Enzymatic Removal of O^6-Ethylguanine *versus* Stability of O^4-Ethylthymine in the DNA of Rat Tissues Exposed to the Carcinogen Ethylnitrosourea: Possible Interference of Guanine-O^6 Alkylation with 5-Cytosine Methylation in the DNA of Replicating Target Cells

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In order to compare the kinetics of their enzymatic elimination from the DNA of liver, kidney, lung, and brain, the alkylation products O^4-ethyl-2′-deoxythymidine (O^4-EtdThd) and O^6-ethyl-2′-deoxyguanosine (O^6-EtdGuo) were quantitated by competitive radioimmunoassay over a period of 48 h after a single pulse of the carcinogen N-ethyl-N-nitrosourea (EtNU) applied i.p. to 10 and 28-day-old BDIX-rats. The content of O^4-EtdThd in the DNA of all organs analyzed remained stable, while O^6-EtdGuo (initially formed in DNA with 3–4 times higher frequency than O^4-EtdThd) was rapidly removed from the DNA of liver, followed by lung and kidney, but persisted strongly in the DNA of brain. At 48 h after the EtNU-pulse, the O^4-EtdThd content of liver DNA exceeded the O^6-EtdGuo content by about a factor of 4. Since both O^6-EtdGuo and O^4-EtdThd are miscoding DNA lesions, the lack of enzymatic removal of O^4-EtdThd is surprising in view of the apparent concern of cells to restore the integrity of the O^6-position of guanine. Genetic consequences more specifically connected with the formation of O^6-alkylguanine in DNA might be considered, *e.g.*, possible alterations of gene expression via interference with enzymatic 5-cytosine methylation in 5′-CpG-3′ sequences of newly replicated DNA.

Introduction

With most chemical carcinogens, initiation of the multi-step process of malignant transformation and tumorigenesis involves structural alterations of target cell DNA [1–3]. A class of carcinogens particularly well characterized with respect to the molecular structure of their reaction products in DNA are the alkylating N-nitroso compounds [3–8]. Their highly reactive, electrophilic derivatives (generated *in vivo* either enzymatically or via non-enzymatic decomposition of the parent compounds) bind covalently to nucleophilic N and O atoms in cellular DNA. Being a function of the type of reaction mechanism characteristic of the respective agent, the relative extent of alkylation on O versus N atoms in DNA varies strongly for different N-nitroso compounds [1, 3–4]. A bimolecular nucleophilic (S_N2) reaction will result in a lower O/N alkylation ratio than an S_N2 mechanism with a tendency towards a unimolecular (S_N1) reaction [4, 9]. The carcinogenic potency of alkylating N-nitroso compounds is positively correlated with their respective O/N ratios, indicating that alkylation of O atoms in DNA is of major importance in terms of the carcinogenic effect [3, 6, 10].

N-ethyl-N-nitrosourea (EtNU), a highly potent carcinogen [10–13] and mutagen [14–16], gives rise to a reactive ethyldiazonium ion via heterolytic decomposition under *in vivo* conditions, and is characterized by one of the highest O/N alkylation ratios (~ 0.7) so far measured in cellular DNA exposed to alkylating agents [17–18]. Although an approximately equal extent of DNA ethylation is initially produced in all pre- and postnatal tissues [12, 19–20], a single application of EtNU to rats results in the development of malignant tumors at high yield almost exclusively in the nervous system, with a strong dependency of the carcinogenic effect on developmental stage at the time of carcinogen exposure [10–12].

Among the ethylation products found in cellular DNA after exposure to EtNU, O^6-ethyl-2′-deoxyguanosine (O^6-EtdGuo) and O^4-ethyl-2′-deoxy-

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thymidine (O^4-EtdThd) are of particular interest since these alkyldeoxynucleosides represent potentially mutagenic structural modifications of DNA [21–22]. Both O^6-alkylguanine (O^6-alkyl-G) and even more so O^4-alkylthymine (O^4-alkyl-T) have been shown to miscode in appropriate test systems for DNA synthesis *in vitro* [23–25]. In the DNA of cells exposed to EtNU, O^4-EtdThd is produced 3–4 times less frequently than O^6-EtdGuo [18]. Contrary to other alkylation products, O^6-EtdGuo persists strongly in the DNA of pre- and postnatal rat brain, but is removed enzymatically from the DNA of other rat tissues, most rapidly from liver DNA [12, 17, 20]. The relative capacity of cells for enzymatic elimination of O^6-alkyl-G from DNA may be an important risk determinant in relation to malignant transformation by N-nitroso carcinogens.

