Down-regulation of human topoisomerase IIα correlates with altered expression of transcriptional regulators NF-YA and Sp1

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Topoisomerase IIα (Topo IIα) is an essential nuclear enzyme with a role in the maintenance of DNA topology. Topo IIα is a target for several anticancer drugs and the levels of activity of this enzyme have been implicated in the development of drug resistance. Our objective was to identify regulatory transcription factors involved in drug-induced down-regulation of Topo IIα. A breast cancer cell line was subjected to a pulsed exposure of doxorubicin and resistant clones propagated. Whole-cell extracts were studied by immunoblotting and RT-PCR for drug-induced changes in the amounts Topo IIα, Sp1, Sp3, NF-Y and MDR1. Topo IIα levels were reduced in six out of eight cell lines. Of these, three showed concomitant changes in the expression of Sp1 and NF-YA. Thus, we provide the first evidence for roles of Sp1 and NF-Y in bringing about the drug-induced down-regulation of Topo IIα gene expression. *Anti-Cancer Drugs* **15:357–362**

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Introduction

Topoisomerases (Topo) are ubiquitous enzymes involved in the maintenance of DNA topology [1], which have also been shown to be the primary target for many commonly used anticancer drugs [2].

In mammals, the type II Topo exist as two isoforms α and β. Topo IIα (174 kDa) and Topo IIβ (182 kDa) share high amino acid sequence identity (~75%) and have a similar gene structure [3]. Topo IIα is an essential enzyme in DNA metabolism that functions by unknotting and untwisting DNA that occurs during replication and cell division [4]. The enzymatic action of Topo IIβ is very similar to that of Topo IIα; however, its role in maintaining DNA topology has yet to be defined.

Topo II-targeted drugs fall into two categories—Topo II poisons and inhibitors. The Topo II poisons stabilize the normally transient Topo II–DNA cleavage complex to turn the enzyme into a cellular toxin [2], while Topo II inhibitors act on other steps of the catalytic cycle. With respect to the Topo II poisons, the levels or activity of the Topo IIα enzyme could determine the amount of drug-induced DNA damage that occurs and in turn may determine the cytotoxicity of the treatment [5,6]. The decreased expression of Topo IIα has been observed in a variety of cell lines that have developed resistance to a range of chemotherapeutic drugs [7–12]. These observations suggest that such a decrease may be a common mechanism of drug resistance to Topo II-targeted drugs.

Mechanisms resulting in the down-regulation of Topo IIα could include transcriptional down-regulation of the Topo IIα gene. While alterations to the Topo IIα mRNA or protein stability are also possible, these mechanisms have yet to be described in resistant cell lines.

The human Topo IIα promoter has been shown to contain two GC boxes, five inverted CCAAT boxes (ICB1–5) and one ATF element among the putative transcription factor binding sites identified [13]. A canonical TATA element was not detected. The ubiquitous transcription factors NF-YA, Sp1 and Sp3 have all been implicated as having roles in the regulation of the Topo IIα promoter [14,15]. NF-YA can bind to ICB1–4, although the affinity for binding at each element varies [16]. NF-YA has been implicated as an activator when bound at ICB1. Sp1 has been identified as a potential activator of the basal Topo IIα promoter activity, and both Sp1 and Sp3 have been shown to bind to GC1 and GC2 [17].

Little is known about the regulation of the Topo IIα promoter in drug-resistant or drug-exposed cells. Sp3 has been implicated as a potential repressor of the Topo IIα

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promoter in etoposide- or teniposide-resistant human epidermoid cells [14] or an activator in a merbarone-resistant CEM cell line [11]. A reduction in NF-YA binding to ICB1–4 has also been implicated in down-regulation of the Topo IIα promoter in drug-resistant cells in several studies [18]. Conversely, an ICRF-8-resistant CEM cell line with increased Topo IIα expression exhibited a decrease in NF-YA binding to ICB3 [19].

