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hMSH3 overexpression and cellular response to cytotoxic anticancer agents

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Mutations or transcriptional silencing of mismatch repair genes have been linked with tumour cell resistance to *O*⁶-guanine methylating agents, 6-thioguanine, cisplatin, doxorubicin and etoposide. Recently, it has been demonstrated that overexpression of the MSH3 protein is associated with depletion of the mismatch binding factor MutSα, and then with a marked reduction in the efficiency of base/base mismatch repair. In the present study we evaluated sensitivity of the HL-60 cell line and its methotrexate-resistant subline HL-60R, which overexpresses the *hMSH3* gene, to a panel of chemotherapeutic agents. Cell growth inhibition induced by temozolomide, 6-thioguanine and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was significantly lower in the *hMSH3*-overexpressing HL-60R cell line as compared with the HL-60 parental line. Moreover, HL-60R cells were more resistant than HL-60 cells to chromosome aberrations induced by either *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine or temozolomide, and to apoptosis triggered by the latter drug. Both cell lines were equally susceptible to growth inhibition induced by cisplatin, etoposide or doxorubicin. In addition, HL-60 and HL-60R cells showed comparable sensitivity to the clastogenic and apoptotic effects of cisplatin and etoposide. These results further confirm that loss of base/base mismatch repair is the most important molecular mechanism involved in cell resistance to *O*⁶-guanine methylating agents and 6-thioguanine. However, the status of the mismatch repair system could still influence tumour cell sensitivity to cisplatin, etoposide and doxorubicin, depending on the specific component of the system that is lost, and on the genetic background of the cell.

Abbreviations: BG, O^6-benzylguanine; BSA, bovine serum albumin; CM, complete medium; DHFR, dihydrofolate reductase; IDLs, insertion/deletion loops; MMR, mismatch repair; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX, methotrexate; O^6-G, O^6-guanine; OGAT, O^6-alkylguanine-DNA alkyltransferase; O^6-MeG, O^6-methylguanine; PBS, phosphate buffered saline; PI, propidium iodide; 6-TG, 6-thioguanine; TMZ, temozolomide, 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one.

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Introduction

The mismatch repair (MMR) system is a DNA repair pathway dedicated to the correction of replication errors that escape processing by the proofreading activity of the replicative DNA polymerase (for reviews, see refs 1 and 2). Mutations in mismatch repair genes confer genetic instability and have been implicated in hereditary non-polyposis colon cancer (1,2).

Biochemical and genetic studies in human cells have defined at least five genes whose products play key roles in mismatch repair: *hMSH2*, *hMSH3*, *hMSH6*, *hMLH1* and *hPMS2* (1,2). The protein complex hMutSα, a heterodimer of hMSH2 and hMSH6, or hMutSβ, consisting of hMSH2 and hMSH3, initially recognize and bind mismatched DNA. After this step, a heterodimer of hMLH1 and PMS2, termed hMutLα, interacts with the DNA-bound hMutSα or hMutSβ to initiate the repair process (1,2).

hMutSα and hMutSβ possess distinct substrate specificities. The hMutSα complex binds to base/base mismatches and one, two and three nucleotide insertion/deletion loops (IDLs), while the hMutSβ heterodimer displays little or no affinity for base/base mismatches, but binds to multiple base IDL-type misalignments with high efficiency (1–3).

Although the MMR system has evolved for the correction of replication errors, it is also implicated in the recognition of other types of DNA damage and in the triggering of events leading to cell death. Indeed, in the past few years, deficiency in MMR was shown to be linked with tumour cell resistance to a number of chemotherapeutic agents (for review, see ref. 4). Inactivation of either *hMSH2* or *hMLH1* has been associated with high levels of resistance to 6-thioguanine (6-TG) (4,5) and O^6-guanine (O^6-G)-methylating agents, such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (4) or 8-carbamoyl-3-methyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one (temozolomide, TMZ) (6,7), as well as with a moderate degree of resistance to cisplatin (4,8), carboplatin (4,9) and etoposide (4,9,10). Loss of *hMLH1* has been shown to confer resistance to doxorubicin (4,9). Mutations in *hMSH6* have been reported to decrease cell sensitivity to O^6-G-methylating agents (4,11,12), 6-TG (1), cisplatin (8), but not to etoposide (11,13). Little is known about the effect of mutations in *hMSH3* and *hPMS2* on drug sensitivity. So far, absence of *hPMS2* expression has been associated with resistance to MNNG (4), cisplatin and carboplatin (14), whereas no changes in sensitivity to cisplatin (8) or MNNG (15) have been found in cells harbouring mutations in *hMSH3*.

Recently it has been demonstrated that mismatch repair deficiency can arise as a result of imbalance in the relative amounts of hMSH3 and hMSH6 proteins (16,17). The *hMSH3* gene is divergently transcribed from the dihydrofolate reductase (*DHFR*) promoter (18). Cells resistant to methotrexate (MTX) as a result of amplification of the *DHFR* locus, also overexpress the hMSH3 protein, which then displaces hMSH6 from its complex with hMSH2. Cells thus became defective in the repair of base/base mismatches and display a mutator phenotype (16,17). However, they retain the ability to correct IDLs.

Methotrexate is widely used for the treatment of human malignancies (19). It is also used in the treatment of some autoimmune diseases and for the prevention of graft-versus-host diseases in transplant patients (19). Resistance to MTX caused by amplification of the *DHFR* gene is frequently observed in tumour cells (20). It appears, therefore, of clinical interest to establish whether the associated co-amplification of the *hMSH3* locus confers cross-resistance to drugs unrelated to MTX, but whose cytotoxic activity could be modulated by the MMR status of the cell.

The chemoresistance pattern of human tumour cells overexpressing the *hMSH3* gene has not been investigated so far. In this study we evaluated the ability of several chemotherapeutic agents to bring about growth inhibition, apoptosis and chromosome aberrations in the MMR-proficient cell line HL-60 and in its MTX-resistant subline HL-60R, which overexpresses the *hMSH3* gene and is deficient in the repair of base/base mismatches (16,17).

