The Mismatch Repair Protein, hMLH1, Mediates 5-Substituted Halogenated Thymidine Analogue Cytotoxicity, DNA Incorporation, and Radiosensitization in Human Colon Cancer Cells

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Abstract

Deficiency in DNA mismatch repair (MMR) is found in some hereditary (hereditary nonpolyposis colorectal cancer) and sporadic colon cancers as well as other common solid cancers. MMR deficiency has recently been shown to impart cellular resistance to multiple chemical agents, many of which are commonly used in cancer chemotherapy. It is therefore of interest to find an approach that selectively targets cells that have lost the ability to perform MMR. In this study, we examine the response of MMR-proficient (hMLH1+) and MMR-deficient (hMLH1−) colon carcinoma cell lines to the halogenated thymidine (dThd) analogues iododeoxyuridine (IdUrd) and bromodeoxyuridine (BrdUrd) before and after irradiation. These dThd analogues are used clinically as experimental sensitizing agents in radioresistant human cancers, and there is a direct correlation between the levels of dThd analogue DNA incorporation and tumor radiosensitization.

In contrast to the well-characterized, marked increase in cytotoxicity (>1 log cell kill) found with 6-thioguanine exposures in HCT116/3-6 (hMLH1−) cells compared to HCT116 (hMLH1+) cells, we found only modest cytotoxicity (10–20% cell kill) in both cell lines when treated with IdUrd or BrdUrd for 1 population doubling. Upon further analysis, the levels of halogenated dThd analogues in DNA were significantly lower (two to three times lower) in HCT116/3-6 cells than in HCT116 cells, and similar results were found in Mlh1−/− spontaneously immortalized murine embryonic fibroblasts and fibroblasts from Mlh1 knockout mice. As a result of the higher levels of the dThd analogue in DNA, there was an increase in radiation sensitivity in HCT116 cells but not in HCT116/3-6 cells after pretreatment with IdUrd or BrdUrd when compared to treatment with radiation alone. Additionally, we found no differences in the cellular metabolic pathways for dThd analogue DNA incorporation because the enzyme activities of dThd kinase and thymidylate synthase, as well as the levels of triphosphate pools were similar in HCT116 and HCT116/3-6 cells. These data suggest that the hMLH1 protein may participate in the recognition and subsequent removal of halogenated dThd analogues from DNA. Consequently, whereas MMR-deficient cells and tumor xenografts have shown intrinsic resistance to a large number of chemotherapeutic agents, the 5-halogenated dThd analogues appear to selectively target such cells for potential enhanced radiation sensitivity.

Introduction

The 5-substituted halogenated dThd3 analogues, including IdUrd and BrdUrd (Fig. 1A), have been recognized as potential radiosensitizing agents since the early 1960s. Although they were initially synthesized as chemotherapeutic agents, these analogues demonstrated no significant clinical activity when tested in a variety of solid tumors and are not approved for clinical use as cancer chemotherapy drugs. However, over the last decade, there has been renewed interest in the clinical testing of these dThd analogues as radiosensitizers in selected cancer patient groups (1). Recent Phase II trials using prolonged continuous or scheduled intermittent i.v. infusions of BrdUrd or IdUrd before and during radiation therapy have focused principally on patients with high-grade brain tumors (2, 3). The results of these recent Phase II clinical trials suggest an improved outcome compared to radiation alone. A therapeutic gain in clinical radiosensitization by these halogenated dThd analogues may also exist for metastatic colorectal cancers and unresectable sarcomas, based on the results of other recent Phase I and/or II trials (4, 5). Incorporation into DNA (Fig. 1B) is a prerequisite for radiosensitization of human tumors by the halogenated dThd analogues, and the extent of radiosensitization (up to three times) correlates directly with the percentage of dThd substitution in DNA (6). Another nucleoside analogue, 6-TG (Fig. 1A), has previously been shown to invoke a cytotoxic response in MMR-proficient cells. This analogue also becomes incorporated into DNA (Fig. 1B), and the extent of DNA incorporation correlates directly with cytotoxicity to 6-TG (7).

