

DNA damage and mutagenesis induced by procarbazine in λ lacZ transgenic mice: Evidence that bone marrow mutations do not arise primarily through miscoding by O^6 -methylguanine

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The DNA damaging and mutagenic activities of procarbazine, a methylating drug employed in cancer chemotherapy and suspected of causing therapy-related leukaemia, were investigated in the liver and bone marrow of λ lacZ transgenic mice (MutaTMMouse). The drug was administered using two different protocols, a 'high-dose' one involving 5 daily doses of 200 mg/kg, expected to cause depletion of the repair enzyme O^6 -alkylguanine-DNA alkyltransferase (AGT) and thus favour the selective accumulation of the premutagenic lesion O^6 -methylguanine (O^6 -meG) relative to other adducts, and a 'low-dose' one involving 10 daily doses of 20 mg/kg procarbazine. Substantial accumulation of O^6 -meG was observed in both tissues examined 6 h after the end of the 'high-dose' treatment, with the liver accumulating somewhat higher levels than the bone marrow (28.0 ± 1.8 fmol/ μ g DNA and 18.5 ± 1.1 fmol/ μ g DNA respectively). However, significant increases in mutant frequency 10 days after the end of treatment were observed only in the bone marrow, reaching a 16-fold increase over background following the 5×200 mg/kg treatment. Sequence analysis of the mutations induced after this treatment revealed a mixed spectrum, in which G:C→A:T transitions (characteristic of O^6 -meG miscoding) were only a secondary feature: Among 20 mutants analysed, only six such mutations were found, including three at CpG sites, which might have arisen from deamination of 5-methylcytosine. The other mutations observed included 1 A:T→G:C transition, five transversions (one G:C→T:A, one double G:C→C:G, two A:T→T:A, one A:T→C:G), five deletions and three insertions. The mechanistic and clinical significance of these findings is discussed.

Introduction

Procarbazine [*N*-isopropyl- α -(2-methylhydrazino)-*p*-toluamide hydrochloride] is a cytostatic drug used in the treatment of a number of human cancers, including Hodgkin's lymphoma where, in the form of combination treatments such as MOPP (a combination of procarbazine and mechlorethamine, vincristine and prednisone), it has made a particularly important contribu-

*Abbreviations: ANLL, acute non-lymphocytic leukaemia; AGT, O^6 -alkylguanine-DNA alkyltransferase; O^6 -meG, O^6 -methylguanine; NT, not tumorigenic; pfu, plaque forming units; O^6 -meG, O^6 -methylguanine; MOPP, mechlorethamine-vincristine-prednisone-procarbazine.

tion to the remarkable progress achieved in terms of long-term survival of patients (1,2). However, this success has been complicated by the appearance, during the 10 years following successful chemotherapy, of an ~5–10% increased risk of acute non-lymphocytic leukaemia (ANLL*), as well as other types of cancer (3,4). Although procarbazine is not the only potentially carcinogenic component of MOPP, its well recognized genotoxicity (5,6), together with evidence from follow-up studies of ex-Hodgkin's lymphoma patients (7), cause concern that it may make a significant contribution to the mechanism of MOPP carcinogenesis. Procarbazine is mutagenic, clastogenic and teratogenic in a wide range of test systems of varying complexity and a wide-spectrum carcinogen in rodents and monkeys, causing tumours of the haemopoietic system, the mammary gland, the lung and the nervous system (8).

