Repair of 2′-*C*-Cyano-2′-Deoxy-1-β-D-*arabino*-Pentofuranosylcytosine–Induced DNA Single-Strand Breaks by Transcription-Coupled Nucleotide Excision Repair

Yaqing Wang,[1] Xiaojun Liu,[1] Akira Matsuda,[2] and William Plunkett[1]

[1]Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas and [2]Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

Abstract

The cytosine nucleoside analogue 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) causes DNA single-strand breaks after its incorporation into DNA. This investigation sought to determine if DNA excision repair pathways were activated to repair this damage. Neither the base excision repair nor the mismatch repair pathway seemed to be involved. Cells deficient in the CSB protein, which initiates transcription-coupled nucleotide excision repair (NER) pathway (TC-NER), exhibited increased clonogenic sensitivity to CNDAC, whereas cells deficient in XPC, which initiates global genome NER, were slightly resistant relative to wild-type cells. The cells lacking either helicase XPB, which unwinds 5′ of the lesion, or endonuclease XPF, which incises 5′ to a lesion, exhibited increased clonogenic sensitivity to CNDAC, as did cells lacking the XPF partner protein ERCC1. This sensitization was independent of p53 function. Repletion of XPF restored sensitivity comparable with the wild type. In contrast, cells lacking either XPD, the 3′-helicase, or the 3′-endonuclease XPG were equally as sensitive as wild-type cells. In comparison, cells deficient in XPF were not sensitized to other cytosine nucleoside analogues, troxacitabine and cytarabine. Thus, the single-strand nick caused by CNDAC is recognized and, in part, repaired by the TC-NER pathway. NER proteins that function in the 5′ direction relative to the UV-induced lesion also participate in the repair of the CNDAC-induced nick, in contrast to proteins that process on the 3′ side of the lesion. [Cancer Res 2008;68(10):3881–9]

Introduction

Upon incorporation into DNA, many nucleoside analogues directly inhibit DNA synthesis, as they are poor substrates for further elongation of nascent DNA strands. DNA chain termination results in stalling of replication forks, activation of the S-phase checkpoint pathway, and arrest of cell growth, an activity typical for the cytosine nucleoside analogues, cytarabine (1-β-D-arabinofuranosylcytosine, ara-C) and gemcitabine (1). In contrast, 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) is a cytosine nucleoside analogue with the novel mechanism of action of causing a single-strand nick after incorporation into DNA (2–5). CNDAC triphosphate is an efficient substrate for DNA polymerase α (V_{max}/K_m = 0.77) relative to the normal substrate, dCTP (V_{max}/K_m = 0.91; ref. 6). However, ligation of the 3′-hydroxyl of the analogue, either chemically or by incorporation of subsequent deoxynucleotides with DNA polymerases, initiates β-elimination. In DNA, this process cleaves the phosphodiester linkage 3′ to the analogue, as the CNDAC nucleotide is rearranged to form 2′-*C*-cyano-2′,3′-didehydro-2′,3′-dideoxycytidine (CNddC). Because CNddC lacks a 3′-hydroxyl group, it is a *de facto* chain terminator that would lead to formation of a single-strand DNA nick. CNddC formation has been documented in DNA of CNDAC-treated cells by both high-performance liquid chromatography and ^{31}P-^{1}H COSY nuclear magnetic resonance spectrum analysis (3–5, 7). This process is unique to CNDAC and, therefore, represents the signature of this DNA self-strand breaking process (5, 7). Unlike ara-C and gemcitabine, which cause an S-phase arrest (8, 9), the single-strand breaks (SSB) induced by CNDAC activate the G_2 checkpoint, which requires both ATR and DNA-PK to activate the Chk1-Cdc25C-Cdk1/cyclin B checkpoint pathway (10, 11).

Mechanisms that may excise DNA chain-terminating analogues have been studied, as they would likely enable reactivation of DNA replication, which may spare the toxicity of drugs and possibly contribute to resistance. The proofreading exonucleases associated with the replicative DNA polymerases δ, ε, and γ have been considered as the first line of defense against misincorporated nucleotides and likely nucleotide analogues (12–15). Given that at least one additional nucleotide must be inserted by the polymerase before the onset of β-elimination to generate a CNddC-3′–terminated nick, it seems unlikely that polymerase-associated exonucleases would have the ability to recognize the nick and then excise in a 5′ direction across the single-strand gap. Similarly, because the rearranged analogue lacks a 3′-hydroxyl group required for ligation, processing in addition to the abilities of the nick repair enzyme polynucleotide kinase would be required, e.g., excision of CNddC before addition of dCMP and sealing of the nick. Therefore, we have focused efforts on the possible participation of the mechanisms that repair DNA damage by other forms of excision, namely base excision repair (BER), mismatch repair (MMR), and nucleotide excision repair (NER).

At least 11 glycosylases of the BER system recognize nucleobases with distinct damage within DNA and remove them by cleaving the glycosidic bond (16). The deoxyribose at the apurinic/apyrimidinic site is removed from phosphodiester linkage, mainly by APE1, and the gap is filled and sealed by the actions of DNA pol β and DNA ligase I or DNA ligase III/XRCC1 (17). The 3′→5′ exonucleolytic activity of APE1 was shown to remove the chain terminating L-nucleoside L-OddC in an oligonucleotide model system (18). MMR functions after passage of the replication fork to recognize

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Y. Wang and X. Liu contributed equally to this work.

Requests for reprints: William Plunkett, Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030. Phone: 713-792-3335; Fax: 713-794-4316; E-mail: wplunket@mdanderson.org.

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noncognate bases and small insertion/deletion errors. After recognition of the lesion by this mechanism, the exonuclease Exo I is activated to processively excise as many as a thousand nucleotides from the misreplicated DNA region before replicative DNA polymerases resynthesize the single strand to close the gap and ligation completes the process (19). Changes in DNA structure caused by adducts or cross-linking are recognized by NER (20). Such alterations are recognized throughout the genome by the global genome NER (GG-NER) mechanism initiated by the heterodimer XPC/HR23B, whereas repair of damage encountered during transcription is recognized by CSA and CSB proteins of the transcription-coupled NER pathway (TC-NER). After this initial recognition step, the two processes proceed identically, with helicases unwinding DNA on either side of the lesion and endonucleases excising the damaged 27 to 29 nucleotide oligomer. This is followed by gap-filling by replicative polymerases and sealing by DNA ligase. Although the resynthesis step is inhibited by incorporation of a nucleotide analogue (21, 22), removal of misincorporated analogues from DNA by NER has not been reported.

The present study compared responses to CNDAC of several genetically related excision repair–deficient cell lines. The results showed that BER and MMR did not seem to be involved in the low level of repair observed. However, TC-NER, but not GG-NER, recognized the CNDAC-induced damage, which was subsequently addressed by the helicase and endonuclease components that function 5′ to a lesion, the lack of which results in a 3-fold to 5-fold sensitization to CNDAC. In contrast, repair pathway components that function 3′ to the lesion were inconsequential to survival.

Materials and Methods

Cell lines and culture conditions. The following cells were purchased from the American Type Culture Collection: the Chinese hamster ovary (CHO) AA8 cell line and its derived mutants EM9 (XRCC1), UV41 (XPF), UV20 (ERCC1), UV135 (XPG), UV24 (XPB), and UV5 (XPD); mouse embryonic fibroblast (MEF) cell lines 92Tag (wild type), 88Tag (pol $\beta^{-/-}$), and 127 Tag (PMS2$^{-/-}$) and the cervical cancer line HeLa CCL2. UV61 (CSB) cells were isolated by Busch and coworkers (23) and obtained from Dr. L. Li (M. D. Anderson Cancer Center).

