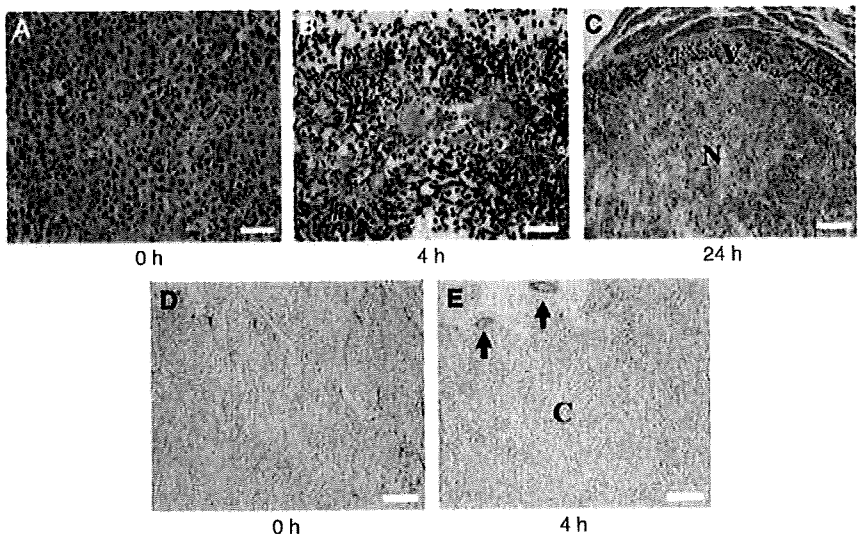

Figure 7 Histological analysis of H460 tumours after treatment with TZT-1027. Mice bearing H460 tumour xenografts were treated with a single dose of TZT-1027 (2.0 mg kg^{-1}), and the tumours were excised at various times thereafter and either stained with hematoxylin-eosin (**A**–**C**) or immunostained for CD31 (**D** and **E**). (**A** and **D**) Control sections of an untreated tumour showing normal capillaries with an intact endothelium and viable tumour cells. (**B** and **E**) Sections of a tumour removed 4 h after administration of TZT-1027. Vascular congestion, with pink deposits of fibrin, and loss of endothelial cells as well as diffuse tumour cell degeneration are apparent in (**b**). Dark immunostaining of intact endothelium (arrows) is apparent in surrounding normal connective tissue, whereas little staining of endothelial cells was observed in the core (**C**) of the tumour (**E**). (**C**) Section of a tumour removed 24 h after TZT-1027 administration, showing extensive central necrosis (N) and a rim of viable cells (V). Scale bars: 50 μm (**A** and **B**), 100 μm (**C**), and 200 μm (**D** and **E**).

TZT-1027 before irradiation induced a marked increase in the proportion of apoptotic cells compared with that apparent with radiation alone. These results thus suggested that potentiation of apoptosis contributed to radiosensitisation by TZT-1027.

Combined treatment with radiation and a single administration of TZT-1027 also inhibited the growth of tumours formed by H460 or A549 cells *in vivo* to a greater extent than did either treatment alone. Tumour microenvironmental factors, such as the vascular supply, are important determinants of sensitivity to radiation therapy *in vivo*. The ability of microtubule-targeting agents to induce a rapid shutdown of the existing tumour vasculature has been recognised by their designation as vascular-targeting agents (VTAs) (Jordan and Wilson, 2004). Treatment with VTAs such as ZD6126 and combretastatin A-4-P typically results in the destruction of large areas of a tumour, with surviving cells remaining only at the tumour periphery (Dark *et al*, 1997; Blakey *et al*, 2002). These peripheral viable tumour cells presumably derive their nutritional support from nearby normal blood vessels that are not responsive to VTA treatment (Li *et al*, 1998; Siemann and Rojiani, 2002). Such support together with a rapid upregulation of angiogenic factors such as vascular endothelial growth factor may directly facilitate the growth and expansion of the remaining tumour cells (Wachsberger *et al*, 2003; Thorpe, 2004). Given that these residual tumour cells are likely well oxygenated (Wachsberger *et al*, 2003), they are an ideal target for radiation therapy. Several studies have recently shown that treatment with VTAs enhances the therapeutic effect of radiotherapy (Li *et al*, 1998; Siemann and Rojiani, 2002, 2005; Horsman and Murata, 2003; Masunaga *et al*, 2004), consistent with the idea that the components of such combination therapy act in a complementary manner, with VTAs attacking the poorly oxygenated cell population in the central region of tumours and radiation killing the well-oxygenated proliferating cells at the tumour periphery (Li *et al*, 1998; Siemann and Rojiani, 2002; Wachsberger *et al*, 2003). TZT-1027 was previously shown to increase vascular permeability and to induce a decrease in tumour blood flow followed by a marked increase in tissue necrosis in the central region of tumour xenografts (Otani *et al*, 2000; Watanabe *et al*, 2006b). We have now shown that TZT-1027 treatment resulted in congestion and occlusion of tumour blood vessels followed by extensive necrosis of the tumour core, with only a thin rim of viable tumour cells remaining, in the H460 tumour model, suggesting that TZT-1027 acts as a VTA. The action of TZT-1027 as a VTA might thus contribute to the radiosensitising effect observed *in vivo* in the present study.

The clinical use of microtubule-interfering agents such as taxanes in combination with radiation has been successful in improving local tumour control. However, taxanes are often of limited efficacy because of the development of cellular resistance such as that mediated by P-glycoprotein-dependent drug efflux (Goodin *et al*, 2004). The action of TZT-1027 has been suggested to be less affected by multidrug resistance factors, including overexpression of P-glycoprotein, than that of other tubulin inhibitors (Watanabe *et al*, 2006a), suggesting that TZT-1027 may be effective in the treatment of taxane-refractory tumours. Further investigations are thus warranted to examine the combined effects of TZT-1027 and ionising radiation on drug-resistant tumour cells. Whether TZT-1027 enhances the tumour response to clinically relevant fractionated doses of radiation such as 2 Gy per fraction also warrants further study.

In conclusion, we have found that the inhibitory effect of TZT-1027 on cell cycle progression is highly specific to M phase. Moreover, TZT-1027 enhanced the effects of radiation on human cancer cells both *in vitro* and in animal models *in vivo*. These preclinical results provide a rationale for future clinical investigations of the therapeutic efficacy of TZT-1027 in combination with radiotherapy.

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ORIGINAL ARTICLE

ZNF143 interacts with p73 and is involved in cisplatin resistance through the transcriptional regulation of DNA repair genes

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Zinc-finger protein 143 (ZNF143) is a human homolog of *Xenopus* transcriptional activator staf that is involved in selenocystyl tRNA transcription. We previously showed that ZNF143 expression is induced by treatment with DNA-damaging agents and that it preferentially binds to cisplatin-modified DNA. In this study, the potential function of ZNF143 was investigated. ZNF143 was overexpressed in cisplatin-resistant cells. ZNF143 knockdown in prostate cancer caused increased sensitivity for cisplatin, but not for oxaliplatin, etoposide and vincristine. We also showed that ZNF143 is associated with tumor suppressor gene product p73 but not with p53. p73 could stimulate the binding of ZNF143 to both ZNF143 binding site and cisplatin-modified DNA, and modulate the function of ZNF143. We provide a direct evidence that both Rad51 and flap endonuclease-1 are target genes of ZNF143 and overexpressed in cisplatin-resistant cells. Taken together, these experiments demonstrate that an interplay of ZNF143, p73 and ZNF143 target genes is involved in DNA repair gene expression and cisplatin resistance.
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Keywords: ZNF143; p73; Rad51; FEN-1; cisplatin; DNA repair

Introduction

Cisplatin is an important chemotherapy drug used in the treatment of many solid tumors (Zamble and Lippard, 1995; Cohen and Lippard, 2001). Its major limitation is the development of resistance (Torigoe *et al.*, 2005). The mechanisms of cisplatin resistance are not completely understood. Cisplatin resistance is influenced by many factors, which affect intracellular drug accumulation (Fujii *et al.*, 1994), levels of cellular thiols (Tew, 1994) and DNA repair activity (Chaney and Sancar, 1996). Drug-induced responses are mediated by transcription factors and include DNA damage signals that lead to the induction of apoptosis in tumor cells by cisplatin (Torigoe *et al.*, 2005; Kohno *et al.*, 2005). Loss of p53 function confers resistance in cancer cell lines (Keshelava *et al.*, 2001). Further, p73 overexpression is associated with cisplatin resistance (Vikhanskaya *et al.*, 2001). Thus, molecular links between transcription factors and drug resistance promises to provide the foundation for novel molecular targeted chemotherapy (Kohno *et al.*, 2005). We previously reported that transcription factor, Y-box binding protein 1 (YB-1), binds preferentially to cisplatin-modified DNA (Ise *et al.*, 1999) and YB-1 expression is upregulated in cisplatin resistance cells (Ohga *et al.*, 1996; Kohno *et al.*, 2003; Kuwano *et al.*, 2004).

We identified the cisplatin-inducible genes such as *activating transcription factor 4* (*ATF4*) (Tanabe *et al.*, 2003) and *Mitochondrial ribosomal protein S11* (*MRP S11*) (Ishiguchi *et al.*, 2004) using differential display (Murakami *et al.*, 2001). ATF4 is upregulated in cisplatin-resistant cells and its expression correlates with cisplatin resistance in lung cancer (Tanabe *et al.*, 2003). Analysis of the *MRP S11* promoter region gene revealed that the zinc-finger transcription factor zinc-finger protein 143 (ZNF143) is involved in the cisplatin induction. ZNF143 is a human homolog of *Xenopus* Staf (Myslinski *et al.*, 1998), and is involved in the transcriptional regulation of small nuclear RNA (snRNA) and snRNA-type genes by RNA polymerase II or III (Schaub *et al.*, 1997; Rincon *et al.*, 1998). It is induced by DNA-damaging agents and binds preferentially to cisplatin-modified DNA (Ishiguchi *et al.*, 2004). In this study, we show that ZNF143 is upregulated in cisplatin-resistant cells. p73 interacts with ZNF143 and promotes the binding of ZNF143 to both ZNF143 binding site and cisplatin-modified DNA. And we also show that ZNF143 plays an important role in the control of DNA repair gene expression.

