The DNA N-Glycosylase MED1 Exhibits Preference for Halogenated Pyrimidines and Is Involved in the Cytotoxicity of 5-Iododeoxyuridine

David P. Turner, Salvatore Cortellino, Jane E. Schupp, Elena Caretti, Tamalette Loh, Timothy J. Kinsella, and Alfonso Bellacosa

Abstract

The base excision repair protein MED1 (also known as MBD4), an interactor with the mismatch repair protein MLH1, has a central role in the maintenance of genomic stability with dual functions in DNA damage response and repair. MED1 acts as a thymine and uracil DNA N-glycosylase on T:G and U:G mismatches that occur at cytosine-phosphate-guanine (CpG) methylation sites due to spontaneous deamination of 5-methylcytosine and cytosine, respectively. To elucidate the mechanisms that underlie sequence discrimination by MED1, we did single-turnover kinetics with the isolated, recombinant glycosylase domain of MED1. Quantification of MED1 substrate hierarchy confirmed MED1 preference for mismatches within a CpG context and showed preference for hemimethylated base mismatches. Furthermore, the kcat values obtained with the uracil analogues 5-fluourouracil and 5-iodouracil were over 20- to 30-fold higher than those obtained with uracil, indicating substantially higher affinity for halogenated bases. A 5-iodouracil precursor is the halogenated nucleotide 5-iododeoxyuridine (5IdU), a cytotoxic and radiosensitizing agent. Cultures of mouse embryo fibroblasts (MEF) with different Med1 genotype derived from mice with targeted inactivation of the gene were evaluated for sensitivity to 5IdU. The results revealed that Med1-null MEFs are more sensitive to 5IdU than wild-type MEFs in both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and colony formation assays. Furthermore, high-performance liquid chromatography analyses revealed that Med1-null cells exhibit increased levels of 5IdU in their DNA due to increased incorporation or reduced removal. These findings establish MED1 as a bona fide repair activity for the removal of halogenated bases and indicate that MED1 may play a significant role in 5IdU cytotoxicity. (Cancer Res 2006; 66(15): 7686-93)

Introduction

Cytosine methylation is the most common epigenetic modification in vertebrate genomes and is essential for the normal development and functioning of organisms through its control of gene activity and epigenetic inheritance (reviewed in ref. 1). Methylation is mediated by a family of DNA methyltransferases, enzymes that add a methyl group to the 5 position of the cytosine pyrimidine ring within a cytosine-phosphate-guanine (CpG) dinucleotide (ref. 2 and references therein). Given the importance of CpG methylation, it is somewhat surprising to find that CpG sequences are potential mutational hotspots within the genome. It has been estimated that approximately one third of all human inherited mutations and cancer-associated mutations occur at CpG sites, including 50% of p53 mutations in Li-Fraumeni syndrome and sporadic colorectal cancer (3) and 50% of hereditary nonpolyposis colorectal cancer mutations (4). Furthermore, aberrant methylation patterns at the promoter region of tumor suppressor genes are associated with the onset of carcinogenesis through gene transcriptional repression (5, 6).

The increased prevalence of mutations at CpG dinucleotides is attributed to the increased tendency of the methylation product, 5-methylcytosine (5-meC), to spontaneously deaminate to thymine. This produces a T-G mismatch, which, if not repaired, results in a C-G to T-A transition mutation and the consequent loss of a methylation site (7, 8). Oxidative damage of cytosine and 5-methylcytosine may lead to G:C to A:T transitions at CpG sites via the formation of uracil- and thymine glycol, 5-hydroxycytosine, and 5-hydroxycytosine, and subsequent incorporation of adenine by bypass polymerases opposite these oxidized bases (9).

