, suggesting that the level of cleavable complexes had reached saturation. However, this could have resulted from suppression of topo II activity at high drug concentrations. Both drugs are DNA intercalators and at low concentrations they intercalate into the DNA and stabilize topo II-DNA complexes. At higher concentrations, it has been reported that suppression of topo II function occurs that could prevent the formation of drug-stabilized cleavable complexes (Pommier, 1997). It is therefore possible that the reduction in immunofluorescence at the highest doses was due to the topo II-suppressing activity of DNA intercalators.

Cytotoxicity studies have been used to assess the role of topo IIβ in inducing cell death. Figure 2c shows that topo IIβ −/− cells are resistant to mitoxantrone at high drug concentrations (50 nM), suggesting that topo IIβ is important for cytotoxicity. Our results confirm previous reports from in vitro (Perrin et al., 1998) and yeast complementation studies (Meczes et al., 1997) that also suggested that both isoforms were important for mitoxantrone cytotoxicity. Studies with sensitive and resistant cell lines have implicated a reduction of topo IIα in mitoxantrone resistance (Son et al., 1998). In contrast, topo IIβ has been implicated in mitoxantrone resistance, although this evidence was inconclusive because the resistant cells that lacked topo IIβ also contained altered topo IIα (Harker et al., 1991, 1995). Also, a cell line resistant to etoposide (due to cytoplasmic location of topo IIα) was not cross-resistant to mitoxantrone, suggesting that topo IIα is not essential for mitoxantrone-induced cell death (Feldoff et al., 1994). These previous studies are inconclusive. The isogenic cell lines used herein have the

