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Amino Acid Asp181 of 5′-Flap Endonuclease 1 Is a Useful Target for Chemotherapeutic Development[†]

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ABSTRACT: DNA alkylation-induced damage is one of the most efficacious anticancer therapeutic strategies. Enhanced DNA alkylation and weakened DNA repair capacity in cancer cells are responsible for the effectiveness of DNA-alkylating therapies. 5′-Flap endonuclease 1 (Fen1) is an important enzyme involved in base excision repair (BER), specifically in long-patch BER (LP-BER). Using the site-directed mutagenesis approach, we have identified an important role for amino acid Asp181 of Fen1 in its endonuclease activity. Asp181 is thought to be involved in Mg^{2+} binding in the active site. Using structure-based molecular docking of Fen1 targeted to its metal binding pocket M2 (Mg^{2+} site), we have identified a potent low-molecular weight inhibitor (LMI, NSC-281680) that efficiently blocks LP-BER. In this study, we have demonstrated that the interaction of this LMI with Fen1 blocked its endonuclease activity, thereby blocking LP-BER and enhancing the cytotoxic effect of DNA-alkylating agent Temozolomide (TMZ) in mismatch repair (MMR)-deficient and MMR-proficient colon cancer cells. The results further suggest that blockade of LP-BER by NSC-281680 may bypass other drug resistance mechanisms such as mismatch repair (MMR) defects. Therefore, our findings provide groundwork for the development of highly specific and safer structure-based small molecular inhibitors targeting the BER pathway, which can be used along with existing chemotherapeutic agents, like TMZ, as combination therapy for the treatment of colorectal cancer.

Colorectal cancer develops in a multistep process involving the functional imbalance among oncogenes, tumor suppressor genes, and DNA repair genes (*1*). Mutations of the adenomatous polyposis coli (*APC*), K_i-*ras*, deleted in colorectal cancer (*DCC*), and *p53* genes play important roles at different stages of colorectal tumorigenesis (*2*). Mutation of the *APC* gene is an early event in familial adenomatous polyposis (FAP), a syndrome in which there is an inherited predisposition to colon cancer. The success of the treatment of colon cancer patients depends on matching the most effective therapeutic regimen with the characteristics of the individual tumor. The primary challenge in achieving this goal is the heterogeneity of the disease. In the past 10 years, the overall survival of colon cancer patients has significantly improved with adjuvant drug trials. However, the recurrence rate over five years is still high. Although a great deal has been learned about the molecular events involved in the initiation and progression of colorectal cancer, surgery still remains the primary treatment, followed by chemotherapy. Thus, there is clearly an urgent need for the development of new chemotherapeutic drugs and strategies.

In chemotherapy, DNA-alkylating agents play a central role in the curative treatment of many tumors. However, one of the major obstacles in chemotherapy is the development of chemoresistance which limits the effectiveness of these agents. The efficacy of the most widely used methylating agent, Temozolomide (TMZ,[1] 4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]-nona-2,7,9-triene-9-carboxamide, NSC-362856), has been attributed to the formation of O^6-methylguanine (O^6-MeG), a DNA lesion repaired by the protein O^6-methyguanine methyltransferase (MGMT). TMZ resistance has been ascribed to elevated levels of MGMT and/or a reduced level of mismatch repair (MMR). Inhibitors of these DNA repair systems have emerged, but they target mainly the MGMT and MMR pathways. However, more than 80% of the DNA lesions induced by TMZ are N-methylated bases [N^7-methylguanine (N^7-MeG), N^3-methylguanine (N^3-MeG), and N^3-methyladenine (N^3-MeA)] that are recognized by DNA glycosylases and are processed efficiently by the base excision repair (BER) system. Therefore, resistance to TMZ may also be due to robust base excision repair. The blockade of the BER pathway has been overlooked, although in the case of several DNA-alkylating drugs, including TMZ, BER is responsible for the repair of 70, 5, and 9% of N^7-MeG, N^3-MeG, and N^3-MeA lesions, respectively (*3*). Any defect in the BER pathway can cause an accumulation of these lesions, resulting in cytotoxicity, a process that can be exploited as a chemotherapeutic target in cancer cells (*4*).

A new and emerging concept is to sensitize cancer cells to DNA-damaging agents by inhibiting various proteins in DNA repair pathways. Low-molecular weight inhibitors (LMIs) have been identified by molecular docking or NMR studies to target

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[1]Abbreviations: APE1, apurinic/apyrimidinic endonuclease 1; Fen1, 5′-flap endonuclease 1; LP-BER, long-patch base excision repair; MMR, mismatch repair; Pol-β, DNA polymerase β; LMI, low-molecular weight inhibitor; TMZ, Temozolomide.

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the BER pathway by inhibiting AP-endonuclaese 1 (APE1) and Pol-β activities (*5*). Although a number of Pol-β inhibitors have been reported in recent years (*5*), more potent and selective inhibitors are still needed. The most active LMI identified for Pol-β by NMR chemical shift mapping is pamoic acid (*6*). However, this LMI, which inhibits the dRP-lyase activity of Pol-β and blocks Pol-β-directed single-nucleotide BER (SN-BER), requires high concentrations of the inhibitor. Since abasic DNA damage can also be repaired by LP-BER, there is a need for agents that can block the LP-BER pathway as well, in which 5′-flap endonuclease 1 (Fen1) plays a major role (*7*). Fen1 recognizes and removes the 5′-flap structure generated by Pol-β during the strand displacement synthesis. The removal of this flap is essential for the joining of the newly synthesized DNA strand with the parent strand by DNA ligase to complete the repair.

