Contribution of ogt-encoded alkyltransferase to resistance to chloroethylnitrosoureas in nucleotide excision repair-deficient Escherichia coli

Nieves Abril, Francisco Ferrezuelo, María-José Prieto-Alamo, Joseph A. Rafferty1, Geoffrey P. Margison1 and Carmen Pueyo2

Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, 14071-Córdoba, España and 1CRC Department of Carcinogenesis, Paterson Institute for Cancer Research, Christie Hospital (NHS) Trust, Manchester M20 9BX, UK

1To whom correspondence should be addressed

We investigated the relative contribution of the two Escherichia coli DNA alkyltransferases (ATases) to the increased sensitivity of ATase-deficient bacteria to the mutagenic and lethal effects of chloroethylnitrosoureas (CNU). The ogt-encoded protein was the principal determinant in resistance to the mutagenic effects of CNU in E. coli. Thus, only when the ogt gene was inactivated was sensitivity to mutagenesis greatly increased; the contribution of inactivation of the ada gene was relatively minor. Furthermore, induction of the adaptive response provided essentially no protection against CNU mutagenesis in either an ogt+ or ogt− background. Finally, overexpression of the ogt gene into ogt− ada− double mutants provided the greatest protection against CNU; introduction of the full-length or truncated ada gene was protective, but to a much lesser extent. Mammalian ATases were not as protective against mutation induction by CNU as Og t, even though they were apparently expressed at higher level. In order of effectiveness the ATases ranked Ogt > human > truncated Ada = Ada > rat. This order was not observed in the protection against killing by 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, where truncated Ada = human > Ogt > rat = Ada. Higher mutation frequency and toxicity were observed in uv− mutants, suggesting that one or more of the potentially mutagenic and/or toxic lesions are also substrates for the excision repair proteins.

Introduction

Chloroethylnitrosoureas (CNU*) are active anti-cancer agents that are being used for treatment of Hodgkin’s and non-Hodgkin’s lymphomas, tumors of the brain, gastrointestinal tract and lung and some other neoplastic diseases. Two representatives of the group are 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine, CCNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU). BCNU was introduced for the treatment of human tumors is a consequence of their ability to modify DNA. CNU are highly reactive compounds that break down by a pathway similar to that described for alkylating agents, to produce the chloroethyl carbonium ion, a bifunctional alkylating species (3). This generates DNA monoadducts, as do monofunctional alkylating agents, but also cyclized derivatives and DNA inter- and intrastrand cross-links (4).

The lethal effects of CNU are thought to be mediated by DNA interstrand cross-links that are formed through the initial alkylation of the O6-position of guanine (4,5). This rapid step is followed by the formation of cyclic O6,N7-ethanoguanine and cross-linking from the N3 position of guanine to the N7 position of a cytosine residue on the opposite strand. DNA repair by mammalian O6-alkylguanine-DNA alkyltransferase (ATase) can attenuate the cytotoxic effects of CNU by recognizing and removing the chloroethyl group from the O6 position of guanine and hence preventing the subsequent cyclization and guanine–cytosine interstrand cross-linking (6). In so doing, the ATase is inactivated and the extent of repair that occurs in a cell is therefore dependent on the amount of repair protein present and the de novo synthesis rate. Another reaction that can occur is the covalent binding of the ATase to the cyclic guanine derivative, but the biological significance of this ATase–DNA complex is not yet established (7,8).

Escherichia coli has two different ATases: the constitutively expressed 19 kDa ogt gene product (9) and the inducible 39 kDa ada gene product (10–12), which is composed of two domains of 18 and 20 kDa respectively. The ogt-encoded protein (Ogt) mediates the irreversible transfer of alkyl groups from O6-alkylguanine and also O4-alkylthymine to a specific cysteine residue in the alkyltransferase itself. The 18 kDa domain of the ada-encoded ATase (Ada) has a similar repair function. However, Ada has an additional active site cysteine residue in the 20 kDa domain that transfers alkyl groups from alkylphosphothriesters. Once alkylated at this position, Ada becomes a positive regulator that induces expression of the ada−alkB operon and other genes during the adaptive response that is initiated by methylating agents (12,13). In wild type, non-adapted E. coli the predominant ATase is that encoded by ogt (14–16).