Materials and methods

Cell lines

Human promyelocytic leukemia cell line HL-60 and its MTX-resistant subline HL-60R were a generous gift of T.Shimada (Nippon Medical School, Tokyo). The *DHFR/MSH3* locus of HL-60R cells is amplified ~200-fold (18) and overproduces the hMSH3 protein (16,17). As a consequence, the cell line does not express the hMutSα heterodimer and is deficient in the repair of base/base mismatches (16,17). O^6-Alkylguanine-DNA alkyltransferase (OGAT) levels were determined in both lines by measuring the transfer of [^{3}H]methyl groups from a DNA substrate to the OGAT protein, as previously described (21). OGAT activity was 973 ± 75 fmol/mg protein and 1010 ± 111 fmol/mg protein in HL-60 and HL-60R cells, respectively.

The cell lines were cultured at 37°C in 5% CO_2 humidified atmosphere and maintained in RPMI-1640 (Hyclone Europe, Cramlington, UK) supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf serum (Hyclone, Logan, UT), 2 mM L-glutamine and antibiotics (Gibco BRL, Life Technologies, Paisley, Scotland) (referred to as complete medium, CM). Once a month, the MTX-resistant line was cultured in the presence of 1 μM MTX for 1 week. Overexpression of the hMSH3 protein in HL-60R cells was periodically verified by Western blot analysis (data not shown).

Drugs and reagents

Temozolomide was kindly provided by Schering-Plough Research Institute (Kenilworth, NJ). Etoposide, 6-TG, O^6-benzylguanine (BG), colchicine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) were purchased from Sigma (St Louis, MO). MNNG was obtained from Aldrich (Milan, Italy). Cisplatin (prontoplatamine, 500 μg/ml in saline, pH 3–5) was purchased from Pharmacia & Upjohn (Milan, Italy). Doxorubicin (adriblastina) was obtained from Pharmacia (Milan, Italy).

Etoposide and MNNG were dissolved in dimethyl sulfoxide. BG and 6-TG were dissolved in ethanol and 0.1 N NaOH, respectively. Colchicine and doxorubicin were dissolved in saline. All these reagents were stored as stock solutions at –80°C, and diluted in CM just prior to use. The final concentrations of dimethyl sulfoxide, ethanol or NaOH did not affect cell growth (data not shown). Temozolomide was always prepared fresh by dissolving the drug in CM. MTT was dissolved at a concentration of 5 mg/ml in phosphate buffered saline (PBS) and stored at 4°C.

Reagents for agarose gel electrophoresis were all purchased from Bio-Rad (Hercules, CA).

Evaluation of cell chemosensitivity by MTT assay

HL-60 and HL-60R cells were suspended in CM at a concentration of 6×10^4 cells/ml and dispensed in 50 μl aliquots into flat-bottom 96-well plates (Falcon; Becton & Dickinson, Frankein Lakes, NJ). Graded amounts of each drug were then added to the wells in 50 μl CM and the plates were incubated at 37°C in a 5% CO_2 humidified atmosphere for 72 h. Four replica wells were used for controls and each drug concentration.

The effects of MNNG and TMZ were also evaluated in the presence of the OGAT inhibitor BG (22) to prevent repair of the methyl adducts at the O^6-G. To this end, cells were suspended in CM containing 10 μM BG, plated as described above and incubated at 37°C for 2 h. Temozolomide or MNNG were then added in 50 μl CM and the plates were maintained at 37°C for 72 h. Cells were therefore also exposed to 5 μM BG during the entire period of drug treatment. Under these conditions a complete abrogation of OGAT activity was obtained before drug treatment and up to the end of the assay (data not shown). Control groups were either untreated or treated with BG alone.

The MTT assay was performed as previously described (23). Briefly, after 72 h of culture, 0.1 mg MTT (in 20 μl PBS) was added to each well and cells were incubated at 37°C for 4 h. Cells were then lysed with buffer (0.1 ml/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).

Cell sensitivity to drug treatment was expressed in terms of IC_{50} (drug concentration 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 cell count

HL-60 and HL-60R cells were suspended in CM at the final concentration of 2×10^5 cells/ml and dispensed in 1 ml aliquots in 24-well plates (Falcon). Graded concentrations of the drugs under investigation were then added to the wells in 1 ml CM and the cells were cultured at 37°C in a 5% CO_2 humidified atmosphere for 72 h. Cell growth was evaluated in terms of viable cell count at the end of the incubation period. Cells were manually counted using a haemocytometer and cell viability was determined by trypan blue exclusion test. All determinations were made in duplicate. The effects of TMZ were evaluated in the presence of BG, which was added at the concentration of 10 μM 2 h before addition of the drug, and maintained in culture at a concentration of 5 μM. Control groups were either untreated or treated with BG alone.

Data were expressed in terms of percentage of cell growth of drug-treated groups with respect to controls. IC_{50} values were calculated on the regression lines in which the number of cells was plotted against the logarithm of drug concentration.

Evaluation of drug-induced chromosome aberrations

HL-60 and HL-60R cells were suspended in CM at the final concentration of 2×10^5 cells/ml and dispensed in 2.5 ml aliquots in 6-well plates (Falcon). TMZ, MNNG, cisplatin or etoposide were then added to the wells in 2.5 ml CM and the cells were cultured at 37°C in a 5% CO_2 humidified atmosphere for 48 h. The cultures were exposed for the last 2 h of incubation to 0.8 μg/ml colchicine. In the case of TMZ and MNNG treatments, BG was added to the cell suspensions at a concentration of 10 μM 2 h before the drug and maintained in culture at a concentration of 5 μM. Control groups were either untreated or treated with BG alone.

At the end of the incubation period, cells were harvested, treated for 15 min with a hypotonic solution (0.075 μM KCl) and fixed by 3:1 (v/v) 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). 100 metaphases, from at least two independent cultures for controls and each drug-treated group, were scored to evaluate the number of chromosome aberrations (chromatid and chromosome breaks) per cell, and the number of cells with chromosome aberrations (aberrant cells). Chromatid (i.e. triradials) and chromosome (i.e. dicentrics) rearrangements were counted as two chromatid and two chromosome breaks, respectively. No evaluation of sister chromatid exchanges was performed since neither cell lines incorporated 5-bromo-2′-deoxyuridine into their DNA (data not shown).

