Selective Radiosensitization of Drug-resistant MutS Homologue-2 (MSH2) Mismatch Repair-deficient Cells by Halogenated Thymidine (dThd) Analogues: Msh2 Mediates dThd Analogue DNA Levels and the Differential Cytotoxicity and Cell Cycle Effects of the dThd Analogues and 6-Thioguanine¹

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ABSTRACT

Mismatch repair (MMR) deficiency, which underlies hereditary non-polyposis colorectal cancer, has recently been linked to a number of sporadic human cancers as well. Deficiency in this repair process renders cells resistant to many clinically active chemotherapy agents. As a result, it is of relevance to find an agent that selectively targets MMR-deficient cells. We have recently shown that the halogenated thymidine (dThd) analogues iododeoxyuridine (IdUrd) and bromodeoxyuridine (BrdUrd) selectively target MutL homologue-1 (MLH1)-deficient human cancer cells for radiosensitization. The levels of IdUrd and BrdUrd in cellular DNA directly correlate with the ability of these analogues to increase the sensitivity of cells to ionizing radiation, and data from our laboratory have demonstrated that MLH1-mediated MMR status impacts dThd analogue DNA levels, and consequently, analogue-induced radiosensitization. Here, we have extended these studies and show that, both in human and murine cells, MutS homologue-2 (Msh2) is also involved in processing dThd analogues in DNA. Using both E1A-transformed Msh2+/+ and Msh2−/- murine embryonic stem (ES)-derived cells (throughout this report we use Msh2+/+ and Msh2−/- to refer to murine ES-derived cell lines that are wild type or mutant, respectively, for the murine Msh2 gene) and human endometrial cancer cells differing in MSH2 status, we see the classic cytotoxic response to 6-thioguanine (6-TG) in Msh2+/+ and human HEC59/2–4 (MSH2+*) MMR-proficient cells, whereas Msh2−/- cells and human HEC59 (MSH2−*) cells are tolerant (2-log difference) to this agent. In contrast, there is very little cytotoxicity in Msh2+/+ ES-derived and HEC59/2–4 cells to IdUrd, whereas Msh2−/- and HEC59 cells are more sensitive to IdUrd. High-performance liquid chromatography analysis of IdUrd and BrdUrd levels in DNA suggests that this differential cytotoxicity may be due to lower analogue levels in MSH2+ murine and human tumor cells. The DNA levels of IdUrd and BrdUrd continue to decrease over time in Msh2−/- cells following incubation in drug-free medium, whereas they remain high in Msh2+/+ cells. This trend was also found in MSH2-deficient human endometrial cancer cells (HEC59) when compared with HEC59/2–4 (hMsh2-corrected) cells. As a result of higher analogue levels in DNA, Msh2−/- cells are selectively targeted for radiosensitization by IdUrd. Fluorescence-activated cell-sorting analysis of Msh2+/+ and Msh2−/- cells shows that selective toxicity of the halogenated nucleotide analogues is not correlated with a G2-M cell cycle arrest and apoptosis, as is found for selective killing of Msh2−/- cells by 6-TG. Together, these data demonstrate MSH2 involvement in the processing of IdUrd and BrdUrd in DNA, as well as the differential cytotoxicity and cell cycle effects of the halogenated dThd analogues compared with 6-TG. Therefore, IdUrd and BrdUrd may be clinically selectively targeted both MLH1- and MSH2-deficient, drug-resistant cells for radiosensitization.

INTRODUCTION

The postreplicative DNA MMR¹ system helps to maintain genomic integrity by correcting naturally occurring mismaps in DNA and has more recently been implicated in the processing of multiple chemotherapy agents as well. MMR facilitates a cytotoxic response in cells to 6-TG (1), temozolomide (2, 3), adriamycin, procarbazine, busulfan, etoposide, cisplatin (2), and carboplatin, and, as a result, MMR-deficient cells are tolerant to these agents (4–16). Mutations in genes coding for the MMR proteins have been found to underlie the hereditary colorectal cancer syndrome HNPCC, with the majority of mutations found in either the hMLH1 gene (30–33%) or the hMSH2 gene (31–43%; Refs. 17 and 18), emphasizing their importance in the eukaryotic MMR process (see Fig. 1).