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Results

ZNF143 is upregulated in cisplatin-resistant cell lines

ZNF143 gene expression was shown to be increased in cisplatin-resistant cells in comparison with the parental

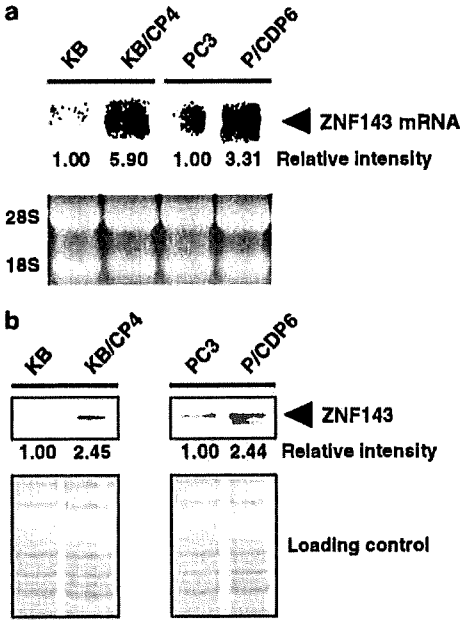


Figure 1 Expression of ZNF143 in cancer cell lines. (a) Northern blotting analysis of ZNF143 mRNA. Total RNA (20 μg/lane) of KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6 were loaded. ZNF143 mRNA (2.6 kb) was detected by Northern blotting analysis. Relative intensity was shown. Gel staining is shown (lower panel). (b) Expression of ZNF143 protein in KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6. Fifty micrograms of sonicated nuclear fractions were subjected by SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 antibody. Relative intensity was also shown. Gel staining with Coomassie Brilliant Blue (CBB) was also shown (lower panel).

cells (Figure 1a). Western blotting analysis revealed that ZNF143 protein was overexpressed in cisplatin-resistant cells when sonicated nuclear fractions were loaded (Figure 1b). However, inverse result was obtained when eluted nuclear extracts using salt buffer were loaded (data not shown), suggesting that ZNF143 may be tightly bound to chromatin in cisplatin-resistant cells.

The effects of ZNF143 expression on cisplatin sensitivity
To determine whether ZNF143 protein plays a role in cisplatin sensitivity, we inhibited its expression using small interfering RNA (siRNA) knockdown. Western blotting analysis showed that three kinds of ZNF143 siRNAs specifically downregulated ZNF143 expression in comparison with control siRNA treatment (Figure 2a). The effect of ZNF143 inhibition on clonogenic survival was also assessed (Figure 2b). Downregulation of ZNF143 expression by three kinds of siRNAs rendered cell sensitive to cisplatin (Figure 2b, left upper panel). Specific inhibition of ZNF143 had significantly sensitized PC3 cells to cisplatin, but not to oxaliplatin, etoposide and vincristine (Figure 2b). Further, downregulation of ZNF143 could partially reverse the cisplatin resistance of P/CDP6 cells (Figure 2c and d).

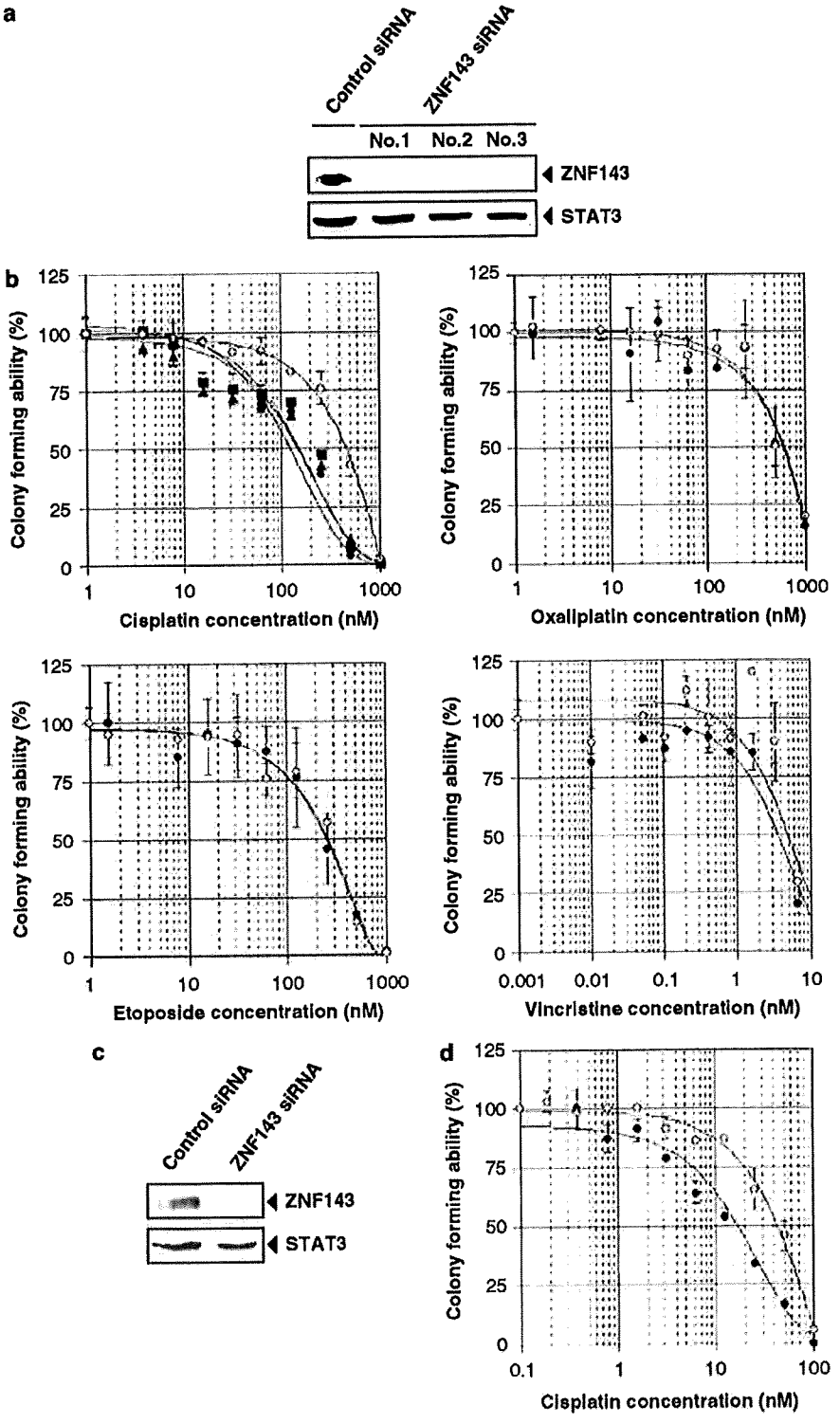
Association of p73 with ZNF143
As damage to DNA increases the nuclear accumulation of tumor suppressor gene products, we next investigated the interaction of ZNF143 with p53 and p73. We employed transient transfection using both 3 × Flag-ZNF143 and hemagglutinin (HA)-p53 or HA-p73-expressing plasmids. The complexes immunoprecipitated with anti-Flag antibody contained HA-p73 (Figure 3a), but not HA-p53 (Figure 3c). And we also verified that the complex contained 3 × Flag-ZNF143 when HA-p73 was reciprocally immunoprecipitated using HA antibody (Figure 3b).

p73 stimulates the DNA binding of ZNF143
We previously reported that ZNF143 preferentially binds to cisplatin-modified DNA (Ishiguchi *et al.*, 2004). To examine the effect of p73 on ZNF143 binding to oligonucleotide containing ZNF143 binding site of human *U6 RNA* promoter and cisplatin-modified DNA, we performed electrophoretic mobility shift assay (EMSA). Both glutathione-*S*-transferase (GST) and GST-p73 could not bind to both ZNF143 binding site (Figure 4a, left panel) and cisplatin-modified DNA (Figure 4b, left panel). The DNA binding of ZNF143 was significantly enhanced by GST-p73 in a dose-dependent manner, but not by control GST. However, p73 did not alter the electrophoretic mobility of the ZNF143 complex formed with DNA.

Potential ZNF143 target genes for DNA repair pathways
More than 150 genes for DNA repair pathways were identified and listed (Wood *et al.*, 2005). A 19 bp consensus sequences for staf binding site was reported (Schaub *et al.*, 1997; Rincon *et al.*, 1998) and was used in a computer search of the human genome database. Initially, we surveyed and selected the putative staf binding sites, which show more than 70% homology in the promoter region containing 1000 bp upstream from the transcriptional start site. Among about 150 DNA repair genes, the putative binding sites were found in the promoter region of 78 genes. As C residues at position 4–6 and 13 are almost invariably conserved more than 95%, this criteria was considered to select the potential ZNF143 binding sites. Finally, we found that the 83 staf binding sites of 62 genes contained these conserved C residues (Supplementary Data). This suggests that ZNF143 functions as the pivotal factor to control gene expressions for DNA repair pathways.

DNA repair-associated gene expression regulated by ZNF143
We found that *Rad51* and *flap endonuclease-1* (*FEN-1*) had putative ZNF143 binding site in the core promoter region as shown in Figure 6a and Supplementary Data. We carried out Western blotting analysis. As shown in Figure 5a, both cellular Rad51 and FEN-1 proteins were upregulated in cisplatin-resistant cells. Reciprocally, cellular Rad51 and FEN-1 proteins were decreased when PC3 cells were treated with ZNF143 siRNA (Figure 5b). To determine whether ZNF143 directly

involves in these gene expressions, we performed EMSA and chromatin immunoprecipitation (ChIP) assay. As shown in Figure 6b, GST–ZNF143 could recognize the putative ZNF143 binding site located in both gene promoters. These signals disappeared after the addition of unlabeled oligonucleotides in the reaction mixture (data not shown). Before the ChIP assay, we established the stable transfectants that expressed 3 × Flag-tagged ZNF143, because no adequate anti-ZNF143 antibody was available for the immunoprecipitation. We



confirmed the ectopic expression of 3 × Flag-ZNF143 by Western blotting (Figure 6c). Stable transfectant expressed slightly larger ZNF143 than endogenous protein in the molecular weight due to the additional tag peptides. The ChIP assay showed that substantial enrichment of the region spanning the ZNF143 binding site in the promoter regions of *Rad51* and *FEN-1* was observed when cells expressing 3 × Flag-ZNF143 were used (Figure 6d, lane 5), but not when cells transfected with vector alone were used (Figure 6d, lane 2). No

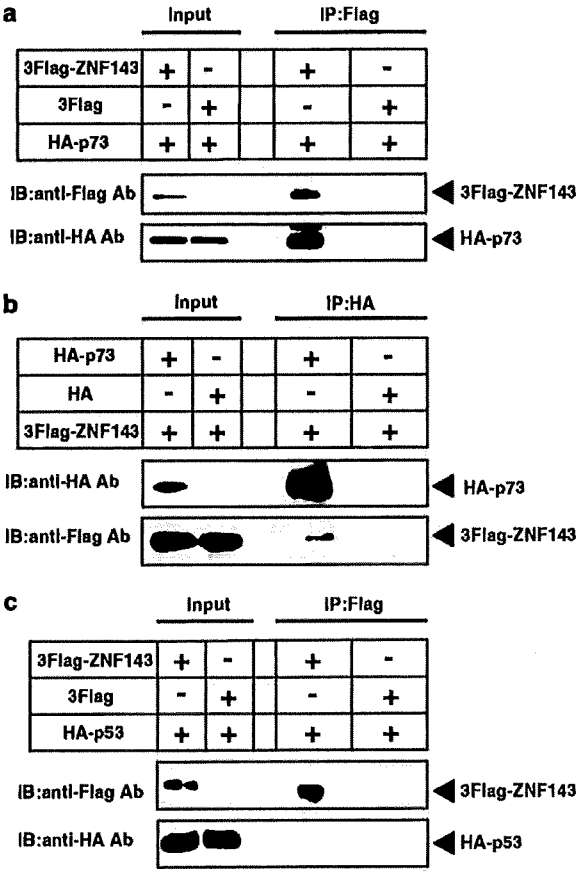


Figure 3 Interaction of p73 with ZNF143. (a) Whole-cell lysates (300 μg) prepared from PC3 cells co-transfected with HA and 3 × Flag expression plasmids were immunoprecipitated with anti-Flag (M2) antibody. The resulting immunocomplexes and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with either anti-Flag or anti-HA antibodies. (b) A reciprocal immunoprecipitation assay and Western blotting were performed. (c) p53 expression plasmid was transfected instead of p73 expression plasmid, and immunoprecipitation assay and Western blotting were performed.

