N7-Methylguanine and O6-methylguanine levels in DNA of white blood cells from cancer patients treated with dacarbazine

Joost H. M. van Delft, Anja Luiten-Schuite, Vassilis L. Souliotis, Soterios A. Kyrtopoulos, Jan Ouwerkerk, H. Jan Keizer and Robert A. Baan

Methyl-DNA adducts are induced by a number of lifestyle, environmental and occupational carcinogens, however knowledge about their kinetics is scarce. Here, *N7*-methylguanine (*N7*-MeGua) and *O*^{6}-methylguanine (*O*^{6}-MeGua) levels were determined in the DNA of white blood cells from eight cancer patients treated iv with the antitumour drug dacarbazine (DTIC). Five of the patients were treated with the drug as a single agent (a single dose of 800 mg m^{-2}) and three on three successive days with dacarbazine (225 mg m^{-2} day^{-1}) in combination with other drugs. The data indicate that maximum adduct levels are reached at 4–8 h after treatment and that the amount of *N7*-MeGua is at least 20-fold higher than that of *O*^{6}-MeGua. The half-life of *N7*-MeGua is 40–96 h and that of *O*^{6}-MeGua 25–27 h. Following treatment on three consecutive days, an accumulation of N7-MeGua was observed but not of *O*^{6}-MeGua. The data show substantial interindividual differences in adduct levels but not in the ratio of *N7*/*O*^{6}-MeGua. This may reflect differences in the metabolism of dacarbazine or in repair capacities.

Keywords: *dacarbazine*, N7-*methylguanine*, O^6-*methylguanine*, N-*nitrosamine*.

Introduction

Controlled human exposure to alkylating agents occurs during treatment of patients with cytostatic drugs directed against various types of tumours. Dacarbazine, i.e. 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC), is a methylating agent used for the treatment of malignant melanoma and soft tissue sarcoma (IARC 1981, Legha 1989). The drug may serve as a model compound for human exposure to methylating genotoxic agents, in particular *N*-nitrosamines such as dimethylnitrosamine, which has a similar metabolic pathway to dacarbazine (Pegg 1980, Meer *et al.* 1986) (see Figure 1). *N*-Nitroso compounds are found among lifestyle, environmental and occupational carcinogens (Hemminki 1990, Searle and Teale 1990). They are supposed to play an important role in mutation induction and tumour formation as a result of their interaction with DNA, either directly or after formation of reactive alkylating metabolites. Biological monitoring studies have indeed demonstrated the presence of methyl-DNA adducts in blood cells from persons with increased exposure to exogenous methylating agents (Mustonen *et al.* 1993a, The Eurogast Study Group 1994). In biomonitoring studies, white blood cells (WBC) are often used as a substitute for the target tissue because blood samples can be obtained relatively easily. Investigations with animal models have already yielded information about the usefulness of WBC data for the prediction of *N*-nitrosamine-induced effects on DNA in target tissues (Bianchini and Wild 1994, Souliotis *et al.* 1995). However, for the evaluation and interpretation of biomonitoring studies and for the extrapolation of animal data to man, knowledge about the kinetics of DNA adducts in man is essential but only scarcely available. The chemotherapy of cancer patients with alkylating drugs offers a unique opportunity to acquire this knowledge.

Dacarbazine, as well as dimethylnitrosamine, is oxidized by microsomal enzymes to an *N*-hydroxymethyl-, *N*-methyl-derivative, which leads in various non-enzymatic steps to the formation of the methyl diazonium ion (Figure 1). Methylation