The absence of MMR results in a phenotype known as MSI² (19–22). Short, repetitive microsatellite sequences in DNA may be copied incorrectly due to polymerase slippage during the replication process. In the absence of MMR, this can lead to expansion or contraction of these sequences throughout the genome. Many types of sporadically occurring tumors have been found to display the MSI phenotype (23–28), indicating that such tumor cells may be deficient in the MMR process. Recent studies have proven that this is, in fact, the case, reporting that the promoter region of the human MLH1 gene is often methylated in MSI tumor cells, leading to the absence of both hMLH1 protein expression and MMR activity. This phenomenon has been found in many of the same tissues in which tumors occur in HNPCC kindreds, including 84% of MSI sporadic colon cancers tested (29), 77% of MSI sporadic endometrial tumors tested (30), and in 10 of 11 high-frequency MSI sporadic gastric carcinomas tested (31). These data clearly indicate that MMR deficiency in cancer is more prevalent than previously thought and, as a result, drug tolerance due to a deficiency in this repair process is becoming an increasing concern.

Although the absence of MMR has been shown to result in resistance to multiple cancer chemotherapy agents that interact with DNA, we have recently shown that the dThd analogues IdUrd and BrdUrd can specifically target MMR-deficient human tumor cells for radiosensitization (32). These halogenated dThd analogues are incorporated into DNA in place of dThd and are thought to radiosensitize cells by forming reactive uracil radicals following irradiation that are capable of inducing strand breaks at adjacent sugar residues (33–36). As a result, the radiosensitization resulting from IdUrd and BrdUrd directly correlates with the levels of dThd analogues in DNA (37, 38). Using both human and murine cell systems, our laboratory has shown

¹The abbreviations used are: MMR, mismatch repair; MLH1, MutL homolog-1; MSH2, MutL homolog-2; HNPCC, hereditary nonpolyposis colorectal cancer; dThd, thymidine; IdUrd, iododeoxyuridine; BrdUrd, bromodeoxyuridine; 6-TG, 6-thioguanine; IR, ionizing radiation; ES, embryonic stem; HPLC, high-performance liquid chromatography; IPUR, 5-ido-2-deoxyurynosin-2'-deoxyribose; SER, sensitizer enhancement ratio; MSI, microsatellite instability; FBS, fetal bovine serum; FACS, fluorescence-activated cell-sorting; PI, propidium iodide.

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that the DNA levels of these dThd analogues are significantly higher in MLH1<sup>+/−</sup>, (MMR-deficient) cells when compared with genetically matched MLH1<sup>+</sup>, (MMR-proficient) cells (32). Consequently, enhanced radiosensitization is seen in MLH1<sup>−/−</sup> cells following exposure to IdUrd or BrdUrd, whereas there is limited toxicity and very little increase in radiosensitization of MMR-proficient cells. We now show that MSH2 is also involved in processing the halogenated dThd analogues in DNA, using isogenic Msh2<sup>+/−</sup> wild-type and Msh2<sup>−/−</sup> E1A-immortalized murine ES cells as a model for primary stem cells from which cancer is believed to originate. In addition, we show this trend in the human parental HEC59 endometrial cancer cells (hMSH2<sup>−</sup>, MMR<sup>−</sup>), and hMSH2-corrected HEC59/2–4 cells. We find that IdUrd selectively targets MMR-deficient cells for radiosensitization at doses that show very little toxicity in MMR-proficient cells. Thus, a combination of halogenated dThd analogue exposure and IR would be a treatment approach to address the emerging problem of drug resistance in MMR-deficient tumors.

MATERIALS AND METHODS

Generation of ES Cells from Msh2<sup>+/−</sup> Wild-Type and Msh2<sup>−/−</sup> Knockout Mice. Msh2<sup>−/−</sup> knockout murine ES cell line dMsh2<sup>−</sup>-9 and isogenic wild-type control line wt-2 (39) were infected with a retrovector expressing the adenovirus E1A gene (40). The resulting cell lines (NW98069, Msh2 wild type, and NW980070 Msh2<sup>−/−</sup>) retained many growth characteristics and the apoptotic responses of the parental ES cell lines but have lost their differentiation potential.

