Cancer Chemother Pharmacol (2003) 52: 185–192
DOI 10.1007/s00280-003-0660-6

ORIGINAL ARTICLE

Patrizia Vernole · Rita Pepponi · Stefania D'Atri

Role of mismatch repair in the induction of chromosomal aberrations and sister chromatid exchanges in cells treated with different chemotherapeutic agents

Received: 28 November 2002 / Accepted: 28 April 2003 / Published online: 19 June 2003

Abstract *Purpose*: The mismatch repair (MMR) system plays a major role in mediating the cytotoxicity and clastogenicity of agents generating O^6-methylguanine in DNA. Loss of MMR has also been associated with tumor cell resistance to the cytotoxic effects of 6-thioguanine and cisplatin and with hypersensitivity to *N*-(2-chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea (CCNU). The aim of the present investigation was to elucidate the role played by the MMR system in the generation of chromosomal damage in cells exposed to 6-thioguanine, cisplatin or CCNU. *Methods*: The MMR-proficient cell lines TK6 and HCT116/3-6, and their MMR-deficient counterparts MT1 and HCT116, were treated with 6-thioguanine, cisplatin or CCNU, and analyzed for cell growth inhibition and chromosomal damage. As a control, similar experiments were performed with the methylating agent temozolomide. *Results*: Cytotoxicity, chromosomal aberrations and sister chromatid exchanges induced by 6-thioguanine and temozolomide were significantly reduced in the MMR-deficient cell lines with respect to their MMR-proficient counterparts. In contrast, although conferring some protection against cytotoxicity, the loss of MMR did not affect cytogenetic damage induced by cisplatin. CCNU produced comparable levels of cytotoxicity, chromosomal aberrations and sister chromatid exchanges in both MMR-proficient and MMR-deficient cell lines. *Conclusions*: The MMR system is involved in the generation of chromosomal damage in cells exposed to 6-thioguanine. The system does not play a relevant role in the generation of chromosomal damage in cells treated with CDDP and does not confer protection against the clastogenic effects of CCNU, at least in the cell lines investigated.

Keywords Mismatch repair · 6-Thioguanine · Cisplatin · *N*-(2-Chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea · Chromosomal damage

Abbreviations *BG* O^6-Benzylguanine · *CCNU* *N*-(2-Chloroethyl)-*N*′-cyclohexyl-*N*-nitrosourea · *CDDP* *cis*-Diamino-di-chloro-platinum (II) · *MGMT* O^6-Methylguanine-DNA methyltransferase · *MMR* Mismatch repair · *MTT* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · *sce* Sister chromatid exchanges · *6-TG* 6-Thioguanine · *TMZ (temozolomide)* 8-Carbamoyl-3-methyl-imidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one

Introduction

Many environmental carcinogens and various antineoplastic drugs produce genotoxic effects through methylation of nitrogen bases in DNA [6]. Among the most important sites of adduct formation are N^7-guanine, N^3-adenine and O^6-guanine. In particular, O^6-methylguanine (O^6-MeG) is considered one of the main biochemical modifications that can lead to cell death, chromosomal aberrations, sister chromatid exchanges (sce) and carcinogenesis [22]. The most efficient repair of methyl adducts at O^6-guanine is due to O^6-MeG-DNA methyltransferase (MGMT), which transfers the methyl group from O^6-MeG to an internal cysteine residue, thus restoring the integrity of the DNA [9, 32]. Indeed, cells with high MGMT levels are more resistant to the cytotoxic and clastogenic effects of DNA-methylating agents than MGMT-deficient cells [9, 32].

Cytotoxicity of O^6-MeG is mediated by the mismatch repair (MMR) system, a DNA repair pathway responsible

P. Vernole (✉)
Department of Public Health and Cell Biology,
University of Rome "Tor Vergata",
Via Montpellier 1, 00133 Rome, Italy
E-mail: vernole@uniroma2.it
Tel.: +39-6-72596060
Fax: +39-6-72596053

R. Pepponi · S. D'Atri
Laboratory of Pharmacology,
Istituto Dermopatico dell'Immacolata (IDI-IRCCS),
Via dei Monti di Creta 104, 00167 Rome, Italy

for the correction of biosynthetic errors occurring during DNA replication [21, 30]. In human cells at least five proteins play a key role in MMR: hMSH2, hMSH3, hMSH6, hMLH1, and hPMS2 [20, 21, 30]. The protein complex hMutSα, a heterodimer of hMSH2 and hMSH6, recognizes and binds to base-base mismatches, while the heterodimer hMutSβ, consisting of hMSH2 and hMSH3, is mainly involved in insertion/deletion loop recognition. After this first step, the heterodimer of hMLH1 and hPMS2, termed hMutLα, interacts with either hMutSα or hMutSβ and thereby initiates the repair process. In cells treated with methylating agents, during DNA duplication, unrepaired O^6-MeG in the template strand forms a mismatch with thymine, which serves as substrate for the MMR. As the repair of base mismatches is always directed to the newly synthesized strand, the result is abortive MMR, i.e. the repetitive misincorporation of thymine opposite to O^6-MeG. It has been proposed that these futile repair attempts of MMR may create DNA single-strand gaps in the first cell duplication, which turn into lethal double strand breaks in the second S-phase [23]. The clastogenic effects of methylating agents have also been related with the unsuccessful attempts of the MMR to repair O^6-MeG mispairs [16, 22, 24]. Indeed, in cells lacking MGMT activity, loss of MMR confers protection against cytotoxicity, chromosomal aberrations and sce induced by methylating agents [5, 16, 24].

