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3-Methyladenine-DNA-glycosylase and O^6-alkyl guanine-DNA-alkyltransferase activities and sensitivity to alkylating agents in human cancer cell lines

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Summary The activities and the expression of 3-methyladenine glycosylase (3-meAde gly) and O^6-alkylguanine-DNA-alkyltransferase (O^6ATase) were investigated in ten human cancer cell lines. Both 3-meAde gly and O^6ATase activities were variable among different cell lines. mRNA levels of the O^6ATase gene, appeared to be related to the content of O^6ATase in different cell lines, whereas no apparent correlation was found between mRNA of 3-meAde gly and the enzymatic activity. No correlation was found between the activity of the two enzymes and the sensitivity to alkylating agents of different structures such as CC-1065, tallimustine, dimethylsulphate (DMSO), *N*-methyl-*N*[1]-nitro-*N*-ntirosoguanidine (MNNG), *cis*-diamminedichloroplatinum (cDDP) and melphalan (L-PAM). The most striking finding of this study is that a correlation exists between the activity of O^6ATase and 3-meAde gly in the various cell lines investigated ($P<0.01$), suggesting a common mechanism of regulation of two DNA repair enzymes.

Keywords: 3-methyladenine glycosylase; O^6 ATase; alkylating agents

Alkylating agents can cause a variety of DNA adducts, the alkylpurines being the main ones (Baranek, 1990; Margison and O'Connor, 1990). These alkylations are eliminated in prokaryotes and eukaryotes (Olsson and Lindahl, 1980; Mehta *et al.*, 1981; Lindahl, 1976; Laval, 1977) through an O^6 alkylguanine alkyltransferase (O^6ATase) acting on O^6 methylguanine (O^6-meGua) and a 3-methyladenine glycosylase (3-meAde gly) acting on a 3-methyladenine (3-meAde) and other alkylpurines.

The molecular effect of alkylation of guanines at the O^6 position is well defined, resulting in a mismatch and, after DNA replication, in a transition mutation (Loveless, 1969). Such DNA lesions can cause lethal events through an abortive mismatch repair (Kat *et al.*, 1993). In this respect the role of O^6ATase has been related to resistance to alkylating agents in various biological systems, from prokaryotes to mammals. Less is known about the 3-alkyladenine in DNA. While in *Escherichia coli* such alkylated bases represent a lethal event (Boiteux *et al.*, 1984; Lindahl *et al.*, 1988), it is still to be confirmed whether it is lethal or mutagenic in mammalian cells. *E. coli* displays two glycosylase activities able to repair 3-alkyladenine, encoded by the *tag* and *alkA* gene products (Bjelland *et al.*, 1993; Nakabeppu *et al.*, 1994).

Mammalian cells seem to have only one type of adenine glycosylase, more like the *alkA* than the *tag* gene product. Recently the mouse, rat and human 3-meAde gly cDNA have been cloned (Engelward *et al.*, 1993; O'Connor and Laval, 1990; Samson *et al.*, 1991; Chakravarti *et al.*, 1991).

The enzyme's precise role in protecting cells from the cytotoxic effects of alkylating agents is still controversial (Klungland *et al.*, 1992; Habraken and Laval, 1993; Imperatori *et al.*, 1994; Ibeanu *et al.*, 1992; Matijasevic *et al.*, 1993). Klungland *et al.* (1992) reported that V79 hamster lung fibroblasts and murine haematopoietic cells permanently expressing the *tag* gene showed increased resistance to killing by methylmethanesulphonate (MMS) and methylnitrosurea (MNU). Habraken and Laval (1993) demonstrated that the expression of the *alkA* or mammalian glycosylase in irs1 cells rendered them resistant to the toxic effects of both MMS and ethylmethanesulphonate (EMS). However, Imperatori *et al.* (1994) and Ibenau *et al.* (1992), who respectively transfected the *tag* and the mammalian glycosylase gene in different cell lines, could not find any increased resistance to the toxic effects of alkylating agents in the stable transfectants, in spite of the high level of glycosylase expression. It was suggested that the level of the endogenous enzyme might account for these differences. Further studies are needed to establish the enzyme's role in the repair of the lesions induced by alkylating agents and in determining tumour resistance to the alkylating agents used in cancer chemotherapy. The lack of data on the enzymatic activity of 3-meAde gly in human tumour cells prompted us to conduct a study in which the enzymatic activity was determined in a number of cancer cell lines together with O^6ATase level and sensitivity to alkylating agents.

