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Repair of *O*-alkylpyrimidines in mammalian cells: A present consensus

(DNA-O^6-methylguanine methyltransferase/DNA-3-methyladenine glycosylase/O^4-alkylthymine/O^2-alkylcytosine/O^2-alkylthymine)

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ABSTRACT **Enzymatic repair of the *O*-alkylpyrimidines (O^2- and O^4-alkylthymine, O^2-alkylcytosine) and alkyl phosphotriesters has been studied in *Escherichia coli*, and the two proteins involved, a glycosylase (DNA-3-methyladenine glycosylase) and a methyltransferase (DNA-O^6-methylguanine:protein-L-cysteine *S*-methyltransferase, EC 2.1.1.63), have been well characterized. In mammals or mammalian cells treated with carcinogenic alkylating agents, loss of these derivatives has been demonstrated repeatedly. Nevertheless, mammalian repair proteins that are analogous to those from *E. coli* do not detectably act on these alkyl derivatives. A variety of techniques has been used by many investigators in the United States and Europe, who conclude here that the mode of *O*-alkylpyrimidine and alkyl phosphotriester repair in mammalian cells differs from that in *E. coli*. New approaches and methods are needed to characterize these processes at the biochemical and molecular level.**

Many methylating and ethylating agents are potent carcinogens when administered to animals. In particular, *N*-nitroso compounds, such as direct acting methylnitrosourea (MeNU) and ethylnitrosourea (EtNU) and the metabolically activated analogous compounds dimethylnitrosamine and diethylnitrosamine, produce tumors in rats, mice, hamsters, etc. (for reviews, see refs. 1 by Montesano and Bartsch and 2 by Preussman and Stewart). The initial amount of alkylpurines in DNA and their persistence, or half-life ($t_{1/2}$), *in vivo* has been measured repeatedly. For O^6-methylguanine (m^6G) or O^6-ethylguanine (e^6G), the half-life has been found to be a function of dose, species, and tissue and cell type; removal of the alkylpurines has been correlated with the DNA-O^6-methylguanine:protein-L-cysteine *S*-methyltransferase (m^6-MT; EC 2.1.1.63) activity present (for reviews, see refs. 3 by Pegg and 4 by Yarosh). This enzyme has been isolated from a variety of mammalian cells and acts on m^6G in a manner similar to the *Escherichia coli* m^6-MT. That is, the enzyme molecule is inactivated by the transfer of the nucleoside alkyl group to a cysteine sulfhydryl group in the enzyme, and there is apparently no regeneration of the inactivated molecule (for review, see ref. 5 by Lindahl).

In common with *E. coli*, mammalian cells also contain a second activity, DNA-3-methyladenine glycosylase, which can remove *N*-3 and *N*-7 alkylpurines from DNA by catalyzing cleavage of the sugar-base bond (6, 7). Both the m^6-MT and the glycosylase are more efficient in acting on methyl derivatives than on ethyl derivatives (8–10). When O^6-modified propyl-, butyl-, isopropyl-, or isobutyldeoxyguanosines are the substrates for m^6-MT, the rate of dealkylation decreases with the size of the alkyl group, and the branched chain adducts are poorly repaired (11).

Most, if not all, of these alkylpurine activities are the same as those found for the analogous enzymes from *E. coli* or *Micrococcus luteus*. However, the specificity of the bacterial repair enzymes differs greatly from repair enzymes isolated from mammalian sources. O^4-Alkylthymine and alkyl phosphotriesters are not detectably removed/repaired *in vitro* by mammalian m^6-MT.

In contrast, the m^6-MT from *E. coli* can dealkylate O^4-methylthymine (m^4T) and one of the two diastereoisomers of the alkyl phosphotriesters (12–15). The latter activity resides in a segment of the 37-kDa enzyme that can be proteolytically cleaved, leaving an 18-kDa enzyme with m^6-MT activity (14) and a 13-kDa protein with alkyl phosphotriester repair activity (16).

One of two *E. coli* glycosylases, DNA-3-methyladenine glycosylase II, produced in *tag* mutants, differs from the tag^+ gene product (DNA-3-methyladenine glycosylase I), in that the former enzyme also excises O^2-methylcytosine (m^2C) and O^2-methylthymine (m^2T). None of the *O*-alkylpyrimidines are repaired as rapidly as the alkylpurines. Nevertheless, such activities of this purified *E. coli* enzyme have been unambiguously demonstrated (12, 13).

In attempts to investigate the mechanism of alkylpyrimidine loss from mammalian cell systems, several research groups have focused on the corresponding repair activities in mammalian cells. This report represents a summary of our attempts to demonstrate repair of *O*-alkylpyrimidines in cell-free mammalian systems.

