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XPA versus ERCC1 as chemosensitising agents to cisplatin and mitomycin C in prostate cancer cells: Role of ERCC1 in homologous recombination repair

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ARTICLE INFO

Article history:
Received 20 January 2006
Accepted 27 April 2006

Keywords:
SiRNA
XPA
ERCC1
Cisplatin
Mitomycin C
DNA repair

Abbreviations:
SiRNA, small interfering RNA
MMC, mitomycin C
ICL, interstrand crosslink
HRR, homologous recombinational repair

ABSTRACT

Nucleotide excision repair is the principal mechanism for the removal of bulky DNA adducts caused by a range of chemotherapeutic drugs, and contributes to cisplatin resistance. In this study, we used synthetic siRNAs targeted to XPA and ERCC1 and compared their effectiveness in sensitising mismatch repair deficient prostate cancer cell lines to cisplatin and mitomycin C. Downregulation of ERCC1 sensitised DU145 and PC3 cells to cisplatin and mitomycin C. In contrast, XPA downregulation did not sensitise either cell line to mitomycin C, and only sensitised DU145 cells to cisplatin. The effects of ERCC1 downregulation may be due to its role in homologous recombination repair. Excision repair of cisplatin adducts in PC3 cells was attenuated to a similar extent by XPA and ERCC1 downregulation. Downregulation of XPA but not ERCC1 caused an increase in the number of cisplatin-induced RAD51 foci in PC3 cells, suggesting that HRR is able to substitute for NER in these cells. We observed co-localisation of ERCC1 and RAD51 in cisplatin treated PC3 cells by immunofluorescence and co-immunoprecipitation, which may represent recruitment of ERCC1/XPF to sites of recombination repair. These results indicate that ERCC1 is a broader therapeutic target than XPA with which to sensitise cancer cells to chemotherapy because of its additional role in recombination repair.

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doi:10.1016/j.bcp.2006.04.025

1. Introduction

NER is the primary repair system for the removal of bulky DNA lesions caused by a variety of chemotherapeutic drugs, reviewed in [1,2]. The core proteins required for NER are XPA, RPA, XPC-hR23B, TFIIH, XPG and ERCC1/XPF [3]. XPA forms part of the pre-incision complex in both global and transcription-coupled repair, and is thought to verify NER lesions and correctly position other NER factors around the lesion [1]. Cells belonging to the XP complementation group A are the most severely impaired in NER and are sensitive to UV and drugs such as cisplatin [1,4]. The ERCC1/XPF heterodimer is a structure-specific endonuclease and its function in NER is to make the 5′-incision on the damaged strand [5,6]. Unlike XPA, which only functions in NER, ERCC1/XPF has also been implicated in the removal of interstrand crosslinks (ICLs) caused by bifunctional alkylating agents [7], and in homologous recombination (HR) [8,9].

Cisplatin resistance is multifactorial (reviewed in [10]), being associated with drug efflux, glutathione levels and DNA repair. The NER pathway is an important factor in cisplatin resistance, as shown by the association between expression levels of NER factors (mainly ERCC1 and XPA), NER and cisplatin resistance [11–19]. Thus, the NER pathway is an attractive target with which to sensitise cancer cells to cisplatin, as well as other DNA crosslinking drugs.

In this study, synthetic siRNAs targeted to XPA and ERCC1 were transfected into the human prostate cancer cell lines PC3 and DU145, and their effectiveness as chemosensitising agents to cisplatin and MMC compared. SiRNA-mediated downregulation of ERCC1 but not XPA sensitised both cell lines to MMC, presumably because of the role of ERCC1 in repairing DNA ICLs. With cisplatin, however, the response to XPA and ERCC1 downregulation differed between the two cell lines. We present evidence that the contribution of HRR to cell survival can be circumvented by ERCC1 downregulation. Our results suggest that because of its dual role in NER and HRR, ERCC1 may be of wider use as a chemosensitising target.

2. Materials and methods

2.1. Cell lines and culture

The prostate cancer cell lines PC3 and DU145 were maintained in RPMI-1640 medium (Invitrogen), supplemented with 8% FCS and 2 mM L-glutamine (complete medium). Cells were grown at 36.5 °C in a 5% CO_2 incubator.

2.2. Transfection with siRNAs

SiRNA Smartpools designed to target human ERCC1 and XPA were purchased from Dharmacon RNA Technologies, catalogue numbers M-006311-00 and M-005067-00 for ERCC1 and XPA, respectively. The catalogue numbers for the single siRNAs used in this study are D-006311-02 and -04 (ERCC1 siRNAs 2 and 4), D-005067-01 and -02 (XPA siRNAs 1 and 2). A non-targeting siRNA pool was used in control experiments and was also purchased from Dharmacon (cat no. D-001206-13).

Transfections were carried out as follows: PC3 and DU145 cells were seeded in six-well plates (1×10^5 cells/well) in complete medium. The following day, the cells were transfected with 100 nM siRNA complexed with 10 μl Oligofectamine (Invitrogen) in 1 ml Opti-mem (Invitrogen). After 4 h the transfection medium was replaced with Opti-mem supplemented with 8% FCS.

2.3. Clonogenic assays

The day following transfection with siRNAs, cells were seeded into 60 mm plates at 600 cells/plate (PC3 cells) and 500 cells/plate (DU145 cells). Triplicate plates were seeded for each drug concentration. The next day, the medium was replaced with complete medium containing a range of concentrations of cisplatin (1 mg/ml injectable aqueous solution from David Bull Laboratories, Australia) or MMC (Sigma) for 1 h. The plates (both drug treated and controls) were washed twice with PBS and then incubated for 11–12 days in drug-free complete medium until colonies contained >50 cells. Colonies were fixed with 95% ethanol, stained with 1% methylene blue and counted. The number colonies in the cisplatin-treated plates compared to the untreated controls was used to calculate the percentage survival. The mean IC50s from at least three independent experiments were determined from exponential plots of survival data, and were used to calculate fold-sensitisation values.

