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Camptothecin enhances the frequency of oligonucleotide-directed gene repair in mammalian cells by inducing DNA damage and activating homologous recombination

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ABSTRACT

Camptothecin (CPT) is an anticancer drug that promotes DNA breakage at replication forks and the formation of lesions that activate the processes of homologous recombination (HR) and nonhomologous end joining. We have taken advantage of the CPT-induced damage response by coupling it to gene repair directed by synthetic oligonucleotides, a process in which a mutant base pair is converted into a wild-type one. Here, we show that pretreating DLD-1 cells with CPT leads to a significant stimulation in the frequency of correction of an integrated mutant enhanced green fluorescent protein gene. The stimulation is dose-dependent and coincident with the formation of double-strand DNA breaks. Caffeine, but not vanillin, blocks the enhancement of gene repair suggesting that, in this system, HR is the pathway most responsible for elevating the frequency of correction. The involvement of HR is further proven by studies in which wortmannin was seen to inhibit gene repair at high concentrations but not at lower levels that are known to inhibit DNA-PK activity. Taken together, our results suggest that DNA damage induced by CPT activates a cellular response that stimulates gene repair in mammalian cells.

INTRODUCTION

The antitumor agent camptothecin (CPT) (1–3) induces DNA breakage at sites often associated with replication forks (4). CPT exerts its effect during S phase by creating a cleavable nucleoprotein complex, a transient intermediate in the topoisomerase I reaction cycle (5–8). This complex interferes with the DNA replication process resulting in DNA damage, which can eventually take the form of double-strand breaks (DSBs). The repair of these lesions is catalyzed by several cellular pathways including nonhomologous end joining (NHEJ) and homologous recombination (HR). It is generally believed that NHEJ precedes HR in damage response as it rejoins broken chromosomal ends to ensure cell viability. The HR response takes longer to engage, but once activated it is responsible for the repair of residual DNA damage. Evidence for the central role played by HR comes from studies in which compounds that retard, stall or inhibit replication induce sister chromatid exchange, a process that utilizes the enzymes of the HR pathway (9–14). In addition, cells deficient in HR are more sensitive to DSBs associated with replication forks than cells deficient in NHEJ (9). The appearance of DSBs, in response to altered replication activities, leads to a relocalization of the HR recombinase, RAD51 (9–11), an indication that DNA lesions have activated the HR pathway. But NHEJ could also play an important role in the repair of lesions at replication forks as shown by Allan *et al*. (15), who demonstrated that inhibition of a critical component of the NHEJ pathway may affect HR initiation. This conclusion is consistent with the observations of Saintigny *et al*. (13) who showed that NHEJ may overlap with the early stages of HR but not with the latter stages that include heteroduplex formation.

We have been studying the mechanism of targeted gene repair directed by modified single-stranded oligonucleotides in yeast and mammalian cell systems [for review see (16–18)]. The current model includes a step in which the oligonucleotide aligns with its complementary DNA sequence within the chromosome, except for a single mismatch located at the center of the paired DNA strands. Recently, we proposed that the initial (pairing) phase of the gene repair reaction is catalyzed by enzymes involved in HR. These enzymes subsequently recruit members of a DNA repair pathway(s) to facilitate the actual base exchange which produces the altered phenotype (19). Results from experiments in yeast support the concept of pairing followed by repairing (18,20,21), and recent data from several groups working on mammalian cells confirm the involvement of HR (22,23 and references therein). DNA replication has also been shown to enhance the frequency of targeted gene repair presumably by destabilizing the chromatin structure (24; Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted), while the addition of hydroxyurea or thymidine, which stall replication forks, elevates the correction efficiency (CE) even further (25,26).

In this study, we utilize CPT to induce DNA lesions and stall replication forks as a way to stimulate the HR pathway. The

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hypothesis is that such action will elevate the level of HR enzymes that not only facilitate lesion repair but also coincidently promote efficient gene repair directed by an oligonucleotide. CPT was chosen as the agent because its impact is seen predominantly at replication forks and because recent studies indicate that the cleavable complex is converted to DSBs during the course of the reaction (4,27,28). This form of DNA damage is known to activate the recombinational repair (NHEJ and HR) pathways. Our results reveal that the addition of CPT to mammalian cells prior to the introduction of the oligonucleotide leads to DSBs, relocalization of RAD51 to the nucleus and an increase in the frequency of targeted gene repair presumably driven by the HR pathway.

MATERIALS AND METHODS

Cell line and culture conditions

The DLD-1 cell line was obtained from ATCC (American Type Cell Culture, VA). Cells were grown in RPMI 1640 medium with 2 mM glutamine (GIBCO, Invitrogen, Carlsbad, CA), 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate and supplemented with 10% FBS. The DLD-1-integrated clone 1 was obtained by integration of the pEGFP-N3 vector (Clontech, CA) containing a mutated enhanced green fluorescent protein (EGFP) gene as previously described (Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted). The mutation introduces a stop codon at position 67 rendering the protein nonfunctional. As directed by the oligonucleotide, the stop codon is converted to a tyrosine (wild-type EGFP) resulting in the expression of a functional EGFP. Clone 1 was maintained under selection in media containing 200 μg/ml Geneticin (Gibco, Invitrogen Co., CA).