A defective MMR has been also linked with tumor cell resistance to the cytotoxic effects of non-methylating chemotherapeutic agents, such as cis-diamine-di-chloro-platinum (II) (CDDP) and 6-thioguanine (6-TG) [14]. In contrast, it has been recently shown that the chloroethylating drug *N*-(2-chloroethyl)-*N′*-cyclohexyl-*N*-nitrosourea (CCNU) is preferentially cytotoxic in cell lines with MMR defects [3]. The relationship between the functional status of the MMR and cell susceptibility to chromosomal aberrations and sce induced by these drugs has not been extensively investigated. In the case of 6-TG, studies demonstrating that loss of MMR confers resistance against chromosomal damage induced by the drug have been performed only in the Chinese Hamster CHO cell line [4]. The protective role of MMR against CCNU clastogenicity has so far been evaluated only by a micronucleus assay in MMR-deficient clones of HeLa cells [15], whereas in the case of CDDP, there is only one report of increased sensitivity of MMR-deficient cells to sce induced by the drug [12].

The aim of the present investigation was to elucidate the role played by MMR in the generation of chromosomal damage in cells exposed to 6-TG, CDDP or CCNU. To this end, two matched pairs of human MMR-proficient and MMR-deficient cell lines were treated with these drugs and analyzed for cell growth inhibition, and induction of chromosomal aberrations and sce. As a control, the same cell lines were tested for sensitivity to the cytotoxic and clastogenic effects of the methylating agent temozolomide (TMZ).

Methods

Cell lines

The human B lymphoblastoid cell lines TK6 and MT1 were a generous gift from W.G. Thilly (Massachusetts Institute of Technology, Cambridge, Mass.). The TK6 line was originally isolated as thymidine kinase heterozygous by Skopek et al. [36]. The MT1 line was obtained from TK6 by treatment with IRC-191 followed by selection for MNNG resistance [17]. Both cell lines are MGMT-deficient [17]. TK6 cells are MMR-proficient, whereas MT1 cells are MMR-deficient [25], harboring different missense mutations in both alleles of the *hMSH6* locus [31]. The cell lines were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and maintained in RPMI-1640 (Hyclone Europe, Cramlington, UK) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone Laboratories, Logan, Utah), 2 m*M* L-glutamine, and antibiotics (GIBCO BRL, Life Technologies, Paisley, UK). The absence of MGMT activity in TK6 and MT1 cells was confirmed using an assay that measures the transfer of [^{3}H]-methyl groups from a DNA substrate to the MGMT protein [40].

The MMR-deficient human colorectal adenocarcinoma cell line HCT116 and its MMR-proficient subline HCT116/3-6 were kindly provided by G. Marra (Institute of Medical Radiobiology, Zurich, Switzerland). HCT116 cells have a hemizygous nonsense mutation in the *hMLH1* gene located on chromosome 3 [31]. The HCT116/3-6 clone was created by microcell chromosome transfer of a single normal human chromosome 3 into HCT116 cells [26]. MGMT activity was 222 ± 17 fmol/mg protein in HCT116 cells and 186 ± 23 fmol/mg protein in HCT116/3-6 cells. The cell lines were maintained in modified McCoy's 5A medium (GIBCO) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, and antibiotics. The chromosome-complemented cell line was grown in medium containing 400 μg/ml of G418 (GIBCO). Experiments were performed in medium without G418. The absence or presence of expression of hMLH1 protein in HCT116 and HCT116/3-6 cells was determined by Western blot analysis (data not shown).

Drugs and reagents

Schering-Plough Research Institute (Kenilworth, N.J.) and Bristol-Myers Squibb (Latina, Italy) kindly provided TMZ and CCNU, respectively. 6-TG, O^6-benzylguanine (BG), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, Mo.). CDDP (Prontoplatamine, 500 μg/ml in saline, pH 3–5) was purchased from Pharmacia (Milan, Italy).

TMZ and CCNU were always freshly prepared. TMZ was dissolved in RPMI-1640, sterilized by filtration through a 0.45-μm membrane filter, and then diluted in culture medium. CCNU was dissolved in ethanol and then diluted in culture medium. 6-TG was dissolved in 0.1 *M* NaOH, stored at −80°C and diluted in culture medium just prior to use. The final concentrations of NaOH or ethanol did not affect cell growth and did not induce sce or chromosomal aberrations (data not shown). MTT was dissolved at a concentration of 5 mg/ml in phosphate-buffered saline (PBS), and stored at 4°C.

Evaluation of cell chemosensitivity by the MTT assay

Exponentially growing TK6 and MT1 cells were suspended in culture medium at a concentration of 1×10^5 cells/ml and dispensed in 50-μl aliquots into flat-bottomed 96-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, N.J.). Graded amounts of the agents under investigation were then added to the wells in 50 μl culture medium and the cells were exposed for 1 h (CDDP and CCNU) or 72 h (6-TG and TMZ). In the first case, at the end of drug treatment, the plates were centrifuged and the drug-containing medium

removed. Culture medium (200 µl) was added to each well and the plates centrifuged again. The cell pellets were then suspended in 100 µl culture medium and the plates incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Four replicate wells were used for controls and each drug concentration.

The MTT assay was performed as previously described [18]. Briefly, at the end of the 72-h incubation period, 0.1 mg MTT (in 20 µl PBS) was added to each well and the cells were incubated at 37°C for 4 h. Cells were then lysed with a buffer (100 µl/well) containing 20% SDS and 50% *N*,*N*-dimethyl formamide, pH 4.7. After an overnight incubation, the absorbance was read at 595 nm using a 3550-UV microplate reader (Bio-Rad, Hercules, Calif.). Cell sensitivity to treatments was expressed in terms of IC_{50} (i.e. drug concentration capable of producing 50% inhibition of cell growth, calculated on the regression line in which absorbance values at 595 nm were plotted against the logarithm of drug concentration).