In vivo procarbazine undergoes a complex series of metabolic changes that result in the generation of a number of chemically reactive species, including methylating agents and free radicals (9). The major circulating metabolite in the serum of animals or humans treated with procarbazine is methylazoxypcarbazine, a relatively stable intermediate that can spontaneously give rise to a methylating species, probably the methyl-diazonium ion $[\text{CH}_3\text{N}_2]^+$. The main type of macromolecular damage known to be produced by procarbazine is DNA methylation, with the liver accumulating the highest amounts of such damage, followed closely by the bone marrow and lymph nodes, two tissues of interest as targets for the carcinogenic and chemotherapeutic action of the drug (10,11). Procarbazine produces in DNA O^6 -methylguanine (O^6 -meG) and *N*7-methylguanine at a ratio of ~0.12, typical of an $\text{S}_{\text{N}}1$ -type methylating agent and similar to that caused by such powerful mutagens and carcinogens as methylnitrosourea and dimethylnitrosamine (10). It is therefore strongly suspected that DNA methylation plays a major role in the mutagenic and carcinogenic activity of this drug. Of the various DNA lesions induced by methylating mutagens and carcinogens, O^6 -meG has particularly important biological activity. It is a directly miscoding lesion, causing G:C→A:T mutations, and is believed to be the cause of the frequent discovery of this type of mutation in activated oncogenes found in animal tumours induced by methylating agents (12,13). In a study of mammary carcinogenesis induced in rats by procarbazine and methylnitrosourea, Fong *et al.* (14) found that the yield of tumours by the two carcinogens correlated approximately with the production in mammary gland DNA of O^6 -meG. In a subsequent study (15), a correlation was reported between accumulation of O^6 -meG and sensitivity to procarbazine-induced tumorigenesis in different tissues, including the mammary gland, the lymph nodes and the thymus. These results support the hypothesis that O^6 -meG plays a significant role in the mechanism of procarbazine carcinogenesis in experimental animals.

Comparative studies of the formation of O^6 -meG in rats and in Hodgkin's lymphoma patients have shown that human susceptibility to procarbazine-induced O^6 -meG accumulation

is similar to that of the rat (16), suggesting that this lesion may play a similar mutagenic role in humans treated with procarbazine as it does in the rat. Evidence compatible with an etiological role of O^6 -meG in the induction of ANLL after MOPP chemotherapy was produced by Sagher *et al.* (17) who found that MOPP-treated individuals who went on to develop ANLL had lower lymphocyte levels of O^6 -alkylguanine–DNA alkyltransferase (AGT), the enzyme responsible for repair of O^6 -meG, than similarly treated individuals who did not develop ANLL. This observation implied that individuals with low levels of AGT may be at higher risk of procarbazine-induced carcinogenesis by accumulating higher amounts of O^6 -meG during MOPP chemotherapy. Further support for this suggestion was obtained through the direct measurement of the formation of O^6 -meG in blood leukocyte DNA of procarbazine-treated patients (18) and the demonstration that individuals with low pre-treatment AGT levels accumulate high amounts of O^6 -meG, an observation also observed in patients treated with another methylating drug, dacarbazine (19,20).

Summarizing, data on the quantitative accumulation of O^6 -meG provide some evidence compatible with this lesion being important in procarbazine-induced carcinogenesis in experimental animals and MOPP-induced ANLL in humans. However, this evidence is far from conclusive. A key event in the initiation of chemical carcinogenesis is the conversion of pre-mutagenic DNA damage into a mutation in the target tissues through the replication of damage-containing cells. The advent of the technology of transgenic rodents with transgenes, which can serve as readily observable targets for chemical mutagens, provides a new way for investigating the ability of carcinogens to induce mutations in tissues susceptible to their carcinogenic effects. Furthermore, the examination of the specific types of sequence changes caused by a mutagen provides a powerful tool for the assessment of the role of specific DNA lesions in the induction of mutagenesis, which are under the full complexity of metabolic and repair pathways that operate under *in vivo* conditions. Procarbazine has been reported to induce a significant increase in mutant frequency in the bone marrow of λ lacZ mice (MutaTMMice) 7 days after the completion of treatment consisting of five daily doses of 200 mg/kg (21). However, no information on the relationship between mutagenesis and DNA adduct induction in any tissue of such treated animals was given in this report. In the present study we have examined the accumulation of O^6 -meG as well as the frequency and types of mutations in the liver and bone marrow of λ lacZ transgenic mice treated with procarbazine. We find that, even though after five daily treatments with 200 mg/kg procarbazine, there were slightly lower amounts of O^6 -meG accumulated in the bone marrow than the liver: a strong mutagenic response was induced only in the former tissue, and the spectrum of induced mutations was not dominated by the characteristic fingerprint of O^6 -meG (G:C→A:T transitions).

Materials and methods

Chemicals

Samples of procarbazine hydrochloride were obtained from Hoffman La Roche, Basle and from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, USA. The drug was stored desiccated in an amber bottle at -20°C and dissolved (protected from light) in physiological saline just before use.