The alkylating agents selected were CC1065 and tallimustine, known to alkylate N3 adenine in a DNA sequence specific manner; *N* - methyl - *N'* - nitro - *N* - nitrosoguanidine (MNNG) and dimethylsulphate (DMS) for their ability to cause a broader alkylation pattern; and two cross-linking agents, *cis*-diamminedichloroplatinum (cDDP) and melphalan (L-PAM).

Materials and methods

Cell culture

The ovarian carcinoma cell lines IGROV, SW626, OVCAR3.S, SKOV-3, the human leukaemia cell lines CEM, U937, K562 and Jurkat were maintained in RPMI-1640 (Gibco Europe, Glasgow, UK); human colon carcinoma cell lines HT-29 and LoVo were maintained respectively in Eagle's minimum essential medium (MEM) (Gibco) and F-12 (Biological Industries) media. All cell lines were supplemented with 10% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel) and 2 mM glutamine (Gibco) and grown at 37°C with 5% carbon dioxide.

Northern blotting analysis

Total RNA from cell lines was separated from exponential growing cells by the quanidium isothiocyanate/caesium

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chloride procedure (Chirgwin *et al.*, 1979). After electrophoresis in a formaldehyde–agarose gel, RNA (10 μg) was transferred to nylon membranes and hybridised separately to the random primed ^{32}P-labelled probes for 16 h at 42°C. The 1.2 kb 3-meAde gly probe was obtained by digesting the puC9-MPG plasmid (generous gift from Professor B Kaina, University of Mainz, Germany) with *Bam*HI and the 0.8 kb O6ATase probe was obtained by digesting the pKT100 plasmid (generous gift from Dr S Mitra, University of Texas, TX, USA) with EcoRI. Membranes were washed in 2 × SSC at room temperature in 2 × SSC /1% sodium dodecyl sulphate (SDS) at 65°C and then autoradiographed.

RNA filters were rehybridised with murine α-actin cDNA.

3-meAde gly assay

Cell pellets ($50-70 \times 10^6$ cells ml^{-1}) were resuspended in 20 mM Tris HCl, pH 7.5 (4°C) containing 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and crude cell extracts were obtained by sonication. Samples were centrifuged to remove cellular debris and stored at −80°C until assay. Crude extracts (0.1–0.2 mg of protein) were incubated in a constant volume of 140 μl at 37°C for variable intervals with aliquots of freshly *N*-[^{3}H] MNU methylated DNA containing (≥) 1000 fmol of 3-[^{3}H]methyladenine; the reactions were stopped on ice at stated times. After addition of the internal standard, DNA was precipitated with sodium chloride–ethanol; supernatants were collected, dried, redissolved and [^{3}H]methyladenine content analysed by radiochromatographic high-performance liquid chromatography (HPLC); similar samples were incubated without protein extracts to assess spontaneous depurination. The results were calculated on the slope of time course of 3-meAde peak radioactivity and were expressed as fmol repaired min^{-1} mg^{-1} DNA.

Total protein concentration was determined by the Bradford dye-binding assay (Bio-Rad).

O6ATase assay

O6ATase was assayed according to the method already published (Catapano *et al.*, 1987). Briefly, extracts of the cell lines were obtained by sonication of the cellular pellets resuspended in cold 50 mM Tris-HCl (pH 7.5), 1 mM DTT and 0.01 mM EDTA. The supernatants were centrifuged before assay. [^{3}H]methylated calf thymus DNA was prepared according to Wiestler *et al.* (1984). The [^{3}H] methylated substrate containing O6-meGua was incubated for 1 h at 37°C with variable amounts of crude cell extracts in 1.7 mM DTT, 72 mM Tris-HCl, pH 8 with or without cell extracts (controls), then the DNA was precipitated by adding cold 1 N perchloric acid. The DNA pellet was hydrolysed by heating at 70°C for 30 min in 0.1 N hydrochloric acid and analysed by HPLC with the Hibar RP8 (4 × 250 mm) CB reversed-phase prepacked column (Merck). Fractions of eluted material were collected and assayed for radioactivity. The sample DNA content was determined with the diphenylamine assay. The results were expressed as fmol μg^{-1} DNA.