A human acute myelogenous leukemia cell line, ML-1, was a gift from Dr. M. Kastan (St. Jude Children's Research Hospital). GM08437B, a human fibroblast line deficient in XPF and transformed by SV40, was purchased from Coriell Cell Repositories. The colon carcinoma cell line HCT116 cells with p53 wild type or null background were provided by Dr. B. Vogelstein (Johns Hopkins Medical School), the HCT116 subline where hMLH1 expression is restored by chromosome 3 transfer (HCT116 + ch3), the hybrid clone of the same cell line HCT116 + ch5 (containing a piece of chromosome 5, MMR deficient), and HCT116 + ch3 + ch5 (MMR proficient) were gifted by Dr. M. Koi (NIEHS). XPC and XPCC cells were created as described before (24) and gifted from Dr. L. Li. (M. D. Anderson Cancer Center). All cells were free of *Mycoplasma*, as determined by an ELISA kit (Life Technologies Myco Test kit).

Chemicals and antibodies. The nucleoside analogue CNDAC was synthesized as described (3). Toxacitabine (L-OddC) was provided by H. Goodreau, and ara-C was purchased from Sigma-Aldrich. The APE1 inhibitor CRT0044876 was from Calbiochem (262015, EMDbiosciences). Methyl methansulfonate (MMS) was from Sigma-Aldrich (129925). Protease Inhibitor Cocktail tablets was from Roche Diagnostics Corporation (11873580001). Sources of antibodies were as follows: mouse monoclonal antibody against XPF (MS1381P, LAB VISION CORP/NEOMARKERS), XPC antibody (GTX70294, Gene Tex, Inc.), and β-actin (A1978, Sigma-Aldrich). IR DYE 680CW conjugated goat anti-mouse IgG (92632220, LI-COR).

Enzymes and plasmid. APE1 (M0282S), T4 polynucleotide kinase (M0201S), and Klenow fragment of DNA pol I (3′ to 5′ exo$^-$; M0212s) were from New England Biolabs, Inc. pcDNA3-XPF plasmid (RDB No. 1785, Tsukuba) was obtained from Dr. Hiraku Takebe through the DNA Bank, RIKEN BioResource Center. pcDNA3 vector was from Invitrogen Life Technologies (A-150228).

Stable transfection. XPF-deficient UV41 was transfected with pcDNA3 vector or pcDNA3-XPF using Lipofectamine 2000 transfection reagent (11668-027, Invitrogen). Human XPF fibroblast line GM08437B was transfected with these plasmids by Amaxa Nucleofector (VPI-1002, Amaxa, Inc.). After 24 to 48 h, cultures were selected in medium supplemented with G418 (200–800 μg/mL). Clones expanded from single colonies were used for biochemical and clonogenic assays.

Preparation of CNDACMP and dCMP primers. 23-mer oligonucleotides primer and 40-mer template were synthesized by Integrated DNA Technologies. The primers were labeled with ^{32}P at their 5′ ends by T4 polynucleotide kinase and annealed to template. The products were extended to 24-mer by Klenow fragment of DNA pol I (3′ to 5′ exo$^-$) in the presence of dCTP or CNDACTP. The products were separated on a sequencing gel, excised, and annealed to a 2-fold to 4-fold excess of 40-mer template again. Oligonucleotides containing either the normal dCMP or CNDACMP at the 3′-terminus *C* of substrate A were used as substrates for excision assays.

Substrate A.

5′-[^{32}P]GTAAAACGACGGCCAGTGCCAAG***C***

3′-CATTTTGCTGCCGGTCACGGTTCGAACGTACGGACGTCCA-5′

To model the β-elimination product, we designed a nicked DNA substrate, with a CNDACMP-terminated (*C*) 24-mer oligo annealed to the 5′ portion, in the presence of a 16-mer oligo with either a 5′-OH (substrate B) or a 5′-phosphate (substrate C) to the 3′ portion of the 40-mer template.

Substrate B.

5′-[^{32}P]GTAAAACGACGGCCAGTGCCAAG***C***^{OH}TTGCATGCCTGCAGGT

3′-CATTTTGCTGCCGGTCACGGTTCGAACGTACGGACGTCCA-5′

Substrate C.

5′-[^{32}P]GTAAAACGACGGCCAGTGCCAAG***C***^{PO_4}TTGCATGCCTGCAGGT

3′-CATTTTGCTGCCGGTCACGGTTCGAACGTACGGACGTCCA-5′

The primer/template complexes were used in a 20-μL reaction mixture containing 10 units of APE1. The primer concentration in each reaction was 1,000 pmol/L. The reactions were carried out at 37°C for up to 40 min, analyzed by electrophoresis, and visualized by phosphoimager (Molecular Dynamics, Inc.).

Small interfering RNA transfections. On-target plus smart pool small interfering RNA (siRNA) targeting human XPC (NM-004628, Dharmacon) or XPF (NM-005236) and control nontargeting siRNA (D-001210-02-05) were used in combination with DharmaFect transfection reagent (T-2001-02) according to the manufacturer's instructions.

Immunoblotting. Lysates of cell pellets were prepared, resolved on SDS-PAGE gels, and transferred onto nitrocellulose membranes as described (10). The blots were visualized by scanning using a Li-Cor Odyssey Imager (Li-Cor).

Clonogenic cell survival assay. Cells (300–500) were seeded in 60-mm dishes 1 d before cells were exposed to a range of concentrations of different cytosine nucleoside analogues for 24 h or MMS for 2 h. In the experiments where APE1 inhibitor CRT0044876 was used, the inhibitor was added 2 h before other drugs and maintained in the medium during cell growth. UVC irradiation was given in medium free of phenol red using a UVGL-25 lamp (UVP, Inc.). The radiation intensity was calibrated using a UVX-25 UV

