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ORIGINAL ARTICLE

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Evaluation of the alkaline comet assay and urinary 3-methyladenine excretion for monitoring DNA damage in melanoma patients treated with dacarbazine and tamoxifen

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Abstract *Purpose*: To develop, using dacarbazine as a model, reliable techniques for measuring DNA damage and repair as pharmacodynamic endpoints for patients receiving chemotherapy. *Methods*: A group of 39 patients with malignant melanoma were treated with dacarbazine 1 g/m^2 i.v. every 21 days. Tamoxifen 20 mg daily was commenced 24 h after the first infusion and continued until 3 weeks after the last cycle of chemotherapy. DNA strand breaks formed during dacarbazine-induced DNA damage and repair were measured in individual cells by the alkaline comet assay. DNA methyl adducts were quantified by measuring urinary 3-methyladenine (3-MeA) excretion using immunoaffinity ELISA. Venous blood was taken on cycles 1 and 2 for separation of peripheral blood lymphocytes (PBLs) for measurement of DNA strand breaks. *Results*: Wide interpatient variation in PBL DNA strand breaks occurred following chemotherapy, with a peak at 4 h (median 26.6 h, interquartile range 14.75–40.5 h) and incomplete repair by 24 h. Similarly, there was a range of 3-MeA excretion with peak levels 4–10 h after chemotherapy (median 33 nmol/h, interquartile range 20.4–48.65 nmol/h). Peak 3-MeA excretion was positively correlated with DNA strand breaks at 4 h (Spearman's correlation coefficient, $r = 0.39$, $P = 0.036$) and 24 h ($r = 0.46$, $P = 0.01$). Drug-induced emesis correlated with PBL DNA strand breaks (Mann Whitney *U*-test, $P = 0.03$) but not with peak 3-MeA excretion. *Conclusions*: DNA damage and repair following cytotoxic chemotherapy can be measured in vivo by the alkaline comet assay and by urinary 3-MeA excretion in patients receiving chemotherapy.

Key words Comet assay · 3-Methyladenine · DNA damage · DNA repair · Melanoma · Dacarbazine · Tamoxifen

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Introduction

Malignant melanoma is frequently resistant to treatment with radiotherapy or chemotherapy. Dacarbazine (DTIC; Dome, Bayer) is the most active cytotoxic drug, producing response rates of between 10 and 20% in patients with metastatic disease [8]. The trigger for apoptotic cell death following cytotoxic chemotherapy is thought to be DNA damage and the subsequent inability of the cell to repair this damage [13, 16, 25]. However, there have been relatively few studies of the potential of measuring DNA damage and repair as a means of monitoring therapeutic intervention [32]. Formation of DNA methyl adducts and subsequent base excision repair provide two possible consequences of dacarbazine therapy that might allow the design of reliable methods for pharmacodynamic monitoring following treatment with the drug.

After intravenous injection, dacarbazine is metabolically activated by microsomal *N*-demethylation to 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide (MTIC) [15, 19]. MTIC methylates DNA to produce a number of methyl lesions including O^6-methylguanine (O^6-MeG), N7-methylguanine (N7-MeG), 3-methyladenine (3-MeA) and other alkyl purines. Alkylating agents can induce p53, cell cycle arrest and apoptosis [14, 23] and it is therefore assumed that methyl lesions directly signal to these pathways. O^6-MeG is thought to be the most cytotoxic lesion and is specifically repaired by

O^6-alkylguanine DNA alkyl transferase (AGT). Depletion of AGT is associated with increased sensitivity to methylating agents [31] whilst elevated levels may lead to resistance to MTIC in vitro [21].