EGFP gene targeting

Cells grown in a complete medium supplemented with 10% FBS were trypsinized and harvested by centrifugation. The pellet was then resuspended in a serum-free medium, at a density of 1.5×10^6 cells/100 μl, and transferred to a 4 mm gap cuvette (Fisher Scientific, PA). The oligonucleotide was added to a final concentration of 4 μM and the cells were electroporated (250 V, 13 ms, 2 pulses, 1 s interval) using a BTX Electro Square Porator^{TM} ECM 830 (BTX Instrument Division, MA). The cells were transferred to a 60 mm dish containing fresh medium supplemented with 10% FBS and incubated at 37°C for 48 h before harvesting for fluorescence activated cell sorter (FACS) analysis.

Drug treatment

Twenty-four hours prior to addition of CPT, caffeine, vanillin or wortmannin, cells were seeded at a density of 1×10^6 in a 100 mm dish. The CPT (Sigma-Aldrich, MO) stock solution was made up to 25 mM in DMSO, the caffeine (Sigma-Aldrich, MO) stock solution to 200 mM in H_2O, the vanillin (Acros Organics, Morris Plains, NJ) stock solution to 250 mM in H_2O and the wortmannin (Sigma-Aldrich, MO) stock solution to 10 mM in DMSO. These agents were added to the cells at the specified concentrations for 24 h prior to the electroporation of the oligonucleotide. In some cases, the cells were treated with CPT for 24 h, then washed twice in 1× PBS (GIBCO, Invitrogen, Carlsbad, CA) followed by incubation for an additional 24 h in fresh medium or in the presence of caffeine.

Flow cytometry analysis

EGFP fluorescence of corrected cells was measured by a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson, NJ). Cells were harvested 48 h after electroporation and resuspended in an FACS buffer (0.5% BSA, 2 mM EDTA, 2 μg/ml propidium iodide in PBS). For cell-cycle analysis, 10^6 cells were plated 24 h before treatment with CPT, after which the cells were trypsinized, resuspended in 300 μl of cold PBS and fixed by adding 700 μl of cold ethanol (70%). Cells were incubated at 4°C for 16 h, then washed and resuspended in 0.5 ml of 1× PBS containing final concentrations of 200 μg/ml RNaseA, 10 μg/ml propidium iodide and 1% FBS and analyzed for DNA content.

Pulsed-field gel electrophoresis (PFGE)

Twenty-four hours before treatment, 10^6 cells were plated in tissue culture flasks and then CPT or VP16 was added for an additional 24 h. The cells were released by trypsinization and melted in agarose inserts. The agarose inserts were incubated in 0.5 M EDTA–1% *N*-laurosylsarcosine–proteinase K (1 mg/ml) at 50°C for 48 h and then washed four times in TE buffer prior to loading on a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad, CA); separation was achieved by PFGE for 24 h, 4 V/cm, 60–240 s switch time (Bio-Rad, CA).

RAD51 foci staining

Cells were seeded onto microscope slides and treated with 30 nM CPT for 24 h. Following treatment, cells were washed twice in PBS and fixed in 3% (w/v) paraformaldehyde for 20 min. Cells were washed again in PBS-T (PBS containing 0.1% (v/v) Triton X-100) and normal goat serum (Zymed Laboratories, San Francisco, CA) was added 30 min prior to incubation with the anti-RAD51 antibody (H92, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1 : 1000 in PBS–3% BSA for 16 h at 4°C. Cells were rinsed four times in PBS-T (15 min each time) and incubated for 1 h at room temperature with a Cy-3-conjugated goat anti-rabbit antibody (Zymed Laboratories, San Francisco, CA) at a dilution of 1 : 500 in PBS–3% BSA. The chambers were washed four times in PBS-T (15 min each wash) and mounted using a SlowFade Antifade Kit containing DAPI (Molecular Probes, Eugene, OR). Images were obtained with a Zeiss 510 NLO inverted confocal microscope using 40× Plan-Neofluar (NA 1.3) oil immersion objectives. A 543 nm helium–neon laser with a 560 long pass emission filter and a 747 nm 5W Mira 900 fs mode-locked Ti:sapphire laser with a 390–465 IR band pass emission filter were used to detect the antibody and DAPI fluorescence, respectively. Nuclei containing more than five foci were classified as RAD51 positive. The same protocol was followed for the experiments in which the cells were treated with both CPT and caffeine.