Evaluation of cell chemosensitivity by the clonogenic assay

HCT116 and HCT116/3-6 cells were seeded in triplicate into 100×20-mm dishes (Falcon) at 250–3000 cells/dish, and incubated overnight at 37°C. Graded concentrations of the chemicals under investigation were then added to the dishes and left in culture for 1 h (CDDP and CCNU) or for the entire period of the assay (i.e. 12 days) (TMZ and 6-TG). In the first case, after drug treatment, the dishes were washed with culture medium and new medium was added. Treatments with TMZ or CCNU were performed in the presence of BG, a specific inhibitor of MGMT [10]. In the case of TMZ treatment, 5 µ*M* BG was added to the dishes 2 h before the drug and on the 6th day of culture. In the case of CCNU, 5 µ*M* BG was added to the dishes 2 h before the drug, with medium replenishment after drug treatment, and on the 6th day of culture. Control groups were exposed to BG alone. MGMT activity of BG-treated cells was undetectable 2 h after the addition of the inhibitor and remained abrogated up to the end of the assay (data not shown).

At the end of the assay, colonies were fixed and stained with crystal violet for visualization. Only those colonies containing 50 or more cells were scored as survival colonies. The relative surviving fraction for each drug-treated group is expressed as the ratio of the plating efficiency in treated groups to that observed in the controls. IC_{50} values were calculated on the regression line in which relative surviving fractions were plotted against the logarithm of drug concentration.

Cytogenetic analysis

Exponentially growing TK6 and MT1 cells were suspended in culture medium at a concentration of 2×10^5 cells/ml and dispensed in 2.5-ml aliquots into flat-bottomed six-well plates (Falcon). Graded amounts of the agents under investigation were then added to the wells in 2.5 ml culture medium, and the plates incubated at 37°C in a humidified atmosphere containing 5% CO_2 for either 1 h (CDDP and CCNU) or 48 h (TMZ and 6-TG). In the first case, after drug exposure, cells were washed and maintained in culture in drug-free medium for an additional 48 h.

HCT116 and HCT116/3-6 cells were plated and treated as described for TK6 and MT1 cells, but allowed to adhere to the wells for 18 h before treatment. The sensitivity of HCT116 and HCT116/3-6 cells to TMZ and CCNU was evaluated in the presence of 5 µ*M* BG, which was added to the wells 2 h before the chemicals under study, and maintained in culture during the assay. Control groups were exposed to BG alone.

To obtain differential staining of sister chromatids for sce evaluation, 2 µg/ml 5-bromodeoxyuridine (BrdU) was added to the cultures, immediately after TMZ and 6-TG, or with the fresh medium after the 1-h treatment with CDDP or CCNU. At the beginning of the study, that BrdU did not interfere with chromosomal damage induced by the drugs under investigation was verified (data not shown).

To analyze chromosomal aberrations, untreated or drug-treated cells were incubated with 2 µ*M* colchicine for the last 2 h of culture, harvested, incubated for 15 min in a hypotonic solution (0.075 µ*M* KCl), and fixed by 3:1 methanol/acetic acid, before dropping them onto clean wet slides. Slides were then stained with a 5% Giemsa solution in Sorensen buffer (pH 6.8). To obtain differential staining of sister chromatids, slides were treated according to the method described by Perry and Wolff [33]. For each treatment, 100 metaphases were examined to evaluate chromosomal damage and at least 30 M2 metaphases to calculate sce.

Statistical analysis

Differences between the IC_{50} values for the MMR-proficient cell lines and those for their MMR-deficient counterparts were subjected to statistical analysis using Student's paired *t*-test. Mean values and standard errors of chromosomal aberrations and aberrant cells were evaluated at least from three different experiments for each drug concentration. Statistical analysis of chromosomal damage was performed using the normal standardized deviate, calculated according to the formula: $u = (x1 - x2)/\sqrt{(x1 + x2)}$, where x1 and x2 are the mean values, expressed as absolute numbers, of chromosomal aberrations in 100 metaphases, or of cells with aberrations out of 100 cells examined [39]. In this way it was possible to compare the damage induced by each treatment with that found in untreated cultures of the same cell line and also the damage induced by the same treatment in the different cell lines.

Statistical evaluation of sce was performed using the Mann-Whitney *U*-test.

Results

Cytotoxicity assays

MMR-deficient MT1 cells have been previously found to be more resistant than the MMR-proficient parental TK6 cells to the cytotoxic effects of TMZ [8]. The results illustrated in Table 1 confirm the previous finding and show that MT1 cells were also more resistant to CDDP and 6-TG than TK6 cells. In contrast, there was no evidence of different susceptibility of the two lines to CCNU.