Assessment of apoptosis by flow cytometric analysis

Cells from control or drug-treated cultures were harvested by centrifugation, washed with PBS and fixed with 70% ethanol at –20°C for 18 h. They were then centrifuged, resuspended in 1 ml hypotonic solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate, 0.1% Triton-X and 10 μg/ml RNAse and incubated in the dark at room temperature for 1 h. Propidium iodide fluorescence was measured on a linear scale using a FACScan flow cytometer (Becton & Dickinson, San Jose, CA). Data from 2×10^4 cells were recorded and analysed using CellQuest software (Becton & Dickinson). Data collection was gated utilizing forward and side light scatter to exclude cell debris and cell aggregates. Apoptotic cells were determined by their hypochromic, sub-G_1 staining profiles (24).

Assessment of apoptosis by DNA fragmentation assay

Cells were collected by centrifugation, washed twice in PBS, resuspended (5×10^6 cells) in 0.2 ml 10 mM Tris–HCl, pH 8.6, 1.5 mM $MgCl_2$, 140 mM NaCl and 0.5% NP-40. Samples were incubated for 5 min on ice and subsequently microfuged for 5 min at 14 000 r.p.m. Supernatants were removed and incubated in the presence of 0.25 mg/ml DNAse-free, RNAse A at 37°C for 1 h. After addition of 0.2 ml 2× proteinase K buffer (0.2 M Tris–HCl, pH 8.0, 25 mM EDTA, 0.3 M NaCl and 2% SDS) samples were incubated for 30 min at 37°C in the presence of 50 μg/ml proteinase K. Low

molecular weight DNA was then extracted once with phenol [buffered with 24:24:1 (v/v/v) 0.1 M Tris–HCl pH 7.4/chloroform/isoamyl alcohol] and precipitated for 24 h in the presence of 0.1 M sodium acetate, pH 5.2, with 1 vol isopropanol. DNA precipitates were recovered by centrifugation at 14 000 r.p.m. using a refrigerated microfuge, and analysed by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide. DNA was visualized and photographed using a UV transilluminator and a Polaroid camera set-up.

Statistical analysis

Statistical analysis was performed, taking into account the results of all independent experiments available. Mean and standard error (SE) of the percentages relative to cell growth and apoptosis were obtained after 'angular transformation' of the calculated percentages, in order to process normally-distributed data. Therefore, statistical analysis, performed according to Student's *t*-test, was carried out by using transformed data.

When the concentration–effect relationship was considered, regression line analysis was carried out on the absorbance values at 595 nm or the number of cells versus the logarithm of drug concentration. Thereafter, the IC_{50} values relative to the drugs under investigation were determined for HL-60 and HL-60R cells in each separate experiment. Differences between IC_{50} relative to parental HL-60 line and those relative to HL-60R subline were then subjected to statistical analysis according to Student's 'pair *t*-test'.

The statistical analysis of HL-60 and HL-60R cell sensitivity to drug-induced chromosome aberrations was performed on the number of aberrant cells, and not on the number of aberrations per cell, because of the variability in chromosome numbers in both lines and the presence of the large amplified region in the HL-60R cell line. The normal standardized deviate was calculated according to the formula

$$U = \frac{n_1 - n_2}{\sqrt{n_1 + n_2}}$$

where n_1 and n_2 are the mean values of cells containing aberrations, considered as absolute numbers on 100 cells. Probability values (P) were then calculated according to 'U' distribution tables.

Results

Chemosensitivity of HL-60 and HL-60R cells

Mutations in *hMSH2* or *hMSH6* have been shown to confer resistance to the cytotoxic effects of MNNG, TMZ, 6-TG and cisplatin. We therefore tested these agents on HL-60 and HL-60R cells to evaluate whether a deficiency of hMutSα, due to overexpression of *hMSH3*, was associated with a similar pattern of chemoresistance. In addition, we evaluated the inhibitory effects on cell growth of doxorubicin and etoposide, which have been shown to be less cytotoxic in cells harbouring mutations in *hMLH1* (doxorubicin) or in either *hMSH2* or *hMLH1* (etoposide). The effects of MNNG and TMZ on cell growth were evaluated both in the absence and in the presence of the OGAT inhibitor BG.

The results illustrated in Table I refer to drug-induced growth inhibition evaluated by the MTT assay. Cell sensitivity to each drug is expressed in terms of IC_{50}. The data show that, in comparison to the MMR-deficient HL-60R cell line, the MMR-proficient HL-60 cells were more susceptible to the inhibitory effects of MNNG (2- and 4-fold in the absence and presence of BG, respectively), TMZ (1.3- and 5-fold in the absence and presence of BG, respectively) and 6-TG (about 2-fold). In contrast, there was no evidence of differential cytotoxicity for doxorubicin, cisplatin and etoposide.

Sensitivity of HL-60 and HL-60R cells to four of the drugs tested by the MTT assay was further confirmed by evaluating cell numbers and viability after 72 h of drug exposure. The results illustrated in Figure 1 indicate that the two types of assays yielded concordant results, with the *hMSH3*-over-expressing cells being more resistant to TMZ, but not to cisplatin, etoposide or doxorubicin, with respect to the parental HL-60 cells.

Table I. Chemosensitivity of HL-60 and HL-60R cells determined by the MTT assay

Drug[a]	IC_{50}[b]		
	HL-60	HL-60R	P[c]
TMZ (μM)	281 ± 24	387 ± 5	<0.05
TMZ + BG	73 ± 3**[d]	358 ± 11	<0.01
MNNG (μM)	4 ± 0.25	8 ± 1.30	<0.05
MNNG + BG	1.4 ± 0.11**	6 ± 1	<0.01
6-TG (μM)	5 ± 0.35	9 ± 0.47	<0.01
CDDP (μM)	0.51 ± 0.04	0.48 ± 0.01	NS[e]
ETO (μM)	0.086 ± 0.007	0.086 ± 0.012	NS
DOXO (nM)	12 ± 2.2	13 ± 1.4	NS

[a]Cells were incubated with graded concentrations of the drug for 72 h and then analysed for cell growth. The effects of MNNG and TMZ were evaluated also in the presence of the OGAT inhibitor BG. Each value represents the mean ± SE of at least three independent experiments performed with quadruplicate cultures.
[b]Drug concentration required to inhibit cell growth by 50%. CDDP, cisplatin; ETO, etoposide; DOXO, doxorubicin.
[c]Probability calculated according to Student's 'pair *t*-test', comparing the IC_{50} values relative to HL-60R with those relative to HL-60.
[d] **, $P < 0.01$, comparing, for each line, the IC_{50} values obtained in the presence of BG with those obtained without the inhibitor.
[e]NS, not significant.

