

# The antipsychotic drug trifluoperazine inhibits DNA repair and sensitizes non-small cell lung carcinoma cells to DNA double-strand break-induced cell death

Anya G. Polischouk,<sup>1</sup> Åsa Holgersson,<sup>1</sup> Dali Zong,<sup>1</sup> Bo Stenerlöv,<sup>3</sup> Hanna L. Karlsson,<sup>2</sup> Lennart Möller,<sup>2</sup> Kristina Viktorsson,<sup>1</sup> and Rolf Lewensohn<sup>1</sup>

<sup>1</sup>Unit of Medical Radiation Biology, Department of Oncology-Pathology, Cancer Centrum Karolinska and

<sup>2</sup>Unit for Analytical Toxicology, Department of Biosciences at Novum, Karolinska Institutet, Stockholm, Sweden; and

<sup>3</sup>Division of Biomedical Radiation Sciences, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden

## Abstract

Trifluoperazine (TFP), a member of the phenothiazine class of antipsychotic drugs, has been shown to augment the cytotoxicity of the DNA-damaging agent bleomycin. In the present study, we investigated the effect of trifluoperazine on (a) survival of bleomycin-treated human non-small cell lung carcinoma U1810 cells, (b) induction and repair of bleomycin-induced DNA strand breaks, and (c) nonhomologous end-joining (NHEJ), the major DNA double-strand break (DSB) repair pathway in mammalian cells. By using a clonogenic survival assay, we show here that concomitant administration of trifluoperazine at a subtoxic concentration enhances the cytotoxicity of bleomycin. Moreover, trifluoperazine also increases the longevity of bleomycin-induced DNA strand breaks in U1810 cells, as shown by both comet assay and fraction of activity released (FAR)-assay. This action seems to be related to suppression of cellular DNA DSB repair activities because NHEJ-mediated rejoining of DSBs occurs with significantly lower efficiency in the presence of trifluoperazine. We propose that TFP might be capable of inhibiting one or more elements of the DNA DSB repair machinery, thereby increasing the cytotoxicity of bleomycin in lung cancer cells. [Mol Cancer Ther 2007;6(8):2303–9]

Received 7/11/06; revised 6/2/07; accepted 6/29/07.

**Grant support:** Swedish Cancer Society (R. Lewensohn and B. Stenerlöv), Stockholm Cancer Society (R. Lewensohn and K. Viktorsson), and Karolinska Institutet (R. Lewensohn and K. Viktorsson).

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**Note:** A.G. Polischouk and Å. Holgersson contributed equally to this work.

**Requests for reprints:** Rolf Lewensohn, Department of Oncology-Pathology, Karolinska Biomics Center, Karolinska Institutet, SE-171 76 Stockholm, Sweden. Phone: 46-8-51773188; Fax: 46-8-51771000. E-mail: Rolf.Lewensohn@ki.se

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doi:10.1158/1535-7163.MCT-06-0402

## Introduction

Phenothiazines constitute a class of widely used antipsychotic drugs (1). In addition, the phenothiazine compound trifluoperazine has been shown to enhance the cytotoxicity of the radiomimetic agent bleomycin (2–5). Regimens that combine bleomycin and trifluoperazine (TFP) have also been evaluated in phase I and phase II clinical trials for the treatment of non-Hodgkin's lymphoma and glioblastoma multiforme, respectively (6, 7). Although TFP clearly can modulate the cytotoxicity of several conventional chemotherapeutic agents, the molecular mechanisms underlying its antitumor activity remain unclear. In leukemic lymphocytes, TFP was shown to enhance bleomycin-induced DNA laddering, cellular shrinkage, and chromatin condensation, all typical signs of apoptotic cell death (4).

The cytotoxicity of bleomycin derives mainly from its ability to cleave DNA, which generates DNA single-strand breaks as well as double-strand breaks (DSB; ref. 8). Studies on cellular responses to ionizing radiation have shown that DSBs are extremely toxic and production of ionizing radiation-induced DSBs correlates with impeded cell survival (9). Mammalian cells repair DNA DSBs predominantly through a process called nonhomologous end joining (NHEJ), in which the two broken strands are directly rejoined end-to-end with no or minimal requirement for sequence homology (10). The core component of NHEJ is the DNA-dependent protein kinase (DNA-PK) complex, which is composed of two subunits, Ku and DNA-PK<sub>cs</sub>. The initial steps of NHEJ involves binding of Ku to free DNA ends (11) and is followed by recruitment of DNA-PK<sub>cs</sub> to the DNA DSB (10). This activates the serine/threonine kinase activity of DNA-PK<sub>cs</sub> and results in phosphorylation of proteins required for further steps of the NHEJ pathway (10). Deficiency in either subunits of DNA-PK causes disruption of NHEJ and hypersensitivity to DSB-inducing agents (12–16).