In keeping with the importance of methylation consensus sequences, cells have evolved specialized DNA repair processes to help protect their integrity within the genome from hydrolytic deamination events that generate T-G and U-G mismatches from 5-meCG and C-G pairs, respectively. In eukaryotes, the base excision repair proteins thymine-DNA glycosylase and MED1 (also known as MBD4) both repair these mismatches that occur at CpG methylation sites by removing the mismatched T and U with their DNA N-glycosylase activity (10-15). DNA N-glycosylases hydrolyze the N-glycosyl bond between the target base and the deoxyribose sugar, resulting in a free base and an apurinic/apyrimidinic site; the latter is further processed by apurine/apyrimidine endonucleases, which cleave the phosphodiester bond in preparation for the gap filling functions of downstream repair proteins (16-20). In addition to the COOH-terminal glycosylase domain, MED1 contains an NH2-terminal 5-methylcytosine binding domain (MBD) and a central linker region of unknown function (12-14, 21, 22).

When in a CpG context, TDG and MED1 have glycosylase activity against thymine glycol paired with guanine (23), suggesting a protective role against oxidative damage as well. MED1 also has activity against the halogenated pyrimidines 5-fluourouracil (5-FU; ref. 13), 5-chlorouracil, and 5-bromouracil (24). Finally, MED1 has weak activity against 3,4-ethylenecytosine (14).

CpG sequences are also susceptible to the damaging effects of alkylation, O (^6)-Methylguanine (O (^6-meG), the most prominent alkylating lesion, also has the potential to form at CpG sequences through
such agents as N-nitroso compounds that act via a unimolecular (S_N1) nucleophilic substitution reaction (25, 26). If unrepairred, \(\text{O}_6\)-mG pairs with thymine in the next round of DNA replication and ultimately results in cell cycle arrest and apoptosis (27, 28). Similar processing is reported following treatment with 6-thioguanine (29, 30). Recently, MED1 has also been assigned a functional role in the activation of DNA damage checkpoints in response to treatment with alkylating agents, such as N-methyl-N′-nitro-N′-nitrosoguanidine, as well as platinum compounds, 5-FU, and irinotecan (31). In the same study, MED1 was shown to display glycosylase activity on thymine paired with \(\text{O}_6\)-mG (31).

Given the importance of the biochemical roles of the MED1 protein and the fact that frameshift mutations of the MED1 gene have been identified in human colorectal, gastric, endometrial, and pancreatic cancer, MED1 seems to be not only a major guardian of methylation site fidelity but also a candidate tumor suppressor gene (21, 32, 33).

Despite several studies on MED1 repair activity, there has been little quantitative assessment of sequence preference or context. We previously conducted a kinetic analysis of MED1 and found that its thymine and uracil glycosylase reactions follow single-turnover, pre-steady-state burst kinetics; this was due not to inactivation of the enzyme after a single reaction cycle but rather to tight binding to the apurinic/apyrimidinic site reaction product (13). However, our previous kinetic data were based on computer-generated simulations and were limited in the variety of substrates analyzed (13). To help elucidate the mechanisms that underlie sequence discrimination by MED1, we have done single-turnover kinetics with the isolated glycosylase domain of MED1 to set benchmarks for future comparisons. For these experiments, we used oligonucleotide substrates that contained a variety of lesions both within a hemimethylated and unmethylated CpG sequence context and in a non-CpG context.

The results revealed a striking preference of the enzyme for halogenated pyrimidines opposite guanine, including 5-iodouracil (5IU). For this reason, we next examined whether MED1 may play a significant role in the cytotoxicity of the halogenated nucleotide 5-iododeoxyuridine (5IdU), a 5IU precursor. For these experiments, we used the isogenic system represented by mouse embryo fibroblasts (MEF) with different \(\text{Med1}\) genotype derived from mice with targeted inactivation of the \(\text{Med1}\) gene (31).