The data illustrated in Table 2 show that HCT116 cells were more resistant than HCT116/3-6 cells to TMZ,

Table 1 Chemosensitivity of TK6 and MT1 cells determined by the MTT assay. Cells were exposed to graded concentrations of drug for 72 h (TMZ and 6-TG) or 1 h (CDDP and CCNU). Each value is the mean ± SE of at least three independent experiments performed with quadruplicate cultures

Drug	IC_{5O} (µ*M*)[a]		*P* value[b]
	TK6	MT1	
TMZ	6.27 ± 0.48	374 ± 63	< 0.01
6-TG	0.92 ± 0.05	1.70 ± 0.11	< 0.01
CDDP	8.67 ± 1.32	15.74 ± 1.17	< 0.01
CCNU	16.75 ± 2.51	17.92 ± 1.73	NS

[a] Drug concentration required to inhibit cell growth by 50%
[b] IC_{50} value for MT1 vs IC_{50} value for TK6; Student's paired *t*-test (*NS* not significant)

Table 2 Chemosensitivity of HCT116 and HCT116/3-6 cells determined by the clonogenic assay. The drug was added to the cells 18 h after seeding and left in culture for the entire period of the assay (i.e. 12 days) (TMZ and 6-TG) or for 1 h (CDDP and CCNU). In the latter case, at the end of treatment the cells were washed and maintained in drug-free medium for 12 days. The effects of TMZ and CCNU were evaluated in the presence of the MGMT inhibitor BG, added to the cultures as described in Materials and methods. Each value is the mean ± SE of three independent experiments performed with triplicate cultures

Drug	IC_{50} (μM)[a]		*P* value[b]
	HCT116/3-6	HCT116	
TMZ + BG	13.66 ± 0.33	233 ± 16	< 0.01
6-TG	0.98 ± 0.16	2.49 ± 0.34	< 0.01
CDDP	3.31 ± 0.48	10 ± 0.70	< 0.05
CCNU + BG	8.61 ± 0.19	8.91 ± 0.32	NS

[a] Drug concentration required to inhibit colony formation by 50%
[b] IC_{50} value for HCT116 vs IC_{50} value for HCT116/3-6; Student's paired *t*-test (*NS* not significant)

6-TG and CDDP, as previously described also by others [1, 19, 27]. On the other hand, as observed for TK6 and MT1 cells, the two lines showed comparable sensitivity to CCNU.

Cytogenetic analysis

To evaluate the sensitivity of TK6 and MT1 cells to chromosomal damage induced by 6-TG, CDDP and CCNU, the cells were initially exposed to drug concentrations in the range of the IC_{50} values observed for the two cell lines in the MTT assay (i.e. 10 and 20 μM for CDDP and 10 and 20 μM for CCNU). In the case of CDDP, only a few metaphases were observed at 10 μM and no metaphases were detected in cells exposed to the higher drug concentration. Similarly, when the cell lines were treated with 20 μM CCNU, no metaphases were observed. Therefore, both TK6 and MT1 cells were treated with 2.5 and 5 μM CDDP, in addition to 10 μM, and with 5 and 10 μM CCNU. For TMZ, since the IC_{50} values for the two cell lines differed 60-fold, we tested the intermediate concentrations of 100 and 200 μM.

Figure 1 illustrates drug-induced chromosomal aberrations and aberrant cells relative to TK6 and MT1 lines. Chromosomal damage was significantly higher ($P < 0.01$) in TK6 than in MT1 cells following treatment with TMZ (Fig. 1a) and with 6-TG (Fig. 1b), while CDDP and CCNU were equally effective in both cell lines (Fig. 1c, d). The former drug, produced equal chromosomal damage in TK6 and MT1 cells also at 10 μM. Indeed, the number of chromosomal aberrations were 59 ± 12 per 100 cells in TK6 cells and 57 ± 10 per 100 cells in MT1 cells.

The results obtained with HCT116 and HCT116/3-6 cells are illustrated in Fig. 2. In this case, the concentrations tested for 6-TG and CDDP were chosen around the IC_{50} values observed for the two lines, since the numbers of analyzable metaphases were found to be sufficient. In the case of CCNU, we used 5 and 20 μM because the IC_{50} values were very similar in the two lines. For TMZ, since the IC_{50} values were very different between the two lines, two intermediate concentrations (100 and 200 μM) were chosen. The MMR-deficient cells were significantly more resistant than the MMR-proficient cells to chromosomal damage induced by TMZ (Fig. 2a) and 6-TG (Fig. 2b). In contrast, both cell lines were equally susceptible to the clastogenic effects of CDDP (Fig. 2c) and CCNU (Fig. 2d). The types of chromosomal aberrations were in a similar proportion