We have previously reported that TFP can inhibit the kinase activity of DNA-PK *in vitro* (17). In the present study, we analyzed the effects of TFP on the induction and repair of bleomycin-induced DNA strand breaks in U1810 human non-small cell lung carcinoma cells. We show here that concomitant addition of a subtoxic concentration of TFP leads to potentiation of bleomycin cytotoxicity. Moreover, TFP also increases the longevity of bleomycin-induced DNA strand breaks in U1810 cells. At least in part, this action involves inhibition of cellular DNA repair activities, because both overall repair of DNA strand breaks and NHEJ-mediated rejoining of DSBs occur with significantly slower kinetics in the presence of TFP. We hypothesize that trifluoperazine is capable of inhibiting one or

more elements of the cellular DNA DSB repair machinery, thereby augmenting the cytotoxicity of bleomycin in lung cancer cells.

## Materials and Methods

### Drugs

Bleomycin (Baxter Medical AB) was dissolved in sodium chloride solution and stored at  $-80^{\circ}\text{C}$ . Fresh working solutions of TFP (ICN Biomedical, Inc.) were prepared in sterilized water immediately before use. Whenever a combination of bleomycin and TFP was used, the drugs were applied concomitantly. Unless otherwise specified, the concentration of TFP used was  $10\ \mu\text{mol/L}$ .

### Cell Culture and Treatments

The U1810 cell line is derived from an undifferentiated human non-small cell lung carcinoma (NSCLC; ref. 18). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum,  $1\ \text{mmol/L}$  L-glutamine,  $100\ \text{IE/mL}$  penicillin, and  $100\ \mu\text{g/mL}$  streptomycin in a humidified atmosphere of 5%  $\text{CO}_2$ . All cell culture supplies were purchased from Life Technologies. For all experiments, cells were seeded at the indicated density 18 to 20 h before drug treatment.

### Clonogenic Survival Assay

Cultures of 100 to 600 cells per dish were exposed to graded concentrations of bleomycin for 1 h in the presence or absence of TFP. Thereafter, cells were washed and incubated in fresh growth medium for 10 days to allow colony formation. Colonies were fixed in 10% formaldehyde solution and stained in Giemsa (BDH). Colonies containing at least 50 cells were scored and all data on viability are given relative to the untreated control.

### Comet Assay

The alkaline variant of the comet assay was done according to Singh et al. (19) with some modifications. Briefly, cells ( $5 \times 10^5$ ) were seeded and exposed to bleomycin in the presence or absence of TFP for 1 h, trypsinized, washed, and resuspended in  $100\ \mu\text{L}$  PBS. Fifteen microliters of this cell suspension were added to  $150\ \mu\text{L}$  of 0.75% low melting point agarose at  $37^{\circ}\text{C}$ , and spread over a microscope slide precoated with 0.3% agarose. Slides were serially immersed in lysis buffer [ $1\%$  Triton X-100,  $2.5\ \text{mol/L}$  NaCl,  $10\ \text{mmol/L}$  Tris,  $0.1\ \text{mol/L}$  EDTA (pH 10)] for 1 h and alkaline DNA unwinding solution ( $0.3\ \text{mol/L}$  NaOH,  $1\ \text{mol/L}$  EDTA) for 40 min, followed by electrophoresis at 25 V ( $\approx 0.86\ \text{V/cm}$ ) for 30 min in a Sub-Cell GT unit. All above steps were done at  $4^{\circ}\text{C}$ . Slides were then neutralized in  $0.4\ \text{mol/L}$  Tris-HCl (pH 7.4), immersed in water, air-dried overnight at room temperature, and fixed in methanol. After staining with ethidium bromide ( $10\ \mu\text{g/mL}$ ), the comets were examined under a  $\times 20$  apochromatic objective of a fluorescence microscope and analyzed using the Komet 4.0 software (Kinetic Imaging, Ltd.). The percentage of DNA in the tail (tail %DNA) was determined for 50 comets in each sample. The background level of the tail %DNA was  $\sim 10\%$ .

### Fraction of Activity Released Assay

Cells were cultured in 35-mm Petri dishes and labeled with  $1,000\ \text{Bq/mL}$  [ $^{14}\text{C}$ ]thymidine (Amersham Biosciences) for 48 h before a 1-h treatment with  $40\ \mu\text{g/mL}$  bleomycin in the absence or presence of TFP. Immediately after drug exposure, cells were washed in ice-cold PBS and either harvested at once or incubated in fresh growth medium for various lengths of time. Harvested cells were mixed with an equal volume of 1.2% low melting point agarose (FMC BioProducts), inserted immediately into precooled plug formers (Bio-Rad), and allowed to solidify for 20 min. To release DNA, plugs with embedded cells (total volume  $100\ \mu\text{L}$ ) were submerged in 1 mL of lysis buffer [ $0.5\ \text{mol/L}$  EDTA (pH 8), 2% *N*-laurylsarcosine,  $1\ \text{mg/mL}$  proteinase K] for  $\geq 20\ \text{h}$  followed by immersion in 2 mL of a high salt buffer [ $1.85\ \text{mol/L}$  NaCl,  $0.15\ \text{mol/L}$  KCl,  $5\ \text{mmol/L}$   $\text{MgCl}_2$ ,  $2\ \text{mmol/L}$  EDTA,  $4\ \text{mmol/L}$  Tris, 0.5% Triton X-100 (pH 7.5)] for 10 to 20 h. All steps for DNA extraction were carried out at  $4^{\circ}\text{C}$ . Kinetics of DNA end rejoining was analyzed by pulse field gel electrophoresis using a protocol optimized for separation of DNA fragments in the size range 1 to 10 Mbp (16), stained with ethidium bromide ( $0.5\ \mu\text{g/mL}$ ), and photographed. The extent of DNA fragmentation was quantified by  $^{14}\text{C}$  scintillation counting of gel slices that were cut at the position of the 5.7 Mbp marker chromosome of *Saccharomyces pombe* (Cambrex Bio Science Rockland, Inc.). The fraction of activity released corresponding to DNA fragments of sizes  $< 5.7\ \text{Mbp}$  is presented in the text as fraction of activity released (FAR)  $< 5.7\ \text{Mbp}$ .