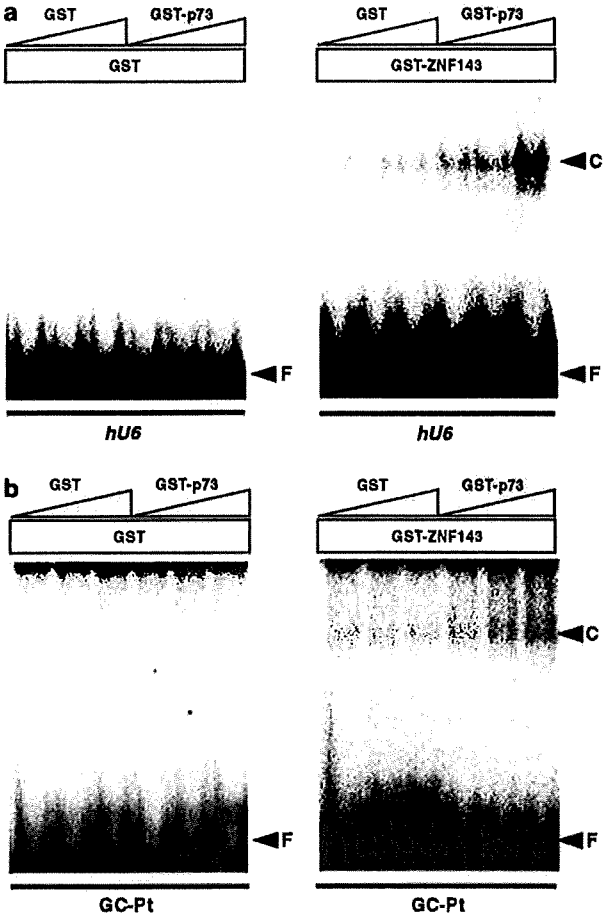


Figure 4 Stimulation of ZNF143 binding to DNA by p73. (a) Enhancement of ZNF143 binding to its binding site of *U6 RNA* promoter by p73. Purified GST or GST-p73 (50, 250 and 500 ng) were mixed with GST (100 ng) or purified GST-ZNF143 (100 ng), and incubated with ^{32}P-labeled *U6 RNA* oligonucleotides containing ZNF143 binding site. The reaction mixtures were resolved by electrophoresis on a 4% polyacrylamide gel with 0.5 × TBE buffer. The gel was dried and analysed by a bio-imaging analyzer (FLA2000). (b) Enhancement of ZNF143 binding to cisplatin-modified DNA by p73. Purified GST-p73 and GST-ZNF143 were incubated with ^{32}P-labeled cisplatin-modified DNA (GC-Pt), and EMSA was performed by the same method as described in (a).

Figure 2 Downregulation by ZNF143 siRNA transfection and drug sensitivity. (a) Downregulation of ZNF143 expression by three kinds of ZNF143 siRNAs (No. 1, No. 2 and No. 3). Control siRNA (50 pmol) or ZNF143 siRNA were transfected into PC3 cells and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 and anti-STAT3 antibodies. (b) Treatment of ZNF143-siRNA sensitized cisplatin. PC3 cells were treated with 50 pmol ZNF143-siRNAs (No. 1; closed circles, No. 2; closed triangle and No. 3; closed square) or 50 pmol control-siRNA (open circles) for 24 h, and exposed to various concentrations of cisplatin, oxaliplatin, etoposide and vincristine for 7 days. The colony number in the absence of drug corresponded to 100%. All values were the mean of least three independent experiments with ± s.d. (c) Downregulation of ZNF143 expression in cisplatin-resistant cells by ZNF143 siRNA. Control siRNA (100 pmol) or ZNF143 siRNA (No. 1) were transfected into P/CDP6 cells and whole-cell lysates (50 μg) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 and anti-STAT3 antibodies. (d) Treatment of ZNF143-siRNA partially reversed cisplatin resistance. P/CDP6 cells were treated with 100 pmol ZNF143 siRNA No. 1 (closed circles) or control siRNA (open circles) for 24 h, and exposed to various concentrations of cisplatin for 7 days. The colony number in the absence of drug corresponded to 100%. All values were the mean of least three independent experiments with ± s.d.

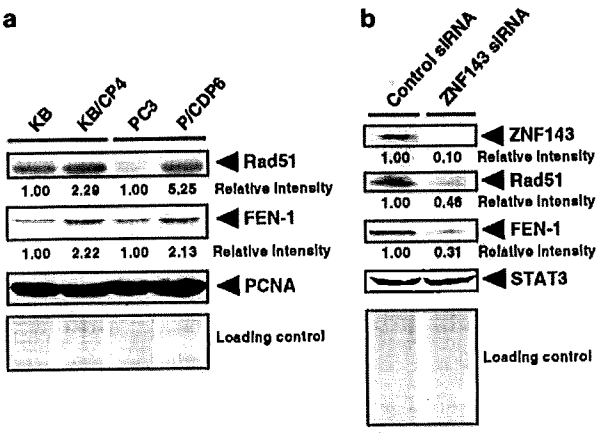


Figure 5 Expression of Rad51 and FEN-1 in cancer cell lines. (a) Expression of Rad51 and FEN-1 protein in KB and PC3 cells and their cisplatin-resistant KB/CP4 and P/CDP6. Nuclear extracts (50 μg) were subjected by SDS–PAGE. Transferred membrane was blotted with anti-Rad51, anti-FEN-1 or anti-PCNA antibodies. Relative intensity was shown at the bottom of each panel. Gel staining with CBB was also shown (lower panel). (b) Down-regulation of ZNF143 expression reduces cellular level of Rad51 and FEN-1. PC3 cells were treated with 50 pmol ZNF143-siRNA or control-siRNA for 72 h, and whole-cell lysates (100 μg) were subjected by SDS–PAGE, and Western blotting with anti-ZNF143, anti-Rad51, anti-FEN-1 and anti-STAT3 antibodies was performed. Relative intensity was shown at the bottom of each panel. Gel staining was also shown.

promoter enrichment was observed when unrelated *peroxiredoxin 4* (*PRDX4*) gene promoter was assayed.

We next performed ChIP assay and reporter assay with transient transfection to gain greater insight into the transcriptional regulation of two DNA repair genes. PC3 cells were co-transfected with the reporter plasmid driven by the promoter of *Rad51* or *FEN-1* genes with p73 expression plasmid. These promoter regions contain ZNF143 binding site. p73 activated both promoter activities (Figure 7a).

Next, we determined whether p73 expression induced by cisplatin treatment enhances the ZNF143 binding to the promoter of these DNA repair genes. We have previously shown that ZNF143 expression was also induced by cisplatin treatment as well as p73 (Ishiguchi *et al.*, 2004; Uramoto *et al.*, 2002). Thus, we employed the stable transfectant for ChIP assay to avoid the effect of the enhanced expression of endogenous ZNF143 by cisplatin treatment on the binding to the promoters. We assessed the effect of cisplatin treatment on p73 expression in PC3 cells. p73 expression by cisplatin treatment was substantially increased relative to untreated control cells (data not shown).

ChIP assay demonstrated that the promoter sequence of both DNA repair genes was concentrated in the immunocomplexes prepared after cisplatin treatment (Figure 7b, lanes 5 and 6). Collectively, these results show that the expression of two DNA repair genes is mediated, at least in part, by ZNF143 binding stimulated by p73 expression after cisplatin treatment.

Discussion

We have previously reported that ZNF143 is induced by cisplatin treatment and that it binds preferentially to cisplatin-modified DNA (Ishiguchi *et al.*, 2004), suggesting that it plays an important role in cisplatin resistance. In the present study, we found that ZNF143 interacts with p73 and is directly involved in cisplatin sensitivity through the regulation of DNA repair gene expression.

ZNF143 is overexpressed at both mRNA and protein levels in cisplatin-resistant cells (Figure 1). Interestingly, an increase in ZNF143 protein was observed when the total nuclear fraction of cisplatin-resistant cells was analysed (Figure 1b), but not when nuclear protein eluted with salt buffer was loaded (data not shown). This indicates that ZNF143 binds tightly to cisplatin-modified chromatin and could not be eluted easily under low salt condition.

Functional analysis of ZNF143 provides considerable insight into the epigenetics of cisplatin-resistance and might be of use in revealing targets for overcoming drug resistance. ZNF143 depletion using siRNA confers cell sensitivity to cisplatin, but not to oxaliplatin, etoposide and vincristine (Figure 2b). Further, downregulation of ZNF143 could partially reverse the cisplatin resistance of P/CDP6 cells (Figure 2c and d). It is noteworthy that ZNF143 does not affect cellular sensitivity to oxaliplatin, which is a third-generation platinum drug that has shown a lack of cross-resistance with cisplatin (Raymond *et al.*, 2002). We previously demonstrated that etoposide can induce ZNF143 expression (Ishiguchi *et al.*, 2004), but the current results suggest that upregulation of ZNF143 by etoposide treatment is not directly involved in etoposide sensitivity. ZNF143 might, however, be involved specifically in DNA repair following DNA damage by cisplatin.

Co-immunoprecipitation assay showed that the tumor suppressor gene product p73 interacts with ZNF143. We previously reported that p53 interacts with high mobility group box 1 (HMGB1) and stimulates the binding of HMGB1 to cisplatin-modified DNA (Imamura *et al.*, 2001). We therefore investigated whether p73 plays a similar role and found, using EMSA, that p73 enhances the cisplatin-modified DNA binding of ZNF143 (Figure 4b). We could not detect a p73 supershift, suggesting that although p73 stimulates ZNF143 binding to cisplatin-modified DNA, it cannot interact stably during electrophoresis. p73 overexpression is associated with resistance to DNA-damaging agents (Vikhanskaya *et al.*, 2001), so both ZNF143 and p73 might be cooperatively involved in cisplatin resistance. p73 also enhances the ZNF143 binding to its binding site located in the promoter region of human *U6 RNA* gene. As both p73 and ZNF143 expression are induced by DNA damage signal, p73 might function cooperatively to activate the ZNF143 target gene expression. Little is known about potential ZNF143 target genes for DNA repair pathways. Among several DNA repair pathways, it has been extensively studied that excision repair cross-complementation group 1 (ERCC1) has the critical role in nucleotide excision repair pathway and high ERCC1