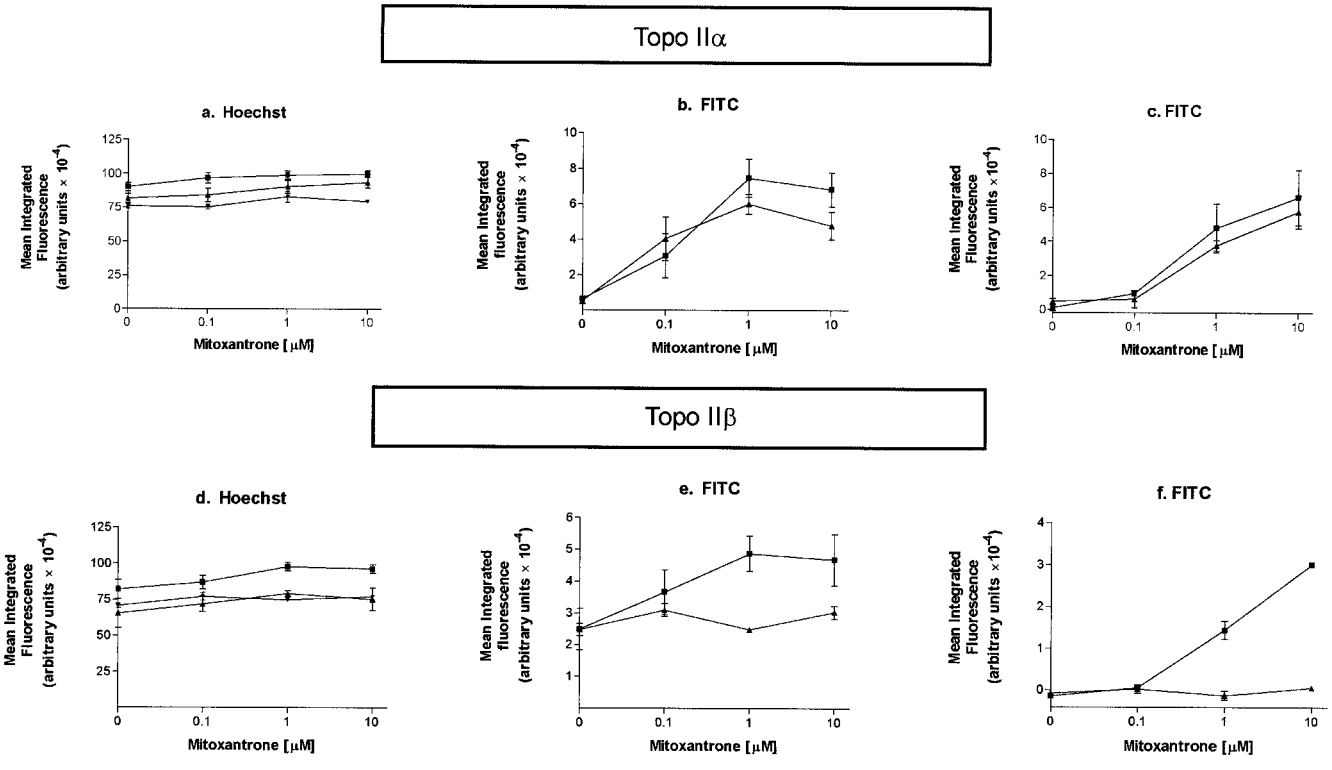

Fig. 4. TARDIS analysis for cells treated with mitoxantrone for 2 h. Plots show means ± S.E., calculated from three to four independent experiments. a and d, Hoechst fluorescence after probing with 18511(α) and 18513(β), respectively. b and c, FITC immunofluorescence in mTOP2β-4/mtop2β-5 and mTOP2β-4/mtop2β-6 cells after probing with 18511(α). e and f, FITC immunofluorescence in mTOP2β-4/mtop2β-5 and mTOP2β-4/mtop2β-6 cells after probing with 18513(β). For (a), (b), (d), and (e), ■, mTOP2β-4; ▲, mtop2β-5; ▼, mtop2β-6. For (c) and (f), ■, mTOP2β-4; ▲, mtop2β-6.

advantage that the only difference between topo IIβ +/+ (mTOP2β-4) and topo IIβ −/− (mtop2β-5 and mtop2β-6) cells is the loss of topo IIβ in the knockout cell lines. This is not the case with drug-resistant cell lines that have acquired resistance after drug exposure.

The mtop2β-5 cells were considerably more resistant to acridine derivatives (mAMSA, AMCA, and mAMCA) than mTOP2β-4 cells over a wide range of drug concentrations; therefore, topo IIβ is a significant cellular target for these compounds. However, although topo IIβ is an important target, it is not the only target because the mtop2β-5 cells still undergo drug-induced cell death. The results presented herein verify previous in vitro work that demonstrated that mAMSA could induce cleavage with both isoforms (Austin et al., 1995). In addition, chinese hamster lung cells resistant to 9-OH ellipticine that contained lower levels of topo IIα and no topo IIβ were resistant to mAMSA. Transfection of topo IIβ into the resistant cells restored mAMSA sensitivity (Dereuddre et al., 1997). Also, a GCL_4 cell line with reduced levels of topo IIβ and no alteration in topo IIα was resistant to mAMSA (Withoff et al., 1996). A recent report has suggested that reduced expression of *TOP2β* may contribute to mAMSA resistance (Herzog et al., 1998). In contrast, two topo IIα mutated cell lines were resistant to mAMSA (Lee et al., 1992) and expression of topo IIα protein has been correlated to mAMSA sensitivity (Houlbrook et al., 1995). Our TARDIS data, obtained with isogenic cell lines, shows that both isoforms are in vivo targets for mAMSA, and survival assays with topo IIβ −/− cells determined that topo IIβ is a significant target for cell killing.

The acridines AMCA and mAMCA are extremely toxic toward noncycling cells and it has been postulated that they might target topo IIβ because this isoform is predominant in nonproliferating cells (Baguely et al., 1997; Moreland et al., 1997). Our results suggest that topo IIβ is targeted by both of these compounds in vivo.

Interestingly, there is a reduction of topo IIα-cleavable complexes formed in mtop2β-5 and mtop2β-6 cells compared with wild-type mTOP2β-4 cells at very high concentrations of mitoxantrone and mAMSA (Figs. 4b and 5b). It could therefore be argued that resistance of mtop2β-5 cells, demonstrated with cytotoxicity studies (Fig. 2, c and d) may not have been because of the absence of topo IIβ but rather because of a reduction in total number of α- and β-cleavable complexes. However, we do not think that this is the case for a number of reasons. First, a significant difference in the levels of FITC immunofluorescence was only observed at extremely high doses of mitoxantrone (~2000-fold higher than IC_{50} values obtained from clonogenic survival studies) when comparing mTOP2β-4 and mtop2β-5 cells. No statistically significant difference was seen for mAMSA at any of the drug doses. Second, topo IIβ-mAMSA and mitoxantrone-stabilized cleavable complexes have been identified in vivo in mTOP2β-4 cells. And third, mTOP2β-4, mtop2β-5, and mtop2β-6 cells contain similar levels of topo IIα. We propose a hypothesis to explain the reduction in topo IIα immunofluorescence in knockout cells at high drug concentrations. Topo II has the ability to alter DNA topology, therefore the absence of the β-isoform in topo IIβ −/− cells could cause these cells to have a different DNA conformation compared with that in the wild-type mTOP2β-4 cells that contain both iso-