### Preparation of Nuclear Extracts

Lysis of cells for *in vitro* DNA strand break repair assay was done essentially as described by Cheong et al. (20). Exponentially growing cells were collected and the packed cell volume (PCV) was determined. The cell pellet was suspended in four PCV equivalents of hypotonic buffer [ $10\ \text{mmol/L}$  Tris-HCl (pH 7.5),  $1\ \text{mmol/L}$  EDTA,  $0.5\ \text{mmol/L}$  DTT,  $170\ \mu\text{g/mL}$  phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Roche)], centrifuged ( $240 \times g$ ), and resuspended in another four packed cell volume equivalents of hypotonic buffer and incubated for 20 min. Disruption of cells was achieved by using a Dounce homogenizer (four strokes, pestle B). High salt buffer [ $10\ \text{mmol/L}$  HEPES-KOH (pH 7.5),  $1.4\ \text{mol/L}$  KCl,  $1.5\ \text{mmol/L}$   $\text{MgCl}_2$ ] was added to the homogenate and centrifuged ( $3,300 \times g$ , 20 min). Pellet containing cell nuclei was resuspended in 0.5 packed cell volume of low salt buffer [ $20\ \text{mmol/L}$  HEPES-KOH (pH 7.9),  $1.5\ \text{mmol/L}$   $\text{MgCl}_2$ ,  $0.02\ \text{mmol/L}$  KCl,  $0.2\ \text{mmol/L}$  EDTA,  $0.5\ \text{mmol/L}$  DTT, and protease inhibitors]. To this solution, 0.25 PCV of high salt buffer was slowly added and the nuclear fraction was extracted under gentle agitation for 1 h. The nuclear extract was subsequently cleared from DNA and nonsoluble nuclear membranes by ultracentrifugation ( $15,000 \times g$ , 30 min) and concentrated to  $25\ \mu\text{L}$  in an ultrafree-0.5 centrifugal filter unit with Biomax-5 membrane (Millipore) at  $11,500 \times g$ . Finally, the sample was dialyzed against a buffer compatible for NHEJ assessment

[20 mmol/L HEPES-KOH (pH 7.9), 100 mmol/L KCl, 0.2 mmol/L EDTA, 20% v/v glycerol, 0.5 mmol/L DTT, and protease inhibitors as above]. All above steps were carried out at 4°C. Protein concentration was determined using the Bio-Rad protocol (Bio-Rad Laboratories) and adjusted to 30 to 40 µg/µL. The extract was snap frozen and stored at -80°C until use.

#### Preparation of DNA Substrates

The pBR322 plasmid (Roche Diagnostics GmbH) was used as DNA substrate for all *in vitro* DNA repair assays. To generate DNA substrate with either compatible 3' overhangs of four nucleotides or with blunt ends, pBR322 was treated with restriction endonucleases PstI and PvuII, respectively (Fermentas). Treatment of plasmid DNA (750 ng) with bleomycin (340 ng/mL) was carried out under the conditions described by Pastwa et al. (21). Cleaved plasmid DNA was purified using a QIAquick gel extraction kit (Qiagen GmbH).

#### Nonhomologous End Joining Assay

The NHEJ reaction was reconstituted in a 10 µL reaction mixture containing 10 ng plasmid DNA substrate, 80 µg nuclear protein extract, 45 mmol/L HEPES-KOH (pH 7.8), 70 mmol/L KCl, 5 mmol/L MgCl<sub>2</sub>, 0.4 mmol/L EDTA, 3% v/v glycerol, 20 µg/mL bovine serum albumin, 1 mmol/L DTT, 2 mmol/L ATP, 40 mmol/L phosphocreatine, 2.5 µg creatine phosphokinase, 2 mmol/L NAD<sup>+</sup>, and 20 µmol/L each of deoxynucleotide triphosphates. When indicated, TFP (200 µmol/L) was added to the reaction mixtures. The reaction was carried out at 30°C for up to 3 h and then stopped by addition of 10 µL of a quenching solution containing 1% SDS and 0.1 mol/L EDTA. After hydrolyzing proteins with proteinase K (10 µg/50 µg protein extract) for 1 h at 37°C, the rejoined and nonrejoined plasmid DNA products were resolved by electrophoresis on a 0.7% agarose gel containing 0.5 µg/mL of ethidium bromide and transferred onto Hybon-XL membranes (Amersham Pharmacia Biotech). For visualization, membranes with bound plasmid DNA material was hybridized to HindIII-digested [ $\alpha$ -<sup>32</sup>P]dCTP-labeled pBR322, and developed on Fuji Medical X-ray films. Radiolabeling of pBR322 was achieved using a multiprimer DNA labeling system (Amersham Biosciences), according to manufacturers' instructions.