A role for the human Mut L homologue 1 protein (hMLH1), an essential component of human MMR, in determining the cellular responses to different types of chemical and physical DNA-damaging agents has been suggested in recent reports in different human tumor cell lines (8–13). One common model system has been used in which parental HCT116 cells were corrected for their deficiency in MMR status (hMLH1−) by microcell fusion of a human chromosome 3 containing a wild-type hMLH1 gene to create HCT116/3-6, DNA MMR-proficient cells (hMLH1+). A second clonally derived cell line, HCT116/2-1, which was created by microcell fusion of human chromosome 2 but is not corrected for hMLH1 expression (hMLH1−), has also been used. Using these cell lines, the MMR system has been implicated in the cytotoxic response to multiple types of DNA-damaging agents including antimetabolites (6-TG), alkylating drugs (MNNG and cis-platinum), and methylating drugs (temozolomide; Refs. 8 and 10–12). In addition, studies using HCT116 and HCT116/3

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The abbreviations used are: dThd, thymidine; MMR, mismatch repair; 6-TG, 6-thioguanine; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; TK, dThd kinase; TS, thymidylate synthase; IR, ionizing radiation; HPLC, high-performance liquid chromatography; FdUrd, fluorodeoxyuridine; MEF, mouse embryonic fibroblast; SER, sensitization enhancement ratio; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; dNTP, deoxynucleotide triphosphate.
Trimethoprim (14), busulfan (15), procarbazine (15), and 6-mercaptopurine (12). It is used chemotherapeutic agents such as etoposide (11), doxorubicin shown to be resistant to these drugs as well as several other commonly used chemotherapeutic agents such as etoposide (11), doxorubicin (14), busulfan (15), procarbazine (15), and 6-mercaptopurine (12). It is therefore of interest to find a means of selectively targeting cells that have lost the ability to carry out MMR.

In this study, we compare the cytotoxicity of the halogenated dThd analogues IdUrd and BrdUrd to that seen with 6-TG in HCT116 and HCT116/3-6 genetically matched human colon carcinoma cell lines. We measure the percentage of dThd substitution in DNA and the 5-substituted dThd analogues are present in exponentially growing tumor cells were exposed to 0 or 5 \( \mu \text{M} \) IdUrd or BrdUrd for 18 h immediately before irradiation (0–6 Gy). Similar colony-forming assays and analyses were performed after 10–14 days as described previously (16). 

**Materials and Methods**

**Cell Lines and Culture Conditions.** Parental HCT116 human colon carcinoma cells are known to have a hemizygous nonsense mutation in the **hMLH1** gene located on chromosome 3. The clone 6 (HCT116/3-6) cell line was created by microcell chromosome transfer of a single normal human chromosome 3 (tagged with pSV2-neo plasmid DNA) into HCT116 cells (8). The HCT116/2-1 cell line (clone 1) was similarly created by microcell fusion and selected for retention of human chromosome 2 (8). HCT116/3-6 cells have been shown to express the hMLH1 transcript and protein (hMLH1*). They are proficient in MMR. HCT116/2-1 cells do not express the hMLH1 transcript and protein (hMLH1*). They are not proficient in MMR, similar to parental cells. HCT116/2-1 cells served as a clonal control for HCT116 parental cells.

Parental HCT116 human colon carcinoma cells, HCT116/2-1, and HCT116/3-6 cells were maintained in \( \alpha \)-MEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 \( \mu \text{g/ml} \)), and streptomycin (100 \( \mu \text{g/ml} \)) and grown in a 95% air-5% CO\(_2\) atmosphere at 37°C. G418 (400 \( \mu \text{g/ml} \); Life Technologies, Inc.) was added to maintain HCT116/3-6 and HCT116/2-1 cells, but experiments were performed in medium that did not contain G418. Our characterization of these cell lines, their population doubling times, and p53 status has been reported previously (13).