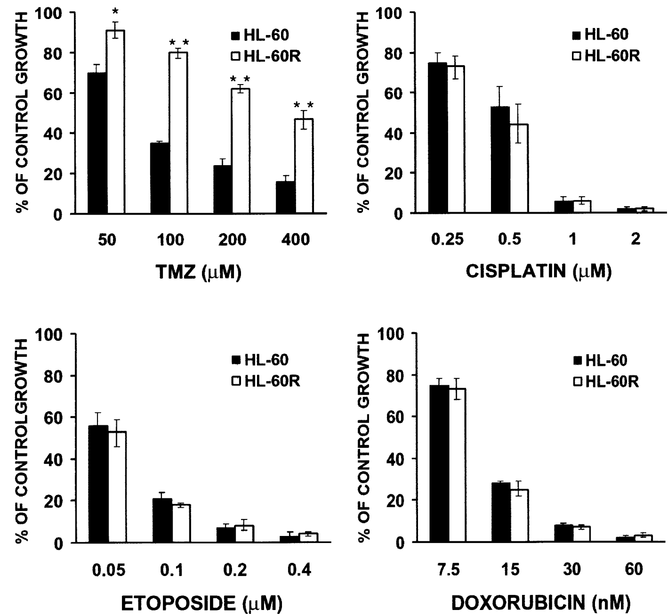

Fig. 1. Inhibition of cell growth induced by TMZ, cisplatin or etoposide in HL-60 and HL-60R cell lines. HL-60 (■) and HL-60R (□) cells (1×10^5 cells/ml) were incubated in CM, either unmodified or containing the indicated concentrations of TMZ, cisplatin, etoposide or doxorubicin, and cell growth was evaluated after 72 h of culture. The effect of TMZ was assayed in the presence of the OGAT inhibitor BG, and the respective control groups were therefore treated with BG alone. Data are expressed in terms of percentage of cell growth of drug-treated groups with respect to controls. Each value represents the mean of three (TMZ and doxorubicin) or four (cisplatin and etoposide) independent experiments performed with duplicate cultures. Bars indicate standard error of the mean. Mean IC_{50} values (± SE) relative to TMZ were 83 ± 7 and 389 ± 58 μM for HL-60 and HL-60R cells, respectively. Mean IC_{50} values (± SE) relative to cisplatin were 0.46 ± 0.06 and 0.42 ± 0.07 μM for HL-60 and HL-60 cells, respectively. Mean IC_{50} values (± SE) relative to etoposide were 0.045 ± 0.01 and 0.039 ± 0.01 μM for HL-60 and HL-60R cells, respectively. Mean IC_{50} values (± SE) relative to doxorubicin were 11.6 ± 0.86 and 10.6 ± 1.2 nM for HL-60 and HL-60R cells, respectively. *$P < 0.05$; **$P < 0.01$.

Table II. Chromosome aberrations induced by TMZ, MNNG, cisplatin (CDDP) or etoposide (ETO) in HL-60 and HL-60R cell lines

Treatment[a]	Aberrations/cell[b]		Aberrant cells/100 cells[c]		
	HL-60	HL-60R	HL-60	HL-60R	*P*[d]
BG	0.09 ± 0.01	0.11 ± 0.02	9 ± 1	10 ± 1	NS[e]
BG +100 μM TMZ	0.42 ± 0.03	0.16 ± 0.05	33 ± 2**	13 ± 3	<0.01
BG +400 μM TMZ	0.58 ± 0.05	0.34 ± 0.03	42 ± 1**	29 ± 5**	<0.05
BG +2 μM MNNG	0.49 ± 0.06	0.18 ± 0.04	39 ± 4**	16 ± 2	<0.01
BG +8 μM MNNG	0.64 ± 0.03	0.39 ± 0.02	43 ± 2**	26 ± 1**	<0.05
None	0.11 ± 0.03	0.12 ± 0.02	11 ± 3	12 ± 2	NS
CDDP (0.5 μM)	0.45 ± 0.06	0.56 ± 0.12	33 ± 1**	31 ± 4**	NS
ETO (0.05 μM)	0.29 ± 0.03	0.35 ± 0.04	27 ± 3**	31 ± 3**	NS

[a]Cells were cultured in the presence of the indicated concentrations of drugs for 48 h, and then processed for evaluation of chromosome aberrations. The clastogenic effects of TMZ and MNNG were evaluated in the presence of the OGAT inhibitor BG, and the respective control groups were therefore treated with BG alone.
[b]Number of chromosome aberrations per cell.
[c]Number of cells with chromosome aberrations out of 100 cells examined.
Each value represents the mean ± SE of three independent experiments, with the exception of the values of control groups treated with BG alone, which represent the mean ± SE of six experiments. 100 metaphases/group were scored for each experiment.
[d]Probability calculated as described in Materials and methods. The number of aberrant cells relative to HL-60R was compared with that of HL-60.
[e]NS, not significant.
$^{**}P < 0.01$, comparing the number of aberrant cells of drug-treated groups with that of controls.