Animal treatment

Male λ lacZ transgenic mice (MutaTMMouse) (16), 9–10 weeks old, purchased from Hazleton, were maintained on normal laboratory chow. Groups of four

mice were treated i.p. with multiple doses of procarbazine (10×20 mg/kg or 5×200 mg/kg at 24-h intervals) or with physiological saline only. One animal from each group was killed 4 h after the last treatment and used for measurement of O^6 -meG in liver and bone marrow DNA, while the remaining three animals were killed 10 days after the last treatment for mutation analysis in the same tissues. Tissues of interest were immediately placed in liquid nitrogen and stored at -70°C until analysis.

Measurement of O^6 -meG

Following DNA extraction from liver and bone marrow of individual animals by a standard method involving multiple proteinase K treatments and phenol–chloroform extractions, O^6 -meG was measured by the competitive repair assay as previously described (22) using *E. coli* AGT (ada protein). Each DNA sample was analysed in duplicate and duplicate measurements agreed to within 10% of each other.

Mutagenesis in the λ lacZ transgenic mouse

High molecular-weight genomic DNA was isolated from liver and bone marrow as previously described (23), dissolved in 10 mM Tris–HCl (pH 7.5) and 4 mM EDTA to a final concentration of 1.5 mg/ml and stored at 4°C . A 5 μl aliquot of each of the DNA solutions was taken and the λ prophages were rescued *in vitro* using the Giga-Pack II Gold packaging extract (Stratagene, La Jolla, CA). Screening for lacZ⁻ mutant phages was carried out by using the phenyl- β -D-galactopyranoside (P-gal)-based positive selection system (24) and all mutants were confirmed on 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) plates. Generally $>3 \times 10^5$ plaque forming units (pfu) were analysed in each case.

For the sequence-characterization of representative mutants, the location of the mutation in the α , β or ω regions of the lacZ gene was determined using a β -galactosidase complementation assay as described by Douglas *et al.* (25). The relevant region was subsequently amplified by PCR and subjected to dideoxy sequencing.

Results

O^6 -Methylguanine accumulation in liver and bone marrow DNA

Animals were treated with two dosing schedules of procarbazine, one involving five daily doses of 200 mg/kg, anticipated to cause substantial depletion of AGT and thus favour the preferential accumulation of O^6 -meG, and one involving 10 daily doses of 20 mg/kg, expected to generate relatively low levels of O^6 -meG. In order to obtain an indication of the adduct levels formed and their tissue distribution, from each group of treated animals one was killed 4 h after the last treatment and O^6 -meG was measured in liver and bone marrow DNA. The results are shown in Table I, where it can be seen that the high-dose treatment resulted in the accumulation of large amounts of O^6 -meG in both the liver and the bone marrow, the latter tissue accumulating approximately two-thirds of the adducts of the liver. Although changes in AGT were not measured, given the pre-treatment levels of AGT in λ lacZ transgenic mice [5.1 fmol/ μg DNA for the liver and 0.7 fmol/ μg DNA for the bone marrow (V.L.Souliotis *et al.*, unpublished results)] it is likely that substantial depletion of this enzyme occurred in both tissues. The death of one of the animals treated with low doses of procarbazine prevented the measurement of the resulting adducts in this case.

Mutant frequency analysis

The remaining animals per group were killed 10 days after the last treatment and mutant frequency in the lacZ transgene was determined in the liver and bone marrow. As can be seen in Table I, no statistically significant increase in mutant frequency was observed in the liver after either treatment, although one animal in each case gave a particularly high mutant frequency value. In the bone marrow, a 5-fold increase was observed in both animals treated with 10×20 mg/kg procarbazine ($P < 0.005$), while a much greater increase occurred in each of the three animals treated with 5×200 mg/

Table I. *O*⁶-meG and mutations in liver and bone marrow of λ lacZ transgenic mice after treatment with procarbazine^a