2.4. Western blotting

Protein extracts were prepared by lysing cells in RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10%, glycerol) plus 5% aprotinin (v/v), 1 mM PMSF, on ice for 30 min. Insoluble material was pelleted at 13,000 *g* for 10 min at 4 °C. Protein concentration was assessed using the direct Lowry method (Pierce and Warriner). Ten micrograms of protein was resolved by SDS-PAGE, and gels were electroblotted onto PVDF membranes (Millipore). The antibodies used for western blotting were: rabbit anti-ERCC1 (FL-297; Santa-Cruz), rabbit anti-XPA (FL-273; Santa-Cruz), mouse anti-lamin A/C (JOL2; Serotec). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from Pierce and Warriner. Bands were visualised using the Pico Enhanced Chemiluminescence System (Pierce and Warriner).

2.5. Immunofluorescent detection of RAD51 foci

Untransfected PC3 and DU145 cells, or siRNA-transfected PC3 cells were seeded onto glass coverslips in 24-well plates. Cells were treated with cisplatin (4 μg/ml in serum-containing RPMI) for 1 h, then washed twice and incubated at 36.5 °C in drug-free medium for 24 h. Cells were washed with PBS and fixed in 3% paraformaldehyde, 2% sucrose in PBS for 10 min at room temperature, then permeabilised with 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, 0.5% Triton X-100 for 5 min at 4 °C, followed by 3 × 5 min washes with PBS. Rabbit anti-RAD51 (H-92, Santa-Cruz), diluted 1:100 in PBS + 2% BSA, was added to the cells, which were then incubated for 20 min at 37 °C. The cells were then washed 3×

with PBS and then incubated for 20 min at 37 °C with goat anti-rabbit FITC-conjugated antibody (Southern Biotechnology, Birmingham, AL), diluted 1:100 in PBS + 2% BSA. The washes were repeated and the coverslips mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence was detected using a CCD camera (Zeiss AxioScop). For scoring, at least three random fields were selected and the RAD51 foci were counted in at least 50 cells. Cells containing 10 or more RAD51 foci were scored as positive. For double-immunofluorescence staining of RAD51 and ERCC1, the above protocol was followed, with the inclusion of mouse anti-ERCC1 (D-10, Santa Cruz), diluted 1/100, and rabbit anti-mouse TRITC-conjugated secondary antibody (Southern Biotechnology). Control coverslips were stained singly with each primary antibody plus both secondary antibodies. Cells were also double-stained with rabbit and mouse anti-ERCC1 to confirm specificity of ERCC1 foci staining.

2.6. Co-immunoprecipitation

Protein extracts from untreated and cisplatin-treated PC3 cells were made using co-immunoprecipitation buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 1 mM EDTA, 0.2% (w/v) NP-40, 5% (v/v) aprotinin, 1 mM PMSF, 2% sodium orthovanadate). Protein extracts were treated with DNase 1 (1 μg/ml) in the presence of 1 mM DTT and 5 mM $MgCl_2$ for 2 h at room temperature prior to immunoprecipitation. Sheep anti-rabbit IgG-coated dynabeads (Dynal) were coated with anti-RAD51 antibody (H92, Santa-Cruz). The coated beads were then incubated with 100 μg protein extract at 4 °C for 1 h in 1 ml wash buffer (1% BSA in PBS). Following extensive washing in wash buffer, the beads were boiled in denaturing sample buffer containing 2-mercaptoethanol and the supernatants subject to SDS-PAGE. Immunoblots were probed with mouse anti-ERCC1 (D-10, Santa-Cruz).

2.7. Measurement of cisplatin intrastrand crosslinks by ELISA

Cells were treated with 16 μg/ml cisplatin for 1 h in serum-containing medium, then incubated in drug-free medium for various times before the cells were harvested and stored at −80 °C. For siRNA transfected cells, cisplatin treatment was carried out 24 h post-transfection. Genomic DNA was extracted using the Qiagen DNeasy Tissue kit, with an RNase digestion step, and DNA concentrations were determined fluorimetrically.

Adduct levels were measured using the competitive ELISA method developed by Tilby et al. [20,21]. The experimental procedure was as described in detail in [22], except that undigested DNA samples were assayed. We used 8 μg of each sample DNA to prepare serial two-fold dilutions, such that the highest concentration was 769 ng/assay well. DNA samples from untreated cells were also assayed in parallel, and samples were assayed immediately after DNA isolation. Cisplatin intrastrand adduct levels were then calculated as described in [20]. The platinated coating DNA, platinated standard DNA and ICR4 antibody were all kindly provided by M.J. Tilby, Newcastle University.

3. Results

3.1. SiRNA-mediated downregulation of XPA and ERCC1 in prostate cancer cell lines

PC3 and DU145 cells were transiently transfected with Smartpool siRNAs directed against ERCC1 and XPA. To assess the level of downregulation, serial dilutions of the control cell extract were included in the Western blots, for comparison with the XPA or ERCC1 siRNA transfected cells. This strategy was used because ECL signals are often not linear, making the extent of protein downregulation difficult to quantify. The XPA Smartpool siRNA was able to downregulate XPA protein by at least 90% (Fig. 1A) and the ERCC1 Smartpool siRNA achieved 75% protein downregulation in PC3 cells (Fig. 1B). Similar downregulation was achieved in DU145 cells (data not shown). The downregulation was found to persist for up to 6 days (data not shown). Individual siRNAs from the Smartpools were also transfected (Fig. 1C and D), and two siRNAs from each pool (XPA siRNAs 1 and 2, and ERCC1 siRNAs 2 and 4) were selected for drug sensitisation studies along with the pooled siRNAs.