Cell Lines and Culture Conditions. Parental HEC59 human endometrial carcinoma cells, which were isolated from a malignant neoplasm (41), have been shown to contain a frameshift mutation in one allele and a truncating mutation in the second allele of the human MSH2 gene (22) and are, therefore, MMR deficient (42). The HEC59/2–4 cell line was established by transfer of both parental ES-derived cells and the human HEC59/2–4 cells were grown in DMEM (Mediatech, Inc., Herndon, VA) supplemented with 10% defined FBS (Hyclone Laboratories, Logan, UT), penicillin (100 mg/ml), and streptomycin (100 mg/ml) and grown in a 90% air-10% CO<sub>2</sub> atmosphere at 37°C. G418 (Life Technologies, Inc., Grand Island, NY) was added to medium to maintain HEC59/2–4 cells, but experiments were carried out in its absence.

Drug Cytotoxicity and IdUrd-induced Radiosensitization Assays. Exponentially growing Msh2<sup>+/−</sup> and Msh2<sup>−/−</sup> were treated with 0.1–3.0 μM 6-TG, in DMEM supplemented with dialyzed FBS (−dThd), for approximately one population doubling (12 h), and were then immediately trypsinized, serially diluted, and plated in drug-free medium supplemented with defined FBS at 37°C. For IdUrd cytotoxicity, exponentially growing cells were treated with 0.1–10.0 μM (murine cells) or 1.0–10.0 μM IdUrd (human cells) in DMEM supplemented with dialyzed FBS (−dThd), for 12 h, followed by a 12-h incubation period in DMEM supplemented with defined FBS (containing dThd; human cells) or with dialyzed FBS (murine cells). Cells were then trypsinized, serially diluted, and plated in 60-mm dishes with drug-free DMEM supplemented with defined FBS (containing dThd), at 37°C. For IdUrd-induced radiosensitization, exponentially growing cells were exposed to 7.5 μM IdUrd for 12 h in DMEM supplemented with dialyzed FBS. After removal of the drug, cells were incubated in drug-free DMEM supplemented with defined FBS for 12 h and then irradiated with 1.0, 2.5, or 5.0 Gy. Four h after irradiation, cells were trypsinized, serially diluted, and plated in 60-mm dishes in drug-free DMEM supplemented with defined FBS at 37°C. Colonies, comprising ≥50 cells, were stained and counted 7–10 days after plating. SERs were calculated, as described previously, at 10% survival (37). All drug cytotoxicity and radiation survival assays were done in duplicate and repeated twice.

HPLC Analysis of IdUrd and BrdUrd DNA Levels. Exponentially growing murine or human cells were exposed to 1–10 μM IdUrd or BrdUrd in DMEM supplemented with dialyzed FBS (−dThd, which would compete with IdUrd and BrdUrd for incorporation into DNA), at 37°C for 4 h (murine cells) or 8 h (human cells). Medium containing the halogenated dThd analogues was then removed, and drug-free DMEM supplemented with defined FBS (containing dThd; human cells) or with dialyzed FBS (murine cells) was added. Cells were left in drug-free medium for 12 h (murine cells) or 18 h (human cells) and were then harvested and processed for HPLC analysis, as described previously (32). HPLC analysis was performed using a Waters 600E multisolvent delivery system and Waters 717 Autosampler, coupled to a C18-mBondclone column (3.9 × 300 mm; Phenomenex, Inc., Torrance, CA). Samples were eluted with 100 mM sodium acetate (pH 5.45) and 7% acetonitrile (mobile phase). Peaks were detected using a Waters 490E Wavelength Detector, and standard curves were generated for dThd, IdUrd, and BrdUrd using authentic samples (Sigma Chemical Co., St. Louis, MO). Waters Millenium<sup>®</sup> Chromatography Manager software was used for analysis of peaks and data quantitation (Waters Corp., Milford, MA).

To analyze the effect of IR on DNA levels of IdUrd, samples were treated in conjunction with the radiosensitization survival assay detailed above. Exponentially growing cells were treated with 7.5 μM IdUrd for 12 h in DMEM supplemented with dialyzed FBS (−dThd), followed by a 12-h washout period in drug-free DMEM supplemented with defined FBS. Cells were then irradiated with 0, 1.0, 2.5, or 5.0 Gy and incubated 4 h at 37°C. Samples were then taken and plated for survival, or processed for HPLC analysis, as detailed above.