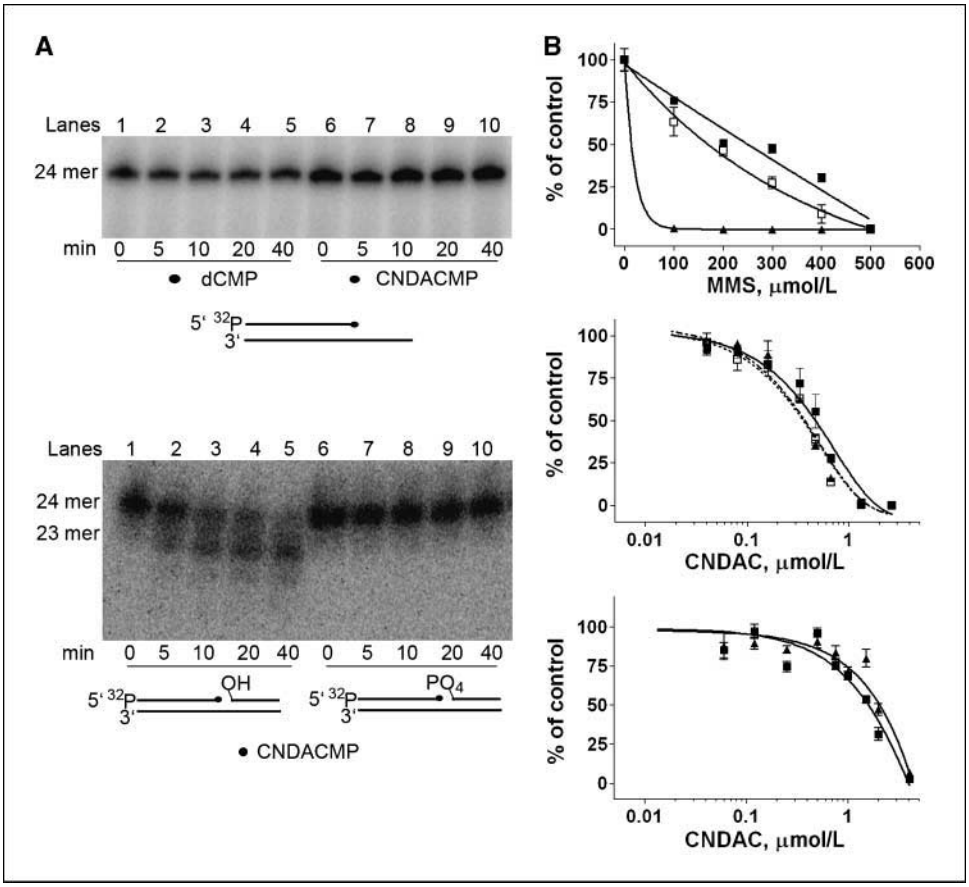

Figure 1. BER does not influence cellular response to CNDAC. *A,* primer excision of CNDACMP by APE1 *in vitro. Top*, 5′-[^{32}P]–labeled 24-mer primers with either dCMP or CNDACMP at its 3′ end were separately annealed to a 40-mer template to generate a recessed substrate (substrate A) to evaluate excision with 10 units APE1 for 0, 5, 10, 20, and 40 min, respectively. The reaction products were analyzed by 15% polyacrylamide sequencing gel and visualized by phosphoimager. *Lanes 1-5,* dCMP-terminated 24-mer/40-mer; *lanes 6-10,* CNDACMP-terminated 24-mer/40-mer. *Bottom*, a substrate containing a nick in the target strand was prepared by annealing the 5′-[^{32}P]–labeled 24-mer primer with CNDACMP at its 3′ terminus to the 40-mer template, as above, in the presence of either of two 16-mer primers complementary to the distal portion of the template. The 5′ terminus of one 16-mer had a phosphate, modeling the β-elimination product (substrate C), whereas the other 16-mer had a hydroxyl, which would reflect a dephosphorylated β-elimination product (substrate B). These two substrates were compared for excision by 10 units of APE1 for 0, 5, 10, 20, and 40 min, respectively. *Lanes 1-5,* substrate B, CNDACMP nicked DNA substrates with 5′-OH 16-mer; *lanes 6-10,* substrate C, CNDACMP nicked DNA substrates with 5′-PO_4 16-mer. Results are representative of three experiments. *B,* the cytotoxicity of CNDAC seems to be independent of the BER pathway. *Top,* AA8 (■) and XRCC1-deficient EM9 cells (▲; 500 cells per 60-mm dish) were treated with various concentrations of MMS for 2 h, then washed into drug-free medium, and incubated for 6 d. AA8 cells in the continuing presence of an APE1 inhibitor CRT0044876 (200 μmol/L; □) were treated similarly. *Middle,* AA8 (with or without CRT0044876) and EM9 cells were treated with various concentrations of CNDAC for 24 h, then washed into CNDAC-free medium, and incubated for 6 d. Symbols, as above. *Bottom,* 92TAg wild-type (■) and 88TAg pol β–deficient (▲) MEF cells were treated with CNDAC, as above. Colonies were counted and normalized as the percentage of control. *Points,* mean of three independent experiments; *bars,* SEM.

meter (UVP, Inc.). Immediately after irradiation, cells were washed into complete medium and incubated for colony formation. Cells were left to grow for 6 to 9 d and stained with crystal violet, and the number of colonies with >50 cells was counted. Survival was expressed as a percentage of the number of colonies formed in the absence of treatment.

Statistical analysis. IC_{50} values were calculated using the GraphPad Prism4 software (GraphPad Software, Inc.) based on at least two independent experiments in triplicates. Intergroup comparisons of clonogenic survival in response to drugs were made using two-tailed paired *t* test.

Results

BER is not involved in removal of CNDAC nucleotide from DNA. We first evaluated the participation of human apurinic/apyrimidinic endonuclease (APE1), an essential enzyme in DNA BER (25), on the stability of CNDAC in DNA. Purified APE1 was unable to excise either dCMP or CNDACMP from the 3′ terminus of a recessed primer (Fig. 1*A, top*). Because the incorporated CNDAC nucleotide must be extended with the addition of subsequent deoxynucleotides to initiate β-elimination, we designed a nicked DNA substrate, with a CNDACMP-terminated 24-mer oligo annealed to the 5′ portion, in the presence of a 16-mer oligo to the 3′ portion of the 40-mer template. When the 16-mer had a hydroxyl group at its 5′ end, CNDACMP could be excised by APE1 over time (Fig. 1*A, bottom, lanes 1–5*), with 62% being excised by 40 minutes (Supplementary Fig. S1). Nevertheless, when the 16-mer had a phosphate group at its 5′ end, which models the β-elimination product, excision of CNDACMP was not detectable (Fig. 1*A, bottom, lanes 6–10*). A similar dependency for excision was observed for dCMP nicked substrates (data not shown).

It was not possible to generate CNddCTP as a potential substrate for the preparation of the CNddC-terminated product for *in vitro* excision. Therefore, as a complementary approach, we used an APE1 inhibitor, CRT0044876 (26), to determine if inhibition of APE1 sensitizes cells to CNDAC. As a control, CRT0044876 (200 μmol/L) sensitized AA8 cells to the response of MMS, an alkylating agent

that causes DNA damage that is repaired by the BER pathway (Fig. 1*B, top*; $P = 0.002$; ref. 27). However, consistent with our *in vitro* data indicating that CNDACMP and CNddC are not substrates for APE1, this concentration of inhibitor did not sensitize AA8 cells to CNDAC ($P = 0.061$; Fig. 1*B, middle*). We extended this approach using the XRCC1-deficient cell line EM9, which were similarly sensitive to CNDAC as parental cells ($P = 0.202$; Fig. 1*B, middle*), although, as previously shown (28), EM9 is supersensitive to MMS (Fig. 1*B, top*). The cell cycle distribution in response to CNDAC was similar for both cell lines (data not shown). Finally, when MEF wild type and DNA pol β-deficient cells were treated with CNDAC as above, the results showed that DNA pol β-deficient cells showed a similar sensitivity to CNDAC as wild-type cells ($P = 0.114$; Fig. 1*B, bottom*). Taken together, these data indicated that the cytotoxicity of CNDAC is independent of the BER pathway.

MMR deficiency does not sensitize cells to CNDAC. The human colon cancer cell line HCT116 lacks hMLH1 function ($hMLH1^{-/-}$) and is therefore incapable of conducting MMR. In contrast, a HCT116 subline in which hMLH1 expression is restored by transfer of chromosome 3, which contains a copy of wild-type hMLH1, is competent in DNA MMR (29). Comparison of changes in the clonogenicity of these lines to increasing concentrations of CNDAC showed similar sensitivities (Fig. 2*A*; $P = 0.056$). Control studies with HCT116 lines containing either chromosome 5, which has no MMR-restoring ability, or with both chromosome 3 and chromosome 5, did not differ from HCT116 responses. The G_2-arresting action of CNDAC in the chromosome-transferred cells (data not shown) was identical to that induced in HCT116 (10). A MEF line deficient in PMS2, which has a less stringent MMR phenotype than does HCT116, was not sensitized to CNDAC compared with wild-type cells (Fig. 2*B*). Thus, lack of MMR capacity did not increase the cytotoxicity of CNDAC, suggesting that it is not involved in repairing damage caused by the analogue.