RESULTS

The assay system

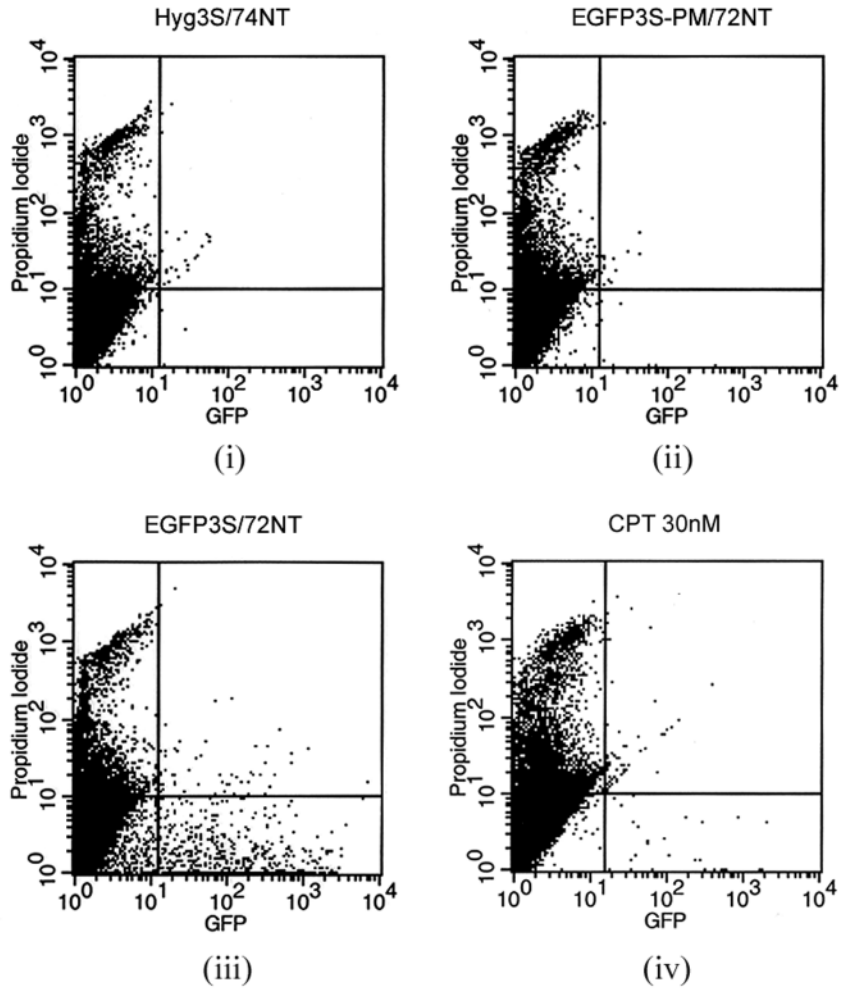
Gene correction was monitored using DLD-1 cells, a colorectal cancer cell line containing three to five copies of an

integrated, mutant EGFP gene (25,29; Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted). The mutation in EGFP is at residue 67 where a stop codon (TAG) has replaced the normal tyrosine codon (TAC). Repair of this point mutation restores the wild-type version of the EGFP gene resulting in the production of green fluorescent protein (Figure 1). The oligonucleotides designed to reverse this null mutation are also shown in Figure 1. EGFP3S/72NT is 72 bases in length, bears three phosphorothioate linkages at each terminus and targets the nontranscribed strand of the gene. A specific mismatched base pair is created when the oligonucleotide aligns with the target site via base complementarity. In this case, a G–G mismatch forms between the two strands and correction of the G residue to a C regenerates the tyrosine codon and functional protein activity. Previous studies have established the nontranscribed strand as being more amenable to gene repair activity than the transcribed strand of EGFP (24,25) although other targets do not exhibit strand bias uniformly (19,30). Also depicted in Figure 1 are a 72-mer bearing perfect complementarity to the nontranscribed strand at the site of the EGFP mutation, and a nonspecific 74-mer bearing no sequence complementarity to the target site.

Expression of EGFP quantitated by FACS analyses was used as a measure of gene repair activity. The validity of this type of assay has already been established in previous studies (24–26,31; Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted); it represents the emergence of a phenotypic change within a genetically altered cell. RT–PCR analyses of the gene demonstrate that transcriptional activity of EGFP is robust in this cell line (data not shown). Gene repair assays were initiated by electroporating the oligonucleotide into a 100 µl volume of fresh media containing 1.5×10^6 cells. The cells were then allowed to recover for 48 h and prepared for FACS analyses (see Materials and Methods). The percentage of cells expressing functional EGFP was calculated by a CellQuest and GFP/PI program. Dual analyses of EGFP (correction) and PI staining (cell death) were performed for each sample, which, in each case, consisted of 50 000 gated cells.

Figure 2 illustrates FACS profiles of DLD-1 cells treated with each type of oligonucleotide. As predicted, the background (control) level of conversion exhibits no detectable EGFP emitting cells as scored in the lower right quadrant (26). The addition of the perfectly matched or the nonspecific oligonucleotide does not induce gene repair at a level detectable by FACS. In contrast, the introduction of EGFP3S/72NT leads to a significant level of gene repair. We have measured conversion events (fluorescent green cells) for both viable and nonviable cells (lower and upper right quadrants) but the percentage correction is calculated using only the viable, corrected cell count. Since CPT is widely used in this study, we also present an addition control. The cells

Figure 1. Sequence of the wild-type, mutant EGFP gene and targeting oligonucleotides. The sequences of the wild-type and mutant EGFP genes are presented with the target codon in bold. Also depicted are the DNA sequences of the 72-mer specific oligonucleotide (EGFP3S/72NT), the 72-mer perfect match oligonucleotide (EGFP3S-PM/72NT) and the 74-mer nonspecific oligonucleotide (Hyg3S/74NT); asterisks indicate phosphorothioate linkages and the oligos are displayed with a 5′ to 3′ polarity. The oligonucleotide EGFP3S/72NT, directed to the nontranscribed strand of the mutant strand, converts the stop codon to a functional, corrected EGFP expressing cell.