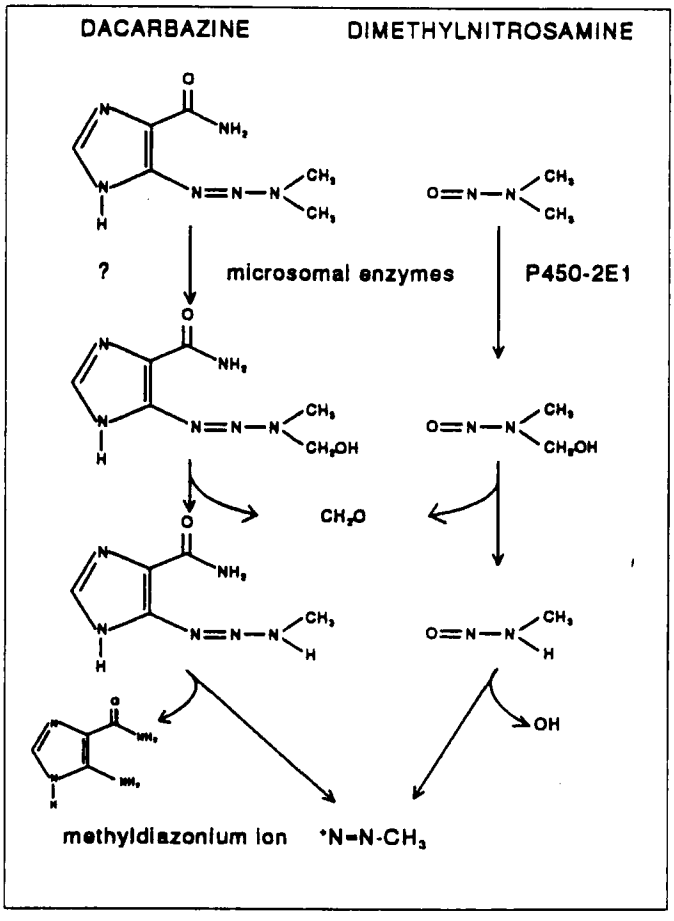

Figure 1. Metabolic pathway for dimethylnitrosamine (DMN) and dacarbazine (DTIC).

Joost H. M. van Delft *(author for correspondence)*, **Anja Luiten-Schuite**, **Robert A. Baan** *are in the Department of Genetic toxicology, Toxicology Division, TNO Nutrition and Food Research Institute, PO Box 360, 3700 AJ Zeist, The Netherlands;* **Vassilis L. Souliotis**, **Soterios A. Kyrtopoulos** *are at the Laboratory of Chemical Carcinogenesis, National Hellenic Research Foundation, 48 Vas. Constantinou Avenue, 11635 Athens, Greece;* **Jan Ouwerkerk** *and* **H. Jan Keizer** *are in the Department of Clinical Oncology, University Hospital, State University of Leiden, PO Box 9600, 2300 RC Leiden, The Netherlands.*

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of DNA by this reactive species leads predominantly to the formation of *N*7-methylguanine (*N*7-MeGua) (≈ 70%). Minor reaction products are *N*3-methyladenine (*N*3-MeAde; ≈ 8%), O^6-methylguanine (O^6-MeGua; ≈ 7%) and methylphosphotriesters (≈ 12%) (Singer and Grunberger 1983).

In only a limited number of studies, the formation of DNA adducts by methylating chemotherapeutic drugs has been described. So far, either only one type of adduct has been investigated, e.g. either *N*7-MeGua following treatment with dacarbazine (Mustonen 1991, van Delft *et al.* 1992, Mustonen *et al* 1993b) or O^6-MeGua after dacarbazine or procarbazine treatments (Souliotis *et al.* 1990, 1991, 1994), or both adducts have been investigated but only for one day after treatment (Philip *et al.* 1995). In the present study both *N*7- and O^6-MeGua levels were determined for several days in WBC DNA from patients who received chemotherapy with the methylating drug dacarbazine.

MATERIALS AND METHODS

Blood sample collection

Samples were obtained from patients receiving chemotherapy with dacarbazine as a single agent or in combination with the antitumour drugs *cis*-diamminedichloroplatinum(II) (CDDP) and bis-(chloroethyl)-nitrosourea (BCNU) (see Table 1). For single agent therapy, dacarbazine was administered at a dose of 800 mg m^{-2} body surface during a 60-min iv infusion. Blood samples were taken before and at several time-points after the dacarbazine infusion. Patients treated with combination therapy received 225 mg dacarbazine per m^2 together with 25 mg CDDP per m^2 and 150 mg BCNU per m^2 during a 30-min iv infusion on each of three successive days. Samples taken at 24 and 48 h after the first treatment were collected just prior to the second and third treatment. Blood samples (2 × 10 ml) were collected in evacuated tubes containing EDTA and immediately stored at −70 °C until analysis of the methyl-DNA adducts. In some cases, lymphocytes were isolated from fresh blood by centrifugation on Lymphoprep (Nycomed AS, Oslo, Norway) and stored at −20 °C. Samples were collected from three patients during two successive chemotherapy cycles, at 3-week intervals.

