Biochem. J. (1994) **298**, 231–235 (Printed in Great Britain)

Differential inactivation of mammalian and *Escherichia coli* O^6-alkylguanine-DNA alkyltransferases by O^6-benzylguanine

Rhoderick H. ELDER,* Geoffrey P. MARGISON and Joseph A. RAFFERTY

CRC Department of Carcinogenesis, Paterson Institute for Cancer Research, Christie Hospital (NHS) Trust, Wilmslow Road, Manchester M20 9BX, U.K.

The action of O^6-benzylguanine (O^6-BzlG) on recombinant mammalian and *Escherichia coli* O^6-alkylguanine-DNA alkyltransferases (ATase; EC 2.1.1.63; methylated-DNA–protein-cysteine methyltransferase) was compared by preincubation of these proteins with the base, followed by measurement of residual ATase activity using [^{3}H]methylated substrate DNA. All of the mammalian proteins examined were inactivated by O^6-BzlG (Chinese hamster: I_{40}, 0.04 μM; human and rat: I_{40}, 0.06 μM); however, the murine ATase was substantially more resistant requiring 4–5 fold higher concentrations of O^6-BzlG to achieve the same levels of inactivation (I_{40}, 0.28 μM). A similar differential inactivation was seen with human and murine ATases when extracts of 3T6 (murine) cells and Raji (human) cells were compared. Of the two *E. coli* ATase proteins, only the *ogt*-encoded protein was inactivated, but approximately 400 times more O^6-BzlG was required to achieve a level of inactivation similar to that seen with the human protein (I_{40}, 24.8 μM). When O^6-BzlG was present in an oligonucleotide, the differential effect on the murine, human and *ogt*-encoded ATases was not seen and only the *ada*-encoded ATase remained refractory under the conditions used.

INTRODUCTION

O^6-Alkylguanine-DNA alkyltransferase (ATase, EC 2.1.1.63; methylated-DNA–protein-cysteine methyltransferase) repairs the mutagenic and toxic DNA lesion, O^6-alkylguanine in DNA by a stoichiometric and autoinactivating reaction involving the transfer of the alkyl group from the DNA to a cysteine residue in the protein (Lindahl et al., 1988; Tano et al., 1990). ATases have been identified in prokaryotes and eukaryotes, and cDNA from several diverse sources have been identified and cloned (see Santibanez-Koref et al., 1992). Examination of the amino acid sequences of these proteins reveals their homologous nature, with a core region of about 70 amino acids encompassing a highly conserved pentet containing the reactive cysteine residue (Santibanez-Koref et al., 1992).

The demonstration that many tumour cells in culture and tumour samples in general have elevated levels of ATase (D'Incalci et al., 1988), which could moderate the toxic effect of methylating and chloroethylating chemotherapeutic agents, has encouraged the search for ATase inhibitors that could be used in conjunction with such agents to potentiate their cytotoxicity. Research in this area has centred on alkylated analogues of guanine, which are thought to act as low-molecular-mass substrates, thereby inactivating the ATase by the irreversible transfer of the alkyl group to the protein. Of inhibitors of this type, O^6-benzylguanine (O^6-BzlG) has been found to be the most potent inactivator of ATase and has been shown to deplete ATase activity in human tumour cells, human ATase transfected *Xeroderma pigmentosum* cells and tumour xenografts in mice (Baer et al., 1993; Dolan et al., 1990a,b, 1991; Friedman et al., 1992; Mitchell et al., 1992; Moschel et al., 1992). When compared with O^6-methylguanine (O^6-MeG), approx. 2000-fold less O^6-BzlG was required to produce 50 % inactivation in HT29 cells (Moschel et al., 1992). This observed difference in the reactivity of O^6-benzyl- and O^6-methyl-guanine is consistent with the protein's proposed mode of action, which envisages a bimolecular displacement (S_N2) reaction (Dolan et al., 1990a; Moschel et al., 1992).

In this report we consider the reaction of O^6-BzlG, both as free base and contained in an oligonucleotide, with a number of highly purified ATases as part of a comprehensive biochemical comparison of substrate specificity. We show for the first time that a higher concentration of O^6-BzlG is required to inactivate the murine ATase than other mammalian ATases, but that this difference is not seen when O^6-BzlG is present in a double-stranded oligonucleotide. Of the two *Escherichia coli* ATases, only the *ogt*-encoded protein is inactivated by O^6-BzlG when present as the free base or in an oligonucleotide. A preliminary report on some of this work has been presented (Elder et al., 1993).