It is a question of considerable interest whether mammalian cells possess (an) enzyme(s) for recognition and removal of O^4-alkyl-T from DNA, analogous to the enzyme activity responsible for the elimination of O^6-alkyl-G [13, 26–33], or whether both alkylation products could be dealt with by the same enzyme(s). Radiochromatographic analyses of DNA after a single dose of [1-^{14}C]-N,N-diethylnitrosamine (DEN) *in vivo*, suggest that O^4-EtdThd (as the alkylation product O^2-ethyldeoxythymidine [34]) remains detectable over an extended time period (600 h) in the DNA of Sprague-Dawley-rat liver [35]. However, more information on the stability of O^4-EtdThd in mammalian DNA is needed, since radiochromatographic data by other authors suggest some degree of elimination of O^4-EtdThd from the DNA of BDIX-rat liver and cultured human fibroblast cell lines [36–37].

Using high-affinity antibodies specifically directed against O^4-EtdThd and O^6-EtdGuo [38, 39] we show here by competitive radioimmunoassay (RIA), that the O^4-EtdThd content of BDIX-rat liver, kidney, lung, and brain DNA does not significantly change over a period of 48 h after application of EtNU while, as expected, the O^6-EtdGuo content of DNA decreases sharply in liver, to a lesser extent in lung and kidney, and least in brain. In view of the fact that O^4-alkyl-T and O^6-alkyl-G share the general property of miscoding during DNA replication and transcription [21–25], the lack of enzymatic removal of O^4-EtdThd from DNA is surprising. We, therefore, discuss the possibility that the particular concern of cells to restore the integrity of the guanine-O^6 position in carcinogen-treated DNA, may relate to genetic consequences specific to this modified base, *e.g.*, a possible interference with 5-cytosine methylation at 5′-CpG-3′ sites in critical gene sequences of replicating target cells.

Materials and Methods

Experimental animals and carcinogen application

Female rats of the inbred BDIX-rat strain [40], 10 or 28 days of age, were used throughout this study. A single pulse of EtNU (Roth; recrystallized twice from methanol), 30, 50, or 75 µg, respectively, per g body weight, was administered intraperitoneally (i.p.) in the form of a freshly prepared 0.1 M solution in 0.1 M sodium acetate buffer, pH 5.0.

Isolation of DNA and quantitation of O^4-EtdThd and O^6-EtdGuo by competitive radioimmunoassay (RIA)

At 1, 24, and 48 h, respectively, after the EtNU-pulse, 3–5 animals per time point were killed in ether anesthesia. Livers, kidneys, lungs, and brains were excised and immediately frozen in liquid N_2. The DNA was then isolated and enzymatically hydrolyzed to deoxynucleosides as described [20, 39]. The contamination of DNA samples with RNA was $< 1\%$. Concentrations of dGuo and dThd in the DNA hydrolysates were determined spectrophotometrically after separation by HPLC according to Ref. [20]. O^4-EtdThd and O^6-EtdGuo concentrations were measured by competitive RIA as previously described [39, 41], using affinity-purified anti-(O^4-EtdThd) antiserum E-30 (antibody affinity constant, 8.5×10^8 l/mol; 38) and anti-(O^6-EtdGuo) antiserum E-3 (antibody affinity constant, 1.8×10^{10} l/mol; 39), respectively. Radioactive tracers O^4-Et-[6-^{3}H]dThd (spec. act. 17 Ci/mmol) and O^6-Et-[8,5′-^{3}H]dGuo (spec. act. 33 Ci/mmol), ~ 2500 dpm/100 µl RIA sample, and the alkylation products O^4-EtdThd, O^4-ethylthymine riboside, O^4-methyl-2′-deoxythymidine, O^2-ethyl-2′-deoxythymidine, 3-ethyl-2′-deoxythymidine, and O^6-EtdGuo, were prepared according to Refs. [38] and [39]. With antiserum E-3, the competitive RIA specifically detects 0.05 pmol of O^6-EtdGuo in a RIA-sample of 100 µl at 50% inhibition of tracer-antibody binding

Table I. Competitive radioimmunoassay (RIA) for O^4-ethyl-2′-deoxythymidine (O^4-EtdThd), using anti-(O^4-EtdThd) antiserum E-30. Inhibition of tracer (O^4-Et-[6-^{3}H]dThd)-antibody binding by various alkylated and natural nucleic acid components.

