Treatment with the PARP-inhibitor PJ34 causes enhanced doxorubicin-mediated cell death in HeLa cells

Natisha Magan[a], Richard J. Isaacs[b] and Kathryn M. Stowell[a]

Adjuvant therapies can incorporate a number of different drugs to minimize the cardiotoxicity of cancer chemotherapy, decrease the development of drug resistance and increase the overall efficacy of the treatment regime. Topoisomerase IIα is a major target of many commonly used anticancer drugs, where cell death is brought about by an accumulation of double-strand DNA breaks. Poly (ADP-ribose) polymerase (PARP)-1 has been extensively studied for its role in the repair of double-strand DNA breaks, but its ability to add highly negative biopolymers (ribosylation) to target proteins provides a vast number of pathways where it can also be important in mediating cell death. In this study, we combine the classical topoisomerase IIα poison doxorubicin with the PARP inhibitor PJ34 to investigate the potentiation of chemotherapeutic efficiency in HeLa cells. We demonstrate that PJ34 treatment has the capacity to increase endogenous topoisomerase IIα protein by about 20%, and by combining doxorubicin treatment with PJ34, we observed a 50% improvement in doxorubicin-mediated cell death in HeLa cells. These results were correlated with the ribosylation of transcription factor specificity factor 1 after doxorubicin treatment, thereby altering its affinity for binding to known regulatory elements within the human topoisomerase IIα promoter. Taken together, these results highlight the synergistic potential of combining PARP inhibitors with classical topoisomerase IIα-targeting drugs. *Anti-Cancer Drugs* **23:627–637 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2012, **23**:627–637

Keywords: chemotherapy, doxorubicin, nuclear factor Y, poly (ADP-ribose) polymerase, ribosylation, specificity factor 1, topoisomerase, transcriptional regulation

[a]Institute of Molecular BioSciences, Massey University, Palmerston North and [b]Regional Cancer Treatment Service, Palmerston North Public Hospital, New Zealand

Correspondence to Dr Natisha Magan, PhD, Institute of Molecular BioSciences, Massey University, Palmerston North 4442, New Zealand
Tel: +64 063 502 564; fax: +64 063 505 688; e-mail: n.magan@massey.ac.nz

Received 19 October 2011 **Revised form accepted** 19 December 2011

Introduction

DNA topoisomerases are essential nuclear enzymes that have the ability to introduce transient double-stranded breaks (DSBs) in DNA, rotate DNA about that break and re-ligate the original break [1,2]. Human DNA type II topoisomerases exist in two isoforms, α (Topo2α, 174 kDa) and β (Topo2β, 182 kDa), in which only Topo2α is essential for cell proliferation [3–5]. Normal cellular processes such as DNA replication, transcription, recombination, DNA repair and chromatin remodelling generate torsional stress in chromosomes that must be corrected by topoisomerase activity. For this reason, rapidly dividing cells exhibit elevated levels of topoisomerases to accommodate their replicative needs and high proliferative status. Among the most commonly used chemotherapy agents are drugs that target cellular topoisomerases, such as amsacrine, etoposide, teniposide, doxorubicin and epirubicin [6,7]. Topoisomerase II-targeting poisons act by converting normal topoisomerase II into a cellular toxin by stabilizing the topoisomerase-mediated DNA DSB; consequently, cell death ensues due to an accumulation of permanent DNA damage. The success in this type of treatment is dependent on high levels of topoisomerase in combination with lethal doses of drug and the inability to efficiently repair DSB in DNA [8–10]. Although cancerous cell lines and tumours often exhibit elevated levels of Topo2α protein [11–13], making them susceptible to topoisomerase-targeting drugs, the high cardiotoxicity of many treatments and the development of drug resistance is still a major clinical problem. The development of doxorubicin resistance can be attributed to a number of different factors including changes in Topo2α protein, changes in cellular drug-efflux mechanisms and enhancement of the natural DNA repair mechanisms inside a cell [14–16]. A single protein or mechanism cannot be held accountable for the development of resistance, making it difficult to provide effective therapy once resistance occurs.

Poly (ADP-ribose) polymerase (PARP-1) is a highly abundant 113–115 kDa predominantly nuclear enzyme. It has low basal enzyme activity, which can be rapidly activated by the presence of DNA breaks and has been classically implicated with roles in DSB repair as well as cell cycle progression and cell death [17–20]. PARP-1 catalyses the polymerization of linear or branched chains of ADP-ribose (pADPr) groups (ribosylation) from donor nicotinamide adenine dinucleotide (NAD^+) onto target proteins. This confers a highly negative charge onto donor proteins, proportional to the degree of ribosylation, thereby affecting protein–protein or protein–DNA interactions. Emerging evidence demonstrates that PARP-1 protein is a major contributor to the posttranslational modification of a wide range of proteins including histones, DNA polymerases, DNA ligases, tumour suppressor p53, RNA polymerase II, Topo2β and PARP-1

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328350900f

itself [21–24]. Most PARP-1 inhibitors act by blocking the natural interaction between donor NAD^+ and PARP-1, thus preventing ribosylation. A range of different PARP-1 inhibitors exist and many more are in development. They vary in the toxicity and range of cellular function during cancer treatment including inhibiting angiogenesis, enhancing sensitivity to radiotherapy and chemotherapy, suppressing cell growth and promoting apoptosis [25–27]. PARP-1 inhibitors also appear to protect against the cardiotoxicity of doxorubicin [28–30]. By combining the two treatments, it may be possible to lower the dosage and reduce the toxic side effects commonly seen with many chemotherapeutic agents.

We and others have previously shown that regulation of the human Topo2α gene is primarily influenced by transcription factors specificity factor 1 (Sp1), specificity factor 3 (Sp3) and nuclear factor Y (NF-Y) [31–37]. These transcription factors bind to DNA sequences within the minimal −617Topo2α promoter. Specifically, Sp1 and Sp3 can bind to either of two GC elements (GC1 and GC2), whereas NF-Y can bind to four of five inverted CCAAT boxes (ICB1-5). In this study, we combined PARP inhibition (using PJ34) with doxorubicin treatment and found an overall improvement in the efficacy of doxorubicin-mediated cell death in HeLa cells. We used the minimal −617Topo2α promoter to show that PARP inhibition has an effect on expression of the Topo2α gene, which was correlated with temporal changes in the amount of Topo2α protein. Our results suggest that this effect is most likely due to changes in the affinity of Sp1 for its cognate sequences within the Topo2α promoter, due to ribosylation of Sp1.

Experimental procedures

Cell culture and drug treatment

HeLa cells were maintained in a modified Eagle's minimal essential reduced serum medium (OPTI-MEM I; Invitrogen, Carlsbad, California, USA) containing 2% v/v foetal-calf serum and 1% v/v penicillin/streptomycin (5000 U/ml penicillin and 5 mg/ml streptomycin sulfate in 0.85% saline). HeLa cells were maintained at all times under humid conditions, 5% CO_2 at 37°C (HERAcell 150, Heraeus; Kendro Laboratory Products GmbH, Langenselbold, Germany). Doxorubicin (50 mg/ml; EBewe, Unterach, Austria) and PJ34 (PARP inhibitor VIII; Calbiochem, EMD Chemicals, Darmstadt, Germany) stock solutions were diluted in complete OPTI-MEM I medium to provide a final concentration of either 0.3, 1.5 or 3 μmol/l doxorubicin and/or 0.25, 0.5, 1 or 2 μmol/l PJ34. Five microlitres of complete OPTI-MEM I media were added as a control.

Immunoprecipitation

Approximately $2–3 \times 10^7$ HeLa cells were washed twice in 2 × (v/v) of ice-cold PBS (0.14 mol/l NaCl, 2.7 mmol/l KCl, 10 mmol/l Na_2HPO_4, 1.8 mmol/l KH_2PO_4; pH 7.2–7.4) and proteins extracted in 1 ml of modified NP40 buffer [50 mmol/l Tris; pH 8, 120 mmol/l NaCl, 0.5% NP40 plus protease inhibitor cocktail (complete mini EDTA free; Roche Applied Science, Mannheim, Germany)]. Each immunoprecipitation reaction was carried out using an NP40 HeLa extract prepared from $2–3 \times 10^7$ HeLa cells along with 2 μg antibody, α-Sp1 (sc-59; Santa Cruz Biotechnology, Santa Cruz, California, USA) or α-pADPr [sc-56198 (10 H); Santa Cruz]. After an overnight incubation at 4°C, immunocomplexes were isolated using Protein G Plus agarose (IP04; Calbiochem), washed five times in ice-cold simplified PBS (pH 7.5; 120 mmol/l NaCl, 11 mmol/l Na_2HPO_4) and proteins were eluted by boiling in 50 μl 1 × SDS treatment buffer (12 mmol/l Tris-HCl; pH 6.8, 1% w/v SDS, 1% v/v β-mercaptoethanol, 5% v/v glycerol and 0.0002% w/v bromophenol blue). Immunoprecipitated proteins were subjected to immunoblotting.