#### Statistical Analysis

Statistical tests on data derived from the comet assay and clonogenic survival assay were based on the two-sample *t* test, taken as significant at *P* < 0.05.

## Results

### TFP Potentiates Bleomycin Cytotoxicity in Non-Small Cell Lung Carcinoma Cells

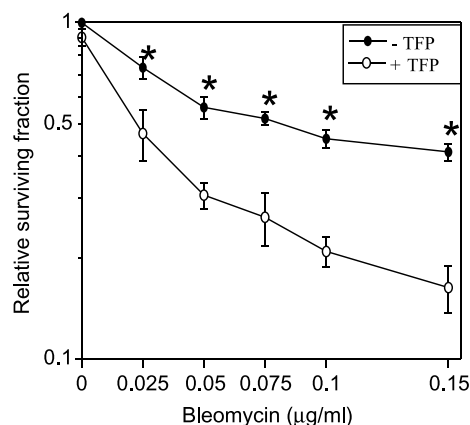
Trifluoperazine per se at 10 µmol/L has previously been shown to be nontoxic in tumor cell lines (22). Here, we examined whether concomitant administration of 10 µmol/L TFP along with bleomycin has any effect on the cytotoxicity of the latter agent in human non-small cell lung carcinoma cells. By using a colony formation assay, we observed that

exposure of U1810 cells to 10 µmol/L TFP alone has minimal effect on cell survival (Fig. 1; bleomycin + trifluoperazine at bleomycin = 0). As expected, exposure to bleomycin results in a dose-dependent decrease in clonogenic survival. Importantly, cells exposed to a combination of bleomycin and 10 µmol/L TFP retain significantly less clonogenic potential compared with cells treated with bleomycin alone (*P* < 0.05; Fig. 1). These results suggest that 10 µmol/L TFP, although nontoxic by itself, can enhance bleomycin cytotoxicity in U1810 cells.

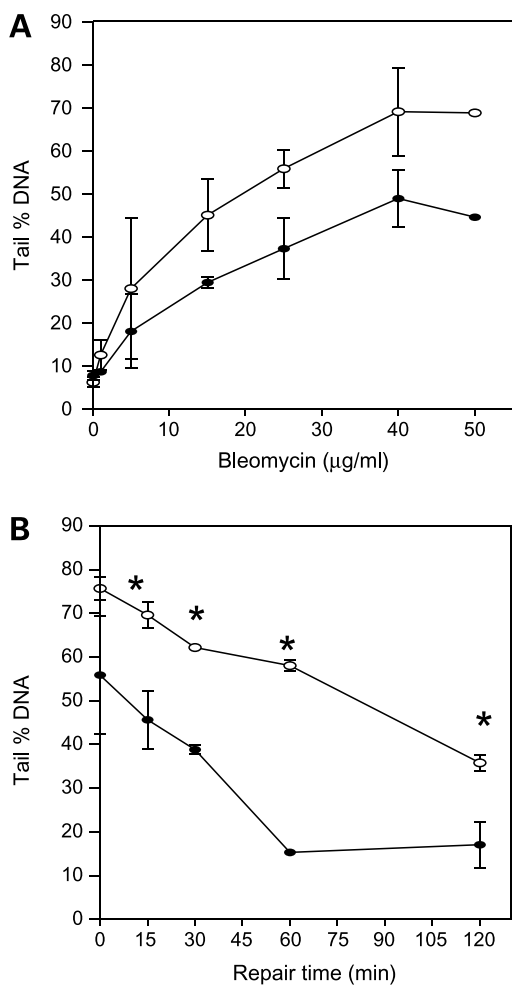
### TFP Enhances the Cellular Level of Bleomycin-Induced DNA Strand Breaks

Bleomycin induces DNA strand breaks (single-strand breaks and DSBs) through a series of complex oxidative reactions (8). Using an alkaline comet assay, we compared the levels of bleomycin-induced DNA strand breaks in U1810 cells in the presence or absence of 10 µmol/L TFP. As illustrated in Fig. 2A, bleomycin per se produces DNA strand breaks (tail % DNA) in a dose-dependent manner. In the presence of 10 µmol/L TFP, the amount of DNA strand breaks induced by bleomycin is markedly increased (Fig. 2A). No appreciable levels of DNA strand breaks are detected in cells exposed to TFP alone (Fig. 2A; bleomycin + TFP at bleomycin = 0).