RESULTS

***In Vivo* Studies.** Based on the lack of mammalian repair activity toward the pyrimidines *in vitro*, it has been suggested that these alkylpyrimidine derivatives are not repaired *in vivo* and that losses occur *only* as a result of cell division. Table 1 is a summary of data on the persistence of

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Abbreviations: MeNU, methylnitrosourea; EtNU, ethylnitrosourea. The base moiety in a polynucleotide or DNA is given by the single letter: A, G, T, C. Modifications are indicated by a superscript for the position and a single letter for the alkyl group. Thus, O^4-methylthymine in DNA is abbreviated as m^4T. The similar abbreviation for O^4-ethylthymine in DNA is e^4T. When an isolated base is meant and the context is not apparent, the full name is used. dThd-*P*(Me)-dThd or dThd-*P*(Et)-dThd, representative alkyl triesters; m^6-MT, DNA-O^6-methylguanine methyltransferase.

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Table 1. Range of observed half-lives of *O*-alkylpyrimidines and representative alkyl triesters *in vivo*

Derivative[a]	$t_{1/2}$ (hours, days, or weeks)[b]	
	Whole rat liver or hepatocytes	Cultured mammalian cells
m^2C	<4 hr (1)	
m^2T	12 hr (1)	
m^4T	≈20 hr (3); ≈80 hr (1)	2–3 hr (1); ≈13 hr (1)
dThd-*P*(Me)-dThd	7–10 days (3)	>20 hr (1)
e^2C	40–78 hr (3)	5, 50 hr[c] (2)
e^2T	>2; 11–20 days[d] (4)	40 hr[c] (1)
e^4T	>2–3; 11–19 days[d] (5)	1–3 days (2)
dThd-*P*(Et)-dThd	5–15 weeks[d] (4)	>8 days (1)

Data are collected from the following references, in chronological order: Shooter and Slade (17), Shooter *et al.* (18), Bodell *et al.* (19), Saffhill and Fox (20), Singer *et al.* (21), Richardson *et al.* (22), Yarosh *et al.* (23), Dyroff *et al.* (24), Den Engelse *et al.* (25), Belinsky *et al.* (26), Boucheron *et al.* (27), Dogliotti *et al.* (28), Den Engelse *et al.* (29), Wani and D'Ambrosio (30) and R.M. (unpublished work). It should be noted the amount of methylation or ethylation differed in most experiments, as did the times of analysis following carcinogen administration. dThd-*P*(Me)-dThd and dThd-*P*(Et)-dThd, representative alkyl triesters.

[a]The approximate proportion of initial total alkylation of each discussed derivative in DNA alkylated *in vivo* is as follows: m^2C, m^2T, m^4T, ≈0.1%; m^6G, 6.3%; e^2C, 3%; e^2T, 7%; e^4T, 2%; e^6G, 8%.

[b]In many experiments, the data on which calculations were made were biphasic, so that the $t_{1/2}$ can depend on the length of time between analytical points. Numbers in parentheses indicate the number of independent findings.

[c]The reported 5-hr half-life for e^2C is in Chinese hamster ovary cells, which are not competent to repair m^6G (28). The 50-hr value is from human fibroblasts in which cell division has been measured, and the data have been corrected (19). The same correction was made for e^2T, for which a $t_{1/2}$ of 55 hr was reported (19).

[d]The wide range of data reflect, to some extent, differences in animal ages, dosage of the carcinogens, and number of experimental points. In a significant number of experiments (not listed) data were insufficient to determine half-lives. Additionally, repair is not noted in all tissues over the reported time period (44, 45).

O-methylated and *O*-ethylated pyrimidines *in vivo* after a single dose of the alkylating agent. Since adult liver does not proliferate rapidly but does contain large amounts of the two enzymes discussed, much of the data relates to that organ or hepatocytes. These data on mammalian cells in culture are primarily from work using cells that have been shown to repair m^6G and *N*-3 methyladenosine (m^3A) efficiently.

The table clearly shows that m^4T is lost fairly rapidly, whereas e^4T has a much longer half-life. In experiments reported by Richardson *et al.* (22), the levels of m^4T and e^4T in liver DNA were adjusted by dosage to be approximately equivalent. They reported the $t_{1/2}$ of m^4T to be 20 hr and the $t_{1/2}$ for e^4T to be 11 days. The $t_{1/2}$ for e^4T was similar after 28 days of diethylnitrosamine exposure, demonstrating that e^4T repair is noninducible in rat liver. It would not seem likely that cell division plays a major role in establishing the $t_{1/2}$ in view of the great difference in the repair rates between methyl and ethyl derivatives and the data on the other adducts. Support for this interpretation is provided by the much longer $t_{1/2}$ for m^7G in the same cells (26) and the shorter $t_{1/2}$ for e^4T, than for [^{3}H]thymidine (11 vs. 35 days) (24). In all reported experiments focusing on pyrimidine repair, the larger ethyl group is more persistent than the methyl group.

