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A novel inhibitor of DNA polymerase β enhances the ability of Temozolomide to impair the growth of colon cancer cells

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Abstract

The recent emerging concept to sensitize cancer cells to DNA-alkylating drugs is by inhibiting various proteins in the base excision repair (BER) pathway. In the present study, we used structure-based molecular docking of DNA polymerase β (Pol- β) and identified a potent small molecular weight inhibitor (SMI), NSC-666715. We determined the specificity of this SMI for Pol- β by using *in vitro* activities of APE1, Fen1, DNA ligase I, and Pol- β -directed single nucleotide (SN)- and long-patch (LP)-BER. The binding specificity of NSC-666715 with Pol- β was also determined by using fluorescence anisotropy. The effect of NSC-666715 on the cytotoxicity of the DNA-alkylating drug, Temozolomide (TMZ), to colon cancer cells was determined by *in vitro* clonogenic and *in vivo* xenograft assays. The reduction in tumor growth was higher in the combination treatment relative to untreated or monotherapy treatment. NSC-666715 showed a high specificity for blocking Pol- β activity. It blocked Pol- β -directed SN- and LP-BER without affecting the activity of APE1, Fen1 and DNA ligase I. Fluorescence anisotropy data suggested that NSC-666715 directly and specifically interacts with Pol- β and interferes with binding to damaged DNA. NSC-666715 drastically induces the sensitivity of TMZ to colon cancer cells both *in vitro* and *in vivo* assays. The results further suggest that the disruption of BER by NSC-666715 negates its contribution to drug-resistance and bypasses other resistance factors, such as mismatch repair defects. Our findings provide the “proof-of-concept” for the development of highly specific and thus safer structure-based inhibitors for the prevention of tumor progression and/or treatment of colorectal cancer.

Keywords

Adenomatous polyposis coli; DNA polymerase β ; small molecular weight inhibitors; colorectal cancer; chemotherapeutic intervention

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Introduction

Colorectal cancers develop through a series of histologically distinct stages from “adenoma to carcinoma to metastasis” (1). A great deal has been learned about the molecular events involved in the initiation and progression of colorectal cancer, but still surgery remains the main stay of its treatment. Since the recurrence rate of colon tumors after surgery is 30–50%, there is an urgent need of the development of new chemotherapeutic approaches suitable for long-term prevention and management of this deadly disease. Mutations of the adenomatous polyposis coli (*APC*), *K-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 (3). The success of treatment of colon cancer patients depends on matching the most effective therapeutic regimen with the prognostic factors of the individual patient.

Modern therapeutic approaches in cancer treatment include targeting signaling pathways, multi-drug resistance, cell cycle checkpoints and anti-angiogenesis (4). In addition to these, a less explored but critical area of cancer chemotherapy is blocking cancer cell's ability to recognize and repair the damaged DNA, which primarily results from the use of chemotherapeutic drugs including DNA-alkylating drugs (5,6). The balance between DNA damage and repair determines the final therapeutic consequences of these drugs. In many cases, an elevated DNA-repair capacity in tumor cells leads to drug resistance and severely limits the efficacy of DNA-alkylating drugs. Thus, the interference with DNA repair has emerged as an important approach to combination therapy against such cancers (7). The chemotherapeutic drugs that induce DNA-alkylation damage elicit lesions that are repaired primarily by the O⁶-methylguanine DNA methyltransferase (MGMT), mismatch repair (MMR), and BER pathways. Inhibitors of these DNA-repair systems have emerged, but they target mainly the MGMT and MMR pathways. The blockade of the BER pathway has been overlooked, although in the case of several DNA-alkylating drugs including Temozolomide (TMZ; 4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide; NSC-362856), BER is responsible for the repair of 70%, 5% and 9% of N⁷-methylguanine (MeG), N³-MeG and N³-methyladenine (MeA) lesions, respectively (8). Any interruption of the BER pathway can cause an accumulation of these lesions, resulting in cytotoxicity; this fact can be exploited further by chemotherapeutic agents for targeting cancer cells (9). Many colon tumors become resistant to DNA-alkylating agents due to overexpression of MGMT or MMR-deficiency (10). The cells deficient in MGMT are unable to process the O⁶MeG during DNA synthesis, and if it is not repaired, then a G:C to G:T transition mutation occurs (11). In previous studies, the role of BER pathway has also been implicated in cellular resistance to TMZ (12,13), which depends on specific BER gene expression and activity (14). Thus, down-regulating the BER pathway can reduce the resistance to DNA-alkylating agents and increase their efficacy to colon cancer cells.