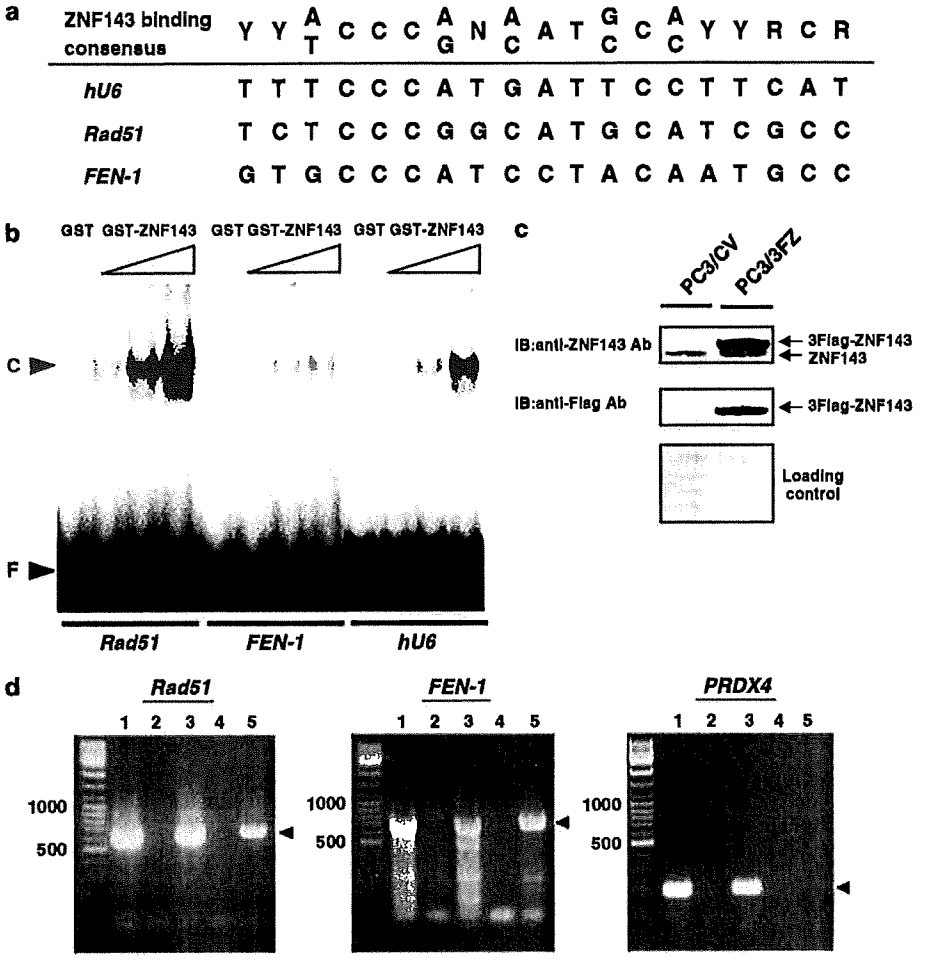


Figure 6 ZNF143 binds to ZNF143 binding sites of DNA repair genes promoter *in vitro* and *in vivo*. (a) Schematic representation of ZNF143 binding sites. ZNF143 binding sites in human *U6 RNA* promoter (*hU6*), human *Rad51* promoter and human *FEN-1* promoter were compared with ZNF143 binding consensus motif (Schaub *et al*., 1997). (b) GST-ZNF143 binding to ZNF143 binding site of both *Rad51* and *FEN-1* gene promoters *in vitro*. Purified GST (500 ng) or GST-ZNF143 (50, 250 and 500 ng) were incubated with ^{32}P-labeled oligonucleotides containing ZNF143 binding sites. The reaction mixtures were resolved by electrophoresis and analysed by a bio-imaging analyzer. (c) Cloning of stable transfectants. Whole-cell lysates (50 μg) of stable transfectant PC3/control vector (PC3/CV) and PC3/3 × Flag-ZNF143 (PC3/3FZ) were subjected to SDS–PAGE. Transferred membrane was blotted with anti-ZNF143 (upper panel) and anti-Flag (middle panel) antibodies. Gel staining with CBB was also shown (lower panel). (d) ZNF143 binding to the promoter *in vivo*. ChIP assay of the PC3/control vector (lanes 1 and 2) and PC3/3 × Flag-ZNF143 (lanes 3–5) was performed with antibodies against Flag (M2) or mouse IgG. Immunoprecipitated DNAs (anti-Flag (M2) in lanes 2 and 5, and anti-mouse IgG in lane 4) and pre-immunoprecipitated DNA (lanes 1 and 3) were amplified by PCR using specific primer pairs for the *Rad51*, *FEN-1* and *PRDX4* promoter regions. Amplification products (682 bp for *Rad51*, 844 bp for *FEN-1* and 157 bp for *PRDX4*) were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The arrowhead indicates amplified PCR fragment containing the promoter region of gene.

expression is associated with cisplatin resistance (Altaha *et al*., 2004). Both BRCA1 and Rad51 have been shown to be involved in recombinational repair and also associated with cisplatin resistance (Bhattacharyya *et al*., 2000; Spiro and McMurray, 2003). The FEN-1 is a 5′ endonuclease and has been implicated in various DNA repair processes (Lieber, 1997). Based on these reports, we searched putative ZNF143 binding site in the promoter region of these DNA repair genes and found that putative ZNF143 binding sites are located in the core promoter region of these genes. We confirmed the role of ZNF143 in the regulation of both *Rad51* and *FEN-1* gene expression by three independent approaches: siRNA strategy, EMSA and ChIP assay as shown in Figures 5b, 6b and d, respectively. In addition, p73 transactivated the promoter activities of two DNA repair genes (Figure 7a) and cisplatin treatment resulted in the enhanced binding of ZNF143 to these promoters (Figure 7b). Thus, p73 interacts with ZNF143 and modulates its function, and therefore has the potential to broadly regulate the DNA repair gene expression.

We also carried out a search of the sequence database to identify the distribution of the ZNF143 binding sites of all DNA repair genes. Surprisingly, we found that a

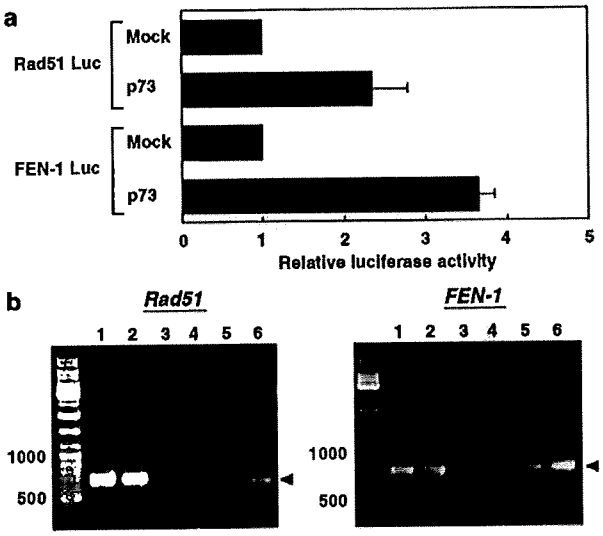


Figure 7 p73 and cisplatin treatment activate the expression of DNA repair genes. (a) Induction of the promoter activity by p73. Rad51 Luc or FEN-1 Luc was transiently co-transfected with p73 expression plasmid and CH110 plasmid (Amersham Biosciences, Piscataway, NJ, USA) expressing β-galactosidase as an internal control. The results are normalized to β-galactosidase activity and are representative of at least three independent experiments. Bars, ±s.d. (b) Enhancement of DNA binding activity of ZNF143 by cisplatin treatment. Soluble chromatin was prepared from Flag-ZNF143 stable transfectant untreated (lanes 1, 3 and 5) or treated (lanes 2, 4 and 6) with cisplatin, and immunoprecipitated with anti-mouse IgG (lanes 3 and 4) or anti-Flag (M2) antibodies (lanes 5 and 6). Extracted DNAs of immunoprecipitation (lanes 3–6) and soluble chromatin (lanes 1 and 2) were amplified using specific primer pairs for the *Rad51* and *FEN-1* promoter regions. Amplification products were subjected by electrophoresis as described in Figure 6d.

number of DNA repair genes contain potential binding site for ZNF143 in their regulatory regions (Supplementary Data). These results suggest that ZNF143 is a positive regulator like a master gene for the expression of DNA repair genes.

In conclusion, our data indicate that ZNF143 is a pivotal transcription factor that regulates the gene expression for DNA repair pathways together with p73 and is involved in cisplatin resistance. Our findings also raise the possibility that inhibition of ZNF143 function might be a target for therapeutic augmentation of cisplatin-based chemotherapy. Further investigation to define the molecular function of ZNF143 will greatly advance our understanding of cisplatin resistance.

Materials and methods

Cell culture

Human epidermoid cancer KB cells and human prostate cancer PC3 cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum. The cisplatin-resistant KB/CP4 and P/CDP6 cells were derived from KB and PC3 as described previously (Tanabe *et al.*, 2003). Seven lung cancer cell lines were obtained as described previously (Sugaya *et al.*, 2002). Cell lines were maintained in a 5% CO_2 atmosphere at 37°C.

Antibodies and drugs

Anti-Flag (M2) monoclonal antibody and anti-Flag (M2) affinity gel were purchased from Sigma (St Louis, MO, USA). Anti-STAT3 (sc-482), anti-proliferating cell nuclear antigen (PCNA) (sc-56) and HA-probe (F-7) AC (agarose conjugate) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Rad51, anti-FEN-1 antibodies and anti-HA-peroxidase (3F10) were purchased from Calbiochem (Darmstadt, Germany), BD Biosciences (BD Biosciences Clontech, Palo Alto, CA, USA) and Roche molecular Biochemicals (Mannheim, Germany), respectively. The anti-ZNF143 antibody was kindly gifted by Dr GR Kunkel (Texas A&M University, TX, USA) (Ishiguchi *et al.*, 2004). Cisplatin, vincristine and etoposide were purchased from Sigma. Oxaliplatin was kindly provided from Yakult Honsha Co., Ltd., Tokyo, Japan.

Plasmid construction

Plasmid construction of pGEX-p53, pGEX-p73 and pGEX-ZNF143 that express GST-p53, GST-p73 and GST-ZNF143 proteins in bacteria, respectively, and pcDNA3-HA-p53 that expresses HA-p53 protein in mammalian cells were described previously (Imamura *et al.*, 2001; Uramoto *et al.*, 2002, 2003). pcDNA3-HA-p73 expression plasmid in mammalian cells was kindly provided by Dr G Melino (University of Rome, Rome, Italy) (De Laurenzi *et al.*, 1998). For construction of pcDNA3–3 × Flag expression plasmid, the following double-stranded oligonucleotides were inserted to pcDNA3 expression plasmid (Invitrogen, San Diego, CA, USA) between *Bam*HI and *Eco*RI sites. Three times Flag oligonucleotides; 5′-ATGGACTACAAAGACCATGACGGTGATTATAAAGAT CATGACATCGATTACAAGGATGACGATGACAAGAAT TGG-3′. To obtain the pcDNA3–3 × Flag ZNF143, full length of ZNF143 cDNA was ligated at C-terminal of 3 × Flag in pcDNA3–3 × Flag mammalian expression plasmid. For construction of pIRES/hygro-3 × Flag ZNF143, *Bam*HI–*Not*I fragment containing 3 × Flag ZNF143 was obtained by digesting the pcDNA3–3 × Flag ZNF143 plasmid with *Bam*HI and *Not*I, and ligated in same sites of pIRES/hygro mammalian expression plasmid (BD Biosciences Clontech). To obtain Rad51 Luc and FEN-1 Luc, *Rad51* and *FEN-1* promoters were amplified by polymerase chain reaction (PCR) with the following primer pairs with restriction enzyme cleavage sites at the 5′ end; 5′-AGATCTGCGATGGTGAGAACTCGCGGA CC-3′ and 5′-AAGCTTCACCCCGCGGGCGTGGCACG-3′ for the *Rad51* promoter (−471 to +211); 5′-AGATCTGTA-CAGAGGCTGTGGGCGCTCC-3′ and 5′-AAGCTTGGTT CGGGGTTGCCCCGGGC-3′ for the *FEN-1* promoter (−525 to +319). These PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). The promoter fragments were gel-purified after *Bgl*II–*Hind*III digestion and ligated into the *Bgl*II–*Hind*III site of pGL3-basic vector (Promega).