The MC3 MEFs contain wild-type Mlh1 (Mlh1\(^{+/+}\)), whereas the MC3 MEFs derived from Mlh1 knockout mice are deficient in both copies of the gene (Mlh1\(^{-/-}\)). These cells have a C57BL/6 background and were generated and generously supplied by Dr. R. M. Liskay (Oregon Health Science University, Portland, OR). Both cell lines were spontaneously immortalized and genotyped in the lab of Dr. R. M. Liskay. MC5 and MC3 fibroblasts were also maintained in \( \alpha \)-MEM supplemented with 10% fetal bovine serum and 50 \( \mu \text{g/ml} \) gentamicin (Life Technologies, Inc.) and grown in 95% air-5% CO\(_2\) at 37°C.

**Drug and Drug Radiation Cytotoxicity.** For clonogenic survival studies after administration of drug alone, exponentially growing human tumor cells (HCT116, HCT116/3-6, or HCT116/2-1) were exposed to 1–10 \( \mu \text{M} \) IdUrd or BrdUrd or to 0.3–30 \( \mu \text{M} \) 6-TG (Sigma Chemical Co., St. Louis, MO) for 18 h (≈1 cell population doubling). After the 18-h exposure, drug-free complete medium was added, and serially diluted cell populations were grown at 37°C. The surviving fraction of cells was determined using a standard colony-forming assay with appropriate controls as described previously (16). A surviving colony was defined as ≅50 cells after 7–10 days of growth after drug treatment. For the IR survival studies, comparable populations of exponentially growing tumor cells were exposed to 0 or 5 \( \mu \text{M} \) IdUrd or BrdUrd for 18 h immediately before irradiation (0–6 Gy). Similar colony-forming assays and analyses were performed after 10–14 days as described previously (16). SERs were calculated, as described previously, at 10% survival (6). Irradiation was performed using a model 109 \( ^{137}\text{Cs} \) irradiator (J. L. Shepherd and Associates, Glendale, CA) using a dose rate of 5.8 Gy/min. Clonogenic survival studies after treatment with drug alone and drug plus radiation were performed in triplicate and repeated at least three times in the human tumor cell lines.

**dThd Levels Present in DNA.** Exponentially growing tumor cell populations were exposed to a 4-h pulse of 1–10 \( \mu \text{M} \) IdUrd or BrdUrd and harvested 14 h after the addition of fresh medium, and MEFs were exposed to 1–10 \( \mu \text{M} \) IdUrd for 18 h at 37°C. The percentage of dThd replacement was determined after treatment with drug alone and drug plus radiation were performed in triplicate and repeated at least three times in the human tumor cell lines. We measure the percentage of dThd substitution in DNA and the in vitro radiosensitivity of these two cell lines after preirradiation exposures for 1 population doubling to clinically achievable plasma concentrations (1–10 \( \mu \text{M} \)) of these dThd analogues. We also determine the in vitro enzyme activities of the two rate-limiting enzymes in the de novo (TS) and salvage (TK) dThd metabolic pathways as well as the triphosphate pools to compare dThd analogue metabolism between these genetically matched human tumor cell lines. Finally, we compare incorporated IdUrd in the DNA of spontaneously immortalized MEFs established from Mlh1 knockout mice and their wild-type siblings after exposure to IdUrd for approximately 1 population doubling. We show that the 5-substituted dThd analogues present in significantly higher levels in the DNA of MMR-deficient cells and that pretreatment with these analogues results in an increase in radiosensitivity in HCT116 MMR-deficient cells but does not result in a significant increase in MMR-proficient HCT116/3-6 cells.

**TK Activity Assay in Human Colon Cancer Cells.** TK activity was determined from cell cytosol prepared by the modification of Lee and Seagjit.

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Fig. 1. Structure and metabolism of 6-TG and the 5'-halogenated pyrimidine (5'-Urd) analogues. A, the R group represents a methyl group in normal dThd or a halogen atom in the halogenated pyrimidine analogues IdUrd and BrdUrd. B, after processing of 6-TG by the purine nucleoside phosphorylase (PNP) or processing of the 5'-dThd analogues by TK, both types of drugs can compete with normal dNTPs for incorporation into DNA.

