Cytotoxicity and Mutagenicity of Frameshift-Inducing Agent ICR191 in Mismatch Repair-Deficient Colon Cancer Cells

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Background: Deficiency of DNA mismatch repair is a common feature of cancers exhibiting instability of microsatellite DNA sequences. Cancers with microsatellite instability are recognizable by their high rate of spontaneous frameshift mutations within microsatellite sequences, their resistance to killing by cytotoxic agents, and their localization to specific tissues, e.g., the proximal colon and stomach. We hypothesized that the mismatch repair deficiency of these cancers would make them vulnerable to environmental or chemical frameshift-inducing agents. This study was undertaken to test whether exogenous frameshift-inducing agents selectively induce mutations in mismatch repair-deficient cells of mutagen-exposed tissues like the colon and whether cytotoxic doses of these agents would preferentially kill those cells. Methods: Cytotoxicity of the acridine mutagen 6-chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxyacridine (ICR191), a DNA frameshift inducer, was determined in the mismatch repair-deficient human colon carcinoma cell line HCT116 versus the repair-reconstituted derivative HCT116+C3. Vulnerability to the mutagenic effects of ICR191 was determined by transfection of HCT116 or HCT116+C3 cells with a frameshift reporter vector, followed by treatment of the cells with ICR191. Alternatively, the reporter vector was reacted ex vivo with ICR191, and the derivatized vector was then transfected into HCT116 or HCT116+C3 cells. Results: ICR191 proved to be fivefold to 10-fold more potent in inducing mutations in mismatch repair-deficient HCT116 cells than in mismatch repair-proficient HCT116+C3 cells. Moreover, at cytotoxic doses of ICR191, repair-deficient HCT116 cells proved to be fivefold more vulnerable to killing than did HCT116+C3 cells. Conclusions: Frameshift-inducing mutagens can selectively induce mutations in mismatch repair-deficient cells versus mismatch repair-proficient cells. Environmental exposures may, therefore, favor development of cancers with microsatellite instability in tissues like the gut. Frameshift-inducing agents can, however, also preferentially kill mismatch repair-deficient cancer cells and, thus, may be promising as model therapeutic compounds. [J Natl Cancer Inst 2000; 92:480–5]

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ceptore type II (RII), which cluster within a 10-base-pair (bp) polyadenine repeat (BAT-RII) in the RII-coding region (8,9). Localization of colon cancers with microsatellite instability to the proximal colon, coupled with the high frequency of BAT-RII mutations, suggests that exogenous cofactors, such as mutagens, can cooperate with defects in mismatch repair in determining the formation of these tumors. To investigate this hypothesis, we examined the vulnerability of mismatch repair-deficient cells to frameshift mutations induced by 6-chloro-9-[3-(2-chloroethylamino)propylamino]-2-methoxy-acridine (ICR191), a member of the acridine family of frameshift mutagens (10–15) and a potent mutagen that preferentially causes single-bp additions in [G] repeats (10–13).

Mammalian cells defective in a DNA repair mechanism are usually more vulnerable to killing by cytotoxic agents (16–18). In contrast, mismatch repair-defective cells often are resistant to killing by cytotoxic agents. Such cells are dramatically refractory to killing by DNA-methylating agents (19–23). In addition, colon cancers with microsatellite instability demonstrate high levels of resistance to 5-fluorouracil (24) and lower degrees of resistance to cisplatin and 2-amino-1-methyl-6-phenyl-imidazo-[4,5-b]-pyridine (PhIP) (25–27). Likewise, cell lines derived from mismatch repair-deficient knockout mice are more resistant to the cytotoxic effects of prolonged low levels or acute high levels of ionizing radiation (28,29). To meet an obvious need for agents that are more toxic for cancers with microsatellite instability, we hypothesized that the vulnerability of mismatch repair-defective cells to frameshift mutations might translate into an enhanced susceptibility to the cytotoxicity of potent frameshift-inducing agents. Therefore, we have examined survival following treatment with ICR191 of the repair-deficient human colon carcinoma cell HCT116 versus its repair-reconstituted derivative HCT116+C3.