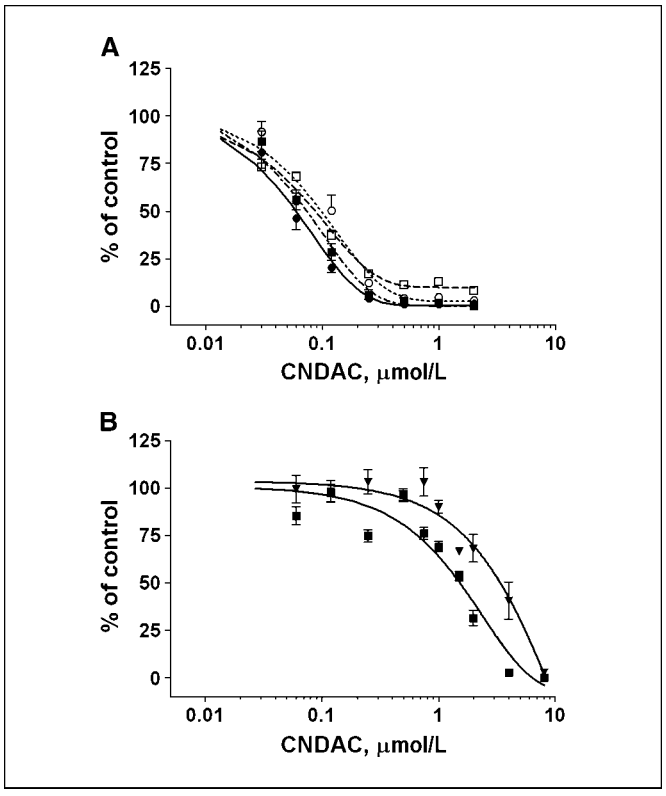

Figure 2. The cytotoxicity of CNDAC is independent of MMR. Cells were exposed to a range of concentrations of CNDAC for 24 h. Cytotoxicity was measured by colony formation and expressed as a percentage of no drug control. *A,* HCT116, ■; HCT116 + CH3, ●; HCT116 + CH5, □; HCT116 + CH3 + CH5, ○. *B,* 92TAg/WT, ■; 127TAg/$PMS2^{-/-}$, ▼. *Points,* mean of three independent experiments; *bars,* SEM.

CNDAC-induced cytotoxicity is influenced by XPF/ERCC1 of the NER pathway. To determine the participation of the NER pathway in addressing DNA damage caused by CNDAC, we focused first on the endonuclease steps in the process. The clonogenic sensitivity to CNDAC of CHO cells lacking XPF (UV41), ERCC1 (UV20), and XPG (UV135) was compared with wild-type cells (AA8). A 2.6-fold to 4.6-fold sensitivity in response to CNDAC was observed in UV20 and UV41 compared with AA8 cells (Fig. 3*A, left*). The IC_{50} values for UV20 was 0.19 μmol/L ($P = 0.003$) and 0.11 μmol/L for UV41 ($P = 0.011$) compared with AA8 cells (0.50 μmol/L). This finding is consistent with the functional association of the two proteins, XPF and ERCC1 (30). In contrast, UV135 cells lacking XPG were equally sensitive, as were wild-type cells ($IC_{50} = 0.41$ μmol/L; $P = 0.356$; Fig. 3*A, left*). However, both UV41 and UV135 exhibited >7-fold sensitivity to UV light (Supplementary Fig. S2). Theoretically, the relative resistance of AA8 to CNDAC may represent a greater ability to repair drug-associated damage.

To compare the capability of removing CNDAC analogues from their DNA in AA8 and UV41 cells, the cellular DNA was labeled with 0.01 μmol/L of [^{3}H]CNDAC, a nontoxic treatment based on clonogenicity studies (Fig. 3*A, left*). AA8 cells had a greater capacity for removal of this analogue from phosphodiester linkage than UV41 cells (Fig. 3*A, right*), a result that indicated less removal of CNDAC was associated with more toxicity to UV41. For evaluating repair in human cells, we labeled ML-1 cells with 0.06 μmol/L [^{3}H]CNDAC, a concentration that did not decrease clonogenicity (data not shown). For comparison, similar determinations were made for ara-C, an analogue that is incorporated principally into phosphodiester linkage in DNA when given at low concentrations, but does not cause a strand break (31). A separate portion of cells was labeled with [^{3}H]thymidine to control for metabolism of bulk DNA. Thymidine was stably incorporated (Supplementary Fig. S3). ara-C was similarly stable; a result that confirms earlier studies that cells have limited capacity for removal of this analogue from phosphodiester linkage (31). Finally, CNDAC nucleotide was removed from DNA slowly, at a rate of ~8% per cell population doubling time (Supplementary Fig. S3). As with ara-C it is unlikely that cells could excise CNDAC nucleotide from an internal position. Thus, it is likely that this rate reflects removal of CNddC from the 3′ terminus of nicks generated by β-elimination.

In a separate approach, UV41 cells were transfected with a plasmid expression vector containing hXPF to generate a stable clone that was isogenic to the UV41 line, as well as empty vector controls. A clone of the selected transfectants was shown to express the human XPF protein (Fig. 3*B, right*) and lost sensitivity to UV (Supplementary Fig. S4). When incubated with CNDAC, the XPF-repleted line reverted to similar sensitivity (IC_{50}, 0.42 μmol/L) to that of AA8 (Fig. 3*B, left*). However, the vector-transfected cells (IC_{50}, 0.19 μmol/L) showed the same phenotype as UV41 cells. A cell line from an individual lacking XPF was sensitized to CNDAC (IC_{50}, 0.12 μmol/L) relative to the same cells following XPF repletion (IC_{50}, 0.78 μmol/L; Fig. 3*C*; $P = 0.002$).

The role of p53 was investigated using HCT116 colon cancer cells with either p53 wild-type or null background that were