Growth inhibition assay

Briefly, cells were plated at a density ranging from 32 to 64×10^3 cells ml^{-1} in 100 μl of complete medium in 96-well plates. After 48 h drugs were added at the concentrations indicated. At the end of 2 h drug treatments, medium was removed, cells were washed with warm phosphate-buffered saline (PBS) and recovered in fresh medium. Ater 72 h surviving fractions were stained with (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) for 4 h then the staining solution was removed by aspiration and cells were dissolved in 100 μl of *l*-propanol/0.025 N hydrochloric acid. Absorbance at 570 nm was measured using a multiwell plate reader (Titertek Multiscan MC340).

The IC_{50}s were determined by interpolation of the dose–response cytotoxic curves. The IC_{50} value for each drug is the mean ± s.e. of at least three different experiments.

Drugs

DMS, MNNG (Sigma, St Louis, MO, USA.) and cDDP (generous gift from Bristol Myers-Squibb, USA) were dissolved in medium; L-PAM (kindly provided by Drug Synthesis and Chemical Branch, Division of Cancer Treatment, NCI, Bethesda, MD, USA) was dissolved in 0.3 N hydrochloric acid; stock solution of CC-1065 (1 mg ml^{-1}) (kindly provided by Upjohn Co, Kalamazoo, MI, USA) in dimethylacetamide (DMA) was diluted in medium just before use; tallimustine (kindly provided by Pharmacia-Farmitalia Carlo Erba, Milan, Italy) was dissolved in dimethylsulphoxide (DMSO) and diluted to the desired concentration in medium just before use.

Results

3-MeAde gly and O6ATase activities in different cell lines

A panel of ten cell lines was selected to measure 3-meAde gly and O6ATase activities. As shown in Table I, the doubling times were similar, except for the colon–rectum HT-29 and LoVo cell lines, which showed longer times (46 and 44 h respectively).

3-MeAde gly activity varied among the cell lines, being lowest in the U937 line (0.11 fmol μg^{-1} DNA min^{-1}) and highest in LoVo cells (3.17 fmol μg^{-1} DNA min^{-1}). O6ATase levels were variable, ranging from undetectable in U937 cells to 24.5 fmol μg^{-1} DNA in LoVo cells. Table I also shows the mean ± s.e. of the DNA repair enzymatic activities of 3-meAde gly and the O6ATase in the cell lines. Although numbers were too small for a statistical comparison, the

Table I Doubling times, 3-meAde glycosylase and O6ATase activities in ten cell lines

Cell line	*Doubling time (h)*	*3-meAde gly activity fmol μg^{-1} DNA min^{-1}*	*mean ± s.e.*	*O6 ATase activity fmol μg^{-1} DNA*	*mean ± s.e.*
Leukaemias					
CEM	35 ± 3.6	0.80 ± 0.079		17 ± 0.5	
JURKAT	29 ± 1	1.41 ± 0.10	0.77 ± 0.26	13 ± 0.9	12 ± 3.5
K562	28 ± 1.1	0.77 ± 0.09		5 ± 0.3	
U937	34 ± 0.6	0.11 ± 0.006		ND	
Ovarian carcinomals					
IGROV	40 ± 2	1.33 ± 0.15		9 ± 0.5	
OVCAR3.S	32 ± 0.9	0.24 ± 0.019		3 ± 0.4	
SKOV-3	31 ± 1	2.10 ± 0.12	1.24 ± 0.38	21 ± 0.6	12 ± 4
SW626	29 ± 0.5	1.28 ± 0.11		14 ± 0.9	
Colon–rectum carcinomals					
HT-29	46 ± 1.1	2.30 ± 0.15	2.74 ± 0.43	17 ± 0.9	21 ± 3
LoVo	44 ± 0.9	3.17 ± 0.25		24 ± 1	

ND, not detectable

levels of both activities were highest in colon–rectum cells, intermediate in ovarian cells and lowest in cells of haematopoietic origin.