Immunoblotting

Twenty to 40 μg of whole-cell extract in extraction buffer (40 mmol/l HEPES; pH 7.9, 0.4 mol/l KCl, 1 mmol/l dithiothreitol, 10% v/v glycerol and protease inhibitor cocktail) was denatured using 1 × SDS treatment buffer and then separated using 7% SDS-PAGE. Proteins were electroblotted onto a PVDF membrane (Roche) and immunoblotted using primary antibodies from a range of sources and diluted as follows: 1/500 α-Sp1 (sc-59; Santa Cruz), 1/2000 α-Sp1 (sc-17824 X; Santa Cruz), 1/1000 α-Sp1 (554129; BD Biosciences, Becton Dickson Ltd, New Jersey, USA), 1/400 α-NF-YA (sc-10779; Santa Cruz), 1/500 α-Sp3 (sc-644; Santa Cruz), 1/700 α-Ku86 (sc-9034; Santa Cruz), 1/600 α-Ku70 (sc-9033; Santa Cruz), 1/1000 α-PARP-1 (sc-8007; Santa Cruz), 1/4000 α-PARP-1 (sc-74469 X; Santa Cruz), 1/100 α-TopoIIα (sc-13058) and 1/4000 α-Tubulin (T 9026; Sigma Chemical Company, St Louis, Missouri, USA). HRP-conjugated secondary α-mouse or α-rabbit antibodies (Sigma) were each diluted 1/4500. Roche POD substrate was used for chemiluminescent signal detection and signals were captured using the Intelligent dark box II LAS 1000 (Fuji Photo Film Co. Ltd, Japan). Immunoblot data were analysed using Image Gauge software ver 4.0 (Science Lab 2001, Fuji Photo Film Co.).

Transient transfections

Reporter gene constructs (−617Topo2αLUC) were prepared and reporter gene assays were carried out as previously described [33]. Briefly, a total of 0.25 μg pCMV SPORT β-galactosidase control vector (Promega Corporation, Madison, Wisconsin, USA) and 0.5 μg of the pGL3B −617Topo2αLUC luciferase reporter gene were transiently transfected using a 3:1 ratio of FuGENE6 (Roche) : plasmid DNA. Each transfection was performed in triplicate, on at least three separate occasions, and harvested 48 h after transfection. PARP-1 inhibitor, PJ34, was added 2 h after transfection.

Impedance-based cell viability assays

Impedance measurements and cell index determinations were carried out using a real time cell analyser (RTCA) SP Station fitted into a cell culture incubator (5% CO_2 at 37°C) including an RTCA Control Unit (xCELLigence; Roche). Briefly, 100 µl of complete OPTI-MEM I media were added to each well of an E-plate 96 (Roche) and used to correct for initial background measurements. The volume in each well was made up to 200 µl with the addition of 1×10^5 HeLa cells in complete OPTI-MEM I media and the impedance was continuously monitored and then converted into a cell index every 15 min over a 72-h period. The cells were allowed to recover and adhere to reach a stable baseline (minimum cell index of 1) before any treatments were added. Each individual experiment was carried out using triplicate sets of HeLa cells and each experiment was performed on at least two separate occasions. Cell index values were converted into normalized cell index by dividing the cell index value at every time point by the cell index at a point close to which the treatment was administered. Data analysis and presentation was carried out using the integrated RTCA software 1.2 (Roche) or by exporting the data into Microsoft Excel (2003).

Dye-based cell viability assays

Cell viability assays were carried out using 7×10^4 HeLa cells in each well of a 96-well plate. Cells were allowed to adhere and stabilize for 18 h and then 5 µl of complete OPTI-MEM I media (no treatment control) or 5 µl PJ34 (diluted in complete OPTI-MEM I media) were added to a final concentration of 0.25, 0.5, 1 or 2 µmol/l. Cytotoxicity of PJ34 was evaluated using the CellTiter96 Aqueous One Solution (Promega), according to the manufacturer's instructions. Each assay was carried out in triplicate on two separate occasions and data were normalized relative to HeLa cell viability at the time of addition of PJ34.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were carried out as previously described [33]. Briefly, each EMSA reaction contained 1 µg of poly dI.dC (1 µg/µl in 50 mmol/l $MgCl_2$), 50% v/v GSB (40 mmol/l Tris pH 7.6, 16% w/v Ficoll, 100 mmol/l KCl, 0.4 mmol/l EDTA, 1 mmol/l DTT) and 10–20 µg of protein along with 0.5 ng of a γ^{32}P-labelled ICB1/GC1 probe (5′ *cgagtcagggattggctggtctgcttcgggcgggctaaaggaag* 3′). Antibodies used in supershift assays were as follows: α-Sp1 (sc-17824 X; Santa Cruz), α-Sp3 (F-7 sc-28305; Santa Cruz), α-PARP-1 (sc-74469 X; Santa Cruz) and α-pADPr (10 H sc-56198; Santa Cruz). Antibodies against the NF-YA subunit (sc-10779; Santa Cruz) were specifically chosen as this subunit of the trimeric protein is essential for an association with DNA [38].

Results

Poly (ADP-ribose) polymerase-1 inhibitor, PJ34, enhances doxorubicin drug efficacy

HeLa cells were subjected to drug treatment and cell growth was monitored using two different techniques to establish whether inhibition of PARP-1 had an effect on HeLa cell viability and doxorubicin drug efficacy.

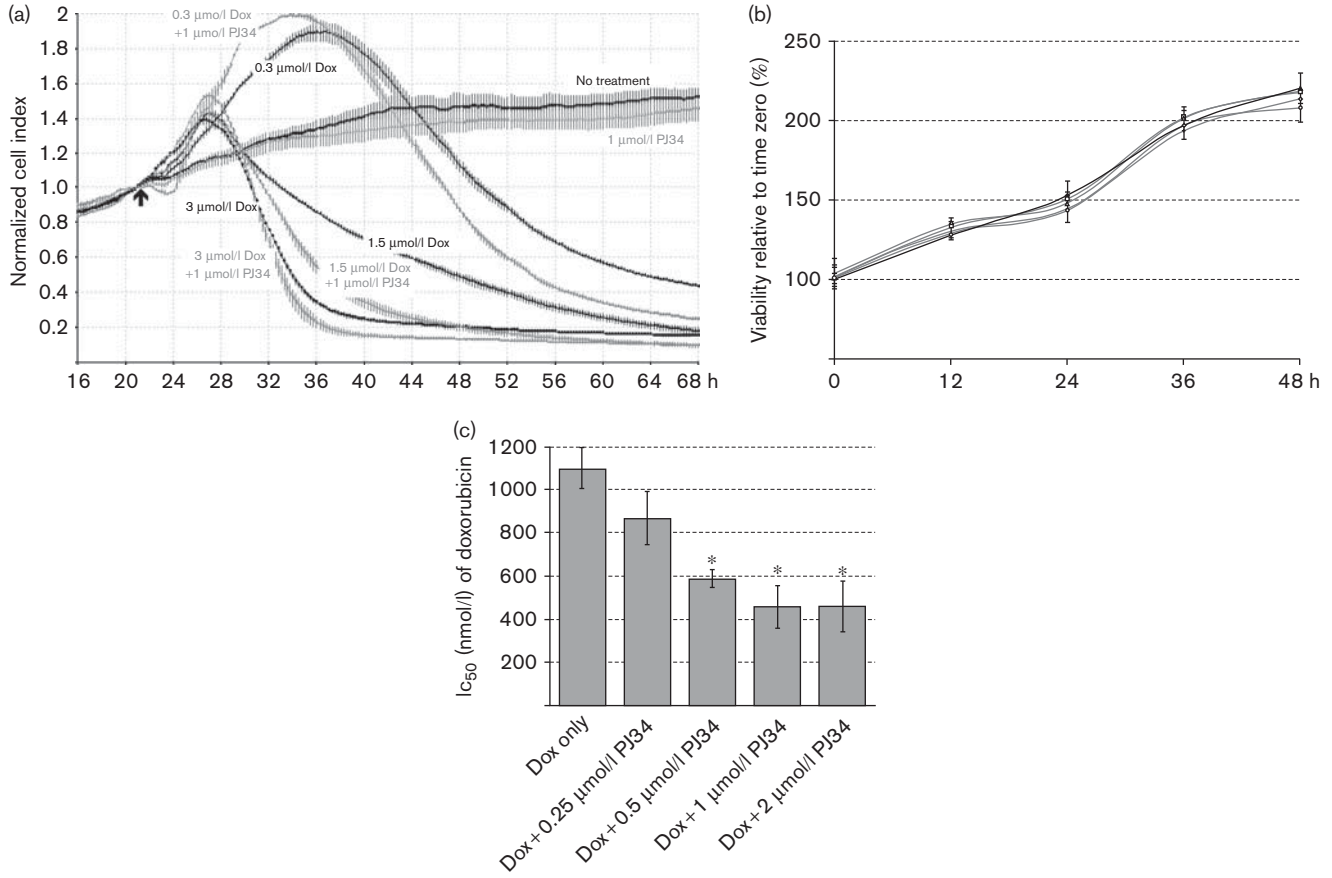
Using a label-free real-time monitoring system, the xCELLigence RTCA measured the impedance of adherent cells and these data were converted into a cell index, normalized and displayed as traces at different time points (Fig. 1a). A steady increase in the cell index indicates cell adherence, growth and spreading, whereas a reduction in the cell index indicates reduced cell viability and proliferation due to detachment from the plate [39]. The normalized cell index traces demonstrated that in the presence of 1 µmol/l PJ34, HeLa cell growth varied little over a 68-h period (Fig. 1a no treatment vs. 1 µmol/l PJ34). In consecutive experiments, addition of 0.25, 0.5 and 2 µmol/l PJ34 also had little effect on HeLa cell growth, even when longer exposure periods of up to 94 h were used (data not shown). PJ34 also had little effect on HeLa cell viability over a 48-h period (Fig. 1b) using the dye-based CellTiter96 Aqueous One proliferation assay.