We show that down-regulation of Topo IIα in doxorubicin-exposed breast cancer cells is concomitant with a decrease in expression of Sp1 and an increase in the expression of NF-YA. These results provide support for a role of Sp1 as a transcriptional activator and NF-YA as a repressor of Topo IIα expression.

Materials and methods

Cell lines and antibodies

The MDA-MB-231 cell line was purchased from the ATCC (Manassas, VA). Doxorubicin was from Delta West (Bently, Western Australia). The following primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA): Sp3 (D-20)-G, Sp1 (PEP2)-G, NF-YA (C-18) and Topo IIα (K-19). The α-tubulin antibody (clone DM 1A) and the anti IgG horseradish peroxidase-conjugated secondary antibody were from Sigma (St Louis, MO).

Exposure of MDA-MB-231 cells to doxorubicin

MDA-MB-231 cells were grown in MEM media (Sigma) supplemented with 10% fetal calf serum and penicillin/streptomycin. The cells were exposed to a single dose of doxorubicin (5 μM) for 1 h. The cells were then washed and the media was changed twice weekly until surviving cells had grown into single clones. The single clones were isolated, amplified, and then analyzed for Topo IIα, Sp1, Sp3 and NF-YA.

Immunoblotting

Whole-cell extracts were prepared as described by Ladias *et al.* [20]. Protein extracts (5 μg) were separated by polyacrylamide (8%) gel electrophoresis in the presence of SDS. Proteins were transferred to nylon membrane by electroblotting in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3) and the membrane was cut prior to blocking for simultaneous probing of the same membrane with multiple antibodies. Membranes were blocked for 2–3 h at room temperature using 1% blocking reagent (Roche, Auckland, New Zealand) in TBST. Primary antibodies were diluted in 0.5% blocking solution (1:1000 for Topo IIα, Sp1 and Sp3; 1:500 for NF-YA; and 1:2000 for α-tubulin). Membranes were incubated overnight at 4°C with the primary antibodies. After washing with TBST in 0.5% blocking solution for 40 min, membranes were incubated with peroxidase-conjugated secondary antibodies (1:10 000 dilution for Topo IIα and Sp1; 1:5000 for α-tubulin; 1:4000 for Sp3; and 1:2500 for NF-YA) for 30 min at room temperature. The membrane was then washed for 1 h with TBST. Bands were developed using chemiluminescent detection reagents (Roche) according to the manufacturer's instructions and then detected and quantified using a LAS 1000 Darkbox (Fuji) and Image Gauge software.

RNA isolation and production of cDNA

RNA, from cells at 40–60% confluence, was extracted using Trizol according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA). RNA samples (6 μg) were treated with 6 U of amplification grade DNase I (Invitrogen Life Technologies) prior to reverse transcription. Reverse transcription was then carried out for 1 h at 37°C using 1 mM dNTPs, 2 U/μl MMLV reverse transcriptase (Invitrogen Life Technologies) in 50 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithioerythritol in a total volume of 40 μl. PCR was carried out using 1 μl of the reverse transcription reaction with 50 ng of each primer, 3 mM dNTPs, 3 U Taq polymerase (Roche). The PCR (for *MDR1* and *GAPDH*) protocol consisted of 50 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min. The *MDR1* primers [21] used were sense 5′-CTGGTGTTTGGAGAAATGACAG-3′ (nt 632–653) and antisense 5′-CCCAGTGAAAAA-TGTTGCCATTGAC-3′ (nt 1009–1033). Primers for GAPDH [22] used as a control for the reverse transcriptase reactions were sense 5′-CGGGAAGCTT-GTGATCAATGG-3′ (nt 252–272) and antisense 5′-GGCAGTGATGGCATGGACTG-3′ (nt 590–609).

Determination of the doubling time

The population doubling time was calculated as described in Davis [23] using a hemocytometer. The average of three cell counts was determined.