Table III. Time course analysis of apoptosis induced by TMZ, cisplatin (CDDP) or etoposide (ETO) in HL-60 and HL-60R cells

Treatment[a]	Percentage of apoptotic cells[b]					
	HL-60			HL-60R		
	24 h	48 h	72 h	24 h	48 h	72 h
BG	10 (12–8)	5 (7–4)	6 (7–5)	8 (9–7)	7 (8–6)	6 (7–8)
BG + 100 μM TMZ	13 (17–9)	38 (43–33)**	33 (38–27)**	9 (11–8)	7 (9–6)	6 (8–5)
BG + 200 μM TMZ	14 (17–10)	52 (59–45)**	42 (47–36)**	11 (14–9)	9 (10–7)	8 (10–6)
None	8 (9–6)	5 (6–4)	4 (5–3)	7 (8–6)	6 (7–5)	6 (7–5)
CDDP (0.5 μM)	12 (13–11)*	15 (17–14)**	12 (14–10)**	11 (12–9)*	15 (19–11)**	17 (21–13)**
CDDP (1 μM)	18 (20–17)**	62 (68–55)**	72 (77–67)**	15 (18–12)**	56 (65–46)**	65 (70–60)**
ETO (0.1 μM)	9 (10–8)	25 (30–19)**	38 (43–34)**	9 (10–8)	25 (30–19)**	41 (45–36)**
ETO 0.2 μM	13 (14–12)*	39 (48–31)**	62 (65–59)**	14 (16–13)**	38 (46–30)**	61 (65–57)**

[a]Cells were cultured in the presence of the indicated concentrations of drugs for 24, 48 or 72 h and then processed for apoptosis evaluation. Induction of apoptosis by TMZ was determined in the presence of the OGAT inhibitor BG, and the respective control groups were therefore treated with BG alone.
[b]The percentage of apoptotic cells was evaluated by flow cytometric analysis of DNA content. Each value represents the mean of four independent experiments, except for the values relative to untreated controls, which refer to eight experiments. In parentheses, mean + SE and mean – SE.
$^{*}P < 0.05$; $^{**}P < 0.01$, comparing the percentages of apoptotic cells of drug-treated groups with those of controls. No significant differences were found when apoptosis induced by either etoposide or cisplatin in HL-60R cells was compared with that induced in HL-60.

Sensitivity of HL-60 and HL-60R cells to chromosome aberrations induced by MNNG, TMZ, cisplatin or etoposide

Cytogenetic analysis of HL-60 and HL-60R cell lines revealed that both strains had karyotypes in the hypotetraploid range (78–84 chromosomes) (data not shown). HL-60R cells showed one or, more seldom, two long markers, not present in the other strain, and containing the amplified region (data not shown).

To evaluate sensitivity of HL-60 and HL-60R cell lines to drug-induced chromosome aberrations, the cells were treated with MNNG (2 and 8 μM), TMZ (100 and 400 μM), cisplatin (0.5 μM) or etoposide (0.05 μM) and then the number of chromosome aberrations per cell, as well as the number of aberrant cells were assessed. These drug concentrations were chosen in the range of the drug IC_{50} values observed for the two lines in cell growth inhibition assays.

The results illustrated in Table II show that the numbers of aberrant cells and chromosome aberrations per cell in the untreated cultures of the two lines were similar. Moreover, there was no increased chromosomal fragility in the HL-60R strain in the chromosomes containing the amplified region (data not shown). When the cells were exposed to MNNG or TMZ (in the presence of BG) a higher number of chromosome aberrations per cell and of aberrant cells was observed in the hMutSα-proficient line with respect to its hMutSα-deficient counterpart (Table II). Statistical analysis performed on the number of aberrant cells showed that the observed differences were statistically significant at all drug concentrations tested. On the other hand, the two lines were not differentially susceptible to the clastogenic effects of cisplatin or etoposide (Table II).

Sensitivity of HL-60 and HL-60R cells to apoptosis induced by TMZ, cisplatin or etoposide

HL-60 and HL-60R cells were incubated with TMZ (100 and 200 μM), cisplatin (0.5 and 1 μM) or etoposide (0.1 and 0.2 μM) for 72 h and apoptosis was evaluated by flow

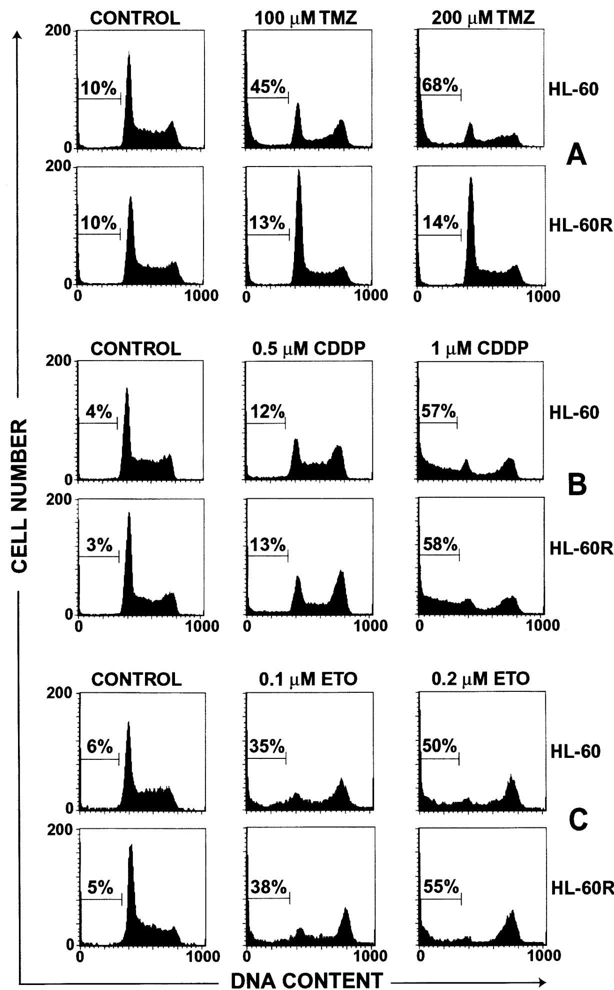

Fig. 2. Induction of apoptosis in HL-60 and HL-60R cells treated with TMZ, cisplatin or etoposide. HL-60 and HL-60R cells were incubated (1×10^5 cells/ml) in CM, either unmodified or containing the indicated concentrations of (**A**) TMZ, (**B**) cisplatin (CDDP) or (**C**) etoposide (ETO). The effect of TMZ was assayed in the presence of the OGAT inhibitor BG and the respective control groups were therefore treated with BG alone. After 48 h of culture, the cells were harvested and apoptosis was evaluated by flow cytometric analysis of DNA content. Data collection was gated utilizing forward and side light scatter to exclude cell debris and cell aggregates. PI fluorescence was measured on a linear scale. The percentage of apoptotic cells (i.e. cells with sub-G_1 DNA content) is shown on each histogram.

cytometry after 24, 48 and 72 h of drug exposure. TMZ treatment was performed in the presence of BG. The results, illustrated in Table III, show that at the concentrations tested TMZ induced apoptosis only in HL-60 cells, whereas the effects of cisplatin and etoposide were comparable in both lines.