Because the red dye associated with doxorubicin interfered with the absorbancies generated using the CellTiter96 Aqueous One proliferation assay, the label-free impedance-based technology (xCELLigence RTCA) could only be used to evaluate doxorubicin toxicity. Figure 1a demonstrates that treatment with 0.3, 1.5 and 3 µmol/l doxorubicin significantly reduced the normalized cell index in a dose-dependent and time-dependent manner, as cell death occurs, causing detachment from the plate. When both doxorubicin and PJ34 were used in combination (grey lines, Fig. 1a), there was a shift in the doxorubicin response curves (black lines, Fig. 1a), indicating that PJ34 may enhance doxorubicin efficacy. The log concentration of doxorubicin was plotted against the normalized cell index for each individual doxorubicin response curve (all data not shown) in the presence of 0.25, 0.5, 1 and 2 µmol/l PJ34 and the relative IC_{50} value was calculated for doxorubicin during drug treatment (Fig. 1c). These data clearly demonstrated that PJ34 caused a progressive reduction in the IC_{50} of doxorubicin from 1100 nmol/l (no PJ34) down to approximately 460 nmol/l with 2 µmol/l PJ34 (Fig. 1c).

Doxorubicin and PJ34 affect the cellular amounts of Topo2α protein

The results from collective xCELLigence assays suggested that treatment with 1 µmol/l PJ34 in conjunction with 1.5 µmol/l doxorubicin caused the greatest enhancement in doxorubicin-mediated cell death (Fig. 1a); therefore, we sought to investigate whether cellular levels of Topo2α protein were altered under these conditions. After a 10-h exposure to 1 µmol/l PJ34 alone, there was a modest, but reproducible increase in the amount of Topo2α protein (Fig. 2). However, treatment with 1.5 µmol/l doxorubicin (with or without 1 µmol/l PJ34) resulted in a 40% reduction in the amount of Topo2α protein. As transcription factor levels have also

Fig. 1



Poly (ADP-ribose) polymerase inhibitor, PJ34, enhances doxorubicin (dox) drug efficacy. (a) Doxorubicin response curves; individual samples were seeded using 1×10^5 HeLa cells and continuously monitored using the xCELLigence real-time cell analyser. Ten hours after seeding, triplicate sets of HeLa cells were treated with 1 µmol/l PJ34 (lighter coloured traces) or media only (no treatment). This was followed by (21 h after seeding) treatment with 0.3, 1.5 or 3 µmol/l doxorubicin (↑). Impedance measurements were normalized close to the 21-h time point and the traces of the normalized cell index are displayed at the time points indicated. (b) PJ34 treatment has no significant effect on HeLa cell viability; HeLa cells were treated with 0.25 µmol/l (□), 0.5 µmol/l (△), 1 µmol/l (+), 2 µmol/l (◇) PJ34 or no treatment (◆ media only). Cytotoxicity of PJ34 was evaluated using Promega CellTiter96 Aqueous One Solution over 48 h. Data are displayed as percentage change in viability relative to HeLa cell viability at time zero (addition of PJ34) for each treatment. (c) Changes in IC_{50} of doxorubicin with PJ34 treatment; duplicate sets of doxorubicin response curves (a) with 0.25, 0.5, 1, 2 µmol/l PJ34 or no treatment (media only) were used in conjunction with the integrated RTCA software 1.2 to calculate the IC_{50} for doxorubicin over a 47-h time period. A summary of the data ($n=6$) is displayed as a percentage change in IC_{50} of doxorubicin in the presence of increasing concentrations of PJ34. *$P<0.01$, significantly different from the doxorubicin-only data set with no PJ34 treatment.

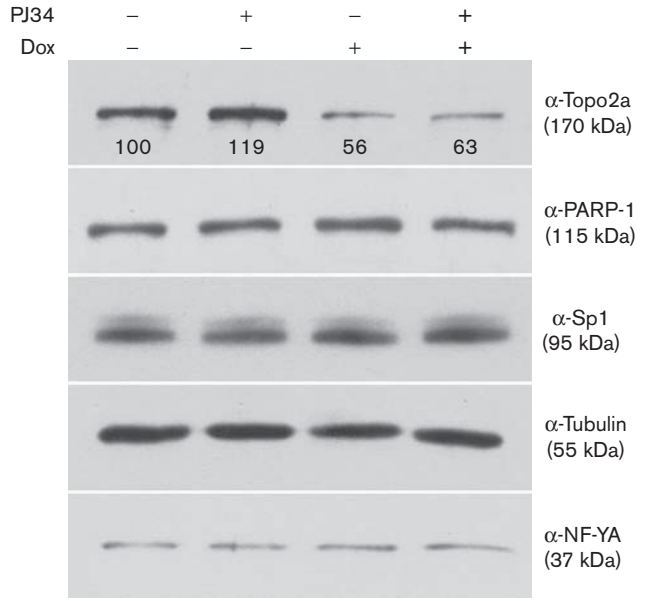
been shown to fluctuate and as a consequence modulate Topo2α gene expression [32,36,37,40], immunoblotting was carried out using antibodies against Sp1 (α-Sp1) and nuclear factor Y (α-NF-Y). No changes in the relative amounts of transcription factors Sp1 or NF-YA were observed. In addition, 1 µmol/l PJ34 did not cause any significant variation in the amount of PARP-1 protein (Fig. 2), demonstrating that inhibition of PARP activity does not affect the cellular levels of PARP-1 protein.

PARP-1 inhibition increases Topo2α promoter activity

PJ34 was shown to consistently enhance Topo2α protein levels in extracts isolated from HeLa cells. Therefore, the effect of PARP-1 inhibition on basal Topo2α promoter activity was examined. HeLa cells were transiently transfected using a luciferase reporter vector pGL3B harbouring the −617Topo2α promoter region of the human topoisomerase IIα gene (−617Topo2αLUC). With the addition of PJ34, luciferase activity increased, demonstrating enhanced promoter activity up to 30% in a dose-dependent manner (Fig. 3a). This suggested that inhibition of PARP-1 activity may result in activation of the topoisomerase IIα promoter.