Analysis of Deoxynucleoside Triphosphate Pool Levels. Cell extract preparation and the conditions for HPLC analysis of dNTP pool measurements were performed as described previously (44). The samples were analyzed using a Waters HPLC system (600E Multisolvent delivery system and controller, 490E Multiwavelength detector, 717 Autosampler, and Millenium Chromatography Manager software). Nucleotides were separated on a 4.6 × 250-mm Partisol-10 SAX column (Whatman, Inc.). 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. dNTPs were quantified by peak heights against authentic standards using the Millenium software.

Cell Cycle Analysis of Msh2<sup>+/−</sup> and Msh2<sup>−/−</sup> Cells. Exponentially growing cells were treated simultaneously for both HPLC analysis (detailed above) and for FACS analysis, with 10.0 μM IdUrd or BrdUrd for 4 h in DMEM supplemented with dialyzed FBS (−dThd) or with 0.1 μM 6-TG for 12 h in DMEM supplemented with defined FBS (±dThd), at 37°C. Medium containing either the halogenated pyrimidine analogues or 6-TG was then removed, and drug-free medium was added (DMEM supplemented with defined FBS, in all cases). Samples were tryptized at the times indicated; cells were pelleted, washed once with PBS, and then fixed in 900 ml of 70% ethanol/100 ml PBS.
The samples were then stored at −20°C until staining. Samples were washed in 0.5 ml of phosphate citric acid buffer [192 ml of 0.2 M Na2HPO4 and 8 ml of 0.1 M citric acid (pH7.8)] at room temperature, pelleted, and resuspended in 0.5 ml of PI solution (1 mg/ml RNase A, 33 mg/ml PI in PBS/0.5 mm EDTA, and 0.2% NP40). Samples were incubated in PI solution overnight at 4°C. FACS analysis was carried out on a Coulter XL Flow Cytometer (Coulter Corp., Miami, FL).

RESULTS

Drug Cytotoxicity. We tested the murine Msh2+/+ and Msh2−/− cells for their response to 6-TG as a positive control to establish that the Msh2-deficient cells display tolerance to this antimetabolite when compared with the Msh2 wild-type cells. These cells had not previously been characterized for their 6-TG response, but, as expected, the MMR-deficient cells survive a dose of 3.0 μM 6-TG, whereas Msh2+/+ cells show a cytotoxic response to 6-TG at doses as low as 0.1 μM (Fig. 2a). These responses closely mimic those of the parental ES cells (data not shown). In contrast, we found essentially no cytotoxicity to IdUrd (0.5–10.0 μM doses) in Msh2+/+ cells, whereas Msh2−/− cells showed ~1 log lower survival at 2.0 μM IdUrd (Fig. 2c). The human endometrial carcinoma cell lines HEC59 and HEC59/2−4 were also compared for cytotoxicity following 6-TG or IdUrd treatment. HEC59/2−4 cells, which have been corrected for their MMR-deficient phenotype by transfer of a human chromosome carrying one wild-type copy of hMSH2, undergo a cytotoxic response to 6-TG, whereas MSH2-deficient HEC59 cells are highly tolerant (Fig. 2c). The human endometrial carcinoma cell lines HEC59 and HEC59/2−4 were also compared for cytotoxicity following 6-TG or IdUrd treatment. HEC59/2−4 cells, which have been corrected for their MMR-deficient phenotype by transfer of a human chromosome carrying one wild-type copy of hMSH2, undergo a cytotoxic response to 6-TG, whereas MSH2-deficient HEC59 cells are highly tolerant (Fig. 2c).

To test whether the difference in dThd analogue levels between the Msh2+/+ and Msh2−/− murine cells might be a cell line-specific effect, and to ensure that this effect could be seen in human MSH2-proficient and MSH2-deficient cells as well, HPLC analysis of dThd analogues in DNA of the two cell lines was done by treating cells with either 6-TG (Fig. 4a) or IdUrd (Fig. 4b), even at doses of up to 3.0 μM. Also similar to the data in the murine cells, the HEC59/2−4 cells demonstrate very little cytotoxicity to IdUrd, whereas the HEC59 cells are more sensitive. However, the trends are not as dramatic with either drug in the human cell lines as well (32). One possible reason for the more moderate differences between the human cell lines in response to 6-TG and IdUrd may be the high propensity of the murine cells to apoptotic responses, whereas the human cells are derived from a malignant carcinoma and may have lost these responses during oncogenesis. Alternatively, it may be due to the presence of only a single wild-type copy of the hMSH2 gene in HEC59/2−4 cells. Despite the variation in the level of response between the human and murine cell lines, these contrasting cytotoxicity data for 6-TG and IdUrd indicate that they are clearly processed quite differently by the MMR system.