Materials and Methods

Protein overexpression and purification. The human MED1 protein or its glycosylase domain (amino acids 427-580 of the full MED1 protein) with an NH\(_2\)-terminal 6\(^{\times}\) histidine tag were cloned in the pET28b expression vector (Novagen, La Jolla, CA; ref. 22). Purification was carried out in \(E\). coli BL21 Codon Plus (DE3)-RIL cells (Stratagene, La Jolla, CA) grown to an \(A_{600}\) of 0.4-0.6 with shaking at 37 °C in Luria-Bertani broth containing kanamycin (30 \(\mu\)g/mL). Protein overexpression was induced by the addition of isopropyl-\(L\)-thio-galactopyranoside to a final concentration of 1 mmol/L and further harvested by centrifugation (2,000 \(\times\)g for 20 minutes) and resuspended in lysis buffer containing 10 mmol/L potassium phosphate (pH 8.0), 500 mmol/L NaCl, 10 mmol/L imidazole, and Complete EDTA-free protease inhibitors (Roche, Mannheim, Germany). Cells were sonicated on ice and the soluble fraction was isolated by centrifugation (10,000 \(\times\)g for 40 minutes). The lysate was then applied by gravity flow to a 2-ml pre-equilibrated \(\text{Ni}^{2+}\)-chelating sepharose column (Amersham, Piscataway, NJ) and washed with 20 mL of wash buffer (10 mmol/L potassium phosphate pH 8.0, 500 mmol/L NaCl, 10 mmol/L imidazole). Protein was eluted via a stepwise gradient of the above wash buffer containing increasing concentrations of imidazole (i.e., 25, 50, 100, and 200 mmol/L, respectively). Fractions were analyzed using SDS-PAGE with Coomassie staining and the fraction containing the MED1 protein was dialyzed against gel filtration buffer (20 mmol/L HEPES [pH 7.6], 50 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L benzamide, 1 mmol/L phenylmethylsulfonyl fluoride). The sample was then applied to a pre-equilibrated Hi-prep 26/60 Sephacyr S-100 gel filtration column (Amersham Biosciences). Protein was eluted at a flow rate of 0.4 mL/min and collected in 2-ml fractions. Fractions were then analyzed using SDS-PAGE with Coomassie staining and the fractions containing the MED1 protein were pooled and concentrated using Ultrafree centrifugal filter devices (Millipore, Bedford, MA). The protein concentration of the glycosylase domain of MED1 was determined by absorbance spectroscopy at 280 nm using the \(\epsilon_{280}\) of 5.60 \(\times\)10\(^{3}\) (mol/L)\(^{-1}\) cm\(^{-1}\) (34). After the addition of 10% glycerol, samples were flash-frozen and stored at −70 °C. MED1 glycosylase domain point mutations were introduced into the active site via overlap mutagenesis (35) or via the QuickChange mutagenesis kit (Stratagene) and overexpressed and purified in the same manner.

Oligodeoxynucleotide synthesis and substrate preparation. Oligodeoxynucleotides were synthesized using standard phosphoramidite chemistry on an automated DNA synthesizer (Applied Biosystems, Foster City, CA) and purified by PAGE as previously described (13, 36). 5-FU- and 3,\(^{\text{N}}\)-ethenocytosine-containing oligonucleotides were purchased from GenSet (La Jolla, CA) whereas 5IU-containing oligonucleotides were purchased from Qiagen (Valencia, CA); these oligos were purified as above. Double-stranded oligonucleotide substrates were prepared by annealing complementary, gel-purified single-strand 37-mer oligonucleotides as follows: 5′-CAACCCTGACTGACA\(\text{Z}_{\text{G}}\)GATGGCCCAATGGCTACTG-5′; 3′-TTAGGATGCGACTGTGYTACACCGGTTCGTAATCG-3′ where \(\text{Z}\) is G (GpG context, in which case the 5′ G pairs with C), or C and \(\text{m}_{6}\text{G}\) (unmethylated and hemimethylated CpG context); and Y is C (no mismatch control), T, or 5FU, as required. For the alkylation assay and 5FU-substrates, the mismatched G in the top strand was replaced by \(\text{O}_6\)-mG and A, respectively. For the 5IU experiments, double-strand oligonucleotide substrates were prepared by annealing complementary, gel-purified single-strand 37-mer oligonucleotides as follows: 5′ CAACCCTGACTGACA\(\text{Z}_{\text{G}}\)GATGGCCCAATGGCTACTG-5′; 3′ TTAGGATGCGACTGTGYTACACCGGTTCGTAATCG-3′ where \(\text{Z}\) is C (no mismatch control), T, or 5FU, as required. For the alkylation assay and 5FU-substrates, the mismatched G in the top strand was replaced by A.