3-MeA and other alkyl purines are removed from DNA either by spontaneous depurination or by the action of alkyl adenine DNA glycosylase (AAG) leaving an apurinic (AP) site which initiates the base excision repair pathway [4]. In mouse embryonic stem cells AAG protects against DNA crosslinking agents such as mitomycin C and BCNU (1,3-bis(2-chloroethyl)-1-nitrosurea) but not against several of the nitrogen mustard chemotherapeutic agents [1, 10]. Urinary 3-MeA excretion has previously been used as a marker of DNA methylation in human volunteers [34, 35, 39] and may provide a non-invasive method for monitoring chemotherapy-induced DNA damage in vivo [37]. It is interesting to note that methyl adducts such as O^6-MeG are cytotoxic by their presence, whereas 3-MeA appears to be cytotoxic by its absence, i.e. removal of 3-MeA gives rise to an abasic site which is pivotal to toxicity [11]. Therefore the measurement of urinary 3-MeA is a relevant marker of a major pathway of toxicity by methylating drugs.

The single cell gel electrophoresis (SCGE), or comet assay, measures DNA strand breaks in mammalian cells [28, 40, 41], and can be used to quantify the transient single strand breaks formed during the base excision repair pathway [12]. This assay has been widely used to screen for genotoxic agents in the environment [2, 33], to look at cell sensitivity to radiation [29, 43], and to study drug sensitivity in vitro [6, 12, 24, 30] and in vivo in animals [9]. The technique has been shown to provide an index of sensitivity to etoposide in xenografted tumours in vivo [17] and therefore lends itself to the evaluation of the pharmacodynamic effects of cytotoxic drugs during clinical cancer therapy.

In this study we assessed two techniques for measuring DNA strand breaks and DNA adducts. These assays provide an indication of the combined effects of DNA damage and repair as well as drug uptake and metabolism in patients receiving cytotoxic chemotherapy. Initially the alkaline comet assay was validated in terms of sample storage and processing of peripheral blood lymphocytes (PBLs). Subsequently we determined the time-course of DNA strand breaks in PBLs from patients with malignant melanoma receiving treatment with dacarbazine alone (cycle 1)[1] and dacarbazine with tamoxifen (cycle 2).

Timed urine collections were performed from the same patient group to measure the time-course of urinary 3-MeA excretion and to correlate this with strand breaks measured by the alkaline comet assay. Both assays reflect the net or cumulative effects of various parameters such as drug uptake, metabolism, and DNA damage and repair, rather than being a measure of a single endpoint. Furthermore, urinary excretion of 3-MeA may be, in part, derived from degraded DNA in apoptotic cells. However, as an indication of total DNA damage, urinary 3-MeA can provide a potentially useful insight into interindividual handling of the same dose of methylating drug [37].

Patients and methods

Patients and treatment

A group of 39 consecutive patients with malignant melanoma receiving dacarbazine and tamoxifen for treatment of metastatic disease (19 patients) or in the adjuvant setting (20 patients) were entered into the study (Table 1). The criteria for offering adjuvant treatment were primary tumour thickness ≥4 mm, presence of two or more regional nodes at surgery or any surgically excised recurrence within 2 years of primary diagnosis. All patients had a staging computed tomography (CT) scan prior to treatment and, where applicable, measurable disease was documented. Eligibility criteria included histologically or cytologically confirmed malignant melanoma, age ≥18 years, World Health (WHO) performance status 0, 1 or 2 and adequate haematological and biochemical function. All patients gave written informed consent and the study was conducted with the approval of the Central Oxford Research Ethics Committee.

Patients were treated with dacarbazine 1 g/m^2 in 500 ml 0.9% saline intravenous infusion over 1 h on day 1 of each cycle. Antiemetic prophylaxis consisted of tropisetron 5 mg on day 1 of treatment, dexamethasone 8 mg twice daily for 3 days and metoclopramide 10 mg twice daily when required. Treatment was repeated every 21 days to a maximum of four cycles for adjuvant patients and six cycles, depending upon response, for patients with metastatic disease. Tamoxifen 20 mg daily was commenced 24 h after the first infusion and continued until 3 weeks after the last cycle of dacarbazine. Treatment was delayed for 1 week in patients with an absolute neutrophil count $\leq 1.5 \times 10^9$/l or platelets $\leq 100 \times 10^9$/l. There was a 25% dose reduction for grade IV toxicity or for a treatment delay of more than 1 week. Toxicity was graded according to the WHO criteria and, for patients with metastatic disease, response was evaluated by computed tomography (CT) scan after every two cycles and at the completion of treatment.