The 5′-flap structure is a common DNA structural intermediate occurring during DNA replication, recombination, and repair. In eukaryotic DNA replication, displacement of an upstream primer by an incoming polymerase can result in the formation of the 5′-flap structure (*8*). Fen1 cleaves the displaced flap at the single-strand−double-strand junction. It also acts as a 5′−3′ exonuclease. By doing so, Fen1 participates in hydrolysis of double-stranded DNA substrates containing a nick, gap, or 3′-overhang. During lagging strand DNA synthesis, RNA primers are removed by RNase H1. However, this enzyme cannot excise the final 5′-terminal ribonucleotide at the RNA−DNA junction. The completion of RNA primer removal by Fen1 is essential for Okazaki fragment processing in reconstituted replication assays (*9*, *10*). The 5′-flap intermediates are also formed during double-stranded break repair, homologous recombination, and excision repair (*11*). Thus, Fen1 is an important enzyme with multiple functions in the cell.

Using the site-directed mutagenesis approach, we identified an important role for amino acid Asp181 of Fen1 in its endonuclease activity. Asp181 is thought to be involved in Mg^{2+} binding in the active site and, thus, important for catalytic activity (*12*). Using structure-based molecular docking of Fen1 and targeting its metal binding pocket M2 (Mg^{2+} site), which is formed by the amino acid residues Asp179, Asp181, and Asp233 (*13*), we have identified a potent low-molecular weight inhibitor (LMI) which efficiently blocks LP-BER. In this paper, we have demonstrated that the interaction of this LMI with Fen1 blocked its endonuclease activity, thereby blocking LP-BER and potentiating the cytotoxic effects of TMZ in both MMR-deficient and MMR-proficient colon cancer cells. Therefore, our findings provide a basis for the development of highly specific and safer structure-based small molecular inhibitors, in combination with existing DNA-alkylating agents, as a novel therapeutic strategy for intervention of colorectal cancer.

MATERIALS AND METHODS

Maintenance of Mammalian Cell Lines. Human colon cancer cell lines HCT-116 (MMR-deficient, ATCC, Manassas, VA) and HCT-116+ch3 (chromosome 3 complementation, MMR-proficient, T. Kunkel, National Institute of Environmental Health Sciences, Research Triangle Park, NC) were grown in McCoy's 5a medium at 37 °C under a humidified atmosphere of 5% CO_2. In each case, the medium was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin, and 100 μg/mL streptomycin.

Oligonucleotides and Chemicals. All oligonucleotides were purchased from Sigma-Genosys (Woodlands, TX). Restriction enzymes and T4 polynucleotide kinase (PNK) were purchased from New England Biolabs (Ipswich, MA), whereas radionuclide [γ-^{32}P]ATP was purchased from MP Biomedicals (Solon, OH).

Molecular Docking. The protein crystal structure used for in silico screening of the structural site in question was that of the human Fen1−PCNA complex (Protein Data Bank entry 1UL1) (*12*). MSROLL was used to generate the molecular surface of 1UL1, which was then used as input for the sphere generation program SPHGEN. A cluster of spheres occupying the site of interest was then selected and manually edited, leaving 50 spheres to be used as the shape-based site points. SHOWBOX was used to construct a three-dimensional rectangle, 4 Å in every direction from the sphere cluster, in which the steric and electrostatic environments of the protein were calculated using GRID. SYBYL 7.0 (Tripos, St. Louis, MO) was used to convert PDB entry 1UL1 into the appropriate mol2 format. DOCK was then used to screen approximately 220000 small molecules from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) within the 1UL1 grid. The output was ranked on the basis of predicted energy scores (composed of electrostatic interactions and van der Waals forces). The top 40 compounds were obtained from the NCI/DTP for testing. All of the programs listed for this procedure with the exception of SYBYL 7.0 were part of the DOCK5.0 suite developed at the University of California (San Francisco, CA) (*14*).

Purification of His-Tagged Human Pol-β, Fen1, and DNA Ligase I Proteins. We purified the hexahistidine-tagged fusion proteins of wild-type Pol-β and Fen1 as described previously with some modifications (*15*). The Fen1(D181N) construct was made using the Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions using the sense strand (5′-AAAGTCTATGCTGCGGCTACCGAGGACATGAATTG-CCTCACCTTC-3′), antisense strand (5′-GAAGGTGAGG-CAATTCATGTCCTCGGTAGCCGCAGCATAGACTTT-3′), and wild-type Fen1 as a template. Human APE1 was obtained from L. Bloom (University of Florida).

Synthesis and Labeling of the Fen1 Substrate. The Fen1 substrate for 5′-flap endonuclease activity was made by annealing an upstream 23-mer (5′-CTAGATGCCTGCAGCTGATGCG-C-3′) and a downstream 51-mer oligonucleotide (5′-FAAC-ATTTTTTTGTACGGATCCACGTGTACGGTACCGAGG-GCGGGTCGACA-3′) to a 63-mer complementary template (5′-GATCTACGGACGTCGACTACGCGACATGCCTAGGT-GCACATGCCATGGCTCCCGCCCAGCTGT-3′). The 51-mer downstream oligonucleotide has a flap of 11 nucleotides (with a teterahydrofuran, F, residue at the 5′-end), which is cleaved by Fen1. The 51-mer downstream oligonucleotide was radiolabeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Bio Lab, Woburn, MA). The labeled probe was purified by using a nick column (GE Healthcare, Piscataway, NJ). All three oligonucleotides were annealed at a molar ratio of 1:1:1.

Fen1 Endonuclease Assay. The in vitro Fen1 endonuclease assay was performed in a 20 μL reaction mixture containing the following final concentrations: 30 mM Hepes (pH 7.5), 30 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, 100 μg/mL BSA, and 5% (v/v) glycerol. Briefly, 0.5 nM Fen1 and different concentrations of LMIs were incubated at room temperature for 5 min, followed by addition of 2.5 nM ^{32}P-labeled 51-mer flapped DNA

substrate. Then, it was incubated for 30 min at 37 °C. Each reaction was terminated by the addition of 20 μL of stop solution [5.0 mM EDTA and 0.4% (w/v) SDS] with 1 μg of proteinase K and 5 μg of carrier tRNA. After incubation for an additional 20 min at 37 °C, the DNA was extracted with an equal volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v) followed by ethanol precipitation. The 11-mer reaction products were resolved on a 15% polyacrylamide−7 M urea gel.