For both the 37- and 34-mers, before annealing, the bottom ssDNA was \(\gamma\)-\(\text{P}^{\text{32}}\)ATP and T4 polynucleotide kinase (37), and salts and excess label were removed using a nucleotide removal kit (Qiagen). DNA \(N\)-glycosylase assay. DNA \(N\)-glycosylase assays were carried out in 20 μL of reaction buffer [20 mmol/L HEPES (pH 7.9), 1 mmol/L DTT, 1 mmol/L EDTA] supplemented with 1 mg/mL bovine serum albumin. Double-stranded oligodeoxynucleotide substrate (5 mmol/L) was incubated with purified wild-type or mutant MED1 glycosylase domain protein (100 mmol/L) at 37 °C for 30 minutes and subsequently treated with NaOH (100 mmol/L) for 20 minutes at 85 °C to break the apurinic/apyrimidinic site. After the addition of denaturing gel loading buffer (formamide containing 8 mol/L urea, 0.5 mol/L EDTA, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol), substrate and product were resolved by denaturing PAGE (15% gels containing 8 mol/L urea) in Tris-borate EDTA buffer [89 mmol/L Tris-borate (pH 8.2), 1 mmol/L EDTA] at 30 W for 40 minutes.

Manual single-turnover repair assay. For single-turnover conditions, the glycosylase assay was typically carried out in 80 μL of reaction buffer [20 mmol/L HEPES (pH 7.9), 1 mmol/L DTT, 1 mmol/L EDTA] supplemented with 1 mg/mL bovine serum albumin. Purified MED1 glycosylase domain (1.6 μmol/L) was incubated with \(\gamma\)-\(\text{P}^{\text{32}}\)ATP and T4 polynucleotide kinase (80 mmol/L) at 37 °C, and 5IU samples were taken at regular time intervals, added to 2 μL of 250 mmol/L NaOH, and immediately frozen on dry ice. Once all the samples had been collected, they were incubated at 85 °C for 20 minutes to cleave the apurinic/apyrimidinic site before the addition of denaturing gel loading buffer. Substrate and product were resolved by denaturing PAGE (15% gels), conducted as described above.

Quench flow single-turnover repair assay. When hydrolysis rates were too fast for a manual assay, we used the quench flow assay to obtain the
timed samples required for accurate $k_d$ measurements. The quench flow assay was carried out using a KimTek RQF-3 chemical quench flow apparatus following previously published protocols (38). Briefly, assays were carried out under the same conditions outlined for the manual assay with 100 mmol/L NaOH used as the quenching solution. Samples were immediately frozen on dry ice and subsequently analyzed as described above for the manual assay.

**Single-turnover data analysis.** Following electrophoresis, radioactivity of each band, representing the amounts of substrate and product, was quantified with a Fuji BAS-2500 bioimaging analyzer using the accompanying software package (Image Gauge 4.0). The counts present in each individual band were calculated as a percentage of the total counts in the lane. Data from the single-turnover experiments were fitted to a first-order rate equation using nonlinear least-squares analysis with the software GraFit. Manual and quench flow single-turnover rates were calculated as the mean of three and two separate experiments, respectively, with the error bars representing ± 1 SD.

**Cell lines.** MEFs with different Med1 genotype (Med1+/− and Med1−/−) were obtained from day 12.5 embryos as previously described (31). To minimize genetic variability, MEF cultures from littermate embryos were used. MEFs were cultured in DMEM supplemented with 15% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L glutamine, 10 units/mL penicillin, and 10 μg/mL streptomycin (31).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. MEFs were seeded in 96-well plates (4,000 per well) and, after 24-hour incubation, were treated with 5IdU at the indicated concentrations for 72 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL solution in PBS) was added and the plates were incubated for 2 hours at 37°C. Cells were lysed in 20% SDS, 50 mM N,N,N-trimethylformamide, 2.5% glacial acetic acid, 2.5% HCl pH 4.7 (39). After overnight incubation at 37°C, plates were analyzed in an ELISA reader (570 nm).