Patient code[a]	Age (years)	Gender	Tumour	Therapy
2	61	F	Melanoma	Combination
3	47	F	Melanoma	Combination
4	51	M	Melanoma	Combination
001	29	F	Melanoma	Single agent
002	62	M	Melanoma	Single agent
003	37	F	Melanoma	Single agent
004	49	M	Melanoma	Single agent
005	30	M	Melanoma	Single agent

Table 1. Description of patients receiving chemotherapy with dacarbazine as single agent or in combination with other drugs.

[a] All patients were treated in Leiden.

Analyses of the methyl-DNA adducts N7-MeGua and O^6-MeGua

After thawing of the blood samples, the red blood cells were lysed by addition of three volumes of cold 0.9% NH_4Cl and incubation for 5 min at 4 °C. WBC were collected by centrifugation (10 min at 450 *g*, 4 °C), whereafter DNA was isolated as described previously (Souliotis *et al.* 1994). For analysis of the *N*7-MeGua levels (all done at TNO), part of the DNA was sonicated and treated with alkali in order to convert *N*7-MeGua into the imidazole ring-open derivative, which can be detected with the monoclonal antibody N7E-026 (van Delft *et al.* 1991). This antibody specifically recognizes ring-open *N*7-MeGua and ring-open *N*7-ethylguanine in DNA, hardly binds to 2-hydroxyethyl lesions and does not show any affinity for sulphur-mustard lesions or O^6-ethylguanine in DNA. Adduct levels in the DNA samples from patients treated with the combination therapy were measured in a direct ELISA (van Delft *et al.* 1992) and from the single-agent therapy group in an immunoslot-blot assay. The procedure for the immunoslot-blot assay has been described elsewhere (van Delft *et al.* 1994). With a detection limit of one *N*7-MeGua per 10^6 nucleotides, the latter assay is more sensitive than the direct ELISA. Each sample was analysed in duplicate. DNA standards were obtained by treatment of DNA with *N*-methyl-*N*-nitrosourea (MNU, Sigma, St Louis, USA) *N*7-MeGua levels therein were analysed by HPLC with electrochemical detection (van Delft *et al.* 1991).

O^6-MeGua in DNA was measured in Athens in the competitive repair assay as described earlier (Souliotis and Kurtopoulos), with *Escherichia coli* O^6-alkylguanine-DNA-alkyltransferase (AGT). Up to 10 μg DNA were employed per assay and each sample was analysed in duplicate.

Results

For the validation of the assays used for the measurements of the *N*7- and O^6-MeGua levels, which were performed at TNO and the National Hellenic Research Foundation, respectively, a series of MNU-treated DNA samples used for the analysis of *N*7-MeGua were also analysed for O^6-MeGua. As can be seen in Table 2, the O^6-MeGua/*N*7-MeGua ratio is about 0.12 (0.10–0.13). This agrees with the known ratio for MNU of 0.10 (Singer and Grunberger 1983), and indicates that data generated by the two methods are reliable.

From three patients who were treated with dacarbazine only, several blood samples were collected between 4 and 72 h after treatment, from two others only one sample was taken at 24 h. The *N*7-MeGua and O^6-MeGua levels in WBC DNA are presented in Figure 2. For both adducts interindividual variations were within a two-fold range. The maximum amount of *N*7-MeGua seemed to be reached slightly later than

MNU (μM)	Adducts per 10^6 nucleotides		
	*N*7-MeGua	O^6-MeGua[a]	O^6/*N*7-ratio
0	0	0	–
10	5.4	0.72	0.13
33	22	2.51	0.12
100	84	8.1	0.10

Table 2. *N*7- and O^6-MeGua levels in DNA treated with *N*-methyl-*N*-nitrosourea (MNU).