There was a significant correlation ($P<0.01$) between 3-meAde gly and O⁶ATase enzymatic activity (Figure 1).

Expression of mRNA coding for 3-meAde gly and O⁶ATase

Figure 2 shows the Northern blot analysis of RNA extracted from the different cell lines. 3-meAde gly transcript was detected in all cells except OVCAR3.S, but the RNA expression did not seem to be quantitatively correlated with the enzymatic activity (Table I and Figure 2). For example, the Jurkat cell line showed quite a high level of enzymatic activity (1.41 fmol μg^{-1} DNA min^{-1}) but very low mRNA expression (Figure 2, lane 3). The correlation between O⁶ATase mRNA and its enzymatic activity was better, as already reported (Fornace *et al.*, 1990; Citron *et al.*, 1993).

Cytotoxic effects of different alkylating agents on the cell lines

Table II shows the IC_{50} of the alkylating agents tested on the ten cell lines selected for this study. The drugs chosen were tallimustine and CC-1065, two alkylating agents known to bind to the minor groove of DNA in a sequence-specific manner (Broggini *et al.*, 1994); DMS and MNNG as methylating agents and cDDP and L-PAM as cross-linking agents. No correlation was found between the levels of O⁶ATase and 3-meAde gly activities and the IC_{50}. For example, Jurkat cells that had high levels of both enzymes, seemed to be the most sensitive to almost all the drugs, except L-PAM. In contrast, OVCAR3.S cells, with low levels of both 3-meAde gly and O⁶ATase, were more resistant to tallimustine, CC-1065, DMS and MNNG than cells with higher enzymatic levels.

Discussion

Ten human cancer cell lines were characterised for their 3-meAde gly and O⁶ATase contents and their susceptibility to different alkylating agents. The O⁶ATase levels varied in the different cell lines, in the range of those reported in previous studies (Fornace *et al.*, 1990; Citron *et al.*, 1993; Walker *et al.*, 1992). For 3-meAde gly, no comparison can be made, as to our knowledge this is the first report describing the activity of this DNA repair enzyme in human cancer cells. A 10-fold difference in 3-meAde gly activity was observed in the cell lines studied, with values ranging from 0.11 to 3.17 fmol μg^{-1} DNA min^{-1} corresponding to 18–165 fmol mg^{-1} protein min^{-1}. These values seem to be much higher than those reported for murine cancer cell lines, which ranged from 0.6 to 17 fmol mg^{-1} protein min^{-1} (Klungland *et al.*, 1992; Imperatori *et al.*, 1994).

Similar interspecies differences have been observed for other DNA repair enzymes: for example O⁶ATase in human tissues is ten times higher than in mouse tissues (Gerson *et al.*, 1986). The cellular content of O⁶ATase correlated with the levels of O⁶ATase mRNA, a finding that confirms previous studies (Fornace *et al.*, 1990; Citron *et al.*, 1993; Walker *et al.*, 1992). However no correlation was found between 3-meAde gly activity and 3-meAde gly mRNA (Table I, Figure 2). There are several potential explanations of these differences. Inactive 3-meAde gly molecules may be synthesised in the non-functional state, or functional protein might be inactivated. On the other hand the protein turnover could be different in the different cell lines, resulting in differences in the enzymatic activities. Interestingly, the simultaneous expression of alternatively spliced 3-meAde gly transcripts in human tissues and cells by reverse transcriptase–polymerase chain reaction (RT-PCR) of total

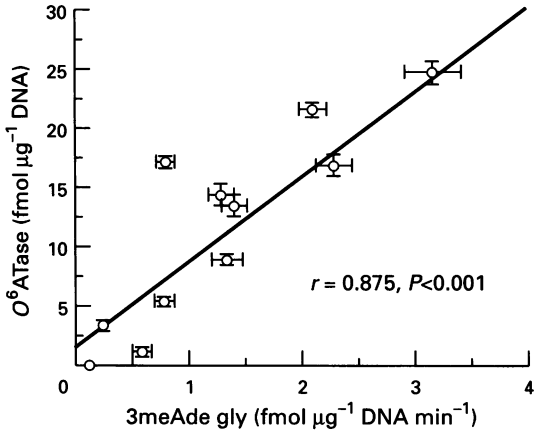

Figure 1 Relationship between the levels of 3-methyladenine-DNA-glycosylase and O⁶-alkylguanine-DNA-alkyltransferase activities. Each point represents the mean ± s.e. of three determinations.