**Colony assay.** MEFs were plated in 10-cm dishes (2 × 106 per dish) and, after 18-hour incubation, were treated with 5IdU at the indicated concentrations for 48 hours. After removing the 5IdU-containing medium, cells were washed with PBS and incubated at 37°C for 7 days in complete DMEM. Colonies (>50 cells) were stained with crystal blue and counted.

5IdU incorporation analysis by high-performance liquid chromatography. MEFs were plated in 10-cm dishes (5 × 105 per dish) and, after 16 hours, were treated with 5IdU concentrations ranging from 2 to 30 μmol/L for 24 hours. Medium was removed after the 24-hour pulse with 5IdU and fresh DMEM with 15% fetal bovine serum and supplements was added to plates. Time points were taken at time of 5IdU removal (time 0), and 6, 12, and 24 hours after 5IdU removal. Time points were taken by trypsinizing cells, centrifuging them to pellet, and washing twice with PBS in a similar manner before freezing the dry pellet at −80°C. DNA was extracted from cells; alkaline RNA hydrolysis was done; and DNA was then digested with DNase I, phosphodiesterase, and alkaline phosphatase, filtered, and then analyzed by high-performance liquid chromatography using authentic standards to determine nanomolar quantities of thymidine and 5IdU as previously described (40).

**Results**

**Single-turnover hydrolysis reveals MED1 preference for substrates in a methylated CpG context.** To date, catalytic rates for the MED1 protein have only been assigned using modeled data resulting from the limited hydrolysis of substrate (13). To obtain more accurate rates from real-time experiments, we did a single-turnover analysis that measures the slowest step of the catalytic cycle, up to and including hydrolysis, but does not include the product release step. These experiments were conducted with the recombinant MED1 glycosylase domain due to the ease of purification and higher activity than the full-length protein. The condition in which our hydrolysis assay meets the criteria required for single-turnover was confirmed by the fact that any increase in protein concentration (i.e., to 2 or 5 μmol/L) failed to show an increase of the hydrolysis rates (results not shown).

Initial single-turnover hydrolysis rates ($k_d$) were obtained for the G:T mismatch in three sequence contexts: hemimethylated and unmethylated CpG and non-CpG (Fig. 1). As summarized in Table 1, the MED1 glycosylase domain showed the highest cleavage rate for a G:T mismatch in which the mispaired G was positioned within a hemimethylated CpG site, producing a $k_d$ value of 0.859 ± 0.01 min⁻¹ (Fig. 1A and B). Removal of the methylated context (Fig. 1C) resulted in a 15% reduction in activity (0.736 ± 0.01 min⁻¹). This finding refines earlier experiments using modeled data, which showed no significant difference in MED1 activity when the mismatched G was in a methylated or unmethylated CpG context (13). On the other hand, in agreement with previous reports (12, 13), removal of the CpG context of the mismatch resulted in an almost 50% reduction in activity, producing a $k_d$ value of 0.483 ± 0.008 min⁻¹ (Fig. 1D). This confirms the designation of MED1 as a guardian of CpG fidelity.

The results obtained with the G:T mismatches were mirrored when T was replaced with U (Table 1; Fig. 2). Whereas the MED1 glycosylase domain showed a 2.5- to 3-fold increase in uracil versus thymine glycosylase activity irrespective of the sequence context, removal of the methyl group again produced a significant 28% reduction in activity (from 2.638 ± 0.14 to 1.908 ± 0.25 min⁻¹; Fig. 2B and C); as for G:T mismatches, removal of the CpG context resulted in a 50% reduction in repair efficiency (1.266 ± 0.05 min⁻¹; Fig. 2D).

Recently, MED1 has not only been shown to remove thymine when paired with the alkylation product O6-meG but has also been implicated in the DNA damage response to alkylating agents and other DNA-damaging drugs (31). We therefore obtained hydrolysis rates for the glycosylase activity of MED1 against T when opposite O6-meG in both a CpG (Fig. 3A) and non-CpG context (Fig. 3B). When in a CpG context, thymine was removed at a higher rate (0.653 ± 0.03 min⁻¹) than in a non-CpG context (0.188 ± 0.02 min⁻¹). Kinetic values for substrates containing 3,4-ethenocytosine were unobtainable due to a low yield of product over time.