MATERIALS AND METHODS

Reagents

O^6-BzlG and the oligodeoxynucleotide duplexes used in this study (Pauly et al., 1988) were generous gifts from Dr. R. C. Moschel, National Cancer Institute, Frederick, MD 21702, U.S.A. Stock solutions of O^6-BzlG (5 mM) were made up in 100 mM HCl and stored at −20 °C for up to 3 months. The 16-mer oligonucleotide duplexes were of the following sequence: 5′-d(GTGGGCGCTGXAGGCG)-3′, where X represents guanine, O^6-MeG or O^6-BzlG (Pauly et al., 1988). The [^{3}H]methylated DNA substrate was prepared by reaction of *N*-[^{3}H]methyl-*N*-nitrosourea (Amersham International) with deproteinized calf thymus DNA (Morten and Margison, 1988).

Purification of recombinant ATases

The cDNA cloning and overexpression of the human (Fan et al., 1991), rat (Potter et al., 1991), murine (Santibanez-Koref et al., 1992), Chinese hamster (Rafferty et al., 1992), *ogt*-encoded (Ogt,

Abbreviations used: O^6-BzlG, O^6-benzylguanine; O^6-MeG, O^6-methylguanine; ATase, O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63; methylated-DNA–protein-cysteine methyltransferase); I_{40}, the concentration of O^6-BzlG required to produce a 40 % reduction in ATase activity.

* To whom correspondence should be addressed.

Potter et al., 1987) and *ada*-encoded (Ada, Brennand and Margison, 1986; Margison et al., 1985) ATases have been reported previously. Purification of the recombinant proteins was achieved by affinity chromatography through a DNA–cellulose column as described previously (Wilkinson et al., 1989, 1990). Protein samples were assessed by Coomassie Blue staining after SDS/PAGE and regularly found to be greater than 95% pure. ATase proteins retained activity for more than 1 year when stored at high concentration at −20 °C in buffer I [50 mM Tris/HCl (pH 8.3), 3 mM dithiothreitol, 1 mM EDTA] and could be thawed and refrozen several times without substantial loss of activity.

Cell culture and preparation of extracts

Murine embryo fibroblast (3T6) cells were grown in minimal essential medium supplemented with 10% fetal calf serum. Raji (Burkitt's lymphoma) cells were grown in suspension culture in RPMI medium supplemented with 10% horse serum. Cell pellets were resuspended in cold (4 °C) buffer I containing 2 μg/ml leupeptin and sonicated for 10 s at 12 μm peak-to-peak distance. After cooling in ice, the cells were sonicated for a further 10 s at 18 μm peak-to-peak distance. Immediately after sonication, 0.01 vol. of 8.7 mg/ml phenylmethanesulphonyl fluoride in ethanol was added and the sonicates were centrifuged at 15000 ***g*** for 10 min at 4 °C to pellet cell debris. The supernatant was kept for determination of ATase activity (see below).

Incubation with O^6-BzlG and ATase assay

ATase proteins to be tested were first titrated in order than 60–75 fmol of active protein could be added to each assay point. In each assay, fixed amounts of ATase were incubated with various amounts of O^6-BzlG in a total volume of 200 μl of buffer I containing 1 mg/ml BSA and 10 μg of calf thymus DNA at 22 °C for 2 h, unless otherwise stated. Under these conditions no loss of activity was observed in any of the ATase samples tested when incubated in the absence of O^6-BzlG. The [^{3}H]methylated-DNA substrate (100 μl; 6.7 μg of DNA containing 100 fmol of O^6-MeG) was added, and incubation was continued at 37 °C for 1 h, until the reaction was completed. After acid hydrolysis of the DNA as previously described (Morten and Margison, 1988) the [^{3}H]methylated protein was recovered and quantified by liquid-scintillation counting. Samples were typically assayed in duplicate and experiments repeated several times.

Oligonucleotides were dissolved in buffer I, heated at 60 °C and cooled slowly to allow annealing.

In order to compare the effects on different ATases, the results are expressed as the percentage activity remaining after preincubation with either O^6-BzlG or an oligonucleotide. The amounts of ATase proteins used varied by up to 15% from experiment to experiment but these small changes made no detectable difference to the observed I_{40} values.