3.2. Downregulation of ERCC1 sensitises prostate cancer cells to cisplatin

To ascertain whether downregulation of XPA and ERCC1 could sensitise prostate cancer cells to cisplatin, we performed clonogenic survival assays on the human prostate cancer cell lines PC3 and DU145 which had been transiently transfected with ERCC1 and XPA siRNA pools, or the control non-targeting (NT) siRNA pool (Fig. 2A and B). Cisplatin sensitivity of the NT-siRNA transfected cells was similar to that of the untransfected cells, indicating that the sensitisation was due to downregulation of the target proteins and not due to non-specific effects induced by siRNA transfection. To prove that sensitisation was not due to chance off-target downregulation by the siRNA pools, clonogenic assays were performed on cells transfected with two individual siRNAs from the pools (Fig. 2C and D). In all cases the degree of sensitisation was similar between the pooled and single siRNAs (Table 1).

Downregulation of XPA and ERCC1 equally sensitised DU145 cells to cisplatin, causing a ~1.6-fold increase in sensitivity (compared to the NT siRNA transfected cells). In

Table 1 – Summary of cisplatin IC50s (mean values from three independent experiments ± S.E.M.) of prostate cancer cells transfected with non-targeting, XPA and ERCC1 siRNAs (see Fig. 2)

Transfection	Cisplatin IC50 (μg/ml)	
	PC3	DU 145
Control	1.9 ± 0.1	4.9 ± 0.1
NT siRNA pool	1.9 ± 0.2	5.0 ± 0.2
XPA siRNA pool	2.2 ± 0.4	3.2 ± 0.1
XPA siRNA 1	N.D.	2.9 ± 0.2
XPA siRNA 2	N.D.	3.0 ± 0.2
ERCC1 siRNA pool	0.9 ± 0.1	3.2 ± 0.1
ERCC1 siRNA 2	0.8 ± 0.1	N.D.
ERCC1 siRNA 4	0.9 ± 0.1	N.D.