Preparation of cells for FACS analysis

Cells were harvested in 1 × trypsin (Invitrogen Life Technologies) in PBSE, centrifuged at 4000 r.p.m. for 30 s, resuspended in 70% ethanol in PBS and left at room temperature for 30 min. The cells were then pelleted by centrifugation at 4000 r.p.m. for 30 s, resuspended in 1 ml staining solution (100 μg/ml RNase, 40 μg/ml propidium iodide in PBS) and incubated for 30 min at 37°C. FACS analysis using a Becton Dickinson (Mountain View, CA) flow cytometer was used to determine the cell-cycle profile.

Transient transfections

Transient transfections were carried out in 12-well tissue culture plates with cells at around 50% confluence using 0.5 μg of reporter vector, 0.5 μg of the pCMVSPORTβ-gal control vector (Invitrogen Life Technologies) plus or minus varying amounts of Sp1 co-expression vector (EF1α/Sp1; gift from Dr Merlin Crossley, University of Sydney) using Fugene 6 (Roche) transfection reagent according to the manufacturer's instructions. After 36 h

the cells were harvested and assayed for luciferase activity using a Luciferase Assay Kit (Promega, Madison, WI) and FLUOStar galaxy detection (BMG LabTechnologies, Melbourne, Australia) according to the manufacturer's instructions. β-Galactosidase activity was measured using a spectrophotometric assay [24]. Relative luciferase activity was calculated from the ratio between relative light units produced from luciferase assays and the absorbance at 420 nm for the β-galactosidase assays. All values were normalized to the wild-type construct that was arbitrarily set at 100%.

Results

Expression of topoisomerase IIα in doxorubicin-treated MDA-MB-231 cells

Six clonal, drug-exposed, cell lines were developed, isolated and propagated as described. Semiquantitative immunoblotting of cell extracts showed decreased amounts of Topo IIα protein in three out of the six drug-treated cell lines when compared to the control (unexposed) cell line (data not shown). The other three cell lines maintained control levels of Topo IIα expression. As transcription factors implicated in the regulation of Topo IIα gene expression, the relative levels of Sp1, Sp3 and NF-YA protein were investigated in each cell line relative to tubulin. Each cell line exhibiting a decrease in Topo IIα also showed a significant decrease in Sp1 and one a significant increase in NF-Y expression. No changes in Sp3 expression were observed in the cell lines with decreased Topo IIα expression (Fig. 1A); however, two of the three with control levels of Topo IIα expression showed a significant reduction in Sp3 protein (data not shown). No changes in Sp1 or NF-Y were observed in these lines. The amounts of Topo IIα, Sp1, Sp3 and NF-Y are expressed as a percent relative to tubulin (Fig. 1B) and normalized to the unexposed cell line which was arbitrarily set at 100%. One selected cell line showed correspondingly reduced levels of Topo IIα mRNA by real-time PCR in comparison to GAPDH mRNA and 18S RNA (results not shown). The other cell lines were not tested in this fashion; however, it is well documented that decreases in Topo IIα expression in doxorubicin-exposed cells occur largely at the transcriptional level [25].

As Sp1 has been shown to be a transcriptional activator in HeLa cells we sought to confirm this functional role in MDA-MB-231 cells transiently transfected with the luciferase reporter vector pGL3 Basic harboring the –617 promoter region of human Topo IIα [17]. This region contains both the distal GC2 and proximal GC1 element as well as all five ICB elements and the transcription start site. Each assay contained the pCMVSPORTβGal vector used as a control for transfection efficiency. The effect of Sp1 on transcription was tested by co-transfecting increasing amounts of an Sp1 expression vector. A 2- to 3-fold activation of transcription was observed (Fig. 2) confirming that Sp1 is a transcriptional activator of human Topo IIα in MDA-MB-231 cells. There was no effect of Sp1 on the vector pGL3 Basic, suggesting this was a specific effect of Sp1 on the Topo IIα promoter.