Tissue	Procarbazine treatment	<i>O</i> ⁶ -meG (mean \pm SD)		pfu $\times 10^5$ counted	Confirmed mutants	Mutant frequency (per 10 ⁶ pfu)	Mutant frequency (per 10 ⁶ pfu) mean (SD)
		fmol/ μ g DNA	μ mol/molG				
Liver	None	Not detected	Not detected	5.50	30	54	46 (10)
				6.05	30	49	
				13.95	49	35	
	10 \times 20 mg/kg	Not done ^b	Not done ^b	3.88	12	31	83 (66)
				5.70	35	61	
				2.03	32	157	
5 \times 200 mg/kg	28.0 \pm 1.8	44.8 \pm 2.8	–	–	– ^c	103 (53)	
			2.95	15	50		
			1.34	21	156		
Bone marrow	None	Not detected	Not detected	3.70	20	54	41 (22)
				3.00	16	53	
				16.26	24	15	
	10 \times 20 mg/kg	not done ^b	Not done ^b	–	–	– ^c	203 (7)
				3.60	75	210	
				2.22	44	196	
	5 \times 200 mg/kg	18.5 \pm 1.1	29.6 \pm 2.5	4.99	350	700	650 (76)
				3.50	240	686	
				1.90	107	562	

^aMeasured 6 h and 10 days, respectively, after completion of treatment.

^bNot measured due to death of animal.

^cNot measured because of poor quality of extracted DNA.

kg procarbazine. The 16-fold average increase from 41 \pm 22 mutants/10⁶ pfu to 650 \pm 76 mutants/10⁶ pfu ($P < 0.0005$) agrees well with the report of Myhr (21) who found a mutant frequency of 772 mutants/10⁶ pfu 7 days after completion of a similar treatment.

Sequence characterization of procarbazine-induced mutations

In view of the relatively small increase in bone marrow mutant frequency observed after the low-dose treatment, sequence characterization of the resulting mutations was impractical. On the other hand, the 16-fold increase observed after the high-dose treatment meant that almost all of these mutants were probably induced by procarbazine and that their sequence analysis could provide useful information for the assessment of the DNA lesions responsible for their induction. Therefore 20 mutants from this group were randomly selected and sequenced (Tables II and III). The DNA sequence changes found were all different from each other, indicating that the mutants were derived from independent mutation events. All possible types of single base substitutions were observed, as well as base insertions and deletions. The most frequent mutations were transitions (7/20), dominated by G:C \rightarrow A:T changes (six out of the seven transitions, including three mutations at CpG dinucleotides). Five transversions were observed (including a GC \rightarrow CG double substitution), as well as five deletions (four of which involved G:C pairs) and three insertions. This mutation spectrum is in broad agreement with that reported by Myhr *et al.* following the analysis of a much smaller number of mutants (21).

Discussion

In an effort to investigate the mechanism of procarbazine mutagenesis and to obtain an indication of the role of *O*⁶-meG in it, we examined adduct and mutation induction in the liver and bone marrow of λ lacZ transgenic mice after treatment with multiple doses of this agent. *O*⁶-Methylguanine was measured 6 h after the last of five or 10 daily treatments. Previous studies with a number of methylating agents, including

Table II. Bone marrow lacZ mutants in mice treated with procarbazine (5 \times 200 mg/kg) and killed 10 days after last treatment

Mutant no.	lacZ region	Mutation	Position	Sequence
1	α	C:G \rightarrow T:A	127	CTGGCGTAA
2	α	G:C \rightarrow T:A	229	GCCGGAAAAG
3	β	C:G \rightarrow A:T	487	CGGGCGCTG
4	β	Insertion C	507–508	CCAGCGACA
5	β	C:G \rightarrow T:A	619	TCAGGATAT
6	β	A:T \rightarrow C:G	621	AGGATATGT
7	β	Deletion T	641	GGCATTTC
8	β	Deletion C	777	ACTACCTAC
9	β	A:T \rightarrow T:A	785	CGGGTAACA
10	β	Deletion C	830	GGCACCGCG
11	β	C:G \rightarrow T:A	928	CGCCGAAAT
12	β	G:C \rightarrow A:T	1164	AGAACAAC
13	β	Insertion TG	1164–1165	AGAATGCAAC
14	β	A:T \rightarrow G:C	1210	GCTGTGGTA
15	β	CG \rightarrow GC	1217, 1218	TACACCGCTGT
16	β	A:T \rightarrow T:A	1242	TGTAATGTGG
17	β	Deletion G	1480	TGAAGGCGG
18	β	Insertion C	1631–1632	AATACCGCC
19	β	C:G \rightarrow T:A	1652	AACAGTCTT
20	ω	Deletion G	2034	ATGTCTGCTC

procarbazine, have shown that repeated daily treatments result in a steady accumulation of *O*⁶-meG, which is reflected in the adduct levels present at the end of the treatment period (11,26,27). Furthermore, *O*⁶-meG appears to have similar lifetimes in the liver and the bone marrow of the rat following AGT-depleting doses of procarbazine or MNU, suggesting that the relative adduct levels observed at 4 h post-treatment are representative of the overall adduct load of the two tissues (11). Finally, the above series of studies has demonstrated a relatively small animal-to-animal variation in the observed adduct levels, typically involving a standard deviation $<20\%$. Thus, although adduct measurements in the present study were obtained at only one time-point and in single animals, and therefore do not provide a basis for quantitative adduct-

