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Pentoxifylline Inhibits Gene-Specific Repair of UV-Induced DNA Damage in Hamster Cells

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SUMMARY We have studied the effect of pentoxifylline (PTX) on DNA repair after ultraviolet radiation (UV) in Chinese hamster ovary cells (CHO). DNA repair of cyclobutane pyrimidine dimers (CPDs) was measured in the dihydrofolate reductase (DHFR) gene and in a downstream nontranscribed genomic region. Pentoxifylline (1 mM) inhibited the repair of CPDs in the hamster DHFR gene by 32% after 8 hr of repair incubation. This decrease in repair of CPDs in the DHFR gene correlated with an enhancement of UV-induced cell killing by PTX. The UV doses required for 37% survival after incubation with 0 and 1 mM PTX were 6.2 J/m^2 and 2.9 J/m^2, respectively. This represents twofold more UV-induced cytotoxicity in irradiated cells in the presence of PTX. We then evaluated the effect of PTX on RNA transcription and cell cycle kinetics. Incubation of UV-irradiated CHO cells with PTX had no effect on the transcription of the DHFR gene. PTX did not produce a significant effect on cell cycle progression during 8 hr after UV-irradiation. However, by 24 hr after irradiation, incubation with PTX induced a distinct block in early S-phase. We conclude that PTX sensitizes CHO cells to UV-irradiation, perhaps because it inhibits DNA repair of active genes. *Radiat Oncol Invest 1996;4:115–121.* **© 1996 Wiley-Liss, Inc.**

Key words: pentoxifylline, DNA repair, cyclobutane pyrimidine dimer, UV light, cell cycle

INTRODUCTION

Pentoxifylline (PTX), a methylxanthine, can potentiate the cytotoxicity in cells exposed to DNA damaging agents. PTX is especially effective as a cell sensitizer for alkylating agents. PTX sensitizes the effect of alkylating agents on hematological malignancies from 2- to 4.2-fold [1]. PTX enhances the antitumor effect of thiotepa against bladder, and breast cancer cells both in vitro and in vivo [2]. Osteosarcoma cells exposed to cisplatin and PTX showed decreased proliferation effects compared to cisplatin alone [3]. PTX also increased the chemosensitization of human cervical and ovarian cell lines to cisplatin [4]. PTX enhancement of cisplatin activity also occurs in vitro and in vivo against breast carcinoma cell lines [5]. PTX potentiation also occurs in more complex drug combinations. For example, PTX and etoposide in combination are effective at increasing antitumor activity of alkylating agents [6]. PTX can also potentiate nonalkylating chemotherapy agents. PTX potentiates the cell killing effect of vincristine in P388 leukemia cells [7]. Finally, PTX can inhibit the release of cytokines [8]. Whether investigators can use these

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Abbreviations: PTX, pentoxifylline; UV, ultraviolet; CPDs, cyclobutane pyrimidine dimers; CHO, Chinese hamster ovary; FdUrd, fluorodeoxyuridine; BrdUrd, bromodeoxyuridine; DHFR, dihydrofolate reductase.

cellular effects to improve anticancer therapy is unknown. Nonetheless, clinicians are now evaluating PTX as an enhancing agent for antitumor chemotherapeutics in human clinical trials [9]. Treatment of cells with caffeine, a related methylxanthine, also enhances the cytotoxicity of some anticancer drugs, X-irradiation, and UV irradiation [10–12]. But PTX, unlike caffeine, has a favorable toxicity profile and can be administered to humans at levels approaching the in vitro concentrations used to potentiate DNA damaging agents.

We have previously demonstrated that caffeine can inhibit the gene-specific repair of UV-induced DNA damage in CHO cells from the active DHFR gene [13]. Our current project sought to determine if PTX could mediate similar effects on DNA repair and cytotoxicity, since PTX may become an important agent in clinical studies. Inhibition of gene-specific DNA repair may be responsible for the increased cytotoxicity observed in the presence of PTX after DNA-damaging treatments. In this work the effect of PTX on gene-specific repair in UV-irradiated CHO cells was examined. We also studied the effects of PTX on cell survival, steady state levels of RNA transcription, and cell cycle progression in UV-irradiated CHO cells. We found that in CHO cells, PTX inhibits the removal of cyclobutane pyrimidine dimers (CPDs) from the DHFR gene at 8 hr after irradiation and decreases cell survival under the same conditions, but has no effect on steady state RNA transcription or cell cycle progression.