Effect of doxorubicin treatment on cell proliferation

As the expression of Topo IIα can be dependent upon proliferation state [26] and has been shown to be cell-cycle dependent [27], we investigated possible alterations in both the doubling time and cell-cycle profile for each of the drug-treated cell lines compared to the parental unexposed cells. No significant changes to either parameter were apparent in any of the cell lines investigated (data not shown).

Expression of MDR1 in doxorubicin-exposed MDA-MB-231 cells

Another major mechanism for the development of drug resistance is through the over-expression of MDR1 [P-glycoprotein (P-gp)]. P-gp is a 170-kDa membrane glycoprotein which acts as an active transporter of drugs such as anthracyclines, epipodophyllotoxins and taxanes [28], and the overexpression of P-gp is thought to have a major role in both the development and maintenance of multidrug resistance [29].

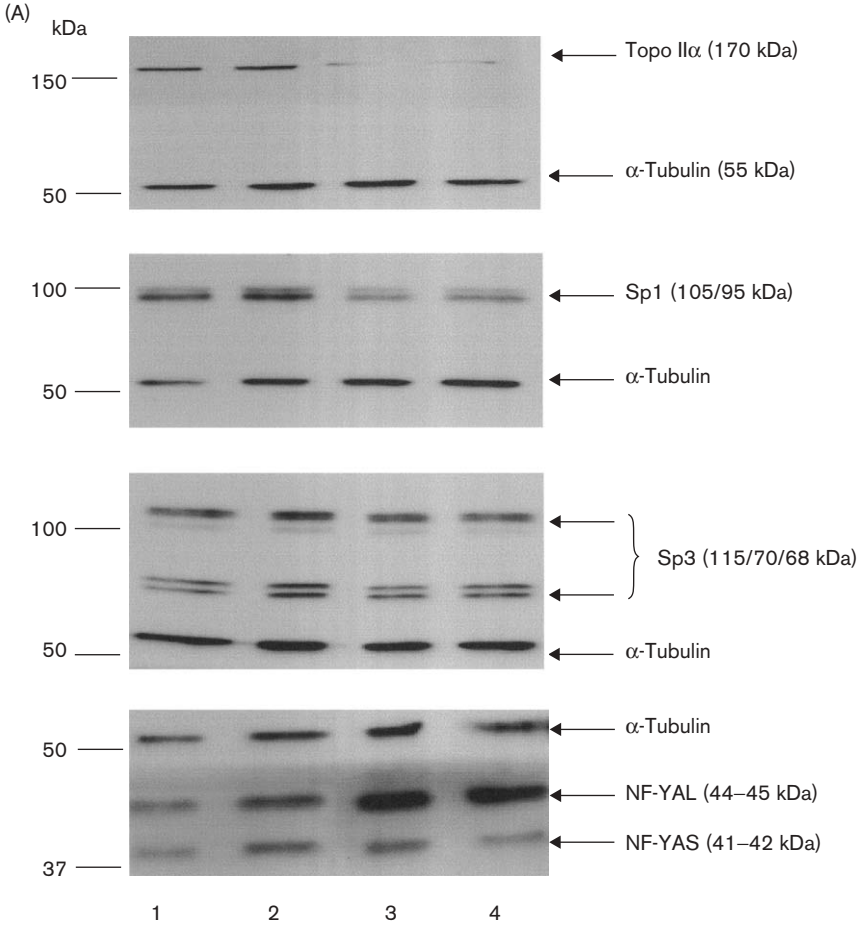
Therefore, the expression of the *MDR1* gene was investigated by RT-PCR in each of the surviving cell lines. RNA from the HepG2 cell line known to express *MDR1* [30] was used as a positive control and *GAPDH* expression was used as an internal control for the reverse transcriptase reaction. MDR1 expression was not detected in any of the surviving cell lines or in the unexposed control cell line but high levels of expression were observed in the HepG2 control (Fig. 3).