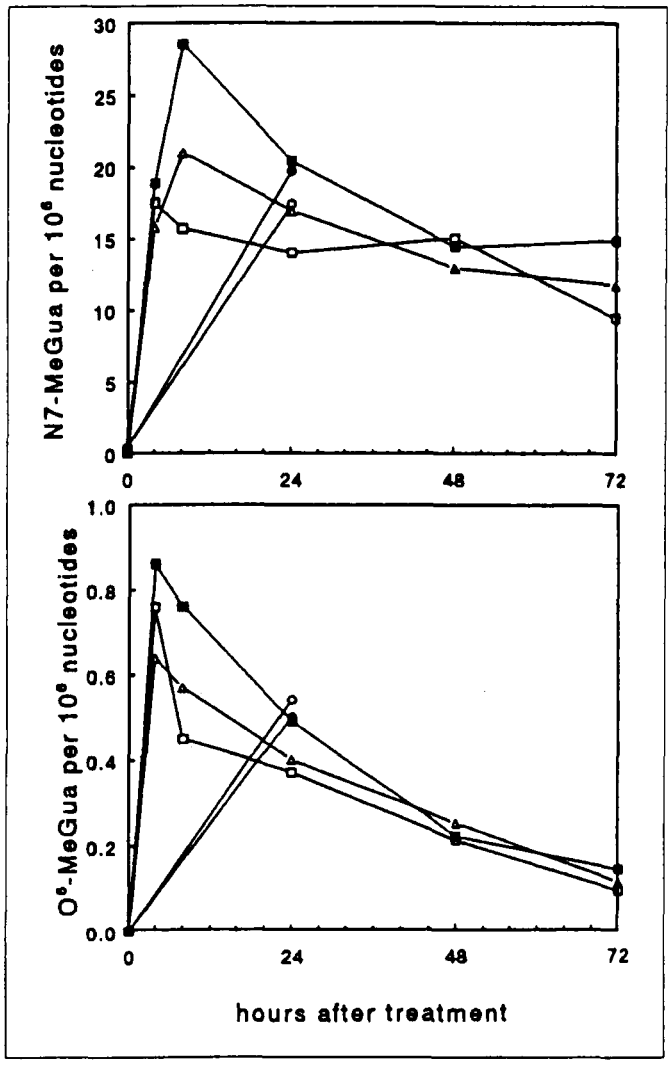

Figure 2. *N*7-MeGua (upper panel) and O^6-MeGua (lower panel) levels in DNA of WBC from five patients treated with dacarbazine only at a single dose of 800 mg m^{-2}. Each symbol represents a different patient.

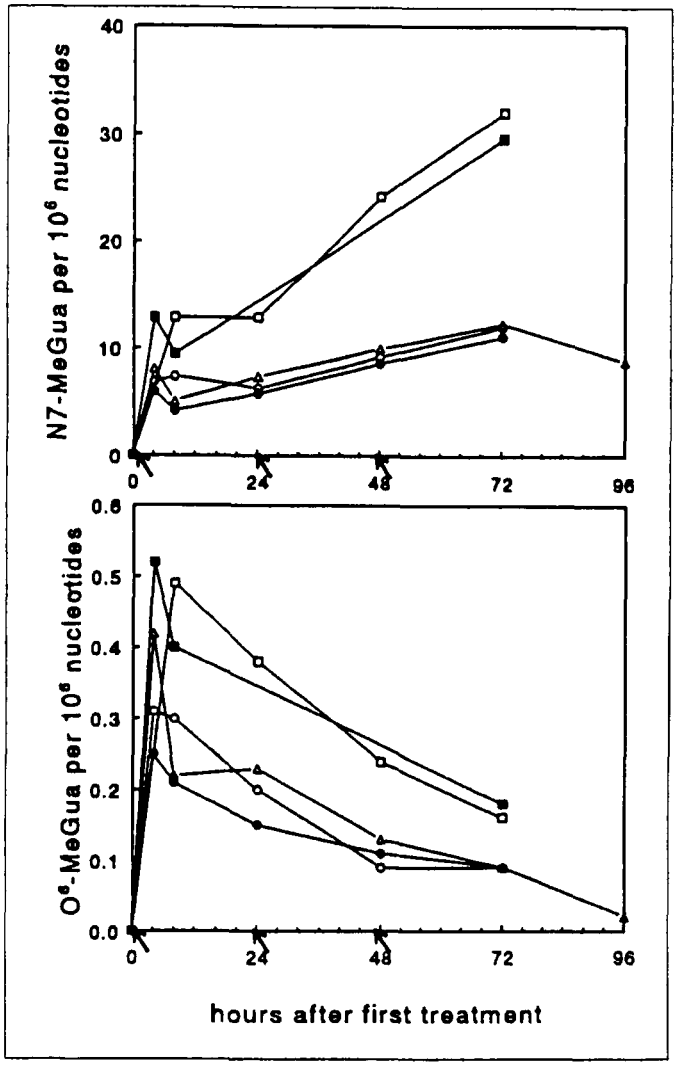

Figure 3. *N*7-MeGua (upper panel) and O^6-MeGua (lower panel) levels in DNA of WBC from three patients treated with dacarbazine in combination with BCNU and CDDP on three successive days. All patients received 225 mg dacarbazine m^{-2} day^{-1}. For two patients data were obtained from blood samples collected during the first and the second chemotherapy cycle, closed and open symbols respectively. Each symbol represents a different patient.