MATERIALS AND METHODS

Isotopes, Enzymes, Chemicals, and Hybridization Probes

[Methyl-^{3}H]thymidine (>80 Ci/mmol) and [^{32}P] dCTP (3,000 Ci/mmol) was purchased from Du Pont-New England Nuclear (Boston, MA). Restriction endonuclease Kpn 1 was purchased from Boehringer Mannheim, New Haven, CT. A 250 mM stock solution of PTX (Sigma Chemical Co., St. Louis, MO) was prepared by dissolving the powdered chemical in sterile, distilled H_2O and stored at −20°C. The PTX (final concentration 1.0 mM) was added to repair media immediately after cells were UV-irradiated. The DNA probes for the DHFR gene (pMB5) and the downstream, noncoding region (cs-14DO) have been previously described [14]. The pMB5 probe detects a 14 kb Kpn I fragment containing the 5′ half of the DHFR gene, and the cs-14DO probe detects a downstream, nontranscribed 14 kb Kpn I fragment.

Cell Culture

The Chinese hamster ovary cell line CHO-B11 that contains an amplified DHFR gene, was grown in Ham's F12 media without glycine, thymidine, or hypoxanthine and supplemented with 10% fetal calf serum (dialyzed) from Gibco (BRL), Gaithersburg, MD [15]. Cells were maintained in 500 nM methotrexate and grown in humidified 5% CO_2, 95% air at 37°C. All experiments were done with cells growing in exponential phase.

Cell Survival Determination

A clonogenic assay was performed under standard tissue culture conditions as described above. Exponentially growing CHO cells in 10 cm dishes were irradiated with 10 or 20 J/m^2 UV light (254 nm) or mock irradiated and then incubated in the absence or presence of PTX (1 mM) for 8 hr at 37°C. The cells were then rinsed, trypsinized, plated (10^2–10^5 cells/dish) and incubated for macroscopic colony formation. Following a 14 day incubation, colonies were fixed with methanol and acetic acid (3:1), stained with crystal violet, and the number of macroscopic colonies containing more than 50 cells were counted. The plating efficiences were determined with and without PTX. All survival points were done in triplicate.

Gene-Specific DNA Repair

The procedure for measuring DNA repair in defined sequences is described in detail elsewhere [16]. Cells were incubated for 3–4 days with 0.3 μCi/ml [^{3}H]thymidine (>80 mCi/mmol) to label the DNA uniformly. The cells were UV-irradiated at 10-20 J/m^2 with a germicidal lamp, and then harvested either immediately or after 8 or 24 hr of repair incubation with or without 1.0 mM PTX. Cells were incubated in media containing 10 μM bromodeoxyuridine (BrdUrd) and 1 μM fluorodeoxyuridine (FdUrd) during repair incubation. The DNA was phenol or salt extracted, treated with RNase and resuspended. DNA was digested with restriction endonuclease Kpn I. Samples were then centrifuged to equilibrium in CsCl gradients, and the fractions containing parental density labeled DNA were pooled, dialyzed, and concentrated by ethanol precipitation. DNA aliquots (2 μg) were removed from each sample, treated with or without T4 endonuclease V, and electrophoresed on 0.5%–0.7% alkaline agarose gels. After standard gel washes, the DNA was transferred to a nylon support membrane (Oncor Inc., Gaithersburg, MD). Pre-hybridization, hybridization, and washes were as previously de-

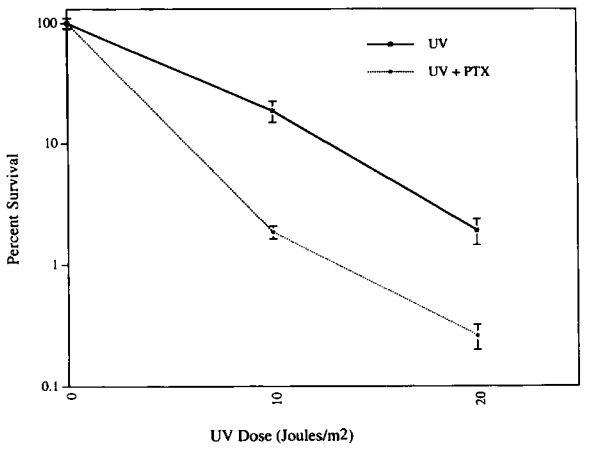

Fig. 1. Hamster cell survival after UV radiation and pentoxifylline. Clonogenic assay was done with exponentially growing CHO cells in dishes irradiated with a dose of 0, 10, or 20 J/m^2 UV and then incubated for 8 hr with (UV + PTX) or without (UV) 1.0 mM PTX at 37°C. The cells were then re-plated and incubated for colony formation. Following a 14 day incubation, colonies were fixed, stained, and counted. All survival points were done in triplicate. Values are plotted as mean ± S.D.