A new and emerging concept is to sensitize cancer cells to DNA-damaging agents by inhibiting various proteins in the DNA repair pathways. Small molecular weight inhibitors (SMIs) have been identified by molecular docking or NMR studies to target the BER pathway by inhibiting apurinic/apyrimidinic endonuclease 1 (APE1) and Pol-β activities. Several Pol-β inhibitors have been reported in recent years (15). The most active SMI identified for Pol-β by NMR chemical shift mapping is pamoic acid (16). However, between the two sub-pathways, i.e., single nucleotide (SN)- and long-patch (LP)-BER (17), the pamoic acid inhibits dRP-lyase activity of Pol-β and blocks only Pol-β-directed SN-BER and is required in high concentrations to achieve its effect. Since abasic DNA damage can also be repaired by LP-BER, there is a need for agents that can specifically block both Pol-β-directed SN- and LP-BER pathways. Based on our findings, APC interacts with Pol-β and Fen1, blocks both SN- and LP-BER

pathways (18–23), and interacts with amino acid residues Thr79, Lys81 and Arg83 of Pol- β (20). We performed molecular docking studies to identify a potent SMI, NSC-666715 [4-chloro-N-(3-(4-chloroanilino)-1H-1,2,4-triazol-5-yl)-2-mercapto-5-methylbenzenesulfonamide], that interacts with Pol- β in such a way that it blocks both SN- and LP-BER activities, without blocking Fen1 activity, and enhances the efficacy of TMZ to reducing the growth of colon cancer cells both *in vitro* and *in vivo*.

A Phase II clinical study of Temozolomide (TMZ) in pre-selected advanced aerodigestive tract cancers (Study P04273AM2), including colorectal neoplasm, is underway by Schering-Plough, Kenilworth, NJ (<http://clinicaltrials.gov/ct2/show/NCT00423150>). In a separate Phase I clinical study of TMZ, a partial response of the drug on metastatic colorectal cancer was observed, suggesting considerable tumor resistance to treatment (24). To overcome the resistance concern of TMZ, another Phase II clinical study was performed in which lomeguatrib was combined with TMZ. These studies also concluded that the efficacy of TMZ to metastatic colorectal cancer was not very significant (25). Thus, there is an urgent need of the development of a new strategy by which the efficacy of TMZ can be increased to colorectal cancers. Our approach of combining NSC-666715 with TMZ shows a promise that at low doses the NSC-666715 can overcome the TMZ-induced resistance and increase its efficacy to colorectal cancer.

Results

Structure-based molecular docking of small molecular weight inhibitors (SMIs) at the APC-binding site of Pol- β

Ideally, a drug should be highly active with only minor to no side effects. In order to achieve these goals, a small molecular weight inhibitor (SMI) should be selected or designed on the basis of structural characteristics that promote highly specific interaction with the intended target site (26). We used a High Performance Computing and Simulation Method to screen small molecular weight molecules targeted to the three-dimensional structure of the APC-binding site of Pol- β (27) in order to identify compounds that would affect its activity (Fig. 1A). The small molecules were ranked based on their overall binding scores (energy scores, sum of polar and non-polar interactions), and the 22 highest scoring SMIs were requested for functional evaluation (Table-1). Based on initial characterization of effects on Pol- β -directed strand-displacement activity *in vitro*, we identified NSC-666715 as the most active SMI. Based on molecular docking, NSC-666715 was predicted to interact with the residues on the surface of Pol- β that extend beyond the APC binding pocket (Fig. 1B).

Screening of the SMIs

In all the inhibitor experiments, we used purified proteins into reconstituted systems. First, we screened 22 top scoring SMIs for their ability to block Pol- β -directed strand-displacement synthesis (data not shown). While some SMIs inhibited Pol- β -directed strand-displacement synthesis at 10 μ M, some were only effective at 10-fold higher concentrations. Furthermore, these SMIs did not affect 1-nt incorporation activity of Pol- β with any of the concentrations tested. The SMI NSC-666715 inhibited Pol- β -directed strand-displacement activity at a concentration of 5 μ M, while higher concentrations of this SMI completely abrogated the displacement product. When the concentrations of NSC-666715 were further increased to 15 μ M, the 1-nt incorporation activity of Pol- β was completely blocked. These results suggest that NSC-666715 is a potent inhibitor of Pol- β activity.

NSC-666715 blocks Pol- β -directed SN- and LP-BER

It has been well established that dRP-lyase activity is a rate-limiting step in SN-BER (17). In the present study, we examined whether NSC-666715 also blocks the dRP-lyase activity of

Pol- β . The results showed inhibition of dRP-lyase activity in a dose-dependent manner with an IC_{50} of 4.1 μ M (Fig. 2A; comparison of lane 3 with lanes 4–7, respectively). Thus, NSC-666715 appears to block dRP-lyase activity. Next, we determined whether the SMI-mediated block of dRP-lyase activity inhibits Pol- β -directed SN-BER. We used 63-mer 32 P-labeled U-DNA as a substrate for these experiments. With this substrate after APE1 incision, an expected 23-mer product was generated (Fig. 2B; comparison of lane 1 with lane 2). The results showed efficient 1-nt incorporation by Pol- β which was ligated with DNA ligase I to generate 63-mer repaired product (Fig. 2B, lane 4). The addition of NSC-666715 blocked Pol- β -directed SN-BER in a dose-dependent manner with an IC_{50} of 3.8 μ M (Fig. 2B; comparison of lane 4 with lanes 5–8, respectively), which is very close to the IC_{50} of dRP-lyase activity.