Expt	# Converted cells/50000	CE (%)	Viability %
1	811	1.62	87.53
2	684	1.37	95.18
3	465	0.93	95.80
Average +/- SD		1.31 +/- 0.35	

Figure 2. FACS analysis of DLD-1 clone-1 corrected cells. Cells were analyzed for EGFP expression and viability by FACS analyses. Cells were electroporated with either the (i) nonspecific oligonucleotide, Hyg3S/74NT, or (ii) a perfectly matched oligonucleotide, EGFP3S-PM/72NT, while (iii) represents cells that were targeted with the specific oligonucleotide, EGFP3S/72NT, and (iv) represents electroporated cells that were treated with CPT (30 nM) for 24 h without oligonucleotide. The table below the FACS profiles represents the average CEs and SDs, when targeted using a specific oligonucleotide (EGFP3S/72NT). Cell death was determined using propidium iodide staining.

are incubated with CPT for 24 h and electroporated without the oligonucleotide. As shown in Figure 2(iv), only a background level of green cells is observed indicating that CPT alone does not induce the expression (nor the correction) of wild-type EGFP. Although we have performed this experiment many times, we list the results of three typical, independent experiments in the table presented as part of Figure 2. The average number of cells and the percentage correction reveal an approximate gene repair frequency of 1.3% with a modest SD. This level of correction is similar to those reported previously for this system (24–26; Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted). The genotypic change has been confirmed via DNA sequence analysis from an isolated EGFP expressing cell

(Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted).

Treatment with CPT induces a higher level of gene repair

The topoisomerase poison, CPT, produces single-stranded lesions predominantly at replication forks (32–36). These lesions arrest the cell cycle in the S and G2 phases and induce HR pathways which direct the repair of the damaged DNA (9,37,38). Since the enzymes involved in HR are important components of gene repair, we sought to increase the frequency by adding CPT to growing cells prior to the electroporation of the oligonucleotide. This protocol enables CPT to induce DNA lesions and activate HR (39 and reference therein, 40,41) before the oligonucleotide initiates the gene repair reaction. Figure 3A presents a concentration curve of CPT and illustrates how each dosage affects the CE. As the final concentration