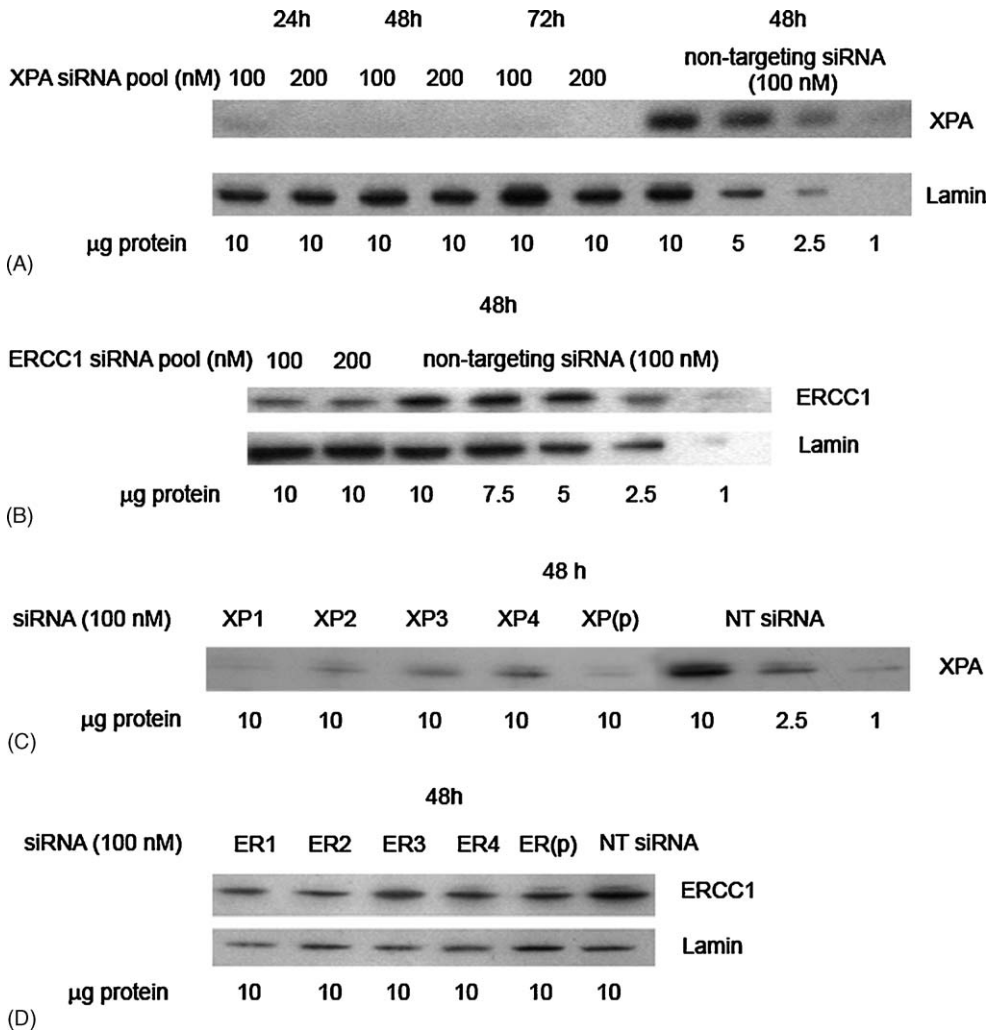

Fig. 1 – SiRNA-mediated downregulation of XPA and ERCC1 protein in prostate cancer cells. PC3 cells were transiently transfected with 100 or 200 nM Smartpool siRNAs directed against XPA (A) and ERCC1 (B). Control cells were transfected with 100 nM non-targeting (NT) siRNA pool. Proteins were harvested at the indicated time points post-transfection, and the degree of downregulation assessed by comparison with serial dilutions of the control cell extracts. Blots were re-probed with anti-lamin A/C as a loading control. Results obtained with single siRNAs are shown in: (C) XPA siRNAs 1–4, plus XPA pool (XP(p)) and (D) ERCC1 siRNAs 1–4 plus ERCC1 pool (ER(p)).

contrast, downregulation of XPA did not sensitise PC3 cells to cisplatin, whereas ERCC1 downregulation caused a ~2.1-fold increase in cisplatin sensitivity.

Double transfections of XPA and ERCC1 siRNAs (100 nM each) in both cell lines were performed to investigate whether the effects would be additive. However, the efficiency of knockdown of both proteins was reduced in these experiments, leading to no increases in sensitisation (data not shown).

3.3. *Downregulation of ERCC1 sensitises prostate cancer cells to MMC*

Because ERCC1 and XPA deficient cells are sensitive to crosslinking agents, we investigated whether siRNA-mediated downregulation of these proteins could sensitise prostate cancer cells to MMC. Results of clonogenic survival assays of prostate cancer cells transfected with pooled and single ERCC1 siRNAs, and the XPA siRNA pool, are shown in Fig. 3 and the calculated IC50 values in Table 2. Downregulation of ERCC1 sensitised both PC3 and DU145 cells to MMC. ERCC1 downregulation increased MMC sensitivity of both cell lines by approximately two-fold (compared to the NT siRNA transfected cells). XPA downregulation did not sensitise PC3 cells to MMC at any dose, whereas there was some sensitisation in DU145 cells (approximately 1.4-fold).

3.4. *PC3 and DU145 cells have similar cisplatin intrastrand adduct repair efficiencies and similar levels of ERCC1 protein*

The differential effect of XPA knockdown on cisplatin sensitivity in PC3 and DU145 cells could be due to PC3 cells already being deficient in NER capacity relative to DU145

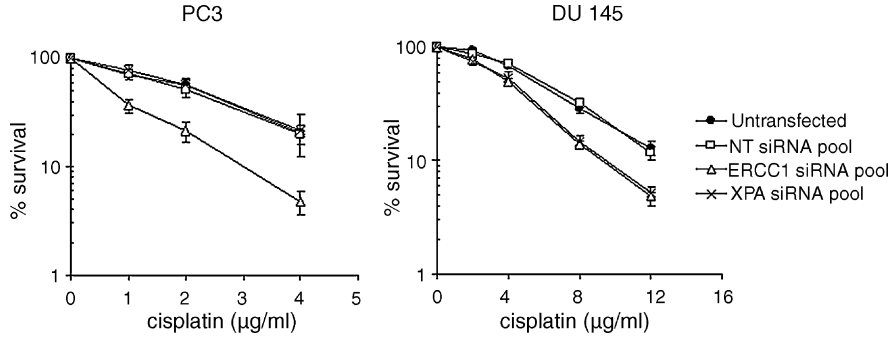

Fig. 2 – Effect of XPA and ERCC1 downregulation on cisplatin sensitivity on prostate cancer cells. Clonogenic survival of PC3 and DU145 cells following transfection with XPA, ERCC1 and non-targeting siRNA pools (100 nM), and treatment with cisplatin (error bars = S.E.M.). The calculated average cisplatin IC50 values (in μg/ml; ±S.E.M.) are summarised in Table 1, along with the results obtained using single ERCC1 and XPA siRNAs (ERCC1 siRNAs 2 and 4; XPA siRNAs 1 and 2). Significant sensitisation ($P < 0.05$) was found with all ERCC1 siRNAs (PC3), and all XPA siRNAs plus the ERCC1 siRNA pool (DU145). Data are the average of three independent experiments ± S.E.M. P values = paired Student's *t*-test with NT-transfected cells as controls.

cells. We therefore used a competitive ELISA method [21] to measure the removal of cisplatin 1,2-intrastrand crosslinks over time (Fig. 4A). PC3 and DU145 cells were exposed to 16 μg/ml cisplatin for 1 h and the adduct levels assayed at 0, 24 and 48 h post-treatment. This concentration of cisplatin was chosen because it gave adduct levels that were easily measured (about 15 fmol/μg), using an amount of DNA that did not give a signal with the non drug-treated DNA samples. Although this cisplatin concentration is higher than the IC50 values obtained from the clonogenic survival experiments, the cells were viable over the time-frame of this assay. The results show that DU145 cells are slightly more efficient than PC3 cells in cisplatin intrastrand adduct removal at 24 h, but by 48 h there is no statistical difference in adduct removal between the two cell lines. Densitometric analysis of western blots showed that DU145 have ~1.5-fold higher levels of ERCC1 protein than PC3 cells, whereas PC3 cells have approximately two-fold higher levels of XPA protein (Fig. 4B).