The results presented above suggest that TFP increases the level of DNA strand breaks in bleomycin-treated cells. However, whether the effect of TFP is on DNA damage induction or repair is not readily discernible. To resolve this issue, we next examined DNA repair kinetics in U1810 cells during a 2-h recovery period after an initial exposure to 40 µg/mL bleomycin in the absence or presence of 10 µmol/L TFP. As in Fig. 2A, immediately after the



**Figure 1.** TFP sensitizes human non-small cell lung carcinoma cells to bleomycin-induced loss of clonogenicity. U1810 cells were exposed to graded concentrations of bleomycin for 1 h in the absence (●) or presence (○) of 10 µmol/L TFP. The cells were thereafter incubated for 10 d in drug-free growth medium to allow colony formation. Clonogenic survival was calculated from the following formula: relative survival fraction = colonies counted / (cells seeded × plating efficiency). Overall plating efficiency was 67%. Points, mean of three separate experiments with triplicate samples; bars, SEM. \*, *P* < 0.05.



**Figure 2.** TFP increases the longevity of bleomycin-induced DNA strand breaks. U1810 cells were exposed to indicated concentrations of bleomycin in the absence (●) or presence (○) of 10 µmol/L TFP for 1 h. The amount of DNA strand breaks induced by bleomycin (tail %DNA) was determined at 1 h postdrug additions using the alkaline comet assay (A). The amount of DNA strand breaks that remain after a 1-h treatment with 40 µg/mL bleomycin in the absence (●) or presence (○) of 10 µmol/L TFP was determined at indicated postincubation times (B). Points, mean of two separate experiments; bars, SEM. \*,  $P < 0.05$ . The background level of tail %DNA was ~10%.

1-h treatment period, cells exposed to bleomycin plus TFP show a higher level of DNA damage than cells exposed to bleomycin only (Fig. 2B;  $t = 0$ ). Upon drug removal, the levels of DNA strand breaks declines with time indicative of ongoing repair. In the absence of TFP, almost background levels of DNA strand breaks remains in bleomycin-treated cells after 1 h repair, at which time 4-fold as much DNA strand breaks can be detected in cells treated with bleomycin plus TFP. Even at 2 h, the last time point of our assay, the level of DNA strand breaks in TFP-treated cells has still not returned to background. These data suggest that trifluoperazine may interfere with the repair of bleomycin-induced DNA damage.

### Trifluoperazine Inhibits Repair of Bleomycin-Induced DNA DSBs

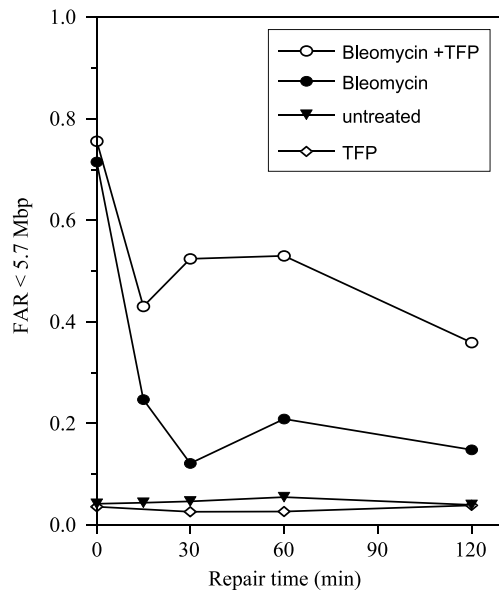
Because accumulation of unrepaired DNA DSBs poses the biggest threat to cell survival, we further characterized the effect of TFP specifically on DNA DSB repair using a pulse field gel electrophoresis (PFGE)-based FAR assay, which measures the level of DSBs in cells. As illustrated in Fig. 3, the induction of DNA DSBs by bleomycin is essentially unaffected by TFP (Fig. 3; bleomycin + trifluoperazine at  $t = 0$ ) and trifluoperazine per se does not give rise to DNA DSBs (Fig. 3). Bleomycin-induced DSBs are more rapidly repaired in the absence of TFP, with ~85% being rejoined after 2 h of repair. In the presence of 10 µmol/L TFP, only about half of bleomycin-induced DSBs are rejoined during the same repair times. These data suggest that TFP may specifically inhibit repair of bleomycin-induced DSBs.

### Trifluoperazine Inhibits Nonhomologous End Joining

We have previously reported that TFP can inhibit the serine/threonine kinase activity of DNA-PK *in vitro* (17). Therefore, we hypothesize that TFP may enhance the cytotoxicity of bleomycin by interfering with NHEJ-mediated DNA DSB repair. Here, we used a plasmid-based *in vitro* DNA repair assay to study the effect of TFP on DNA DSB repair in more detail. In this assay, DNA DSBs with blunt, cohesive, or random ends were introduced into supercoiled bacterial plasmids (pBR322) using restriction enzymes or bleomycin, respectively, and the capacity for DNA end-to-end joining was studied in nuclear protein extracts. In the absence of TFP, nuclear extract of U1810 cells promotes efficient end-joining of linearized pBR322 with blunt and cohesive ends such that after 3 h of repair, 80% of PvuII-cleaved and almost all of (~95%) PstI-cleaved linear monomers are ligated into multimers and/or circular forms of the plasmid (Fig. 4A; -TFP). Inclusion of 200 µmol/L TFP in the reaction mixtures results in significantly lower yield of ligated products (Fig. 4A; +TFP). When bleomycin was used as the plasmid DNA-cleaving agent, successive increases of TFP concentrations up to 100 µmol/L fail to increase the amount of bleomycin-induced DNA strand breaks (Fig. 4B), which rules out the possibility of a direct genotoxic effect of TFP. Hence, TFP seems to impair DNA DSB repair by NHEJ without affecting DSB generation.

