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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 anti-cancer therapeutic strategies. Increased DNA alkylation and decreased DNA repair capacity in cancer cells is 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 (LP)-BER. Using the site-directed mutagenesis approach, we have identified an important role for amino acid Asp181 of Fen1 in its endonuclease activity. The Asp181 is thought to be involved in Mg²⁺ binding in the active site. Using structure-based molecular docking of Fen1 targeted to its metal-binding pocket M2 (Mg²⁺ site), we have identified a potent small molecular weight inhibitor (SMI; NSC-281680) that efficiently blocks the LP-BER. In this study, we have demonstrated that the interaction of this SMI with Fen1 blocked its endonuclease activity, thereby blocking the 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.

Keywords

Base excision repair; 5'-flap endonuclease 1; small molecular weight inhibitor; colorectal cancer; chemotherapeutic intervention

Introduction

Colorectal cancer develops in a multi-step process involving the functional imbalance between oncogenes, tumor suppressor genes, and DNA repair genes (1). Mutations of the adenomatous polyposis coli (*APC*), *Ki-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

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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; 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 O⁶-methylguanine (O⁶-MeG), a DNA lesion repaired by the protein O⁶-methylguanine methyl transferase (MGMT). TMZ resistance has been ascribed to elevated levels of MGMT and/or reduced 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 [N⁷-Methylguanine (N⁷-MeG), N³-Methylguanine (N³-MeG) and N³-Methyladenine (N³-MeA)] which 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 N⁷-MeG, N³-MeG, and N³-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. Small molecular weight inhibitors (SMIs) have been identified by molecular docking or NMR studies to target the BER pathway by inhibiting AP-endonuclease 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 SMI identified for Pol- β by NMR chemical-shift mapping is pamoic acid (6). However, this SMI, which inhibits the dRP-lyase activity of Pol- β and blocks Pol- β -directed single-nucleotide (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 long-patch (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, Asp 181 and Asp233 (13), we have identified a potent small molecular weight inhibitor (SMI) which efficiently blocks the LP-BER. In this paper, we have demonstrated that the interaction of this SMI 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; Dr. Tom Kunkel, NIEHS, 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 U/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 (RCSB Protein Data Bank code: 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. The SHOWBOX program was used to construct a 3-dimensional rectangle, 4 Angstroms in every direction from the sphere cluster, in which the steric and electrostatic environments of the protein were calculated using the GRID program. SYBYL 7.0 (Tripos, St. Louis, MO) was used to convert the PDB file of 1UL1 into the appropriate mol2 format. DOCK was then used to screen approximately 220,000 small molecules from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) within the 1UL1 grid. The output was ranked based on 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 UCSF (14).

Purification of His-tagged human Pol- β , Fen1 and DNA ligase I proteins

We purified the hexa-histidine 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, USA) according to the manufacturer's instructions using the sense strand (5'-AAAGTCTATGCTGCGGCTACCGAGGACATGA ATTGCCTCACCTTC-3'), anti-sense

strand (5'-GAAGGTGAGGCAATTCATGTCCTCGGTAGCCGCAGCATAGACTTT-3') and the wild type Fen1 as a template. The human APE1 was obtained from Dr. Linda Bloom (University of Florida, Gainesville, FL).