Cloning of stable transfectants

Two micrograms of pIRES/hygro vector or pIRES/hygro-3 × Flag ZNF143 was transfected into 1×10^5 cells of PC3 using 10 μl Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After 24 h of transfection, the cells were trypsinized and plated on 100-mm dishes with dilution to form colonies and cultured with maintenance medium containing 250 μg/ml hygromycin. The resulting colonies were isolated using cloning cylinders and transferred to 24-well plates. Cellular expression level of

3 × Flag ZNF143 in each clones was investigated by following Western blotting with anti-Flag antibody. Stable transfectant with expression of 3 × Flag ZNF143 was named PC3/3 × Flag ZNF143 (PC3/3FZ) and was used in this study. PC3/control vector (PC3/CV) was also selected by the transfection with pIRES/hygro vector alone.

Northern blotting
Northern blotting analysis was performed as described previously (Murakami *et al.*, 2001; Ishiguchi *et al.*, 2004). Briefly, total RNA was isolated using the Sepasol reagent (Nacalai Tesque, Kyoto, Japan). RNA samples (20 μg/lane) were separated on a 1% formaldehyde agarose gel and transferred to a hybond N^+ membrane (GE Healthcare Bio-Science, Piscataway, NJ, USA) with 10 × SSC. ZNF143 cDNA fragments were labeled with random primers using the Megaprime DNA labeling kit (GE Healthcare Bio-Science). After prehybridization and hybridization, signal intensities were quantified using a bio-imaging analyzer (FLA2000, Fuji Photo Film, Tokyo, Japan).

Western blotting
Whole-cell lysates and eluted nuclear extracts were prepared as described previously (Uramoto *et al.*, 2002). To prepare whole-nuclear protein, isolated nuclei was directly sonicated for 10 s and designated as nuclear fractions. The indicated amounts of whole-cell lysates, nuclear extracts or nuclear fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred to a polyvinylidine difluoride microporous membranes (Millipore, Bedford, MA, USA) using a semi-dry blotter. The blotted membrane was treated with 5% (w/v) skimmed milk in 10 mM Tris, 150 mM NaCl, 0.2% (v/v) Tween 20 and incubated for 2 h at 4°C with a 1:5000 dilution of anti-ZNF143, a 1:10 000 dilution of anti-Flag (M2), a 1:2000 dilution of anti-PCNA, a 1:1000 dilution of anti-STAT3, a 1:500 dilution of anti-FEN-1 and a 1:1000 dilution of anti-Rad51 antibodies. The membrane was then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody or a 1:5000 dilution of anti-HA-peroxidase. It was treated with an ECL kit (GE Healthcare Bio-Science) and exposed to Kodak X-OMAT film by autoradiography. The intensity in each signal was assessed numerically by NIH image program (NIH, Bethesda. MD, USA).

Transient transfections and co-immunoprecipitation assay
Transient transfection and immunoprecipitation assay were performed as described previously (Uramoto *et al.*, 2002; Izumi *et al.*, 2003). Briefly, 1 × 10^5 PC3 cells were seeded into six-well tissue-culture plates. The following day, both 1 μg HA and 3 × Flag expression plasmids were transfected using Superfect reagent (Qiagen) according to the manufacturer's instructions. Three hours post-transfection, the cells were washed with phosphate-buffered saline, cultured at 37°C for 48 h in fresh medium and then lysed in buffer X containing 50 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 1 μM $ZnCl_2$. After incubating for 30 min on ice, the lysates were centrifuged at 21 000 *g* for 10 min at 4°C. The supernatant (300 μg) was incubated for 2 h at 4°C with anti-Flag M2 affinity gel or HA-probe (F-7) AC, and the beads were washed three times with buffer X. Immunoprecipitated samples and pre-immunoprecipitated samples (50 μg) were separated by SDS–PAGE, and Western blotting analysis was performed with anti-Flag antibody and anti-HA-peroxidase as described above.

Knockdown analysis using siRNAs
Three kinds of double-stranded ZNF143 RNA 25 bp oligonucleotides were generated from Stealth Select RNAi (Invitrogen) No. 1. 5′-UAACCAUAGCAACAGAGUGCGUUCC-3′ and 5′-GGAACGCACUCUGUUGCUAUGGUUA-3′; No. 2. 5′-UAAUUUGUUGCACUGGCAAAUGCCC-3′ and 5′-GGGCAUUUGCCAGUGCAACAAAUUA-3′; and No. 3. 5′-AUAAGCUGUGGUACCAUCUUCCAGC-3′ and 5′-GC UGGAAGAUGGUACCACAGCUUAU-3′. siRNA transfections were performed according to the manufacturer's instructions (Invitrogen). Briefly, 1 μl lipofectamine transfection reagent (Invitrogen) was diluted in 250 μl Opti-minimum essential medium (MEM) I medium (Invitrogen) and incubated for 5 min at room temperature. Next, 50 or 100 pmol ZNF143 siRNA and Stealth RNAi negative control with medium GC (Invitrogen) diluted in 250 μl Opti-MEM I were added gently and incubated for 20 min at room temperature. Oligomer–Lipofectamine complexes and aliquots of 2 × 10^5 PC3 or P/CDP6 cells in 2 ml culture medium were combined and incubated for 10 min at room temperature. Aliquots of 4 × 10^2 PC3 cells or 6 × 10^2 P/CDP6 cells were used for a colony-formation assay as described below. The remaining cells were seeded in 35 mm dishes with 2 ml culture medium and harvested after 72 h culture for Western blotting analysis as described above.

Cytotoxicity assay
For the colony-formation assay, 4 × 10^2 PC3 or 6 × 10^2 P/CDP6 cells transfected with siRNAs were seeded in 35 mm dishes with 2 ml culture medium. The following day, the cells were treated with indicated concentrations of cisplatin, oxaliplatin, etoposide and vincristine. After 7 days, the number of colonies was counted.

Purification of GST fusion protein
Induction and purification of GST fusion proteins were described previously (Ise *et al.*, 1999). Briefly, *Escherichia coli* cells transformed with GST expression plasmids were induced by isopropyl-1-thio-γ-D-galactopyranoside for 1 h and sonicated for 10 s in buffer X as described above. Soluble fractions were obtained by centrifugation at 21 000 *g* for 10 min at 4°C. GST fusion proteins were bound to 10 μl glutathione-sepharose 4B in a 50% slurry in buffer X for 4 h at 4°C, washed three times with buffer X and eluted with 50 mM Tris-HCl (pH 8.0) and 20 mM reduced glutathione according to the manufacturer's protocol (GE Healthcare Bio-Science).

Electrophoretic mobility shift assay
The sequences of the oligonucleotides used for the probes in EMSAs were as follows: human *U6 RNA* oligo, 5′-GCC TATTTCCCATGATTCCTTCATATTTGC-3′ and 5′-GGGC AAATATGAAGGAATCATGGGAAATAGG-3′; human *Rad51* oligo, 5′-GGTACATCTCCCGGCATGCATCGCCG GCG-3′ and 5′-GGCGCCGGCGATGCATGCCGGGAGAT GTA-3′; human *FEN-1* oligo, 5′-GGACCCGTGCCCATCC TACAATGCCCTGG-3′ and 5′-GGCCAGGGCATTGTAG GATGGGCACGGGT-3′; GC oligo for modification of cisplatin, 5′-GGCCGGGGCGGGGCGATCGGGGCGGGGGC-3′ and 5′-GGGCCCCCGCCCCGATCGCCCCGCCCCGG. The ZNF143 binding sites in these oligonucleotide probes were underlined (see Figure 6a). Preparation of the ^{32}P-labeled oligonucleotide probes were described previously (Imamura *et al.*, 2001; Ishiguchi *et al.*, 2004). Briefly, the oligonucleotides were annealed with complementary strands. The double-stranded products were end-labeled with [α-^{32}P] deoxycytidimine triphosphate (GE Healthcare Bio-Science) using the

Klenow fragment (Fermentas, Vilnius, Lithuania) and purified from gel. For preparation of cispaltin-modified DNA, labeled GC oligonucleotide probe was treated with 0.3 mM cisplatin at 37°C for 6 h and purified by ethanol precipitation. EMSAs with purified GST fusion proteins were performed as described previously (Imamura *et al.*, 2001; Ishiguchi *et al.*, 2004). Briefly, GST fusion proteins were incubated for 5 min at room temperature in a final volume of 20 μl containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 10% glycerol, 0.05% Nonidet P-40 and 4 ng ^{32}P-oligonucleotide probe. The reaction mixtures were resolved by electrophoresis on a 4% polyacrylamide gel (polyacrylamide/bisacrylamide, 80:1) by 10 V/cm for 90–120 min at room temperature with 0.5 × tris-borate-EDTA (TBE) buffer (45 mM Tris base, 45 mM boric acid and 1 mM EDTA). The gel was dried and analysed by a bio-imaging analyzer (FLA2000).

ChIP assay

The ChIP assay was performed as described previously (Uramoto *et al.*, 2002). Both PC3/CV and PC3/3FZ stable transfectants were treated with or without 20 μM cisplatin for 12 h. Briefly, protein–DNA crosslinking was performed by incubating PC3/control vector and PC3/3 × Flag-ZNF143 cells with formaldehyde. The cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 1 mM phenylmethylsulfonyl fluoride) and the lysates were sonicated. Soluble chromatin from 1 × 10^6 cells was incubated with 5 μg/ml anti-Flag (M2) affinity gel or anti-mouse immunoglobulin G (IgG) with protein A/G agarose (Santa Cruz) by rotation for 2 h at 4°C. Immune complexes were collected by centrifugation. They were then treated with 0.2 M NaCl to reverse protein–DNA crosslinking, and were digested with proteinase K and RNase A. The purified DNA was dissolved with 20 μl dH_2O. The DNA (1 μl) was then used for PCR analysis with the following primer pairs for the *Rad51* promoter region (−471 to +211): 5′-AG ATCTGCGATGGTGAGAACTCGCGGACC-3′ forward primer and 5′-AAGCTTCACCCCGCGGGCGTGGCACG-3′ reverse primer; for the *FEN-1* promoter region (−525 to +319): 5′-AGATCTGTACAGAGGCTGTGGGCGCTCC-3′ forward primer and 5′-AAGCTTGGTTCGGGGTTGCCCCGGGC-3′ reverse primer; the *PRDX4* promoter region (−121 to +36): 5′-AGATCTGCCCACGTGGCGGGGCGGGGAGC-3′ forward primer and 5′-CTCGAGCGCAGAAACACGTCCCTT GGCG-3′ reverse primer. The PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide.