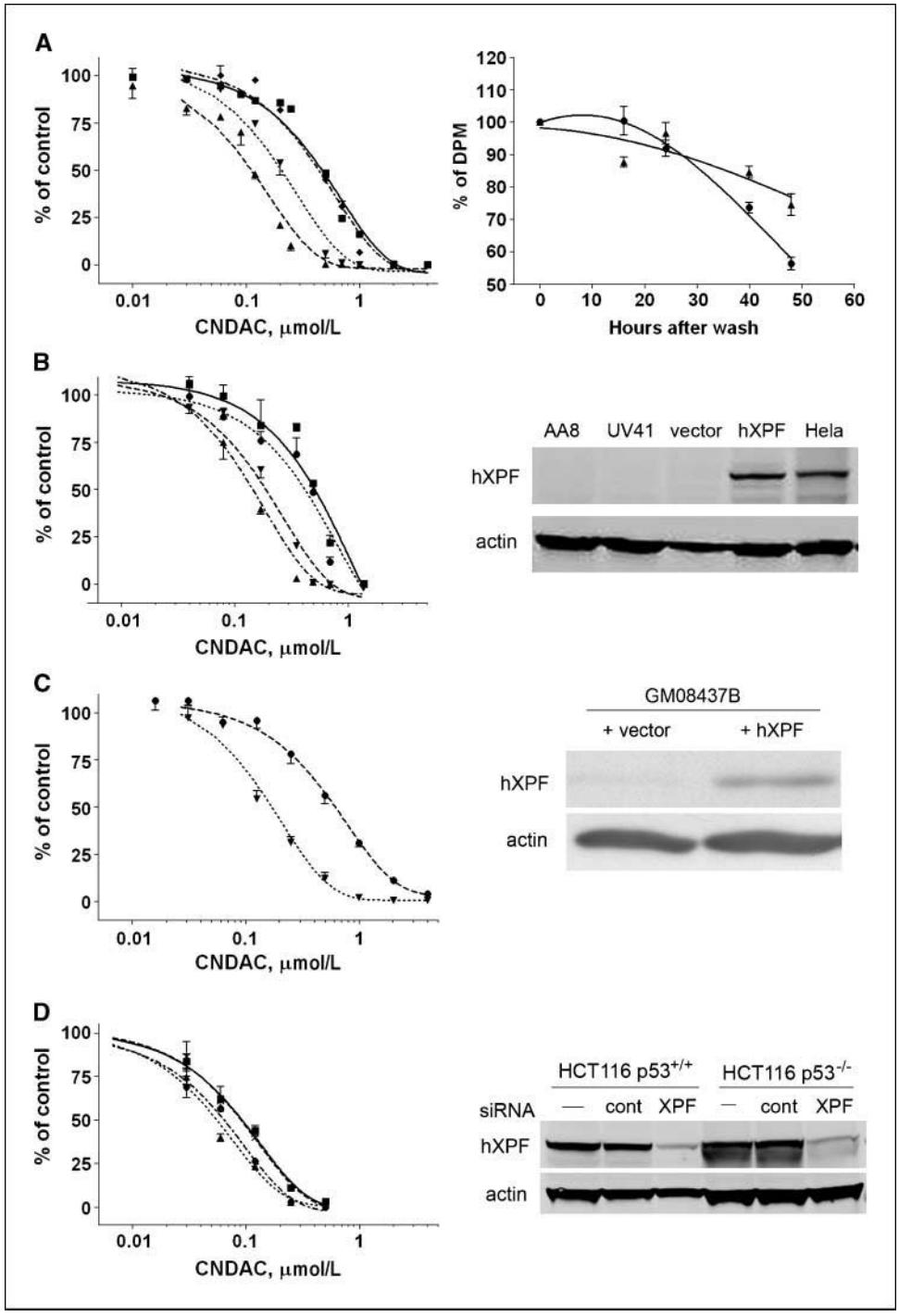

Figure 3. The effect of CNDAC on the cytotoxicity of NER-deficient CHO cells (*A* and *B*) and human cells (*C* and *D*). *A, left*, AA8 and NER mutant cell lines (500 cells/60-mm dish) were treated with various concentrations of CNDAC for 24 h, then washed into drug-free medium and incubated for 6 d. Colonies were counted and normalized as the percentage of controls. AA8, ■; UV41, ▲; UV20, ▼; UV135, ♦. *Right*, AA8 (●) and UV41 (▲) cells were incubated with 0.01 μmol/L [^{3}H]CNDAC for 24 h and washed into drug-free medium. This drug concentration did not affect clonogenic survival. Aliquots were taken at the indicated times, and DNA was extracted. The rates of removal from DNA were determined by quantitating the amount of each labeled nucleoside that remained in DNA by liquid scintillation counting. *DPM,* disintegration per minute. *B, left,* a human XPF gene cDNA expression plasmid, pcDNA3-XPF, was stably transfected into the UV41 cells and the clonogenic assays were then performed. The vector pcDNA3 plasmid was also transfected as a control. AA8, ■; UV41, ▲; UV41 transfected with pcDNA3-XPF, ●; UV41 transfected with vector, ▼. *Right*, immunoblotting of the cell lysates prepared from AA8, UV41, pcDNA3 transfected (vector), and pcDNA3-XPF transfected (hXPF) cells. As a positive control, the cell lysates from the HeLa cells were used in the assay. *C,* pcDNA3-XPF was stably transfected into the human XPF fibroblast GM08437B cells and the clonogenic assays were then performed. Cells transfected with pcDNA3-XPF, ●; cells transfected with vector, ▼. *Right*, protein expression level of hXPF. *D,* sensitivity of XPF knockdown cell line to CNDAC is independent of p53. *left*, XPF protein in HCT116 p53 wild-type and null cells was knocked down by transient siRNA silencing and then treated with CNDAC for 24 h. Cell survival was assessed in clonogenic cell growth assays. p53 wild-type cells transfected with control siRNA, ■; p53 wild-type cells transfected with siRNA targeting XPF, ▲; p53 null cells transfected with control siRNA, ×; p53 null cells transfected with siRNA targeting XPF, ●. *Points,* mean of three independent experiments; *bars,* SEM. *Right*, Western blot analysis of total protein content from control cells, nontargeting control siRNA, and XPF siRNA knockdown cells.

incubated with CNDAC after knocking down XPF protein using a human specific siRNA. The results showed that depletion of XPF protein sensitized both p53 wild-type (P = 0.046) and null HCT116 cells (P = 0.041) to CNDAC (Fig. 3*D, left*). Immunostaining indicated that XPF protein was depleted 75% and 78%, respectively, in p53 wild-type and null cells (Fig. 3*D, right*). Taken together, these results provide evidence that loss of XPF/ERCC1 can contribute to sensitivity to CNDAC independent of p53 status.

ara-C and L-OddC are not excised by XPF. Two other cytidine nucleoside analogues, ara-C and L-OddC, were examined to determine whether or not the contribution of XPF is specific to the single-strand nick induced by CNDAC. The similar clonogenic responses indicated that XPF did not participate in the repair of ara-C (P = 0.144; Fig. 4*A*) and L-OddC (P = 0.080; Fig. 4*B*). Thus, the role of XPF seems to be unique to the nick created through β-elimination following CNDAC incorporation, but not to the termination of nascent DNA chains at the replication fork caused by L-OddC (32) or the replication fork-stalling actions of ara-C (33).

Participation of the components upstream of the XPF/ERCC1 incision step of the NER pathway in CNDAC-induced cytotoxicity. Whereas both the GG-NER and TC-NER pathways require the endonucleolytic actions of XPF/ERCC1, different mechanisms for

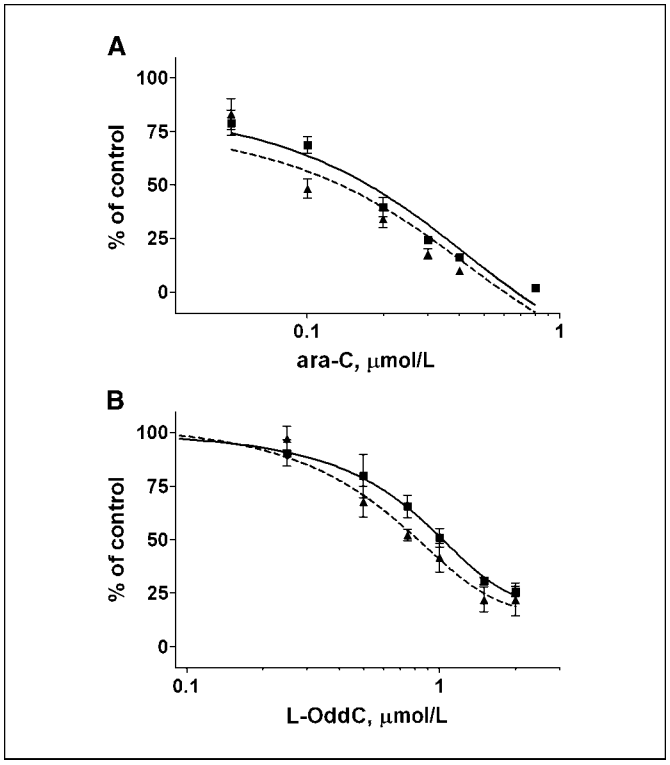

Figure 4. Cytotoxicity of ara-C and L-OddC to XPF-deficient CHO cells. AA8 and UV41 cells were treated with various concentrations of ara-C (*A*) or L-OddC (*B*) for 24 h, then washed into drug-free medium, and incubated for 6 d. *Points,* mean of three independent experiments; *bars,* SEM. AA8, ■; UV41, ▲.

recognizing damaged DNA distinguish GG-NER and TC-NER (34). Therefore, we compared survival of the XPC and CSB mutant lines with their isogenic wild-type cells, respectively, following CNDAC treatment using clonogenic survival assays. Consistent with an earlier report (24), repletion of fibroblasts derived from a patient with XPC syndrome with the human gene exhibited resistance to UV compared with XPC-deficient cells (Figs. 5*A* and 6*A*). Surprisingly, XPC repleted cells (IC_{50}, 0.53 μmol/L) exhibited 2-fold more sensitivity to CNDAC compared with the deficient cells (IC_{50}, 0.91 μmol/L; $P = 0.025$; Figs. 5*B* and 6*A*). This observation was also investigated by knocking down XPC protein using siRNA in colon cancer HCT116 cells ($P = 0.016$; Fig. 5*C*). CSB mutant cells exhibited 1.7-fold sensitivity to CNDAC (IC_{50} = 0.31 μmol/L), compared with AA8 (IC_{50} = 0.52 μmol/L; $P = 0.023$; Figs. 5*D* and 6*A*), indicating that CSB participated in the recognition of DNA damage.