Dose-Response of IdUrd and BrdUrd DNA Levels in Human and Murine Cells. HPLC analysis of DNA from murine cells following exposure to IdUrd indicates that the difference in IdUrd-induced toxicity between these cell lines may be due to differences in the accumulation of dThd analogue in DNA (Fig. 3a). Cells were exposed to various doses of dThd analogue for a 4-h pulse, followed by a chase with excess dThd-containing medium. After treatment, Msh2−/− cells show roughly 3-fold higher levels of IdUrd or 2-fold higher levels of BrdUrd in DNA when compared with Msh2+/+ cells

Fig. 2. Murine Msh2−/− knockout and Msh2+/+ wild-type cells and human HEC59 MSH2− cells are resistant to 6-TG, but not to IdUrd. Survival assays in the murine Msh2+/+ and Msh2−/− ES-derived cells were carried out by treating cells with either 6-TG (a) or IdUrd (b) for 12 h, then plated immediately (6-TG) or allowed a period of ~12 h in drug-free medium before plating (IdUrd). Colonies were stained 7 days later and counted, with ~50 cells representing a colony. Human endometrial cancer cell lines were treated with either 6-TG (c) or with IdUrd (d) for 24 h, then plated immediately (6-TG) or allowed ~24 h in drug-free medium before plating (IdUrd). Colonies were stained 14 days later and counted, with ~50 cells representing a colony.
IdUrd and BrdUrd DNA levels between MLH1-proficient and -deficient cells were not due to differences in dNTP pool levels or nucleotide metabolism between the two cell lines. We found that intrinsic dNTP pool levels, dThd kinase activity, and thymidylate synthase activity were very similar in human HCT116 (MLH1-deficient) colon cancer cells and HCT116/3–6 (MLH1-proficient) cells (32). Together, these data suggest that the differences in halogenated dThd analogue levels in DNA are not due to differences in the dNTP pools between the cell lines, but are likely due to the MMR status.

**Time Dependence of BrdUrd and IdUrd DNA Levels in Murine Msh2<sup>+/−</sup> and Msh2<sup>−/−</sup> Cells.** We analyzed the levels of both dThd analogues, over time, in the murine cells, as well as their cell cycle patterns. Cells were treated under conditions identical to those in Fig. 3 and, after harvesting, were processed either for HPLC or FACS analysis. BrdUrd levels were found to be similar in the DNA of both Msh2<sup>+/−</sup> and Msh2<sup>−/−</sup> cells at the end of the pulse period (Fig. 5a), suggesting that the differing levels of analogue seen in Fig. 3 are not due to less halogenated dThd analogue being incorporated into the DNA of MMR-proficient cells, but rather a subsequent decrease in analogue levels in MMR-proficient cells. Also, over time, the levels of BrdUrd increase in the DNA of both cell lines (8 h and 12 h) before declining in Msh2<sup>+/−</sup> cells or continuing to rise in Msh2<sup>−/−</sup> cells. Fig. 5b shows the same trend following treatment with IdUrd, as well, and likely reflects intracellular levels of dThd analogue, which, in the absence of a discriminating MMR system, result in increasing levels of BrdUrd or IdUrd in DNA even after the drug is no longer being administered. These data suggest that MSH2, while determining cytotoxicity of some nucleotide analogues (6-TG), is involved in the protection of the genome from the toxic effects of other nucleotide analogues (such as IdUrd and BrdUrd).