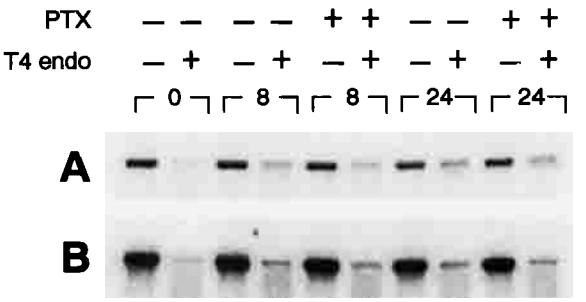

Fig. 2. Effect of pentoxifylline on gene-specific repair in hamster cells after UV radiation. DNA was isolated from CHO cells after irradiation with 20 J/m^2 UV light and cells were then allowed to repair for 8 or 24 hr with or without PTX. Samples were run in parallel (+) or (−) T4 endonuclease treatment with (+) or without (−) PTX (1.0 mM) as indicated. **A:** Support membrane probed for 14 kb region containing the 5′ portion of the DHFR gene and **(B)** 14 kb region containing downstream of the DHFR gene.

scribed [16]. Hybridization was carried out at 45°C in 5 ml Hybrisol (Oncor Inc.), using random primed ^{32}P-labelled DNA probes (approximately 1 × 10^9 cpm/μg) in the amount of 4 × 10^7 cpm. After standard washes of the support membrane, the final wash was in 0.1 × SSPE, 0.1% SDS at 65°C. Support membranes were exposed to Kodak XAR5 film. Autoradiograms were scanned by a Shimadzu densitometer or the membranes were analyzed directly using a Betagen blot analyzer (Betagen Corp., Waltham, MA). The calculations of CPDs frequencies were determined as previously described [16].

Cell Cycle Analysis

Samples were fixed and stained according to the procedure published by Nicoletti et al. [17]. Briefly, cells (1–2 × 10^6) were UV-irradiated or mock irradiated and incubated with or without PTX. At 0, 8, or 24 hr post UV-irradiation, the cells were harvested and resuspended in 1.5 ml 0.1% sodium citrate and 0.1% Triton X100 (Sigma Chemical Co., St. Louis, MO) staining solution containing 50 μg/ml propidium iodide (PI) (Sigma). Samples were left overnight at 4°C and then filtered through a 60 μm nylon mesh to remove any debris. DNA data was collected using a Coulter Epics V cell sorter (Coulter Inc., Hileah, FL). The PI was excited using the 488 nm line of an argon ion laser while the PI fluorescence was detected using 515/530 nm interference and long pass filters. Histograms were generated from cytometric analysis of approximately 1.5–2 × 10^4 events (cells).

RNA Isolation and Northern Analysis

RNA was isolated by a single step acid guanidinium thiocyanate procedure as previously described [18]. Ten microgram of total RNA was electrophoresed on 1% agarose gels containing formalin, then stained with ethidium bromide, and blotted onto Nylon membranes (Sure Blot, Oncor, Gaithersburg, MD). Hybridization was carried out overnight in Hybrisol (Oncor) to either pMB5 (DHFR) or actin probe (Lofstrand Labs, Gaithersburg, MD). Membranes were washed and subjected to autoradiography. Densitometric analysis was performed and the level of DHFR gene transcription was calculated relative to actin gene RNA.