3-6 cells have also implicated the MMR system, and hMLH1 in particular, in a G\(_2\)-M-phase arrest after treatment with 6-TG or IR (9, 13). Consequently, cells lacking hMLH1 protein expression have been shown to be resistant to these drugs as well as several other commonly used chemotherapeutic agents such as etoposide (11), doxorubicin (14), busulfan (15), procarbazine (15), and 6-mercaptopurine (12). It is therefore of interest to find a means of selectively targeting cells that have lost the ability to carry out MMR.
Cheng’s original method (18) by Fisher et al. (19). Briefly, exponentially growing HCT116, HCT116/3-6, and HCT116/2-1 cells were harvested, scraped, and pelleted by centrifugation at 600 × g for 10 min at 4°C. Matching cultures of cells were resuspended by trypsinization and counted to determine the specific enzyme activity per million cells. The pellet was resuspended in 2 volumes of buffer containing 5 mM Tris-HCl (pH 7.5), 10 mM KCl, 5 mM DTT, 5 mM ATP, 5 mM MgCl2, and 10% (v/v) glycerol and homogenized using a Dounce homogenizer. Cell debris was removed by centrifugation at 12,000 × g for 20 min at 4°C. The supernatant was recovered and placed on ice, and the enzyme reaction was carried out as described previously (19). TK activity was expressed as pmol of TMP produced/106 cells/min.

Measurement of TS Activity in Intact Human Colon Cancer Cells. TS activity was measured in intact HCT116, HCT116/3-6, and HCT116/2-1 cells as described previously (20). In addition to measuring intrinsic levels of TS activity in exponentially growing cells, activity after treatment with dThd analogues was also measured. For these data, exponentially growing cells were plated and treated in 6-well cell culture plates. Treatment involved an 18-h exposure to 0 or 5 μM IdUrd or BrdUrd or a 2-h exposure to 0 or 5 μM FdUrd. FdUrd was used as a positive control because it effectively inactivates (binds) TS with shorter exposures, as reported previously by our laboratory (20). Cell density at the time enzyme activity was measured ranged from 1.5–2.0 × 106 cells/dish/10. TS activity was calculated as pmol of 3H released/106 cells/min.

Measurement of Triphosphate Pools. Cell extract preparation and the conditions for HPLC analysis of dNTP pool measurements were performed as described previously (20). The samples were analyzed using a Waters HPLC system (600E Multisolvent delivery system and controller, 490E Multiwavelength detector, 717 Autosampler, and Milenium chromatography manager software). Nucleotides were separated on a 4.6 × 250-mm Partisil-10 SAX column (Whatman). The mobile phase consisted of 0.35 M NH4H2PO4 (pH 3.0) with H3PO4 at a flow rate of 2 ml/min. Peaks were detected at 254 nm. Retention time of dCTP, dTTP, dATP, and dGTP were 10.6, 12.4, 14.5, and 26.8 min, respectively. dNTPs were quantified by peak heights against authentic standards using the Millenium software. The percentage IdUrd DNA incorporation was calculated as follows: [(IdUrd)/[(dThd) + (IdUrd)]] × 100.

**Results**

**Drug Cytotoxicity.** The clonogenic survival data of parental HCT116 (hMLH1+) cells and the corrected (hMLH1−) subline HCT116/3-6 cells after 18-h exposures to 6-TG (Fig. 2A; 0.3–30 μM), and IdUrd (Fig. 2B; 1–10 μM) are shown in Fig. 2. As reported previously (8, 11, 13), the HCT116/3-6 cells showed ≥1 log lower survival after exposure to 0.1 and 1.0 μM 6-TG compared to the parental HCT116 cells. In contrast, HCT116/3-6 cells showed little cytotoxicity (<1 log cell kill) after the 18-h exposure to 1–10 μM IdUrd and also have a consistently higher survival compared to parental HCT116 cells. Comparable survival data were found for the HCT116 and HCT116/3-6 cells after exposure to BrdUrd as well (data not shown). Clonogenic survival after the three separate drug treatments (6-TG, IdUrd, and BrdUrd) is similar in HCT116 and HCT116/2-1 cells, which are both hMLH1+. (IdUrd) subclone (HCT116/3-6) shows a 2–3-fold lower level of dThd analogue in DNA after IdUrd exposure than HCT116 cells. There was a 1.5–2.0-fold lower level of BrdUrd in HCT116/3-6 cells than in HCT116 cells, similar to the data for IdUrd. For example, after exposure to a clinically relevant dose of 2 μM BrdUrd, HCT116/3-6 cells and HCT116 cells showed 4.8 ± 0.1% and 8.6 ± 0.1% incorporation levels, respectively. As a control, the percentage of dThd...
DNA incorporation values were the same in HCT116 and HCT116/2-1 cells after IdUrd and BrdUrd exposures (data not shown). Additionally, MC5 (Mlh1+/+) spontaneously immortalized MEF cells show lower levels of IdUrd present in their DNA than MC3 (Mlh1−/−) MEF cells derived from Mlh1 knockout mice (Fig. 3B), supporting our results using the human colon carcinoma cells.