In Vitro LP-BER Assays with Purified Proteins. The LP-BER reaction mixture contained the following final concentrations: 30 mM Hepes (pH 7.5), 30 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, 100 μg/mL BSA, 0.01% (v/v) Nonidet P-40, 0.5 mM ATP, and dATP, dCTP, dGTP, and dTTP (20 μM) in a final volume of 20 μL. Briefly, 0.5 nM Fen1 and different concentrations of LMIs were incubated at room temperature for 5 min followed by addition of 2.5 nM ^{32}P-labeled 63-mer F-DNA (preincubated with 1 nM APE1 to create an incision at the repair site), 5 nM Pol-β, and 0.4 nM DNA ligase I. The structure of the F-DNA substrates has been described in our previous studies (*16*, *17*). The incubation period for LP-BER was 30 min at 37 °C. Each reaction was terminated by the addition of 20 μL of stop solution [5.0 mM EDTA and 0.4% (w/v) SDS] with 1 μg of proteinase K and 5 μg of carrier tRNA. After incubation for an additional 20 min at 37 °C, the DNA was extracted with an equal volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1, v/v) followed by ethanol precipitation. The reaction products were resolved on a 15% polyacrylamide−7 M urea gel.

In Vitro LP-BER Assays with the Nuclear Extract. The in vitro LP-BER assay was performed using the APE1-nicked ^{32}P-labeled 63-mer F-DNA, and a nuclear extract served as a source of BER proteins. The nuclear extract was prepared using the procedure of Shapiro et al. (*18*). Here, 5 μg of the nuclear extract from HCT-116 cells was incubated at 37 °C for 60 min.

Clonogenic Assay. A single-cell suspension of HCT-116 and HCT-116+ch3 cells was plated (200 cells/well) in triplicate in a six-well plate. Cells were pretreated with varying concentrations of LMI or vehicle (0.1% DMSO) for 2 h followed by the treatment with different concentrations of TMZ for 48 h. After the treatment, the cultures were replaced with fresh medium and cells were allowed to grow for a further 8 days. Visible colonies of more than 100 cells were stained with methylene blue and counted for viability.

RESULTS

Importance of Amino Acid Asp181 of Fen1 in Its 5′-Flap Endonuclease Activity. The active site of human Fen1 is located at the central cleft with two possible metal ion binding sites, which are formed by two clusters of conserved acidic residues. Four residues (Asp34, Asp86, Glu158, and Glu160) form the first metal ion-binding site (M1). Three residues (Asp179, Asp181, and Asp233) form the second metal ion-binding site (M2) (*13*). These acidic residues are conserved in all known Fen1 enzymes, and the prevailing catalytic mechanism is thought to be universal. M1 is known to play an important role in catalysis, probably in the nucleophilic attack of the phosphodiester bonds of DNA, while it has been suggested that M2 is involved in DNA binding (*12*). Using site-directed mutagenesis, we created a mutant of Fen1 in which the Asp181 of metal-binding site M2 was changed to Asn (D181N). The 5′-flap endonuclease assay for this mutant protein (Figure 1B) and

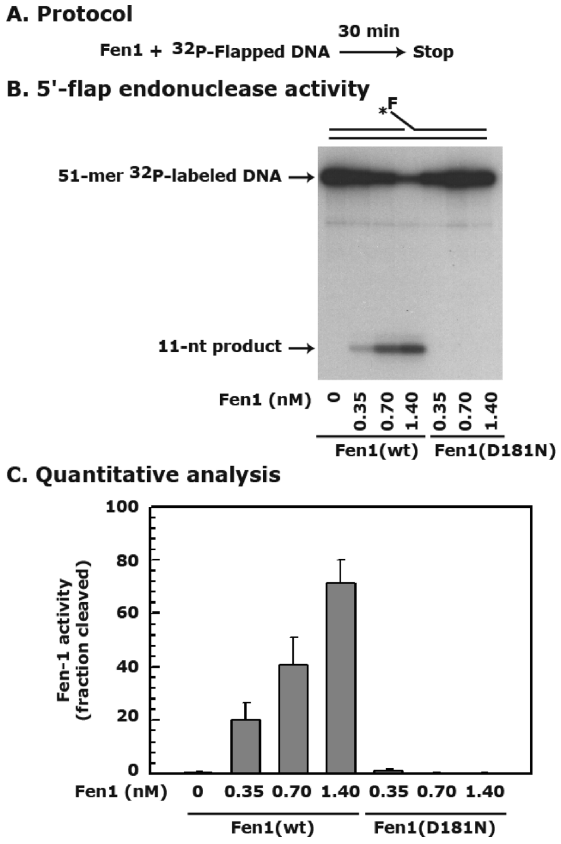

FIGURE 1: Asp181 is an important amino acid for Fen1 endonuclease activity. Panel A shows the schematic representation of the experimental protocol. Panel B shows the effect of varying concentrations of the wild-type and mutant Fen1(D181N) protein on its endonuclease activity. Panel C is the quantitative analysis of endonuclease activity of Fen1 proteins. The fraction of cleavage was calculated as the percent radioactivity present in the cleaved product as follows: % cleavage = [11-nt/(51-mer + 11-nt)] × 100. The data are the means ± standard error of three different experiments.

quantification of the cleaved product (Figure 1C) revealed that it is functionally inactive, indicating the importance of D181 in its catalytic function. Therefore, M2 was used as a target for the identification of low-molecular weight inhibitors (LMIs) by structure-based molecular docking.