**MED1 activity on halogenated bases.** Previously, MED1 has been shown to have significant activity against the halogenated uracil analogues 5-FU (13), 5-chlorouracil, and 5-bromouracil (24). Interestingly, in our study, initial assays using 5-FU as a substrate produced very rapid hydrolysis rates, which could not be measured manually. Therefore, a quenched flow apparatus was used to obtain a catalytic rate for this substrate. In these experiments, the difference in hydrolysis rates between the different sequence contexts was more defined and showed a higher rate of hydrolysis for a mismatch situated within a hemimethylated CpG context. Hydrolysis of a hemimethylated CpG site containing a G:5-FU mismatch (Fig. 4A) produced a 20-fold increase in activity compared with unhalogenated uracil (59.62 ± 3.79 and 2.638 ± 0.14 min⁻¹, respectively). Removal of the methyl group reduced activity by almost 60% (26.85 ± 9.12 min⁻¹) and positioning the mismatch within a non-CpG context reduced activity by almost 80% (10.49 ± 2.2 min⁻¹). The wild-type full-length MED1 protein also exhibits preference for the halogenated uracil over the nonhalogenated uracil (Fig. 4B).

Recently, the halogenated nucleotide, 5IdU, and its oral produg 5-ido-2-pyrimidine-2′-deoxyribose have been employed as preclinical and clinical radiation sensitizers in human cancers.
5IdU is sequentially phosphorylated intracellularly to 5IdUTP and competes with dTTP for incorporation (43). The extent of radiosensitization correlates with the percent of thymine replacement in DNA by 5IU (44). Based on prior studies on mutagenesis in λ phage and E. coli (45, 46), 5IdU can be incorporated into DNA to form either the pair A:5IU or the mispair G:5IU. The former is the result of incorporation of 5IdU opposite A during DNA synthesis. On the other hand, the mispair G:5IU may originate from misincorporation of 5IdU opposite G during DNA synthesis or, alternatively, from misincorporation of G opposite 5IdU.

Given the high affinity MED1 shows for 5-FU, we repeated the hydrolysis assays with an oligodeoxynucleotide substrate containing a single 5IU:G mismatch in a CpG context. Incubation of this substrate with the MED1 glycosylase domain produced a single-turnover \( k_{cat} \) value similar to that observed with its 5-FU counterpart (i.e., 35.44 ± 4.12 min\(^{-1}\) compared with 26.85 ± 9.12 min\(^{-1}\); Fig. 4C). As the iodine atom at the 5 position of the pyrimidine ring has a similar van der Waals radius as the methyl group in thymine (43), the single-turnover rates for 5IU:G are >40-fold compared with the hemimethylated and unmethylated T:G mismatch.

Whereas we detected elevated activity of MED1 on the G:5IU mispair, a very low activity was detected on an A:5IU pair (Fig. 4D).

Interestingly, similar results (i.e., G:5IU mispair processed with greater affinity than a G:T mispair and lack of recognition of the A:5IU mispair) were reported by our group with the mismatch repair recognition complex hMutSα (47). We also confirmed (13) that MED1 has undetectable activity on an A:5-FU substrate (Fig. 4D).

**Sensitivity of Med1-null cells to 5IdU.** The 20- to 30-fold higher \( k_{cat} \) values observed for MED1 with oligodeoxynucleotide substrates containing the 5IU:G mismatch versus U:G and T:G mismatches suggest that MED1 is a major repair activity for 5IU that may play a significant role in 5IdU cytotoxicity. To functionally test this hypothesis, we evaluated the sensitivity of cultures of MEFs with different Med1 genotype derived from mice with targeted inactivation of the Med1 gene (31).