**Cell Cycle Effects of 6-TG and the dThd Analogues in Msh2<sup>+/−</sup> and Msh2<sup>−/−</sup> Cells.** To investigate the cellular basis of the differential cytotoxicity of IdUrd and 6-TG in the Msh2-proficient and -deficient cell lines, we analyzed cell cycle effects following treatment with 6-TG and with the halogenated dThd analogues. It was of particular interest to determine whether IdUrd or BrdUrd caused the cell cycle alterations (45–48) or apoptosis (1–2, 10, 48) commonly seen with 6-TG, IR, cisplatin, and N-methyl-N'-nitro-N-nitrosoguanidine in MMR-proficient human endometrial cancer cells HEC59 and HEC59/2−4 (b) were harvested, and HPLC analysis was used to determine their intrinsic dNTP pool levels.
Flow cytometry shows that 6-TG causes a G2-M arrest and an apoptotic sub-G1 population, selectively in Msh2+/- cells, whereas Msh2+/+ cells show no cell cycle alterations in response to 6-TG treatment (Fig. 6). The cell cycle changes occur 48 h after the addition of drug, or roughly three to four population doublings later, and are still present at 54 h (data not shown). Similar to the cytotoxicity data, there was a difference in the impact of 6-TG and dThd analogues on the cell cycle pattern of these cell lines as well. Cell cycle analysis of both cell lines following a 4-h treatment with IdUrd (Fig. 6b) or BrdUrd (Fig. 6c) do not reveal a G2-M arrest or significant alterations in the normal pattern at 48 h when compared with untreated cells. Cells were also analyzed at 4, 8, 12, 24, and 36 h, as well, without significant cell cycle perturbations, despite differences between the cell lines in dThd analogue levels in DNA (Fig. 5). Taken together, these data show that levels of both dThd analogues decrease over time selectively in Msh2-proficient cells, without a G2-M arrest.

IdUrd-induced Radiosensitization of Msh2+/+ and Msh2-/- Cells. From our data, it is apparent that there is a more pronounced effect of MMR status on the DNA levels of IdUrd, than of BrdUrd, to study radiosensitization in these cell lines to determine whether there is enhanced dThd analogue-induced radiosensitization in MMR-deficient cells compared with MMR-proficient cells. Pretreatment with 7.5 μM IdUrd results in a small increase in radiosensitization of Msh2+/+ cells when compared with radiation alone, but results in up to a 1–2-log enhanced kill in Msh2-/- cells over 2.5–5.0 Gy radiation alone (SER value of ~2.0 measured at 10% survival; Fig. 7, a and b). Because IR might invoke a repair response that would alter the levels of IdUrd in DNA, independently of MMR status, we have also used HPLC to analyze dThd analogue levels in DNA taken from samples treated simultaneously with those in the survival assay. Fig. 7c clearly shows that under these conditions, IR does not have an observable impact on the DNA levels of IdUrd in either cell line.

In addition, the impact of a combined treatment of IdUrd and IR results in cell kill that is clearly synergistic (Fig. 8). Because IdUrd alone demonstrates virtually no toxicity in Msh2-/- cells, and IR induces very little, it is apparent that the cell kill from a combined approach is greater than additive (Fig. 8), even when analogue levels are low in MMR-proficient cells. This effect is even more dramatic in Msh2-/- cells. As a result of both the direct correlation between two cell lines (Figs. 3 and 5). Consequently, we have used IdUrd, rather than BrdUrd, to study radiosensitization in these cell lines to determine whether there is enhanced dThd analogue-induced radiosensitization in MMR-deficient cells compared with MMR-proficient cells. Pretreatment with 7.5 μM IdUrd results in a small increase in radiosensitization of Msh2+/+ cells when compared with radiation alone, but results in up to a 1–2-log enhanced kill in Msh2-/- cells over 2.5–5.0 Gy radiation alone (SER value of ~2.0 measured at 10% survival; Fig. 7, a and b). Because IR might invoke a repair response that would alter the levels of IdUrd in DNA, independently of MMR status, we have also used HPLC to analyze dThd analogue levels in DNA taken from samples treated simultaneously with those in the survival assay. Fig. 7c clearly shows that under these conditions, IR does not have an observable impact on the DNA levels of IdUrd in either cell line.

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![Figure 5](image-url)

Fig. 5. Msh2 status impacts dThd analogue levels in DNA over time. Msh2+/+ and Msh2-/- cells were treated with a 4-h pulse of 10.0 μM BrdUrd (a) or 10.0 μM IdUrd (b). Following the treatment, drug-free medium was added and samples were collected at the times indicated. IdUrd, BrdUrd, and dThd levels in DNA were analyzed by HPLC.

![Figure 6](image-url)

Fig. 6. The halogenated dThd analogues do not disrupt the cell cycle or cause apoptosis in Msh2+/+ cells. Murine Msh2+/+ and Msh2-/- cells were treated with 0.1 μM 6-TG for 12 h (a), 10.0 μM IdUrd (b), or 10.0 μM BrdUrd (c) for 4 h. Drug-free medium was then added, and samples were collected at regular intervals following the addition of drug and analyzed by flow cytometry. The time point shown for all samples is 48 h after drug addition.