Kinetic experiments with synthetic oligonucleotides containing modified bases have revealed important differences between the two bacterial DNA ATases. Thus, Ogt has been found to be 173 times faster than Ada in removing O6-ethylguanine, both repair proteins acting, however, at approximately the same rate on O6-methylguanine (17). In agreement with such in vitro substrate specificity studies, we have demonstrated that inactivation of the ogt gene sensitizes the bacterial cells to mutagenesis by ethylating (18) and also propylating compounds (19). In comparison, sensitization to

*Abbreviations: CNU, chloroethylnitrosoureas; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ATase, DNA alkyltransferase; MNG, N-nitroso-N'-methyl-N-nitrosoguanidine; EMS, ethyl methanesulfonate; Ara, L-arabinose.
methylating agents was particularly marked in the absence of the Ada protein (18,20). The *E. coli* ATases also show gross structural differences from their mammalian counterparts (21), suggesting potential differences in substrate specificity. Indeed, it has recently been shown that repair of $O^6$-alkylthymine is not ATase mediated but excision repair-dependent in human cells (22).

We recently demonstrated that *ogt*<sup>-</sup> *ada*<sup>-</sup> null mutants had increased sensitivity to both the mutagenic and lethal effects of CCNU. The mutation spectra induced by CCNU in the *lacI* gene of bacteria proficient (*ogt*<sup>+</sup> *ada*<sup>+</sup>) or deficient (*ogt*<sup>-</sup> *ada*<sup>-</sup>) ATases were obtained. All genetic manipulations and strain constructions were performed as described by Curtis (31). Media were according to Davis (32).

### Materials and methods

**Bacterial strains and plasmids**

The *E. coli* K-12 strains and the plasmids used in this study are described in Table I. UC8310 (*uvr<sup>-</sup>*), UC755 (*ogt*) and UC838 (*uvr<sup>-</sup>* *ogt*) were previously isolated in our laboratory (24,25). UC1033 (used as wild type), UC1012 (*uvr<sup>-</sup>* *ada*<sup>-</sup>) and UC1011 (*uvr<sup>-</sup>* *ogt*<sup>-</sup> *ada*<sup>-</sup>) are derivative strains constructed by P1vir-mediated transduction. UC978 is equivalent to UC1011 except for the *ogt* deficiency; UC978 and *ogt*- strains are isogenic for *lacI* disruption mutation and *uvr<sup>-</sup>* and *ata*<sup>-</sup> deletion. Strains overproducing bacterial (UC1063, UC1065 and UC1095) or recombinant rat (UC1094) or human (UC1089) ATases were isolated by transformation of UC978 with the plasmids listed in Table I. Control strains (UC1064 and UC1086) carrying cloning vectors were also obtained. All genetic manipulations and strain constructions were performed as described by Curtis (31). Media were according to Davis et al. (32).

### Mutagens

CCNU was purchased from Aldrich (Milwaukee, WI). BCNU was kindly provided by Bristol-Myers Squibb Co. (Evansville, IN). N-nitroso-N'-methyl-N-nitrosoguanidine (MNNG) and ethyl methanesulfonate (EMS) were from Sigma (St Louis, MO). Mutagens were dissolved in *N,N*-dimethylformamide (CCNU), absolute ethanol (BCNU) or dimethyl sulfoxide (MNNG and EMS). Solvents were from Merck (Darmstadt, Germany).