To determine whether NSC-666715 blocked LP-BER as well, we used 32 P-F-DNA as a substrate and assembled the reaction mixture as shown in Figure 3A. Pol- β showed the strand-displacement synthesis activity (Fig. 3B, lane 3), which was stimulated by Fen1 (Fig. 3B, lane 4) and then repaired by DNA ligase I (Fig. 3B, lane 5) by LP-BER. Interestingly, NSC-666715 blocked the LP-BER in a dose-dependent manner (Fig. 3B; comparison of lane 5 with lanes 6–10, respectively). These results suggest that NSC-666715 blocks both Pol- β -directed SN- and LP-BER activities.

NSC-666715 has no effect on APE1, Fen1 and DNA ligase I activities

To determine whether NSC-666715 interacts with and blocks the activity of Pol- β only (Fig. 4A), we also determined the effect of this SMI on other BER enzyme activities. Results showed that NSC-666715 did not affect the activity of APE1, Fen1 and DNA ligase I up to 15 μ M concentrations (Fig. 4B–D, respectively). We concluded that the inhibitory effect of NSC-666715 was highly specific to Pol- β , and did not affect other BER enzymes.

NSC-666715 abolishes the binding of Pol- β with gapped DNA

The fact that SN-BER was inhibited by NSC-666715 suggested that the SMI inhibited dRP-lyase activity of Pol- β . However, LP-BER was also inhibited at relatively higher concentrations (Fig. 3). We inferred that the latter effect was due to a conformational alteration of the Pol- β structure after the binding of the SMI in the proposed docking site (Fig. 1). If so, the inhibitor should affect general characteristics of Pol- β in addition to the specific inhibition of the AP-lyase activity. We sought to probe the validity of this inference, by determining the DNA binding constant of Pol- β in the absence and presence of the SMI. For this purpose, we measured fluorescence anisotropy (FAN) using 5'-fluorescein labeled 63-mer DNA with a 5-nt gap (28). In the absence of SMI, Pol- β showed a binding isotherm which resulted in a K_d value of 58.7 nM (Fig. 5A). However, the DNA binding was significantly decreased in the presence of 10 μ M SMI (Fig. 5A). The result is consistent with the previous results (Fig. 3) in which the LP-BER was relatively resistant to the inhibitor at lower concentrations, while inhibition was increasingly visible at more than 10 μ M of the SMI, even though the AP-lyase activity of Pol- β is not required for the LP-BER. At the same concentration, on the other hand, NSC-666715 did not interfere APE1 with its AP-site binding activity (Fig. 5B), excluding the possibility that the inhibitor merely bound to DNA which might indirectly interfere Pol- β with its reaction. These results support our hypothesis that NSC-666715 directly and specifically interacts with Pol- β , among other BER proteins, in such a way that it blocks both SN- and LP-BER activities, which is critical for increasing the chemotherapeutic efficacy of DNA-alkylating drugs. Whether NSC-666715 binds with other cellular proteins is currently not known.

The cytotoxic effect of TMZ on MMR-deficient and MMR-proficient colon cancer cells is enhanced by NSC-666715: in vitro cell culture studies

In these experiments, we first treated MMR-deficient (lacking *hMLH1* expression) cell lines HCT-116-APC(WT, wild-type APC expression) and HCT-116-APC(KD, stably knocked-down APC with shRNA), with different concentrations of TMZ alone or in combination with different concentrations of NSC-666715. We determined the IC₅₀ of TMZ using a clonogenic assay (20). The cellular toxicity results showed that NSC-666715 was able to increase the cytotoxicity of TMZ in both HCT-116-APC(WT) and HCT-116-APC(KD) cell lines. However, the cytotoxicity of this compound was greater in HCT-116-APC(WT) than in HCT-116-APC(KD) cells (Fig. 6A and B, respectively). The cytotoxicity of NSC-666715 in the absence of TMZ was very low at concentrations up to 100 μ M (Fig. 6D). The IC₅₀ values of TMZ alone or in combination with SMI are given in Table-2. These results suggest that NSC-666715 increases the sensitivity of TMZ in APC-knockdown and MMR-deficient colon cancer cells. Thus, the SMI NSC-666715 can be useful in enhancing the cytotoxicity of TMZ in colon cancer cell lines that express either wild-type APC or truncated APC (lacking the DRI-domain) and are MMR-deficient.

Next, we extended our studies to determine the effect of APC on TMZ-induced cytotoxicity in MMR-proficient HCT-116-APC(WT)+ch3 cells. In this cell line, 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 (Fig. 6C and Table-2) as compared to the MMR-deficient HCT-116(WT) and HCT-116-APC(KD) cells (Fig. 6A and B, respectively, and Table-2). The combination of different concentrations of NSC-666715 further reduced the IC₅₀ of TMZ in a dose-dependent manner in these cells (Fig. 6C, and Table-2). These results suggest that NSC-666715 can be useful chemotherapeutic agent for the intervention of tumor progression of both MMR-deficient and MMR-proficient colorectal tumors.