Transient transfection and luciferase assay

Transient transfection and a luciferase assay were performed as described previously (Uramoto *et al.*, 2002). Briefly, 5 × 10^4 PC3 cells were seeded into 12-well tissue-culture plates. The following day, 0.2 μg of *Rad51* or *FEN-1* reporter plasmid was transfected with 1.2 μg of p73 expression plasmid using 3 μl/well Superfect reagent (Qiagen) according to the manufacturer's instructions. Three hours post-transfection, the cells were washed and cultured at 37°C for 48 h in fresh medium. Luciferase activity using cell lysated with lysis buffer and brief centrifugation was detected by a Picagene kit (Toyoinki, Tokyo, Japan), and the light intensity was measured with a luminometer (Luminescencer JNII RAB-2300; ATTO, Japan) according to the manusfacturer's instructions.

ZNF143 binding site analysis

The search for ZNF143 binding site was performed using the DataBase of Transcriptional Start Sites software version 5.2. 0 (http://dbtss.hgc.jp) developed by Dr Sumio Sugano and Dr Yutaka Suzuki and the Searching Transcription Factor Binding Sites software version 1.3 (http://mbs.cbrc.jp/research/db/TFSEARCH.html) developed by Dr Yutaka Akiyama. Briefly, each 1000 bp upstream region from transcriptional Start Site was obtained by the DataBase of Transcriptional start sites software with GeneID number of Entrez Gene database in National Center for Biotechnology Information. Then, staf binding site (19 bp consensus sequence), which is the same as ZNF143 binding site, was searched by the Searching Transcription Factor Binding Sites software with 70 threshold score. More than 150 genes associated with DNA repair were searched, and 83 ZNF143 binding sites in the 62 genes were found. These results were listed in a Supplementary Data.

Acknowledgements

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ORIGINAL REPORT

Dofequidar Fumarate (MS-209) in Combination With Cyclophosphamide, Doxorubicin, and Fluorouracil for Patients With Advanced or Recurrent Breast Cancer

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ABSTRACT

Purpose
To evaluate the efficacy and tolerability of dofequidar plus cyclophosphamide, doxorubicin, and fluorouracil (CAF) therapy in comparison with CAF alone, in patients with advanced or recurrent breast cancer. Dofequidar is a novel, orally active quinoline derivative that reverses multidrug resistance.

Patients and Methods
In this randomized, double-blind, placebo-controlled trial, patients were treated with six cycles of CAF therapy: 28 days/cycle, with doxorubicin (25 mg/m^2) and fluorouracil (500 mg/m^2) administered on days 1 and 8 and cyclophosphamide (100 mg orally [PO]) administered on day 1 through 14. Patients received dofequidar (900 mg PO) 30 minutes before each dose of doxorubicin. Primary end point was overall response rate (ORR; partial or complete response). In total, 221 patients were assessable.

Results
ORR was 42.6% for CAF compared with 53.1% for dofequidar + CAF, a 24.6% relative improvement and 10.5% absolute increase (P = .077). There was a trend for prolonged progression-free survival (PFS; median 241 days for CAF *v* 366 days for dofequidar + CAF; P = .145). In retrospectively defined subgroups, significant improvement in PFS in favor of dofequidar was observed in patients who were premenopausal, had no prior therapy, and were stage IV at diagnosis with an intact primary tumor. Except for neutropenia and leukopenia, there was no statistically significant excess of grade 3/4 adverse events compared with CAF. Treatment with dofequidar did not affect the plasma concentration of doxorubicin.

Conclusion
Dofequidar + CAF was well tolerated and is suggested to have efficacy in patients who had not received prior therapy.

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INTRODUCTION

Despite the advances in chemotherapeutic intervention, many cancers are either inherently resistant or develop resistance to chemotherapy.[1,2] Consequently, multidrug resistance (MDR) remains a major obstacle to the successful treatment of cancer.[1,3,4] One mechanism by which MDR operates is via the increased cellular efflux of cytotoxic compounds due to increased expression of membrane transport proteins such as P-glycoprotein (P-gp) and MDR-associated protein (MRP).[1,4,5] MDR affects many structurally and functionally unrelated agents including cytotoxic drugs that are hydrophobic, natural products, such as taxanes, vinca alkaloids, anthracyclines, epipodophyllotoxins, topotecan, dactinomycin, and mitomycin.[1,6,7] These represent some of the most commonly used chemotherapeutic agents.

In tumors with low levels of P-gp expression at baseline or diagnosis, P-gp expression increases after exposure to chemotherapy agents, thus leading to the development of MDR. In breast cancer patients who had received prior chemotherapy, P-gp expression has been shown to increase from 11% in untreated patients to 30% after chemotherapy.[8] Furthermore, compared with P-gp–negative tumors, a significant increase in resistance to paclitaxel and doxorubicin was reported in P-gp positive breast cancer tissue, irrespective of prior therapy.

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The degree of P-gp expression also strongly correlated with the degree of drug resistance observed.[8]

Chemotherapy remains the treatment of choice for women with hormone receptor–negative and hormone-refractory breast cancer disease.[9-11] However, many tumors that are initially responsive to chemotherapy frequently relapse and develop resistance to the broad spectrum of cytotoxic drugs currently employed.[8,12,13] Consequently, MDR remains a major reason for treatment failure in patients with metastatic breast cancer and highlights the urgent need for MDR modifiers in breast cancer chemotherapy.

Since the discovery of verapamil as an MDR-reversing agent,[14] many compounds have been investigated as MDR inhibitors.[14-16] Dofequidar fumarate (Fig 1), is a novel, orally active, quinoline-derived inhibitor of MDR.[17] In preclinical studies, dofequidar reversed MDR in P-gp– and MRP-1–expressing cancer cells in vitro (1 to 3 μmol/L), as well as enhancing the antitumor effects of doxorubicin in MDR tumor–bearing mice.[17-19] A phase I trial in healthy volunteers showed dofequidar to be well tolerated (10 to 1,200 mg) with no dose-limiting toxicities and an effective plasma concentration was maintained for 8 hours at 900 mg (data on file, Schering AG, Berlin, Germany). In a phase II combination trial in patients with recurrent breast cancer, dofequidar potentiated the antitumor effects of CAF (cyclophosphamide, doxorubicin, and fluorouracil) therapy; patients who had not responded to treatment with three cycles of CAF responded to subsequent treatment with dofequidar plus CAF. The numbers of patients with an objective response were two of seven at 600 mg and two of six at 900 mg dofequidar, though dose escalation was stopped at 1,200 mg due to increased hematologic toxicity (data on file, Schering AG). On the basis of this result, this phase III study was conducted to compare the efficacy and safety of dofequidar plus CAF with placebo plus CAF in patients with advanced or recurrent breast cancer.

PATIENTS AND METHODS

Study Design

This was a randomized, multicenter, double-blind, placebo-controlled trial conducted at 46 centers across Japan, comparing the efficacy and safety of dofequidar plus CAF with placebo plus CAF. Female patients (age 20 to 70 years) with advanced (stage IV at diagnosis with an intact primary tumor) or recurrent breast cancer were enrolled onto the study. Other inclusion criteria included a histologically defined, measurable or assessable primary lesion; two or fewer regimens of prior chemotherapy in both neo/adjuvant and metastatic settings, (excluding prior endocrine or single-agent fluorouracil therapy); 180 mg/m^2 anthracyclines (doxorubicin equivalent) or less previously; a performance status of 0 to 2; and adequate bone marrow, renal, hepatic and cardiac functions. Patients who progressed or had a recurrence in less than 6 months with anthracycline-containing chemotherapy, and those who had a history of major cardiac disease, uncontrolled hypertension, symptomatic brain metastasis, or simultaneous malignancy were excluded. The trial was approved by the institutional review board and was conducted in accordance with the Declaration of Helsinki (1996). All patients provided written informed consent before study entry.

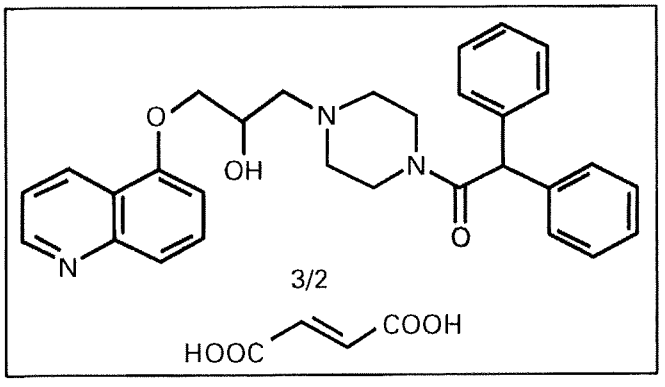

Fig 1. Structure of dofequidar (MS-209)

Dosing and Dose Modification for Toxicity

Patients were treated with six cycles of CAF therapy with dofequidar or placebo, and each treatment cycle lasted for 28 days; drugs were administered as follows: days 1 and 8, doxorubicin (25 mg/m^2) and fluorouracil (500 mg/m^2), each infused over 15 minutes; days 1 through 14, cyclophosphamide (100 mg orally [PO]); dofequidar (900 mg/d; 3 × 300 mg tablets) or placebo administered 30 minutes before each doxorubicin dose to ensure adequate blood concentration of dofequidar. The doses of doxorubicin and fluorouracil were reduced to 20 mg/m^2 and 400 mg/m^2, respectively, if any of the following criteria were met: grade 3 nonhematologic toxicity (except nausea and vomiting); grade 3 or worse neutropenia (< 1,000/mm^3) maintained for at least 5 days with an episode of fever of 38.5°C or higher; grade 3 or worse thrombocytopenia (< 50,000/mm^3); and grade 4 neutropenia (< 500/mm^3). The next cycle was postponed for 3 weeks unless the patient had a WBC count of at least 4,000/mm^3, or a neutrophil count of at least 2,000/mm^3 and a platelet count of at least 100,000/mm^3. Patients were followed up for 3 months after completion or discontinuation of treatment.

Treatment Assignment

Patients were randomly assigned to their treatment by the Trial Register Center. Treatment assignment was securely stored and coded until completion of the study. Investigators were also blinded to the assigned treatment. Patients were stratified by the number of prior chemotherapy regimens, including adjuvant chemotherapy, by a history of prior use of anthracyclines, and by the presence of liver metastases.

Efficacy

The primary study end point was the overall response rate (ORR) in the full analysis set (FAS; all patients who received treatment at least once and met all inclusion/exclusion criteria). Efficacy assessment by lesion and ORR assessment were made at each treatment cycle (every 4 weeks) and at treatment completion. Objective responses were assessed through blinded reading of radiographs by an independent expert panel. The secondary study end points included complete response rate (CR), time to treatment failure (TTF), time to progression (TTP), and progression-free survival (PFS).

Subgroup analyses were conducted to assess PFS within specific patient subpopulations, including premenopausal women, patients who had no prior therapy, and patients who had advanced primary breast cancer.

Safety and Tolerability

Adverse events (AEs) were recorded at the end of each treatment cycle and at the end of the study period using data from the safety population (all patients who received treatment at least once in the study). AEs were categorized according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) Version 2. The incidence of significant decreases in left ventricular ejection fraction (LVEF) and serious AEs were recorded. The CBC was evaluated weekly. Serum chemistries and urinalysis were evaluated every 2 weeks. The minimum hematology values and LVEF in each treatment cycle were also recorded and analyzed in the per-protocol set (PPS; all patients who received treatment at least once and had no protocol deviations).