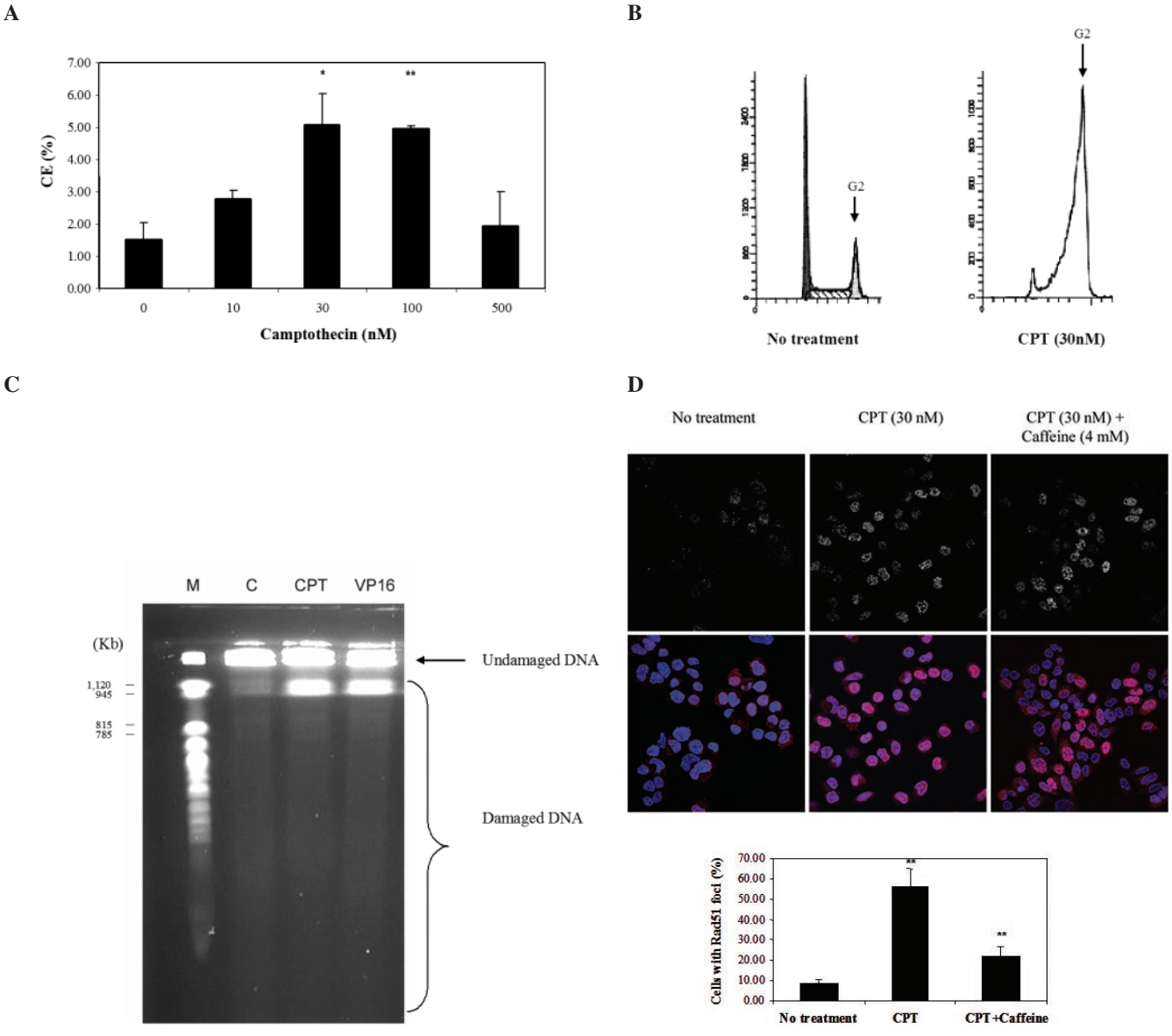


Figure 3. CPT treatment increases CE. (**A**) CPT dose curve. CPT was added to the cells at concentrations of 10, 30, 100 or 500 nM, for 24 h prior to the electroporation of the oligonucleotide. Cells were allowed to recover for 48 h before FACS analysis and the mean and SDs of three independent experiments are shown. *$P < 0.05$, **$P < 0.01$. (**B**) Cell cycle analysis of CPT treated cells. Cells were treated with 30 nM CPT for 24 h and then processed for cell cycle analysis; the profiles were generated using the ModFit LT software. Samples treated with CPT show an increased number of cells in the S and G2 phases (indicated by the arrow) as compared with the untreated samples. (**C**) DSBs are induced in DLD-1 cells exposed to CPT. Cells were treated for 24 h with CPT (30 nM) before harvesting and prepared for PFGE (see Materials and Methods). The gel was run for 24 h and subsequently stained with ethidium bromide. Samples were treated with 30 nM CPT or 3 μM VP16 which was used as a positive control to demonstrate DNA double-strand breakage. In lane M the chromosomal DNA from *S.cerevisiae* was used as size markers presented in kb. (**D**) Enhanced levels of RAD51 nuclear localization and foci formation in cells treated with CPT. Cells were treated with 30 nM CPT or with CPT plus caffeine (4 mM) for 24 h before staining with an antibody directed against RAD51. The top panels represent the RAD51 foci only, as shown in black and white. The lower panels represent the RAD51 antibody as is visible by red fluorescence while the counter stain is DAPI (blue) highlighting the nucleus. The bar graph below represents the number of cells containing more then five RAD51 foci (**$P < 0.01$). The data represents >500 nuclei counted from three independent experiments.

rises through 100 nM, there is a coordinated rise in CE but the level is then reduced as the concentration reaches 500 nM. The dose dependency mimics the stimulatory effects of CPT on HR as measured by more traditional DNA exchange reactions (40).

We also measured the effect of CPT on cell cycle progression, replication activity and genome integrity under conditions that promote gene repair. After treatment with CPT for 24 h, we examined the sample for actively replicating DNA templates using BrdU staining (data not shown). Previous studies have shown that cells treated with CPT respond by halting or retarding the rate of DNA synthesis (42–44). As shown in Figure 3B, untreated DLD-1 cells exhibit a normal cell cycle profile with predictable population distributions in the G1, S and G2 phases. In contrast, cells treated with CPT present a profile in which the population is heavily shifted toward the late S/early G2 phase. These results are not surprising since CPT gives rise to DSBs in the S phase, which must be repaired before the cells proceed through the cell cycle. Evidence for the presence of DSBs is provided in Figure 3C where the status of the genomic DNA, extracted from cells treated with CPT for 24 h, was examined by PFGE. Damaged DNA is evident in the lanes containing samples from CPT-treated cells as well as from cells treated with VP16, a topoisomerase II poison known to induce DSBs (45) and used here as a control. Previous studies have shown that DSBs induced by CPT are not repaired until the drug is washed out so that repair does not actually take place during the 24 h pretreatment period (40). Thus, the DNA breaks observed with CPT reflect the integrity of the genomic DNA at the time of electroporation of the oligonucleotide.