Figure 2 illustrates flow cytometric analysis of a representative experiment showing apoptosis induced in HL-60 and HL-60R cell lines by a 48 h treatment with TMZ (100 and 200 μM), cisplatin (0.5 and 1 μM) or etoposide (0.1 and 0.2 μM). Drug-induced apoptosis is indicated by the appearance of a cell population with sub-G_1 DNA content.

Apoptosis was further confirmed by analysis of DNA fragmentation in control and drug-treated cells. Figure 3 illustrates the results of a representative experiment in which HL-60 and HL-60R cells were exposed to TMZ (50, 100 and 200 μM) or cisplatin (0.5 and 1 μM) for 48 h and the apoptotic DNA ladder was analysed by conventional agarose gel electrophoresis. The results show that cisplatin caused comparable DNA fragmentation in both lines, whereas TZM treatment was effective only in HL-60 cells.

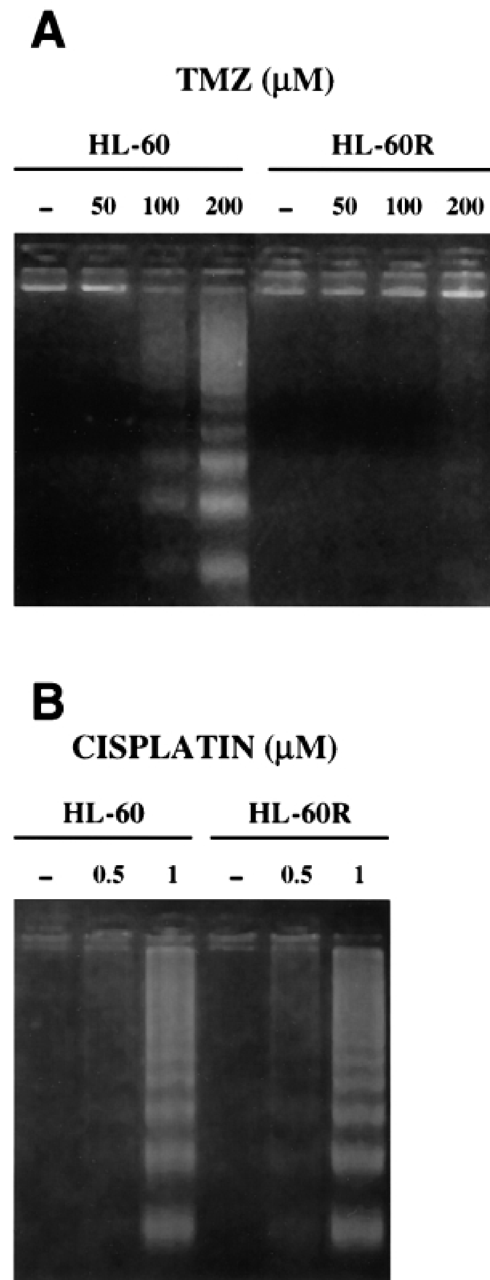

Fig. 3. Apoptosis-associated DNA degradation in HL-60 and HL-60R cells treated with TMZ or cisplatin. HL-60 and HL-60R cells were incubated (1×10^5 cells/ml) in CM, either unmodified or containing the indicated concentrations of (**A**) TMZ or (**B**) cisplatin. The effect of TMZ was assayed in the presence of the OGAT inhibitor BG, and the respective control groups were therefore treated with BG alone. After 48 h of culture, low molecular weight DNA was extracted from untreated or drug-treated cells and apoptotic fragmentation was analysed by agarose gel electrophoresis. The figure is a photograph of an agarose gel stained with ethidium bromide. The experiment is representative of two independent experiments.

Discussion

We have previously demonstrated that in the human lymphoblastoid cell line MT1, which harbours missense mutations in both alleles of the *hMSH6* locus, tolerance to TMZ resulted

from the inability to activate programmed cell death (11). The results presented here show that the apoptotic response of HL-60R cells to TMZ is similarly compromised (Table III; Figures 2 and 3). In agreement with the results of Hickman and Samson (12) who have shown that Chinese hamster cells overexpressing *MSH3* are more resistant than parental cells to MNNG (12), the growth of HL-60R cells was significantly less affected by MNNG (Table I) or TMZ (Table I and Figure 1) than that of the parental HL-60 line. Both MT1 and HL-60R cells are deficient in the correction of base/base mismatches. However, their MMR is otherwise functional, as witnessed by their ability to repair IDLs (17). Therefore, it may be assumed that the increased resistance to killing by methylating agents is due to their respective defects in hMutSα. This hypothesis was further confirmed by the finding that abrogation of OGAT activity by BG increased the sensitivity of HL-60, but not of HL-60R cells, to MNNG and TMZ (Table I). Thus, the cytotoxic effects of the two drugs are essentially mediated by the methylation of O^6-G and the consequent recognition of O^6-MeG/C and O^6-MeG/T mispairs by the hMutSα heterodimer.

The clastogenic effects of O^6-G-methylating agents also appear to be linked with the attempts of the MMR system to repair base pairs containing O^6-MeG. Defects in hMutSα have been associated with reduced cell susceptibility to chromosome aberrations induced by MNNG and MNU (25). In agreement with these findings, our present data show that upon treatment with MNNG or TMZ (both tested in the presence of BG), a higher number of chromosome aberrations occurred in HL-60 cells with respect to the HL-60R cell line (Table II), indicating that MMR is involved in the generation of this kind of lesion.

That a functional hMutSα complex is absolutely required for the processing of base pairs containing methylated nucleotides was further supported by our finding that the HL-60R cells were also more resistant than parental cells to 6-TG (Table I). This base analogue is incorporated into DNA and successively methylated at the S^6 position by endogenous *S*-adenosylmethionine. DNA replication gives rise to S^6-methyl-TG/T and S^6-methyl-TG/C mispairs, which are assumed, like O^6-MeG/T and O^6-MeG/C mispairs, to be recognized by hMutSα and thus subjected to 'futile' processing by MMR, which eventually results in cell death (26).