RESULTS

The effect of preincubation of the human, rat, murine and Chinese hamster ATases with various amounts of O^6-BzlG on residual ATase activity measured by [^{3}H]methyl transfer from [^{3}H]methylated substrate DNA is shown in Figure 1. The murine ATase was more resistant to inactivation by O^6-BzlG and required a 4–5-fold higher concentration to elicit 40% inactivation (Table 1). Similarly, on incubation with O^6-BzlG for various times at a concentration that produced 40% inactivation of the human ATase after 2 h, the murine protein exhibited a much lower rate of reaction than the other mammalian proteins which were indistinguishable in their rates of inactivation (Figure 2).

To determine if the differences observed for the murine ATase were due to some inherent property of the recombinant protein, extracts of murine (3T6) and human (Raji) cells expressing endogenous ATase were assayed after incubation with O^6-BzlG. Figure 3 shows the inactivation profile of the endogenous and recombinant human ATases which are essentially the same, with an I_{40} of 0.06–0.08 μM. The murine protein has a significantly higher I_{40} for O^6-BzlG (0.28–0.42 μM), indicating that the endogenous and recombinant proteins have similar properties, although the I_{40} was reproducibly higher with the 3T6 extracts than for the recombinant protein (Figure 3).

The effect of preincubation of the *E. coli* Ada and Ogt proteins with O^6-BzlG is shown in Figure 4. Whereas the ATase activity

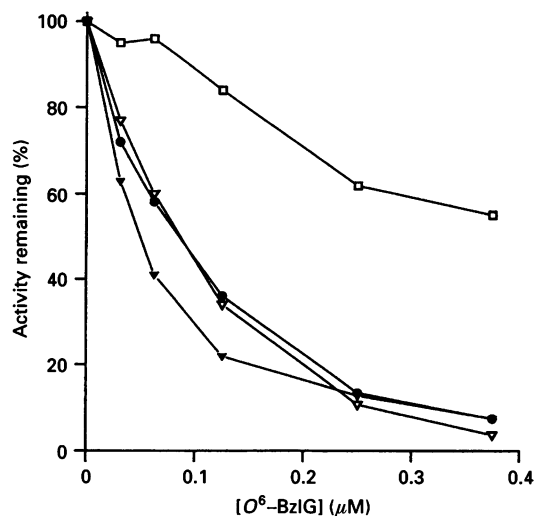

Figure 1 Effect on the residual ATase activity of preincubation of recombinant murine (□), rat (▽), human (●) and Chinese hamster (▼) ATases with increasing concentrations of O^6-BzlG

Duplicate samples were assayed as described in the Materials and methods section.

Table 1 Comparison of O^6-BzlG inactivation of mammalian and *Escherichia coli* ATases

Data are expressed as the concentration, in μmol/l of O^6-BzlG required to produce a 40% reduction in ATase activity, after preincubation at 22 °C for 2 h. Values for the molar ratio are based on 70 fmol of ATase.

ATase	I_{40} (μM)	Molar ratio (BzlG/ATase)
Human	0.06	170
Rat	0.06	170
Chinese hamster	0.04	115
Murine	0.28	800
Ogt	24.8	71000
Ada	> 125	> 360000
3T6	0.42	1200
Raji	0.06	170

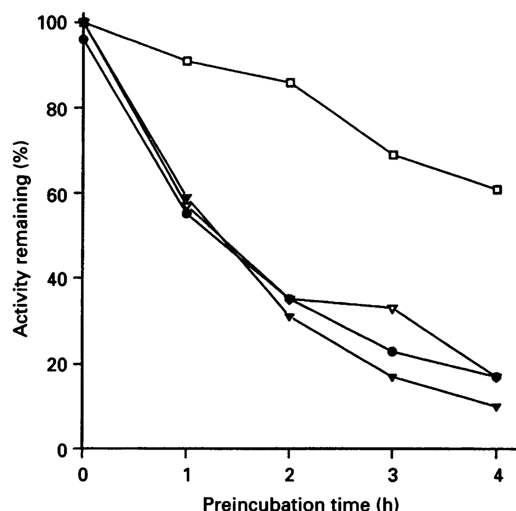

Figure 2 Rate of inactivation of recombinant murine (□), rat (▽), human (●) and Chinese hamster (▼) ATases by O^6-BzlG as measured by residual ATase activity

Duplicate samples were incubated with O^6-BzlG for different periods at 22 °C before determination of ATase activity as described in the Materials and methods section.

