Differential regulation of DNA repair protein Rad51 in human tumour cell lines exposed to doxorubicin

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Radiotherapy and chemotherapy often induce DNA double-strand breaks in both normal and malignant cells. The proteins involved in the repair of such lesions are central to cancer prognosis and treatment, as they can be overexpressed in many cancers, accelerating malignant transformation and increasing repair capacity, potentially leading to cellular resistance. If malignant cells can be selectively targeted repair proteins could also be candidates for targeted therapy. In this study, two keyplayers in eukaryotic DNA double-strand break repair, Rad51 and DNA-dependent protein kinase catalytic subunit, were analysed in noncancerous human breast cells (MCF12A) and the breast cancer cell lines (MDA MB 231 and MCF7) in response to treatment with doxorubicin. A cell cycle-independent increase in Rad51 protein levels (a recombinase involved in homologous recombination repair) was observed 24 and 48 h after treatment in MDA MB 231 and MCF12A when exposed to low levels of doxorubicin, whereas MCF7 cells displayed a continuous decrease in Rad51 protein with increasing drug concentration. DNA-dependent protein kinase catalytic subunit, which is involved in nonhomologous end joining of DNA lesions, remained unaltered under all conditions tested. Topoisomerase II-α protein, the primary target of doxorubicin, was upregulated at low concentrations of doxorubicin in all cell lines tested. Here we show that Rad51 protein levels can be differentially regulated in normal and malignant breast cell lines in response to doxorubicin, independent of cell cycle state. These observations have direct relevance to chemosensitivity and add an additional prognostic factor that could be taken into account when designing targeted therapeutic regimes. *Anti-Cancer Drugs* **18:419–425 © 2007 Lippincott Williams & Wilkins.**

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Introduction

In humans and other eukaryotes double-strand breaks (DSB) can be caused by endogenous factors, e.g. reactive oxygen species, V(D)J recombination and replication-associated damage, and exogenous factors such as mechanical stress, chemicals, chemotherapeutic agents and ionizing radiation. If left unrepaired, DSBs can lead to chromosome aberrations, higher radiation sensitivity and cell death [1]. Two main DSB repair mechanisms exist: homologous recombination repair (HRR) and nonhomologous end joining (NHEJ). Cell cycle state is critical in determining which of these pathways is utilized for the repair process. NHEJ is predominantly responsible for DNA DSB repair during G_0, G_1 and early S phases, whereas in late S and G_2, both HRR and NHEJ seem to be of equivalent importance [2]. The NHEJ repair pathway is inherently more error prone than HRR, causing the loss of nucleotides from the site of breakage. DNA-dependent protein kinase (DNA-PK) plays an important role in apoptotic signalling, telomere maintenance and NHEJ, during which, the Ku heterodimer facilitates the recruitment and binding of the 465 kDa DNA-PK catalytic subunit (DNA-PKcs) to the site, forming an activated DNA-PK complex which requires auto-phosphorylation in the process of repair [3]. Silencing of DNA-PKcs using small interfering RNA has resulted in sensitizing cancer cells to ionizing radiation and chemotherapeutic agents [4,5].

HRR is the most precise form of repair, but it is comparatively slow and may not always be feasible as a chromosome template is required for repair. Rad51 is critical for catalysing homologous strand pairing and exchange during HRR [6–9]. Overexpression of Rad51 has been implicated in a variety of cancers, including breast cancer and is thought to contribute to resistance mechanisms, and tumour progression in these cells [9–11]. Increasing evidence has been obtained linking the rate of DNA damage repair with levels of DNA-PK and Rad51, which contributes to cell resistance against ionizing radiation (IR) and chemotherapy [12,13]. Alterations in expression or activity of proteins involved in either NHEJ or HRR have been implicated in tumourigenesis and IR/chemoresistance mechanisms.