The XPB and XPD helicases unwind the DNA on the 5′ and 3′ sides of the damage, respectively. Treatment of cells deficient in these proteins showed that cells lacking XPB were sensitized 3-fold (IC_{50}, 0.14 μmol/L) compared with AA8 cells ($P = 0.049$), whereas XPD-deficient cells exhibited similar sensitivity (IC_{50}, 0.45 μmol/L) as the wild-type cells ($P = 0.213$; Figs. 5*D* and 6*A*), indicating that sensitization was associated with proteins know to function of the 5′ side of a UV lesion.

Finally, we compared the contributions of NER proteins to resistance of CNDAC relative to their importance to surviving UV irradiation (Fig. 6*A* and Supplementary Fig. S2). Whereas these results indicate the importance of each of these proteins to sensitizing cells to UV, it is clear that the damage caused by CNDAC is recognized by the TC-NER pathway and proteins that function only in the 5′ direction relative to the lesion in the repair of UV damage also participate in the repair of CNddC nucleotide. The relative importance of each protein to cellular responses to CNDAC is illustrated in the model in Fig. 6*B*.

Discussion

Whereas the mechanism of action of most nucleosides involves their incorporation into DNA (35–38), the toxic action of CNDAC of nick formation in DNA and the cellular responses to this damage

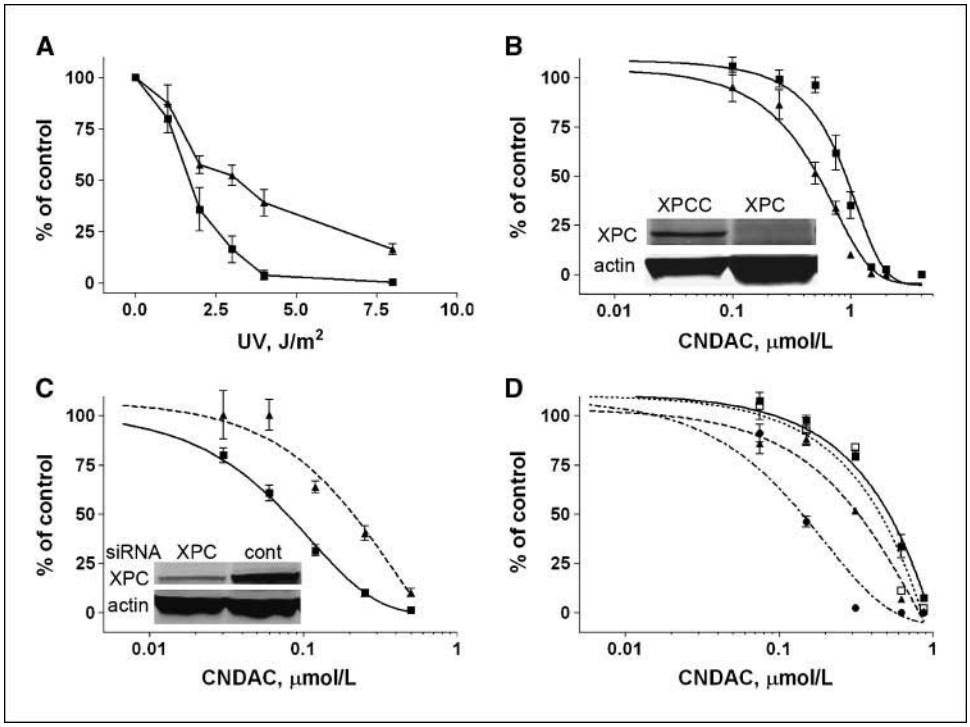

Figure 5. The cytotoxicity of CNDAC is independent of GG-NER but dependent on TC-NER. XPC (■) and XPCC (▲) cells were examined for their UV (*A*) and CNDAC (*B*) sensitivity, respectively. *XPC,* human fibroblast cell line derived from a patient with XPC syndrome; *XPCC,* XPC cells repleted with a human XPC cDNA. Protein level is shown in inset. *C,* XPC protein level was knocked down by siRNA transfection in HCT116 cells and cell survival was examined using clonogenic assay. HCT116 transfected with siRNA targeting XPC gene (▲) and transfected with nontargeting control siRNA (■). Protein level is shown in inset. *D,* clonogenicity of AA8 (■), UV61 (▲), UV5 (□), and UV24 (●) cells. *Points,* mean of three independent experiments; *bars,* SEM.

A

Contribution of NER proteins to resistance of CNDAC and UV

Cell lines	CNDAC IC_{50} (μmol/L)	CNDAC Fold of sensitive	CNDAC P value vs. AA8	UV IC_{50} (J/m^2)	UV Fold of sensitive
AA8	0.50±0.18	1		16.3	1
CSB	0.31	1.7	0.023	4.4	3.7
XPC[a]	0.91	0.5	0.025	1.7	1.9
XPB	0.14	3.6	0.049	2.1	7.6
XPD	0.45	1.1	0.213	3.3	4.9
XPF	0.11	4.6	0.011	1.4	11.7
ERCC1	0.19	2.6	0.003	ND	ND
XPG	0.41	1.2	0.356	2.3	7.2

NOTE: [a] XPC is derived from patient and compared with XPC repleted cells. ND, not detected

B

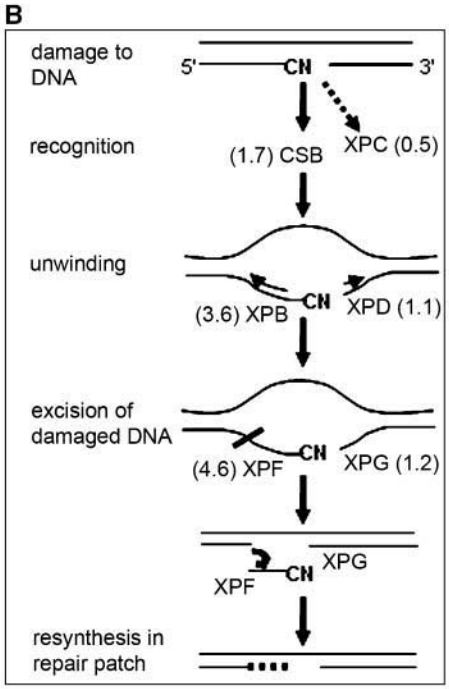

Figure 6. *A,* this table summarizes the contribution of NER proteins to resistance of CNDAC and UV. Note that all mutants exhibited UV sensitivity but only CSB, XPB, XPF, and ERCC1 exhibited CNDAC sensitivity, relative to AA8 wild-type cells. *B,* this model summarizes the participation of NER components in processing DNA damage caused by CNDAC. Both GG and TC-NER are required in order to excise the damage caused by UV, but TC-NER is the only pathway in processing the damage induced by CNDAC incorporation. The damage generated following incorporation of CNDAC actually is a CNddC-terminated nick created through β-elimination. When this nick is encountered by RNA polymerase II during transcription, CSB will be recruited to define this damage and further recruit helicase XPB for unwinding DNA helix in a 3′ to 5′ polarity and allowing access of XPF/ERCC1 to incise from 5′ to lesion. The XPD helicase and XPG endonuclease are not necessary due to the existence of the nick. This could explain the sensitivity of UV61 (CSB), UV24 (XPB), and UV41 (XPF) and lack of effect for UV5 (XPD) and UV135 (XPG) in response to CNDAC compared with AA8 (wild type). Numbers indicate fold of sensitive. *CN,* CNddC.

seem to be unique. First, the data presented indicate that CNddC, the DNA chain-terminating β-elimination product of CNDAC after incorporation into DNA, is long lived in the DNA of whole cells. This provides the possibility that the single-strand nick will be processed into a double-strand break upon subsequent DNA replication. Second, the TC-NER pathway is likely responsible for recognition of the nick, but repair of the damage by the NER pathway is of limited capacity and is conducted in an asymmetrical fashion. Proteins that function 5′ relative to the lesion in the repair of UV damage also participate in the repair of CNddC nucleotide. In contrast, proteins that participate in processes on the 3′ side of the lesion are dispensable to CNDAC-induced damage.