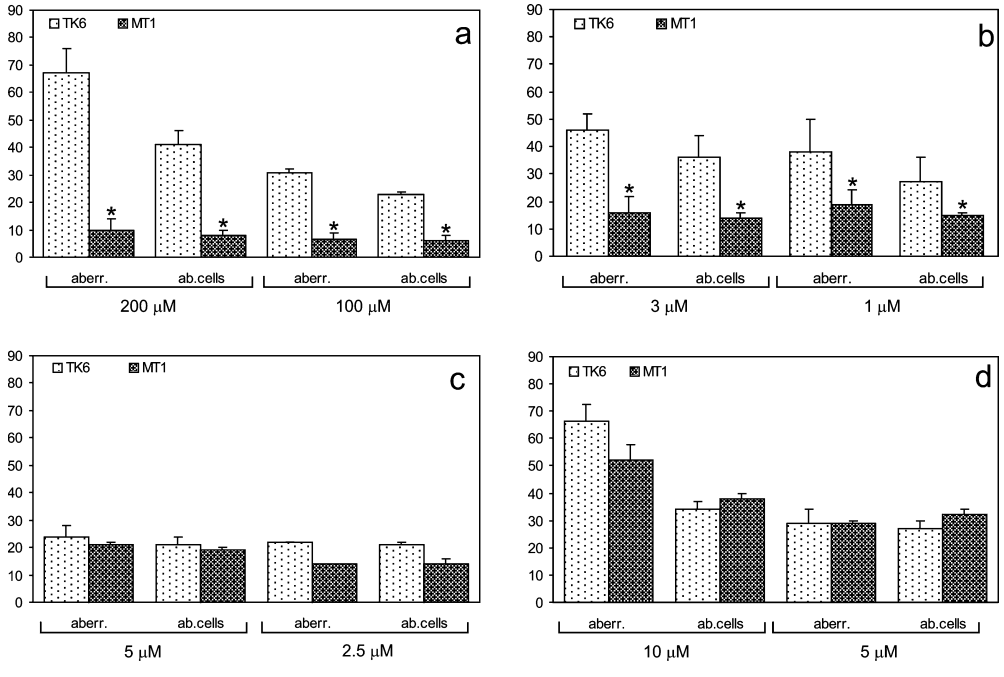

Fig. 1a–d Drug-induced chromosomal damage in TK6 and MTI cells. Cells were exposed to the indicated concentrations of TMZ (**a**), 6-TG (**b**), CDDP (**c**) or CCNU (**d**) for 1 h (CDDP, CCNU) or 48 h (TMZ, 6-TG), and then analyzed for chromosomal damage. The data are expressed in terms of the number of aberrations per 100 cells (*aberr.*) and the number of aberrant cells per 100 cells examined (*ab.cells*). Each value is the mean ± SE of at least three independent experiments. In each experiment the number of chromosomal aberrations found in the untreated cultures (two to six aberrations per 100 cells) was subtracted from that observed in the drug-treated cultures. *$P < 0.01$

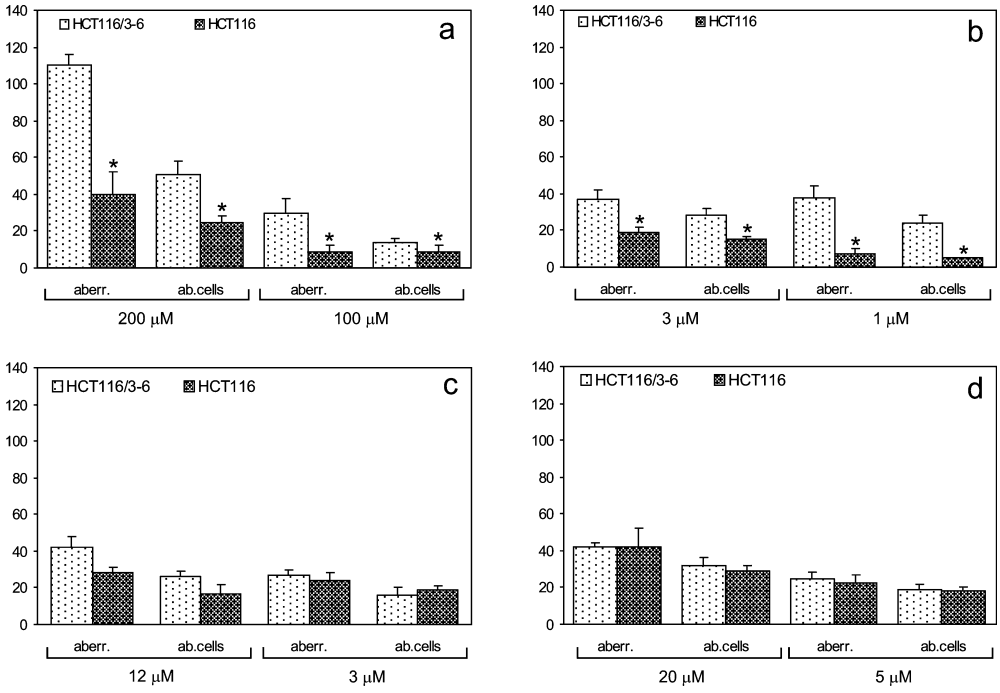

Fig. 2a–d Drug-induced chromosomal damage in HCT116 and HCT116/3-6 cells. Cells were exposed to the indicated concentrations of TMZ (**a**), 6-TG (**b**), CDDP (**c**) or CCNU (**d**) for 1 h (CDDP, CCNU) or 48 h (TMZ, 6-TG), and then analyzed for chromosomal damage. The data are expressed in terms of the number of aberrations per 100 cells (*aberr.*) and the number of aberrant cells per 100 cells examined (*ab.cells*). Each value is the mean ± SE of at least three independent experiments. In each experiment the number of chromosomal aberrations found in the untreated cultures (zero to eight aberrations per 100 cells) was subtracted from that observed in the drug-treated cultures. $*P < 0.01$

after all treatments in all cell lines, with a majority of chromatid breaks.

To further confirm the equal sensitivity of HCT116 and HCT116/3-6 cells to chromosomal damage induced by CDDP and CCNU, both cells lines were treated with 20 μ*M* CDDP or with 50 and 100 μ*M* CCNU. In the case of CDDP, the number of breaks per 100 cells was 52 ± 7 in HCT116 cells and 64 ± 10 in HCT116/3-6 cells. In the case of 50 μ*M* CCNU, the number of breaks per 100 cells was 159 ± 20 in HCT116 cells and 139 ± 15 in HCT116/3-6 cells. Finally, when the cells were treated with 100 μ*M* CCNU, the number of breaks per 100 cells was 270 ± 51 in HCT116 cells and 232 ± 40 in HCT116/3-6 cells. Thus, the two cell lines were also not significantly different in their susceptibility to the clastogenic effects of CDDP and CCNU when exposed to high concentrations of these drugs.