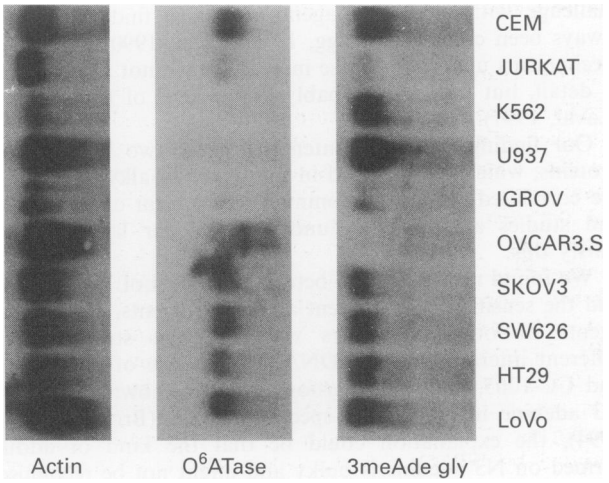

Figure 2 Northern blot analysis of 3-methyladenine-DNA-glycosylase and O⁶-alkylguanine-DNA-alkyltransferase and α-actin expression in ten cell lines.

Table II IC_{50} of various agents on ten human cell lines

Cell line	Tallimustine (uM)	CC-1065 (uM)	DMS (uM)	MNNG (uM)	cDDP (uM)	L-PAM (uM)
CEMM	0.06 ± 0.005	4.3 ± 0.8	364 ± 19	60 ± 5	9 ± 2	1 ± 0.2
JURKAT	2.3 ± 1.3	0.4 ± 0.16	216 ± 56	26 ± 4	8 ± 2	6 ± 1
K562	24 ± 7	11 ± 1	350 ± 66	40 ± 8	68 ± 5	111 ± 20
U937	9 ± 3	3 ± 0.6	310 ± 44	9 ± 2	23 ± 3	10 ± 0.3
IGROV	15 ± 1	6 ± 0.8	976 ± 75	131 ± 20	28 ± 2	151 ± 33
OVCAR3.S	83 ± 16	4 ± 3	835 ± 26	130 ± 51	35 ± 5	65 ± 6
SKOV-3	8 ± 3	6 ± 1	866 ± 105	66 ± 7	83 ± 12	102 ± 3
SW626	9.5 ± 1	8 ± 1	886 ± 54	67 ± 23	62 ± 5	128 ± 26
HT-29	19 ± 11	25 ± 8	1503 ± 159	370 ± 31	253 ± 13	184 ± 26
LOVO	> 136	6 ± 0.6	960 ± 5	78 ± 5	99 ± 14	88 ± 6

RNA has been demonstrated recently (Pendlebury *et al.*, 1994). The functional role, if any, of these two isoforms has yet to be established. They might display different substrate specificity, even if there are no data yet to confirm this hypothesis. The probe we used for the 3-meAde gly mRNA detection is homologous for both these isoforms, so that the discrepancy between the mRNA expression and the enzymatic activity, based on the ability to remove labelled-3-meAde from DNA, could also be explained by the different substrate specificity of the two isoforms.