**Materials and Methods**

**Cell culture.** The mismatch repair-deficient human colon carcinoma cell line HCT116, defective in the mismatch repair gene hMLH1, and its mismatch repair-proficient derivative HCT116+C3, complemented with a wild-type hMLH1 gene on an exogenous human chromosome 3, were provided by Dr. Richard Boland (University of California, San Diego) (21). The cells were cultured in minimum essential medium (MEM) (obtained from the Tissue Culture Medium Facility of the Comprehensive Cancer Center of Case Western Reserve University, Cleveland, OH) supplemented with 1-glutamine, nonessential amino acids, sodium selenite, 10 mg/L bovine insulin, 2 mg/L human transferrin, 1 mg/L hydrocortisone, and 10% bovine calf serum (Hy-Clone Laboratories, Inc., Logan, UT). Monolayer cultures were grown at 37 °C in a 5% CO2 atmosphere. HCT116+C3 cells were grown in the presence of genetecin (G418) but were cultured free of G418 during and after all exposures to ICR191. Western blot analysis confirmed that the hMLH1 expression was maintained in the absence of G418.

**Preparation of ICR191.** The acridine mutagen ICR191 (Sigma Chemical Co., St. Louis, MO) was dissolved at 4 mg/mL in 0.01 M HCl, adjusted to 400 µg/mL in MEM without serum, filtered under sterile conditions, and diluted to the appropriate concentration in MEM with additives for the experiment. Fresh drug was prepared immediately before each experiment.

**Evaluation of the cytotoxicity of ICR191.** ICR191-induced cytotoxicity was evaluated by a clonogenic assay, which assesses cell killing. In this experiment, cells were seeded at 500 cells per well of a six-well dish. Then 12–16 hours later, the medium was replaced with serum-free medium containing ICR191, and incubated for 2 hours at 37 °C. After the 2-hour treatment with ICR191, the drug-containing medium was removed and the cultures were returned to MEM with serum, incubated at 37 °C for 7–10 days, and then evaluated for colony number after visualization with methylene blue and automated counting on an Alpha Innotech Imaging System (Alpha Innotech Corp., San Leandro, CA).

**Vector preparation.** Three vectors were prepared in our laboratory for this study. The second-generation pCAR vector, pCMV-CAR, a gift from Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD), was modified to create these vectors. This second-generation 14.6-kilobase vector, described by Parsons et al. (30), contains the complete coding region of β-galactosidase and the eu-karyotic promoter derived from cytomegalovirus, both of which allow direct visualization of mammalian cells expressing this gene product. The vector also contains an ampicillin-resistance gene and a leu2 promoter that permit selection of the vector and production of β-galactosidase in bacteria. In the three vectors constructed for this study, the CA repeat sequence in the pCAR vector has been replaced with a 10-base homopolymeric run of G residues 45 bp downstream from the ATG start site for the β-galactosidase gene. This region of these three vectors is shown in Fig. 1. One of these vectors is designed to place the β-galactosidase messenger RNA template strand 1 bp out-of-frame in the [−1] direction and requires a +1 frameshift for expression of β-galactosidase [designated the out-of-frame (+1) vector]. A second vector is designed to place the start site codon [+1] bp out-of-frame and requires a −1 frameshift for expression of β-galactosidase [designated the out-of-frame (−1) vector]. A control vector was also constructed, in which the run of G residues is in-frame, allowing constitutive synthesis of β-galactosidase following transfection into human colon cell lines or *Escherichia coli* (designated the in-frame vector).

**Ex vivo treatment of vector DNA with ICR191.** Plasmid DNA was directly exposed to increasing doses of ICR191 for 30 minutes in 0.5x phosphate-buffered saline (PBS). Treated DNA was then purified from the drug with the use of a Microcon-50 column (Amicon; Millipore Corp., Bedford, MA). In this procedure, 100 µL of vector DNA and drug-containing solution was loaded on the Microcon-50 column, 400 µL of deionized water was added, and the column was spun at room temperature for 10 minutes at 2940g. After two more washes with 500 µL of water, the column was inverted into a new vial and centrifuged identically, and approximately 100 µL containing the purified DNA was collected and verified for purity on an agarose gel. This vector DNA was then transfected into colon cancer cells and analyzed for mutation as described below.