In the single experiment measuring the loss of m^4T after uptake of the nucleoside into V79 cellular DNA (20), only a few molecules of the derivative were present per cell. This direct measurement of a $t_{1/2}$ of 2–3 hr indicates active removal/repair. In the study by Bodell *et al.* (19) on loss of ethylated derivatives, data were corrected for cell division, with resulting $t_{1/2}$ values of 40–60 hr for e^4T, e^2T, and e^2C.

Comparable data (to those in Table 1) for the $t_{1/2}$ of m^6G compared with e^6G have been repeatedly obtained. Regardless of the carcinogen dose, below the saturation level of m^6-MT, the $t_{1/2}$ of m^6G is shorter than that of e^6G. Similarly, data from rat liver show that m^6G is repaired faster than *O*-alkylpyrimidines.

Montesano and colleagues (unpublished work) find that the hamster liver does not repair m^6G at high dosages of dimethylnitrosamine but does remove/repair m^4T similarly to rat liver given comparable doses of dimethylnitrosamine ($t_{1/2}$ hamster liver ≈80 hr). In contrast, similar administration of the carcinogen (20 mg/kg) to rats did not saturate the m^6-MT, as already known. Nevertheless, repair/removal of m^4T occurred at about the same rate as in hamster liver.

***In Vitro* Studies.** On the basis of the *in vivo* data, the authors of this report have used a variety of substrates and enzymes or extracts from mammalian sources and, nevertheless, have failed to find unambiguous or reproducible *in vitro* *O*-alkylpyrimidine repair activity.

Some data are published; some data are unpublished due to negative conclusions; and some data are mentioned in reviews but are not separately published. The following experimental procedures have been used.

One technique takes advantage of the irreversible inactivation of m^6-MT when an alkyl group is bound. Thus, quantitation of the residual m^6-MT activity can measure the affinity of an alkyl group. Brent (31) found that the m^6-MT purified from human cultured lymphoblasts (CCRF-CEM line) was not depleted when incubated with MeNU-treated poly(dA·dT). Conversely, the same incubation using MeNU-treated poly(dG·dC) resulted in complete depletion. The amount of methylated poly(dA·dT) used to test the reaction with m^6-MT contained about 150 times more m^4T than the amount of m^6G in the methylated poly(dG·dC). Brent thus concluded that the m^6-MT did not recognize m^4T by a factor of at least 10^{-2} compared to that of m^6G.

Yarosh *et al.* (23) examined repair of m^2T, m^4T, and methyl phosphotriesters in human cell extracts, using as substrate [^{3}H]MeNU-treated poly(dT) that was then annealed to poly(dA). In addition to the published experiments on human liver (4), this substrate was incubated with sonicated cell extracts prepared from human tumor strain A1235 (mer^-), human fibroblast strains GM10 (mer^-), and GM11

(mer$^+$), and from Chinese hamster ovary cell extracts containing little or no m^6-MT. As controls, repair of m^{6}G was measured using [^{3}H]MeNU-treated DNA and the cell extracts; or by using an extract from *E. coli* BS21, which contains m^6-MT, on both the DNA and poly(dT)·poly(dA) substrates. None of the human cell or human liver extracts repaired the *O*-alkylpyrimidines. In contrast, the extract from cell line GM11, which is high in m^6-MT content repaired m^{6}G, and the extract from *E. coli* BS21 repaired m^{6}G and the pyrimidines, as expected.

Yarosh (D.B.Y., unpublished data) also incubated a partially purified extract of human liver with the [^{3}H]MeNU-treated DNA and [^{3}H]MeNU-treated poly(dT)·poly(dA). The amount of m^{2}T or m^{4}T in the methylated polymer was equivalent to the amount of m^{6}G in the methylated DNA. The triester concentration in both substrates was higher. The incubated samples were then electrophoresed on a $NaDodSO_4$/polyacrylamide gel and, on autoradiography, the only band appeared in the m^{6}G-containing DNA lane, at ≈25 kDa; this result indicated that the transfer of a labeled alkyl group to an acceptor protein did not occur at a detectable level with a m^{4}T-containing polymer.