Sce in untreated cultures were similar in all cell lines (2.92–3.71 sce/cell). Figure 3a shows that treatments with 100 μ*M* TMZ or 1 μ*M* 6-TG induced a higher number of sce per cell in TK6 than in MT1 cells, whereas 5 μ*M* CDDP and 5 μ*M* CCNU were equally effective in the two cell lines. Similar numbers of sce were also detected in TK6 and MT1 cells exposed to 10 μ*M* CDDP (i.e. 15 ± 2.1 in TK6 cells and 12.5 ± 1.5 in MT1 cells). Comparable results were obtained with HCT116 and HCT116/3-6 cells, as the latter cell line showed a higher number of sce than the HCT116 line only when exposed to TMZ or 6-TG (Fig. 3b).

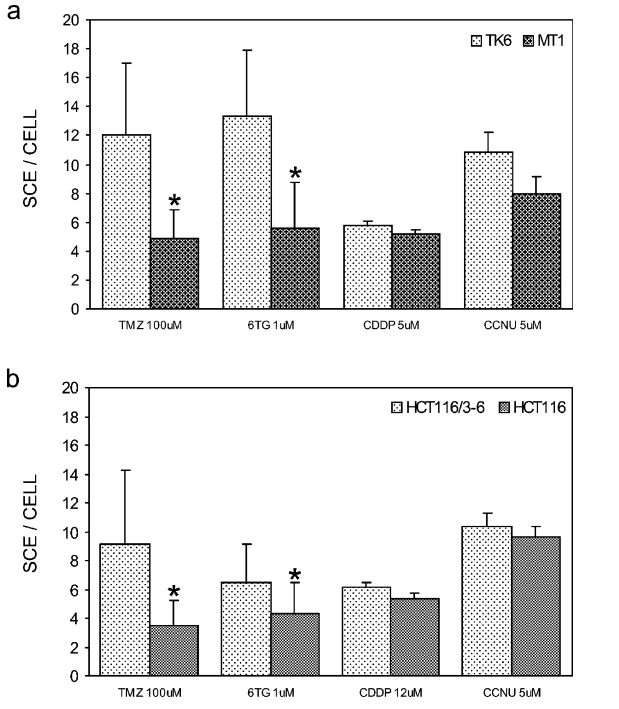

Fig. 3a, b Drug-induced sce in TK6 and MT1 (**a**) and in HCT116/3-6 and HCT116 (**b**) cell lines. Cells were treated with the indicated concentrations of TMZ or 6-TG (for 48 h), or CDDP or CCNU (for 1 h) and then the numbers of sce per cell were determined. Each value is the mean ± SE of at least three independent experiments. $*P < 0.01$

Discussion

The aim of the present study was to obtain further insights into the possible involvement of the MMR system in the generation of chromosomal breakage and sce in cells exposed to 6-TG, CDDP and CCNU. We used two matched pairs of human MMR-proficient and MMR-deficient cell lines not previously analyzed for differential sensitivity to the clastogenic activity of these drugs. As a

control, growth inhibition and chromosomal damage induced by the methylating agent TMZ were evaluated in the same cell lines.

As expected, the sensitivity to cytotoxicity, chromosomal damage and sce induced by TMZ was significantly reduced in both MT1 and HCT116 cells, with respect to their MMR-proficient counterparts. The results of our study are also consistent with the involvement of the MMR system in cytotoxicity and chromosomal damage produced by 6-TG in human cell lines. An increased resistance to the cytotoxic effects of 6-TG has been demonstrated in cell lines with a defective MMR. This base analogue is incorporated into DNA and successively methylated at the S^6 position by endogenous S-adenosylmethionine. DNA replication causes S^6-methyl-TG/T mispairs, which are assumed, like O^6-MeG/T mispairs, to be recognized by the MMR system and thus subjected to "futile" processing, which eventually results in cell death [4, 37]. Armstrong and Galloway [4] have shown that MMR-deficient hamster cells resistant to killing by 6-TG also show fewer chromosomal aberrations than cells with normal MMR. Our data confirm, for the first time, this finding in two couples of human MMR-proficient and MMR-deficient cell lines. Both MT1 and HCT116 cells were indeed more resistant than their MMR-proficient counterparts to cell growth inhibition, chromosomal aberrations and sce induced by 6-TG.

CDDP is a bifunctional agent that can generate crosslinks between DNA and proteins and inter- and intrastrand crosslinks in DNA [34]. Recently, MMR defects have been linked with resistance to this agent [13]. The hMutSα complex and hMSH2 alone have been reported to bind CDDP adducts in DNA [11, 29]. Moreover, an increased resistance to the drug has been documented in several cell lines harboring mutations in *hMSH2*, *hMSH6*, *hMLH1* or *hPSM2* [13, 38]. The differences in sensitivity between MMR-proficient and MMR-deficient cell lines are generally not greater than twofold, and it has been suggested that the MMR system may promote cell death by preventing recombinational repair of double-strand breaks that arise as secondary lesions of CDDP-induced DNA modifications [23]. On the other hand, no data are presently available in the literature on differential susceptibility of MMR-proficient and MMR-deficient cells to the clastogenic effects of CDDP. In this study, we confirmed that loss of MMR confers a moderate degree of protection against the cytotoxic effects of CDDP. Indeed, both MT1 and HCT116 cell lines were more resistant to killing by the drug with respect to their MMR-proficient counterpart. On the other hand, the finding that comparable numbers of chromosomal aberrations and sce were produced by the drug in the MMR-proficient and MMR-deficient cell lines suggests that this DNA repair pathway is not particularly involved in the generation of chromosomal damage after CDDP treatment.