Compound (inhibitor)	Amount of inhibitor required for 50% inhibition of tracer-antibody binding[a]	
	Picomol	Multiple of O^4-EtdThd
O^4-ethyl-2′-deoxythymidine	0.36	1
O^4-ethylthymine riboside	0.36	1
O^4-methyl-2′-deoxythymidine	7	19
O^2-ethyl-2′-deoxythymidine	1000[b]	$> 3 \times 10^4$
3-ethyl-2′-deoxythymidine	1000[b]	$> 3 \times 10^4$
O^6-ethyl-2′-deoxyguanosine	210	600
2′-deoxythymidine	n.i.d.	$> 10^7$
2′-deoxycytidine	n.i.d.	$> 10^7$
enzymatic DNA hydrolysate (deoxynucleosides)	n.i.d.	$> 10^7$

n.i.d.: No inhibition detectable.
[a] In RIA-samples of 100 µl total volume.
[b] < 10% inhibition at this concentration.

[39], while antiserum E-30 detects 0.36 pmol of O^4-EtdThd under the same RIA conditions (Table I and Ref. [38]). In the case of anti-(O^6-EtdGuo) antiserum E-3, a concentration of O^4-EtdThd ~ 2800-fold higher than of O^6-EtdGuo is required for 50% inhibition of tracer-antibody binding in the competitive RIA [38]. The O^6-EtdGuo/dGuo molar ratios determined by RIA were corrected for the respective concentrations of DNA in the test samples with the use of calibration curves for O^6-EtdGuo measured in the presence of different concentrations of DNA hydrolysates [39]. Contrary to the O^6-EtdGuo/dGuo values, the O^4-EtdThd/dThd molar ratios did not require correction for DNA concentration, as significant changes in the values for 50% tracer-antibody binding did not occur in the presence of concentrations of $\leqq 2$ mg of hydrolyzed DNA/ml in the test samples.

To obtain an estimate of the extent of reduction of the O^4-EtdThd and O^6-EtdGuo contents in DNA due to DNA replication (as opposed to specific removal of these alkylation products from DNA by cellular enzymes), and in analogy to a recently published study [20], the average DNA content of BDIX-rat liver, kidney, lung, and brain, was determined at 1 and 48 h, respectively, after i.p. administration of 30 µg of EtNU/g to 10-day-old BDIX-rats (5–10 animals/time point).

Results and Discussion

Table II shows the O^4-EtdThd/dThd and O^6-EtdGuo/dGuo molar ratios determined in liver, kidney, lung, and brain of 28-day-old and 10-day-old BDIX-rats, respectively, over a period of 48 h after exposure to EtNU. The O^4-EtdThd/dThd molar ratio in DNA was measured after i.p. administration of 75 µg of EtNU/g body weight, while the O^6-EtdGuo/dGuo molar ratio was determined after i.p. doses of 30 and 50 µg of EtNU/g, respectively. For comparison, the table also contains literature data for O^6-EtdGuo/dGuo obtained by radiochromatographic analyses of liver and brain DNA after i.p. administration of 75 µg of [1-^{14}C]-EtNU/g to 10-day-old BDIX-rats [12, 17]. In none of the organs analyzed did the increase in average DNA content measured at 48 h following the lowest (*i.e.*, least DNA synthesis-inhibiting; [42]) EtNU-dose (30 µg/g) exceed a factor of 1.16 (liver of BDIX-rats 10 days of age the time of the EtNU-pulse). The corresponding factors for kidney, lung, and brain were 1.08, 1.11, and 1.08, respectively. Therefore, no correction coefficient [20] was introduced to compensate the reduction in the measured O^4-EtdThd/dThd and O^6-EtdGuo/dGuo molar ratios by DNA replication in the course of the kinetic analyses.

The data compiled in Table II clearly show that the ethylation product O^4-EtdThd is not enzymatically removed to any significant degree from the DNA of BDIX-rat liver, kidney, lung, and brain, during an observation period of 48 h after i.p. exposure to 75 µg of EtNU/g body weight. Indeed, all O^4-EtdThd/dThd molar ratios measured in these organs at 1, 24, and 48 h are within ± 22% (S.D.) of a common value of 4.97×10^{-6}. While the initial (1 h) O^6-EtdGuo/dGuo molar ratios after EtNU-doses of 75, 50, and 30 µg/g in liver and brain DNA, and after 30 µg of EtNU/g in the DNA of kidney and lung, are all higher than the initial O^4-EtdThd/dThd molar ratios, the O^6-EtdGuo/dGuo values for the DNA of liver, kidney, and lung (but not brain) have decreased below the mean O^4-EtdThd/dThd value at the 24 and 48 h time points (Table II). The stability of O^4-EtdThd in the DNA of BDIX-rat liver, kidney, lung, and brain, as reflected by the present measurements, is in agreement with recent radiochromatographic data for the liver of female Sprague-Dawley rats exposed to the hepatocarcinogen DEN [35], which have suggested a high degree