In this isogenic system, cell cultures differ only for their wild-type or null Med1 status. Because 5IdU cytotoxicity is related to proliferation and DNA synthesis, we confirmed that wild-type and Med1-null MEFs exhibit similar cell cycle profile and proliferation rate (31).
MEF cultures were treated with increasing doses of 5IdU for 3 days and their surviving fraction was measured with the MTT assay. Med1−/− MEFs displayed reduced survival in comparison with wild-type cultures (Fig. 5A). To corroborate these findings, we measured the colony-forming potential of wild-type and Med1-null MEFs: 5IdU cytotoxicity that would affect sensitive cells, preventing cell growth and colony formation. MEFs of different genotype were treated with increasing doses of 5IdU for 48 hours and then grown in drug-free medium for 7 days to allow the formation of colonies. In comparison with wild-type MEFs, Med1-null MEFs showed a dramatic reduction in colony number (Fig. 5B) and size (data not shown).

**Increased 5IdU incorporation in Med1-null cells.** We next determined whether the increased cytotoxicity and reduced colony formation of Med1-null cells might be related to augmented 5IdU incorporation in their DNA. To this end, we conducted high-performance liquid chromatography analyses on DNA samples extracted from Med1+/+ and Med1−/− MEFs after a 24-hour treatment with 5IdU (2-30 μmol/L). The results indicated that Med1−/− MEFs incorporated 5IdU in their DNA at higher levels than Med1+/+ MEFs, both at time of 5IdU removal and at 6, 12, and 24 hours after 5IdU removal (Fig. 6). These data suggest that the increased sensitivity of Med1-null cells to 5IdU is associated with augmented incorporation or reduced removal of 5IdU.

![Figure 2](image1.png)

![Figure 3](image2.png)
Discussion

Recent publications provide compelling evidence that the MED1 glycosylase is a multifunctional protein involved not only in the excision repair of mismatched bases but also in the cellular response to DNA damage (31, 48, 49). MED1 recognizes a series of DNA lesions that result from a wide range of DNA damage, including deamination, oxidation, and alkylation. However, despite the growing significance and wide substrate spectrum of this enzyme, little quantitative assessment of its sequence preference or context has been examined. The only known kinetic data available identify the preference of MED1 for G:T and G:U mismatches that occur at CpG methylation sites (13). The same study failed to show any preference for the methylation status of the CpG site containing the lesion. This is contrary to the data outlined in the present article. Whereas a

Figure 4. MED1 glycosylase domain hydrolysis of oligonucleotides containing a halogenated U:G mismatch. A, single-turnover analysis of the glycosylase activity of MED1 on a 5-FU:G mismatch in a hemimethylated CpG context. B, wild-type full-length MED1 also exhibits preference for halogenated uracil. Denaturing PAGE of the substrate and product produced on incubation of wild-type full-length MED1 with 5-FU:G and U:G mismatches in an unmethylated CpG context over time. Percent product generation is indicated. C, single-turnover analysis of the glycosylase activity of MED1 on a 5IU:G mismatch in an unmethylated context. D, very low and undetectable activity of MED1 on 5IU:A (34-mer) and 5-FU:A (37-mer) pairs, respectively. Denaturing PAGE of the substrate and product produced on incubation for 20 minutes of wild-type full-length MED1 (fl) and its glycosylase domain (gd), or no enzyme (−), with the indicated halo-U:A pairs and halo-U:G mismatches (positive controls). Only one of two independent experiments is shown for each substrate.

Figure 5. Med1 status affects sensitivity to 5idU. A, survival analysis conducted with the MTT assay. Wild-type and Med1-null MEFs were treated with the indicated doses of 5idU for 72 hours and the surviving fraction was detected by measuring the absorbance of metabolized MTT. The sensitivity of cells to 5idU was detected by colony assay formation. Wild-type and Med1-null MEFs were treated with the indicated doses of 5idU for 48 hours and then allowed to form colonies in drug-free medium for 7 days. Colonies were stained with crystal blue and their number was plotted with respect to untreated control cultures. Representative of an isogenic pair of cell lines (wild-type and Med1-deficient); similar results were obtained with several independent wild-type and Med1-null cell lines.
sequences, as recently shown for the values obtained here may not necessarily be applicable to all of the top strand guanine. It should be emphasized that the kinetic is increased to similar for T:G and U:G (lines, the ratio between unmethylated and non-CpG contexts is the nature of the mismatched pyrimidine itself. Along the same imparts a defined and specific effect on catalysis independent of recognition of the top strand opposite the mismatched pyrimidine 5-FU, and 5IU, respectively; see Table 1). This suggests that hemimethylated versus unmethylated CpG contexts were similar present study, virtually all of the substrate was turned into product, a kinetic simulation program to assign kinetic values (13). In the of the substrate was converted into product and therefore required explained by the fact that in the initial investigation, only 30% to 50% of the substrate was converted into product and therefore required a kinetic simulation program to assign kinetic values (13). In the present study, virtually all of the substrate was turned into product, allowing a direct real-time measurement of catalytic turnover.