Pol- β activity blockade by NSC-666715 can be used as a potential chemotherapeutic target to augment tumor cell death by TMZ: in vivo xenograft studies

In these studies, we used SCID mouse-model for the preclinical testing of the efficacy of TMZ and NSC-666715, as described in Figure 7A. The results showed an increase in the tumor volume in the control set in a time-dependent manner for all the cell lines, *i.e.*, HCT-116-APC(WT), HCT-116-APC(KD) and HCT-116+ch3. The tumor volume reached a maximum of 1,120 mm³ within 42 days of xenograft implant (Fig. 7B; comparison of *a*, *b* and *c*). The tumor growth in the HCT-116-APC(KD) and HCT-116+ch3 xenograft control sets were similar to the HCT-116-APC(WT) xenograft set (Fig 7B). The anti-tumor effect of TMZ alone was less pronounced but a significant decrease was seen with the MMR-proficient tumors than the MMR-deficient tumors. TMZ treatment did not result in significant differences in the tumor volume of HCT-116-APC(WT) and HCT-116-APC(KD) cell lines. It is possible that TMZ-induced expression of APC in HCT-116-APC(WT) xenograft models may not be sufficient to block Pol- β activity; thus, the effect of TMZ remains the same in both HCT-116-APC(WT) and HCT-116-APC(KD) xenografts. Furthermore, the effect of TMZ on MMR-proficient and MMR-deficient xenografts is not as prominent as is seen with clonogenic assays (Fig. 6). This could be due to the use of a concentration of TMZ that may not have been high enough to show the difference in the sensitivity of MMR-proficient and MMR-deficient cells. This argument is supported by previous studies in which the treatment of even a two-fold higher concentration of TMZ (40 mg/kg body weight) showed no differential effect on the xenograft tumor growth of MMR-proficient and MMR-deficient colon cancer cells (29). Treatment with NSC-666715 in the absence of TMZ treatment resulted in a significant decrease in the growth of xenograft tumors of all the three cell lines (37% to 54% relative to control; $p < 0.001$) (Fig. 7B). Once the treatment of TMZ was combined with NSC-666715, the tumor growth was greatly reduced (66–71%; $p < 0.001$) as compared to that observed in the absence of TMZ (Fig. 7B). Since this

SMI is specific to Pol- β -binding, its effect on tumor growth can be seen in the presence of alkylation damage. At present, we can propose two explanations for this effect: (i) xenograft tumors produce more abasic lesions than cells in culture, which sensitizes these cells to treatment with NSC-666715; and/or (ii) NSC-666715 blocks critical cell survival pathways which are active in xenograft tumors but not in culture cells. These possibilities will be explored in our future studies.

On day 42 of the experiment, the efficacy of the combination of TMZ with NSC-666715 treatment in the reduction of tumor volume as compared to vehicle control was as follows: HCT-116+ch3 (71%) > HCT-116-APC(WT) (66%) > HCT-116-APC(KD) (62%) (Fig. 7B). The gain in body weight of control and treated animals with NSC-666715 and TMZ alone or in combination was the same (Supplementary Fig. S1). It seems that the 20 mg/kg body weight dose of TMZ and 10 mg/kg body weight doses of NSC-666715 were well tolerated and did not cause any obvious adverse side effects in the mice, which is highly encouraging for future studies. These results suggest that xenograft tumors originating from the MMR-proficient system respond more efficiently to TMZ and SMI combination treatment. The MMR-deficient system also works well but to a lesser degree during this short period of treatment.

Discussion

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 (30), and the therapeutic efficacy is determined by the balance between DNA damage and repair (5). In many cases, an elevated DNA repair capacity in tumor cells leads to drug resistance and severely limits the efficacy of these agents. Thus, the combination of interference with DNA repair with the DNA-alkylating agents has emerged as an important strategy (7). The alkylation damage-induced lesions are repaired by BER, MGMT and MMR pathways. The inhibitors that have been developed as anticancer drugs target primarily the MGMT and MMR; however, many colon tumors become resistant to alkylating drugs due to a deficiency in MMR (31). Thus, the blockade of the BER pathway becomes equally important in inducing cellular toxicity as the major alkylation lesions created by TMZ are repaired by the BER pathway (8,^{9,32}).