Using [^{3}H]MeNU-treated poly(dT) annealed to poly(dA) (12), Laval (F.L., unpublished data) found removal of neither m^{4}T nor m^{2}T using sonicated extracts or partially purified extracts from H4 (rat liver fibroblasts, mer$^+$), LICH (human liver fibroblasts, mer$^+$), normal human lymphocytes (mer$^+$), or Chinese hamster ovary cells (mer$^-$). No activity toward m^{4}T and m^{2}T was detected in H4 cells either pretreated with low *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine doses, which increased the m^6-MT activity by a factor of about 5-fold (32, 33), or treated with various agents that also increased the m^6-MT activity (34). As a control, the removal of m^{4}T by *E. coli* extracts was measured under the same experimental conditions and was found to be proportional to the protein concentration used.

Singer and Fraenkel-Conrat (B.S. and H.F.-C., unpublished work) used m^6-MT purified from human lymphoblasts (31) and purified human placental DNA-3-methyladenine glycosylase (6). Both enzymes were assayed for their activity toward e^{6}G or *N*-3-ethyladenosine (e^{3}A) in DNA before use. The substrate was very highly labeled EtNU-treated poly(dA·[^{3}H]dT). The enzymes were used over a wide range of concentrations and for varying time periods. Detection levels for loss of e^{2}T, e^{4}T, or e^{2}C were sensitive enough to measure 1/100th the rate, or extent of loss, of the ethylated pyrimidines, compared with the ethylated purines. Neither enzyme was detectably active toward the substrate.

Ammonium sulfate fractions of relatively crude extracts from human lymphoblasts (CCRF-CEM line) were also tested by the same methods. None of the five fractions (0–20%, 20–40%, 40–60%, 60–80%, or 0–80%) had reproducibly measurable activity toward any *O*-ethylpyrimidine. Nevertheless, there was equivocal evidence of dealkylase activity toward e^{2}T. This result would not be in accord with the type of repair occurring in *E. coli*. However, McCarthy *et al.* (13) do not preclude such a possibility.

Using a variety of substrate polynucleotides treated with [^{3}H]MeNU of a high specific activity, Hall and Karran (35) investigated methyltransferase activity towards m^{4}T in unfractionated extracts of a variety of mer$^+$ or mer$^-$ human lymphoid cell lines, when very high concentrations of cell extracts were unable to release m^{2}T or m^{2}C from methylated DNA. m^6-MT from calf thymus (3382-fold purification, 5100 units per mg) was assayed for its ability to act on [^{3}H]MeNU-treated poly(dA·dT). Hall and Karran (35) report this highly purified m^6-MT does not recognize m^{4}T, using an excess of the mammalian enzyme, whereas the same unitage of the *E. coli* m^6-MT completely demethylated the derivative.

Hall and Karran (35) also tested crude extracts from a variety of lymphoma and lymphoblastoid cell lines for glycosylase activity against three substrates: [^{3}H]MeNU-DNA, [^{3}H]MeNU-poly(dA·dT), and [^{3}H]MeNU-poly(dT) subsequently annealed to poly(dA). Extracts from Burkitt lymphoma Raji (mex$^+$) and its TK$^-$ subline (mex$^-$) or the lymphoblastoid cell lines contained measured activity toward m^{3}A but were unable to release measurable amounts of m^{2}T or m^{4}T from the substrates, whereas the *E. coli* BS21 extract was highly proficient in releasing m^{3}A, m^{2}C, and m^{2}T. From experiments using very highly labeled substrate, it could be calculated that the specific activity of any m^{2}T glycosylase in human cells is <0.04% of the *E. coli* enzyme activity.

Using a substrate made by reaction of [^{3}H]*N*-methyl-*N*-nitrosourea with poly(dT) followed by addition of poly(dA), Dolan *et al.* (36) and Domoradzki *et al.* (37) found no significant repair of m^{4}T upon incubation with partially purified rat liver extracts or crude extracts prepared from transformed human fibroblasts (XP12BE). These extracts were active in the removal of m^{6}G. This work was extended to include studies with calf thymus DNA methylated with ^{3}H-labeled *N*-methyl-*N*-nitrosourea as a substrate (38). An excess of m^6-MT over the total of m^{6}G (7.5% of total methylation) and m^{4}T (0.06% total methylation) was added. All of the m^{6}G was removed, but there was no demethylation of the m^{4}T in the DNA by the rat liver m^6-MT. The m^6-MT isolated from *E. coli* BS21 was able to repair m^{4}T in these substrates (36–38).