Synthesis and labeling of Fen1 substrate

Fen1 substrate for 5'-flap endonuclease activity was made by annealing an upstream 23-mer (5'-CTAGATGCCTGCAGCTGATGCGC-3') and a downstream 51-mer oligonucleotide (5'-FAACATTTTTTTGTACGGATCCACGTGTACGGTACCGAGGGCGGGTTCGACA-3') to a 63-mer complementary template (5'-GATCTACGGACGTCGACTACGCGACATGCCTAGGTGCACATGCCATGGCTCCCGCCCAGCTGT-3'). The 51-mer downstream oligonucleotide has a flap of 11-nt (with a tetrahydrofuran, 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 the 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 μ l reaction mixture containing the final concentrations of 30 mM Hepes, pH 7.5, 30 mM KCl, 8.0 mM MgCl₂, 1.0 mM DTT, 100 μ g/ml BSA and 5% (v/v) glycerol. Briefly, 0.5 nM Fen1 and different concentrations of SMIs were incubated at room temp for 5 min, followed by addition 2.5 nM of 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 μ l of stop solution (5.0 mM EDTA, 0.4% (w/v) SDS) with 1 μ g of proteinase K and 5 μ g carrier tRNA. After incubation for an additional 20 min at 37°C, the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (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 final concentrations of 30 mM Hepes, pH 7.5, 30 mM KCl, 8.0 mM MgCl₂, 1.0 mM DTT, 100 μ g/ml BSA, 0.01 % (v/v) Nonidet P-40, 0.5 mM ATP, and 20 μ M each of dATP, dCTP, dGTP, dTTP in a final volume of 20 μ l. Briefly, 0.5 nM Fen1 and different concentrations of SMIs were incubated at room temp for 5 min followed by addition of 2.5 nM 32 P-labeled 63-mer F-DNA (pre-incubated 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 (16–18). The incubation period for LP-BER was 30 min at 37°C. Each reaction was terminated by the addition of 20 μ l of stop solution (5.0 mM EDTA, 0.4% (w/v) of SDS) with 1 μ g of proteinase K and 5 μ g carrier tRNA. After incubation for an additional 20 min at 37°C, the DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (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 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.* (19). Here, 5 μ g 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 pre-treated with varying concentrations of SMI 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 its metal-binding site M2 was changed to Asn (D181N). 5'-Flap endonuclease assay for this mutant protein (Fig.1B) and quantification of the cleaved product (Fig.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 small molecular weight inhibitors (SMIs) by structure-based molecular docking.

Structure-based molecular docking of small molecules

Ideally, a drug should be highly active with only little or no side effects. In order to achieve these goals, small molecular weight inhibitors (SMIs) should be selected or designed on the basis of structural characteristics that promote specific interaction with the intended target site (20). We used a high performance computing simulation method to screen approximately 220,000 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 the 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 (Fig. 2A; RCSB Protein Data Bank code: 1UL1) (12). After screening, 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) (Fig. 2B and C).

NSC-281679, NSC-337807 and NSC-281680 block Fen1 endonuclease activity

Initially, we screened 40 different SMIs for their ability to block Fen1 endonuclease activity. We selected three SMIs, namely NSC-281679, NSC-337807 and NSC-281680 for further studies. Results obtained using these SMIs are shown in Figure 3. These SMIs blocked Fen1 mediated endonuclease activity in a dose-dependent manner (Fig. 3B; Lane 2–7, lane 8–13 and lane 14–19, respectively). The IC_{50} of these SMIs to inhibit Fen1 endonuclease activity was also calculated (Fig. 3C). The SMI NSC-281680 inhibited Fen1 endonuclease activity in a dose dependent manner and blocked it completely at 4.5 μM , while a similar effect was observed for NSC-337807 and NSC-281679, but at 10-times higher concentrations.

NSC-281679, NSC-337807 and NSC-281680 blocked LP-BER Activity

The SMIs 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 SMIs blocked Fen1-mediated strand-displacement (Fig. 4B, lane 6–9, lane 14–17, and lane 22–25) as well as complete LP-BER activities in a dose-dependent manner (Fig. 4B lane 10–13, lane 18–21, and lane 26–29). Similarly, using nuclear extracts (NEs) from HCT-116 cells, these SMIs blocked of LP-BER in a dose-dependent manner (Fig. 5B, lane 3–7, lane 8–12, and lane 13–17). By comparison, these results revealed that out of three SMIs, NSC-281680 was the most potent. Thus, the SMI 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 showed a greater cytotoxic response to TMZ treatment as compared to the MMR-deficient HCT-116 cells (Fig. 6A and B). The combination of different concentrations of NSC-281680 with TMZ further reduced the IC₅₀ of TMZ in a dose-dependent manner in these cells (Fig. 6A and B). When applied alone NSC-281680 exhibited no cytotoxic effects on either the HCT-116 or HCT-116+ch3 cell lines (Fig. 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 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 anti-tumor agents relies on random and semi-empirical screening procedures that have proven 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 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 (21). 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 (22). Importantly, a phase-II clinical trial study of TMZ in pre-selected 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 (23), 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 (24). 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 due to a deficiency in MMR (25). 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 (3,4,26).