IR Survival. We also compared clonogenic survival after IR in these same three tumor cell lines (HCT116, HCT116/3-6, and

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IR Survival. We also compared clonogenic survival after IR in these same three tumor cell lines (HCT116, HCT116/3-6, and
HCT116(2-1) with or without a prior 18-h exposure to 5 μM IdUrd or BrdUrd. In agreement with the direct correlation between the percentage of dThd DNA replacement by the halogenated analogues and dThd analogue-induced radiosensitization (6), we found a significant increase in radiosensitization (SER = 2.0) after exposure to 5 μM IdUrd and subsequent irradiation compared to exposure to irradiation alone in the HCT116 cells. Essentially no radiosensitization resulted after preirradiation with IdUrd in HCT116/3-6 cells (Fig. 4, A and B). An intermediate effect (SER = 1.32) on radiation survival was noted with a preirradiation exposure to 5 μM BrdUrd. Again, no differences in radiation survival (± preirradiation exposures to IdUrd/BrdUrd) were found in the HCT116/2-1 cells compared to parental HCT116 cells (data not shown).

**TK and TS Enzyme Activities in Human Colon Cancer Cells.** To evaluate whether the differences in cytotoxicity and dThd levels in DNA after the 18-h exposures to IdUrd or BrdUrd might be related to differences in drug metabolism (Fig. 1B), we measured the enzyme activities of TK and TS in exponentially growing cell lines. Both TK and TS enzyme activities were modestly higher in HCT116/3-6 cells compared to HCT116 cells (Fig. 5A). The TK levels were 1.991 ± 0.002 and 1.548 ± 0.003 pmol/TMP/10^6 cells/min in cytosolic extracts, whereas the TS activity levels in intact cells were 0.304 ± 0.005 and 0.208 ± 0.003 pmol of ^3H released/10^6 cells/min for HCT116/3-6 cells and HCT116 cells, respectively. Furthermore, measurements of TS activity in these two genetically matched cell populations showed only a modest inhibition (10–30%) after an 18-h exposure to 5 μM IdUrd or BrdUrd compared to a marked (90%) inhibition after a 2-h exposure to 5 μM 5-FdUrd, which is known to be a strong inhibitor of TS (Fig. 5A). TK and TS enzyme activities were similar in the two hMLH1^- cell lines, HCT116 and HCT116/2-1 (data not shown).

**dNTP Pools in Human Colon Cancer Cells.** dNTP pool levels in HCT116, HCT116/2-1, and HCT116/3-6 cells were also examined to determine whether variations could account for the differences observed in survival and dThd analogue incorporation between the hMLH1^- and hMLH1^+ cell lines. However, dNTP levels were similar between the hMLH1^- and hMLH1^+ cell lines (Fig. 5B), showing slightly higher levels overall in the HCT116/3-6 cells (hMLH1^+). These higher dNTP levels in HCT116/3-6 cells correlate with the slightly higher, measured enzyme activities of TK and TS (Fig. 5A).