In the clinic, doxorubicin and other topoisomerase (topo) II-α poisons are commonly used to treat breast cancers in combination with alkylating agents and microtubule

poisons. Topo II poisons primarily cause cell death by producing DNA DSBs, but also form reactive oxygen radicals. Although many studies have investigated the effects of doxorubicin on cell cycle and cell viability, little is known about the response of proteins involved in the repair of doxorubicin-generated DNA damage, which represent major factors in a cell's struggle for survival and drug resistance. A better understanding of the response of normal and malignant cells to this cellular assault is essential for the development of targeted and more effective approaches to eliminating cancer cells.

In this study, we investigated the DNA damage repair response to doxorubicin of estrogen receptor (ER)-negative, dysfunctional p53 and transforming growth factor (TGF)-β MDA MB 231 and ER-positive, functional p53 and TGF-β MCF7 breast cancer cells, and of an immortalized noncancerous human breast cell line, MCF12A, by monitoring the expression of two key proteins, Rad51 and DNA-PKcs. We show that the response of Rad 51 to doxorubicin exposure is cell-type-specific and independent of the cell cycle.

Materials and methods

Cell culture

MDA MB 231 and MCF7 cells were grown in Opti-MEM (Gibco/BRL, Invitrogen, Auckland, New Zealand) supplemented with 2% fetal calf serum, 2.4 mg/ml sodium bicarbonate, and 50 units/ml of penicillin and streptomycin. MCF12A were cultured in a 1 : 1 mixture of Ham's F12 (Sigma-Aldrich, Castle Hill, New South Wales, Australia) and Dulbecco's modified Eagle's medium (Sigma) supplemented with 2.4 mg/ml sodium bicarbonate, 20 ng/ml epidermal growth factor (Sigma), 500 ng/ml hydrocortisone (Sigma), 5% horse serum (Gibco/BRL), and 50 units/ml of penicillin and streptomycin (Gibco/BRL). MCF7 and MCF12A were grown in medium containing 10 μg/ml insulin (Roche Diagnostics, Mt. Wellington, Auckland, New Zealand). All cells were grown in humid conditions at 37°C in a 5% CO_2 incubator and experiments were carried out during exponential growth phase. Cells were passaged using 0.25% Trypsin (Gibco/BRL) in phosphate-buffered saline (PBS).

Drug exposure

Cells were seeded at 25–30% confluence and left undisturbed for 24 h before exposure to doxorubicin (Pharmacia & Upjohn, Auckland, New Zealand) for 1 h. The medium was then replaced with doxorubicin-free conditioned medium and cells were left undisturbed until harvested.

Cell viability assay

Cells were seeded at 100 000 (MDA MB 231 and MCF12A) and 50 000 (MCF7) per well in 12-well plates. For analyses, growth media and trypsinized cells were collected and centrifuged at 480 *g* for 5 min. Cell pellets were resuspended in PBS and diluted 1 : 1 with 10 mg/ml erythrosin B (British Drug Houses, Poole, UK) to distinguish between viable and dead cells.

Immunoblotting

Proteins were extracted with 40 mmol/l *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid pH 7.9, 0.4 mol/l KCl, 1 mmol/l dithiothreitol, 10% glycerol, 1 mmol/l ethylenediaminetetraacetic acid, Complete mini (Roche) protease inhibitor and quantified using BioRad (Hercules, California, USA) Bradford reagent according to the manufacturer's instructions. Twenty micrograms or 40 μg of protein were loaded onto 5–10% gradient sodium dodecyl sulphate–polyacrylamide gel electrophoresis mini gels. Proteins were electroblotted onto nitrocellulose membrane (Roche) and immunolabeled using 1 : 250 Rad51 (Santa Cruz Biotechnology, Santa Cruz, California, USA) and 1 : 4000 DNA-PKcs (Santa Cruz), 1 : 250 topo II-α (Santa Cruz), 1 : 250 poly (ASP-ribose) polymerase (PARP)-1 (Santa Cruz) and 1 : 2000 α-tubulin (Sigma) primary antibodies and appropriate secondary antibodies [antirabbit, 1 : 2500; antigoat and antimouse, 1 : 5000 (Sigma)]. Roche POD substrate was used for chemiluminescent signal detection according to the manufacturer's instructions. Images were acquired using a Fuji LAS 1000 and quantified using ImageJ software (http://rsb.info.nih.gov/ij).