Structure-Based Molecular Docking of Small Molecules. Ideally, a drug should be highly active with only weak or no side effects. To achieve these goals, low-molecular weight inhibitors (LMIs) should be selected or designed on the basis of structural characteristics that promote specific interaction with the intended target site (*19*). We used a high-performance computing simulation method to screen approximately 220000 small molecules for their ability to interact with the crystal structure of Fen1. Specifically, the screen was targeted to the Fen1 metal-binding pocket (Mg^{2+}-binding site), which is formed by amino acid residues Asp179, Asp181, and Asp233, for the purpose of identifying a small molecule to block its 5′-flap endonuclease activity. The protein crystal structure used for in silico screening of the structural site in question was that of the human Fen1−PCNA complex (Figure 2A) (Protein Data Bank entry 1UL1) (*12*). After being screened, the 40 highest-scoring compounds were obtained from the NCI/DTP for testing. One such small molecule (NSC-281680) is shown interacting with amino acid Asp181 of the Fen1 metal-binding pocket (M2) (Figure 2B,C).

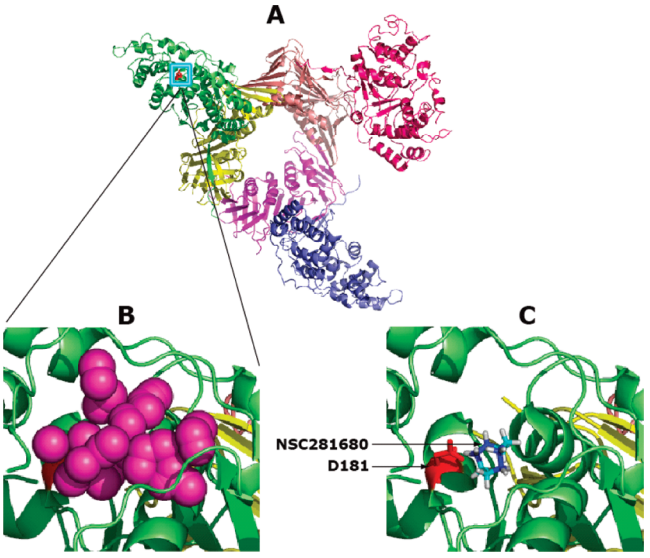

FIGURE 2: Identification of small molecules interacting with Fen1. (A) Crystal structure of the human Fen1−PCNA complex (green, pink, and blue for Fen1 and yellow, purple, and salmon for PCNA) (PDB entry 1UL1). This structure was used as the basis for in silico molecular screening. (B) The screening area of Fen1 is designated by site point spheres (purple) to which compounds from the NCI/DTP database were docked. (C) Structure of NSC-281680 docked into the Fen1 pocket. The key amino acid residue D181 (red) is in the proximity of the compound.

NSC-281679, NSC-337807, and NSC-281680 Block Fen1 Endonuclease Activity. Initially, we screened 40 different LMIs for their ability to block Fen1 endonuclease activity. We selected three LMIs, namely, NSC-281679, NSC-337807, and NCS-281680, for further studies. Results obtained using these LMIs are shown in Figure 3. These LMIs blocked Fen1-mediated endonuclease activity in a dose-dependent manner (Figure 3B, lanes 2−7, lanes 8−13, and lanes 14−19, respectively). The IC_{50} of these LMIs for inhibiting Fen1 endonuclease activity was also calculated (Figure 3C). The LMI NSC-281680 inhibited Fen1 endonuclease activity in a dose-dependent manner and blocked it completely at 5 μM, while a similar effect was observed for NSC-337807 and NSC-281679, but at 10-fold higher concentrations.

NSC-281679, NSC-337807, and NSC-281680 Blocked LP-BER Activity. The LMIs NSC-281679, NSC-337807, and NSC-281680 were further tested for their inhibitory effects on LP-BER by using purified proteins. Results showed that these LMIs blocked Fen1-mediated strand displacement (Figure 4B, lanes 6−9, 14−17, and 22−25, respectively) as well as complete LP-BER activities in a dose-dependent manner (Figure 4B, lanes 10−13, 18−21, and 26−29, respectively). Similarly, using nuclear extracts (NEs) from HCT-116 cells, these LMIs blocked LP-BER in a dose-dependent manner (Figure 5B, lanes 3−7, 8−12, and 13−17, respectively). By comparison, these results revealed that of the three LMIs, NSC-281680 was the most potent. Thus, the LMI NSC-281680 was used in subsequent studies.

The Cytotoxic Effect of Temozolomide (TMZ) on MMR-Deficient and MMR-Proficient Colon Cancer Cells Is Enhanced by NSC-281680. We determined the effect of NSC-281680 on TMZ-induced cytotoxicity in MMR-deficient (HCT-116) and MMR-proficient (HCT-116+ch3) colon cancer cells. In MMR-proficient HCT-116+ch3 cells, a single copy of chromosome 3 harboring the *hMLH1* gene has been inserted. As expected, these cells exhibited a greater cytotoxic response to