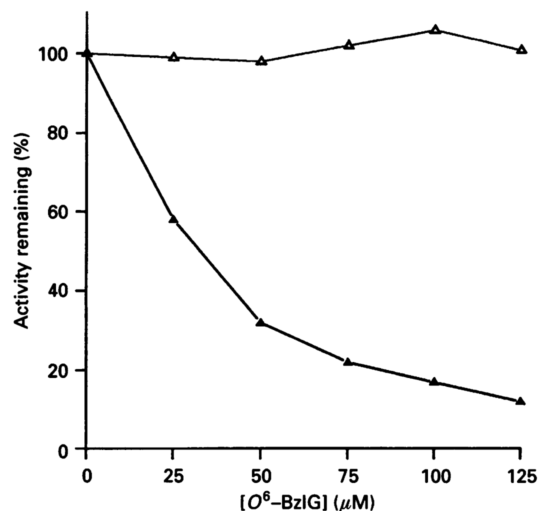

Figure 4 Effect on the residual ATase activity of the *E. coli* Ada (△) and Ogt (▲) ATases of preincubation with increasing concentrations of O^6-BzlG

Duplicate samples were assayed as described in the Materials and methods section.

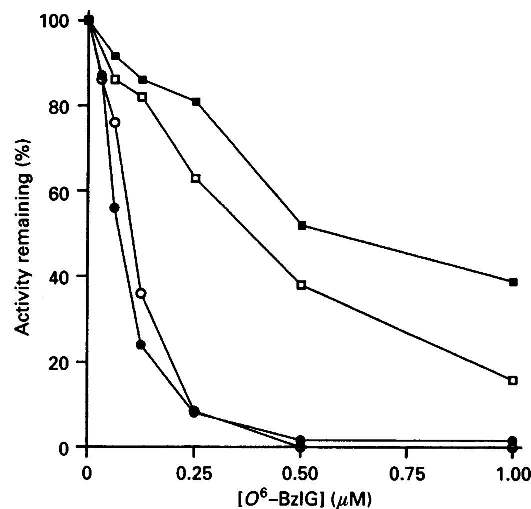

Figure 3 Effect on the residual ATase activity of extracts of murine 3T6 (■) and Raji (○) cells and recombinant human (●) and murine (□) ATases of preincubation with increasing concentrations of O^6-BzlG

Duplicate samples were assayed as described in the Materials and methods section.

of Ada was not affected by O^6-BzlG, the Ogt protein was inactivated, albeit at concentrations approximately 400-fold greater than those required to produce similar inactivation of the human protein (Table 1).

In order to assess the effect of preincubation of the ATases with O^6-BzlG when incorporated into double-stranded DNA, short (16-mer) oligonucleotide duplexes containing either O^6-MeG or O^6-BzlG at a single site in one strand were used. Oligonucleotides which contained unmodified guanine at the appropriate site, but which otherwise had the same nucleotide sequence, were used as controls and these produced no inactivation of any of the ATases over the concentration range used (results not shown).

Reactions of the human and murine ATases with both the O^6-MeG and O^6-BzlG-containing oligonucleotides were indistinguishable, resulting in very similar dose–responses (Figure 5a and 5b) and in the complete inactivation of the ATase activity at the highest level of oligonucleotide added (275 fmol).

Both of the *E. coli* proteins were inactivated by the oligonucleotide containing O^6-MeG to an extent that was closely similar to that for the mammalian ATases. However, the reaction with the O^6-BzlG-containing oligonucleotide differed: whereas preincubation of Ogt with the O^6-BzlG-containing oligonucleotide resulted in a loss of activity similar to that seen for the mammalian proteins (Figure 5c), Ada retained all of its original activity after incubation with 275 fmol of the O^6-BzlG-containing oligonucleotide (Figure 5d).

In summary, the rate of reaction of the ATases with O^6-BzlG when present as a free base clearly differs from that when it is incorporated in duplex DNA. Under the experimental conditions used, 40 % inactivation of the human ATase by O^6-BzlG requires a 170-fold molar excess of the inhibitor and this increases to 800-fold for the murine, and 71000-fold for Ogt, whereas Ada is not affected by the free base (Table 1). However, with the exception of the Ada protein, when incorporated in a short double-stranded oligonucleotide, near stoichiometric amounts of O^6-BzlG are sufficient to achieve similar levels of inhibition under the same incubation conditions.