3.5. *SiRNA-mediated downregulation of XPA and ERCC1 retards the repair of cisplatin intrastrand crosslinks in PC3 cells*

The fact that downregulation of XPA failed to sensitise PC3 cells to cisplatin was surprising, given that there was more siRNA-mediated suppression of XPA than ERCC1. However, since PC3 cells have higher levels of XPA protein expression than DU145 cells (Fig. 4B), and it has been suggested that low levels of XPA are sufficient for NER [23], it is possible that residual levels of XPA protein after siRNA-mediated knock-down could be sufficient for NER in PC3 cells. To see whether the siRNA-mediated XPA downregulation was sufficient to perturb NER of cisplatin adducts in PC3 cells, we measured 1,

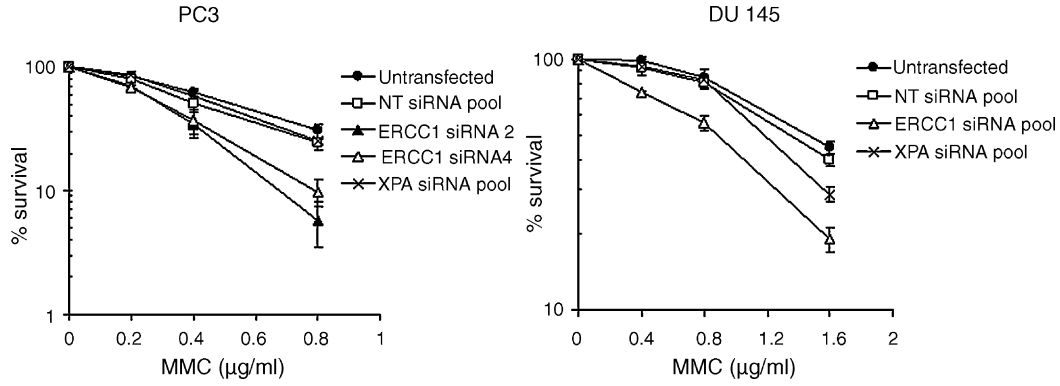

Fig. 3 – ERCC1 downregulation sensitises prostate cancer cells to MMC. Clonogenic survival of PC3 and DU145 cells following transfection with XPA, ERCC1 and non-targeting siRNAs (100 nM), and treatment with MMC (error bars = S.E.M.). ERCC1 siRNAs 2 and 4 but not the XPA siRNA pool sensitised PC3 cells to MMC, with ERCC1 siRNA 2 causing approximately two-fold increase in sensitivity ($P = 0.031$). In DU145 the ERCC1 siRNA pool caused a ~1.8-fold sensitisation ($P = 0.001$). The XPA siRNA pool caused slight sensitisation of DU145 cells (~1.4-fold ($P = 0.009$)). The calculated MMC IC50s of single and pooled siRNA transfected cells are summarised in Table 2. Data are the average of three independent experiments ± S.E.M. P-values = paired Student's *t*-test with NT-transfected cells as controls.

Table 2 – Summary of MMC IC50s (mean values from three independent experiments ± S.E.M.) of prostate cancer cells transfected with non-targeting, XPA and ERCC1 siRNAs (see Fig. 3)

Transfection	MMC IC50 (μg/ml)	
	PC3	DU145
Untransfected	0.53 ± 0.05	1.6 ± 0.1
NT siRNA pool	0.45 ± 0.02	1.4 ± 0.1
XPA siRNA pool	0.43 ± 0.02	1.0 ± 0.1
ERCC1 siRNA 2	0.23 ± 0.01	N.D.
ERCC1 siRNA 4	0.24 ± 0.03	N.D.
ERCC1 siRNA pool	N.D.	0.79 ± 0.05

2-intrastrand adduct removal in NT, XPA and ERCC1 siRNA transfected PC3 cells as described above (Fig. 5). We found that downregulation of both XPA and ERCC1 attenuated the rate of 1,2-intrastrand adduct removal to a similar extent compared to the control NT siRNA-transfected cells. We conclude from this that PC3 cells are able to tolerate attenuation of NER of cisplatin intrastrand adducts. The finding that ERCC1

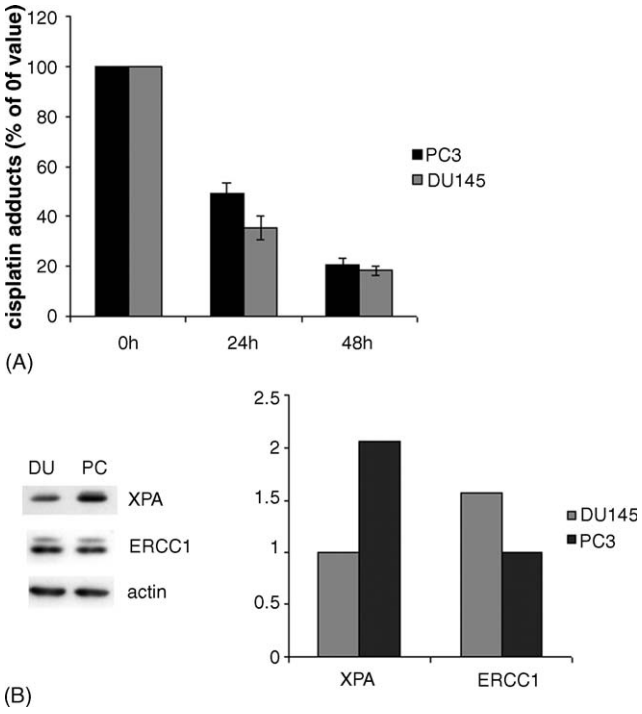

Fig. 4 – Repair of cisplatin intrastrand crosslinks and NER factor levels in PC3 and DU145 cells. (A) PC3 and DU145 cells were treated for 1 h with cisplatin (16 μg/ml). The adduct level in 795 ng DNA extracted at 0, 24 and 48 h post-treatment was determined by ELISA (see Section 2). Equivalent amounts of control DNA (from untreated cells) did not produce a detectable signal in these experiments. The adduct levels are expressed as a percentage of the 0 h value in each case. Data are from three independent experiments ± S.E.M. (B) Western blot of PC3 and DU145 cell extracts probed with XPA, ERCC1 and β-actin antibodies. Levels of XPA and ERCC1 protein normalised to β-actin were determined by densitometry and shown graphically in arbitrary units.