**Transfection of ex vivo treated vector DNA into bacteria.** A total of 0.1 µg of purified vector DNA (treated or untreated) was transfected into Subcloning Efficiency DH5αcompetent *E. coli* cells (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD) to determine the mutational effect of ICR191 on the out-of-frame vectors. We determined the number of successful transforms by using the in-frame vector and monitoring colony growth on Luria Broth-ampicillin plates containing X-gal (i.e., 5-bromo-4-chloro-3-indolyl β-galactopyranoside). Colonies with frameshifts in the out-of-frame vector...
frameshift and out-of-frame vectors, we calculated the frequency of frameshifts induced by a given concentration of ICR191 by dividing the number of β-galactosidase-producing cells obtained with the out-of-frame vector by the number of β-galactosidase-producing cells obtained with the in-frame vector for each dose of ICR191. A mock-transfection control was also included at each ICR191 dose to ensure that neither the ICR191 treatment nor the transfection procedure resulted in production of endogenous β-galactosidase.

Statistical methods. All drug treatments were done in multiple replicates. Results are shown as mean values calculated for each drug dose, with 95% confidence intervals calculated as 1.96 times the standard error of the means.

RESULTS

Increased Toxicity of ICR191 in Mismatch Repair-Deficient Cells Versus Mismatch Repair-Proficient Cells

The inability of mismatch repair-defective cells to repair lesions causing frameshifts raises the possibility that agents leading to these lesions may exert more toxicity for mismatch repair-defective cells than for mismatch repair-proficient cells. To test this hypothesis, we used a clonogenic survival assay to compare the toxicity of ICR191 in the HCT116 human colon cancer cell line, which bears only an inactive mutant hMLH1 mismatch repair gene, with that in HCT116+C3 cells, in which mismatch repair has been reconstituted with a wild-type hMLH1 gene transferred on an exogenous chromosome 3 (Fig. 2). The results demonstrate that ICR191 is more toxic to mismatch repair-deficient cells than to mismatch repair-proficient cells. The LD50 dose of ICR191 (i.e., the dose at which clonogenic survival fell by 50%) was increased fivefold from 1 μg/mL in the sensitive HCT116 cells to 5 μg/mL in the more resistant HCT116+C3 cells. Similarly, the cytotoxicity of a single 5-μg/mL dose of ICR191 increased from LD50 in the resistant HCT116+C3 cells to LD50 in the sensitive HCT116 cells. In contrast, a derivative of HCT116+C3 cells, in which the wild-type hMLH1 gene has been lost [clone M2 (21)], demonstrated restored sensitivity to killing by ICR191, with an ICR191 LD50 dose decreased back to a level identical to that of the HCT116 cells. The fivefold increased sensitivity of HCT116 cells to killing by the frameshifting agent was greater than the twofold change in resistance that these cells have demonstrated to killing by the chemotherapeutic agent cisplatin (25,31). While numerically modest, such changes in drug sensitivity are within the range of alterations that can directly affect clinical responsiveness of cancers.

Poor Repair of ICR191-Induced [+1] Frameshift Mutations in Mismatch Repair-Deficient Human Colon Cancer Cells