### Discussion

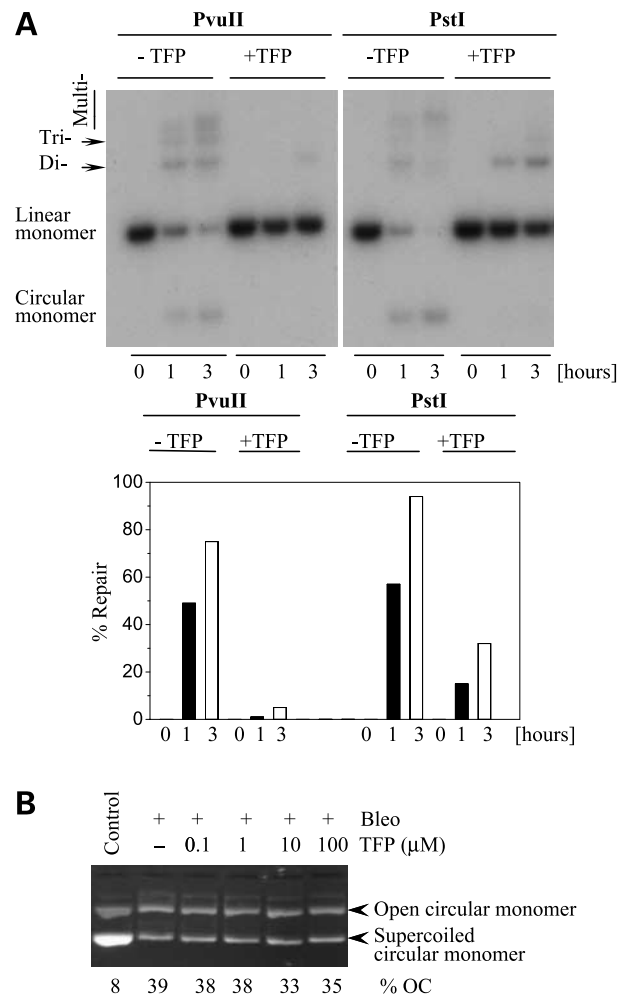
The cytotoxicity of bleomycin is mainly related to its ability to generate DNA breaks, in particular DNA DSBs (8). An important factor that governs tumor cell responsiveness to bleomycin is the cellular DNA DSB repair capacity, and, in line with this, increased sensitivity to bleomycin is observed in cells deficient in NHEJ (15, 23, 24). TFP, a phenothiazine class antipsychotic drug, has been reported to enhance bleomycin-induced cytotoxicity by a poorly characterized mechanism (2, 4, 5, 22). We have previously shown that TFP can inhibit the kinase activity of the NHEJ core component DNA-PK *in vitro* (17). In this study, we



**Figure 3.** TFP inhibits repair of bleomycin-induced DNA DSBs. [ $^{14}\text{C}$ ]thymidine-labeled U1810 cells were exposed to 40  $\mu\text{g}/\text{mL}$  bleomycin in the absence (●) or presence (○) of 10  $\mu\text{mol}/\text{L}$  TFP for 1 h. Kinetics of DNA DSB end rejoining was determined by PFGE-based FAR assay. The fraction of radioactivity corresponding to DNA of sizes <5.7 Mbp was quantified by scintillation counting and expressed as fraction of activity released <5.7 Mbp. Data from one of two independent experiments are shown.

examined whether trifluoperazine can interfere with NHEJ-mediated repair *in vivo* and thereby enhance bleomycin-induced cytotoxicity. In agreement with previous reports, we show here that although 10  $\mu\text{mol}/\text{L}$  TFP per se is nontoxic to non-small cell lung carcinoma U1810 cells, combining TFP with bleomycin results in potentiation of the latter agent (Fig. 1). Results presented in Fig. 2B suggest that TFP may interfere with the repair of bleomycin-induced DNA strand breaks. However, both Fig. 2A and B also show that the initial levels of DNA strand breaks (i.e., after 1 h bleomycin treatment) are influenced by TFP. There are two likely and nonmutually exclusive explanations for this. One is that TFP may enhance the formation of bleomycin-induced DNA strand breaks. Because onset of the cellular DNA damage response is very rapid, the other possibility is that during the 1-h exposure to bleomycin, DNA repair is also taking place and the higher levels of initial damage in TFP-treated cells imply an inhibitory effect of TFP on DNA repair. Although the comet assay detects overall DNA strand breaks, it does not differentiate DNA single-strand breaks from DSBs. As accumulation of DSBs induces cell death, we investigated the effect of TFP on the fate of bleomycin-induced DNA DSBs. Importantly, slower kinetics of DNA DSB rejoining are observed in the presence of trifluoperazine when the DSB inducer was bleomycin (Fig. 3), restriction endonucleases (Fig. 4), or ionizing radiation (data not shown). Our data clearly

show that comparable levels of bleomycin-induced DNA DSBs are detected regardless of TFP exposure or nonexposure, suggesting that the induction of DNA DSB by bleomycin is a TFP-independent event. Results from Fig. 4 indicate a negative effect of TFP on NHEJ *in vitro* because end joining of linearized plasmid DNA, a process that requires functional NHEJ, is markedly inhibited by TFP. Direct ligation of some simple DSB ends with local sequence microhomology can occur without DNA-PK