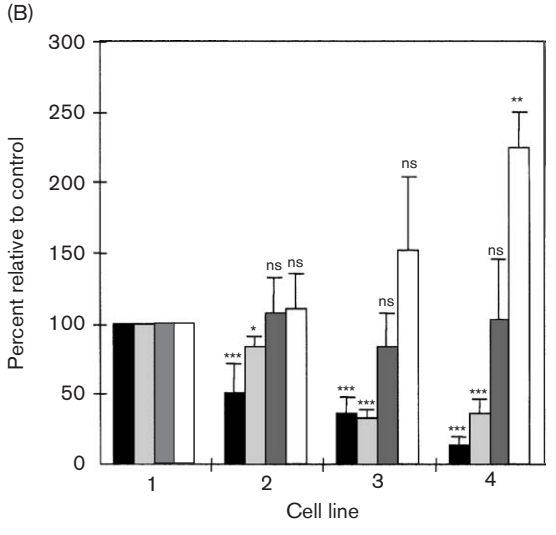
Discussion

Three out of six cell lines surviving a single 1-h exposure to 5 μM doxorubicin showed reduced amounts of Topo IIα protein which correlated to a reduction in Topo IIα mRNA. This indicates that drug-induced down-regulation in Topo IIα occurs in only a subset of cells, which supports other reports [31]. Sp1 protein levels were reduced in all cell lines that also had reduced Topo IIα, but there were no differences in Sp1 expression in cell lines with normal Topo IIα expression. In addition, Sp1 was shown to be a transcriptional activator of Topo IIα in MDA-MB-231 corroborating the results of Magan *et al.* [17] in HeLa cells.

NF-Y exists as a trimer containing NF-YA (CBF-B), NF-YAB (CBF-A) and NF-YAC (CBF-C). NF-YA was investigated as DNA binding of the NF-Y trimer has been correlated with NF-YA levels [32]. Levels of NF-YA increased after a single exposure to doxorubicin in one cell line only. All other cell lines showed no significant

Fig. 1





(A) Immunoblots of whole-cell extracts with antibodies against Topo IIα, Sp1, Sp3 and NF-YA. (NF-YAL, NF-YA long form; NF-YAS, NF-YA short form). Lane 1, unexposed cells; lanes 2–4, doxorubicin exposed cells. Tubulin was included as a control in each assay. The positions of the molecular weight markers are indicated on the left-hand side of each immunoblot. Identities of the immunoreactive bands are indicated by arrows on the right-hand side. (B) Quantification of immunoblot data from at least three experiments. Topo IIα (black bars), Sp1 (light grey bars), Sp3 (dark grey bars) and NF-YA (white bars). The amount of each protein was calculated relative to tubulin and normalized to the untreated control, which was arbitrarily set at 100%. Results are presented as average ± SEM where $n=3$ or 4. $^{ns}p>0.05$, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ were obtained by comparing the relative amounts of each protein in doxorubicin treated cells to the control untreated cells using Student's *t*-test.

changes in amounts of NF-YA protein. These results suggest that the reduction in Topo IIα protein observed in drug-treated cells may be due a direct effect of alterations in regulatory transcription factors, in particular to a drug-induced decrease in the transcriptional activator Sp1.

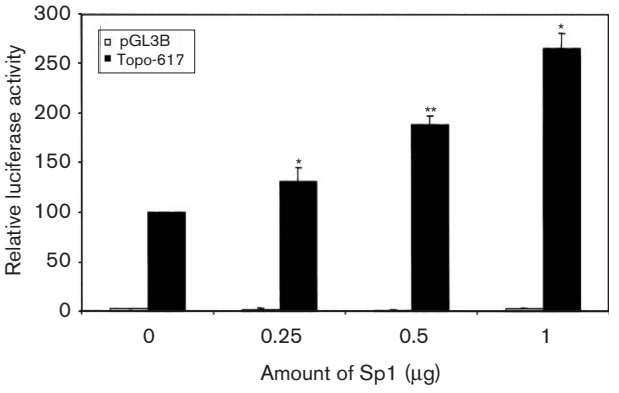
The effect of NF-Y on Topo IIα levels is less clear. A report by Morgan *et al.* [19] suggested that NF-Y acts a repressor at ICB3 and NF-Y has also been reported to be a repressor of Topo IIα acting at ICB2 during confluence arrest [15]. The cells used in the current study were harvested at around 60% confluence and therefore were likely to be freely proliferating when harvested. We have shown previously that NF-Y acting at ICB1 of Topo IIα is a transcriptional activator, but its effect depends upon the relative occupancy of the GC1 and GC2 elements by Sp1 (and/or Sp3) [17]. While the absolute amounts of Sp3 in cell lines with reduced Topo IIα did not alter due to doxorubicin treatment, the ratio of Sp1 to Sp3 may be more important in determining the level of expression of Topo IIα. This, coupled with moderate increases in NF-Y protein levels, may be sufficient to cause the observed down-regulation of Topo IIα.