RESULTS

Effect of PTX and UV Radiation on Hamster Cell Survival

CHO cells were UV-irradiated or mock irradiated, then incubated in the absence or presence of PTX (1 mM) for 8 or 24 hr. Results of these survival experiments are summarized in Figure 1. Cells were then re-plated and incubated for colony formation. Incubation of cells in PTX without UV radiation resulted in no reduction in cell survival. After 10 J/m^2 of UV radiation, incubating the culture with 1.0 mM PTX caused a significant increase in cell

Table 1. Effect of Pentoxifylline on Gene-Specific Repair in Hamster Cells†

		DHFR		Noncoding region (cs-14DO)	
Time (hr)	PTX (mM)	CPDs	% Repair	CPDs	% Repair
0	0	1.93 ± .25	0	1.75 ± .04	0
8	0	1.20 ± .01*	38	1.57 ± .25	10
8	1.0	1.42 ± .04*	26	1.70 ± .13	2.9
24	0	0.84 ± .04	56	1.60 ± .16	8.5
24	1.0	0.81 ± .07	58	1.73 ± .40	1.0

†All values are determined from 2 biological experiments and are expressed as mean CPDs per DNA fragment ± S.D.
*Significant difference with $P = 0.02$. P value determined by unpaired t-test between CPDs remaining after 8 hr from UV-irradiated cells incubated with PTX to CPDs remaining in UV-irradiated cells incubated without PTX.

killing (18.6% survival decreased to 1.84%). A similar increase occurs at 20 J/m^2. The UV radiation dose required for 37% survival after incubation with 0 and 1 mM PTX were extrapolated to be 6.2 and 2.9 J/m^2, respectively. The dose modification factor (the ratio of the doses which result in 37% survival) revealed a twofold increased enhancement in UV-induced cytotoxicity in the presence of PTX.

Effect of PTX on CPDs Repair of the Hamster DHFR Gene and Downstream Region

Repair analysis was performed in the parental (non-replicated) DNA from exponentially growing Chinese hamster ovary cells that were exposed to 20 J/m^2 and incubated for up to 24 hr after irradiation in the presence of BrdUrd with or without PTX. The frequency of CPDs was measured in both the transcriptionally active, DHFR gene (Fig. 2A) and an inactive region downstream of DHFR (Fig. 2B). CPD repair was measured as the removal of T4 endonuclease V sensitive sites as a function of time. The number of CPDs remaining after 8 hr of repair incubation with 1.0 mM PTX was significantly higher than the CPDs remaining in irradiated cells incubated without drug ($P = 0.02$ by unpaired t-test). By 24 hr of repair incubation, the level of repair was comparable to that found in cells incubated without PTX. The results in Table 1 show inhibition of gene-specific DNA repair by PTX at 8 hr with 32% inhibition of the repair of CPDs from the DHFR gene. The difference in number of CPDs remaining in the downstream region (cs-14DO) after 8 or 24 hr of repair incubation with or without PTX (1.0 mM) was not statistically significant in UV-irradiated cells (Table 1). There was no or little repair of the inactive region.

Effect of PTX on DHFR Gene Transcription in UV-Irradiated CHO Cells

To determine whether the effect of PTX on repair was mediated through an effect on DHFR gene transcription, Northern analysis of RNA transcription was performed on the total RNA from exponentially growing Chinese hamster ovary cells that were exposed to 20 J/m^2 and incubated for up to 24 hr after irradiation in the presence of BrdUrd with or without PTX (Fig. 3). The relative steady state levels of transcripts from the DHFR gene compared with the actin gene, after UV damage with and without PTX did not vary significantly (Table 2). Thus, the PTX related inhibition of CPDs gene-specific repair was not associated with a decrease in steady state RNA transcripts at a level detectable by Northern analysis.

Effect of PTX on Cell Cycle in UV-Irradiated CHO Cells

To determine whether the effect of PTX on repair was related to altered progression through the cell cycle, flow cytometry was performed on irradiated populations in the presence or absence of PTX (Fig. 4). These histograms indicated little cell cycle changes occurring 8 hr after UV exposure (Fig. 4B); 24 hr after irradiation it appeared that a G_2/M block (as evidenced by the increased G_2/M peak) and possibly a slower S-phase transit time (as evidenced by the increased S phase fraction) occurred in the irradiated cells without PTX (Fig. 4C). At 8 hr after UV-irradiation, there were no significant differences in cell cycle progression between cells incubated without compared to with 1 mM PTX (Fig. 4B and E). At 24 hr after irradiation, however, PTX produced a distinct block in early S phase in the