DISCUSSION

The isolation, expression in *E. coli* and purification of several mammalian and bacterial ATases was undertaken in order to compare the biochemical characteristics of these proteins. In the present report we have investigated the ability of O^6-BzlG, both as a free base and when present in a short oligonucleotide duplex, to inactivate recombinant ATases.

The use of alkylating agents in certain chemotherapy regimens (D'Incalci et al., 1988; Lee et al., 1991, 1993a,b) has focused

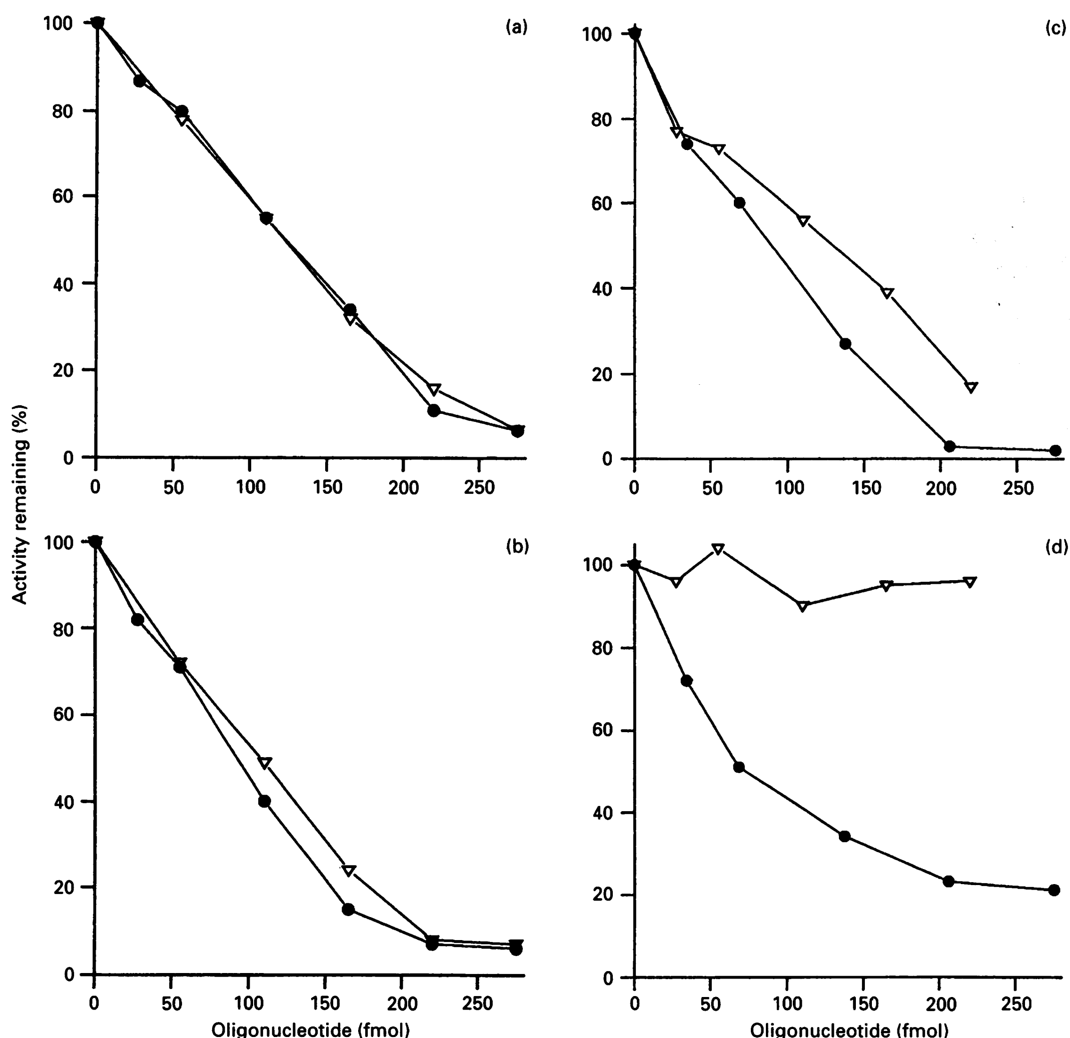

Figure 5 Effect on the activity of recombinant ATases of preincubation with oligonucleotides containing O^6-MeG (●) or O^6-BzlG (▽)