Mismatch repair defects have been linked also with resistance to cisplatin. The hMutSα complex and hMSH2 alone were reported to bind cisplatin adducts in DNA (27–30) and increased resistance to this drug has been documented in several cell lines harbouring mutations in *hMSH2*, *hMSH6*, *hMLH1* or *PSM2* (4,8,14), but not *hMSH3* (8). On this basis it has been suggested that both hMutSα and hMutLα, but not hMutSβ, are required for the lethal processing of cisplatin adducts by the MMR. We were thus somewhat surprised to find that this drug induced similar levels of growth inhibition (Table I and Figure 1), chromosome aberrations (Table II) and apoptosis (Table III, Figures 2 and 3) in both HL-60 and HL-60R cells. One hypothesis that might explain our results is that hMutSβ recognizes cisplatin adducts as efficiently as hMutSα. Although hMutSβ is usually much less abundant than hMutSα (16,17,31), in cells overexpressing *hMSH3* the concentration of the former heterodimer is substantially increased (16,17). Thus, if the hMutSβ heterodimer were able to recognize cisplatin adducts, it could compensate for the loss of hMutSα. However, even though hMutSβ appears to be capable of recognizing DNA damage induced by bulky chemical carcinogens as efficiently as hMutSα (32), the ability of hMutSβ to bind cisplatin intrastrand cross-links has not been tested to date.

Resistance to cisplatin is multifactorial and can be due to decreased drug accumulation, inactivation of the drug by thiol compounds, an increase in metallothionein levels and accelerated DNA repair (33,34). Moreover, HL-60 and HL-60R cell lines do not express p53 (35), and the latter cell line has a mutator phenotype. Thus, although unlikely, we cannot rule out the possibility that HL-60R cells might have acquired changes in cellular factors that regulate sensitivity to cisplatin and are thus able to counteract the effects of hMutSα deficiency, or that the absence of p53 in both cell lines outweighs the influence of the different MMR status on drug sensitivity.

In the present study, we also failed to detect differential susceptibility of HL-60 and HL-60R cells to growth inhibition induced by etoposide and doxorubicin (Table I and Figure 1). Moreover, both lines were also equally susceptible to the clastogenic and apoptotic effects of etoposide (Tables II and III; Figure 2). Although it is reasonable to hypothesize that inactivation of *hMSH6* itself may not be sufficient to confer resistance to etoposide and doxorubicin, as discussed for cisplatin, we cannot ignore the fact that the absence of p53, or the presence of additional molecular mechanisms of resistance (for review, see refs 36 and 37), might override the effects of MMR status on HL-60 and HL-60R cell sensitivity to these agents.

In conclusion, our data demonstrate that *hMSH3* overexpression induces a significant increase in cell resistance to the cytotoxic effects of O^6-G methylating agents and 6-TG, as previously described for mutations in the genes *hMSH2*, *hMSH6*, *hMLH1* and *hPMS2*. This finding has clinical relevance because MTX is routinely employed in the treatment of childhood acute lymphocytic leukemia, as well as other tumours, such as breast cancer. Amplification of the *DHFR* locus under MTX-selective pressure may indeed lead to a rapid appearance of a multi-drug-resistant phenotype in the tumour. It must, however, be taken into consideration that the degree of *DHFR* locus amplification in tumours may be limited to two to four extra copies. Although it is possible that even a small increase in hMSH3 levels could lower mismatch repair efficiency, further investigations are required to establish the pattern of drug resistance in tumours with such a moderate degree of *hMSH3* amplification.

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References

1. Modrich,P. (1997) Strand-specific mismatch repair in mammalian cells. *J. Biol. Chem.*, **272**, 24727–24730.
2. Jiricny,J. and Nystrom-Lahti,M. (2000) Mismatch repair defects in cancer. *Curr. Opin. Genet. Dev.*, **10**, 157–161.
3. Palombo,F., Iaccarino,I., Nakajima,E., Shimada,T. and Jiricny,J. (1996) hMutSβ, a heterodimer of hMSH2 and hMSH3, binds to insertion/deletion loops in DNA. *Curr. Biol.*, **6**, 1181–1184.
4. Fink,D., Aebi,S. and Howel,S.B. (1998) The role of DNA mismatch repair in drug resistance. *Clin. Cancer Res.*, **4**, 1–6.