Table III. Summary and comparison of spontaneous and procarbazine-induced lacZ mutation spectra in bone marrow

Type of mutation	Spontaneous Myhr <i>et al.</i> (21) <i>n</i> = 20	Procarbazine-induced Myhr <i>et al.</i> (21) <i>n</i> = 8	This work <i>n</i> = 20
Transitions	16	2	7
G:C→A:T	15 (14 ^a)	1	6 (3 ^a)
A:T→G:C	1	1	1
Transversions	2	6	5
G:C→T:A	2	–	1
G:C→C:G	–	1	1 (double)
A:T→T:A	–	3	2
A:T→C:G	–	2	1
Deletions	2	–	5
Insertions	–	–	3

^aWithin CpG dinucleotide.

mutagenesis comparisons, they provide an adequate indication of the relative magnitude of DNA damage that occurred in the two tissues examined.

Despite the fact that the liver accumulated the highest levels of *O*⁶-meG (up to 28.0 ± 1.8 fmol/μg DNA, corresponding to ~53 000 residues of *O*⁶-meG per 10⁶ lacZ transgenes, after the high-dose treatment), no statistically significant increase in liver mutagenesis could be detected with either dosing regimen. It has been suggested that the induction of toxicity-related liver cell proliferation may be a necessary prerequisite for mutagenesis (28). Although no specific information on the hepatotoxicity of procarbazine is available, the liver does not appear to be among the main targets of the toxicity of this compound, which are the gonads, the lung and the haematopoietic system (8). It thus seems possible that the low rate of liver cell proliferation after procarbazine treatment did not favour the efficient conversion of these adducts into mutations.

In contrast to what was observed with the liver, a dose-related increase in bone marrow mutagenesis was induced by both procarbazine treatments employed. Thus, despite the accumulation after the high dose treatment of 30% lower amounts of *O*⁶-meG in the bone marrow than the liver (18.5 ± 1.1 fmol/μg DNA, corresponding to 35 000 adduct residues per 10⁶ transgenes), a 16-fold increase of mutagenesis over background was observed in the former tissue. The much greater susceptibility of the bone marrow to procarbazine mutagenesis is probably due in part to its greater basal rate of cell proliferation. In addition, the bone marrow is a major target for the acute toxicity of procarbazine (8,29,30), which is expected to lead to restorative cell proliferation and favour mutation fixation, thus accounting for the susceptibility of the bone marrow to procarbazine mutagenesis. A number of studies (reviewed in reference 8) have shown that the haematopoietic system is also a major target of procarbazine carcinogenesis in the mouse. Interestingly, the same appears to be true in the monkey (31), in contrast to the rat where induction of leukaemia was relatively rare (8).

In addition to the rate of cell proliferation being an important factor that determines the relative tissue susceptibility to procarbazine mutagenesis, it is also possible that the accumulation of *O*⁶-meG is not a good measure of the critical pre-mutagenic damage induced by procarbazine, i.e. that miscoding by this lesion is not the primary mechanism of mutagenesis by this agent. Such an explanation would be compatible with