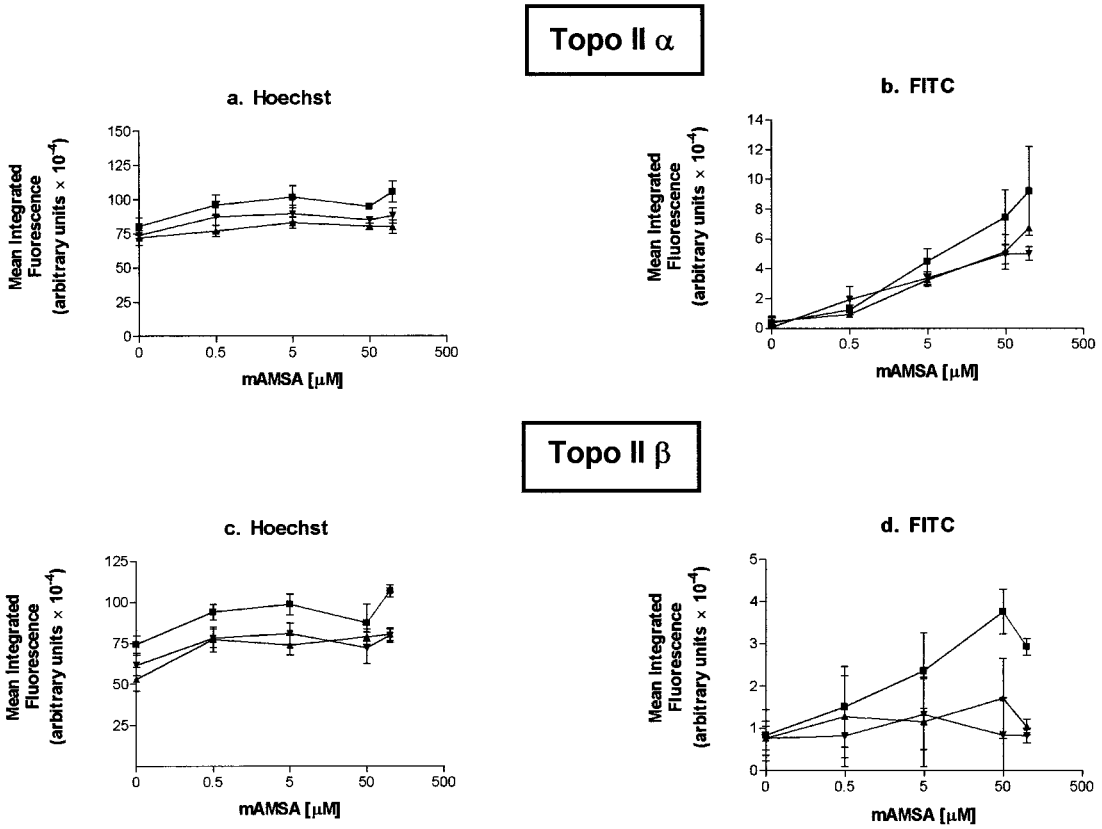

Fig. 5. TARDIS analysis for cells treated with mAMSA for 2 h. Each graph shows the means ± S.E., calculated from four to six independent experiments. a and c, mean Hoechst fluorescence values for cells treated with 18511(α) or 18513(β), respectively. In addition, (b) and (d) show average FITC immunofluorescence values after staining with 18511(α) or 18513(β), respectively. ■, mTOP2β-4; ▲, mtop2β-5; ▼, mtop2β-6.

forms. We observed that mtop2β-5 and mtop2β-6 cells consistently show less Hoechst staining than mTOP2β-4 cells, particularly after drug treatment, suggesting that the DNA conformation was altered in the knockout cells. The difference in DNA conformation might alter the way drugs intercalate into the DNA at high concentration and affect the levels of topo IIα drug-stabilized cleavable complexes in topo IIβ $-/-$ cells compared with wild-type cells. We do not think that the reduction in topoIIα cleavable complexes after treatment with high concentrations of mAMSA and mitoxantrone is responsible for the drug resistance of mtop2β-5 cells. However, we cannot disregard this factor completely.

In conclusion, murine topo IIβ $-/-$ cell lines have been used for the first time to investigate the role of topo IIβ in the cytotoxic action of several topo II inhibitors and to investigate the in vivo formation of topo IIα- and topo IIβ-stabilized cleavable complexes. These studies confirm that both topo IIα- and β-stabilized cleavable complexes are formed in vivo after treatment with etoposide, mitoxantrone, and mAMSA, suggesting that both isoforms could be responsible for the cytotoxic effects of these compounds. Cytotoxicity studies, with these isogenic cell lines, have shown that topo IIβ does play a statistically significant role in the cytotoxic action of the three acridine derivatives investigated, mAMSA, AMCA, and mAMCA, and also the anthraquinone mitoxantrone.

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