Collection of samples

Venous blood (20 ml) was taken into glass Vacutainer tubes (Becton Dickinson) containing EDTA and stored in the dark on

Table 1 Patient characteristics

Number of patients	
Male	22
Female	17
Total	39
Adjuvant	20
Metastatic	19
Evaluable for toxicity	38
Evaluable for response	19
Age (years)	
Median	54
Range	26–81
Site of metastases[a] (number of patients)	
Skin	5
Lymph nodes	8
Lungs	6
Liver	7
Bone	2
Adrenal	1
Brain	1

[1] Cycles are repeated every 21 days

[a] Some patients more than one site

crushed ice until processing. All samples were processed within 4 h of collection. PBLs were separated by density centrifugation over 5 ml Lymphoprep (Nicomed, Majorstua, Norway) at 4 °C, washed twice in phosphate-buffered saline (PBS) and snap-frozen in 0.5-ml aliquots at −70 °C in 10% dimethyl sulphoxide (DMSO) in PBS. During the initial validation of the technique, patient blood samples were taken pretreatment and at 1, 2, 4, 8 and 24 h after dacarbazine treatment. These were assayed by the comet assay to obtain a profile of DNA damage and repair in vivo prior to rationalizing time-points for collection of further PBL samples.

Timed urine collections were obtained from patients in the study on cycles 1 and 2 of treatment prior to chemotherapy and for up to 24 h after chemotherapy. Timed urine collection periods were pretreatment, 0–4 h, 4–10 h, 10–20 h and 20–24 h. All urine excreted during each time period was collected into plain sterile containers without preservative and the total volume measured. A 20-ml aliquot of urine from each collection was frozen at −70 °C until analysis.

Comet assay

The method used was a modification of that described by Olive [26] with assay conditions optimized for measuring substantial DNA damage after chemotherapy. Stored PBLs were thawed, diluted 1:4 in PBS, and mixed with an equal volume of 2% low melting point agarose at 40 °C. A 1-ml aliquot of the suspension was layered onto polylysine-coated slides (BDH Laboratory Supplies, Poole, UK), lowered into lysis buffer (0.3 *M* NaOH/1 *M* NaCl/0.1% *N*-lauroylsarcosine, pH 11.5) and left in the dark for 1 h to lyse cells and remove most of the proteins. Salt was removed with two 30-min washes in 0.3 *M* NaOH/2 m*M* EDTA before the DNA was electrophoresed at 0.55 V/cm (40 mA) for 25 min in the same buffer at pH 11.5. This wash time provided 1 h DNA unwinding time, which has been shown to be important in the detection of DNA damage [22]. After electrophoresis the DNA was stained with propidium iodide and examined under a confocal microscope (MRC 600, excitation wavelength 480 nm, magnification ×100). The area and mean pixel intensity of the head and the tail of the comets were measured to determine the percentage DNA in the tail for the individual cell [5, 27]. In each patient sample a total of 30 cells, from two slides, were measured and the mean DNA damage was determined from all the cells examined.

Validation of techniques

The effect of storing blood samples prior to processing was assessed by comparing DNA damage in samples which had been stored on ice for 4 h with those which had been processed immediately. Similarly assays were carried out on both fresh cells and those frozen at −70 °C for control samples and cells containing DNA damage.

A dose-dependent increase in DNA strand breaks following treatment with temozolamide (a generous gift from Prof. M. Stevens, University of Nottingham, UK) was evaluated in PBLs from a normal volunteer. Temozolamide is an imidazotetrazine derivative that is degraded to the active metabolite MTIC by mild alkaline hydrolysis [42]. PBLs were treated in vitro for 1 h with temozolamide (concentration range 1–1000 μg/ml) before assessment of DNA strand breaks by the comet assay. The time-course of DNA strand breaks was assessed in vitro by exposure of PBLs to temozolamide (100 μg/ml) for 24 h with comet assays performed on aliquots taken pretreatment and at 0.5, 1, 2, 4, 6, 8 and 24 h.