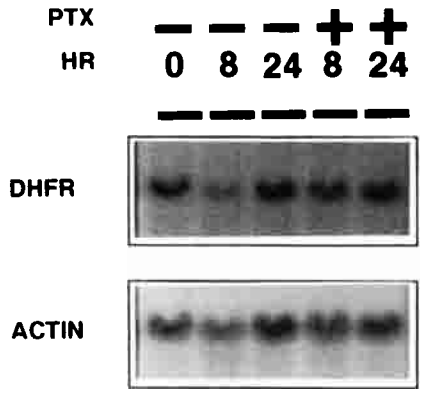

Fig. 3. Northern analysis of pentoxifylline on cells after UV irradiation. Autoradiograms of Northern blot derived from exponentially growing CHO cells irradiated with 20 J/m^2 UV and then incubated for 0, 8, or 24 hr with (+) or without (−) 1.0 mM PTX at 37°C. The same membrane was probed with the pMB5 probe to detect the DHFR gene transcript **(top)** and the actin probe **(bottom).** PTX: pentoxifylline; HR: hour.

Table 2. Northern Analysis of DHFR Gene Expression*

Time (hr)	PTX (mM)	DHFR (relative expression)
0	0	1.07 ± .07
8	0	0.83 ± .27
8	1.0	1.09 ± .11
24	0	0.92 ± .06
24	1.0	1.06 ± .26

*Values for DHFR gene relative expression were determined by dividing the mean band intensities obtained from 3 different membranes for the DHFR gene by the mean band intensities from 2 of those same membranes stripped and probed for actin gene expression. Values are expressed as mean ± S.D.

irradiated cells (Fig. 4F), but not in the irradiated cells incubated without PTX.

DISCUSSION

PTX potentiates the cytotoxicity of a number of DNA-damaging, chemotherapeutic agents including: alkylating agents [1,2,10], cisplatin [3,4,5], and vincristine [7]. The mechanism(s) of this enhancement is not clearly established, because the drug has complex effects on different cellular mechanisms. Our previous report demonstrated that caffeine, another methylxanthine, inhibited gene-specific repair of the DHFR gene in CHO cells [13]. We sought to explore the role that DNA repair inhibition might play in the potentiation of DNA-damaging agents by PTX. In CHO cells, repair of CPDs only occurs in actively transcribed sequences (transcription-coupled repair). In this study, we found that PTX inhibited the DNA repair of UV-induced CPDs from the active DHFR gene in CHO cells. Our prior experiments did not demonstrate overall repair inhibition of CPDs by caffeine in CHO cells, but these data do not exclude the possibility that PTX might also inhibit overall DNA repair [13]. In our experiments, PTX only inhibited the gene-specific repair at 8 hr after UV-irradiation. Therefore, PTX inhibits the rate of CPDs repair but not the final extent of repair. This gene-specific repair inhibition in CHO cells correlates with a twofold enhancement of UV-induced cell killing by PTX. Some alkylating agents (nitrogen mustard) also demonstrate gene-specific repair, while others agents do not (dimethyl sulfate) [19,20]. Thus, the enhancement of tumor cell cytotoxicity by PTX after alkylating treatment (with nitrogen mustard) might be secondary to an inhibition of DNA repair [10].

Caffeine has been shown in vitro to inhibit the ability of certain bacterial recognition proteins (UvrA) to bind to UV-induced DNA damage [21]. Thus, methylxanthines can specifically inhibit DNA binding proteins and in this way might affect transcription and/or (transcription-coupled) repair. However, our Northern analysis did not show any effect of PTX on DHFR gene transcription, indicating that our observed slower rate of (transcription-coupled) repair in the presence of PTX is not due to a decreased rate of transcription. Thus, the likely scenario is that PTX inhibits nucleotide excision repair processes, perhaps interfering with the transcription-repair coupling factor (TCRF) that links gene-specific repair to transcription [22].