APC interacts with Pol- β at amino acid residues T79, K81 and R83 (23). The interaction surface area and the involved amino acid residues of Pol- β for both APC and NSC-666715 appear to be different. This becomes more apparent when we find that APC blocks Pol- β -directed SN-BER, but requires Fen1 to block LP-BER. However, NSC-666715 blocks both SN- and LP-BER without blocking Fen1 activity. It is unclear though whether both APC and NSC-666715 simultaneously bind to Pol- β and synergistically inhibit Pol- β activity. Our hypothesis is that NSC-666715 interacts with Pol- β , as shown with our fluorescence anisotropy-binding data, and changes its structural configuration in such a way that they block both SN- and LP-BER. To address these questions more precisely it will be necessary to co-crystallize NSC-666715 with Pol- β and then determine the amino acid residues involved in binding. Furthermore, NSC-666715 did not block the *in vitro* activity of APE1, Fen1 and DNA ligase I suggesting that Pol- β -targeted cytotoxicity of colon cancer cells is highly specific. Since Pol- β is a ubiquitously expressed protein and BER is the main DNA repair pathway for alkylation-induced lesions, it may affect normal colonic epithelial cells as well. However, its effect will be much higher in rapidly proliferating cancer cells than in normal cells. This view is supported by our *in vivo* animal data in which the tumor growth was reduced after treatment with TMZ and NSC-666715 without producing any adverse effect on the animal body weights during the study period. This hypothesis is also supported by other studies (33). It is well known that most of the commonly used cancer chemotherapeutic drugs target DNA for cytotoxicity. Thus, the

DNA damage response to the chemotherapeutic drugs in both malignant and normal cells/tissues determines the therapeutic index of the treatment. This involves a complex set of cell processes such as multiple pathways of DNA repair, cell cycle regulation, and cell death/survival with both damage specificity and coordination of the DNA damage response to different types of DNA damage (5). Studies are in progress to identify these complex cellular and molecular events involved in the DNA damage response in colonic tumors and normal colonic epithelial cells/tissues.

Many colon tumors become resistant to DNA-alkylating agents due to overexpression of MGMT or MMR-deficiency (10). The cells deficient in MGMT are unable to process the O⁶MeG during DNA synthesis. If the lesion is not repaired, then a G:C to G:T transition mutation occurs (29). The G:T mismatch is then repaired by MMR pathway (34). However, if the O⁶MeG is not repaired before the re-synthesis step in MMR, the thymine is likely to be reinserted opposite the lesion. It is believed that the repetitive cycle of futile MMR results in a generation of tertiary lesions, most likely gapped DNA. This then gives rise to double-stranded breaks (DSBs) in DNA that elicits a cell death response (34). 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-666715 with TMZ seems to effectively block the growth of both MMR-proficient and MMR-deficient colon cancer cells *in vitro* and *in vivo*. This suggests that the blockade of the repair of TMZ-induced N⁷-MeG, N³-MeG, N³-MeA lesions by NSC-666715 causes much higher cytotoxicity than the lesions of O⁶-MeG alone. 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 (12), and clinical studies indicate that MMR-deficiency may not be the main cause of TMZ-induced resistance in adult malignant glioma (35). Our results support these findings and clearly suggest the importance of targeting BER and argue strongly in favor of mechanistic studies for the development of inhibitors of BER. Thus, we expect that the use of NSC-666715 in combination with TMZ will be a highly effective strategy to provide the pre-clinical framework for the development of novel and advanced chemotherapeutic agents and facilitate the improvement of conventional colon cancer treatments.

Materials and Methods

Maintenance of mammalian cell lines

Human colon cancer cell lines HCT-116-APC(WT), HCT-116-APC(KD) and HCT-116+ch3 were grown in McCoy's 5a medium at 37°C under a humidified atmosphere of 5% CO₂. In each cell line, the medium was supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) 100 U/ml of penicillin, and 100 µg/ml of streptomycin.

Molecular docking

We utilized the crystal structure of human Pol-β (PDB code 1BPZ) to provide the basis for molecular docking. To prepare the site for docking, all water molecules were removed. Protonation of Pol-β residues was performed with SYBYL (Tripos, St. Louis, MO). Intermolecular AMBER energy scoring (vdw + columbic), contact scoring and bump filtering were implemented in DOCK5.1.0 (36). SETOR (37) and GRASP (38) were used to generate molecular graphic images. The 140,000 small molecules from the National Cancer Institute (NCI) database were positioned in the selected structural pocket (which includes amino acid residues T79/K81/R83 of Pol-β) and scored based on predicted polar (H-bond) and non-polar (van der Waals) interactions. Each of the small molecules was positioned in the selected site in 100 different orientations. The best orientation and scores (contact and electrostatic) were calculated. The twenty two highest-scoring compounds for the selected structural pocket were obtained for use in Pol-β inhibition assays from the Developmental Therapeutics Program

(DTP) of National Cancer Institute (NCI). Docking calculations were performed with the DOCK v5.1.0 (39). The molecular surface of the structure was explored using sets of spheres to describe potential binding pockets. The spheres fill in the available pocket spaces where a ligand might be able to form a complex. DOCK uses the spheres as a guide to search for orientations of each molecule that fit into the selected sites. The sites selected for molecular docking were defined using the SPHGEN program and filtered through the CLUSTER program (39). The SPHGEN program generates an unbiased grid of points that reflect the actual shape of the selected site. The CLUSTER program groups the selected spheres to define the points that are used by DOCK to match (superimpose) potential ligand atoms with spheres. Each compound in the NCI/DTP database was positioned in the selected site in 100-different orientations. Intermolecular AMBER energy scoring (van der Waals + columbic), contact scoring, and bump filtering were implemented in DOCK v5.1.0 (39). PYMOL was used to generate molecular graphic images (40).