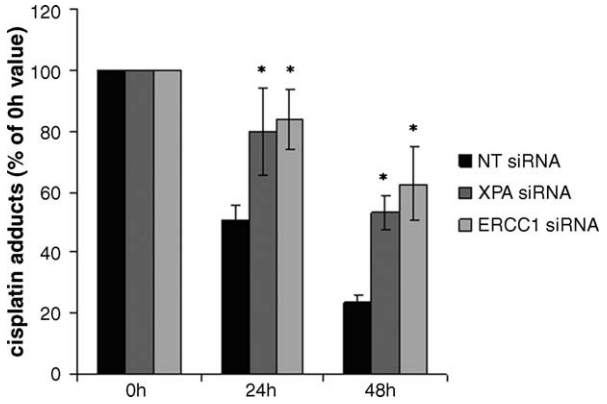

Fig. 5 – Repair of intrastrand cisplatin adducts in PC3 cells is impaired by downregulation of either XPA or ERCC1. PC3 cells were transfected with XPA, ERCC1 or non-targeting (NT) Smartpool siRNAs and treated for 1 h with cisplatin (16 μg/ml). The adduct level in 795 ng DNA extracted at 0, 24 and 48 h post-treatment was determined as described in Fig. 4, and expressed as a percentage of the 0 h value in each case. Data are from four independent experiments ± S.E.M. Asterisks denote values that differed significantly from those of the NT siRNA-transfected cells ($P < 0.05$; paired Student's t-test).

downregulation sensitised PC3 cells to cisplatin may reflect the additional role of ERCC1 in HRR of cisplatin lesions.

3.6. *XPA downregulation leads to an increase in RAD51 foci in response to cisplatin in PC3 cells*

There is evidence to suggest that recombinational bypass of cisplatin lesions is suppressed by MMR proteins [24]. Previous studies have shown that prostate cancers and prostate cancer cell lines are deficient in mismatch repair (MMR) activity [25–27], and defects in MMR are associated with cisplatin resistance in cancer cells (reviewed in [28]). To determine whether recombinational bypass is the mechanism by which PC3 cells are able to tolerate the increased levels of cisplatin adducts in response to XPA downregulation, we immunostained XPA, ERCC1 and NT siRNA transfected cells 24 h post-cisplatin treatment for RAD51. RAD51 is a key protein involved in HRR (reviewed in [29]) that forms foci at sites of double strand breaks and in response to cisplatin treatment, and therefore the cells were scored for RAD51 foci. The 24 h time-point was selected because the percentage of cisplatin-induced RAD51 foci-positive cells is maximal in both DU145 and PC3 cells at this point (Fig. 6A). The rate of clearance of RAD51 foci was also similar between the two cell lines, suggesting that rates of HRR of cisplatin lesions are similar.

Whereas ERCC1 downregulation did not cause a statistically significant difference in the number of RAD51 foci formed in response to cisplatin treatment, XPA downregulation led to a significant increase in the number of cisplatin-induced RAD51 foci compared to the control NT-transfected cells (Fig. 6B). These findings suggest that recombinational bypass of cisplatin adducts may be a mechanism by which PC3 cells are able to tolerate the increased levels of cisplatin adducts