The vector, out-of-frame (+1), carrying a 10-bp G′C repeat sequence constituted to facilitate detection of [+1] frame-shifts, was used as a target for the mutational effects of ICR191. After direct exposure of this vector to varying concentrations of ICR191 between 1 and 10 μg/mL (2–22 μM), the drug-exposed vector was transfected into HCT116 and HCT116+C3 human colon cancer cells. Concentrations of ICR191 that cause no expression of mutations in the out-of-frame (+1) vector when transfected into repair-proficient HCT116+C3 cells induced substantial numbers of mutations in the out-of-frame (+1) vector when transfected into mismatch repair-deficient HCT116 cells (Fig. 3). Since ICR191 was reacted with vector DNA and not directly with HCT116 cells, the increased mutations demonstrated in HCT116 cells reflect an intrinsic vulnerability of these cells to ICR191-induced mutations and not an increase in the level of ICR191 drug uptake or accumulation. At ICR191
cells are specifically sensitive to ICR191—mutations were demonstrated in mismatch repair-deficient HCT116. Dose-dependent ICR191-induced frameshift mutations within the G repeat results reported in previous studies in bacteria. Sequence was altered to detect [−1] frameshift in the [−1] vector in which the G repeat was directly susceptible to a frameshift mutagen, HCT116 and HCT116+C3 cells were transiently transfected with the out-of-frame (+1) vector harboring the [+1] frameshift reporter; 24 hours later, the transfected cells were treated with ICR191. As shown in Fig. 4, mutations in the [+] frameshift—sensitive vector increased in a dose-dependent manner in both the mismatch repair-proficient and mismatch repair-deficient cell lines. However, at all doses of ICR191, the mismatch repair-defective HCT116 cell line demonstrated a fivefold to 10-fold greater induction of mutations than did its mismatch repair-proficient counterpart, the HCT116+C3 cells. Moreover, in HCT116 cells, 8 µg/mL ICR191 induced a 45-fold increase in vector mutation frequency above the spontaneous vector mutation frequency of 1.8 × 10⁻⁵. Thus, repair-deficient cells may carry a markedly elevated total mutational burden arising from both an increased basal mutation susceptibility of HCT116 cells to ICR191—Induced Mutations In Vivo

To confirm that repair-deficient living cells were directly susceptible to a frameshift mutagen, HCT116 and HCT116+C3 cells were transiently transfected with the out-of-frame (+1) vector harboring the [+1] frameshift reporter; 24 hours later, the transfected cells were treated with ICR191. As shown in Fig. 4, mutations in the [+1] frameshift—sensitive vector increased in a dose-dependent manner in both the mismatch repair-proficient and mismatch repair-deficient cell lines. However, at all doses of ICR191, the mismatch repair—defective HCT116 cell line demonstrated a fivefold to 10-fold greater induction of mutations than did its mismatch repair—proficient counterpart, the HCT116+C3 cells. Moreover, in HCT116 cells, 8 µg/mL ICR191 induced a 45-fold increase in vector mutation frequency above the spontaneous vector mutation frequency of 1.8 × 10⁻⁵. Thus, repair—deficient cells may carry a markedly elevated total mutational burden arising from both an increased basal mutation rate and an increased sensitivity of these cells to induced mutations generated by specific chemical or environmental agents.

Discussion

Our findings demonstrate a potentially important role for frameshift—inducing agents in mismatch repair—deficient cancers. The increased susceptibility of mismatch repair—deficient cells to induced frameshift mutations suggests that environmental agents may play an important role in promoting malignant progression of repair—deficient neoplasms and in determining the tissue specificity of these cancers. In addition, mismatch repair—deficient cells are also more susceptible to the cytotoxic action of a frameshift—inducing agent. This specific vulnerability may potentially be exploited for development of targeted therapeutic strategies. A number of mechanisms could underlie the increased sensitivity of HCT116 cells to ICR191—mediated cytotoxicity. The high mutation rates typical of mismatch repair—deficient cells may, by generating a high rate of deleterious mutations, impair the ability of these cells to survive. In this model, the increased sensitivity of HCT116 cells to killing by ICR191 directly arises from the increased number of ICR191—induced mutations accumulating in these cells (32). Alternatively, mismatch repair may play a direct role in the normal repair of lesions induced by intercalating agents such as ICR191, and recognition or abortive repair of these lesions by alternative repair pathways may provide signals that trigger an apoptotic or other toxic response. This model is consistent with observations that the mismatch repair enzymes are capable of directly recognizing and binding to some drug—DNA cross—links, such as those generated by cisplatin (33), and with our findings that mismatch repair seemingly is involved in repair of ICR191—induced mutations in eukaryotic cells.