5. Buermeyer,A.B., Wilson-Van Patten,C., Baker,S.M. and Liskay,M. (1999) The human MLH1 cDNA complements DNA mismatch repair defects in Mlh1-deficient mouse embryonic fibroblasts. *Cancer. Res.*, **59**, 538–541.
6. Liu,L., Markowitz,S. and Gerson,S.L. (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Res.*, **56**, 5375–5379.
7. Levati,L., Marra,G., Lettieri,T., D'Atri,S., Vernole,P., Tentori,L., Lacal,P.M., Pagani,E., Bonmassar,E., Jiricny,J. and Graziani,G. (1998) Mutation of the mismatch repair gene *hMSH2* in a human T-cell leukemia line tolerant to methylating agents. *Gene Chromosom. Cancer*, **23**, 159–166.
8. Vaisman,A., Varchenko,M., Umar,A., Kunkel,T.A., Risinger,J.I., Barrett,J.C., Hamilton,T.C. and Chaney,S.G. (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**, 3579–3585.
9. Fink,D., Nebel,S., Norris,P.S., Aebi,S., Kim,H.K., Haas,M. and Howell,S.B. (1998) The effect of different chemotherapeutic agents on the enrichment of DNA mismatch repair-deficient tumour cells. *Br. J. Cancer*, **77**, 703–708.
10. de las Alas,M.M., Aebi,S., Fink,D., Howell,S.B. and Los,G. (1997) Loss of DNA mismatch repair: effects on the rate of mutation to drug resistance. *J. Natl Cancer Inst.*, **89**, 15437–15441.
11. D'Atri,S., Tentori,L., Lacal,P.M., Graziani,G., Pagani,E., Benincasa,E., Zambruno,G., Bonmassar,E. and Jiricny,J. (1998) Involvement of the mismatch repair system in temozolomide-induced apoptosis. *Mol. Pharmacol.*, **54**, 334–341.
12. Hickman,M.J. and Samson,L.D. (1999) Role of DNA mismatch repair and p53 in signaling induction of apoptosis by alkylating agents. *Proc. Natl Acad. Sci. USA*, **96**, 10764–10769.
13. Duckett,D.R., Bronstein,S.M., Taya,Y. and Modrich,P. (1999) hMutSα- and hMutLα-dependent phosphorylation of p53 in response to DNA methylator damage. *Proc. Natl Acad. Sci. USA*, **96**, 12384–12388.
14. Fink,D., Nebel,S., Aebi,S., Nehme,A. and Howell,S.B. (1997) Loss of DNA mismatch repair due to knockout of MSH2 or PMS2 results in resistance to cisplatin and carboplatin. *Int. J. Oncol.*, **11**, 539–542.
15. Hinz,J.M. and Meuth,M. (1999) MSH3 deficiency is not sufficient for a mutator phenotype in Chinese hamster ovary cells. *Carcinogenesis*, **20**, 215–220.
16. Drummond,J.T., Genschel,J., Wolf,E. and Modrich,P. (1997) DHFR/MSH3 amplification in methotrexate-resistant cells alters the hMutSα/hMutSβ ratio and reduces the efficiency of base–base mismatch repair. *Proc. Natl Acad. Sci. USA*, **94**, 10144–10149.
17. Marra,G., Iaccarino,I., Lettieri,T., Roscilli,G., Delmastro,P. and Jiricny,J. (1998) Mismatch repair deficiency associated with overexpression of the MSH3 gene. *Proc. Natl Acad. Sci. USA*, **95**, 8568–8573.
18. Fujii,H. and Shimada,T. (1989) Isolation and characterization of cDNA clones derived from the divergently transcribed gene in the region upstream from the human dihydrofolate reductase gene. *J. Biol. Chem.*, **264**, 10057–10064.
19. Allegra,C.J. and Grem,J.L. (1997) Antimetabolites. In De Vita,V.T., Hellman,S. and Rosemberg,S.A. (eds) *Cancer: Principles and Practice of Oncology.* Lippincott–Raven, Philadelphia–New York, pp. 432–452.
20. Gorlick,R., Goker,E., Trippett,T., Waltham,M., Banerjee,D. and Bertino,J.R. (1996) Intrinsic and acquired resistance to methotrexate in acute leukemia. *N. Engl. J. Med.*, **355**, 1041–1048.
21. Watson,A.J. and Margison,G.P. (1999) O^6-Alkylguanine-DNA alkyltransferase assays. In Brown,R. and Boeger-Brown,U. (eds) *Cytotoxic Drug Resistance Mechanisms.* Humana Press, Totowa, NJ, pp. 167–178.
22. Dolan,E.M., Moshel,R.C. and Pegg,A.E. (1990) Depletion of O^6-alkylguanine-DNA alkyltransferase activity by O^6-benzylguanine provides a means to evaluate the role of this protein in protection against carcinogenic and therapeutic alkylating agents. *Proc. Natl Acad. Sci. USA*, **87**, 5368–5372.
23. Hansen,M.B., Nielsen,S.E. and Berg,K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods*, **119**, 203–210.
24. Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F. and Riccardi,C. (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Methods*, **139**, 271–279.
25. Galloway,S.M., Greenwood,S.K., Hill,R.B., Bradt,C.I. and Bean,C.L. (1995) A role for mismatch repair in production of chromosome aberrations by methylating agents in human cells. *Mutat. Res.*, **346**, 231–245.
26. Swann,P.F., Waters,T.R., Moulton,D.C., Xu,Y.Z., Zheng,Q., Edwards,M. and Mace,R. (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. *Science*, **273**, 1109–1111.
27. Fink,D., Nebel,S., Aebi,S., Zheng,H., Cenni,B., Nehmé,A., Christen,R.D. and Howell,S.B. (1996) The role of DNA mismatch repair in platinum drug resistance. *Cancer Res.*, **56**, 4881–4886.
28. Mello,J.A., Acharya,S., Fishel,R. and Essingmann,J.M. (1996) The mismatch-repair protein hMSH2 binds selectively to DNA adducts of the anticancer drug ciplatin. *Chem. Biol.*, **3**, 579–589.
29. Duckett,D.R., Drummond,J.T., Murchie,A.I., Reardon,J.T., Sancar,A., Lilley,D.M. and 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. USA*, **93**, 6443–6447.
30. Yamada,M., O'Regan,E., Brown,R. and Karran,P. (1997) Selective recognition of a cisplatin–DNA adduct by human mismatch repair proteins. *Nucleic Acids Res.*, **25**, 491–495.
31. Drummond,J.T., Li,G.M., Longley,M.J. and Modrich,P. (1995) Isolation of an hMSH2–p160 heterodimer that restores DNA mismatch repair to tumour cells. *Science*, **268**, 1909–1912.
32. Wu,J., Gu,L., Wang,H., Geacintov,N.E. and Li,G.M. (1999) Mismatch repair processing of carcinogen–DNA adducts triggers apoptosis. *Mol. Cell. Biol.*, **19**, 8292–8301.
33. Andrews,P.A. (1994) Mechanisms of acquired resistance to cisplatin. In Goldstein,L.J. and Ozols,R.F. (eds) *Anticancer Drug Resistance*. Kluwer Academic Press, Boston, pp. 217–248.
34. Akiyama,S., Chen,Z.S., Sumizawa,T. and Furukawa,T. (1999) Resistance to cisplatin. *Anticancer Drug Des.*, **14**, 143–151.
35. Wolf,D. and Rotter,V. (1985) Major deletions in gene encoding the p53 tumour antigen cause lack of p53 expression in HL-60 cells. *Proc. Natl Acad. Sci. USA*, **82**, 790–794.
36. Rubin,E.H., Li,T., Duan,P. and Liu,F.L. (1996) Cellular resistance to topoisomerase poisons. In Hait,W.N. (ed.) *Drug Resistance*. Kluwer Academic Press, Norwell, pp. 243–260.
37. Nielsen,D., Maare,C. and Skovgaard,T. (1996) Cellular resistance to anthracyclines. *Gen. Pharmacol.*, **27**, 251–255.

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