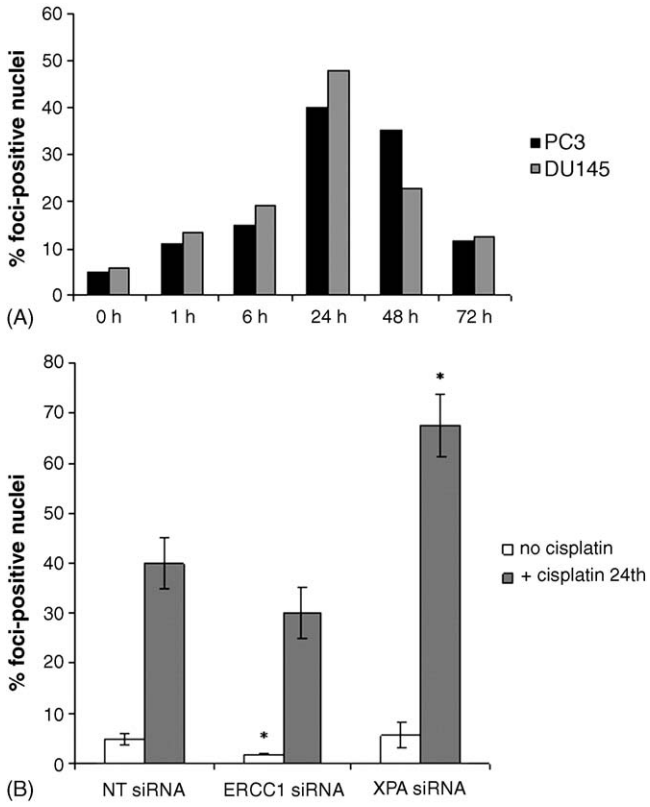

Fig. 6 – XPA downregulation increases cisplatin-induced RAD51 foci. (A) DU145 and PC3 cells were either untreated or treated with 4 μg/ml cisplatin for 1 h, then fixed and stained for RAD51 at various times post-treatment and the percentage of RAD51-foci positive nuclei was scored as described in Section 2, with nuclei containing 10 or more foci counted as positive (data are the mean of two independent experiments). RAD51 foci induction was maximal at 24 h in both cell lines. (B) PC3 cells transfected with XPA, ERCC1 or non-targeting (NT) siRNA Smartpools were either untreated or cisplatin-treated as above then fixed and stained for RAD51 at 24 h post-treatment. The percentage of RAD51 foci-positive nuclei was scored as above. Data are from three independent experiments ± S.E.M. Asterisks indicate a statistically significant difference ($P < 0.05$; paired Student's t-test) from the control cells.

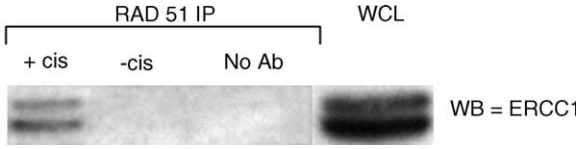

Fig. 8 – Cisplatin-induced co-immunoprecipitation of RAD51 and ERCC1. Untransfected PC3 cells were treated with 4 μg/ml cisplatin for 1 h, or were untreated. Lysates were made 24 h post-treatment and were immunoprecipitated with anti-RAD51. Co-precipitation of ERCC1 was observed with RAD51 in the cisplatin-treated cells (+cis) but not the untreated cells (−cis). A control immunoprecipitation with non-coated beads plus lysate from cisplatin treated cells (−Ab) was also loaded, plus 10 μg whole cell lysate (WCL) from PC3 cells.

caused by downregulation of XPA. The fact that ERCC1 downregulation did not lead to an increase in cisplatin-induced RAD51 foci, even though NER of cisplatin adducts was significantly perturbed, may reflect the additional role of ERCC1 in HRR.

3.7. *Co-localisation of RAD51 and ERCC1 foci in cisplatin-treated PC3 cells*

To investigate whether ERCC1/XPF interacts with HRR complexes, we carried out double immuno-staining of untreated and cisplatin treated PC3 cells for ERCC1 or XPA with RAD51. We found that ERCC1, but not XPA, forms foci in treated and untreated cells. We also found clear localisation of some of the ERCC1 foci with RAD51 foci in cisplatin treated cells (Fig. 7). We did not find any co-localisation in untreated cells. However, we could not rule out such an association since RAD51 foci are much less frequent in the untreated cells. Only a minority of the ERCC1 foci associated with RAD51 foci, which suggests that ERCC1 foci can exist independently, or are part of other repair complexes. Co-immunoprecipitation experiments with protein extracts from PC3 cells also showed ERCC1 to associate with RAD51 (either directly or indirectly) in response to cisplatin treatment (Fig. 8). We estimate from the co-immunoprecipitation experiments that ~0.005% of the total ERCC1 is associated with RAD51 in the cisplatin-treated cells,

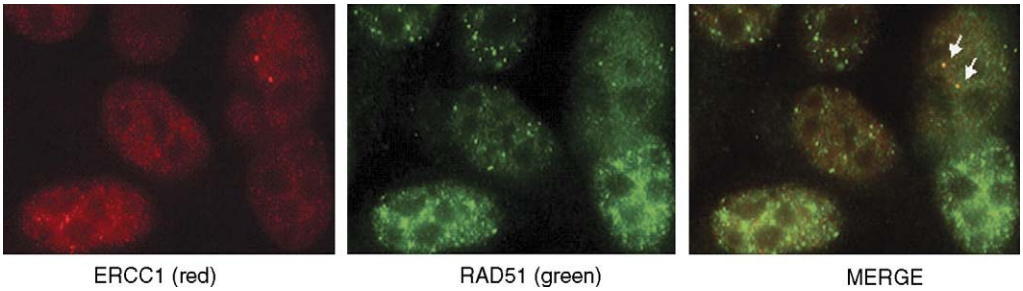

Fig. 7 – Co-localisation of ERCC1 and RAD51 foci. Untransfected PC3 cells were treated with 4 μg/ml cisplatin for 1 h, and then immunostained with rabbit anti-RAD51 and mouse anti-ERCC1 24 h post-treatment. Red and green images were superimposed (merge). Arrows indicate co-localisation of RAD51 and ERCC1 foci. Cells were also immunostained singly for ERCC1 and RAD51 as a control.

which suggests a transient interaction. Others have previously identified ERCC1/XPF foci in mammalian cells, but these workers ruled out a role for these foci in NER [30]. Our data show that ERCC1 is capable of interacting directly or indirectly with RAD51, and this may reflect its role in HRR of cisplatin adducts.

4. Discussion

In this study we have explored the use of XPA and ERCC1 as potential therapeutic targets to sensitise prostate cancer cells to the chemotherapeutic drugs MMC and cisplatin. We found that ERCC1 downregulation sensitised both PC3 and DU145 cells to MMC, by approximately two-fold. XPA downregulation did not sensitise PC3 cells to MMC, but did slightly sensitise DU145 cells. MMC is a bifunctional DNA alkylating agent that results in ICLs and double strand breaks (DSBs). MMC ICLs are recognised and removed by NER machinery [31–33]. However, helical distortion caused by MMC ICLs is minimal [34], and the majority of MMC ICLs are thought to be repaired by HRR (reviewed in [35]). It has recently been shown that DSBs form in S phase as an intermediate of MMC ICL repair (even in ERCC1−/− cells), and that ERCC1/XPF function in HRR of these DSBs [36]. ERCC1 and XPF mutant CHO cell lines are hypersensitive to MMC compared to other XP mutant cells [7]. Our data indicate that ERCC1 is a better target than XPA with which to sensitise cancer cells to MMC, and demonstrate the potential of ERCC1 as a therapeutic target in the treatment of prostate cancer in combination with DNA crosslinking agents.