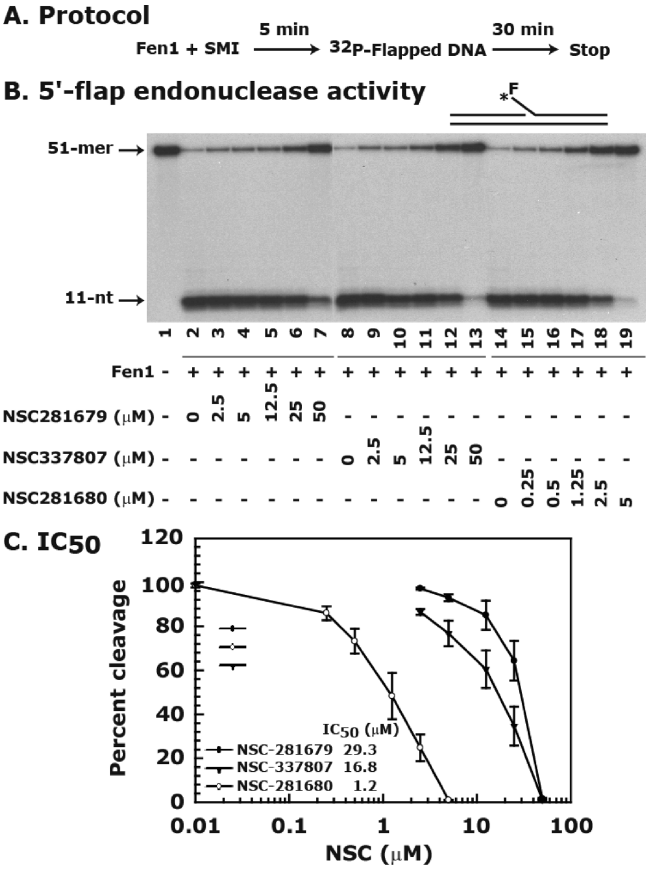

FIGURE 3: Screening of LMIs for blocking the 5′-flap endonuclease activity of Fen1. Panel A shows the schematic representation of the experimental plan and conditions that are described in detail in Materials and Methods. Panel B shows the effect of varying concentrations of LMIs on the 5′-flap endonuclease activity of Fen1. Lane 1 contained ^{32}P-labeled 51-mer F-DNA substrate and lane 2 the 11-mer cleaved product; lanes 3−7, 8−13, and 14−19 show the dose-dependent inhibition of Fen1 endonuclease activity by NSC-281679, NSC-337807, and NSC-281680, respectively. The arrows indicate the positions of 51-mer substrate and 11-mer product. Panel C shows the IC_{50} values of NSC-281679, NSC-337807, and NSC-281680. The data are means ± standard error of three different experiments.

TMZ treatment than the MMR-deficient HCT-116 cells (Figure 6A,B). The combination of different concentrations of NSC-281680 with TMZ further reduced the IC_{50} of TMZ in a dose-dependent manner in these cells (Figure 6A,B). When applied alone, NSC-281680 exhibited no cytotoxic effects on either the HCT-116 or HCT-116+ch3 cell line (Figure 6C), suggesting its clinical usefulness in combination with TMZ. These results suggest that NSC-281680 interacts with Fen1 and blocks LP-BER activity, which in turn increases the size of the DNA damage burden caused by TMZ treatment and results in cell death. Thus, this strategy can be useful for chemotherapeutic intervention in both MMR-deficient and MMR-proficient colorectal tumors.

DISCUSSION

The current approach for the discovery of antitumor agents relies on random and semiempirical screening procedures that have proven to be largely ineffective in treating complicated diseases, including colorectal cancers. The failure of such drugs could be due to an insufficient understanding of their pharmacology and their impact on the biochemistry and molecular genetics of normal and cancerous cells. Thus, there is a critical

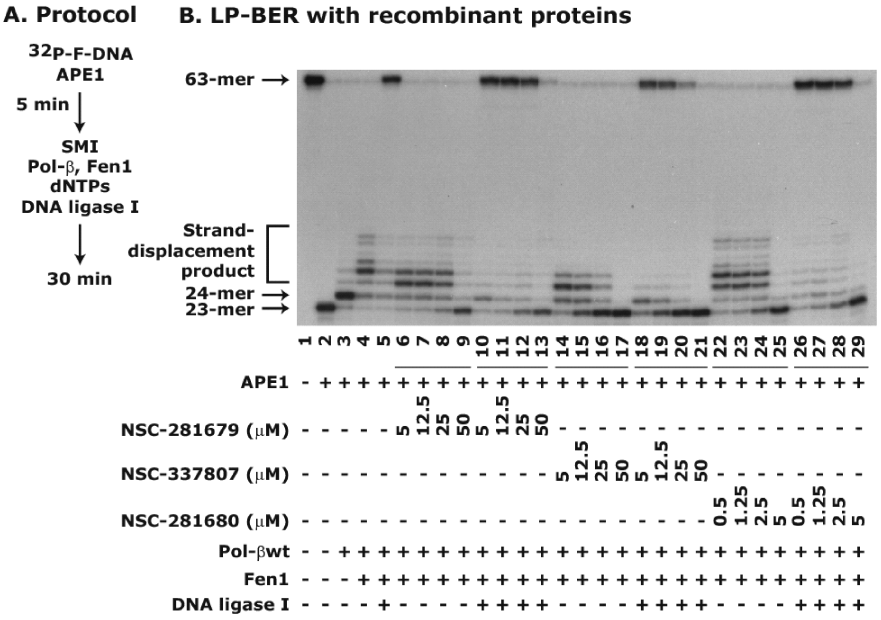

FIGURE 4: NSC-281679, NSC-337807, and NSC-281680 block LP-BER activity using purified proteins. Panel A shows the schematic representation of the experimental protocol. Panel B shows the effect of varying concentrations of LMIs on LP-BER activity using purified proteins. The LP-BER reaction mixture in a volume of 20 μL contained the following final concentrations: 30 mM Hepes (pH 7.5), 30 mM KCl, 8.0 mM $MgCl_2$, 1.0 mM DTT, 100 μg/mL BSA, 0.01% (v/v) Nonidet P-40, 0.5 mM ATP, and dATP, dCTP, dGTP, dTTP (20 μM each). Briefly, 0.5 nM Fen1 and different concentrations of LMIs were incubated at room temperature for 5 min followed by addition of 2.5 nM ^{32}P-labeled 63-mer F-DNA (preincubated with 1 nM APE1 to create an incision at the repair site), 5 nM Pol-β, and 0.4 nM DNA ligase I. The incubation period for LP-BER was 30 min at 37 °C. The data are representative of three independent experiments.