Pharmacokinetics

To assess the effect of concomitant dofequidar use on the pharmacokinetics of doxorubicin, the plasma doxorubicin concentration on day 1 of cycle 1 was compared between treatment groups. Blood samples were taken at baseline and at 15 minutes, 30 minutes, and 1, 2, 4, and 6 hours after the start of doxorubicin administration. Plasma doxorubicin concentrations were determined by reversed-phase high-performance liquid chromatography. Area

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under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule.

Statistical Analyses

The primary end point was analyzed using the Fisher's exact test at a significance level of 2.5% in a one-sided test. A difference in response rates of 20% between the two treatment groups was used as the basis for a statistically significant difference. CR, TTF, TTP and PFS were analyzed by the log-rank test at a significance level of 5% in a two-sided test. The CR, TTF, TTP and PFS were analyzed in the FAS, and the pharmacokinetic data analyzed in the PPS.

RESULTS

Patient Characteristics

A total of 227 patients were recruited onto the study (Fig A1, online only), of which 225 patients were included in the safety analysis (n = 113 for the dofequidar group; n = 112 for the placebo group); two patients did not receive the study treatment and were thus excluded. Four patients did not meet the inclusion/exclusion criteria; therefore, the FAS consisted of 221 patients (n = 113 for the dofequidar group; n = 108 for the placebo group). The PPS consisted of 199 patients (n = 100 for the dofequidar group; n = 99 for the placebo group). There were 22 patients excluded from the PPS analysis due to protocol deviations. Baseline patient characteristics were well balanced between the two treatment arms (Table 1). Most patients had predominantly recurrent disease and had received prior chemotherapy plus endocrine therapy. Also, many patients who had advanced primary breast cancer had received no prior therapy.

Table 1. Patient Demographics (full analysis set)

Characteristic	Dofequidar + CAF (n = 113)		Placebo + CAF (n = 108)	
	No.	%	No.	%
Age, years				
Mean	54.4		52.4	
SD	7.69		8.97	
Medical history known	65	57.5	60	55.6
Weight, kg				
Mean	56.2		54.1	
SD	7.52		7.73	
Height, cm				
Mean	154.7		154.7	
SD	5.71		5.61	
Body surface area, m^2				
Mean	1.5		1.5	
SD	0.11		0.11	
Disease state				
Recurrent	81	71.7	80	74.1
Advanced	32	28.3	28	25.9
Prior therapy				
Radiotherapy + chemotherapy + endocrine therapy	32	22.1	32	29.6
Chemotherapy + endocrine therapy	55	48.7	54	50.0
Radiotherapy	1	0.9	1	0.9
No prior therapy	25	22.1	21	19.4
Menopausal status				
Premenopausal	24	21.2	26	24.1
Postmenopausal	88	77.9	79	73.1

Abbreviations: CAF, cyclophosphamide, doxorubicin, and fluorouracil; SD, standard deviation.

Efficacy

The ORR, rated as CR or partial response rate, was 42.6% for CAF plus placebo versus 53.1% for dofequidar plus CAF (Table 2). Although this represents a 24.6% relative improvement and a 10.5% absolute increase in response rate for patients receiving dofequidar plus CAF compared with those receiving CAF plus placebo, this response was not statistically significant ($P = .077$). A higher value was observed in the dofequidar treatment group for all secondary end points compared with placebo, though these results were not statistically significant. Among them, Figure 2 shows a trend for prolonged PFS (median, 241 days for CAF plus placebo *v* 366 days for dofequidar plus CAF; $P = .145$).

Dofequidar plus CAF significantly improved PFS in several patient subgroups, including patients who were premenopausal ($P = .046$; Fig 3A), patients who had not received prior therapy ($P = .0007$; Fig 3B), and patients who had advanced primary breast cancer ($P = .017$; Fig 3C). An extended follow-up showed that dofequidar plus CAF also significantly improved overall survival ($P = .0034$; Fig 3D) in patients who had no prior therapy.

Safety and Tolerability

A similar number of patients completed six treatment cycles in both groups (n = 53 for the dofequidar group; n = 51 for the placebo group). The mean number of treatment cycles was 4.5 in the dofequidar group and 4.3 in the placebo group. More than half of patients in both groups included in each cycle from cycle 2 onward had a delay in treatment, mostly due to prolonged hematologic toxicities.

Dofequidar plus CAF was well tolerated throughout the study. No statistically significant excess of grade 3/4 AEs, except for neutropenia ($P = .006$) and leukopenia ($P = .005$), was found in the dofequidar group compared with placebo (Table A1, online only). Importantly, there was no marked difference in the incidence of neutropenia-related morbidity, such as febrile neutropenia or infection, between the two treatment groups. No significant differences in the incidence of cardiac AEs were found between the two treatment groups. In addition, dose intensities of chemotherapeutic agents were similar in both treatment arms. No significant difference in the incidence of serious AEs (SAEs) was observed between either group. However, there was a trend for a higher incidence of SAEs from leukopenia in the dofequidar group than in the placebo group ($P = .060$; Fisher's exact test); five leukopenia cases were reported for dofequidar, whereas no such case was reported for placebo.

A total of 124 patients discontinued the study (n = 61 for the dofequidar group; n = 63 for the placebo group). The major reasons for discontinuation were progressive disease (n = 23 for the dofequidar group; n = 28 for the placebo group), grade 4 hematologic toxicity (n = 20 for the dofequidar group; n = 6 for the placebo group), failure to meet treatment continuation criteria (n = 6 for the dofequidar group; n = 8 for the placebo group), and consent withdrawal (n = 6 for the dofequidar group; n = 12 for the placebo group). Of the 225 patients who received treatment in the study, 14 patients died during the treatment period (n = 3), the follow-up period (n = 2), or the follow-up period after study termination (n = 9). There were 49 other serious AEs in 32 patients during the study and follow-up period.

Pharmacokinetics

The mean plasma concentrations of doxorubicin in the dofequidar- and placebo-treatment groups at 15 minutes postadministration reached 0.997 μg/mL and 1.259 μg/mL, respectively, followed by biphasic elimination in both treatment groups. Mean plasma concentrations in

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Table 2. Response Rates for Patients Treated With Dofequidar Plus CAF (n = 113) or Placebo Plus CAF (n = 108)

Treatment Group	Parameter (No. of patients)					Overall Response Rate (%)	95% CI
	Complete Response	Partial Response	No Change (stable disease)	Progressive Disease	Not Assessable		
Dofequidar	5	55	40	10	3	53.1	43.5 to 62.5
Placebo	4	42	41	14	7	42.6	33.1 to 52.5

NOTE. Odds ratio = 1.53 (range, 0.87-2.69); P = .077 for dofequidar *v* placebo.
Abbreviation: CAF, cyclophosphamide, doxorubicin, and fluorouracil.

the dofequidar and placebo groups remained similar at 1, 2, 4, and 6 hours after the start of doxorubicin administration. Thus the elimination pattern for the first 6 hours after the start of administration was similar in both groups. The plasma concentrations of doxorubicin in the terminal phase (4 and 6 hours postadministration) were slightly higher in the dofequidar group compared with placebo (1.2- to 1.3-fold). However, AUC (0 to 6 hours) values showed no statistically significant difference between the dofequidar and placebo groups (mean, 0.480 $\mu g \cdot h/mL$; standard deviation [SD], 0.324; range, 0.237-1.692; and mean, 0.407 $\mu g \cdot h/mL$; SD, 0.062; and range, 0.289-0.500, respectively). Therefore, treatment with dofequidar did not affect the plasma concentrations of doxorubicin in patients (Fig 4).

DISCUSSION

Chemotherapy remains the preferred adjuvant treatment for patients with hormone receptor–negative disease and for patients with more aggressive, hormone receptor–positive tumors.[11,20] However, despite the use of conventional adjuvant chemotherapy regimens, a significant proportion of patients with breast cancer still experience disease recurrence because of inherent or acquired drug resistance.[12] In this randomized phase III trial, the efficacy and safety of the multidrug resistance inhibitor dofequidar plus CAF was compared with CAF plus placebo in patients with recurrent or advanced breast cancer. Although, there was an observed relative improvement and absolute increase in response rate for patients who received dofequidar plus CAF, these results did not reach statistical significance. This improvement in response rate may have been reflected in the observation that there was a trend for prolonged PFS, which favored patients in the dofequidar plus CAF group.

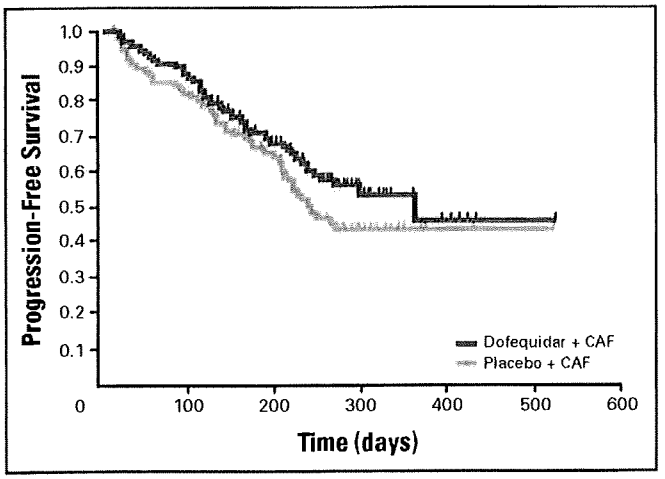

Fig 2. Progression-free survival in patients treated with dofequidar plus cyclophosphamide, doxorubicin, and fluorouracil (CAF) and placebo plus CAF (P = .145).

To date, only two randomized trials have examined the efficacy of a P-gp inhibitor in combination with chemotherapy in breast cancer patients. Wishart et al[21] examined quinidine combined with epirubicin in patients with advanced breast cancer, but failed to show any significant difference in overall survival or PFS compared with placebo. In a more recent prospective study of patients with anthracycline-resistant metastatic breast cancer (n = 99), verapamil combined with vindesine and fluorouracil resulted in a significantly longer overall survival and a higher response rate compared with patients who did not receive the P-gp inhibitor (median survival, 323 *v* 209 days; P = .036, respectively; ORR, 27% *v* 11%; P = .04, respectively).[22]

In the subgroup analyses, dofequidar in combination with CAF displayed a significantly increased PFS in patients who had not received prior therapy, who had advanced primary breast cancer or who were premenopausal. In addition, dofequidar also significantly improved overall survival in the patient group who had no prior therapy. Although the patient numbers in these analyses were small, the results remain important within these clinically significant patient populations. Both preclinical and clinical data have indicated that newer-generation MDR modulators can prevent the development of resistance.[23,24] A phase I/II trial in patients with acute myeloid leukemia showed that dosing with cyclosporine before and in combination with daunorubicin prevented chemotherapy resistance, while also resulting in a decrease in MDR-1 RNA expression.[24] Our results may highlight one potential treatment approach to MDR tumors that has not yet been fully exploited in the clinical environment, specifically the prevention of the emergence of resistance through the early use of P-gp inhibitors.[1-3] It seems reasonable that agents such as dofequidar may be useful in the adjuvant or even neoadjuvant setting with the goal of preventing or delaying the induction of MDR associated with chemotherapy.