Transcription factor Sp1 also has the ability to activate Topo2α gene expression through a direct interaction with cognate GC1 and GC2 elements [33,37]. To determine whether this PJ34-mediated promoter activation was dependent on the presence of Sp1, both the GC1 and the GC2 elements were mutated within the −617Topo2α LUC reporter gene construct (GC1mt/GC2mt −617Topo2αLUC) and reporter gene assays were carried out as shown in Fig. 3a. There was no significant change in promoter activity in the presence of PJ34 when both GC

Fig. 2



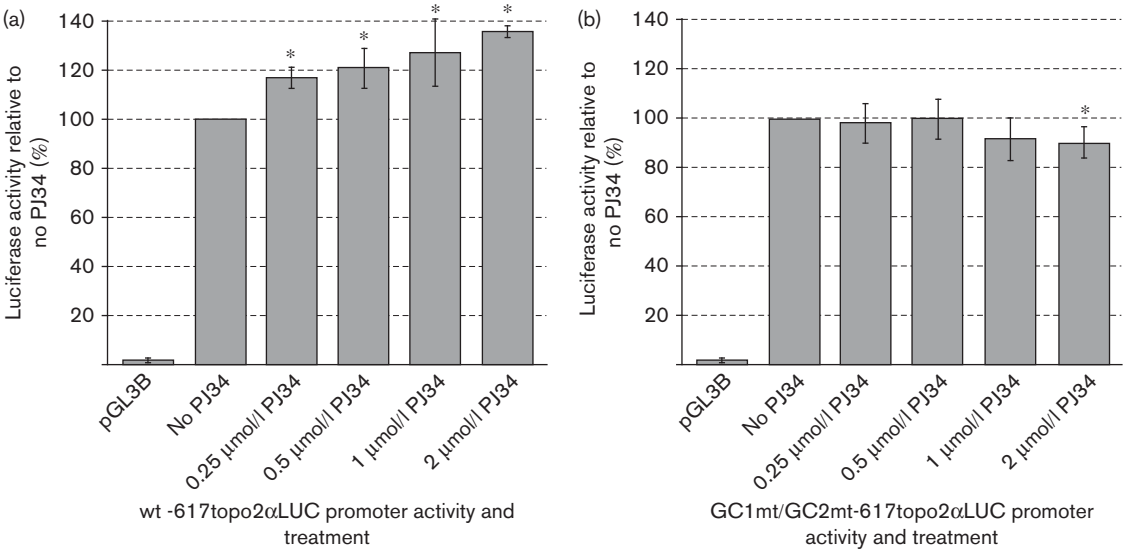
Doxorubicin (dox) and PJ34 affect the cellular amounts of topoisomerase IIα (Topo2α) protein. Immunoblot comparing the changes in the amounts of Topo2α protein when HeLa cells were exposed initially to 1 μmol/l PJ34 for 10 h and then 1.5 μmol/l doxorubicin for 6 h. Data from five separate immunoblots were quantified and normalized against each respective tubulin signal. The numbers represent the percentage change in signal intensity/protein relative to no treatment (100%). NF-Y, nuclear factor Y; PARP-1, poly (ADP-ribose) polymerase-1; Sp1, specificity factor 1.

elements were mutated (Fig. 3b), suggesting that PJ34 may mediate its effect through Sp1 binding to the Topo2α promoter. In addition, a 5–10% decrease in luciferase activity was observed in the presence of 2 μmol/l PJ34, suggesting that promoter repression could occur when higher concentrations of PJ34 were used and this effect could be Sp1-independent.

PJ34 causes Sp1 to bind more strongly to DNA

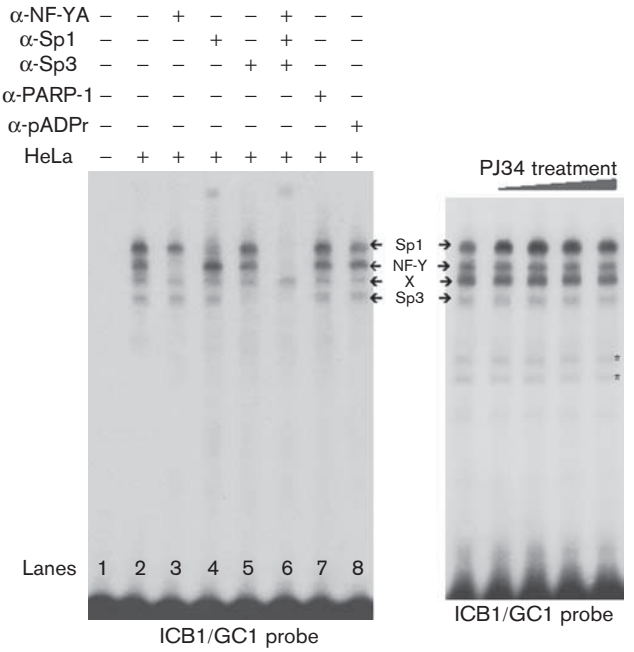
Previous work examining the −617Topo2α promoter has shown that both the ICB1 and the GC1 elements exhibit the greatest influence on transcriptional regulation of the Topo2α gene [33]. Using a ^{32}P-labelled probe encompassing both ICB1 and GC1 elements in-vitro binding assays (EMSA), we asked whether PARP-1 could directly interact with these elements. Transcription factors Sp1, Sp3 and NF-Y bound specifically to the ICB1/GC1 probe (Fig. 4a; lanes 4, 5 and 3, respectively); however, PARP-1 did not appear to associate with either the ICB1 or the GC1 element directly (Fig. 4a; lane 7). With the addition of antibody against the posttranslational modification ribosylation (Fig. 4a; lane 8 α-pADPr), a reproducible reduction in the band that corresponds to the Sp1/probe complex was observed, suggesting that Sp1 could potentially be ribosylated. An increase in the amount of the Sp1/probe complex was observed when protein extracts from HeLa cells treated with increasing amounts of PJ34 were used in EMSA (Fig. 4b, compare the uppermost band Sp1 in lane 1 with 2–5). This suggests

Fig. 3



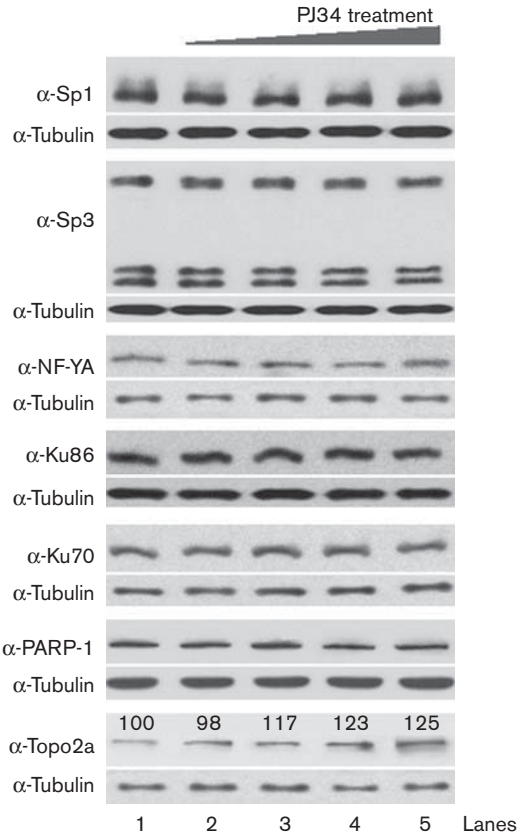
Poly (ADP-ribose) polymerase-1 inhibition increases topoisomerase IIα (Topo2α) promoter activity. HeLa cells were transiently transfected ($n=9$) with a −617Topo2αLUC promoter reporter gene construct and increasing concentrations of the poly (ADP-ribose) polymerase-1 inhibitor, PJ34 (as indicated). An empty pGL3B plasmid represents the no-promoter control. *$P<0.03$, moderately different from the −617Topo2αLUC promoter data set with no PJ34 treatment. (a) Enhanced Topo2α promoter activity with PJ34 treatment; normalized luciferase activities are displayed as percentages relative to the wt −617Topo2αLUC promoter activity with no PJ34 treatment (100%). (b) Mutation of GC1 and GC2 elements within the Topo2α promoter abolishes the enhanced Topo2α promoter activity observed with PJ34 treatment; normalized luciferase activities are displayed as percentages relative to the GC1mt/GC2mt −617Topo2αLUC promoter activity with no PJ34 treatment (100%).

Fig. 4



PJ34 causes specificity factor 1 (Sp1) to bind more strongly to DNA. Proteins present in the whole-cell HeLa extract that bind to the Topo2α regulatory elements ICB1 and GC1 (probe/DNA) were examined using electrophoretic mobility shift assay (EMSA). Four typical protein–DNA interactions were observed (←); labelled Sp1, nuclear factor Y (NF-Y) and specificity factor 3 (Sp3), X represents an uncharacterized complex. *Non-specific protein–DNA interactions. Figures are representative of four similar experiments. Left panel: Poly (ADP-ribose) polymerase-1 (PARP-1) does not bind directly to the IBC1/GC1 regulatory elements; antibodies against proteins demonstrate a supershift and identify specific protein–DNA interactions. Right panel: EMSA using a PJ34-exposed HeLa extract; HeLa cells were exposed to 0.25, 0.5, 1 and 2 μmol/l PJ34 for 48 h and the whole-cell HeLa extracts were used in EMSA.