A study by Durant et al. [12] has shown that CDDP induces a higher level of sce in the MMR-deficient cell line A2780/cp70/ch3 with respect to the MMR-proficient parental A2780 cell line. According to the authors, the lower sensitivity of MMR-proficient cells to CDDP-induced sce is dependent on MMR-mediated inhibition of recombinational bypass of DNA adducts produced by CDDP. In the MMR-deficient cell lines used in our study, we failed to detect increased sensitivity to CDDP-induced sce. It is possible that in these cell lines, and in their MMR-proficient counterparts, recombinational bypass of DNA adducts produced by CDDP does not occur to a significant extent, and therefore sce formation is not affected by the MMR status.

CCNU is a chloroethylating drug that produces different DNA adducts, including O^6-(2-chloroethyl)guanine, which is corrected by MGMT [28, 35]. This DNA adduct rearranges into O^6,N^1-ethanoguanine which, if not removed by 3-methyladenine DNA glycosylase, reacts with the N^3 of cytosine in the complementary strand to form an interstrand DNA crosslink [2]. This is considered one of the major drug-DNA interactions responsible for cell death, sce and chromosomal aberrations, since the levels of MGMT are usually inversely correlated with cell sensitivity to the alkylating nitrosourea [7]. Recently, it has been shown in some cell lines that loss of MMR is associated with hypersensitivity to killing by CCNU [3, 15]. It has also been demonstrated that MMR-deficient clones of HeLa cells are more susceptible to micronucleus induction by CCNU than the MMR-proficient parental cells [15]. In this regard, it has been hypothesized that the MMR system participates in the removal of the interstrand crosslinks generated by unrepaired O^6-(2-chloroethyl)guanine, thus providing protection against the cytotoxic and clastogenic effects of the drug [3, 15].

In our study, MMR-proficient and MMR-deficient cells were equally sensitive to cell growth inhibition and chromosomal damage induced by CCNU. These results are not in agreement with those described by others in different couples of MMR-proficient and MMR-deficient cells [3, 15]. It is possible that in the cell lines used in the present investigation, other repair pathways play a more relevant role in protection against CCNU toxicity than the MMR system. Indeed, besides O^6-(2-chloroethyl)guanine, other DNA monoadducts are produced by CCNU, which can contribute to cytotoxicity and chromosomal damage. These adducts can be removed by base and nucleotide excision repair, and therefore the efficiency of these DNA repair pathways may also affect cell sensitivity to the drug. Moreover, O^6,N^1-ethanoguanine itself can be removed by 3-methyladenine DNA glycosylase [2]. Thus the activity of this enzyme might determine the amount of interstrand crosslinks generated by unrepaired O^6-(2-chloroethyl)guanine with respect to other products of CCNU-DNA interaction and the actual relative contribution of the MMR to protection against the alkylating agent.

In conclusion, the results of the present study indicate that: (a) the MMR system plays a major role in the generation of cell death signals, chromosomal

aberrations and sce in human cells treated with 6-TG; (b) the MMR system does not play a relevant role in the induction of clastogenic damage and sce in cells exposed to CDDP (however, loss of this DNA repair pathway can provide a low level of resistance to the drug); and (c) MMR did not confer protection against the clastogenic action of CCNU in our system. Probably different genetic backgrounds in the target cells contribute to determine whether loss of MMR results in increased sensitivity to chromosomal aberrations and cytotoxicity produced by this drug.

Acknowledgements The authors thank Prof. E. Bonmassar for his valuable advice, and Mr. G. Bonelli for his skilful technical assistance. The research was partly supported by grants from M.U.R.S.T to P.V., and partly by the Italian Ministry of Health.