Long-patch (LP)-BER is the sub-pathway 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 (27,28). Since Asp181 is located in the active site of Fen1 along with Asp179 and Asp233 that constitute the Mg²⁺ binding-pocket M2 (12,13), we selected this amino acid to identify small molecular weight inhibitors (SMIs) predicted to interact with Fen1 and blocking its activity. By using structure-based molecular docking we have identified a potent SMI NSC-281680, which efficiently blocks the LP-BER. Although the mechanism by which it blocks Fen1 activity can be more precisely determined by co-crystallization of NSC-281680 with Fen1, our studies suggest that interaction of NSC-281680 with Fen1 may cause some structural changes and the loss of Mg²⁺ binding that may be leading to loss of its endonuclease activity. In earlier studies, it is suggested that the position of D181 is not crucial for cleavage capability of Fen1, but it also acts as a ligand for Mg²⁺ binding (27).

NSC-281680 does not block the *in vitro* activity of APE1, Pol- β and DNA ligase I (data not shown), suggesting its specificity toward 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 higher in rapidly proliferating cancer cells than in normal cells, as supported by previous studies (29). 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 due to overexpression of MGMT or MMR-deficiency (30). Cells deficient in MGMT are unable to process O⁶MeG during DNA synthesis, and if it is not repaired, then a G:C to G:T transition mutation occurs (31). The G:T mismatch is then repaired by the MMR pathway (32). However, if the O⁶MeG is not repaired before the re-synthesis step in MMR, a thymine is likely to be re-inserted 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 (32). 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 N⁷-MeG, N³-MeG, N³-MeA lesions by NSC-281680 causes much higher cytotoxicity than the lesions of O⁶-MeG. The results of previous studies suggest that N⁷-MeG, N³-MeG and N³-MeA lesions can be toxic in both MMR-deficient and MMR-proficient cells if the BER pathway is interrupted (33), and clinical studies indicate that MMR-deficiency may not be the main cause of TMZ-induced resistance in adult malignant glioma (34). 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 pre-clinical framework for the development

of novel and advanced chemotherapeutic agents and facilitate the improvement of conventional colon cancer treatments.

Abbreviations

APE1	apurinic/aprimidinic endonuclease 1
Fen1	5'-flap endonuclease 1
LP-BER	long-patch base excision repair
MMR	mismatch repair
Pol- β	DNA polymerase β
SMI	small molecular weight inhibitor
TMZ	Temozolomide

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Figure1. 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 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 = $[11\text{-nt}/(51\text{-mer} + 11\text{-nt})] \times 100$. The data are the mean $\pm$ SE of three different experiments.

Figure 2. Identification of Fen1 interacting small molecules

Panel A, crystal structure of the human Fen1/PCNA complex (Green, Pink and Blue: Fen1; Yellow, Purple and Salmon: PCNA) (RCSB code: 1UL1). This structure was used as the basis for *in silico* molecular screening. **Panel B**, the screening area of Fen1 is designated by site-point spheres (Purple) to which compounds from the NCI/DTP database were docked. **Panel C**, The structure of the compound NSC-281680 docked into the Fen1 pocket. The key amino acid residue D181 (Red) is in close proximity to the compound.

Figure 3. Screening of SMIs 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 under Materials and Methods. **Panel B** shows the effect of varying concentrations of SMIs on the 5'-flap endonuclease activity of Fen1. Lane 1 represents ^{32}P -labeled 51-mer F-DNA substrate, Lane 2 shows the 11-mer cleaved product, and 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, respectively. **Panel C** shows the IC₅₀ of SMIs NSC-281679, NSC-337807 and NSC-281680. The data are the mean $\pm$ SE of three different experiments.

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 SMIs on LP-BER activity using purified proteins. The LP-BER reaction mixture in a 20 μl contained the final concentrations of 30 mM Hepes, pH 7.5; 30 mM KCl, 8.0 mM MgCl₂, 1.0 mM DTT, 100 μg/ml BSA, 0.01 % (v/v) Nonidet P-40, 0.5 mM ATP, and 20 μM each of dATP, dCTP, dGTP, dTTP. Briefly, 0.5 nM Fen1 and different concentrations of SMIs were incubated at room temp for 5 min followed by addition of 2.5 nM ³²P-labeled 63-mer F-DNA (pre-incubated 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 under Materials and Methods. **Panel B** shows the effect of varying concentrations of SMIs 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.

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 by clonogenic survival assay. **Panel A** and **B** show the enhanced cytotoxic effect of NSC-281680 to enhance 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 the mean $\pm$ SE 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 = $P < 0.014$ and e = $P < 0.009$, respectively, as indicated in the Figure).