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

We purified the hexa-histidine fusion proteins of the wild-type Pol- β and Fen1 as described previously with some modifications (20). We obtained human APE1 from Dr. Linda Bloom (University of Florida, Gainesville, FL) and human UDG from New England Biolabs (Ipswich, MA).

In vitro BER assays with purified proteins

The BER reaction mixture contained 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. The following additions were made to the above mixture: (i) For SN-BER, 2.5 nM of ³²P-labeled 63-mer U-DNA (containing uracil at the 24th position, pre-incubated with 1 unit of UDG to create an abasic site and 1 nM APE1 to create an incision at the 5' end of the repair site), 5 nM of Pol- β and different concentrations of SMIs (20); (ii) For strand-displacement synthesis, the reaction mixture was assembled similarly as with SN-BER, except 2.5 nM of ³²P-labeled 63-mer F-DNA (pre-incubated with 1 nM of APE1 to create an incision at the 5' end of the repair site) and 0.5 nM of Fen1 were used. The F-DNA is an AP-site analogue (3-hydroxy-2-hydroxymethyltetrahydrofuran) containing substrate, in which the F is inserted at the 24th position of the DNA (20); and (iii) For LP-BER, the reaction mixture was assembled similarly as for strand-displacement synthesis, except the repair was initiated with 0.4 nM of DNA ligase I.

dRP-lyase activity

A 63-mer U-DNA was labeled at the 3'-end by terminal deoxynucleotidyltransferase using [α -³²P]ddATP and annealed to the complementary 63-mer oligonucleotide. To remove uracil, the 3'-end labeled double-stranded oligonucleotide (2.5 nM) was treated with UDG (2 units) for 20 min at 37°C in a 20 μ l buffer containing 30 mM Hepes, pH 7.5, 30 mM KCl, 8.0 mM MgCl₂, 1.0 mM DTT, 100 μ g/ml of bovine serum albumin, 0.01% (v/v) Nonidet P-40, and 0.5 mM ATP. After incubation, the mixture was supplemented with 1.0 nM of APE1 and further incubated for 10 min to generate the substrate for dRP-lyase activity. A concentration of 2.5 nM of Pol- β protein was preincubated with varying concentrations of SMI for 5 min at 22°C. The reaction was initiated by the addition of preincubated mixture of protein and SMI with dRP-lyase substrate and incubated at 37°C for 15 min. After incubation, NaBH₄ was added to a final concentration of 340 mM, and kept on ice for 30 min. The stabilized (reduced) DNA products were ethanol precipitated. The reaction products were resolved on a 15% polyacrylamide-7 M urea gel.

Fluorescence anisotropy

A five nucleotide (5-nt) gap-containing 63-mer DNA (41) labeled with fluorescein at the 5' end was synthesized. The 10 nM of the 5'-fluorescein-labeled DNA was incubated with different concentrations of Pol- β in 50 mM Hepes, pH 7.5 and 1 mM EDTA. For APE1:AP-site binding assay, purified APE1 (0–200 nM) (42) was incubated with 5'-fluorescein labeled-duplex oligonucleotide containing a synthetic AP-site analog, tetrahydrofuran (42,⁴³). The sequence of the oligonucleotides were:

5'-F-AGGCCAATGATCGGTATXAAGTCGCGGGATAA-3'

5'-TTATCCCGCGACTTCATACCGATCATTGGCCT-3'

In these fluorescein and tetrahydrofuran are indicated as 'F' and 'X', respectively. Fluorescence anisotropy was determined at 25 °C using Varian Eclipse fluorescence polarizer. The apparent K_d values were calculated by non-linear regression analysis (28) using Mathematica 6.

Clonogenic assay

A single cell suspension of HCT-116-APC(WT) and HCT-116-APC(KD) and HCT-116+ch3 cells were plated (200 cells/well) in triplicate in a six-well plate. Cells were pretreated for 1 h with SMIs or vehicle (0.1% DMSO) followed by treatment with varying concentrations of TMZ for 48 h. After the treatment, the culture was replaced with fresh medium and cells were allowed to grow for a further 8 days. Visible colonies of more than 50 cells were stained with methylene blue and counted for viability (20,⁴⁴).

Xenograft studies

Female homozygous, 6-week old, severe combined immunodeficient mice (SCID; lacking functional T and B cells) were used in the study. Our choice for female mice was based on recent study describing that the estimated new cases of colon cancer in 2009 will be higher in females compared to males (45). HCT-116-APC(WT), HCT-116-APC(KD) and HCT-116+ch3 cells were harvested and a single cell suspension with >95% viability (5×10^6 cells) diluted in equal volume of Matrigel (BD Biosciences) were injected subcutaneously into the right flank of each mouse. After the tumors were established, as determined by caliper measurements (approximately 50–75 mm³ in size 10 days after cell injection), the mice were randomized into the following six groups (n = 6–4): (a) Vehicle control, (b) TMZ, (c) NSC-666715, and (d) TMZ plus NSC-666715. TMZ (20 mg/kg body weight) and NSC-666715 (10 mg/kg body weight). Drugs were administered intraperitoneally (*i.p.*) every day for 5 consecutive days. Tumor volume was measured weekly in each group. All mice were euthanized when the tumor volume in the control mice reached approximately 1,000 mm³ (day 42). The mice were housed and maintained under sterile conditions in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH.