![Figure 7](image-url)
analogue levels in DNA and radiosensitivity, and the synergistic effect of the two treatments, a MMR-associated 2.5-fold difference in analogue levels between cell lines results in a $10^\text{th}$-fold difference in the percentage of survival between cell lines following the combined treatment versus radiation alone. These results suggest that MSH2-mediated MMR status plays an important role in dThd analogue levels in DNA and resulting radiosensitivity and can, therefore, be used to selectively target MMR-deficient tumors for radiosensitization.

**DISCUSSION**

Together, both hMLH1 and hMSH2 mutations account for 60–70% of HNPCC kindreds (17, 18), and MMR-deficiency has currently been linked to many types of sporadically arising tumors as well. Both MLH1- and MSH2-deficient cells are tolerant to a large number of anticancer drugs (4–16), and, as a result, MSH2- and MLH1-mediated MMR status may be an important factor in resistance to cancer chemotherapy (12, 13, 49). The first clinical evidence correlating chemotherapy responses with the *in vitro* data has come from studies of ovarian and breast cancer. Brown *et al.* (10) have shown that there is an increase in the number of ovarian tumor cells that score negative for hMLH1 expression following treatment with cisplatin when compared with untreated tumors. More recent studies have also correlated tumor response and lower survival with loss of hMLH1 expression in breast tumors following neoadjuvant chemotherapy (49). In addition, decreased expression of hMutSα and hMLH1 has been associated with head and neck cancer (50), and a study by Friedman *et al.* (51) found that low levels of hMLH1 and hMSH2 proteins in malignant gliomas may correlate with resistance to temozolomide. For these reasons, treatment protocols that would selectively target MMR-deficient cells will be of great value for the treatment of both HNPCC-related and sporadic MMR-deficient cancers. Previously, we have shown that the halogenated dThd analogues IdUrd and BrdUrd selectively radiosensitize hMLH1-deficient human tumor cells. Here, we extend this work to show in both human and murine cells that these analogues can be used to target MSH2-deficient cells for radiosensitization as well.

**Fig. 7.** IdUrd treatment selectively targets MMR-deficient Msh2$^{-/-}$ cells for radiosensitization. Msh2$^{-/-}$ (a) and Msh2$^{++/}$ (b) ES-derived cells were exposed to 7.5 μM IdUrd for a period of 12 h, followed by a 12-h washout period in drug-free medium containing dThd. Cells were then treated with varying doses of radiation and allowed 4 h before plating. Colonies ($\geq 50$ cells) were counted ~14 days after plating. c, samples taken jointly and analyzed by HPLC to determine whether IR affects the level of IdUrd in DNA.

**Fig. 8.** IdUrd and IR-mediated toxicity is synergistic. Msh2$^{-/-}$ and Msh2$^{++/}$ ES-derived cells were treated in conditions identical to those in Fig. 6. The difference in the percentage of survival for Msh2$^{++/}$ cells with the combined approach compared with 2.5 Gy IR alone is 1.95-fold, whereas there is a 39.4-fold decrease in the percentage of survival in Msh2$^{-/-}$ cells with the combined treatment over 2.5 Gy IR alone (a). With 5.0 Gy IR, there is a 2.5-fold decrease in cell survival in Msh2$^{++/}$ cells with IdUrd pretreatment, whereas the Msh2$^{-/-}$ cells demonstrate a 54.4-fold decrease in the percentage of survival following IdUrd pretreatment, compared with 5.0 Gy IR alone (b).
the cytotoxicity of various chemotherapeutic drugs. In addition, we have used a human endometrial cancer cell system to confirm that the halogenated dThd analogues IdUrd and BrdUrd can be used to selectively radiosensitize MSH2-deficient human tumor cells. Moreover, we demonstrate a direct toxicity of IdUrd toward Msh2-deficient, but not Msh2-proficient, ES-derived cells that is further enhanced by exposure to IR, the combined treatment resulting in a significant enhancement of cell kill of MMR-deficient cells (SER, 2.0 at 10% survival) when compared with the wild-type controls. This differential cytotoxicity is correlated with increased levels of the analogue in the Msh2-proficient cells, suggesting a direct role of MMR in the removal of these dThd analogues. In addition, Fig. 5 indicates that levels of the analogue continue to accumulate in the DNA of MMR-deficient cells, after removal of analogue. This suggests that there are intracellular reserves of the drug remaining and, in cells that lack an intact MMR system, IdUrd and BrdUrd levels continue to increase in DNA even in the absence of the drug, resulting in increased toxicity to these cells from halogenated analogue exposure alone (Fig. 2), as well as a potential for greater radiosensitization. Therefore, a short exposure of the radiosensitizers, followed by a “recovery period” may allow for an increased therapeutic index, with lower toxicity to proliferating normal tissues and an increased potential for radiosensitization of MMR-deficient tumor cells. Currently, we are testing this drug schedule concept to enhance radiosensitization in athymic mice with MMR− and MMR+ matched tumor xenografts.