**Mutagenesis and survival assays**

Mutagenesis was assayed by means of the so-called L-arabinose (Ara) resistance test (33,34). The Ara test uses a strain mutated in gene *araD* (Ara<sup>r</sup>) as an indicator of mutagenicity and selects forward mutations to L-arabinose resistance (Ara<sup>r</sup>) in a medium containing L-arabinose and a carbon source (glycerol) which is unable to repress *araDAB* operon expression. The mutagenesis protocol was carried out basically as previously described (34,35). Briefly, bacteria were grown at 37°C for 12 h with shaking (90 r.p.m.) in LB nutrient medium (32). Cells were then harvested by centrifugation and resuspended in 0.2 M phosphate buffer, pH 7.4. An aliquot of bacterial suspension (containing ~4×10<sup>8</sup> cells) and the mutagen to be tested were preincubated at 37°C for 40 min with shaking (90 r.p.m.) in 1 ml phosphate buffer. Aliquots of 0.25 ml (~10<sup>7</sup> bacteria) were then combined in 2 ml molten top agar containing a trace amount of 0.5 mg D-glucose, for full expression of Ara<sup>r</sup> mutants (33), and the contents poured on selective plates. Pre-incubations with the mutagen solvent were included for spontaneous counts. Selective plates for Ara<sup>r</sup> mutants were as follows: VB salts (32) containing Difco agar (17 g/l), L-arabinose (2 g/l), glycerol (2 g/l), thiamine (5 mg/ml), nicotinic acid (5 mg/ml) and adenine (100 mg/ml). Bacterial colonies were counted automatically (Analytical Measuring System Ltd, UK; model 40-10). All data represent averages from at least two duplicate plates. Each mutagenesis assay was repeated on at least two separate occasions using a wide range of mutagen concentrations. A treatment was considered mutagenic when a dose-response curve was produced and when more than twice the spontaneous background number of mutants were induced at, at least, one dose. For survival determinations, aliquots of 0.1 ml were withdrawn from the pre-incubation mixtures after 40 min exposure. Bacteria were then diluted in VB salts and plated in LB nutrient agar. Strains with plasmids were grown in the presence of ampicillin (50 mg/ml).

### Results

The *E. coli uvrB<sup>-</sup>* strain (UC8310) was more sensitive to both the mutagenic effects, as measured by the induction of forward mutations to L-arabinose resistance (Ara<sup>r</sup>), and the lethal effects of CCNU than was the *uvrB<sup>-</sup>* wild-type (UC1033) (Figure 1). This agrees with previous studies in which *uvrA* mutant
contribution of the UvrABC excision pathway to the repair of DNA damage induced by CNU and their biological effects was circumvented by using *E. coli* carrying a Δ(uvrB-bio) mutation.

In such a *uvr* - genetic background, *E. coli ada* - mutants (UC1012) had no or only a slight increase in susceptibility to mutagenesis by either CCNU or BCNU (Figure 2). In contrast, *ogt* deletion (UC838) caused a marked increment in mutation induction by these compounds. An additional *ada* - mutation to produce *ogt* - *ada* - double mutants (UC1011) had relatively little impact on mutation induction by both CNU (Figure 2). Since mutagenesis by CCNU and BCNU was attenuated to the greatest extent by the presence of the *ogt*-encoded ATase, it is not unreasonable to suggest that this DNA repair protein plays the principal role in protection against the mutagenic effect of these agents. The role of both DNA alkytransferases in preventing cytotoxicity by CCNU and BCNU was also assessed (Figure 2). Inactivation of *ada* gene had little or no effect on susceptibility to the lethal effects of CNU; *ogt* - mutants were markedly more sensitive, but *ogt* - *ada* - double mutants showed the greatest sensitivity.

Failure of CNU-induced damages to induce the synthesis of the *ada*-encoded ATase (38) might explain the lack of enhanced sensitivity to CNU mutagenesis of *ada* - bacteria as compared with *ada* + wild-type cells. To exclude this possibility, the role of Ada protein in mutagenesis by CNU was further investigated by adapting exponential phase cells by growth in low concentration of MNNG (Table II), according to a protocol optimized by others (39). As anticipated, the induction of the adaptive response provided extensive protection against the mutagenic effects of a challenging dose of MNNG. In contrast, the mutagenicity of CCNU remained essentially unchanged, either in the presence or the absence of the *ogt*-encoded ATase.