Cell cycle effects are important for other methylxanthines such as caffeine [23]. Pentoxifylline abrogates the cell cycle arrest induced by aphidocolin or nitrogen mustard in human lymphoma cells [24]. PTX reverted the activity of cyclin A- and B1-kinases in nitrogen mustard treated cells to approximately normal, through a disruption of the signalling pathway that regulates cell cycle arrest after DNA damage [24]. Thus, PTX may permit cell cycle progression despite growth arrest signals after DNA damage. This may result in mutations, chromosome aberrations, or cell death. Since active genomic regions are repaired first, if insufficient time passes for repair to take place then this damage remains (or its repair is delayed). Other evidence also suggests a connection between the cell cycle and DNA repair. A strand bias of DNA repair and mutations has been described for both hamster and human cells [25–27]. Therefore an effect of PTX on cell cycle kinetics could be the reason for our observed inhibition of repair. However, there was no effect

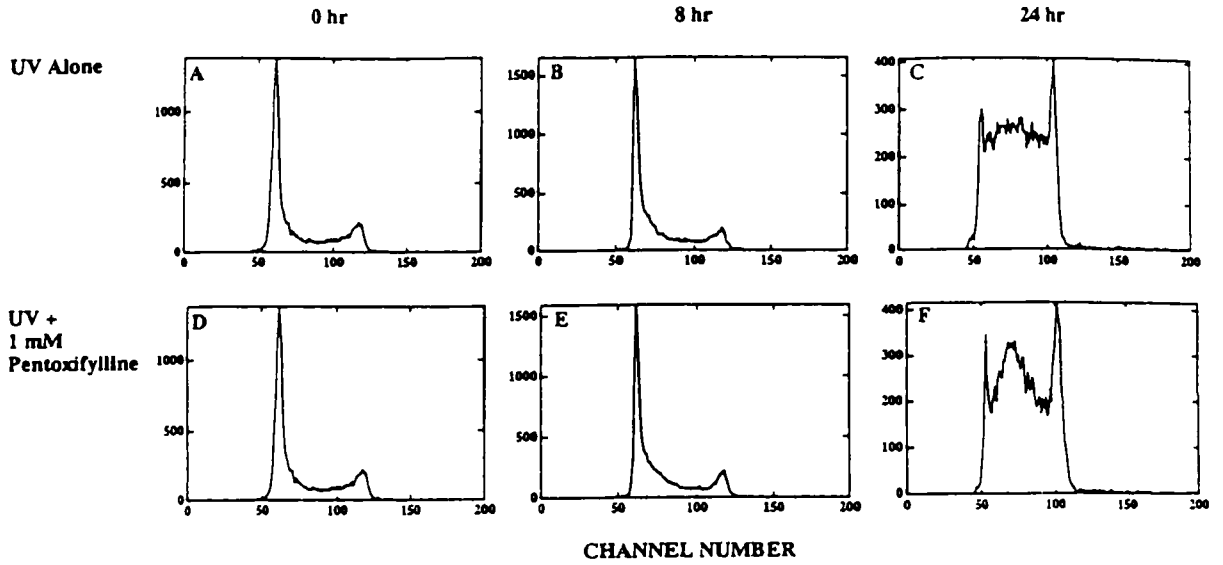

Fig. 4. Cell cycle effects of pentoxifylline on cells after UV irradiation. Single parameter DNA analysis of **(A)** control cells immediately after UV exposure, **(B)** cells 8 hr after a 20 J/m^2 pulse of UV irradiation, **(C)** cells 24 hr after the UV pulse, **(D)** control cells + 1.0 mM PTX immediately after the UV pulse, **(E)** cells + 1.0 mM PTX 8 hr after the UV pulse and, **(F)** cells + 1.0 mM PTX 24 hr after the UV pulse. X-axis represents increasing DNA content while the y-axis represents cell number.

of PTX (1.0 mM) on cell cycle distribution at 8 hr after UV irradiation, while gene-specific repair was inhibited during this interval. However, limited cell cycle progression occurred by 8 hr after UV irradiation and this might have made the evaluation of subtle PTX effects on cell cycle progression at 8 hr difficult to observe. Nonetheless, the observation suggest that the decreased DNA repair is due to more direct effects on repair processes, not to major PTX-induced change in cell cycle progression.

Our experiments illustrate that changes in gene specific repair affect survival of cells, i.e., the gene specific repair is a significant determinant of the biological outcome. More detailed examination of CPD repair at nucleotide resolution during different parts of the cell cycle might also provide insight, and such techniques have recently become available [28]. Our finding of DNA repair inhibition by PTX in CHO cells may have relevance in designing improved cancer therapy. Clinical attempts to potentiate the cytotoxicity of DNA-damaging chemotherapeutic agents by PTX are now underway [29]. In clinical trials, the achieved dose of PTX should approach the concentrations at which PTX can sensitize a cell to DNA damaging agents [2]. Further understanding of the effects of PTX on DNA repair may aid these efforts.

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