Fig. 5



Immunoblots of HeLa protein extracts with increasing PJ34 treatment. Thirty micrograms of whole-cell HeLa extract (exposed to 0.25, 0.5, 1 and 2 μmol/l PJ34 for 48 h) was subjected to SDS-PAGE and individual protein levels were monitored by immunoblotting. Data from four individual immunoblots were quantified and normalized against each respective tubulin signal. The numbers represent the percentage change in signal intensity/protein relative to no-treatment HeLa protein extracts (100%). NF-Y, nuclear factor Y; PAGE, polyacrylamide gel electrophoresis; PARP-1, poly (ADP-ribose) polymerase-1; Sp1, specificity factor 1; Sp3, specificity factor 3.

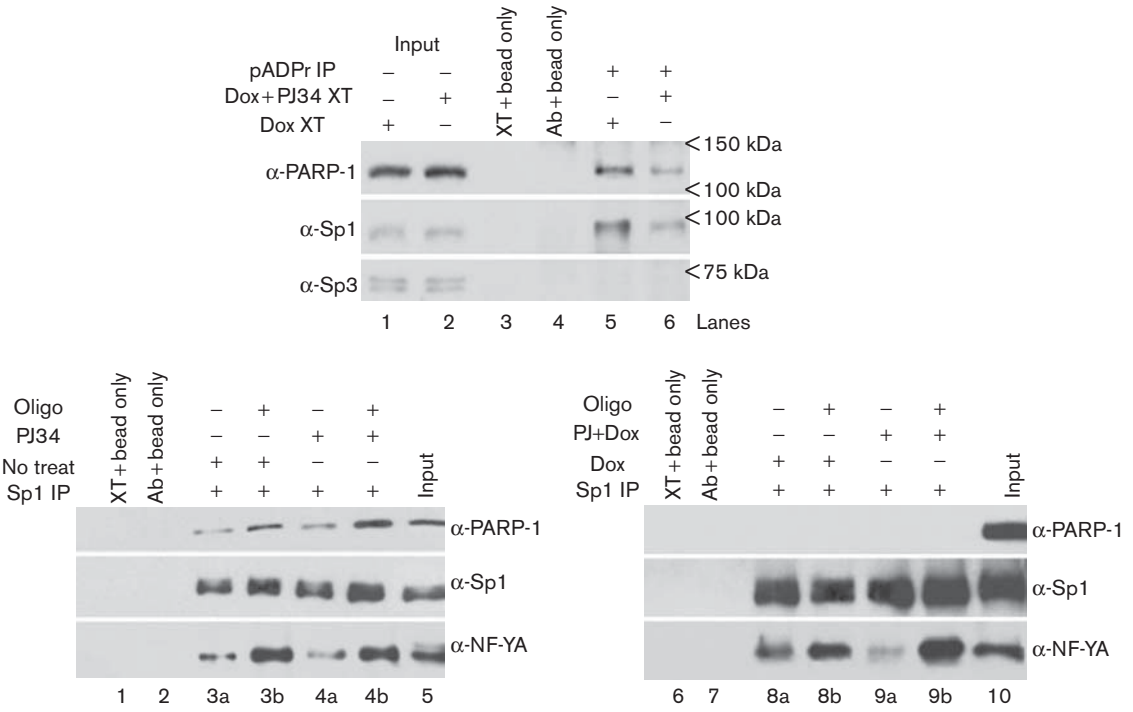
that there is enhanced binding of Sp1 to DNA upon treatment with PJ34 as ribosylation is inhibited. Taken together, these results suggest that PJ34 decreases ribosylation of Sp1 by PARP-1 and nonribosylated Sp1 may bind more strongly to the Topo2α promoter.

In order to eliminate the possibility that the observed increase in Sp1 binding to the ICB1/GC1 probe was not due to temporal changes in the amount in Sp1 present in these PJ34-treated extracts, the same HeLa extracts were subjected to analysis by immunoblotting (Fig. 5). There was no significant variation in the amount of Sp1 present in these extracts (Fig. 5, compare lane 1 with lanes 2–5), suggesting that the increase observed in the EMSA (Fig. 4b) was most likely to be due to an increased affinity for DNA rather than any change in the amount of Sp1 protein. Consistent with earlier results (compare Fig. 2 with Fig. 5), inhibition of PARP-1 activity did not influence the levels of NF-Y or PARP-1 protein. These conditions did not affect the amount of transcription factor Sp3 either (Fig. 5). Proteins Ku86 and Ku70 were also examined as they are known to interact with PARP-1 protein as part of the DSB repair pathway [41]. No changes were observed for any of these proteins; however, there was an increase in the amount of Topo2α protein (Fig. 5) with increasing PJ34 treatment, consistent with previous experiments.

Ribosylation of interacting proteins Sp1 and PARP-1

In order to establish whether treatment with doxorubicin could stimulate PARP-1 activity, immunoprecipitation reactions were carried out using antibodies specifically against pADPr residues (Fig. 6a). Extracts were prepared from HeLa cells treated with only 1.5 μmol/l doxorubicin (input lane 2) or a combination of both 1 μmol/l PJ34 and 1.5 μmol/l doxorubicin (input lane 1), and immunocomplexes were examined for the presence of PARP-1, Sp1 and Sp3 (lanes 5 and 6). The results of these assays demonstrate that both PARP-1 and Sp1 formed immunocomplexes with the pADPr antibody, which were