The possibility that Ogt ATase might be inactivated upon CCNU treatment was investigated by measuring the effect of CCNU pre-treatment on mutation induction by EMS (Table

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**Fig. 1. Effects of Uvr excision repair and Ogt ATase on survival and mutation induction by CCNU.** The percentages of survival and the absolute numbers (total minus the corresponding spontaneous values) of *Ara* + induced mutants per selective plate were plotted as function of the tested dose of mutagen. The spontaneous background numbers of mutants were 165 ± 40, 216 ± 42, 120 ± 57 and 130 ± 30 *Ara* + mutants/10^7 bacteria plated for the *uvr* + *ogt* + (UC1033, ◦), *uvr* + *ogt* - (UC8310, ○), *uvr* + *ogt* - (UC575, △) and *uvr* - *ogt* - (UC838, □) strains respectively.

**Fig. 2. Comparative effects of Ogt and Ada ATases on survival and mutation induction by CCNU and BCNU.** The percentages of survival and the absolute numbers (total minus the corresponding spontaneous values) of *Ara* + induced mutants per selective plate were plotted as a function of the tested dose of mutagen. The spontaneous background numbers of mutants were 216 ± 42, 264 ± 4, 154 ± 11 and 198 ± 18 *Ara* + mutants/10^7 bacteria plated for the *ogt* + *ada* + (UC8310, ◦), *ogt* + *ada* - (UC838, □), *ogt* + *ada* + (UC1012, △) and *ogt* + *ada* - (UC1011, ○) strains respectively.

**Fig. 3. Comparative effects of bacterial and mammalian ATases on survival and mutation induction by CCNU.** The percentages of survival and the absolute numbers (total minus the corresponding spontaneous values) of *Ara* + induced mutants per selective plate were plotted as a function of the tested dose of mutagen. The spontaneous background numbers of mutants were 376 ± 51, 263 ± 49, 412 ± 106, 472 ± 122, 307 ± 72 and 253 ± 8 *Ara* + mutants/10^7 bacteria plated for bacteria carrying the plasmid pUC8.1 (UC1086, ◦), pUCOgt (UC1063, ◦), pSV2ada (UC1065, △), pTada (UC1095, ◦), pUC8.1AT (UC1094, △) and pUC8.1hAT (UC1099, □) respectively. Plasmids are in the genetic background of UC978 (*ogt* + *ada* -). The *ogt* gene is cloned into pUC19, the *ada* gene into pSV2 and the truncated version of *ada* and the cDNA mammalian sequences into pUC8.1. Data are referred to control strain with pUC8.1, since other control bacteria gave similar results.
Table II. Effect of pre-adaptation with MNNG on the mutagenicity by CCNUa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Ara' mutants/107 bacteriab</th>
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<tbody>
<tr>
<td></td>
<td>UC8310 (ogt')</td>
</tr>
<tr>
<td></td>
<td>Unadapted</td>
</tr>
<tr>
<td></td>
<td>Adapted</td>
</tr>
<tr>
<td>None MNNG</td>
<td>246</td>
</tr>
<tr>
<td>34 nmol CCNU</td>
<td>16 190 (15 944)</td>
</tr>
<tr>
<td>107 nmol CCNU</td>
<td>1327 (1081)</td>
</tr>
<tr>
<td>856 nmol CCNU</td>
<td>2490 (2244)</td>
</tr>
</tbody>
</table>

aThe adaptive response was induced by growing the bacteria in minimal medium in the presence of 0.1 μg/ml MNNG for 4 h at 32°C. Unadapted and adapted bacteria (~4×10^9 cells) were treated in VB salts with challenge doses of MNNG for 5 min at room temperature or CCNU for 40 min at 37°C. Around 10^7 bacteria were spread per selective plate.
bFor untreated, unadapted bacteria, number of spontaneous Ara' mutants/10^7 bacteria; for untreated, adapted bacteria, number of Ara' mutants (spontaneous + induced during MNNG pre-adaptation)/10^7 bacteria. For treated, unadapted bacteria, number of Ara' mutants (spontaneous + induced by the mutagen)/10^7 bacteria, with the induced value in parentheses; for treated, adapted bacteria, number of Ara' mutants (spontaneous + induced during MNNG pre-adaptation + induced by the mutagen)/10^7 bacteria, with the induced value by mutagen value in parentheses.