Interestingly, the ratios between catalytic rates for mismatches in hemimethylated versus unmethylated CpG contexts were similar with different substrates (~1.2, 1.4, 2.2, and 1.9 for thymine, uracil, 5-FU, and 5Iu, respectively; see Table 1). This suggests that recognition of the top strand opposite the mismatched pyrimidine imparts a defined and specific effect on catalysis independent of the nature of the mismatched pyrimidine itself. Along the same lines, the ratio between unmethylated and non-CpG contexts is similar for T:G and U:G (~1.5 for both substrates; see Table 1) but is increased to ~3.5 for T-O6mG (see Table 1), a likely consequence of altered hydrogen bonding due to methylation of the O6 position of the top strand guanine. It should be emphasized that the kinetic values obtained here may not necessarily be applicable to all sequences, as recently shown for the N-methylpurine-DNA glycosylase (50). However, similar results were obtained with longer (63-mer) substrates (data not shown).

The MED1 preference for hemimethylated lesions mirrors that observed for the bacterial VSR protein, which also displays a preference for a mismatched context within a CpG context that was hemimethylated over an unmethylated CpG context. This preference was observed with all of the relevant substrates analyzed. These contradicting data may be explained by the fact that in the initial investigation, only 30% to 50% of the substrate was converted into product and therefore required a kinetic simulation program to assign kinetic values (13). In the present study, virtually all of the substrate was turned into product, allowing a direct real-time measurement of catalytic turnover.

Interestingly, the ratios between catalytic rates for mismatches in hemimethylated versus unmethylated CpG contexts were similar with different substrates (~1.2, 1.4, 2.2, and 1.9 for thymine, uracil, 5-FU, and 5Iu, respectively; see Table 1). This suggests that recognition of the top strand opposite the mismatched pyrimidine imparts a defined and specific effect on catalysis independent of the nature of the mismatched pyrimidine itself. Along the same lines, the ratio between unmethylated and non-CpG contexts is similar for T:G and U:G (~1.5 for both substrates; see Table 1) but is increased to ~3.5 for T-O6mG (see Table 1), a likely consequence of altered hydrogen bonding due to methylation of the O6 position of the top strand guanine. It should be emphasized that the kinetic values obtained here may not necessarily be applicable to all sequences, as recently shown for the N-methylpurine-DNA glycosylase (50). However, similar results were obtained with longer (63-mer) substrates (data not shown).

The MED1 preference for hemimethylated lesions mirrors that observed for the bacterial VSR protein, which also displays a preference for a mismatched context within the cytosome methylation sequence of the dcm-methyltransferase (51). As both proteins prefer mismatches that occur at the sites of methylation, it is reasonable to assume that a hemimethylated context would be highly sensitive to the cytotoxic and radiosensitizing activity of 5IdU. Future experiments will address the role of MED1 in 5IdU radiosensitization of mammalian cells.

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This article is dedicated to the memory of our friend and colleague Elizabeth (Betty) Travaglini, who was among the first to investigate the kinetics of incorporation of complementary and non-complementary nucleotides by DNA polymerase, and was exemplary in placing the highest value upon friendship, tolerance and science above all, before fighting with dignity and serenity her battle against cancer.

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References


The DNA N-Glycosylase MED1 Exhibits Preference for Halogenated Pyrimidines and Is Involved in the Cytotoxicity of 5-Iododeoxyuridine

David P. Turner, Salvatore Cortellino, Jane E. Schupp, et al.


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