Fluorescence-activated cell sorting analysis

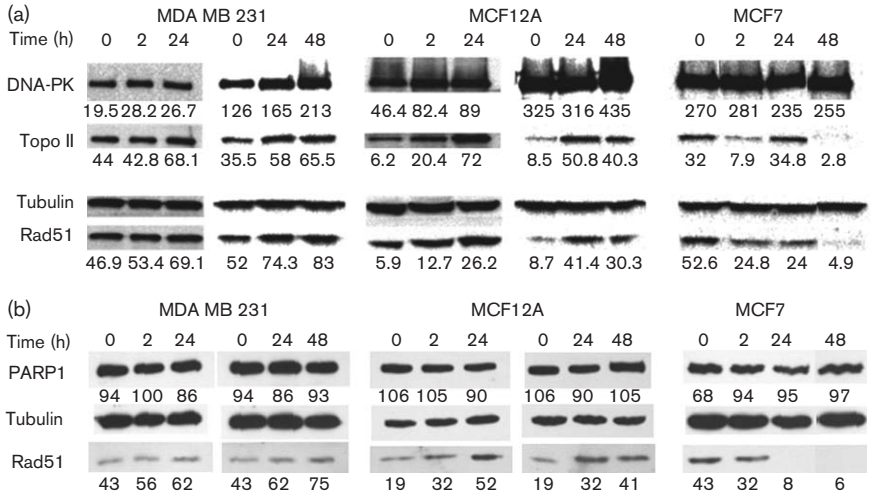
Before protein extraction 10^6 cells were set aside for fluorescence-activated cell sorting (FACS) analysis. Cells were resuspended in 50 μl PBS and fixed with 600 μl 70% ethanol at 4°C for at least 1 h or stored for up to 1 week at 4°C. Cells were then washed in PBS twice before adding 100 μl of 1 mg/ml RNAse (Sigma) for 15 min at room temperature and staining in 200 μl of 100 μg/ml propidium iodide (Sigma) for 2–24 h. FACS analysis was performed on a Becton Dickinson FACScalibur (BD Biosciences, New South Wales, Australia) counting at least 10^5 cells per sample.

Results

Initially, MDA MB 231 cells were treated with 3 μmol/l doxorubicin to approximate clinical dosages. This dosage allowed a small percentage of cells to survive, thus selecting for drug-tolerant and potentially resistant cells. In an effort to analyse the time course of response to the drug in noncancerous breast and breast cancer cells, relative amounts of DNA-PKcs, topo II-α, α-tubulin and Rad51 were analysed by immunoblotting 2, 24 and 48 h after drug exposure (Fig. 1a).

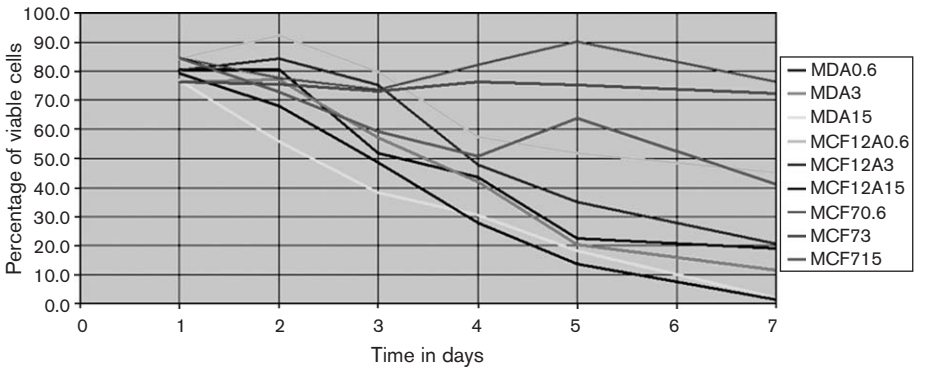
In both MDA MB 231 and MCF12A an increase in Rad51 and topo II-α levels was observed 24 and 48 h after drug exposure. In MCF7, however, Rad51 was downregulated