Duplicate samples of 70 fmol of recombinant human (**a**), murine (**b**), Ogt (**c**) and Ada (**d**) were incubated with increasing concentrations of the appropriate oligonucleotide, before measurement of the residual ATase activity as described in the Materials and methods section.

attention on the human protein for the prospects of modulating the effect of such treatments. The fact that many tumours have elevated levels of ATase activity, which may render them resistant to the toxic effects of such agents, combined with the auto-inactivating nature of the reaction, has led to the search for drugs able to inhibit, and/or deplete the ATase activity in cells. O^6-MeG, as a free base, is a relatively poor substrate for both mammalian and *E. coli* proteins (Moschel et al., 1992). However, it has recently been shown that O^6-BzlG can deplete ATase activity in human tumour cell lines or xenografts and in CHO cells transfected with the cDNA for human ATase (Baer et al., 1993; Dolan et al., 1990a,b, 1991; Friedman et al., 1992; Mitchell et al., 1992; Moschel et al., 1992).

The experiments described here show that human, rat and Chinese hamster ATases are inactivated to the same extent by O^6-BzlG but that the murine protein differs from the other mammalian proteins in that it is relatively more resistant to inactivation by O^6-BzlG. However, this difference is not apparent when the benzylated base is present in double-stranded DNA. The two *E. coli* ATase proteins can also be differentiated by their inactivation by O^6-BzlG, the Ada ATase being refractory and depletion of the Ogt activity occurring only at concentrations 400-fold greater than those required to inactivate the mammalian ATases. This differential between Ogt and the mammalian proteins was not observed when O^6-BzlG was present in a short oligonucleotide duplex: whereas a large excess of free base was required to inhibit even the most sensitive ATase, near-stoichiometric concentrations of modified oligonucleotide were sufficient to cause substantial ATase inactivation under the same experimental conditions.

With the exception of the human and Ada ATases, previous investigations on the effectiveness of O^6-BzlG in depleting activity have been carried out on cellular extracts, or partially purified proteins. This study is, to our knowledge, the most wide ranging and the first carried out with essentially pure proteins. The results indicate that the bacterial proteins are not suitable models for testing ATase inhibitors of this type to be used in mammalian systems. However, that Ada is refractory and Ogt is less susceptible to inactivation by O^6-BzlG may be of potential use in gene therapy which could, by overexpression of one or other of the *E. coli* ATases in haematopoietic cells (Jelinek et al., 1988), achieve protection not only against the toxic effects of temo-

zolomide and related agents but also against the sensitization to such agents that O^6-BzlG produces on inactivation of the human ATase (L. Fairbairn, A. J. Watson, J. A. Rafferty, R. H. Elder and G. P. Margison, unpublished work).

The finding that the murine ATase is more resistant to inactivation by O^6-BzlG is interesting given the homologous nature of the mammalian proteins, especially amongst the rodents. Although the observed difference might be due to a single amino acid change, the murine ATase peptide sequence differs from those of the other three mammalian proteins at seven positions and combinations of these differences might be responsible for the observed effects. On the other hand, two of the differences are in the C-terminal region which we have shown not to be essential for ATase functional activity (Elder et al., 1992), and, of the rest, in only one case is the amino acid residue in the other three proteins identical. Site-directed mutagenesis of this or other residues may help to elucidate the structural features that are important in the action of these proteins.

The differential inactivation of human and murine ATase may also have important implications for studies of the effect of O^6-BzlG on the sensitivity to methylating or chloroethylating agents of human tumour xenografts in mice (Friedman et al., 1992; Mitchell et al., 1992). Any ATase-inactivation-mediated increases in the cytotoxic effects of the alkylating agents in normal murine tissues might not be as extensive as in the same human tissues and thus changes in the chemotherapeutic effectiveness of such combinations might be non-representative of effects in cancer patients.

This work was supported by the Cancer Research Campaign.