Table III. Effect of CCNU pre-treatment on mutagenicity by EMSa

<table>
<thead>
<tr>
<th>Mutagenic treatments</th>
<th>Number of Ara' mutants/107 bacteriab</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNU (nmol)</td>
<td>EMS (μmol)</td>
</tr>
<tr>
<td></td>
<td>UC1012 (ogt' ada')</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>107</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
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<td>0</td>
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<td>107</td>
<td>10</td>
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<td>0</td>
<td>107</td>
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<td>0</td>
<td>107</td>
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</table>

aCCNU treatment was as described in Materials and methods. After CCNU treatment, bacteria were harvested by centrifugation and resuspended in 0.5 ml 0.2 M phosphate buffer, pH 7.4. This bacterial suspension (containing ~8×10^7 cells) was subsequently treated with EMS for 20 min at 37°C. Aliquots of 0.125 ml (~10^7 bacteria) were then spread per selective plate. Controls (0 dose) contained the mutagen solvents.
bNumber of Ara' mutants/10^7 bacteria with the induced value in parentheses.

III. As previously shown (18,19) EMS was considerably more mutagenic in an ogt' strain than in an ogt' strain. Pre-treatment of ogt' bacteria with CCNU greatly increased the sensitivity to mutation induction by EMS, though not to the level caused by inactivating the ogt' gene (Table III).

The ability of Ogt to protect E.coli against the deleterious effects of CNU was considered with that of Ada, a truncated Ada version and the human and rat ATases by introducing multicopy plasmids encoding these proteins into ogt'-ada' double mutants (Figure 3). The Tada version retains the O^-alkylguanine/O^-alkylthymine ATase domain (but not the alkylphosphotriester ATase segment) (40). Comparison between the full-length E.coli 39 kDa Ada protein and the truncated version is mandatory, since important differences have recently been observed in the in vitro repair of O^-ethylguanine substrate by the 39 kDa Ada protein as compared with the catalytic C-terminus alone (41). None of the ATases were as effective as Ogt in protecting against the mutagenic effects of CCNU, even though they were apparently expressed at higher level (Table IV). Indeed, the rat ATase cDNA-transformed E.coli exhibited more than 200 times more ATase activity than the ogt transformed clone but this provided less protection than any of the other ATases, assuming that all of this protein is available for DNA repair in vivo. In order of effectiveness the ATases ranked Ogt > human > truncated Ada = Ada > rat. This order was not observed in the protection against killing by CCNU, where truncated Ada = human > Ogt > rat = Ada (Figure 3).

Discussion

The involvement of mammalian O^-alkylguanine-DNA alkyltransferase in promoting resistance to CNU became apparent from the landmark paper of Erickson et al. (5). This knowledge has been used to increase the sensitivity of resistant mammalian cells by depleting their ATase content. Here we study the contribution of the two E.coli ATases to bacterial cytotoxicity and mutagenesis due to CNU.

The data indicate that of the two E.coli ATases, the ogt'-encoded protein is the principal determinant in resistance to the mutagenic effects of CNU in E.coli. Thus, only when the ogt' gene was inactivated was sensitivity to mutagenesis by both CCNU and BCNU greatly increased; the contribution of inactivation of the ada gene was relatively minor. Since the ogt'-encoded protein accounts for ~30% of ATase molecules in unadapted cells. Here we study the contribution of the two E.coli ATases to bacterial cytotoxicity and mutagenesis due to CNU.
contribution of both ATases was further analyzed by induction of the adaptive response. Adaptation, which produced a very substantial protection against the mutagenic effects of MNNG (thus showing that the response had indeed been induced), provided essentially no protection against CCNU mutagenesis in either an ogt+ or ogt− background. Finally, overexpression of high levels of the full-length or truncated ada+ gene was somewhat protective against mutation induction by CCNU, but to a much lesser extent than overexpression of the ogt+ gene. In fact, bacteria overproducing Ada or Tada retained, on average, ~35% of the mutagenic sensitivity of the parental cells, whereas overexpression of Ogt completely suppresses sensitivity of the ogt− ada− double mutants to CCNU mutagenesis. These results are in agreement with previous data showing that the pre-mutagenic lesions induced by BCNU involve DNA repair mechanisms that are independent of Ada (37).