Finally, formation of nuclear foci containing the mammalian RecA analog, RAD51, has, by and large, been taken as evidence that DNA damage requiring a response from the HR pathway has occurred inside the cell (46,47). RAD51, a prominent member of the HR response pathway, relocalizes and concentrates into the nucleus in response to DNA damage signals. It is important, however, to note that RAD51 also accumulates in cells not exposed to external agents because forks are naturally stalled during the replication process. When the DLD-1 cells are examined for the presence of RAD51 foci, much of the red fluorescence is scattered throughout the cell population (Figure 3D). But in response to CPT, a higher level of nuclear foci appears within the population, providing evidence that DNA damage has activated the RAD51 relocalization response (Figure 3D). Cells treated with CPT and caffeine show a reduction in RAD51 foci when compared with those treated with CPT alone. As predicted, caffeine reduces HR activation (48,49, and the section below), reflected here by the occurrence of lower level of RAD51 foci formation in the nucleus. Cells treated with CPT display a clear enhancement of nuclear relocalization that can be seen by immunofluorescence using antibodies directed against RAD51. Taken together, our data suggest that an enhancement of gene repair frequency occurs in cells that have been pretreated with CPT. Under these reaction conditions, the cells are arrested in the late S/early G2 phase, accumulate DNA damage in the form of DSBs and exhibit nuclear localization of RAD51. Thus, the cell's capacity to respond to DNA damage, perhaps as a function of HR activation, may result in a higher gene repair activity.

Caffeine, but not vanillin, blocks the CPT-induced elevation of gene repair activity

Caffeine, a xanthine derivative and radiosensitizer, has been shown to inhibit p53 (ser-15) phosphorylation by ATM and as a result reduce HR between 60 and 90%; it has little effect on NHEJ (45 and references therein). In contrast, vanillin inhibits the activity of DNA-PK, a critical component in the NHEJ pathway (46). Since our experimental results point to a role for HR in response to the presence of CPT, we established a series of reactions using caffeine or vanillin, to test the inhibition or suppression of the gene repair reaction. For all reactions (Figure 4A), except the controls (I and II), CPT was incubated with the cells for 24 h prior to electroporation and the stimulation of gene repair was once again observed (III). When caffeine is present with CPT, in the preincubation phase of the reaction, no elevation of gene repair activity is seen, in fact, a decrease in the CE is observed (IV). This decrease also appears in a reaction where caffeine is present but CPT is not (II); both results are predictable if one assumes that HR is involved in the gene repair process. In the next reaction (V), CPT was washed out after the 24 h preincubation phase and the cells were allowed to recover for 24 h prior to the introduction of the oligonucleotide. The level of gene repair is enhanced six-fold relative to samples that have not been treated with CPT. However, when caffeine is present in the sample during this 24 h recovery period (VI), no stimulation is observed. These results suggest that caffeine inhibits the pathway(s) induced by CPT that contribute to the stimulation of gene repair activity. In reactions VII and VIII, respectively, we substituted vanillin for caffeine under two reaction conditions (II and IV) in which caffeine had been seen to act as an inhibitory agent. As stated above, vanillin is known to block the activity of DNA-PK, a critical enzyme in the NHEJ pathway. Gene repair activity stimulated by CPT is unaffected by the presence of vanillin, thus, suggesting that DNA-PK and, perhaps, NHEJ, in general, play a less significant role in the gene repair pathway.

Wortmannin blocks gene repair activity at concentrations that inhibit HR

Wortmannin is a fungal metabolite that strongly inhibits members of the phosphatidylinositol 3-kinase-related kinases (PIKK), a group of enzymes that include DNA-PK and the damage signaling proteins ATM and ATR (50). This reagent provides a differential blockage of NHEJ and HR at separate, but defined, concentrations (50). We examined gene repair within this concentration range by adding wortmannin to DLD-1 cells 24 h prior to the electroporation of the oligonucleotide. As shown in Figure 4B, wortmannin has no effect on the CPT-induced elevation of gene repair activity at lower concentrations, in fact, a statistically significant enhancement of gene repair is observed at 300 nM. At higher concentrations, a marked reduction in activity is observed. Taken together, these data are consistent with the notion that the HR pathway participates in the CPT-induced gene repair response.

DISCUSSION

DNA replication forks are disabled when they encounter the topoisomerase I–DNA complexes that are stabilized by the