Interestingly, we found a significant correlation ($P < 0.01$) between the levels of enzymatic activity. To our knowledge no data have been published so far on the expression and activity of these two DNA repair enzymes in the same human cell lines. In *E. Coli*, both enzymes exist as constitutive (respectively the *tag* and *otg* gene products) (Sakumi *et al.*, 1986; Bjelland and Seeberg, 1987; Pegg and Byers, 1992) or inducible forms (respectively the *AlkA* and *ada* gene products) (Nakabeppu *et al.*, 1984; Karran *et al.*, 1982; Evensen and Seeberg, 1982). This induction, which is termed the adaptive response, is an efficient system that enables *E. Coli* to respond to damage by alkylating agents. In *Saccaromyces cerevisiae* the alkylation damage is repaired by a methyltransferase (encoded by *MTG1*) and 3-meAde gly (encoded by *MAG*) that releases O6meGua, 3meAde and 7meAde from alkylated DNA (Chen *et al.*, 1990; Xiao and Samson, 1992). *MAG* but not *MTG1* is inducible by DNA damage (Chen and Samson, 1991; Xiao *et al.*, 1991). A decamer consensus sequence has been recently identified in the promoters of the *MAG*, *MGT1* and several other genes involved in the DNA repair and metabolism (Xiao *et al.*, 1993), suggesting a common mechanism of regulation. In mammalian cells induction of alkyltransferase activity has been shown in some cases, but the changes are much more modest than the ones seen in *E. Coli*, up to 10-fold. Induction of O6ATase has been reported in human cancer cells after challenge with alkylating agents, but this finding has not always been confirmed (Pegg, 1990; Laval, 1990, 1991). The mechanisms underlying these increases have not been studied in detail, but they are probably at the level of transcription (Laval, 1991).

Our finding that the contents of these two DNA repair proteins, which are involved in the repair of alkylated DNA, are correlated suggests a common mechanism of regulation, and studies are currently underway in our laboratory to clarify this.

We found no correlation between the level of 3-meAde gly and the sensitivity to different alkylating agents, methylating agents or other alkylators with different structure and different interactions with DNA. In the case of tallimustine and CC-1065, two minor groove binders known to alkylate N3 adenine in a sequence-specific manner (Broggini *et al.*, 1994), the explanation could be that the kind of adduct formed on N3 adenine is bulky and might not be recognised and cleaved by the enzyme. For the cross-linking agents L-PAM and cDDP too, the cytotoxic lesions appear to be the cross-links between two adjacent guanines or guanines located on opposite strands, so the alkylation of N3 adenine might be relatively unimportant for their cytotoxicity. In fact it has been shown that the nucleotide excision repair plays an important role in the repair of the lesions caused by cDDP and L-PAM treatment (Lee *et al.*, 1993; Larminat and Bohr, 1994).

For methylating agents (DMS and MNNG) the lack of correlation between the levels of the DNA repair protein and the sensitivity confirmed previous findings (Imperatori *et al.*, 1994) on murine cells expressing the *E. Coli tag* or human *MPG* gene, which did not become resistant to the toxic effects of methylating agents. As already proposed, the endogenous levels of 3-meAde gly might be the crucial factor governing the sensitivity to methylating treatment. In the ten lines selected for this study, the 3-meAde gly contents were relatively high, possibly sufficient *per se* to repair the 3-meAde adduct caused by treatment with alkylating agents. In fact, resistance to methylating treatment has been reported in cell lines transfected with the *tag* gene, with an increase in the level of repair activity from 0.6 to 4.3 fmol mg^{-1} protein min^{-1} (O'Connor and Laval, 1990).

The other major factor for resistance to methylating agents is the cellular content of O6ATase. However, no correlation between the transferase activity and cellular sensitivity to different alkylating agents, particularly with MNNG and DMS, which are known to cause the highest proportion of O6meGua was found. It has already been shown that the sensitivity to MNNG is not necessarily associated with O6ATase level (Lefebvre and Laval, 1993). Recently a defective mismatch repair has also been implicated in resistance and tolerance to some alkylating agents (such as MNU and MNNG). In this case O6-meGua persists in tolerant cells but is no longer lethal. The current underlying hypothesis is that methylation tolerance arises through the loss of a mismatch repair system that is defective in many tolerant cells (Karran and Bignami, 1994).

In conclusion, the present findings suggest that the two repair enzymes, 3meAde gly and O6ATase, are not necessarily vital to the sensitivity to various alkylating agents. It is likely that after DNA damage a cascade of events occurs, resulting in a certain degree of survival. For example, two cell lines may have the same level of DNA damage and the same rate of adduct removal but the activation of different pathways for cell survival, for example activation of different proteins involved in cell growth and survival (e.g. p53), so that no single factor is likely to be responsible for the sensitivity or resistance to a certain drug.

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