that of O^6-MeGua, i.e. between 4 and 8 h, and after 4 h, respectively. When plotted semi-logarithmically, the apparent half-life of *N*-7MeGua was calculated to be around 65 h (range: 40–96 h) and the half-life of O^6-MeGua was around 26 h (range: 25–27 h). At 4 h after treatment the *N*7/O^6-ratio was 23, and it continuously increased thereafter. For two patients adduct levels were also determined in purified lymphocytes. Compared with total WBC, the *N*7-MeGua levels in the lymphocytes were slightly higher but the O^6-MeGua levels were about the same (data not shown).

In addition, blood samples were studied from three patients who received dacarbazine on three consecutive days in combination with BCNU and CDDP. From two of these patients samples were taken during two successive cycles with 3-week intervals. After the first treatment, blood samples were collected between 4 and 96 h. The data, depicted in Figure 3, show that *N*7-MeGua accumulated after each of the three doses, whereas the level of O^6-MeGua reached a maximum at 4–8 h after the first treatment and did not increase following the dosages on the next 2 days. In one patient the treatments resulted in roughly two-fold higher adduct levels, both for *N*7- and O^6-MeGua, than in the blood samples from the other two patients. For all patients individually, however, hardly any differences in adduct levels were observed between the two chemotherapy cycles.

All available data on *N*7- and O^6-MeGua levels in WBC collected at 8 h and 24 h after dacarbazine treatment are plotted in Figure 4. It demonstrates that in all cases the *N*7- and O^6-adduct levels were strongly correlated.

Discussion

For the first time the analysis is described of both the *N*7- and O^6-MeGua levels after several days in DNA from WBC of patients receiving chemotherapy with a methylating agent.

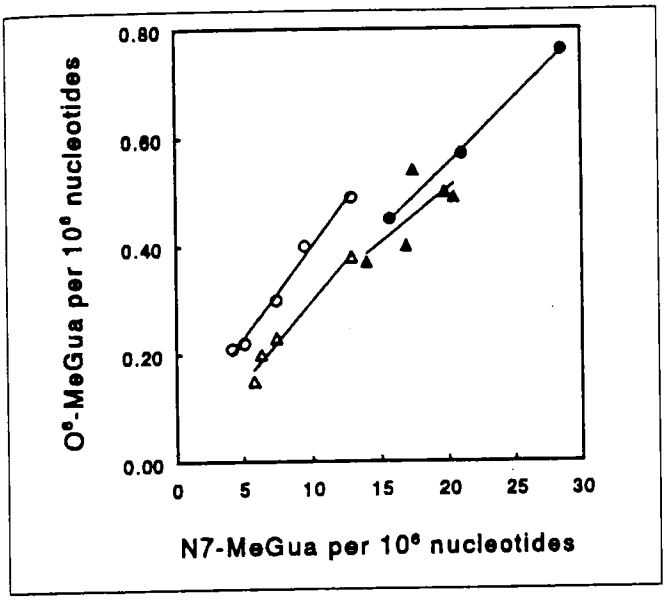

Figure 4. O^6-MeGua versus *N*7-MeGua levels in DNA from blood samples collected at 8 h (circles) and at 24 h (triangles) after dacarbazine treatment. Closed symbols are data from patients treated with 800 mg m^{-2} and the open symbols after a first treatment with 225 mg m^{-2}.

Knowledge about the response of individuals exposed to methylating agents is needed for the extrapolation of animal data to man, as well as for the evaluation and interpretation of biomonitoring studies. Cancer patients who receive chemotherapy under well-controlled conditions offer a unique opportunity to acquire this knowledge.