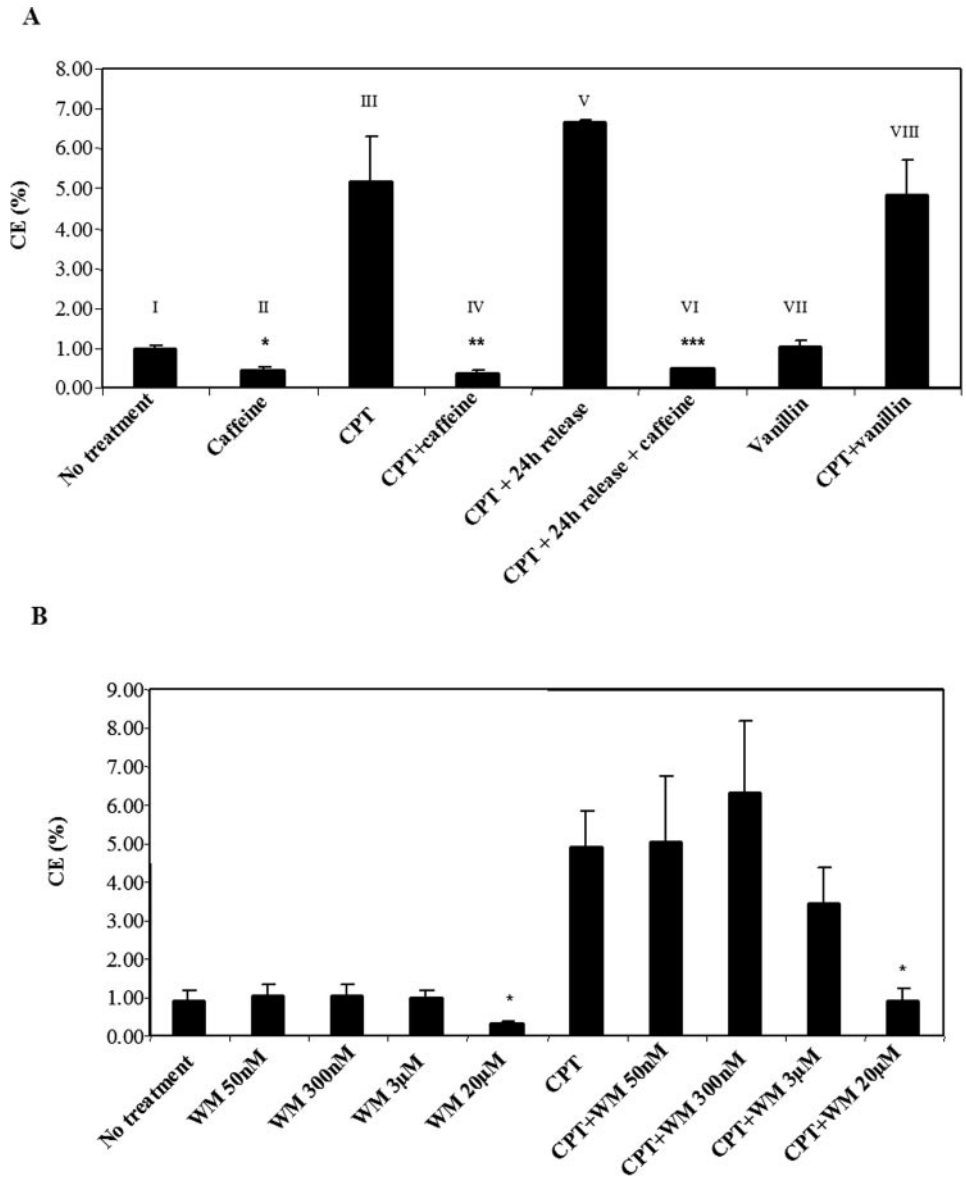


Figure 4. Gene repair activity is suppressed by the addition of caffeine. (**A**) Gene repair activity is suppressed by the addition of caffeine but not vanillin. Cells were plated 24 h before treatment followed by electroporation of EGFP3S/72NT (4 μM) where correction of the EGFP mutation is analyzed 48 h later by FACS. I, control with no addition of any compound; II, caffeine (4 mM) was present in the sample from the time of initial treatment; III, CPT (30 nM) was present in the sample at the time of treatment after 24 h of plating; IV, CPT and caffeine were both present at the start of the reaction; V, CPT was present at the start of the reaction and was washed out after 24 h (release); VI, CPT was present from the beginning of the reaction and washed out after 24 h; caffeine was added subsequently for 24 h; VII, vanillin (1 mM) was present at the start of the reaction; VIII, CPT and vanillin were present at the start of the reaction. The oligonucleotide was electroporated after 24 h of incubation with each of the compounds described above. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, when comparing II with I, IV with III and VI with V, respectively. The means and SDs from three experiments are shown. (**B**) Wortmannin (WM) inhibits gene repair activity at concentrations that specifically block HR. Cells were treated with increasing concentrations of WM (50 nM, 300 nM, 3 μM, 20 μM) alone or in combination with 30 nM CPT for 24 h before electroporation of the oligonucleotide. CPT (30 nM) was added to the reaction for 24 h prior to electroporation of the oligonucleotide; CPT+WM (50 nM, 300 nM, 3 μM, 20 μM), CPT plus the indicated concentrations of WM were incubated together for 24 h prior to the electroporation of the oligonucleotide. The cells were analyzed by FACS, 48 h after electroporation, $*P < 0.05$. The means and SDs from three independent experiments are presented.

presence of CPT. DSBs are eventually created at these locations, which are then recognized and repaired by HR (9,27,28,51,52). In essence, a stalled fork containing DNA damage induces the HR response and serves as a substrate or template upon which the repair of DNA lesions can take place. The work reported herein explores the role of HR in oligonucleotide-directed gene repair, taking advantage of the DNA damage induced by CPT. Based on our results, it seems likely that the induction of HR leads to a rise in coincident activities, DSB repair and gene repair.