Table II. Kinetics of elimination of O^4-ethyl-2′-deoxythymidine (O^4-EtdThd) and O^6-ethyl-2′-deoxyguanosine (O^6-EtdGuo) from the DNA of liver, kidney, lung, and brain, after a single pulse of the carcinogen N-ethyl-N-nitrosourea (EtNU) applied intraperitoneally to 10-day-old (O^6-EtdGuo determinations) and 28-day-old BDIX-rats (O^4-EtdThd determinations). O^4-EtdThd and O^6-EtdGuo were quantitated by competitive radioimmunoassay (RIA).

Organ	Dose of EtNU [μg/g]	Time after EtNU-pulse (h)					
		1	24	48	1	24	48
		O^4-EtdThd/dThd × 10^6			O^6-EtdGuo/dGuo × 10^6		
Liver	75	4.6	3.5	4.6	16.5[a]	3.6[a]	1.6[a]
	50	–	–	–	12.0	2.0	0.87
	30	–	–	–	7.0 ± 0.1[b]	1.2	0.4
Kidney	75	4.8	5.7	4.4	–	–	–
	30	–	–	–	6.2	–	3.2 ± 0.2[b]
Lung	75	5.0	4.1	3.6	–	–	–
	30	–	–	–	6.9 ± 0.4[b]	–	3.2 ± 0.4[b]
Brain	75	6.9	6.4	6.0	11.8[a]	8.2[a]	6.9[a]
	50	–	–	–	10.0	7.0	6.3
	30	–	–	–	6.3 ± 0.2[b]	–	5.2 ± 0.3[b]

[a] Data from Refs. 12, 17, and 42 (measured by radiochromatography after i.p. application of [1-^{14}C]-EtNU).
[b] Mean value ± S.D.

of persistence of O^4-EtdThd in the DNA of this rat organ over an extended time period. As already well documented in the literature [10, 12–13, 20], the data compiled in Table II further show that O^6-EtdGuo is most persistent in the DNA of BDIX-rat brain and most rapidly removed from the DNA of liver, followed by lung and kidney.