Here we show that after treatment with dacarbazine *N*7-MeGua is induced at much higher levels than O^6-MeGua, that *N*7-MeGua has a two- to three-fold longer half-life, and that—in contrast to O^6-MeGua—*N*7-MeGua accumulates upon treatments on successive days. These data suggest that for monitoring of environmental, life-style or occupational exposure to methylating agents, *N*7-MeGua may be a more suitable marker than O^6-MeGua. However, the analysis method for O^6-MeGua is far more sensitive (presently one per 10^8 nucleotides; unpublished data from Souliotis and Kyrtopoulos) than that of *N*7-MeGua (one per 10^6 nucleotides). In addition, O^6-MeGua is a more relevant measure of the mutagenic and carcinogenic theory of methylating agents (Frei *et al.* 1978, Van Zeeland *et al.* 1985).

The half-life of *N*7-MeGua in the present study, ranging from 40 to 96 h, is comparable to that published in a previous study (van Delft *et al.* 1992) and corresponds to that in liver (60 h; Frei *et al.* 1978) of mice. The O^6-MeGua levels found in this investigation are of the same order as those in a previous study, when taking into account the different exposure levels (Souliotis *et al.* 1991). The half-life of O^6-MeGua, about 26 h, is comparable to that in another study (Souliotis *et al.* unpublished data). Comparable half-life values have been observed in several organs and in WBC of rats and mice (Frei *et al.* 1978, Valavanis *et al.* 1994). In cells, O^6-MeGua is mainly repaired by the suicide enzyme AGT; it transfers the methyl group from the DNA to one of its cysteines, which results in the inactivation of the enzyme. Indeed, a reduction of the AGT activity in lymphocytes from patients treated with dacarbazine at doses > 500 mg kg^{-1}, has been described (Lee *et al.* 1991, Philip *et al.* 1995). This may also be true in the current study, and may have affected the repair rate of O^6-MeGua.

At 4 h after dacarbazine treatment, when the plasma concentration is reduced by more than 90% (Buesa and Urzechaga 1991), the ratio of *N*7-MeGua to O^6-MeGua is about 24 instead of 10, which has been determined following reaction of the methyl diazonium ion with DNA *in vitro* (Singer and Grunberger 1983). This discrepancy is most likely due to fast initial repair of O^6-MeGua and indicates that roughly 60% of this adduct has already been removed within the first 4 h after dacarbazine treatment. It suggests that the initial half-life of O^6-MeGua is much shorter than the 26 h found for the repair period between 4 h and 3 days (see above).

In patients treated on three successive days with dacarbazine in combination with BCNU and CDDP, the *N*7-MeGua levels accumulated with the dose. However, the O^6-MeGua level showed no increase: it reached a maximum at 4–8 h after the first infusion and thereafter it gradually decreased. This finding contrasts with the observations in patients who received the methylating drug procarbazine as part of a combination chemotherapy (Souliotis *et al.* 1990, 1994). In these studies a continuous accumulation of O^6-MeGua was observed throughout the exposure period of 10 days with three oral dosages per day. In the lymphocytes of these patients no decrease in AGT content was seen during these periods, probably due to the induction of much lower levels of O^6-MeGua compared with treatment with dacarbazine. In the rat, procarbazine causes a prolonged depletion of AGT, while dacarbazine does not have such a prolonged effect (Valavanis *et al.* 1994, Souliotis *et al.* unpublished data). At this moment no conclusive explanation is available for these contrasting findings on O^6-MeGua accumulation. It may be that combination therapy with CDDP and BCNU, which can also form DNA-adducts, results in a stimulation of the repair of O^6-MeGua or competes with the adduct formation by dacarbazine. Another explanation can be that procarbazine, which is metabolized to the methyldiazonium ion (Prough and Tweedie 1988) but can also form methyl radicals (Goria-Gatti *et al.* 1992), induces different adduct spectra and distribution than dacarbazine. Indeed, methyl radicals are able to generate 8-methylguanine residues in DNA (Augusto *et al.* 1990), while this lesion is not known to be induced by methyldiazonium ions. It cannot be excluded that differences in O^6-MeGua accumulation are due to differences in timing of treatment (one per day versus three per day for dacarbazine and procarbazine, respectively) and blood collection (24 h after treatment versus 2–4 h after the day's first intake), and in kinetics of the metabolism of both drugs.

Although the data in this study are from a limited number of patients, they do show substantial interindividual differences. The differences are similar for both *N*7-MeGua and O^6-MeGua. This may reflect interindividual variations in the metabolisms of dacarbazine, or in repair capacities. However, to see whether these differences have a predictive value for the tumour responses to the chemotherapy, more data are required.

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