Fig. 1



Immunoblots of MDA MB 231, MCF12A and MCF7 cell protein extracts. Twenty micrograms of total protein were separated on a 5–10% polyacrylamide gel to compare temporal changes in topoisomerase (topo) II-α, DNA repair proteins Rad51 and DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase relative to α-tubulin 0, 2, 24 and 48 h after 3 μmol/l doxorubicin treatment. Numbers indicate signal intensity as a percentage, relative to tubulin (100%). The results are representative of three experiments.

Fig. 2



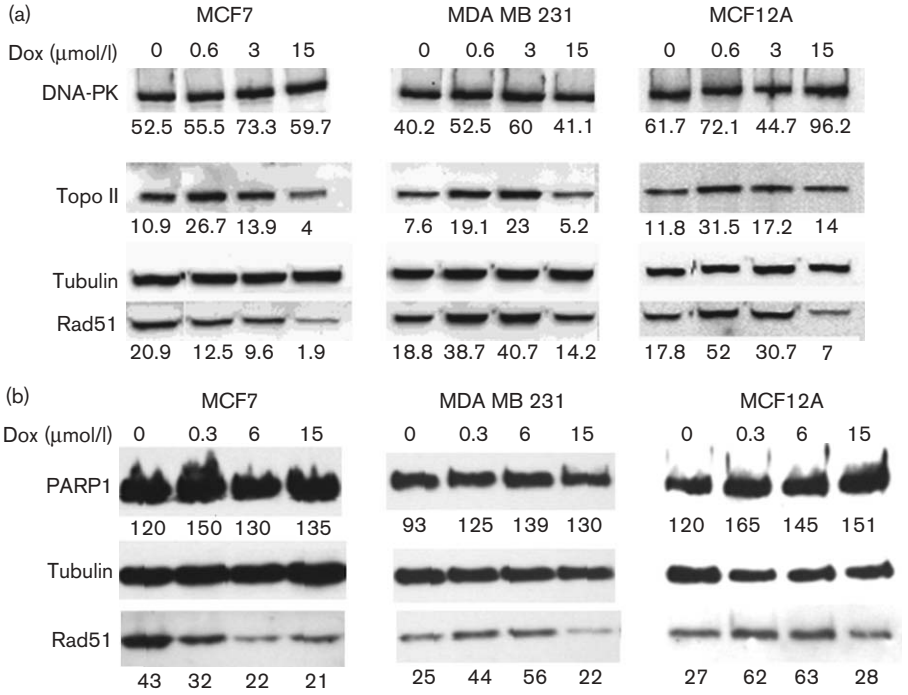
Cell viability assay for the three tested cell lines using varying amounts of doxorubicin. The impact of drug treatment on MDA MB 231, MCF12A and MCF7 cell survival using 0, 0.6, 3 and 15 μmol/l doxorubicin was monitored over 1 week with samples taken every 24 h after initial exposure. No sample was taken on day 6. Results are averages of duplicate (in some cases triplicate) counts of two, independently grown and identically treated samples.

at all time points. DNA-PKcs signals varied slightly between experiments, essentially remaining unaltered. Cell survival could be due to a decrease in apoptosis, associated with increased DNA repair in MDA MB 231 and MCF12A cell lines. Therefore these experiments were repeated to compare the expression of Rad 51 with PARP (Fig. 1b). Decreased levels of PARP are generally associated with an increase in apoptosis [14]. For MDA MB 231 and MCF12A, PARP levels did not change markedly, but an increase in PARP expression was observed for MCF7 cells. The absolute values of Rad51 in this set of experiments were slightly different to the first set but the trends over the time course and for each cell line were identical.