Statistical analysis

The statistical significance between experimental groups and control was determined by Student's '*t*' test. For mouse xenograft study the statistical significance of differential findings between experimental groups and control was determined by two-way ANOVA as implemented by GraphPad StatMate (GraphPad software, La Jolla, CA). $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	adenomatous polyposis coli
APE1	apurinic/apyrimidinic endonuclease 1
BER	base excision repair
Fen1	5'-flap endonuclease 1
LP	long-patch
MMR	mismatch repair
Pol- β	DNA polymerase β
SMI	small molecular weight inhibitor
SN	single-nucleotide
TMZ	Temozolomide

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FIGURE 1. Molecular docking of Pol-β

Panel A shows the molecular surface of Pol-β (gray color) indicating the selected site for docking (purple spheres). **Panel B** shows the display of the SMI NSC-666715. Residues that are essential for the binding of APC are colored orange (T79), blue (K81) and purple blue (R83). NSC-666715 exhibits high molecular docking scores of -29.774746 kcal/mol.

FIGURE 2. NSC-666715 blocks Pol-β-directed dRP-lyase and SN-BER activities
Panel A represents dRP-lyase activity. The upper panel of the figure shows the schematic representation of dRP-lyase DNA substrate and product. Autoradiogram shows the effect of varying concentrations of NSC-666715 on dRP-lyase activity of Pol-β. Lane 1 shows uncut 63-mer ³²P-labeled U-DNA, Lane 2 shows 3'-labeled 40-mer dRP-lyase substrate, Lane 3 shows 3'-labeled 40-mer dRP-lyase product, and Lanes 4–7 contained 1–10 μM of NSC-666715. **Panel B** depicts SN-BER. The experimental protocol is depicted on the top of the autoradiogram. Lane 1 shows uncut 63-mer ³²P-labeled U-DNA, Lane 2 shows 23-mer product after APE1 incision, Lane 3 shows 1-nt incorporation by Pol-β, Lane 4 shows the 63-mer ligated product, which is blocked by NSC-666715 in a dose-dependent manner (compare lane 4 with 5–8, respectively). Data is a representative of three different experiments.

A. Protocol

B. LP-BER

FIGURE 3. NSC-666715 blocks Pol-β-directed LP-BER

Panel A shows the protocol for LP-BER. **Panel B** shows the autoradiogram for a typical LP-BER. Lane 1 shows uncut 63-mer ³²P-labeled F-DNA, Lane 2 shows 23-mer product after APE1 incision, Lane 3 shows strand-displacement synthesis and the lane 4 shows the strand-displacement stimulated by Fen1, Lane 5 shows the 63-mer ligated product, which is blocked by NSC-666715 in a dose-dependent manner (compare lane 5 with 6–10, respectively). Data is a representative of three different experiments.

A. Pol- β activity **B. APE1 activity** **C. Fen1 activity** **D. DNA ligase I activity**

FIGURE 4. NSC-666715 does not block the activity of APE1, Fen1 and DNA ligase I in reconstituted *in vitro* assays

Panel A shows the effect of NSC-666715 on Pol- β -directed strand-displacement synthesis. Lane 1 represents 32 P-labeled 63-mer F-DNA, Lane 2 shows 23-mer product after APE1 incision, and Lane 3 shows displacement activity in the presence of Pol- β . Lanes 4–6 contained increasing concentrations of NSC-666715. The arrows indicate the positions of 63-mer substrate and the products. **Panel B** shows the effect of NSC-666715 on APE1 activity. A 2.5 nM of 32 P-F-DNA was incubated for 10 min with 1 nM of APE1 without (lane 2) or with different concentrations of NSC-666715 (0–15 μ M, lanes 3–6). Lane 1 shows the position of uncut substrate. **Panel C** shows the effect of NSC-666715 on Fen1 activity. A 2.5 nM of 32 P-labeled flapped-DNA was incubated for 15 min with 1 nM of Fen1 without (lane 2) or with different concentrations of NSC-666715 (0–15 μ M, lanes 3–6). Lane 1 shows the position of 51-mer labeled oligonucleotide. **Panel D** shows the effect of NSC-666715 on DNA ligase I activity. A 2.5 nM of 32 P-labeled nicked DNA was incubated for 30 min with 1 nM of DNA ligase I without (lane 2) or with different concentrations of NSC-666715 (0–15 μ M, lane 3–6). Lane 1 shows 23-mer labeled oligonucleotide. Data are representative of three different experiments.