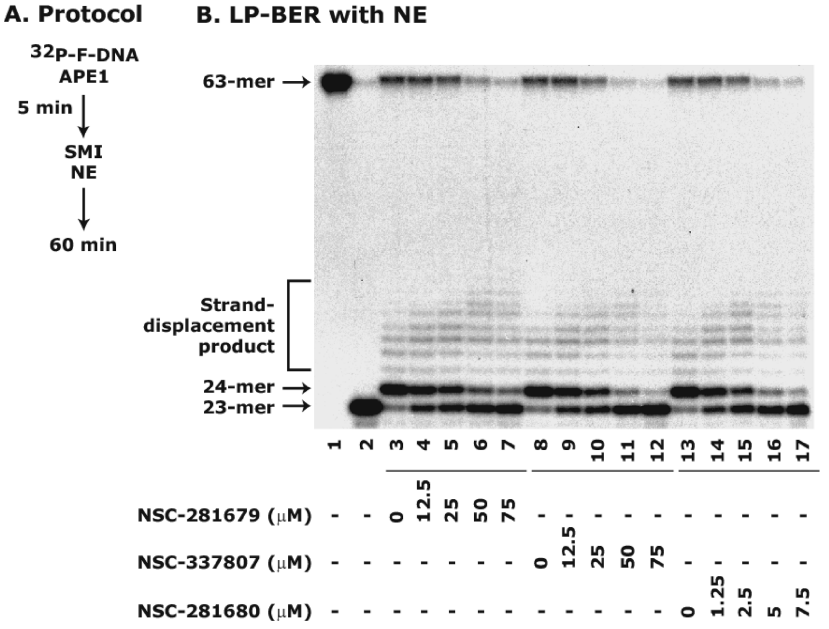

FIGURE 5: NSC-281679, NSC-337807, and NSC-281680 block LP-BER activities using nuclear extract. Panel A shows the schematic representation of the experimental plan and conditions that are described in detail in Materials and Methods. Panel B shows the effect of varying concentrations of LMIs on LP-BER activity. The in vitro LP-BER assays with nuclear extract were performed as described above using the APE1-nicked 63-mer F-DNA, except that instead of purified individual BER proteins, a nuclear extract was used as a source of BER proteins. The data are representative of three independent experiments.

need for rational design of mechanism-based drugs with well-characterized specific targets. Most chemotherapeutic drugs are DNA-alkylating agents. Some of these are procarbazine (PCB), dacarbazine (DIC), streptozotocin (STZ), Temozolomide (TMZ), chloroethylnitrosourea (CENU), carmustin (BCNU), lomustine (CCNU), nimustine (ACNU), and fotemustine (Muphoran). The efficacy of these alkylating agents can be improved by blocking DNA repair pathways (*20*). These agents react with DNA via an S_N1 mechanism to form an electrophilic carbonium ion, which covalently binds to nucleophilic sites on the DNA. Among the alkylating agents, TMZ has been assessed in clinical trials involving patients with renal cell carcinoma, soft tissue sarcoma, pancreatic carcinoma, advanced nasopharyngeal carcinoma, prostate cancer, and brain metastases from a variety of solid tumors and melanoma (*21*). Importantly, a phase II clinical trial study of TMZ in preselected

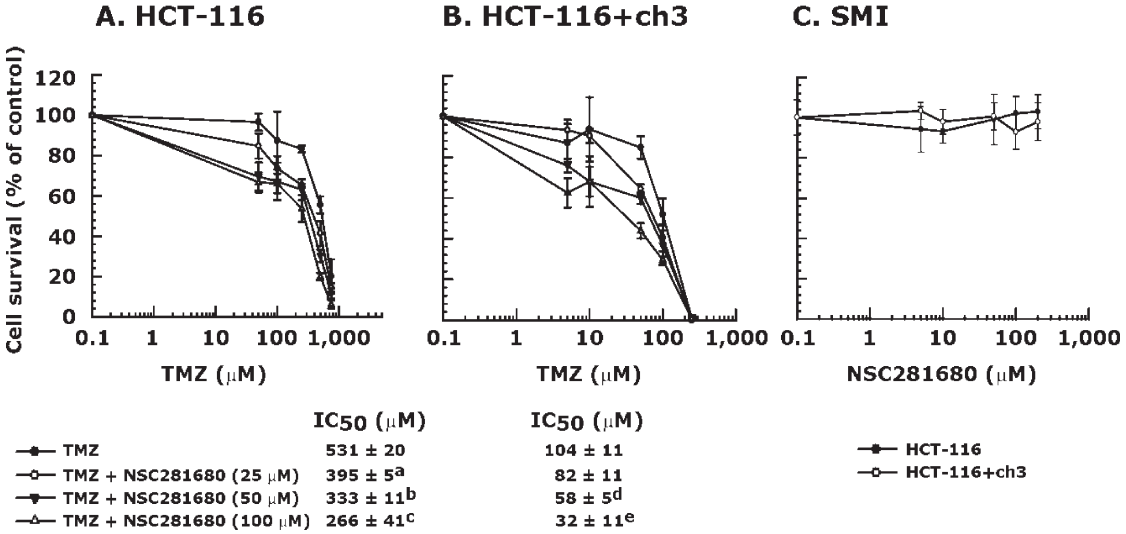

FIGURE 6: Efficacy of NSC-281680 in enhancing the cytotoxicity of TMZ to HCT-116 and HCT-116+ch3 colon cancer cell lines. Cells were pretreated for 2 h with different concentrations of NSC-281680 followed by treatment with different concentrations of TMZ for 48 h. The survival of the cells was determined after 8 days with a clonogenic survival assay. Panels A and B show the enhanced cytotoxic effect of NSC-281680 that enhances the cytotoxicity of TMZ in HCT-116 and HCT-116+ch3 cells, respectively. Panel C shows the cytotoxic effect of different concentrations of NSC-281680 alone in HCT-116 and HCT-116+ch3 cells. The data are means ± standard error of three different experiments. Results are statistically significant as compared with TMZ (a, $P < 0.002$; b, $P < 0.001$; c, $P < 0.004$; d, $P < 0.014$; e, $P < 0.009$).

advanced aerodigestive tract cancers, including colon cancer, is already in progress (Schering-Plough, http://clinicaltrials.gov/ct2/show/NCT00423150).