The potential clinical significance of P-gp and MRP expression in breast cancer is supported by the results from a number of studies. For example in a study of primary breast cancer patients (n = 259), MRP expression was associated with an increased risk of treatment failure in patients with small tumors (T1) and node-positive patients who received adjuvant cyclophosphamide, methotrexate, and fluorouracil (CMF) chemotherapy but not in node-negative patients.[25] Burger et al[12] reported that the expression of MDR1 mRNA in primary breast tumors was inversely correlated with the efficacy of first-line chemotherapy. Additionally, the high level of MDR1 expression was suggested to be a significant predictor of poor prognosis in patients

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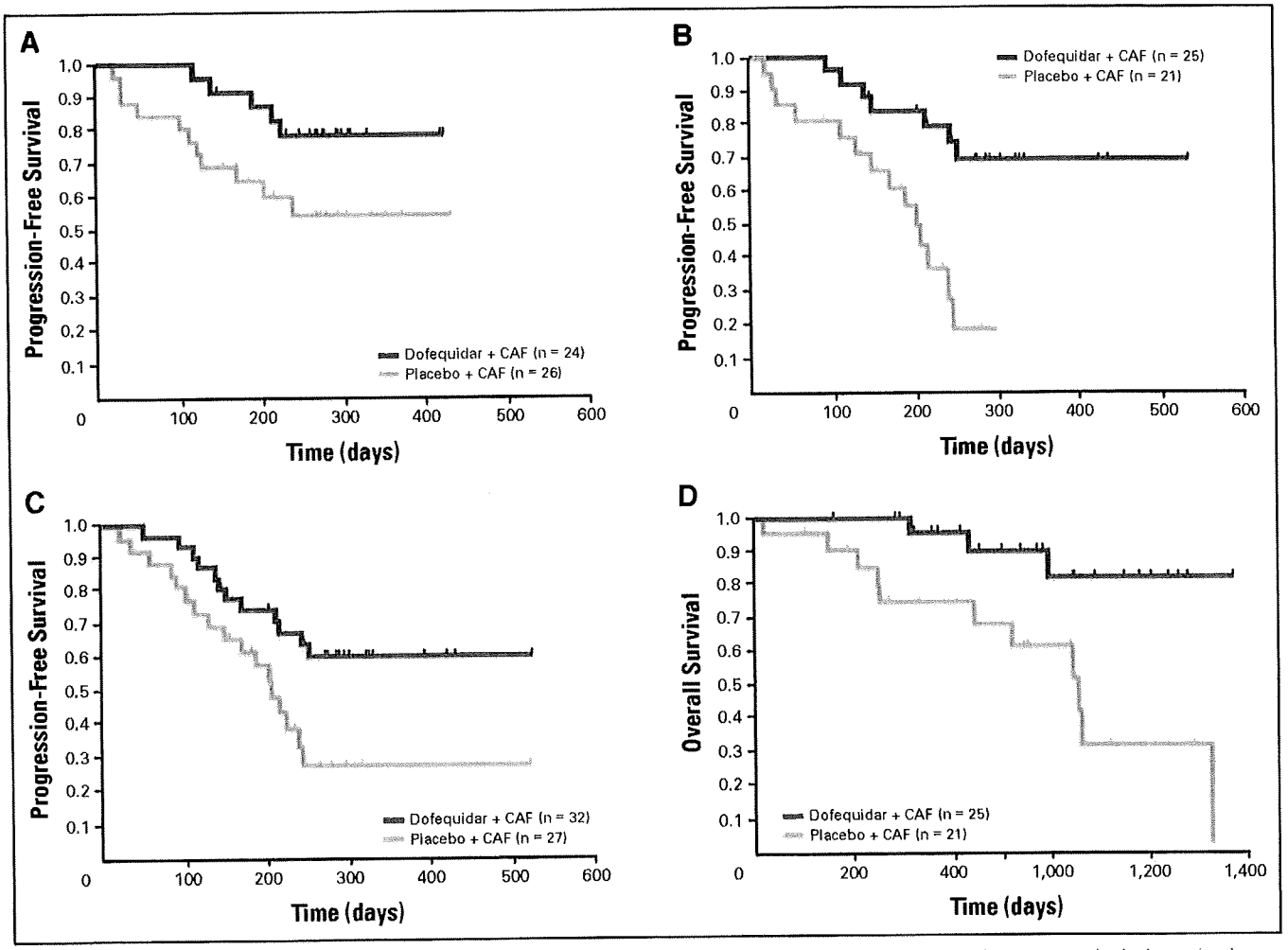

Fig 3. Subgroup analyses (A) Progression-free survival in premenopausal patients ($P = .046$); (B) progression-free survival in patients who had no prior therapy ($P = .0007$); (C) progression-free survival in patients who were stage IV at diagnosis with an intact primary tumor ($P = .017$), and (D) overall survival in patients who had no prior therapy ($P = .0034$)

with advanced disease.[12] Significantly increased expression of P-gp and MRP-1 has also been reported in an immunohistochemical study of patients treated with preoperative chemotherapy, whereas pretreatment expression of MRP-1 was associated with significantly shorter PFS in patients.[26] In a more recent study, MRP-1 expression was shown to be an independent predictor for shorter relapse-free survival and overall survival, after adjuvant CMF treatment, in premenopausal, hormone receptor–positive patients.[27] However, MRP-1 expression did not affect patients' response to adjuvant tamoxifen plus goserelin treatment.[27]

These findings and our results support the view of Leonard et al,[3] who indicate that future patients will need to be carefully selected for the identification and development of effective drug-resistance modulators. Patient populations who may derive maximal benefit from MDR inhibition, for example, the no-prior-therapy, advanced-disease, or premenopausal patient group in the present study, could quite easily be overlooked or lost within a large, heterogeneous trial population.[3] Furthermore, by refining future clinical trials to incorporate specific disease and patient characteristics, a clearer picture of drug resistance in cancer will be obtained and the most effective MDR inhibitor/chemotherapeutic agent(s) selected.

Many MDR inhibitors have required high serum concentrations for MDR reversal, which resulted in unacceptable toxicity, thereby limiting their clinical impact.[7,28-32] Although more recent agents have shown improved tolerability profiles, this has been countered by unpredictable pharmacokinetic interactions with other transporter molecules (eg, cytochrome P450–mediated drug metabolism and excretion, necessitating dose reductions in chemotherapy agents and leading to inconsistent chemotherapy dosing among patients).[1,5] Similarly, the addition of the MDR-modulating agent valspodar (PSC 833) to chemotherapy agents did not improve treatment outcome.[33,34] Toxicity was increased in the valspodar-treated group compared with chemotherapy agents alone, despite the reduction of chemotherapy doses in the valspodar-containing regimen. In our study, dofequidar was well tolerated, with no indication of the unacceptable toxicity associated with early MDR inhibitors. Importantly, dofequidar did not affect the plasma concentrations of doxorubicin in patients during the study and displayed an acceptable pharmacokinetic profile.

In conclusion, this study suggests that treatment with dofequidar resulted in possible clinical benefit for patients who had not received prior therapy, who were premenopausal, or who were stage IV at diagnosis with an intact primary tumor. Dofequidar was also well

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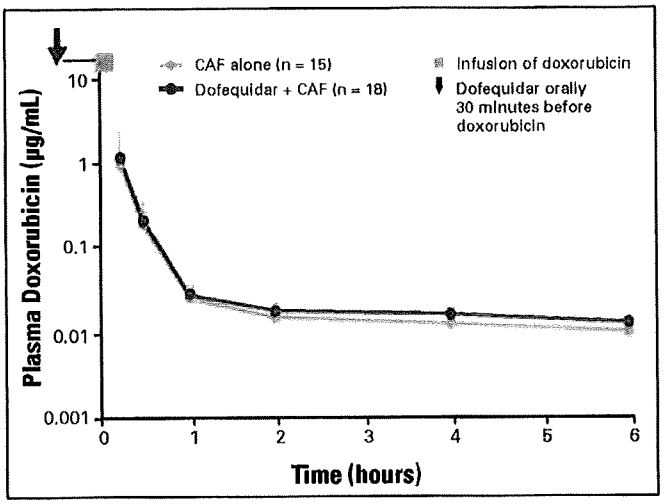

Fig 4. Plasma levels of doxorubicin in patients receiving dofequidar or placebo CAF, cyclophosphamide, doxorubicin, and fluorouracil

tolerated in the clinical setting and had no impact on doxorubicin pharmacokinetics. Further studies are merited to assess the effect of dofequidar in specific patient populations with breast cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

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Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: results of the West Japan Thoracic Oncology Group trial (WJTOG0403)

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The purpose of this study was to evaluate the efficacy of gefitinib and the feasibility of screening for epidermal growth factor receptor (*EGFR*) mutations among select patients with advanced non-small cell lung cancer (NSCLC). Stage IIIB/IV NSCLC, chemotherapy-naive patients or patients with recurrences after up to two prior chemotherapy regimens were eligible. Direct sequencing using DNA from tumour specimens was performed by a central laboratory to detect *EGFR* mutations. Patients harbouring *EGFR* mutations received gefitinib. The primary study objective was response; the secondary objectives were toxicity, overall survival (OS), progression-free survival (PFS), 1-year survival (1Y-S) and the disease control rate (DCR). Between March 2005 and January 2006, 118 patients were recruited from 15 institutions and were screened for *EGFR* mutations, which were detected in 32 patients – 28 of whom were enrolled in the present study. The overall response rate was 75%, the DCR was 96% and the median PFS was 11.5 months. The median OS has not yet been reached, and the 1Y-S was 79%. Thus, gefitinib chemotherapy in patients with advanced NSCLC harbouring *EGFR* mutations was highly effective. This trial documents the feasibility of performing a multicentre phase II study using a central typing laboratory, demonstrating the benefit to patients of selecting gefitinib treatment based on their *EGFR* mutation status.
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Keywords: epidermal growth factor receptor (*EGFR*) mutation; gefitinib; non-small cell lung cancer (NSCLC); multicentre prospective phase II; central laboratory

Gefitinib, a tyrosine kinase inhibitor (TKI), is an orally active small molecule that functions as a selective epidermal growth factor receptor (*EGFR*) inhibitor (Ranson *et al*, 2002). Two phase II trials (Fukuoka *et al*, 2003; Kris *et al*, 2003) for previously treated non-small cell lung cancer (NSCLC) (IDEAL-1 and -2, respectively) have documented favourable objective responses in 14–18% of patients. However, in a phase III trial (Thatcher *et al*, 2005), no survival benefit of gefitinib was observed when compared with best-supportive care (BSC) for previously treated NSCLC. In contrast, we have seen a significant survival benefit of erlotinib compared with BSC as a salvage therapy (BR21); erlotinib is also an *EGFR*-TKI and its chemical structure, which is based on quinazoline, is quite similar to that of gefitinib (Shepherd *et al*, 2005). Although we do not know whether differences between gefitinib and erlotinib were responsible for these different outcomes, appropriate patient selection to identify good responders is likely crucial for revealing the clinical benefits of the *EGFR*-TKI family.

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