Fig. 6

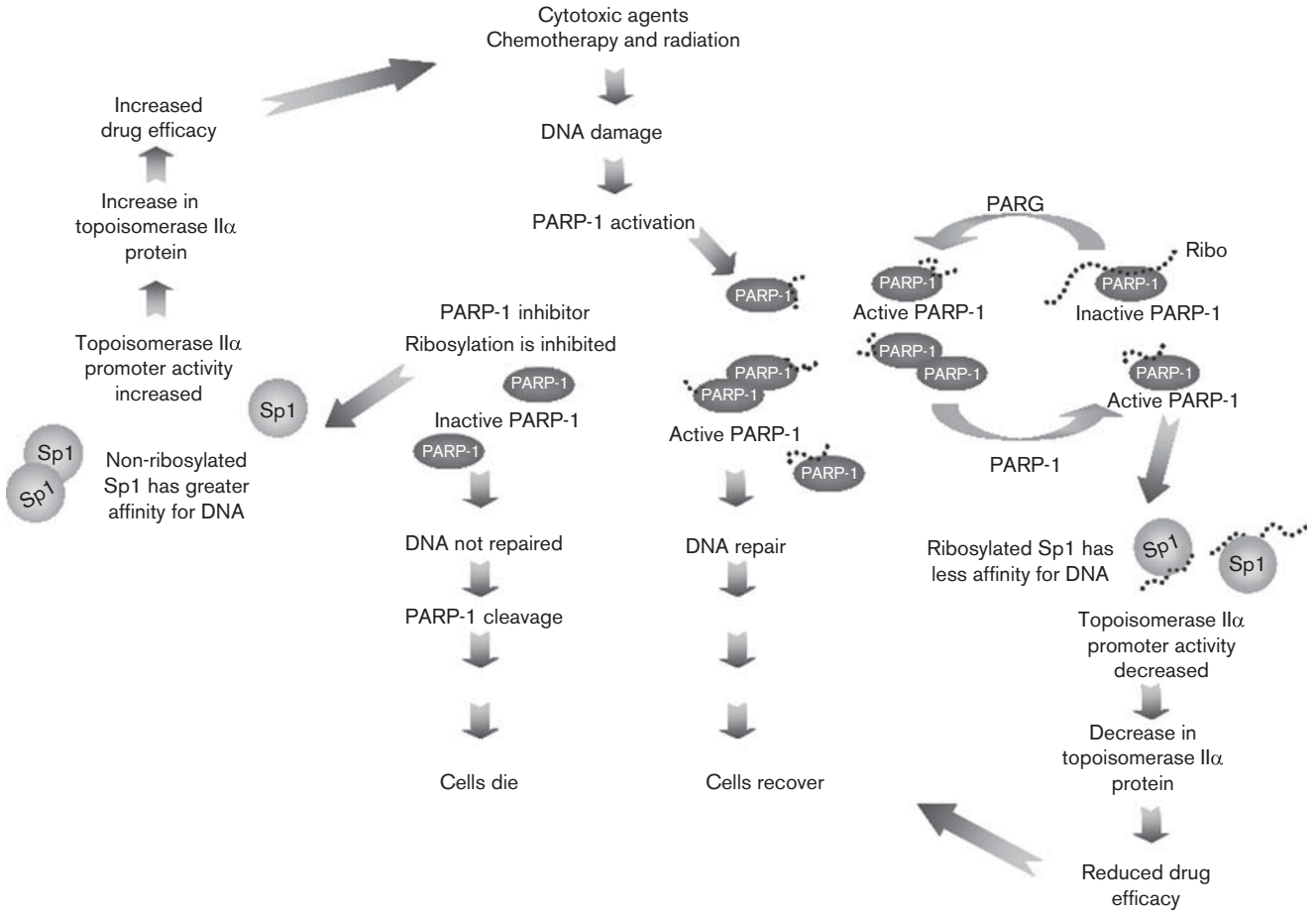


Ribosylation of interacting proteins specificity factor 1 (Sp1) and poly (ADP-ribose) polymerase-1 (PARP-1). (a) Both PARP-1 and Sp1 can be ribosylated through treatment with doxorubicin; using an antibody against ribosyl groups (α-pADPr), proteins were immunoprecipitated from extracts (XT) isolated from HeLa cells treated with 1.5 μmol/l doxorubicin (dox) (6 h, lane 5) or a combination of both 1 μmol/l PJ34 (10 h) and 1.5 μmol/l doxorubicin (6 h, lane 6). Twenty micrograms of NP40 HeLa extract (input lanes 1 and 2) or the total volume of an immunoprecipitation reaction was separated using 7% SDS-PAGE and immunoblotted using antibodies against PARP-1 (top panel), Sp1 (middle panel) and Sp3 (bottom panel). (b) Changes in Sp1 and PARP-1 interactions: results of Sp1 immunoprecipitation using extracts isolated from HeLa cells treated with 1 μmol/l PJ34 for 10 h, 1.5 μmol/l doxorubicin (10 h) or both. Sp1 immunoprecipitation reactions were also supplemented with 5 μg ICB1/GC1 oligonucleotide (lanes 3b, 4b, 8b and 9b) to enhance for the binding of Sp1 to DNA and PARP-1 coimmunoprecipitation. Twenty micrograms of NP40 HeLa extract (XT), along with half (lanes 3–4) or the total volume (lanes 8–9) of an immunoprecipitation reaction, was separated using 7% SDS-PAGE and immunoblotted using antibodies against PARP-1 (top panel), Sp1 (middle panel) and nuclear factor Y (NF-YA) (bottom panel). The figure is representative of duplicate similar experiments. PAGE, polyacrylamide gel electrophoresis.

considerably reduced in the presence of PJ34 (top and middle panels, compare lanes 5 and 6). This result suggests that doxorubicin treatment could stimulate the addition of ribosyl groups onto both PARP-1 and Sp1. Sp3 did not appear to form immunocomplexes with the antibodies against pADPr (α-Sp3 panel, lanes 5 and 6), indicating that Sp3 might not be ribosylated under these conditions.

In order for PARP-1 to ribosylate Sp1, these proteins must interact. Sp1 immunoprecipitation reactions were modified to not only investigate the conditions that may influence a PARP-1–Sp1 interaction but also determine the requirement for the cognate Sp1 DNA-binding site. Sp1 immunoprecipitation reactions were carried out in the presence (Fig. 6b, lanes 3b, 4b, 8b and 9b) and absence (Fig. 6b, lanes 3a, 4a, 8a and 9a) of the composite ICB1/GC1 oligonucleotide with extracts isolated from HeLa cells subjected to a 10-h exposure with 1 μmol/l PJ34 (input lane 5), a 6-h treatment with 1.5 μmol/l doxorubicin or a combination of both (input lane 10). Small amounts of PARP-1 protein were found to coimmunoprecipitate with Sp1 (Fig. 6b; lanes 3a and 3b); however, the presence of the ICB1/GC1 oligonucleotide significantly increased the amount of PARP-1 that immunoprecipitated with Sp1 protein (Fig. 6b; compare lane 3a with 3b, 4a with 4b). In order to monitor whether enhancement or inhibition of specific proteins occurs when immunocomplexes form in the presence of the ICB1/GC1 oligonucleotide, immunoprecipitates were also screened for the presence of ICB1-binding protein NF-YA (bottom panels with α-NF-YA). Figure 6b demonstrates that immunocomplexes containing the ICB1/GC1 oligonucleotide can enhance the amount of Sp1 and NF-Y that is immunoprecipitated (compare lanes 3a with 3b and 4a with 4b). In comparable experiments using mutated oligonucleotides, reduced coimmunoprecipitation of NFY-A was observed when the ICB1 element was mutated (data not shown). In addition, reduced Sp1 was observed when the GC1 element was mutated (data not shown), substantiating the specific nature of these DNA:protein:antibody immunocomplexes.

Fig. 7



Simplified representation demonstrating two of the processes by which poly (ADP-ribose) polymerase (PARP) inhibitors could improve topoisomerase IIα (topo2α)-targetting drug-mediated cell death. A rapid induction of PARP-1 activity and ribosylation (Ribo ⋯) occurs with the introduction of DNA damage (double-strand breaks). Autoribosylation and dimerization of PARP-1 activates PARP-1; excessive autoribosylation inactivates PARP-1. Ribosylation has a short cellular half-life, where the balance of cellular ribosylation is maintained by poly (ADP-ribose) glycohydrolase (PARG). PARP inhibitors are classically thought improve chemotherapy-mediated cell death through inhibition of double-strand break repair. This study demonstrates that PARP inhibitors could also reduce the amount of ribosylated specificity factor 1 (Sp1), which in turn increases the amount of cellular topo2α protein and improves the efficacy of topo2α-targetting chemotherapies.

A major target of PARP-1 ribosylation is PARP-1 itself, where PARP-1 is thought to be activated upon ribosylation, causing dimerization [21]. When ribosylation was inhibited by PJ34, PARP-1 was still observed to interact with Sp1 (Fig. 6b; compare lanes 3a and 4a, in presence of PJ34), indicating that PARP-1 activity might not be required in order for it to interact with Sp1. However, the interaction between Sp1 and PARP-1 was completely lost when HeLa cells were treated with doxorubicin (Fig. 6b; compare lanes 3a and 4a with 8a and 9a) and was not restored even in the presence of PJ34 (compare lanes 8a and 8b with 9a and 9b). Taken together with data from Fig. 6a, this suggests that the treatment of HeLa cells with 1.5 μmol/l doxorubicin is sufficient to induce ribosylation of both Sp1 and PARP-1 itself. This posttranslational modification affects their ability to interact with each other and their cognate DNA sequence. Activation of the ribosylation activity of PARP-1 is most likely due to the induction of DSB upon doxorubicin treatment. This is consistent with the current understanding that the addition of pADPr groups confers a highly negative charge to Sp1 and leads to electrostatic repulsion between DNA and protein, thereby affecting protein–protein and protein–DNA interactions [42–44].

Discussion

HeLa cells were treated with PJ34 in a range of concentrations (0.5–1 μmol/l) previously reported in other studies [44,45] to effectively inhibit PARP-1 catalytic activity. The effect of PJ34 on cell viability was then assessed using impedance-based technology [46–48]. The degree of change in impedance is primarily influenced by the number of cells that are seeded, the cell–cell interactions and the quality of the interaction as the cells adhere to the surface of the

electrodes, as well as the morphology of the cells. In this study, HeLa cells were initially titrated (data not shown) to optimize the number of cells to use for these experiments. Using 1×10^5 HeLa cells, it typically took 9–10 h to reach a stable baseline (a minimum cell index of 1) and cells were monitored for a further 10 h to establish growth curves, before drug treatment commenced. The data represented by the dye-based CellTiter96 Aqueous One proliferation assay and the xCELLigence impedance data (compare Fig. 1a and b) were consistent in demonstrating that a single dose of PJ34 alone did not significantly affect HeLa cell viability. Interestingly, when doxorubicin was added to adherent cells (Fig. 1a), there was a rapid dose-dependent increase in the cell index that was not observed when PJ34 or media only were administered. This rapid increase in the cell index may indicate an induction of cell growth, but is more likely to be due to an overall change in cell morphology and cell cycle arrest as the HeLa cells responded to the toxic doxorubicin treatment. The increase in the cell index was followed by a decline (Fig. 1a), which was again dose dependent and was correlated with a loss in cell adherence and viability when measured microscopically. Using impedance-based technology, the calculated IC_{50} for doxorubicin without any PJ34 treatment was 1100 nmol/l (± 95 nmol/l), similar to other reports using HeLa cells [49,50]. Significantly, the IC_{50} of doxorubicin reduced as HeLa cells were also treated with increasing concentrations of PJ34. The most marked reduction in IC_{50} was observed with 0.5 µmol/l PJ34 (588 ± 37 nmol/l) and 1 µmol/l PJ34 (458 ± 97 nmol/l), indicating that inhibition of PARP activity can improve the efficacy of doxorubicin-mediated cell death (Fig. 1c).