Cisplatin reacts with the N7 atom of purine bases and forms several types of adduct, including 1,2- and 1,3-intrastrand crosslinks and ICLs [37]. Intrastrand crosslinks are repaired by NER, although 1,2-intrastrand crosslinks are more poorly recognised by the NER machinery [38], and therefore may be the critical cytotoxic lesion. Disrupting the function of NER proteins such as XPA [39,40] and ERCC1 [41] has been shown to sensitise some cancer cell lines to cisplatin. However, the efficacy of XPA and ERCC1 as targets has not been compared previously. We have shown that siRNA-mediated downregulation of ERCC1 caused an increase in cisplatin sensitivity in both PC3 and DU145 cell lines. The degree of sensitisation was greatest in PC3 cells, with an approximately two-fold decrease in IC50. In DU145 cells, downregulation of XPA or ERCC1 resulted in equal sensitisation. This observation was despite the fact that the extent of XPA downregulation is greater than that of ERCC1. In PC3 cells, however, XPA downregulation did not increase cisplatin sensitivity. The fact that both XPA and ERCC1 downregulation inhibited cisplatin intrastrand crosslink repair suggests that failure of XPA downregulation to sensitise PC3 cells to cisplatin, was not simply due to a lower threshold requirement for XPA to carry out NER.

Microsatellite instability, a phenotype associated with defects in mismatch repair (MMR), is detectable in some prostate cancers (reviewed in [42]). Defects in mismatch repair have been associated with resistance of cancer cells to cisplatin [43]. Both PC3 and DU145 cell lines have deficiencies in MMR proteins [25,26]. PC3 cells had a higher frequency of genomic instability than DU145 cells in a microsatellite mutation assay [26], which may partially account for the differences observed in cisplatin sensitisation in response to XPA downregulation. Additional factors such as the expression of anti-apoptotic proteins or the efficiency of translesion DNA synthesis [44–46] may also play a role in determining the degree of sensitisation achieved by XPA downregulation in PC3 and DU145 cells. Unrepaired cisplatin intrastrand adducts are recognised by the mismatch repair heterodimer hMSH2/hMSH6. Binding of the mismatch repair complex is thought to bring about cell death either by the initiation of futile cycles of MMR or by direct signalling to the apoptotic machinery [28,47]. Mismatch repair proteins are also thought to decrease cisplatin adduct tolerance by inhibiting recombination-dependent lesion bypass in yeast and mammalian cells [24]. The observation that downregulation of XPA in the MMR-deficient PC3 cell line led to an increase in RAD51 foci formation in response to cisplatin treatment is consistent with this model. This also suggests HRR of un-repaired cisplatin adducts at stalled replication forks to be a mechanism of cellular resistance to cisplatin in a NER-deficient, MMR-deficient background. The ERCC1/XPF structure-specific nuclease has a role in the repair of cisplatin adducts that is additional to its function in NER and ability to uncouple cisplatin ICLs, and that role may be in the recombinational processing of cisplatin adducts [22]. Previous studies have shown HRR to play a major role in the repair of cisplatin DNA damage in mammalian cells [48,49]. Because of its probable role in HRR of cisplatin adducts, ERCC1 is an attractive therapeutic target, especially in MMR-deficient cancers, where increased recombinational bypass of cisplatin lesions enhances resistance to this drug. We have demonstrated that siRNA-mediated downregulation of ERCC1 significantly sensitises the MMR-deficient PC3 line to cisplatin, where inhibition of the NER pathway had no effect. Thus, ERCC1 has potential as a therapeutic target in the treatment of MMR-deficient prostate cancers in combination with cisplatin.

The precise role of ERCC1/XPF in the recombinational repair of cisplatin adducts remains to be elucidated. Interestingly, DSBs do not appear to be intermediates in the repair of cisplatin ICLs, in contrast with other DNA crosslinking drugs such as nitrogen mustard and MMC. It is possible that homology searching is initiated prior to recruitment of ERCC1/XPF in the recombinational repair of cisplatin lesions at stalled replication forks [22]. Co-localisation of ERCC1 foci and RAD51 foci in response to cisplatin treatment has not previously been observed, and may represent recruitment of ERCC1/XPF to sites of recombinational repair. Further studies are necessary to determine whether this interaction is direct and whether recruitment is dependent on protein-protein interactions. ERCC1/XPF has been shown to interact with the HR protein RAD52 [50]. However, we observed no co-localisation of RAD52 with ERCC1 or RAD51 foci and, moreover, RAD52 does not appear to be required for HRR of damaged DNA in vertebrate cells [51]. Because of the requirement for ERCC1/XPF in HRR of cisplatin lesions, and the importance of this pathway in determining cisplatin sensitivity, targeted therapies that either reduce expression of these proteins or disrupt their interaction with HRR complexes have the potential to sensitise cancer cells to this drug.

Acknowledgements

We are very grateful to Dr. Mike Tilby, Newcastle University for his advice in setting up the cisplatin intrastrand crosslink assay, and for providing the platinated standards and ICR4 antibody. This work was supported by the Orchid Cancer Appeal.

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