Our observation that human cells deficient in mismatch repair are susceptible to induced mutations caused by an intercalating agent is consistent with findings that mismatch repair—deficient bacteria show an increased susceptibility to mutations induced by ICR191 (34), by the chemically related noncovalently binding acridine derivatives 9—aminoacridine and proflavin, and by iPr—OPC, an intercalating oxazolopyridocarbazole (35).
The mismatch repair system recognizes single-strand loops that arise by strand slippage during replication (36), and a mechanistic basis for involvement of mismatch repair in the prevention of ICR191-induced mutation is suggested by models in which aminoacidines stabilize stem loop structures (37–39) that give rise to strand slippage events that lead to frameshift mutations (40,41). Consistent with the previously demonstrated specificity of ICR191 to induce mutations in homopolymeric G: C repeat sequences, we did not observe a substantial increase in ICR191-induced mutation rates in studies using the pCAR reporter vector that bears a CA repeat (30) or employing a pCAR-derived reporter vector containing a poly A:T repeat (30).

Mismatch repair-defective colon cancers account for an estimated 12% of colon cancer cases annually (3). These cancers can arise from either inheritance of germ line defects in mismatch repair genes or somatic inactivation of mismatch repair genes (1–4) that most commonly is due to methylation and silencing of the hMLH1 gene (42–44). An unexplained conundrum has been that both inherited and sporadic repair-deficient colon cancers demonstrate a marked predilection for the proximal colon. We and other investigators (5–9,45) have previously demonstrated that mismatch repair deficiency elevates the spontaneous mutation rate of expressed homopolymeric sequences by several orders of magnitude. We now find in human colon cancer cells that mismatch repair deficiency also dramatically elevates the susceptibility of these sequences to induced frameshift mutations. In this context, our data are consistent with the hypothesis that susceptibility to an environmental agent may target repair-deficient cancers to the proximal colon over other sites, even in individuals who bear germ line mutations in mismatch repair genes throughout their bodies. Importantly, such exposures would represent a risk factor in these cancer-prone individuals, which could be reduced by intervention. Moreover, even a twofold increase in mutation rate due to environmental agents present in the colon could theoretically lead to a marked preference for tumor formation in the colon. Since five or more mutations are required to transform a normal epithelial cell (3), a twofold increase in the rate of each mutation would increase by 2^5, or 32-fold, the overall rate of cancer formation. Thus, while we have used a single high-dose exposure to a mutagen, ICR191, chronic low-level exposures could also substantially alter the risk of cancer arising in mutagen-exposed versus unexposed tissues. This effect would be particularly pronounced for mutagens that target key sequence motifs, such as the RII polyA tract.

In conclusion, this study shows that mismatch repair-defective cells exhibit increased sensitivity to both the toxicity and mutagenicity of specific DNA-binding agents. These observations imply that some environmental agents could have an impact on tumor development from mismatch repair-defective progenitor cells. This study also proves in principle that a mismatch repair defect can lead to selective sensitivity to the cytotoxic effects of certain agents. Therefore, drugs similar to the frameshift mutagen ICR191 may provide strategies for selective targeting of mismatch repair-defective tumors. We note with interest that amisacrine, an investigational chemotherapeutic agent used in human clinical trials, is an acridine derivative with frameshift-inducing activity (46). In addition, the bisimidazoacridones, which are acridine-related compounds, have been noted to be active against HCT116 cells in both cell line and xenograft models (47). Moreover, a number of new acridine-derived compounds are currently being evaluated for anticancer activity in human trials (48,49). Further elucidation of environmental agents that potentially promote malignant progression of repair-deficient colon cells and of therapeutic agents that exploit the vulnerability of these cells to killing by frameshift mutagens should have clear implications for the care of individuals at risk for or affected by this class of malignancies.

REFERENCES

(20) Goldmacher VS, Cuzick RA Jr, Thilly WG. Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methyltransferase and N-


(35) René B, Auclair C, Paolotti C. Frameshift lesions induced by oxazolopyridocarbazoles are recognized by the mismatch repair system in Escherichia coli. Mutat Res 1988;193:69–73.


Notes

S. D. Markowitz, W. D. Sedwick, and M. L. Veigl contributed equally to this work.

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