Response to drug dosage

As clear changes in expression of both Rad51 and topo II-α were evident 24 h after treatment with 3 μmol/l doxorubicin, we investigated dosage-dependent changes in protein levels. Doxorubicin was used at a moderate to low (0.6 μmol/l), moderate to high (3 μmol/l) and high concentration (15 μmol/l) for MDA MB 231 cells, as these were the most sensitive of the three cell lines used (Fig. 2).

Fig. 3



Immunoblots of MDA MB 231, MCF12A and MCF7 cell protein extract. Twenty micrograms of total protein was separated on a 5–10% polyacrylamide gel to investigate a drug-dose response of DNA-dependent protein kinase (DNA-PK), topoisomerase (topo) II-α, poly(ADP-ribose) polymerase, α-tubulin and Rad51 to 0, 0.6, 3 and 15 μmol/l doxorubicin (Dox) in the cell lines tested. Numbers indicate signal intensity as a percent relative to tubulin (100%) of the same lane. The results are representative of three experiments.

As in the time-course experiments, DNA-PKcs levels remained largely unaltered across all concentrations of doxorubicin, as compared with the unexposed control (Fig. 3a). Rad51 and topo II-α expression in both MDA MB 231 and MCF12A were markedly increased in response to 0.6 and 3 μmol/l doxorubicin. Indeed all cell lines displayed significantly higher topo II-α levels 24 h after exposure to 0.6 μmol/l doxorubicin than did the control. Interestingly, in MCF7 cells Rad51 levels exhibited a completely opposite response as they declined significantly with increasing drug concentration. Hence the Rad51 response of MCF7 cells is contrary to that seen in the MDA MB 231 cell line.

A dose of 15 μmol/l appeared to reduce topo II-α and Rad51 levels compared with lower doses. The use of high drug concentrations resulted in fewer viable cells and a higher number of cells with an abnormal morphology compared with cells that had received lower drug concentrations (data not shown). One possible explanation could be that higher amounts of drug may result in more cells undergoing necrosis or apoptosis more quickly, which may impair protein synthesis and decrease levels of certain proteins, such as Rad51 and topo II-α, because of proteolysis.

These experiments were also repeated to monitor the expression of PARP in response to increased dosage of doxorubicin. Apart from a slight increase in PARP expression at all concentrations of doxorubicin compared with the control in each of the three cell lines, PARP levels were essentially the same with each concentration of doxorubicin (Fig. 3b). The dose-dependent trends for Rad 51 expression shown in Fig. 3(a and b) were identical.

Fluorescence-activated cell sorting analysis

Rad51 and topo II-α expression increase in late S and G_2/M phases of the cell cycle [15,16]. FACS analysis was employed to establish whether the observed changes in protein levels were due to simply a change in cell cycle distribution or a genuine response to drug exposure.

During the time-course experiments, FACS profiles for MCF12A remained unaltered after drug exposure (Table 1), indicating that changes in Rad51 and topo II-α levels are independent of cell cycle state in this cell line treated with 3 μmol/l doxorubicin. Drug treatment of MDA MB 231 produced a strong increase in the proportion of S and G_2/M cells with a concomitant decrease of cells in G_1 compared with the control. Neither MCF7 nor MCF12A, however, showed any significant changes in cell cycle distribution 24 h after being exposed to varying amounts of doxorubicin (Table 2). Consequently, the changes in Rad51 and topo

Table 1 Cell cycle phase distribution (as percentage) of MDA MB 231, MCF12A and MCF7 cells at 0 and 24 and 48 h after 3 μmol/l doxorubicin treatment