**Figure 4.** TFP inhibits NHEJ-mediated DNA repair. *PvuII*- or *PstI*-cleaved plasmid DNA was incubated with U1810 nuclear extract in the absence (–TFP) or presence of 200  $\mu\text{mol}/\text{L}$  TFP (+TFP) for the indicated times (A). The 0 time points represent aliquots taken immediately after the assembly of the complete reactions on ice. Di-, tri-, and multi-, denoted dimer, trimer, and multimers, respectively; are visualized by Southern blotting (top). Repair efficiency (% repair) is determined by densitometric analysis of gel pictures and is given as the fraction of ligated products (multi-, tri-, di-, and circular monomers) in relation to the total monomeric forms of the plasmid (bottom). Cleavage of plasmid DNA by bleomycin (*Bleo*) was assessed in the presence of increasing concentrations of TFP and expressed as the fraction (%OC) of relaxed open circles (DNA cleavage) in relation to supercoiled circles (intact DNA; B). All diagrams are representative of two separate experiments.

(25). We observe that unlike plasmids with blunt ends (PvuII-cut), plasmids having compatible 3'-overhang (PstI-cut) are still rejoined to some extent (30% after 3 h) in the presence of TFP. Taken together, the available data suggest that TFP may possess NHEJ-suppressive activity consistent with an inhibition on DNA-PK. Whether TFP also inhibits NHEJ *in vivo* is an issue that needs to be clarified because the concentration of TFP found to inhibit DNA-PK (200  $\mu\text{mol/L}$ ) *in vitro* is much higher than the 10  $\mu\text{mol/L}$  required to chemosensitize U1810 cells to bleomycin. Interestingly, results presented in Fig. 2 raise the possibility that TFP may also affect DNA single-strand breaks. Under the conditions used here, however, it is difficult to conclude whether TFP acts on the induction and/or the repair phase.

Phenothiazines are well-characterized calmodulin antagonists (26, 27), and this led Chafouleas et al. (2) to propose that TFP might augment bleomycin cytotoxicity by inhibiting a putative calmodulin-regulated DNA repair pathway. Recent studies have shown that inositol phosphates serve as cofactors for DNA-PK<sub>cs</sub> during NHEJ (28–30). Notably, inositol hexakisphosphate (InsP<sub>6</sub>) has been shown to regulate the mobility of Ku proteins and depletion of InsP<sub>6</sub> by the TFP-related compound chlorpromazine causes a reduction in the mobile fraction of Ku (31). Therefore, one might speculate that the inhibitory action of TFP on DNA DSB repair shown in this study could be a consequence of calmodulin antagonism. Although the present work did not address the involvement of calmodulin, other investigators have shown that repair of DNA DSBs is in fact calmodulin dependent, at least under certain circumstances (32, 33). On the other hand, Eriksson et al. (17) have shown that TFP also suppresses the kinase activity of purified DNA-PK *in vitro*, suggesting that TFP may also directly influence DNA DSB repair capacity by modulating DNA-PK activity.

In summary, we show here that TFP enhances the cytotoxic effects of bleomycin in U1810 cells, which may be partially mediated by inhibition of DNA DSB repair. Given that phenothiazines such as TFP are routinely used in the clinical management of psychiatric disorders with tolerable side effects, we believe that TFP and similar compounds are suitable lead substances for development of the next generation of chemosensitizers and radiosensitizers.

#### Acknowledgments

We thank laboratory assistant Birgitta Mörk for excellent assistance with PFGE sample preparation and Dr. Jenny Forshed for valuable statistical help.