Topo2α expression has previously been correlated with the proliferative state of a tumour and the relative response to adjuvant chemotherapy [8–13,32,51]. Treatment with 0.25–2 µmol/l PJ34 did not change the relative amounts of Sp1, Sp3, NF-YA, Ku86, Ku70 or PARP-1 (Fig. 5); however, treatment with 1 µmol/l PJ34 (Figs 2 and 5) was sufficient to generate a reproducible 20% increase in cellular Topo2α protein. We observed a significant 40% reduction in the amount of Topo2α protein 6 h after treatment with 1.5 µmol/l doxorubicin, which did not change in the presence of PJ34 (Fig. 2). The reduction in Topo2α protein levels may be reflective of an early cellular response that influences the down-regulation of Topo2α and the subsequent development of drug resistance. This warrants further study.

We have previously shown that a mutation in the ICB1 element that interacts with NF-Y reduces basal promoter activity by approximately 70% and overexpression of Sp1 can activate transcription in a dose-dependent manner [33]. Sp3, however, can repress Sp-1 mediated transcriptional activation by competing for binding to the GC elements [37]. The ICB1 element is adjacent to GC1 within the topoisomerase IIα promoter and the two elements are thought to work synergistically to regulate transcription through the recruitment of transcription factors to their respective elements through mechanisms that allow chromatin reorganization, DNA bending and an interaction with RNA polymerase II [32,37,52,53]. In this study, a 20% increase in Topo2α protein (Figs 2 and 5) and a 30% increase in Topo2α promoter activity (Fig. 3a) were observed after HeLa cells were treated with PJ34. These changes did not correlate with any fluctuations in the cellular levels of defined transcription factors (Fig. 5), nor was PARP-1 found to directly interact with the regulatory ICB1/GC1 elements (Fig. 4a, lane 7). In addition, enhancement of Topo2α promoter activity was abolished when mutations in both GC1 and GC2 were incorporated (Fig. 3b). Collectively, these data suggest that that even though fluctuations in Sp1 protein were not observed, Sp1 may be posttranslationally modified by PARP-1 activity to modulate Topo2α gene expression. The EMSA data provided preliminary evidence (Fig. 4a, lane 8) that inhibition of ribosylation could effectively enhance the amount of Sp1 that can associate with DNA (Fig. 4b). Immunoprecipitation reactions using antibody against ribosyl groups (α-pADPr) demonstrated that both PARP-1 and Sp1 could be ribosylated and ribosylation was inhibited in the presence of PJ34 (Fig. 6a). Doxorubicin treatment for 6 h was sufficient to induce PARP-1 activity and detect ribosylation of PARP-1 and Sp1 (Fig. 6a, compare lanes 5 and 6). This is consistent with the understanding that the amount of pADPr formed in living cells is in proportion to the extent of DNA damage, which may take time to accumulate due to doxorubicin uptake and catabolism in living cells [43,54]. Sp1 immunoprecipitations (Fig. 6b) demonstrated that significant enhancement of a PARP-1–Sp1 interaction occurred in the presence of the ICB1/GC1 composite oligonucleotide (Fig. 6b comparing lane b with a). This interaction was abolished when extracts from doxorubicin-exposed HeLa cells were used (Fig. 6b, top panel, lanes 8–10). Taken together, these data suggest that doxorubicin-mediated ribosylation could inhibit the interaction between PARP-1 and Sp1. Together with the alterations in Sp1 binding to its cognate elements (Figs 4b and 6b), this study highlights an additional mechanism by which PARP-1 inhibitors could improve the efficacy of Topo2α-targeting chemotherapeutic agents (Fig. 7).

Conclusion

In this study, HeLa cells that were treated with PARP inhibitor PJ34 exhibited an increase in Topo2α promoter activity and an increase in Topo2α protein. These results correlated with an increase in binding of the activating transcription factor Sp1 to its cognate sequence within the Topo2α promoter in the absence of ribosylation. Conversely, treatment with doxorubicin enhanced ribosylation of Sp1 and reduced its association with DNA. Most strikingly, by combining doxorubicin treatment with

PJ34, doxorubicin-mediated cell death increased by approximately 50%. This demonstrates the potential for using PARP-1 inhibitors to reset cellular sensitivity to topo2-poisons by enhancing the amount of Topo2α protein present in cells and highlights that PARP-1 inhibitors have the potential to improve current chemotherapy regimes in a multifactorial manner.

Acknowledgements

The authors thank the Palmerston North Medical Research Foundation (PNMRF) for financial support during the course of this study, and also the Institute of Molecular Biosciences (Massey University, Palmerston North, New Zealand) for the financial support to complete this manuscript. N.M. was supported by a Massey University Doctoral Scholarship, additional funding from the Todd Foundation for Excellence and the Edward and Isobel Kidson Scholarship.

Conflicts of interest

There are no conflicts of interest.