We demonstrate that CPT stimulates the frequency of gene repair in a dose-dependent reaction which relies on the specific oligonucleotides designed to direct the nucleotide exchange reaction. Oligonucleotides that bear perfect complementarity to the target sequence or ones that bear no complementarity to the target gene sequence exhibit no activity. As shown

previously, cells treated with CPT accumulate DSBs particularly during the S phase (51–57) and we observe DNA damage in the form of DSBs in DLD-1 cells as well (Figure 3C). We also observe a relocalization of RAD51 to the nucleus after CPT treatment, a hallmark of the HR response (14,58 and references therein) as well as an accumulation of cells in the S phase (late) and G2 phase. These cells are stalled in their progression through the cell cycle as they await the resolution or repair of the induced DNA lesions. Targeted mutagenesis (59) and HR (9,60,61) can occur during this period as the cell surveys the DNA lesions and initiates repair (62). Taken together, we propose a tentative link between the elevated levels of gene repair and the activation of HR.

Further support for this notion comes from inhibition studies using caffeine, vanillin and wortmannin. Caffeine inhibits $p53^{ser\text{-}15}$ phosphorylation via ATM kinase activity reducing HR by 60–90% (48,63–67), and the presence of caffeine in a cell culture incubated with CPT lowers the gene repair frequency. In contrast, vanillin is a potent inhibitor of DNA–PK, which blocks the NHEJ pathway (49). Our studies indicate that pretreatment with vanillin has minimal effect on gene repair activity as a lower level of inhibition is seen compared with the effects of caffeine (Figure 4A). The results from studies using a single inhibitor must however be interpreted cautiously (41,68). For example, Block *et al*. (68) report that caffeine inhibits CPT-induced RPA-32 phosphorylation and suggest that it cannot be used to discriminate solely between NHEJ and HR. But these studies emphasize the inhibition of the autophosphorylation of ATM using *in vivo* studies and do not utilize a gene repair readout. In our case, when the data from multiple inhibitors are considered in total, the hypothesis that the HR pathway plays an important role in the process of gene repair in mammalian cells appears to be supported. Furthermore, this interpretation is consistent with genetic data obtained in *Saccharomyces cerevisiae* (18,21) and is strengthened by the data reported in Figure 4B wherein wortmannin blocks the gene repair activity at higher concentrations (50,69). This is not to say that NHEJ has no role in this reaction since the possibility still exists that the NHEJ activity may still precede HR response (13) in the gene repair process. But at concentrations of wortmannin that are known to inhibit DNA-PK activity, no detectable effect on the reaction was observed.

As described above, cells treated with CPT exhibit a number of different responses including a reduced progression through the S phase, the acquisition of DNA damage, which activates homologous pathways, and the stalling of replication forks (4,27,28,51,52). Each of these responses individually or together may induce higher levels of gene repair activity. Since it is unlikely that the stalled fork or DNA lesion is located at the site of the oligonucleotide–target gene pairing, we propose that the stalled fork itself acts as the signal to elevate the cellular concentration of HR proteins that coincidently repair the lesion and catalyze the oligo-directed nucleotide exchange. In other words, we believe that gene repair reactions are the beneficiary of the cell's effort to repair its damaged DNA at distal sites. This would explain why gene repair is most active in the S phase without the addition of exogenous signals (59). During the normal course of DNA synthesis, inaccurate replication events may cause the fork to stall and induce recombinational repair (14 and references therein; Y. Hu, H. Parekh-Olmedo, M. Drury, M. Skogen and E. B. Kmiec, manuscript submitted). Augmenting the stalling of the fork by adding drugs such as CPT only intensifies the HR response which apparently leads to robust gene repair activity.

Two specialized forms of recombinational repair—gene conversion and single-strand annealing—utilize the mismatch repair (MMR) genes *MSH2* and *MSH3* (70–72) which act during the initialization of DSB repair. Since the latter phase(s) of the gene repair reaction must involve a nucleotide exchange process, it may be fortuitous that activation of the HR pathway in some cases may stimulate the MMR pathway. Evidence has been presented that *MSH2* assists in the nuclear relocalization of RAD51 or the formation of RAD51 foci after DNA damage (58,73). Presumably, a coordinated activation of these two proteins would also promote the gene repair activity, which is manifest in our studies by an increase in the frequency. It should be noted that while MMR has been implicated as one of the repair pathways that operate in some gene repair systems (71), data suggesting the involvement other pathways have also been reported (72).

Finally, it is important to note that the stimulation of gene repair activity by CPT may not rely on the presence of totally intact NHEJ, HR or MMR pathways. Since our assay system is designed to measure the correction of single base mutations, the efficiency and/or specificity with which CPT induced DSBs may not be pertinent to the gene repair reaction itself. To this end, DLD-1 cells have been reported to have a reduced level of recombinational frequency (73), but the assay used in this study differs dramatically from the gene repair assay utilized herein. Conclusions drawn from studies in tumor cell lines must still be interpreted with caution since critical imbalances of key regulatory factors may exist. Nevertheless, the data presented in this paper, based on the CPT induction of DNA damage response, point to the HR pathway as a keystone feature of targeted gene repair.

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