#### References

- Shen WW. A history of antipsychotic drug development. *Compr Psychiatry* 1999;40:407–14.
- Chafouleas JG, Bolton WE, Means AR. Potentiation of bleomycin lethality by anticalmodulin drugs: a role for calmodulin in DNA repair. *Science* 1984;224:1346–8.
- Hait WN, DeRosa WT. Calmodulin as a target for new chemotherapeutic strategies. *Cancer Invest* 1988;6:499–511.
- Sullivan GF, Garcia-Welch A, White E, Lutzker S, Hait WN. Augmentation of apoptosis by the combination of bleomycin with trifluoperazine in the presence of mutant p53. *J Exp Ther Oncol* 2002;2:19–26.
- Hait WN, Lazo JS, Chen DL, Gallichio VS, Filderman AE. Antitumor and toxic effects of combination chemotherapy with bleomycin and a phenothiazine anticalmodulin agent. *J Natl Cancer Inst* 1988;80:246–50.
- Hait WN, Morris S, Lazo JS, et al. Phase I trial of combined therapy with bleomycin and the calmodulin antagonist, trifluoperazine. *Cancer Chemother Pharmacol* 1989;23:358–62.
- Hait WN, Byrne TN, Piepmeier J, et al. The effect of calmodulin inhibitors with bleomycin on the treatment of patients with high grade gliomas. *Cancer Res* 1990;50:6636–40.
- Chen J, Stubbe J. Bleomycins: towards better therapeutics. *Nat Rev Cancer* 2005;5:102–12.
- Iliakis G. The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. *BioEssays* 1991;13:641–8.
- Hefferin ML, Tomkinson AE. Mechanism of DNA double-strand break repair by non-homologous end joining. *DNA Repair (Amst)* 2005;4:639–48.
- Walker JR, Corpina RA, Goldberg J. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. *Nature* 2001;412:607–14.
- Blunt T, Finnie NJ, Taccioli GE, et al. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. *Cell* 1995;80:813–23.
- Kirchgessner CU, Patil CK, Evans JW, et al. DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science* 1995;267:1178–83.
- Taccioli GE, Gottlieb TM, Blunt T, et al. Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination. *Science* 1994;265:1442–5.
- Allalunis-Turner MJ, Zia PK, Barron GM, Mirzayans R, Day RS III. Radiation-induced DNA damage and repair in cells of a radiosensitive human malignant glioma cell line. *Radiat Res* 1995;144:288–93.
- Stenerlow B, Karlsson KH, Cooper B, Rydberg B. Measurement of prompt DNA double-strand breaks in mammalian cells without including heat-labile sites: results for cells deficient in nonhomologous end joining. *Radiat Res* 2003;159:502–10.
- Eriksson A, Yachnin J, Lewensohn R, Nilsson A. DNA-dependent protein kinase is inhibited by trifluoperazine. *Biochem Biophys Res Commun* 2001;283:726–31.
- Bergh J, Nilsson K, Ekman R, Giovannella B. Establishment and characterization of cell lines from human small cell and large cell carcinomas of the lung. *Acta Pathol Microbiol Immunol Scand [A]* 1985;93:133–47.
- Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184–91.
- Cheong N, Perrault AR, Iliakis G. *In vitro* rejoining of DNA double strand breaks: a comparison of genomic-DNA with plasmid-DNA-based assays. *Int J Radiat Biol* 1998;73:481–93.
- Pastwa E, Neumann RD, Winters TA. *In vitro* repair of complex unligatable oxidatively induced DNA double-strand breaks by human cell extracts. *Nucleic Acids Res* 2001;29:E78.
- Lazo JS, Hait WN, Kennedy KA, Braun ID, Meandzija B. Enhanced bleomycin-induced DNA damage and cytotoxicity with calmodulin antagonists. *Mol Pharmacol* 1985;27:387–93.
- Hendrickson EA, Qin XQ, Bump EA, Schatz DG, Oettinger M, Weaver DT. A link between double-strand break-related repair and V(D)J recombination: the scid mutation. *Proc Natl Acad Sci U S A* 1991;88:4061–5.
- Tanaka T, Yamagami T, Oka Y, Nomura T, Sugiyama H. The scid mutation in mice causes defects in the repair system for both double-strand DNA breaks and DNA cross-links. *Mutat Res* 1993;288:277–80.
- Collis SJ, DeWeese TL, Jeggo PA, Parker AR. The life and death of DNA-PK. *Oncogene* 2005;24:949–61.
- Hait WN, Gesmonde JF, Lazo JS. Effect of anti-calmodulin drugs on the growth and sensitivity of C6 rat glioma cells to bleomycin. *Anticancer Res* 1994;14:1711–21.

27. Matsushima N, Hayashi N, Jinbo Y, Izumi Y.  $\text{Ca}^{2+}$ -bound calmodulin forms a compact globular structure on binding four trifluoperazine molecules in solution. *Biochem J* 2000;347 Pt 1:211–5.
28. Hanakahi LA, Bartlet-Jones M, Chappell C, Pappin D, West SC. Binding of inositol phosphate to DNA-PK and stimulation of double-strand break repair. *Cell* 2000;102:721–9.
29. Hanakahi LA, West SC. Specific interaction of IP6 with human Ku70/80, the DNA-binding subunit of DNA-PK. *EMBO J* 2002;21:2038–44.
30. Ma Y, Lieber MR. Binding of inositol hexakisphosphate (IP6) to Ku but not to DNA-PKcs. *J Biol Chem* 2002;277:10756–9.
31. Byrum J, Jordan S, Safrany ST, Rodgers W. Visualization of inositol phosphate-dependent mobility of Ku: depletion of the DNA-PK cofactor InsP6 inhibits Ku mobility. *Nucleic Acids Res* 2004;32:2776–84.
32. Ori Y, Herman M, Chagnac A, Malachi T, Gafter U, Korzets A. Spontaneous DNA repair in human mononuclear cells is calcium-dependent. *Biochem Biophys Res Commun* 2005;336:842–6.
33. Wang Y, Mallya SM, Sikpi MO. Calmodulin antagonists and cAMP inhibit ionizing-radiation-enhancement of double-strand-break repair in human cells. *Mutat Res* 2000;460:29.

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*Mol Cancer Ther* 2007;6:2303-2309.

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