REFERENCES

Baer, J. C., Freeman, A. A., Newlands, E. S., Watson, A. J., Rafferty, J. A. and Margison, G. P. (1993) Br. J. Cancer **67**, 1299–1302
Brennand, J. and Margison, G. P. (1986) Carcinogenesis **7**, 2081–2088
D'Incalci, M., Citti, L., Taverna, P. and Catapano, C. V. (1988) Cancer Treat. Rev. **15**, 279–292
Dolan, M. E., Moschel, R. C. and Pegg, A. E. (1990a) Proc. Natl. Acad. Sci. U.S.A. **87**, 5368–5372
Dolan, M. E., Stine, L., Mitchell, R. B., Moschel, R. C. and Pegg, A. E. (1990b) Cancer Commun. **2**, 371–377
Dolan, M. E., Mitchell, R. B., Mummert, C., Moschel, R. C. and Pegg, A. E. (1991) Cancer Res. **51**, 3367–3372
Elder, R. H., Tumelty, J., Douglas, K. T., Margison, G. P. and Rafferty, J. A. (1992) Biochem. J. **285**, 707–709
Elder, R. H., Margison, G. P. and Rafferty, J. A. (1993) Proc. Am. Assoc. Cancer Res. **34**, 140
Fan, C.-Y., Potter, P. M., Rafferty, J. A., Watson, A. J., Cawkwell, L., Searle, P. F., O'Connor, P. J. and Margison, G. P. (1991) Nucleic Acids Res. **18**, 5723–5727
Friedman, H. S., Dolan, M. E., Moschel, R. C., Pegg, A. E., Felker, M., Rich, J., Bigner, D. D. and Schold, C. S., Jr. (1992) J. Natl. Cancer Inst. **84**, 1926–1931
Jelinek, J., Kleibl, K., Dexter, T. M. and Margison, G. P. (1988) Carcinogenesis **9**, 81–87
Lee, S. M., Thatcher, N. and Margison, G. P. (1991) Cancer Res. **51**, 619–623
Lee, S. M., Thatcher, N., Dougal, M. and Margison, G. P. (1993a) Br. J. Cancer **67**, 216–221
Lee, S. M., Margison, G. P., Woodcock, A. A. and Thatcher, N. (1993b) Br. J. Cancer **67**, 1356–1360
Lindahl, T., Sedgwick, B., Nakabeppu, Y. and Sekiguchi, M. (1988) Annu. Rev. Biochem. **57**, 133–157
Margison, G. P., Cooper, D. P. and Brennand, J. (1985) Nucleic Acids Res. **13**, 1939–1952
Mitchell, R. B., Moschel, R. C. and Dolan, M. E. (1992) Cancer Res. **52**, 1171–1175
Morten, J. E. N. and Margison, G. P. (1988) Carcinogenesis **9**, 45–49
Moschel, R. C., McDougall, M. G., Dolan, M. E., Stine, L. and Pegg, A. E. (1992) J. Med. Chem. **35**, 4486–4491
Pauly, G. T., Powers, M., Pei, G. K. and Moschel, R. C. (1988) Chem. Res. Toxicol. **1**, 391–397
Potter, P. M., Wilkinson, M. C., Fitton, J., Carr, F. J., Brennand, J., Cooper, D. P. and Margison, G. P. (1987) Nucleic Acids Res. **15**, 9177–9193
Potter, P. M., Rafferty, J. A., Cawkwell, L., Wilkinson, M. C., Cooper, D. P., O'Connor, P. J. and Margison, G. P. (1991) Carcinogenesis **12**, 727–733
Rafferty, J. A., Elder, R. H., Watson, A. J., Cawkwell, L., Potter, P. M. and Margison, G. P. (1992) Nucleic Acids Res. **20**, 1891–1895
Santibanez-Koref, M., Elder, R. H., Fan, C. Y., McKie, J. H., Douglas, K. T., Margison, G. P. and Rafferty, J. A. (1992) Mol. Carcinogenesis **5**, 161–169
Tano, K., Shiota, S., Collier, J., Foote, R. S. and Mitra, S. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 686–690
Wilkinson, M. C., Potter, P. M., Cawkwell, L., Georgiadis, P., Patel, D., Swann, P. F. and Margison, G. P. (1989) Nucleic Acids Res. **17**, 8475–8484
Wilkinson, M. C., Cooper, D. P., Southan, C., Potter, P. M. and Margison, G. P. (1990) Nucleic Acids Res. **18**, 13–16

Received 28 July 1993/24 September 1993; accepted 28 September 1993