The extent of DNA damage incurred plays a role in determining the cell's response. The cells either attempt to continue to repair the DNA damage or, in the face of extensive damage, switch to an apoptotic response. The use of alkylating agents as chemotherapeutic drugs is based on their ability to trigger the apoptotic response (*22*), and the therapeutic efficacy is determined by the balance between DNA damage and repair. In many cases, an elevated DNA repair capacity in tumor cells leads to drug resistance and severely limits the efficacy of these agents. Thus, interfering with DNA repair in combination with DNA-alkylating agents has emerged as an important strategy (*23*). The alkylation damage-induced lesions are repaired by BER, MGMT, and MMR pathways. The inhibitors that have been developed as anticancer drugs mostly target the MGMT and MMR pathways, and many colon tumors become resistant to alkylating drugs because of a deficiency in MMR (*24*). However, the blockade of the BER pathway is equally important in inducing cellular toxicity as the major alkylation lesions created by TMZ are repaired by the BER pathway (*3*, *4*, *25*).

LP-BER is the subpathway of BER in which Fen1 plays a critical role by removing the 5′-flap generated by Pol-β during the strand displacement synthesis. In our lab, using a site-directed mutagenesis approach, we identified Asp181 as an important amino acid in Fen1 endonuclease activity, which has been supported by other studies (*26*, *27*). Since Asp181 is located in the active site of Fen1 along with Asp179 and Asp233 that constitute the Mg^{2+}-binding pocket M2 (*12*, *13*), we selected this amino acid to identify low-molecular weight inhibitors (LMIs) predicted to interact with Fen1 and blocking its activity. By using structure-based molecular docking, we have identified a potent LMI NSC-281680, which efficiently blocks LP-BER. Although the mechanism by which it blocks Fen1 activity can be more precisely determined by cocrystallization of NSC-281680 with Fen1, our studies suggest that interaction of NSC-281680 with Fen1 may cause some structural changes and the loss of Mg^{2+} binding that may be leading to loss of its endonuclease activity. In earlier studies, it was suggested that the position of D181 is not only crucial for the cleavage capability of Fen1, but it also acts as a ligand for Mg^{2+} binding (*26*).

NSC-281680 does not block the in vitro activity of APE1, Pol-β, or DNA ligase I (data not shown), suggesting its specificity toward the Fen1-targeted cytotoxicity of TMZ toward colon cancer cells. Since Fen1 is a ubiquitously expressed protein and BER is a primary DNA repair pathway of alkylation-induced lesions, it may also affect normal colonic epithelial cells. However, its effect will be much stronger in rapidly proliferating cancer cells than in normal cells, as supported by previous studies (*28*). We expect that TMZ alone or in combination with NSC-281680 reversibly targets normal cells and preferentially targets the tumor cells. This will be explored in our future studies involving in vivo xenograft tumor growth of MMR-proficient and MMR-deficient colon cancer cells.

Many colon tumors become resistant to DNA-alkylating agents because of overexpression of MGMT or MMR deficiency (*29*). Cells deficient in MGMT are unable to process O^6-MeG during DNA synthesis, and if it is not repaired, then a G:C to G:T transition mutation occurs (*30*). The G:T mismatch is then repaired by the MMR pathway (*31*). However, if the O^6-MeG is not repaired before the resynthesis step in MMR, a thymine is likely to be reinserted opposite the lesion. It is believed that the repetitive cycle of futile MMR results in the generation of tertiary lesions, most likely gapped DNA. This then gives rise to double-stranded breaks (DSBs) in DNA that elicit a cell death response (*31*). Thus, a chemotherapeutic strategy which can induce cell death in both MMR-proficient and MMR-deficient colon cancer cells is highly desirable. Our results indicate that the strategy of combining NSC-281680 with TMZ seems to effectively block the growth of both MMR-proficient and MMR-deficient colon cancer cells. This suggests that the blockade of the repair of TMZ-induced N^7-MeG, N^3-MeG, and N^3-MeA lesions by NSC-281680 causes much higher cytotoxicity than the lesions of O^6-MeG. The results of previous studies suggest that N^7-MeG, N^3-MeG, and

N^3-MeA lesions can be toxic in both MMR-deficient and MMR-proficient cells if the BER pathway is interrupted (*32*), and clinical studies indicate that MMR deficiency may not be the main cause of TMZ-induced resistance in adult malignant glioma (*33*). Our results support these findings and clearly suggest the importance of targeting BER and argue in favor of mechanistic studies for the development of inhibitors of BER. Thus, we expect that NSC-281680 can be used in combination with TMZ as a highly effective strategy that will provide the preclinical framework for the development of novel and advanced chemotherapeutic agents and facilitate the improvement of conventional colon cancer treatments.