References

1. Aebi S, Fink D, Gordon R, Kim HK, Zheng H, Fink JL, Howell SB (1997) Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. Clin Cancer Res 3:1763
2. Allan MJ, Engelward BP, Dreslin AJ, Wyatt MD, Tomasz M, Samson LD (1998) Mammalian 3-methyladenine DNA glycosylase protects against the toxicity and clastogenicity of certain chemotherapeutic DNA cross-linking agents. Cancer Res 58:3965
3. Aquilina G, Ceccotti S, Martinelli S, Hampson R, Bignami M (1998) N-(2-chloroethyl)-N′-cyclohexyl-N-nitrosurea sensitivity in mismatch repair-defective human cells. Cancer Res 58:135
4. Armstrong MJ, Galloway SM (1997) Mismatch repair provokes chromosome aberrations in hamster cells treated with methylating agents or 6-thioguanine, but not with ethylating agents. Mutat Res 373:167
5. Bean CL, Bradt CI, Hill R, Johnson TE, Stallworth MV, Galloway SM (1994) Chromosome abs: persistence of alkylation damage and modulation of O6-alkylguanine-DNA alkyltransferase. Mutat Res 307:67
6. Beranek DT (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 231:11
7. Bodell WJ, Aida T, Berger MS, Rosenblum ML (1986) Increased repair of 1 O^6-alkylguanine DNA adducts in glioma-derived human cells resistant to the cytotoxic and cytogenetic effects of 1,3-bis(2-chloroethyl)-1-nitrosurea. Carcinogenesis 7:879
8. D'Atri S, Tentori L, Lacal PM, Graziani G, Pagani E, Benincasa E, Zambruno G, Bonmassar E, Jiricny J (1998) Involvement of the mismatch repair system in temozolomide-induced apoptosis. Mol Pharmacol 54:334
9. D'Incalci M, Citti L, Taverna P, Catapano CV (1988) Importance of DNA repair enzyme O^6-alkyltransferase (AT) in cancer chemotherapy. Cancer Treat Rev 15:279
10. Dolan EM, Moshel RC, Pegg AE (1990) Depletion of O6-alkylguanine-DNA alkyltransferase activity by O6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. Proc Natl Acad Sci U S A 87:5368
11. Duckett DR, Drummond JT, Murchie AI, Reardon JT, Sancar A, Lilley DM, Modrich P (1996) Human MutSα recognizes damaged DNA base pairs containing O-6-methylguanine O-4-methylthymine, or the cisplatin-d (GpG) adduct. Proc Natl Acad Sci U S A 93:6443
12. Durant ST, Morris MM, Illand M, McKay HJ, McKormick C, Hirst GL, Borts RH, Brown R (1999) Dependence on RAD52 and RAD1 for anticancer drug resistance mediated by inactivation of mismatch repair genes. Curr Biol 9:51
13. Fink D, Nebel S, Aebi S, Zheng H, Cenni B, Nehmè A, Christen RD, Howell SB (1996) The role of DNA mismatch repair in platinum drug resistance. Cancer Res 56:4881
14. Fink D, Aebi S, Howell SB (1998) The role of DNA mismatch repair in drug resistance. Clin Cancer Res 4:1
15. Fiumicino S, Martinelli S, Colussi C, Aquilina G, Leonetti C, Crescenzi M, Bignami M (2000) Sensitivity to DNA cross-linking chemotherapeutic agents in mismatch repair-defective cells in vitro and in xenografts. Int J Cancer 85:590
16. Galloway SM, Greenwood SK, Hill R, Bradt CI, Bean CL (1995) A role for mismatch repair in production of chromosome aberrations by methylating agents in human cells. Mutat Res 346:231
17. Goldmacher VS, Cuzick RA, Thilly WG (1986) Isolation and partial characterization of human cell mutants differing in sensitivity to killing and mutation by methylnitrosurea and N-methyl-N′-nitro-N-nitrosoguanidine. J Biol Chem 261:12462
18. Hansen MB, Nielsen SE, Berg K (1989) Reexamination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203
19. Hawn MT, Umar A, Carethers JM, Marra G, Kunkel TA, Boland CR, Koi M (1995) Evidence for a connection between the mismatch repair system and the G2 cell cycle checkpoint. Cancer Res 55:3721
20. Jiricny J (1998) Eukaryotic mismatch repair: an update. Mutat Res 409:107
21. Jiricny J, Nystrom-Lahti M (2000) Mismatch repair defects in cancer. Curr Opin Genet Dev 10:157
22. Kaina B, Ziouta A, Ochs K, Coquerelle T (1997) Chromosomal instability, reproductive cell death and apoptosis induced by O6-methyl-guanine in Mex−, Mex+ and methylation-tolerant mismatch repair compromised cells: facts and models. Mutat Res 381:227
23. Karran P (2001) Mechanisms of tolerance to DNA damaging therapeutic drugs. Carcinogenesis 22:1931
24. Karran P, Bignami M (1992) Self-destruction and tolerance in resistance of mammalian cells to alkylation damage. Nucleic Acids Res 20:2933
25. Kat A, Thilly WG, Fang WH, Longley MJ, Li GM, Modrich P (1993) An alkylation-tolerant mutator human cell line is deficient in strand-specific mismatch repair. Proc Natl Acad Sci U S A 90:6424
26. Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N′-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res 54:4308
27. Liu L, Markowitz S, Gerson SL (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis (2-chloroethyl)nitrosourea. Cancer Res 56:5375
28. Ludlum DB (1990) DNA alkylation by haloethylnitrosureas: nature of modifications produced and their enzymatic repair or removal. Mutat Res 233:117
29. Mello JA, Acharya S, Fishel R, Essingmann JM (1996) The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug cisplatin. Chem Biol 3:579
30. Modrich P (1997) Strand-specific mismatch repair in mammalian cells. J Biol Chem 272:24727
31. Papadopoulos N, Nicoladesi NC, Wei YF, Ruben SM, Carte KC, Rosen CA, Haseltine WA, Fleishmann RD, Fraser CM, Adams MD, Venter JC, Hamilton SR, Petersen GM, Watson P, Lynch HT, Peltomaki P, Mecklin JP, de la Chapelle A, Kinzler KW, Vogelstein B (1994) Mutation of a mutL homolog in hereditary colon cancer. Science 263:1625
32. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51:167
33. Perry P, Wolff S (1974) New Giemsa method for the differential staining of sister chromatids. Nature 251:156

34. Roberts JJ, Friedlos F (1987) Quantitative estimation of cis-platinum induced DNA interstrand cross-links and their repair in mammalian cells: relationship to toxicity. Pharmacol Ther 34:215
35. Robins P, Harris AL, Lindahl T (1983) Cross-linking of DNA induced by chloroethylnitrosurea is prevented by O^6-methylguanine-DNA methyltransferase. Nucleic Acids Res 22:7743
36. Skopek TR, Liber HL, Penman BW, Thilly WG (1978) Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: possibility for a rapid human cell mutation assay. Biochem Biophys Res Commun 84:411
37. Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, Edwards M, Mace R (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. Science 273:1109
38. Vaisman A, Varchenko M, Umar A, Kunkel TA, Risinger JI, Barrett JC, Hamilton TC, Chaney SG (1998) The role of hMLH1, hMSH3, and hMSH6 defects in cisplatin and oxaliplatin resistance: correlation with replicative bypass of platinum-DNA adducts. Cancer Res 58:3579
39. Vernole P, Tedeschi B, Caporossi D, Maccarrone M, Melino G, Annicchiarico-Petruzzelli M (1998) Induction of apoptosis by bleomycin in resting and cycling human lymphocytes. Mutagenesis 13:209
40. Watson AJ, Margison GP (1999) O^6-Alkylguanine-DNA alkyltransferase assays. In: Brown R, Boeger-Brown U (eds) Cytotoxic drug resistance mechanisms. Humana Press, Totowa, p 167