In hopes of addressing MMR status in cancer chemotherapy, recent testing has been aimed at finding protocols that would be feasible in the clinic to determine the MMR status of tumor cells. Currently, different methods are being used to identify defects in MMR genes or protein expression in sporadic human tumors to attempt to correlate MMR status with response to different chemotherapy agents, and to identify mutations in hMLH1 or hMLH1 among HNPCC kindred members to determine who may need close surveillance. The result of using sequence analysis of the genes has been the identification of pathogenic mutations in hMLH1 and hMSH2 in a significant proportion of HNPCC kindred members (17, 18, 53) and in a small number of African Americans with colon cancer (54). An alternative assay to test for MSI+ at certain loci has demonstrated good correlation with MMR protein expression (55) and deficiency in in vitro functional MMR assays (21). Testing for MMR protein expression in tumors has revealed an inverse correlation with survival in breast cancer (49), the response of patients with malignant glioma to temozolomide (51), and risk for head and neck cancer (50). Ultimately, these tests may be used to determine what types of cancer therapy might be most beneficial, based on the MMR status of tumors. In cases where MMR-deficient tumors are identified, a combined approach of halogenated dThd analogues and radiation therapy may provide improved benefit. This approach has been used in previous clinical trials on various tumor types with good results. A Phase I trial of i.v. BrdUrd, combined with radiation therapy for pancreatic cancer, yielded a pathological complete response (56), and positive results have also been found with IdUrd and radiation therapy for sarcomas (57) and for colorectal metastases to the liver (58–60). Phase I trials for intraarterial IdUrd, combined with radiation therapy of colorectal liver metastases, demonstrated that IdUrd alone had antitumor activity before radiation (59), and Phase II/I studies of IdUrd, in combination with radiation therapy, have shown increased survival in patients with anaplastic astrocytoma (61, 62). More recent data indicates improved outcome for patients with glioblastoma multiforme following treatment with BrdUrd and IR (63). In addition, we now have an oral prodrug, IPdR, that is metabolized to IdUrd by an aldohexide oxidase enzyme in liver cells and has an increased therapeutic index when compared with oral or continuous infusion IdUrd. Our group has shown two to three times higher IdUrd levels in DNA of HCT116 and HT29 human colon cancer and U251 human glioblastoma xenografts in athymic mice following oral IPdR compared with oral or continuous infusion IdUrd, while simultaneously demonstrating >2-fold lower IdUrd DNA levels in proliferating normal tissues (bone marrow, intestine) with oral IPdR (64–66). As a result, oral IdUrd and IPdR have a better therapeutic index than i.v. IdUrd and IPdR, and our in vivo data now indicate that this combined treatment should be tested to target MMR-deficient tumors. With numerous current studies indicating that in vitro resistance of MMR-deficient cell lines and tumor xenografts to various cancer chemotherapy agents may extend into the clinic, it is important to find a treatment that would selectively target such cells. Using molecular tests to identify mutations in MMR genes, expression of MMR proteins, or MSI− in patients may enable clinical preevaluation of tumors and subsequent planning of a treatment protocol such as IdUrd (or IPdR) and IR, which will specifically target MMR-deficient tumor cells.

REFERENCES


Selective Radiosensitization of Drug-resistant MutS Homologue-2 (MSH2) Mismatch Repair-deficient Cells by Halogenated Thymidine (dThd) Analogues: Msh2 Mediates dThd Analogue DNA Levels and the Differential Cytotoxicity and Cell Cycle Effects of the dThd Analogues and 6-Thioguanine

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