In related experiments, [^{14}C]EtNU-treated DNA, containing 2.5% e^{4}T and 7.8% e^{6}G (of the total ethylation), was incubated for 8 hr with enough partially purified rat liver m^6-MT to remove all e^{6}G in 1 hr. No loss of e^{4}T was detected (36), nor was e^{2}T or e^{2}C lost from the DNA (B.S., unpublished data). Pegg and Singer (unpublished data) also found that a crude rat or human liver m^6-MT also could not act on ethylated pyrimidines in DNA. The amounts of enzymes used were greatly in excess of those needed to remove the e^{6}G. Thus, competition for limited enzyme molecules was not the cause of failure to dealkylate e^{4}T.

Pegg, Dolan, and colleagues (39, 40) have also exploited the use of defined oligomers containing a single methylated base to measure repair by rat liver and human tumor cell m^6-MT. Self-complementary dodecamers containing m^{6}G were excellent substrates for the m^6-MT (39, 40), but similar oligomers containing m^{4}T were not repaired by the mammalian cell extracts. In these experiments the m^{4}T-containing dodecamer was labeled at the 5′ end with ^{32}P by reaction with polynucleotide kinase and [^{32}P]ATP at a very high specific activity (5–6 Ci/μmol; 1 Ci = 37 GBq). Repair of the m^{4}T lesion was determined by quantitating the amount of labeled, demethylated dodecamer after separation on a reverse-phase column. There was complete repair of m^{4}T by the purified *E. coli* m^6-MT; yet, no detectable product formed upon incubation with crude extract prepared from two human cell lines, HeLa and HT29 (41). Crude rat liver extract also did not result in repair of m^{4}T, although there was some nonspecific degradation of the substrate with this extract. These results contrast sharply with those from similar studies with dodecamers containing m^{6}G. In these experiments (40) there was repair of m^{6}G by the m^6-MT from several mammalian sources including rat liver and human cell lines, even though the concentrations of dodecamer containing m^{6}G and of m^6-MT were 3000-fold less and 500-fold less, respectively, than in the experiments with dodecamers containing m^{4}T. This approach to studying repair is at least an order of magnitude more sensitive than previously described protocols.

Although all experiments with relatively pure enzyme preparations are negative, Hall and Karran (35) raise the

possibility, based on using crude Raji cell extracts, that there may be a m^4T repair mechanism that is either inactivated or removed during enzyme purification. This tentative clue is similar to that indicated by Singer and Fraenkel-Conrat, also using crude cell fractions. Such a second or labile protein may also account for the reported slight loss of m^4T by crude monkey liver extracts (42). Becker and Montesano (42) do not identify the product after enzyme treatment but suggest that crude preparations might contain a separate enzyme for pyrimidine repair.

Potter *et al.* (43) have identified a second m^6-MT from *E. coli*, the *ogt* gene product, which does not act on alkyl phosphotriesters in methylated DNA, nor apparently on m^4T (P.M.P. and G.P.M., unpublished data). This methyltransferase, which is expressed at low levels in unadapted *E. coli*, may be more closely related to the mammalian m^6-MT than is the *ada* gene product. Moreover, the *ogt* gene product could explain the presence of m^6-MT activity toward m^6G in various ada^- mutants (P.M.P. and G.P.M., unpublished work).

DISCUSSION

E. coli has long represented a model system for the study of the repair of alkylation damage. The major cytotoxic and mutagenic lesions introduced into DNA by alkylating agents, m^3A and m^6G, are repaired by DNA glycosylase and DNA methyltransferase actions, respectively, in *E. coli* and mammalian systems. The data reviewed here indicate that for repair of other *O*-alkyl DNA lesions, mammalian cells lack these well-defined *E. coli* mechanisms. Thus, no m^6-MT from mammalian cells acts on m^4T or alkyl phosphotriesters. The mammalian DNA-3-methyladenine glycosylases, although recognizing several methylated purines in DNA, are not active on m^2C or m^2T to any measurable extent.

However, *in vivo*, all these alkylated products are lost with half-lives varying with the derivative and cell type (Table 1), but not primarily as a result of cell turnover or chemical instability. The recent *in vivo* studies by Belinsky *et al.* (26), showing that the relative loss of m^6G and m^4T in DNA from rat liver, lung, and nasal mucosa differs markedly, point to the existence of two separate repair enzymes.

The number of investigators from different laboratories coauthoring this paper indicates the wide range of independent experiments that agree, in principle, that repair of alkylated pyrimidines is not the same in mammalian cells as in prokaryotes. We hope scientists interested in the mechanism of such DNA repair will develop new approaches to understanding how mammalian cells handle these lesions that affect survival, mutagenesis, and carcinogenesis.

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