References

1 Roca J. Topoisomerase II: a fitted mechanism for the chromatin landscape. *Nucleic Acids Res* 2009; **37**:721–730.
2 Deweese JE, Osheroff N. The DNA cleavage reaction of topoisomerase II: wolf in sheep's clothing. *Nucleic Acids Res* 2009; **37**:738–748.
3 Mirski SE, Bielawski JC, Cole SP. Identification of functional nuclear export sequences in human topoisomerase IIalpha and beta. *Biochem Biophys Res Commun* 2003; **306**:905–911.
4 Sakaguchi A, Kikuchi A. Functional compatibility between isoform alpha and beta of type II DNA topoisomerase. *J Cell Sci* 2004; **117**:1047–1054.
5 Linka RM, Porter AC, Volkov A, Mielke C, Boege F, Christensen MO. C-terminal regions of topoisomerase IIalpha and IIbeta determine isoform-specific functioning of the enzymes in vivo. *Nucleic Acids Res* 2007; **35**:3810–3822.
6 Fortune JM, Osheroff N. Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice. *Prog Nucleic Acid Res Mol Biol* 2000; **64**:221–253.
7 Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol Ther* 2003; **99**:167–181.
8 Coon JS, Marcus E, Gupta-Burt S, Seelig S, Jacobson K, Chen S, *et al*. Amplification and overexpression of topoisomerase IIalpha predict response to anthracycline-based therapy in locally advanced breast cancer. *Clin Cancer Res* 2002; **8**:1061–1067.
9 Kellner U, Rudolph P, Parwaresch R. Human DNA-topoisomerases – diagnostic and therapeutic implications for Cancer. *Onkologie* 2000; **23**:424–430.
10 Burgess DJ, Doles J, Zender L, Xue W, Ma B, McCombie WR, *et al*. Topoisomerase levels determine chemotherapy response *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* 2008; **105**:9053–9058.
11 Cardoso F, Durbecq V, Larsimont D, Paesmans M, Leroy JY, Rouas G, *et al*. Correlation between complete response to anthracycline-based chemotherapy and topoisomerase II-alpha gene amplification and protein overexpression in locally advanced/metastatic breast cancer. *Int J Oncol* 2004; **24**:201–209.
12 Uesaka T, Shono T, Kuga D, Suzuki SO, Niiro H, Miyamoto K, *et al*. Enhanced expression of DNA topoisomerase II genes in human medulloblastoma and its possible association with etoposide sensitivity. *J Neurooncol* 2007; **84**:119–129.
13 O'Malley FP, Chia S, Tu D, Shepherd LE, Levine MN, Bramwell VH, *et al*. Topoisomerase II alpha and responsiveness of breast cancer to adjuvant chemotherapy. *J Natl Cancer Inst* 2009; **101**:644–650.
14 Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SP, Deeley RG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 1994; **54**:357–361.
15 Bredel M. Anticancer drug resistance in primary human brain tumors. *Brain Res Brain Res Rev* 2001; **35**:161–204.
16 Saxena D, Yiu GK, Ni X, Huang KC, Mantovani R, Jacquemin-Sablon AG, *et al*. Characterization of promoter elements involved in the down-regulation of topoIIα expression in a drug-resistant cell line. *Gene* 2004; **342**:145–155.
17 Wielckens K, George E, Pless T, Hilz H. Stimulation of poly(ADP-ribosyl)ation during Ehrlich ascites tumor cell starvation and suppression of concomitant DNA fragmentation by benzamide. *J Biol Chem* 1983; **258**:4098–4104.
18 Alvarez-Gonzalez R, Althaus FR. Poly(ADP-ribose) catabolism in mammalian cells exposed to DNA-damaging agents. *Mutat Res* 1989; **218**:67–74.
19 Gagne JP, Hendzel MJ, Droit A, Poirier GG. The expanding role of poly(ADP-ribose) metabolism: current challenges and new perspectives. *Curr Opin Cell Biol* 2006; **18**:145–151.
20 Heeres JT, Hergenrother PJ. Poly(ADP-ribose) makes a date with death. *Curr Opin Chem Biol* 2007; **11**:644–653.
21 Mendoza-Alvarez H, Alvarez-Gonzalez R. Poly(ADP-ribose) polymerase is a catalytic dimer and the automodification reaction is intermolecular. *J Biol Chem* 1993; **268**:22575–22580.
22 D'Amours D, Desnoyers S, D'Silva I, Poirier GG. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* 1999; **342**:249–268.
23 Gagné J, Isabelle M, Lo KS, Bourassa S, Hendzel MJ, Dawson VL, *et al*. Proteome-wide identification of poly(ADP-ribose) binding proteins and poly(ADP-ribose)-associated protein complexes. *Nucl Acids Res* 2008; **36**:6959–6976.
24 Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. PARP inhibition: PARP1 and beyond. *Nat Rev Cancer* 2010; **10**:293–301.
25 Cosi C. New inhibitors of poly(ADP-ribose) polymerase and their potential therapeutic targets. *Expert Opin Ther Patents* 2002; **12**:1047–1071.
26 Tentori L, Lacal PM, Muzi A, Dorio AS, Leonetti C, Scarsella M, *et al*. Poly(ADP-ribose) polymerase (PARP) inhibition or PARP-1 gene deletion reduces angiogenesis. *Eur J Cancer* 2007; **43**:2124–2133.
27 D'Onofrio G, Tramontano F, Dorio AS, Muzi A, Maselli V, Fulgione D, *et al*. Poly(ADP-ribose) polymerase signaling of topoisomerase 1-dependent DNA damage in carcinoma cells. *Biochem Pharmacol* 2011; **2**:194–202.
28 Pacher P, Liaudet L, Bai P, Virag L, Mabley JG, Hasko G, Szabo C. Activation of poly(ADP-ribose) polymerase contributes to development of doxorubicin-induced heart failure. *J Pharmacol Exp Ther* 2002; **300**:862–867.
29 Curtin NJ. PARP inhibitors for cancer therapy. *Expert rev Mol Med* 2005; **7**:15.
30 De Soto JA, Wang X, Tominaga Y, Wang RH, Cao L, Qiao W, *et al*. The inhibition and treatment of breast cancer with poly (ADP-ribose) polymerase (PARP-1) inhibitors. *Int J Biol Sci* 2006; **2**:179–185.
31 Hochhauser D, Stanway CA, Harris AL, Hickson ID. Cloning and characterization of the 5′-flanking region of the human topoisomerase II alpha gene. *J Biol Chem* 1992; **267**:18961–18965.
32 Isaacs RJ, Harris AL, Hickson ID. Regulation of the human topoisomerase IIalpha gene promoter in confluence-arrested cells. *J Biol Chem* 1996; **271**:16741–16747.
33 Magan N, Szremska AP, Isaacs RJ, Stowell KM. Modulation of DNA topoisomerase II alpha promoter activity by members of the Sp (specificity protein) and NF-Y (nuclear factor Y) families of transcription factors. *Biochem J* 2003; **374**:723–729.
34 Joshi AA, Wu Z, Reed RF, Suttle DP. Nuclear factor-Y binding to the topoisomerase IIalpha promoter is inhibited by both the p53 tumor suppressor and anticancer drugs. *Mol Pharmacol* 2003; **63**:359–367.
35 Bronner C, Hopfner R, Mousli M. Transcriptional regulation of the human topoisomerase IIalpha gene. *Anticancer Res* 2003; **22**:605–612.
36 Allen KA, Williams AO, Isaacs RJ, Stowell KM. Down-regulation of human topoisomerase IIalpha correlates with altered expression of transcriptional regulators NF-YA and Sp1. *Anticancer Drugs* 2004; **15**:357–362.
37 Williams AO, Isaacs RJ, Stowell KM. Down-regulation of human topoisomerase IIalpha expression correlates with relative amounts of specificity factors Sp1 and Sp3 bound at proximal and distal promoter regions. *BMC Mol Biol* 2007; **8**:36.
38 Liang SG, Maity SN. Pathway of complex formation between DNA and three subunits of CBF/NF-Y. Photocross-linking analysis of DNA-protein interaction and characterization of equilibrium steps of subunit interaction and DNA binding. *J Biol Chem* 1998; **273**:31590–31598.
39 Atienza JM, Yu N, Kirstein SL, Xi B, Wang X, Xu X, *et al*. Dynamic and label-free cell based assays using the real-time electronic sensing system. *Assay Drug Dev Technol* 2006; **5**:597–607.
40 Kubo T, Kohno K, Ohga T, Taniguchi K, Kawanami K, Wada M, *et al*. DNA topoisomerase II alpha gene expression under transcriptional control in etoposide/teniposide-resistant human cancer cells. *Cancer Res* 1995; **55**:3860–3864.

41 Tuteja R, Tuteja N. Ku autoantigen: a multifunctional DNA-binding protein. *Crit Rev Biochem Mol Biol* 2000; **35**:1–33.
42 Rouleau M, Aubin RA, Poirier GG. Poly(ADP-ribosyl)ated chromatin domains: access granted. *J Cell Sci* 2004; **117**:815–825.
43 Kim MY, Zhang T, Kraus WL. Poly(ADP-ribosyl)ation by PARP-1: PAR-laying NAD+ into a nuclear signal. *Genes Dev* 2005; **19**:1951–1967.
44 Zaniolo K, Desnoyers S, Leclerc S, Guerin SL. Regulation of poly(ADP-ribose) polymerase-1 (PARP-1) gene expression through the post-translational modification of Sp1: a nuclear target protein of PARP-1. *BMC Mol Biol* 2007; **8**:96.
45 Luo HR, Hattori H, Hossain MA, Hester L, Huang Y, Lee-Kwon W, *et al*. Akt as a mediator of cell death. *Proc Natl Acad Sci USA* 2003; **100**: 11712–11717.
46 Xiao C, Lachance B, Sunahara G, Luong JH. An in depth analysis of electrical cell-substrate impedance sensing to study the attachment and spreading of mammalian cells. *Anal Chem* 2002; **74**:1333–1339.
47 Atienza JM, Zhu J, Wang X, Xu X, Abassi Y. Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. *J Biomol Screen* 2005; **10**:795–805.
48 Atienzar FA, Tilmant K, Gerets HH, Toussaint G, Speeckaert S, Hanon E, *et al*. The use of real-time cell analyzer technology in drug discovery: defining optimal cell culture conditions and assay reproducibility with different adherent cellular models. *J Biomol Screen* 2011; **16**:575–587.
49 Lu DY, Huang M, Xu CH, Yang WY, Hu CX, Lin LP, *et al*. Anti-proliferative effects, cell cycle G2/M phase arrest and blocking of chromosome segregation by probimane and MST-16 in human tumor cell lines. *BMC Pharmacol* 2005; **5**:11.
50 Draganov AB. Novel rhein analogues a potential anticancer agents and a novel metal free synthesis of 6H-isoindol[2,1-Aindol-6-one. *Chem Thesis* 2011 Paper 40.
51 Asano T, An T, Mayes J, Zwelling LA, Kleinerman ES. Transfection of human topoisomerase II alpha into etoposide-resistant cells: transient increase in sensitivity followed by down-regulation of the endogenous gene. *Biochem J* 1996; **319**:307–313.
52 Kadonaga JT, Jones KA, Tjian R. Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem Sci* 1986; **11**:20–23.
53 Coustry F, Hu Q, de Crombrugghe B, Maity SN. CBF/NF-Y functions both in nucleosomal disruption and transcription activation of the chromatin-assembled topoisomerase IIalpha promoter. Transcription activation by CBF/NF-Y in chromatin is dependent on the promoter structure. *J Biol Chem* 2001; **276**:40621–40630.
54 Peralta-Leal A, Rodríguez-Vargas JM, Aguilar-Quesada R, Rodríguez MI, Linares JL, de Almodóvar MR, *et al*. PARP inhibitors: new partners in the therapy of cancer and inflammatory diseases. *Free Radic Biol Med* 2009; **47**:13–26.