As preliminary studies suggest that CNDAC is a poor substrate for excision (39), it seemed unlikely that the 3′ strand terminated by CNddC would be a substrate for exonucleolytic processing. Therefore, our studies investigated the possibility that either BER, MMR, or NER may function to remove the chain-terminating analogue, using clonogenic sensitization to CNDAC of cells lacking these repair functions as an indicator of involvement. The endonuclease APE1, a key enzyme that is responsible for repairing apurinic/apyrimidinic sites generated in the BER mechanism (40) with a 3′→5′ exonuclease activity (18), was not able to remove the CNDACMP from the 3′ terminus *in vitro* of either a recessed oligonucleotide or a nicked substrate that modeled the β-elimination product (Fig. 1*A*). Consistent with this, a specific APE1 inhibitor (26) did not sensitize cells to CNDAC (Fig. 1*B*). Neither deficiency in the BER scaffold protein, XRCC1, nor lack of DNA pol β (41) sensitized cells to CNDAC, although cells lacking these enzymes were highly sensitized to a monofunctional alkylating agent (Fig. 1*B*). Thus, we conclude that BER is not involved in repair of CNDAC-induced DNA damage, as this function did not spare the toxicity of CNDAC.

MMR processes in eukaryotic cells are dependent upon a nick in the target strand of DNA (42). As this is the lesion generated subsequent to CNDAC nucleotide incorporation, it seemed possible that MMR could be involved in its repair. Furthermore, the principal exonucleolytic enzyme, ExoI, may excise in the 3′→5′ direction from the nick providing that proliferating cell nuclear antigen and MutLα, a heterodimer composed of MLH1 and PMS2, are present (43). Finally, the 3′→5′ proof-reading exonuclease of DNA polymerase δ may participate in excision to a limited extent (44). However, when the clonogenic survival of cells lacking hMLH1 or PMS2 was evaluated, the results indicated that neither line was sensitized to CNDAC (Fig. 2). Whether this could be due to lack of recognition of the nick or to inability of ExoI to remove CNddC remains to be determined.

Our initial studies showed that cells lacking XPF/ERCC1, the endonuclease in NER that incises 5′ to the lesion, were 2-fold to >4-fold sensitized to CNDAC relative to wild-type cells (Fig. 3). In contrast, cells lacking XPG, which incises 3′ to the lesion, were not affected. These results provided the first indication NER is involved in repair of CNDAC-induced damage, albeit in an asymmetrical manner. Furthermore, the effect of CNDAC-induced damage on clonogenic survival was independent of p53 function (Fig. 3*D*).

Additional investigations revealed that CNDAC-induced DNA damage is repaired in a transcription-coupled manner, not in a genome-wide process. Both of XPC-deficient cells derived from a patient and siRNA knockdown of XPC showed 2-fold resistance relative to XPC repleted or wild-type cells, respectively (Fig. 5*B* and *C*). Although the function of XPC is likely to be complex due to the cross-network between different repair pathways (45), our data suggested that XPC may be involved in initiating cell death signals, but not DNA repair, in response to CNDAC.

The finding that nicked DNA cannot be bypassed during transcription (46) is consistent with our observation that TC-NER initiates the cellular repair response to CNDAC. Presumably, when this damage is encountered by RNA pol II during transcription, CSB is recruited and binds to the lesion. The transcription factor TFIIH contributes subunits to the DNA repair process as the associated helicase XPB unwinds the DNA helix 5′ of the nick which subsequently allows XPF/ERCC1 incision 5′ to the CNddC lesion. This was illustrated by the observation that cells deficient in the CSB and XPB showed 1.7-fold to 3.6-fold greater sensitivity to CNDAC (Fig. 5*D*). In contrast, cells lacking XPD, which unwinds the lesion in the 3′ direction in the NER mechanism, were equally sensitive as wild-type cells. Taken together, we conclude that the nick itself is representative of the XPG excision function, so that neither the XPD helicase nor the XPG endonuclease are mandatory participants in the process.

The generation of SSBs after β-elimination after CNDAC incorporation is likely a stochastic process because the analogue is present in DNA both as the CNDAC nucleotide in 5′ to 3′ phosphodiester linkage in DNA and as CNddC at the 3′ termini (5). Internal CNDACMP is unlikely to be repaired based on our observation of ara-C *in vivo* (Supplementary Fig. S3) and earlier reports (31). If so, the removal rate that we measured reflects solely for the excision of CNddC. Overall, removal of the analogue from DNA is a relatively slow process (Fig. 3*A, right* and Supplementary Fig. S3). The rate of CNddC removal was associated with relative greater sensitivity in XPF-deficient UV41 compared with wild-type AA8 cells. This also supports the hypothesis that cells lacking XPF would be sensitized to CNDAC.

In contrast, loss of XPF did not sensitize UV41 to the other two cytosine nucleoside analogues, ara-C and L-OddC (Fig. 4). Under the experimental conditions, ara-C was mainly internally incorporated, so its susceptibility to DNA repair mechanisms would be strongly dependent upon their ability to recognize the analogue. In this respect, it was somewhat more surprising that UV41 has similar sensitivity to the chain terminator L-OddC as was AA8. This suggests that mechanisms that recognize a nick that is not a substrate for ligation are different from those activated by *de facto* DNA chain termination during replication and illustrates the precise responses of cellular defense mechanisms to two analogues that terminate DNA chains but in different biological contexts. Concentrations of CNDAC that greatly reduce clonogenicity have little effect on DNA replication, but the incorporated analogue generates a nick that cannot be ligated and blocks the passing of RNA polymerase II, initiating TC-NER. Presumably, a second round of replication across a nick would generate a double-strand break, although we have not excluded the possibility that a slow repair capability of blocked termini can be bypassed (47, 48). In contrast, the chain terminating L-OddC analogue may stall the replication fork, which, if not excised by APEI, may activate replication restart processes (49).