the mutation spectrum observed (Table III), in which G:C→A:T transitions (the fingerprint of *O*⁶-meG miscoding) constitute only 30% of the total. For comparison, it is noted that AGT-depleting doses of the methylating agents *N*-nitrosodimethylamine or *N*-methyl-*N*-nitrosourea gave rise to 80% and 95% G:C→A:T transitions in the liver and the spleen, respectively, of lacI transgenic mice (28,32). As three of the six G:C→A:T mutations induced by procarbazine were at CpG sites, it is possible that some or all of these might have arisen by deamination of 5-methylcytosine, thus decreasing the proportion of mutations likely to have derived from direct *O*⁶-meG miscoding even further. On the other hand, two of the remaining G:C→A:T mutations were at the 3'-G of pu-G dinucleotides (a site known to be favoured for *O*⁶-meG mutagenesis) and can probably be attributed to this lesion. Although *O*⁶-meG was the only adduct measured in the present study, it is expected that other DNA adducts (particularly *N*7-methylguanine and *N*3-methyladenine) were also produced in substantial amounts during the multiple treatments. Furthermore, procarbazine is known to undergo a complex pattern of metabolism, which in addition to the production of methylating intermediate(s), also leads to the formation of free radical species (9). If genotoxic species other than a methylating agent are also formed during the metabolism of procarbazine, they might well undergo substantial reaction with guanine (the most nucleophilic base in DNA) and give rise to some of the G-centred sequence changes observed. If alternative pathways of procarbazine metabolism result in the addition to DNA of the bulky side chain attached to the second hydrazine nitrogen, the resulting lesions might, for example, account for the frequent observation of frameshifts at or next to G (seven out of eight insertions or deletions), including four instances of frameshifts at GG dinucleotides, sites known to favour polymerase slippage (33).

A mixed mutation spectrum with a high proportion of frameshifts, especially those associated with polymerase slippage, is characteristically observed in mutants derived from cells deficient in mismatch repair (34,35). Methylating agents such as *N*-methyl-*N*-nitrosourea are well known to readily cause mutational inactivation of mismatch repair genes in mammalian cells, giving rise to mutants that, in addition to being hypermutable, are methylation tolerant and thus would have a growth advantage in a background of toxic methylation insult (36). The mixed mutation spectrum and the relatively high frequency of frameshifts observed in this study (Table III) would thus be compatible with the possibility of an additional pathway of procarbazine mutagenesis involving the inactivation of mismatch repair and subsequent generation of procarbazine-resistant cells prone to replication errors. While compatible with our observations and in line with known mutagenic properties of methylating agents, this hypothesis would need to be tested by specially designed experiments. An alternative possibility to consider is that the repetitive cycles of abortive mismatch repair that take place on the strand opposite *O*⁶-meG (36), believed to be the main basis of the cytotoxicity of this lesion, may give rise to DNA rearrangements in cells that survive this process.

Mutations in the *ras* oncogene have been detected in blood leukocytes of patients who had undergone cytotoxic therapy for lymphoma, which probably included procarbazine (38). Of the nine mutations found, seven were transversions (five G:C→T:A, one G:C→C:G, one A:T→C:G and two A:T→G:C transitions). Although the small number of mutations characterized and the uncertain nature of the administered chemotherapy

do not permit any definitive conclusions, it is evident that this mutation spectrum does not resemble the one observed in the present study. On the other hand, the absence of *ras* gene G:C→A:T mutations is in concordance with the low proportion of such mutations in the present study and supports the suggestion that direct miscoding by *O*⁶-meG may not be the main source of procarbazine-induced mutations. If confirmed by further studies, this suggestion might have important implications regarding the possibility of modification of chemotherapeutic protocols involving procarbazine with the aim of enhancing their therapeutic efficacy. Since there is evidence that *O*⁶-meG contributes substantially to the toxicity of procarbazine (39,40), in principle it would be possible to enhance this drug's cytostatic efficacy through the depletion of AGT, along lines similar to those currently the subject of extensive experimental studies and clinical trials with nitrosourea-based drugs (41). However, in contrast to the latter drugs that bring about their mutagenic effects primarily via DNA cross-links, rather than *O*⁶-alkylguanine-type lesions, any enhancement of procarbazine-induced *O*⁶-meG accumulation would carry with it the undesirable risk of a substantial increase in mutagenicity. If, on the other hand, *O*⁶-meG plays only a minor role in procarbazine mutagenesis, as implied by the results reported here, such risks may be smaller than until now anticipated. This would open the way to protocol modifications that might be particularly valuable in the treatment of brain tumours, where procarbazine-mediated chemotherapy has not met with the success that it has in the case of Hodgkin's lymphoma.

In conclusion, the data reported here indicate that the mouse bone marrow is highly susceptible to procarbazine mutagenesis and that mutations induced may not be primarily derived from direct miscoding by *O*⁶-meG. The molecular mechanism of the observed mutagenesis, and whether the same mechanism determines mutagenesis in genes involved in procarbazine carcinogenesis in man, are important questions that remain to be elucidated.

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