Sp3 levels were decreased in two drug-treated cell lines, with no concomitant change in Topo IIα. While Sp3 has been suggested to be a transcriptional repressor of Topo IIα [14], this association is not supported by these data, although it may interact with other factors as suggested above. Since these cell lines showed no change in Topo IIα, alternative mechanisms must account for drug resistance, which is in accordance with previous reports [33]. While we cannot rule out RNA stability as a factor in the drug-induced down-regulation of Topo IIα, the observed alterations in amounts of key regulatory transcription factors suggest that the effect is at the level of transcription.

The reduction in the amount of Topo IIα that occurred upon drug treatment was not due to changes in the doubling time or to cell-cycle profile. In addition, the major multidrug resistance protein MDR1 did not contribute to survival of the cells to doxorubicin exposure.

This report provides the first direct evidence of a role for Sp1 in drug-induced down-regulation of Topo IIα gene expression.

Fig. 2

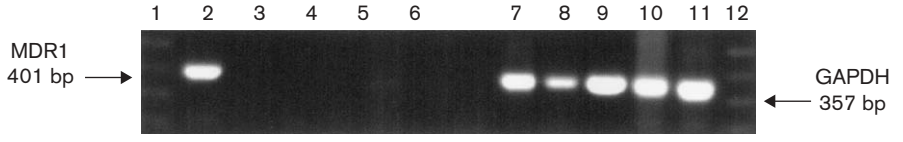


Reporter gene assays. Relative luciferase activity is plotted against amount of Sp1 expression vector in each transfection. All transfections were carried out with 0.5 μg of the −617wt Topo IIα reporter plasmid. Results are presented as average ± SEM where $n=9$. *$p<0.005$, **$p<0.001$ were obtained by comparing relative luciferase activities plus and minus Sp1 using Student's *t*-test.

Conclusion

A single dose of doxorubicin resulted in decreased amounts of Topo IIα protein in surviving cell lines. Concomitant with this decrease, alterations in the amounts of transcription factors Sp1 and NF-YA were also observed. This provides direct evidence that the transcriptional regulation of Topo IIα gene expression is altered by doxorubicin exposure and that the primary effect may be in the regulation of expression of the transcription factors themselves. As Sp1 and NF-Y are ubiquitous transcription factors, the effects of altered expression are likely to far reaching. This may in part account for the multifactorial nature of doxorubicin-induced development of drug resistance after chemotherapy.

Fig. 3



Expression of *MDR1* in doxorubicin-treated MDA-MB-231 cells. RT-PCR was used to detect the presence of *MDR1* gene expression with *GAPDH* as a constitutively expressed control. Lanes 1 and 12, 1 kb Plus ladder (Invitrogen Life Technologies); lanes 2–6, PCR products using primers specific for MDR1; lanes 7–12, PCR products using primers specific for GAPDH; lanes 2 and 7, PCR products using cDNA from HepG2 RNA as template (MDR1 positive control); lanes 3 and 8, PCR products from control MDA-MB-231 RNA; lanes 4–6 and 9–11, PCR products using RNA from doxorubicin-treated cells.

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