The results obtained from this study provide insights into the molecular mechanism of potential tumor cell drug resistance by activating this DNA damage repair pathway and provide a rationale for designing inhibitors of NER. An orally bioavailable derivative of CNDAC is currently in clinical investigation (50).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Sampath D, Rao VA, Plunkett W. Mechanisms of apoptosis induction by nucleoside analogs. Oncogene 2003;22:9063–74.
2. Matsuda A, Nakajima Y, Azuma A, Tanaka M, Sasaki T. Nucleosides and nucleotides. 100. 2′-C-cyano-2′-deoxy-1-β-D-arabinofuranosyl-cytosine (CNDAC): design of a potential mechanism-based DNA-strand-breaking antineoplastic nucleoside. J Med Chem 1991;34:2917–9.
3. Matsuda A. 2′-C-Cyano-2′-deoxy-1-b-d-arabinofuranosyl-cytosine(CNDAC): a mechanism-based DNA-strand-breaking antitumor nucleoside. Nucleosides Nucleotides 1995;14:461–71.
4. Hayakawa Y, Kawai R, Otsuki K, Kataoka M, Matsuda A. Evidence supporting the activity of 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentafuranosylcytosine as a terminator in enzymatic DNA-chain elongation. Bioorg Med Chem Lett 1998;8:2559–62.
5. Azuma A, Huang P, Matsuda A, Plunkett W. 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine: a novel anticancer nucleoside analog that causes both DNA strand breaks and G(2) arrest. Mol Pharmacol 2001;59:725–31.
6. Azuma A, Huang P, Matsuda A, Plunkett W. Cellular pharmacokinetics and pharmacodynamics of the deoxycytidine analog 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC). Biochem Pharmacol 2001;61:1497–507.
7. Hanaoka K, Suzuki M, Kobayashi T, et al. Antitumor activity and novel DNA-self-strand-breaking mechanism of CNDAC (1-(2-C-cyano-2-deoxy-β-D-arabino-pentofuranosyl) cytosine) and its N4-palmitoyl derivative (CS-682). Int J Cancer 1999;82:226–36.
8. Sampath D, Cortes J, Estrov Z, et al. Pharmacodynamics of cytarabine alone and in combination with 7-hydroxystaurosporine (UCN-01) in AML blasts *in vitro* and during a clinical trial. Blood 2006;107:2517–24.
9. Shi Z, Azuma A, Sampath D, Li YX, Huang P, Plunkett W. S-phase arrest by nucleoside analogues and abrogation of survival without cell cycle progression by 7-hydroxystaurosporine. Cancer Res 2001;61:1065–72.
10. Liu X, Guo Y, Li Y, et al. Molecular basis for G2 arrest induced by 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine and consequences of checkpoint abrogation. Cancer Res 2005;65:6874–81.
11. Liu X, Matsuda A, Plunkett W. Ataxia-telangiectasia and Rad3-related and DNA-dependent protein kinase cooperate in G2 checkpoint activation by the DNA strand-breaking nucleoside analogue 2′-C-cyano-2′-deoxy-1-D-arabino-pentofuranosylcytosine. Mol Cancer Ther 2008;7:133–42.
12. Kamiya K, Huang P, Plunkett W. Inhibition of the 3′→5′ exonuclease of human DNA polymerase ε by fludarabine-terminated DNA. J Biol Chem 1996;271:19428–35.
13. Kramata P, Downey KM, Paborsky LR. Incorporation and excision of 9-(2-phosphonylmethoxyethyl)guanine (PMEG) by DNA polymerase δ and ε *in vitro*. J Biol Chem 1998;273:21966–71.
14. Perrino FW, Mazur DJ, Ward H, Harvey S. Exonucleases and the incorporation of aranucleotides into DNA. Cell Biochem Biophys 1999;30:331–52.
15. Johnson AA, Ray AS, Hanes J, et al. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J Biol Chem 2001;276:40847–57.
16. Krokan HE, Standal R, Slupphaug G. DNA glycosylases in the base excision repair of DNA. Biochem J 1997;325:1–16.
17. Lindahl T. Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. Mutat Res 2000;462:129–35.
18. Chou KM, Kukhanova M, Cheng YC. A novel action of

human apurinic/apyrimidinic endonuclease: excision of L-configuration deoxyribonucleoside analogs from the 3′ termini of DNA. J Biol Chem 2000;275:31009–15.
19. Iyer RR, Pluciennik A, Burdett V, Modrich PL. DNA mismatch repair: functions and mechanisms. Chem Rev 2006;106:302–23.
20. Chaney SG, Sancar A. DNA repair: enzymatic mechanisms and relevance to drug response. J Natl Cancer Inst 1996;88:1346–60.
21. Yamauchi T, Nowak BJ, Keating MJ, Plunkett W. DNA repair initiated in chronic lymphocytic leukemia lymphocytes by 4-hydroperoxycyclophosphamide is inhibited by fludarabine and clofarabine. Clin Cancer Res 2001;7:3580–9.
22. Sandoval A, Consoli U, Plunkett W. Fludarabine-mediated inhibition of nucleotide excision repair induces apoptosis in quiescent human lymphocytes. Clin Cancer Res 1996;2:1731–41.
23. Busch DB, Cleaver JE, Glaser DA. Large-scale isolation of UV-sensitive clones of CHO cells. Somat Cell Genet 1980;6:407–18.
24. Legerski R, Peterson C. Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. Nature 1992;360:610.
25. Doetsch PW, Cunningham RP. The enzymology of apurinic/apyrimidinic endonucleases. Mutat Res 1990; 236:173–201.
26. Madhusudan S, Smart F, Shrimpton P, et al. Isolation of a small molecule inhibitor of DNA base excision repair. Nucleic Acids Res 2005;33:4711–24.
27. Wyatt MD, Pittman DL. Methylating agents and DNA repair responses: Methylated bases and sources of strand breaks. Chem Res Toxicol 2006;19:1580–94.
28. Thompson LH, Brookman KW, Dillehay LE, et al. A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. Mutat Res 1982;95:427–40.
29. Koi M, Umar A, Chauhan DP, et al. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 1994;54:4308–12.
30. Gaillard PH, Wood RD. Activity of individual ERCC1 and XPF subunits in DNA nucleotide excision repair. Nucleic Acids Res 2001;29:872–9.
31. Kufe DW, Munroe D, Herrick D, Egan E, Spriggs D. Effects of 1-β-D-arabinofuranosylcytosine incorporation on eukaryotic DNA template function. Mol Pharmacol 1984;26:128–34.
32. Kukhanova M, Liu SH, Mozzherin D, Lin TS, Chu CK, Cheng YC. L- and D-Enantiomers of 2′,3′-dideoxycytidine 5′-triphosphate analogs as substrates for human DNA polymerases. Implications for the mechanism of toxicity. J Biol Chem 1995;270:23055–9.
33. Townsend AJ, Cheng YC. Sequence-specific effects of ara-5-aza-CTP and ara-CTP on DNA synthesis by purified human DNA polymerases *in vitro*: visualization of chain elongation on a defined template. Mol Pharmacol 1987;32:330–9.
34. Wood RD. Nucleotide excision repair in mammalian cells. J Biol Chem 1997;272:23465–8.
35. Kufe DW, Major PP, Egan EM, Beardsley GP. Correlation of cytotoxicity with incorporation of ara-C into DNA. J Biol Chem 1980;255:8997–900.
36. Major PP, Egan EM, Beardsley GP, Minden MD, Kufe DW. Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. Proc Natl Acad Sci U S A 1981; 78:3235–9.
37. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 1991;51:6110–7.
38. Huang P, Chubb S, Plunkett W. Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine. A mechanism for cytotoxicity. J Biol Chem 1990;265: 16617–25.
39. Wang Y, Matsuda A, Plunkett W. Excision of 2′-C-cyano-2′-deoxy-1-β-D-arabino-pentofuranosylcytosine (CNDAC) residues from DNA [abstract 516]. In: Proceedings of the 97th Annual Meeting of the American Association for Cancer Research; AACR; 2006. p. 122.
40. Robson CN, Hickson ID. Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in E. coli xth (exonuclease III) mutants. Nucleic Acids Res 1991;19:5519–23.
41. Sobol RW, Horton JK, Kuhn R, et al. Requirement of mammalian DNA polymerase-β in base-excision repair. Nature 1996;379:183–6.
42. Fang WH, Modrich P. Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J Biol Chem 1993;268:11838–44.
43. Dzantiev L, Constantin N, Genschel J, Iyer RR, Burgers PM, Modrich P. A defined human system that supports bidirectional mismatch-provoked excision. Mol Cell 2004;15:31–41.
44. Constantin N, Dzantiev L, Kadyrov FA, Modrich P. Human mismatch repair: reconstitution of a nick-directed bidirectional reaction. J Biol Chem 2005;280: 39752–61.
45. Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. Mutat Res 2002;511:145–78.
46. Saxowsky TT, Doetsch PW. RNA polymerase encounters with DNA damage: transcription-coupled repair or transcriptional mutagenesis? Chem Rev 2006; 106:474–88.
47. Poltoratsky V, Horton JK, Prasad R, Wilson SH. REV1 mediated mutagenesis in base excision repair deficient mouse fibroblast. DNA Repair (Amst) 2005;4: 1182–8.
48. Friedberg EC, Wagner R, Radman M. Specialized DNA polymerases, cellular survival, and the genesis of mutations. Science 2002;296:1627–30.
49. Heller RC, Marians KJ. Replication fork reactivation downstream of a blocked nascent leading strand. Nature 2006;439:557–62.
50. Kantarjian H, Garcia-Manero G, Faderi S, et al. Phase I study of sapacitabine, an oral nucleoside analogue, in patients with advanced leukemias or myelodysplastic syndromes (MDS) [abstract]. Blood 2007;110:270a.