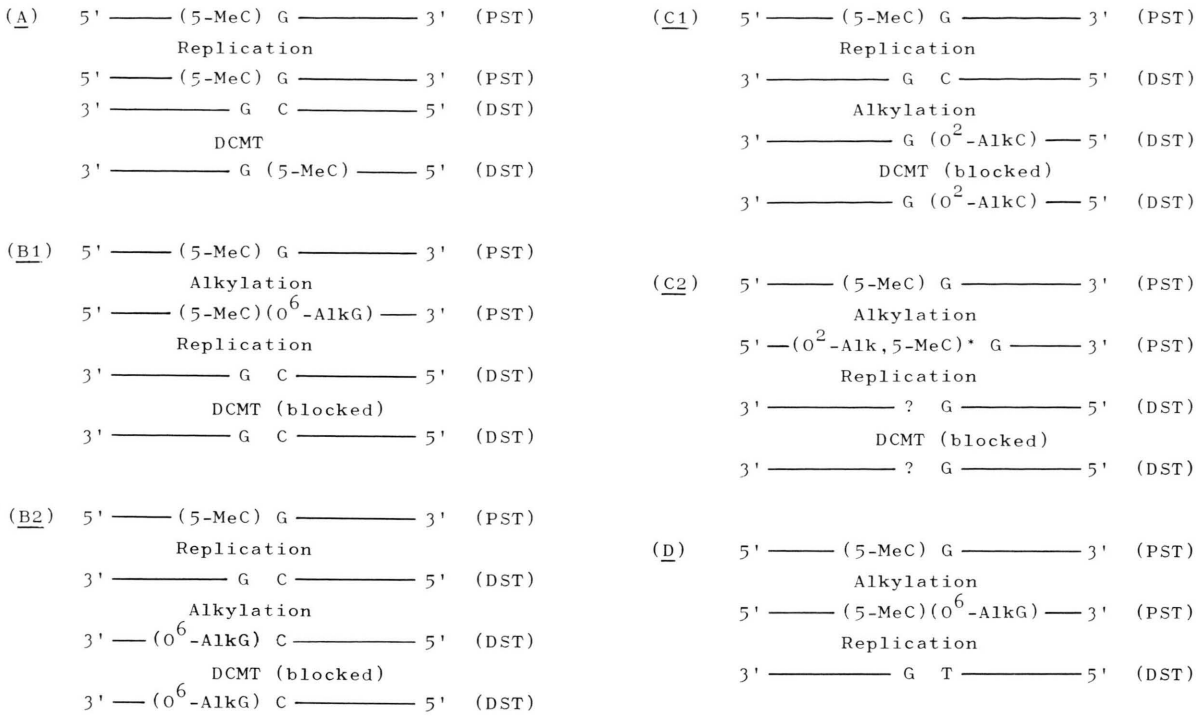
It should be noted that the present analyses were carried out on DNA isolated from whole organs composed of multiple subpopulations of cells of different types and states of development/differentiation and proliferation. The measured O^4-EtdThd/dThd and O^6-EtdGuo/dGuo molar ratios are thus equivalent to mathematical averages for all cells of a given organ (with little relevance for individual cells). Regardless of whether such whole organ data indicate persistence of a given alkylation product in DNA or rapid enzymatic removal, they will be strongly biased against smaller subpopulations of cells with elimination capacities significantly different from those reflected by the measured overall kinetics. No information can be derived, *e.g.*, on the frequencies of rare elimination-negative variant cells within large elimination-positive cell populations and *vice versa*. Even major constituent cell populations of a given tissue can be severely misrepresented by average values for whole tissue DNA, when they differ strongly with respect to the expression of enzymes capable of removing specific, carcinogen-modified components from DNA. This is evidenced, *e.g.*, by the high overall competence for enzymatic elimination of O^6-methylguanine from DNA in the case of the parenchymal cell population of rat liver as opposed to non-parenchymal liver cells [43]. Recently developed sensitive immunoanalytical methods for DNA structurally modified by DNA-reactive carcinogens [44], including the use of high-affinity monoclonal antibodies in conjunction with electronically intensified, direct immunofluorescence for the quantitation of very low amounts of specific alkyldeoxynucleosides in the DNA of individual cells [45–46], will permit more precise information on cellular repair capacity to be obtained in the future.

It has not yet been fully established whether a single enzyme, or perhaps more than one enzyme, is responsible for the specific elimination of O^6-alkyl-G from DNA in mammalian cells. However, it is clear that both bacteria and mammalian cells express, to varying degrees, a rapidly-acting

O^6-methylguanine-DNA transmethylase of molecular weight 18000 and 21000 to 22000, respectively, which transfers a methyl group from the O^6-atom of guanine in DNA to one of its cysteine residues and thereby becomes inactivated ("suicide enzyme" [26–33]). The bacterial enzyme [27], and to a large degree also similar mammalian enzymes from rat liver [33] and human lymphoid cells [32], have recently been purified and characterized. Although less efficiently, these DNA transmethylases can also remove an ethyl group from the O^6-atom of guanine [28, 33]. It is surprising that in the response to alkylation damage at least of the rat organs analyzed here (and possibly of a much greater number of mammalian cell types), enzymatic removal of O^6-alkyl-G from DNA is not accompanied by a significant degree of elimination of O^4-alkyl-T, although the latter alkylation product miscodes during DNA replication and transcription to an even higher extent [23–25]. The fact that O^4-alkyl-T is formed in the DNA of cells exposed to alkylating N-nitroso compounds to a lower degree than O^6-alkyl-G, does not provide a convincing explanation for this phenomenon, since in cells capable of eliminating O^6-alkyl-G from DNA, persisting O^4-alkyl-T gradually becomes the predominant miscoding DNA lesion (Table II).