Cell cycle phase	Time (h)		
	0	24	48
MDA MB 231			
G_1	62	28	26
S	12	25	20
G_2/M	26	47	54
MCF12A			
G_1	88	87	83
S	3	3	5
G_2/M	9	10	12
MCF7			
G_1	56	45	45
S	19	12	7
G_2/M	25	43	48

Table 2 Cell cycle phase distribution (as percentage) of MDA MB 231, MCF7 and MCF12A cells 24 h after exposure to 0, 0.6, 3 and 15 μmol/l doxorubicin

Cell cycle phase	Doxorubicin (μmol/l)			
	0	0.6	3	15
MDA MB 231				
G_1	72	22	32	54
S	10	15	30	22
G_2/M	18	63	39	25
MCF7				
G_1	57	56	58	59
S	15	9	9	21
G_2/M	29	35	33	21
MCF12A				
G_1	69	63	68	69
S	10	9	12	18
G_2/M	22	29	20	13

II-α expression in these cell lines were not cell cycle state-specific, but a genuine response to drug exposure. Results were more difficult to interpret for the MDA MB 231 cell line. A similar immunoblotting response was observed in cells exposed to 0.6 and 3 μmol/l of doxorubicin, with the latter containing 50% more cells in G_1 (Fig. 3a). If Rad51 expression was indeed restricted to the late stages of the cell cycle, this should have resulted in a much weaker signal in samples treated with 3 μmol/l compared with 0.6 μmol/l doxorubicin, but in fact the observed signal was actually slightly stronger. Hence, it seems likely that changes in Rad51 and topo II-α protein levels in MDA MB 231 cells are also independent of cell cycle distribution, at least under these conditions.

Discussion

Proteins involved in DNA repair pathways have been proposed as targets for selectively sensitizing tumour cells to radio and chemotherapy. A better understanding of the response to chemotherapeutic agents of two key DNA double-strand break repair proteins, DNA-PKcs and Rad51, would help determine the feasibility of such an approach.

Rad51 is overexpressed in many tumours compared with normal tissue, leading to increased levels of drug resistance [9,17] and has been proposed as one of the most suitable targets for selective sensitisation. There have been conflicting results, however, as to whether Rad51 is radiation or drug-inducible [18,19]. In our study, Rad51 levels were significantly altered after doxorubicin treatment in both malignant and normal breast cell lines, but with a variable pattern of expression. Rad 51 levels correlate with topo II-α levels in both a temporal and dose-dependent manner in all cell lines tested. This response was cell cycle-independent as well as cell line-specific. MDA MB 231 and MCF12A showed increased Rad51 levels upon drug exposure 24 and 48 h after treatment. It seems plausible that to repair the DSB lesions Rad51 expression is increased, especially if NHEJ is saturated. The availability of DNA-PK is likely to be a limiting factor for NHEJ and thus levels of this enzyme may not be increased with drug-induced DSB [20]. Although DNA-PKcs analysis showed some inconsistencies, we found no significant alterations because of drug exposure, in accordance with these observations. No clear correlations between Rad 51 and PARP levels in MCF12A or MDA MB 231 cell lines were observed over the time course of doxorubicin treatment. This suggests that there is no change in apoptosis in these lines over the time course of doxorubicin treatment, but the increases observed in Rad51 may in fact reflect *bona fide* increases in DNA repair and hence cell survival.

Downregulation of Rad51 however, as seen in MCF7 is not easily explained. One of the most striking differences between MCF7 and MDA MB 231 cells is that the latter express a mutant p53, a mutant TGF-β and are ER-negative. In particular, p53 [21–24] and TGF-β [21] act as regulators of Rad51-mediated DNA repair. BRCA2 [22] and c-Abl [25,26] are also known to interact with Rad51, but the exact mechanisms of Rad51 regulation are not yet fully understood. An imbalance and/or dysfunction of p53 and TGF-β could influence Rad51 expression, as a response to the increased need for DNA repair. On the other hand, MCF12A cells express normal p53 and TGF-β, but respond to doxorubicin treatment in a similar manner to that observed in MDA MB 231 cells, suggesting an alternative mechanism is likely to be associated with increased Rad51 protein expression in response to chemotherapeutic treatment. The mechanisms controlling this variable response in expression will need to be identified to develop cell-specific targeted therapies for malignancy.