Protection against the toxic effects of CNU did not follow exactly the same pattern as mutation induction. Thus, ogt− ada− double mutants were more sensitive to the lethal effects of both CCNU and BCNU than ogt− ada+ bacteria, although inactivation of the ada gene in an ogt+ background had little or no influence on susceptibility to killing. In contrast, ogt− single mutants were markedly more sensitive than the corresponding ogt+ parental cells. Furthermore, introduction into ogt− ada− E. coli of plasmids containing the entire ada coding region provided less protection than plasmids containing the ogt gene, but overexpression of the truncated ada gene yielded the highest resistance.

The mechanism of Ogt protection against CNU mutagenesis is likely to be mainly the removal of the chloroethyl group from the O6 position of guanine, thus preventing mismatching with thymine upon DNA replication. Such mismatching is envisaged as the source of GC→AT transitions which are the predominant type of mutations induced in uvr+ bacteria by chloroethylating compounds (23). As the same initial lesions are considered to be principally responsible for the toxic effects of CNU, it might be anticipated that protection against killing would follow that against mutation induction, but this does not appear to actually be the case in these studies.

Around 4% of CCNU-induced mutations are targeted at A:T base pairs in both prokaryotic (23) and eukaryotic cells, where they increase up to 13% with CCNU concentration (44). A:T targeted mutations, but in particular AT→GC transitions, are attributed to a putative O6-chloroethylthymine lesion (44). As the ogt-encoded ATase repairs O6-methylcytosine and longer alkyl chain derivatives of O6-alkylguanine much more efficiently than the ada ATase (17), one possible explanation for the different outcomes that Ogt or Ada deficiency have on mutation and killing by CCNU is that the postulated O6-chloroethylthymine lesion contributes more to mutation than to toxicity. Alternatively, the DNA sequence context in which particular O6-chloroethylguanine lesions are located may be a critical factor in determining their mutability and cytotoxicity and also the repair efficiency by different ATases (23). Indeed, when mammalian ATases were expressed in an ogt− ada− background, apparently very high levels of expression were not as protective against mutation induction as much lower levels of Ogt, so that substrate or sequence context effects may also apply for the mammalian ATases. Such effects may have important implications in chemotherapy, modulating both the therapeutic (cytotoxicity) and the carcinogenic/mutagenic properties of anti-tumor CNU.

Expression of Ogt in endogenous ATase-deficient mammalian cells has been shown to be more effective than expression of Ada in protecting against the growth inhibitory effects of CNU and related agents, including N-ethyl-N-nitrosourea and N-butyl-N-nitrosourea (45). This is consistent with the more efficient repair of the corresponding O6-alkylguanine and/or O6-alkylthymine lesions in DNA. In the view of the present results, it will now be interesting to examine the effects of prokaryotic ATase expression on mutation induction in mammalian cells.

In this study recombinant human ATase overexpressed in ogt− ada− defective bacteria was close to Ogt in suppressing both cytotoxicity and mutagenesis by CCNU, suggesting that this bacterial protein might be a suitable model for extrapolation to situations involving human cells exposed to chloroethylating drugs. Comparatively, rat ATase turned out to be much less efficient than the human counterpart, suggesting functional differences between these two proteins, although they do not show differential sensitivity to in vitro inactivation by O6-benzylguanine (46).

The mechanism of the contribution of the excision repair proteins in CNU-induced mutation and killing processes should also be considered in E.coli. The higher mutation frequency and toxicity in uvr− mutants suggests that one or more of the potentially mutagenic and/or toxic lesions might be substrates for these enzymes. This repair pathway may be particularly relevant to the in vivo fate of the ATase–DNA crosslinks that have been demonstrated in chloroethylated DNA in vitro (7,8). Such complexes might be substrates for the Uvr system and the efficiency of their repair might be influenced by the ATase involved and/or the sequence context in which they are formed. It will therefore be essential to take into account the effectiveness of the equivalent mammalian systems when considering the mechanism of cell killing and mutagenesis by CNU and related agents in the context of cancer chemotherapy.

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that covalent complex formation between BCNU-treated oligonucleotides and \textit{E. coli} alkyltransferases requires the O6-alkylguanine function. \textit{Nucleic Acids Res.}, 18, 3961–3966.