Rather unexpectedly, recent analyses of the DNA of rat liver exposed *in vivo* to high doses of non-alkylating hepatotoxins (*e.g.* hydrazine [47]), and of purified DNA incubated *in vitro* with the major cellular methyl donor S-adenosylmethionine (SAM [48]), have indicated the presence of 7-methyl- and O^6-methylguanine [47], and of small amounts of 7-methylguanine and 3-methyladenine [48], in the respective DNA preparations. Partly in connection with these findings, it has been argued that cellular enzymes eliminating methylated bases from DNA may have evolved, and been conserved in evolution, (i) in response to a permanent low "background" of undesired base methylation by SAM and perhaps by non-specific action of DNA methyltransferases normally concerned with 5-cytosine methylation in DNA; or (ii) because methylated bases such as those detected might in fact represent normal DNA constituents occurring much more transiently than 5-methylcytosine (5-MeC), the only modified base so far known in mammalian DNA [49–51]. These considerations seem to be inconsistent with the lack of enzymatic removal of O^4-ethyl-T from DNA, as observed in the present study in 4 different rat tissues, unless, for unkown reasons, O^4-methylthymine cannot be aberrantly formed (or does not occur normally) in DNA, or an enzymatic activity exists in mammalian cells that will remove O^4-methyl- but not O^4-ethyl-T from DNA.

In search of particular reasons for ensuring selectively the integrity of the O^6-position of guanine in DNA, one might look for genetic consequences more specifically connected with O^6-alkyl-G, *i.e.*, different from those shared with O^4-alkyl-T on the basis of their common miscoding character. One property of guanine that distinguishes is from thymine is its particular role in relation to DNA methylation. In mammalian DNA $\sim 3\%$ of all cytosines are methylated in the 5-position, and $> 90\%$ of the 5-MeC occur in the nucleotide sequence 5′-CpG-3′ (Fig. 1, A); *i.e.*, depending on species, cell type, and state of cellular differentiation and proliferation, 50–70% of the CpG sites in a mammalian genome contain 5-MeC [49–50]. Enzymatic methylation of cytosines in specific mammalian gene sequences can block the expression of genes, probably via altered DNA-protein interactions based on a signal function of 5-MeC *per se* and/or the induction or stabilization of local changes in DNA secondary structure [51–53]. After a dose of 50 µg of EtNU/g (equivalent to about 20% of the LD_{50} in rats [11, 42]), which produces O^6-EtdGuo in DNA at an O^6-EtdGuo/dGuo molar ratio of $\sim 10^{-5}$ (Table II), a diploid rat genome will contain $\sim 10^3$ O^6-EtdGuo molecules located in 5′-(5-MeC)p(O^6-EtG)-3′ sites. As detailed in Fig. 1 (B1 and B2), it could be speculated that an O^6-alkyl-G either persisting unrepaired through DNA replication 3′ of a 5-MeC in the parental DNA strand, or formed 3′ of the corresponding C in the daughter strand immediately after replication but prior to the action of the DNA cytosine 5-methyltransferase on the newly replicated DNA, might mislead or block this enzyme. This could lead to an undermethylation of critical gene sequences, *i.e.*, possibly facilitate inappropriate gene expression. A potential 5-MeC site would also be eliminated from the daughter strand (*i.e.*, replaced by thymine) when an O^6-alkyl-G located 3′ of a 5-MeC in the parental strand mispairs with thymine in DNA replication (Fig. 1, D). A further candidate alkylation product for interference with DNA methylation is O^2-alkyl-cytosine, which is formed in



PST, parental DNA strand. DST, DNA daughter strand. DCMT, DNA cytosine 5-methyltransferase.

*, Formation of this product in cellular DNA exposed to alkylating agents not documented.

Fig. 1. Alkylation of the O^6-position of guanine in the DNA of replicating target cells: Possible mechanisms resulting in reduced 5-cytosine methylation. (A): Regular mechanism of postreplicational 5-cytosine methylation by DNA cytosine 5-methyltransferase (DCMT) in a 5′-CpG-3′-sequence of the newly synthesized DNA daughter strand (DST). (B, 1–2): Possible interference of O^6-guanine alkylation with 5-cytosine methylation by DCMT. (C, 1–2): Possible interference of O^2-cytosine alkylation with 5-cytosine methylation by DCMT. (D): Elimination of a 5-MeC site from DST DNA due to (O^6-alkyl-G):T mispairing in the replication of a 5′-(5-MeC)p(O^6-alkyl-G)-3′ sequence in the parental strand (PST).

DNA exposed to EtNU at a similar frequency as O^4-alkyl-T [18], but requires further study with respect to the degree of its persistence in the DNA of different types of cells (Fig. 1, C1 and C2). Interestingly, an overall inhibitory effect on DNA methylation in cells exposed to various carcinogenic chemicals has been reported by several authors (see Ref. [54] for review). If verified by experiments currently in progress, mechanisms of the type discussed here would be of considerable interest in relation to the molecular basis of malignant transformation by alkylating N-nitroso carcinogens and might provide some explanation for the specific importance of a structurally modified guanine-O^6-position in DNA.

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