The downregulation of Rad51 in MCF7 cells implies that HRR may not play a critical role in repair of doxorubicin-induced DNA damage, at least in some malignant clones. One consequence of this downregulation would be a decrease in the ability of the cell to perform chromosomal recombination. Overexpression of Rad51 is seen in

immortalized human cell lines [27] and in tumour cells [17], and has been shown to have a dominant negative effect on cell survival [28,29]. Indeed, MCF7 overexpress Rad51 [30], but according to our data MCF12A showed an even more elevated expression and MDA MB 231 feature the highest levels of the three cell lines tested. A further increase as a response to DSB could possibly be more harmful leading to hyper-recombination and/or increased apoptosis, however, our results do not support a marked increase in apoptosis as PARP levels appear relatively constant with the decrease in Rad51. In this respect, the downregulation of Rad51 may enhance doxorubicin tolerance concomitantly distributing DNA repair duties to other Rad51-independent pathways. Indeed, MCF7 cells are the most doxorubicin tolerant cell line tested here, suggesting the involvement of other DNA repair pathways. It has been suggested that NHEJ may be the predominant repair pathway for topo II poison-induced DNA lesions [31]. Further studies in chicken cells (DT 40) have indicated the contribution of HRR to the removal of IR-induced DNA DSBs to be marginal. These authors also suggest a model in which HRR may not be involved in the repair of DSBs until after the breaks have been sealed [32].

Our findings also illustrate that Rad51 expression in response to doxorubicin is cell line-dependent, which is in agreement with a study by Russell *et al.* [18] who found Rad51 expression to be inducible in glioma tumour cells but not in normal fibroblasts. Levels of topo II-α were upregulated in a cell cycle-independent manner 24 h after exposure to low to moderate drug concentrations in all cell lines used. It is possible that topo II-α levels are elevated to compensate for drug-bound topo II-α protein or owing to an increased need for topological rearrangement and maintenance triggered by DNA DSB repair. Hence, Rad51 as well as topo II-α are doxorubicin 'inducible' in MDA MB 231 and MCF12A cell lines, resulting in higher protein levels.

As both topo II-α and Rad51 are inducible by low doxorubicin concentrations in certain cell lines, it is possible that a combination of topo II poisons and a Rad51 inhibitor may be more potent in killing susceptible tumour cells than current procedures. We have also found that levels of Rad51 in primary human breast cancers may be either upregulated or downregulated compared with the surrounding normal tissue, whereas expression of DNA-PKcs was found to be upregulated, but not downregulated in some tumours (data not shown). With this in mind, a combination therapy with specific inhibitors may be most effective when treating those tumours that initially overexpress Rad51 or DNA-PKcs. Our results, however, also demonstrate that targeting Rad51 in combination with doxorubicin treatment may be problematic, as Rad51 expression can be increased in non-cancerous cells and/or decreased in tumour cells in response to chemotherapy. In some cases, this could result in increased toxicity of normal compared with tumour cells. Hence, it will be particularly important to define further differences between normal and malignant cells, as modified DNA repair in normal cells could result in unacceptable clinical toxicity, rather than an improvement in elimination of cancer cells. It is perhaps most likely that the increasing use of molecular profiling of tumours, including determining expression of DNA repair proteins, will identify those tumours most susceptible to repair inhibitors and other targeted therapies. Clearly, more information is needed to unravel the complex regulatory mechanisms involved in DNA repair, particularly Rad51 expression and the precise role it plays in response to chemotherapeutic treatment.

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