**Discussion**

A significant difference in the levels of halogenated dThd analogues present in DNA was found in parental HCT116 colon carcinoma cells, which are known to be hMLH1^- and hMLH1^-/^- cells, which are proficient in MMR due to microcell fusion of a wild-type copy of hMLH1. A similar trend was seen after exposure to BrdUrd. A second clonally derived cell line, HCT116/2-1, which contains a normal human chromosome 2 as a control for microcell fusion and remains MMR (hMLH1^-/-) deficient, showed a pattern of IdUrd and BrdUrd DNA incorporation similar to that of the parental hMLH1^- cells. Additionally, spontaneously immortalized MEF cells expressing Mlh1 demonstrated lower levels of IdUrd in DNA than spontaneously immortalized Mlh1^-/- knockout MEF cells. Compared to the HCT116/3-6 cells, parental HCT116 (and HCT116/2-1) cells, which show significantly higher levels of IdUrd in DNA, also show a modest increase in cytotoxicity and a significant increase in drug-induced radiosensitization after exposure to 1–10 μM IdUrd and BrdUrd for 1 population doubling. In general, no significant differences in the triphosphate pools or in the enzyme activities of TK and TS were found in the hMLH1^- cells compared to the hMLH1^+ cells. Collectively, these results strongly suggest that the corrected hMLH1^- cells are capable of removing the incorporated dThd analogues as a consequence of their proficient MMR phenotype.

These results were somewhat surprising because the dThd analogues would not be expected to result in the formation of mismatches upon their incorporation into DNA. Because the halogen atom in the dThd analogues has been substituted at the 5 position of the pyrimidine ring, the dThd analogues do not vary from normal dThd in their ability to form hydrogen bonds (Fig. 1A). However, it has been shown that mispairs can be generated during replication across DNA containing halogenated dThd analogues (21). It is therefore interesting to speculate whether the MMR system recognizes the analogues or distortions in DNA resulting from their initial incorporation or whether it recognizes mispairs generated during subsequent rounds of DNA replication. Current data from studies on the role of MMR in recognition and the cytotoxicity of chemically induced adducts in DNA, as well as the tolerance of MMR-deficient cells for alkylating agents, have supported a model that favors the latter hypothesis (22). After interaction of various alkylating agents such as MNNG, methyl-nitrosourea, and the antimetabolite 6-TG with DNA, cells undergo replication, and mispairing may result from the adducts in DNA. The resulting mispairs are then thought to be recognized by the MMR proteins, leading to futile cycles of DNA repair in the newly replicated strand. This futile cycling is hypothesized to ultimately result in cellular cytotoxicity.

However, we found substantial differences in the level of IdUrd and BrdUrd within the DNA of hMLH1^- cells compared to that of hMLH1^+ cells after only 1 population doubling, indicating that the
MMR machinery recognizes the analogues or distortions in DNA introduced upon initial halogenated dThd analogue incorporation. Both mismatched bp arising during normal cellular processes and the halogenated dThd analogues appear to be removed from DNA by the MMR system without significant cytotoxicity. However, other adducts such as those induced by 6-TG and cisplatin, which are recognized by the MMR system, appear to result in significant cellular cytotoxicity. Consequently, there may be structural differences in these types of adducts that determine whether the MMR machinery will recognize and remove the adduct or take part in a cascade that ultimately results in cytotoxicity. Structural studies have been done on different naturally occurring mispairs in a B-DNA undecamer, and a correlation was found between helical distortion caused by mispairs and the efficacy of MMR (23). Bases involved in G-T and A-C mismatches, which are repaired most efficiently by the MMR system, are displaced into the major and minor grooves but remain an integral part of the helix rather than looping out from the helix. However, in the case of A-G and C-T mismatches, which are repaired at much lower frequencies by MMR (23), the bases involved in the mismatch loop out from the helix. These data suggest that differences in structural alterations in DNA resulting from mispairs may account for the varying efficacy of MMR. It has also been shown that single bp mismatches are recognized by a different complex of MMR proteins than mispairs in small insertion/deletion loops (24), indicating that structural differences dictate which MMR proteins are involved in recognition. Given the different responses by MMR-proficient cells to single base mismatches and incorporated halogenated pyrimidines compared to agents such as MNNG, 6-TG, and cisplatin, it also seems possible that structural differences may account for the type of response elicited by the MMR system.

The fact that the DNA levels of halogenated pyrimidines appear to remain higher in MMR-deficient tumor cells may have clinical relevance as well. MMR deficiency, due to mutations in one of the known hMLH1 STATUS AFFECTS THE LEVELS OF dThd ANALOGUE IN DNA because of the type of response elicited by the MMR system.

References

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