SMI	K_d (nM)
(DMSO)	58.7 ± 10.3
NSC-666715 (10 μ M)	$(>1.78 \times 10^7)^*$

SMI	K_d (nM)
(DMSO)	67.4 ± 11.2
NSC-666715 (10 μ M)	63.7 ± 13.2

FIGURE 5. Effect of NSC-666715 on the Pol- β -binding affinity for gap-containing DNA
Panel A shows the 5'-fluorescein-labelled 63-mer 5-nt gap-containing DNA (10 nM) was incubated with the wild-type Pol- β in 50 mM Hepes, pH 7.5 and 1 mM EDTA to determine fluorescence anisotropy. **Panel B** shows no effect of SMI on APE1 binding to AP-site DNA. A 32-mer duplex DNA (10 nM) containing a single tetrahydrofuran was incubated with variable concentration of APE1 in the presence or absence of SMI to measure fluorescence anisotropy. Values are the mean $\pm$ SD of triplicate estimations. Apparent K_d values are shown in the tables below the figures. (*) A light scattering effect became obvious at higher Pol- β WT concentrations.

FIGURE 6. Efficacy of NSC-666715 in enhancing the cytotoxicity of TMZ against HCT-116-APC (WT), HCT-116-APC(KD) and HCT-116+ch3 colon cancer cell lines in culture

Panel A and B show the data with MMR-deficient cell lines HCT-116-APC(WT) and HCT-116-APC(KD), respectively, and **Panel C** with MMR-proficient cell line HCT-116+ch3. **Panel D** shows the effect of NSC-666715 alone on the cytotoxicity of HCT-116-APC(WT), HCT-116-APC(KD) and HCT-116+ch3 cell lines. The survival of the cells was determined by clonogenic assay. Data presented are the mean $\pm$ SE of three different estimations.

A. Protocol **B. Tumor volume**

FIGURE 7. Effect of NSC-666715 in combination with TMZ on the growth of tumors in a xenograft model

Panel A shows the schematic representation of the experimental protocol. **Panel B** shows the change in tumor volume from the day of transplant to the 42nd day of the experiment. Data are the mean $\pm$ SD of four to six animals in each group. *, significantly different than control; †, significantly different than NSC-666715. $P < 0.05$.

Table 1

Molecular docking scores of small molecule inhibitors selected by interactions with a structural pocket of DNA polymerase β at and around amino acid residues T79/K81/R83.

ZINC Number	NSC Number	Molecular Weight	Energy score	Non-polar VDS score	Polar ES score
01581700	351093	140	-89.029320	24.749727	-113.779045
01712438	10730	248	-40.963669	-16.336159	-24.627512
06006521	674711	278	-36.608250	-14.154952	-22.453299
01676297	43656	332	-34.261108	-23.879509	-10.381598
01857358	680472	247	-34.217258	-16.810270	-17.406988
01574931	89296	199	-33.672127	-14.079313	-19.592812
05080395	124854	435	-32.218060	-17.747440	-14.470618
04016970	18477	1265	-31.091694	-12.727660	-18.364033
05575424	303853	149	-30.338240	-10.957428	-19.380812
01642331	666715	430	-29.774746	-26.206652	-3.568094
01577652	21371	234	-29.692141	-13.306726	-16.385414
03869916	160172	259	-27.969837	-13.967024	-14.002814
01611158	609422	298	-25.709021	-14.753160	-10.955860
04430831	44254	410	-25.702097	-14.428201	-11.273896
05372445	274937	324	-25.606686	-14.801039	-10.805646
01726446	141842	303	-22.577610	-12.593750	-9.983859
01637754	660669	288	-21.624624	-7.856870	-13.767754
05762908	668472	575	-20.783243	-17.747669	-3.035574
05519455	360215	301	-20.581064	-10.825204	-9.755860
01558491	263659	304	-19.474409	-13.520727	-5.953682
01727649	143995	236	-16.814648	-14.094140	-2.720507
01592704	91855	230	-16.181042	-13.209587	-2.971454

Compounds are ranked according to the overall Energy Score (which is a sum of VDS and ES scores). DOCK v5.1.0 calculates polar (ES score) and non-polar (VDS score) contacts in kcal per mole based on posed interactions between small molecular weight compounds and selected structural pocket in Pol- β .

VDS, van der Waals
ES, electrostatic

Table 2

IC₅₀ of NSC-666715 for MMR-deficient [HCT-116-APC(WT) and HCT-116-APC(KD)] and MMR-proficient (HCT-116+ch3) colon cancer cell lines.

HCT-116-APC(WT)		HCT-116-APC(KD)	HCT-116+ch3
IC ₅₀ (μM) of TMZ			
NSC-666715 (μM)			
0	312	491	128
25	156	205	100
50	107	178	24
100	55	154	

Cells were pretreated for 1 h with different concentrations of TMZ followed by treatment with different concentrations of NSC-666715. After 48h, the survival of the cells was determined by clonogenic assay. Data presented has been calculated from Figure 6.