REFERENCES

1. Fearon, E. R., and Vogelstein, B. (1991) A genetic model for colorectal tumorigenesis. *Cell 61*, 759–767.
2. Narayan, S., and Roy, D. (2003) Role of APC and DNA mismatch repair genes in the development of colorectal cancers. *Mol. Cancer 2*, 41.
3. Liu, L., and Gerson, S. L. (2004) Therapeutic impact of methoxyamine: Blocking repair of abasic sites in the base excision repair pathway. *Curr. Opin. Invest. Drugs 5*, 623–627.
4. Adhikari, S., Choudhury, S., Mitra, P. S., Dubash, J. J., Sajankila, S. P., and Roy, R. (2008) Targeting base excision repair for chemo sensitization. *Anticancer Agents Med. Chem. 8*, 351–357.
5. Horton, J. K., and Wilson, S. H. (2003) Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency. *DNA Repair 6*, 530–543.
6. Hu, H. Y., Horton, J. K., Gryk, M. R., Prasad, R., Naron, J. M., Sun, D. A., Hecht, S. M., Wilson, S. H., and Mullen, G. P. (2004) Identification of small molecule synthetic inhibitors of DNA polymerase β by NMR chemical shift mapping. *J. Biol. Chem. 279*, 39736–39744.
7. Beard, W. A., and Wilson, S. H. (2006) Structure and mechanism of DNA polymerase β. *Chem. Rev. 106*, 361–382.
8. Bambara, R. A., Murante, R. S., and Henrichsen, L. A. (1997) Enzymes and reactions at the eukaryotic DNA replication fork. *J. Biol. Chem. 272*, 4647–4650.
9. Robins, R., Pappin, D. J. C., Wood, R. D., and Lindahl, T. (1994) Structural and functional homology between mammalian DNase IV and the 5′-nuclease domain of *Escherichia coli* DNA polymerase I. *J. Biol. Chem. 269*, 28535–28538.
10. Waga, S., Bauer, G., and Stillman, B. (1994) Reconstitution of complete SV40 DNA replication with purified replication factors. *J. Biol. Chem. 269*, 10923–10934.
11. Kim, C. Y., Shen, B., Park, M. S., and Olah, G. A. (1999) Structural changes measured by X-ray scattering from human flap endonuclease-1 complexed with Mg^{2+} and flap DNA substrate. *J. Biol. Chem. 274*, 1233–1239.
12. Shen, B., Nolan, J. P., Sklar, L. A., and Park, M. S. (1996) Essential amino acids for substrate binding and catalysis of human flap endonuclease 1. *J. Biol. Chem. 271*, 9173–9176.
13. Sakurai, S., Kitano, K., Hirito, Y., Hamada, K., Okada, K., Fukuda, K., Uchida, M., Ohtsuka, E., Morioka, H., and Hakoshima, T. (2005) Structural basis for recruitment of human flap endonuclease 1 to PCNA. *EMBO J. 24*, 683–693.
14. Moustakas, D. T., Lang, P. T., Pegg, S., Pettersen, E., Kuntz, I. D., Brooijmans, N., and Rizzo, R. C. (2006) Development and validation of a modular, extensible docking program: DOCK 5. *J. Comput.-Aided Mol. Des. 20*, 601–619.
15. Balusu, R., Jaiswal, A. S., Armas, M. L., Bloom, L. B., and Narayan, S. (2007) Structure/function analysis of the interaction of adenomatous polyposis coli (APC) with DNA polymerase β and its implications for base excision repair. *Biochemistry 46*, 13961–13974.
16. Narayan, S., Jaiswal, A. S., and Balusu, R. (2005) Tumor suppressor APC blocks DNA polymerase β-dependent strand displacement synthesis during long patch but not short patch base excision repair and increases sensitivity to methylmethane sulfonate. *J. Biol. Chem. 280*, 6942–6949.
17. Jaiswal, A. S., Balusu, R., Armas, M. L., Kundu, C. N., and Narayan, S. (2006) Mechanism of adenomatous polyposis coli (APC)-mediated blockage of long-patch base excision repair. *Biochemistry 45*, 15903–15914.
18. Shapiro, D. J., Sharp, P. A., Wahli, W. W., and Keller, M. J. (1988) A high-efficiency HeLa cell nuclear transcription extract. *DNA 7*, 47–55.
19. Hait, W. N. (2009) Targeted cancer therapies. *Cancer Res. 69*, 1263–1267.
20. Middleton, M. R., and Margison, G. P. (2003) Improvement of chemotherapy efficacy by inactivation of a DNA-repair pathway. *Lancet Oncol. 4*, 37–44.
21. Payne, M. J., Pratap, S. E., and Middleton, M. R. (2005) Temozolomide in the treatment of solid tumours: Current results and rationale for dosing/scheduling. *Crit. Rev. Oncol. Hematol. 53*, 241–252.
22. Sawyers, C. (2004) Targeted cancer therapy. *Nature 432*, 294–297.
23. Ding, J., Miao, Z. H., Meng, L. H., and Geng, M. Y. (2006) Emerging cancer therapeutic opportunities target DNA repair systems. *Trends Pharmacol. Sci. 27*, 338–344.
24. Liu, L., Markowitz, S., and Gerson, S. L. (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to Temozolomide but not to 1,3-bis(2-chloroethyl) nitrosourea. *Cancer Res. 56*, 5375–5379.
25. Kaina, B., Christmann, M., Naumann, S., and Roos, W. P. (2007) MGMT: Key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents. *DNA Repair 6*, 1079–1099.
26. Kim, C. Y., Park, M. S., and Dyer, R. B. (2001) Human flap endonuclease-1: Conformational change upon binding to the flap DNA substrate and location of the Mg^{2+} binding site. *Biochemistry 40*, 3208–3214.
27. Zheng, L., Li, M., Shan, J., Krishnamoorthi, R., and Shen, B. (2002) Distinct roles of two Mg^{2+} binding sites in regulation of murine flap endonuclease-1 activities. *Biochemistry 41*, 10323–10331.
28. Keyomarsi, K., and Pardee, A. B. (2003) Selective protection of normal proliferating cells against the toxic effects of chemotherapeutic agents. *Prog. Cell Cycle Res. 5*, 527–532.
29. Liu, L., and Gerson, S. L. (2006) Targeted modulation of MGMT: Clinical implications. *Clin. Cancer Res. 12*, 328–331.
30. Kawate, H., Sakumi, K., Tsuzuki, T., Nakatsuru, Y., Ishikawa, T., Takahashi, S., Takano, H., Noda, T., and Sekiguchi, M. (1998) Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes. *Proc. Natl. Acad. Sci. U.S.A. 95*, 5116–5120.
31. Branch, P., Aquilina, G., Bignami, M., and Karran, P. (1993) Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature 362*, 652–654.
32. Liu, L., Taverna, P., Hitacre, C. M., Chatterjee, S., and Gerson, S. L. (1999) Pharmacologic disruption of base excision repair sensitizes mismatch repair-deficient and -proficient colon cancer cells to methylating agents. *Clin. Cancer Res. 5*, 2908–2917.
33. Maxwell, J. A., Johnson, S. P., McLendon, R. E., Lister, D. W., Horne, K. S., Rasheed, A., Quinn, J. A., Ali Osman, F., Friedman, A. H., Modrich, P. L., Bigner, D. D., and Friedman, H. S. (2008) Mismatch repair deficiency does not mediate clinical resistance to Temozolomide in malignant glioma. *Clin. Cancer Res. 14*, 4859–4868.