3-Methyladenine excretion

3-MeA was determined in 2-ml aliquots of urine from each time period as described previously [35]. Briefly, the urine pH was adjusted to pH 7.4 and [^{3}H]-3-MeA (1000 dpm) was added as internal standard. The urine was eluted through an immunoaffinity column (IgG protein A-sepharose with an IgG fraction from an anti-3-MeA rabbit serum). After a series of washes with PBS (5 ml) and water (10 ml), 3-MeA was eluted with 1 *M* acetic acid (2 ml) and recovery calculated by counting the radioactivity in 1 ml of the sample. The remaining 1 ml was reconstituted in PBS for analysis by competitive ELISA.

Statistical methods

Results from the comet assay and 3-MeA excretion did not demonstrate a normal distribution and were therefore analysed by nonparametric statistical methods. Matched pairs of data for DNA strand breaks and 3-MeA excretion were compared with baseline data at each time-point in cycles 1 and 2 using a signed rank's test. DNA damage measured by the comet assay at 4 and 24 h on cycle 1 was assessed against peak 3-MeA excretion by Spearman's correlation coefficient. Comparison of DNA damage with toxicity from treatment was analysed by the Wilcoxon rank-sum (Mann-Whitney) test.

Results

Comet assay

Sample storage and in vitro validation

Storage of blood on ice for up to 4 h prior to PBL separation did not cause DNA strand breaks in cells with undamaged DNA ($n = 5$ normal nonsmoking adults, $n = 3$ melanoma patients, mean percentage DNA in the tail was 0% in paired samples processed immediately or stored on ice for 4 h). Similarly the level of DNA damage in previously damaged cells was not altered by storage on ice ($n = 3$, mean ± SD percentage DNA in the tail was 47.6 ± 11.8% for immediate assay and 45.3 ± 12.0% after 4 h on ice) or at −70 °C for 2 days (mean percentage DNA in the tail 45.5 ± 10.0%). There was no significant increase in damage in samples stored for up to 28 days (undamaged: $n = 1$, mean percentage DNA in the tail, fresh 0%, frozen 0%; damaged: $n = 1$, mean percentage DNA in the tail, fresh 39.9 ± 19.7%, frozen 57.5 ± 21.3%). The median storage time for samples in this study was 19 days, although PBLs were stored at −70 °C for up to 43 days with no detectable damage in comparison with pretreatment samples.

In vitro exposure of PBLs to temozolamide for 1 h resulted in increasing numbers of strand breaks with doses up to 1000 μg/ml (Fig. 1). The time-course of strand breaks in PBLs exposed to temozolamide showed a peak in damage at 90 min with a reduction in strand breaks at subsequent time-points (Fig. 2a). There was substantial variation in DNA damage between individual cells leading to a wide standard deviation.

Comet assay in vivo

PBLs were collected at 0, 1, 2, 4, 8 and 24 h from eight patients to determine the time-course of DNA damage following treatment with dacarbazine (Fig. 2b). Subse-

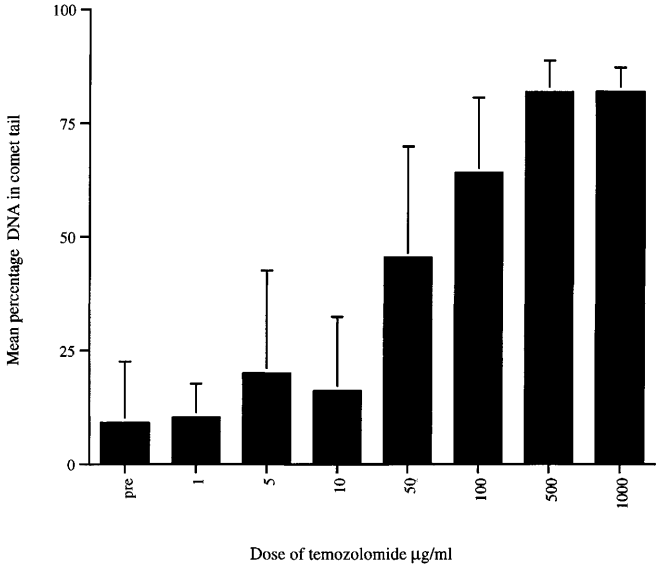
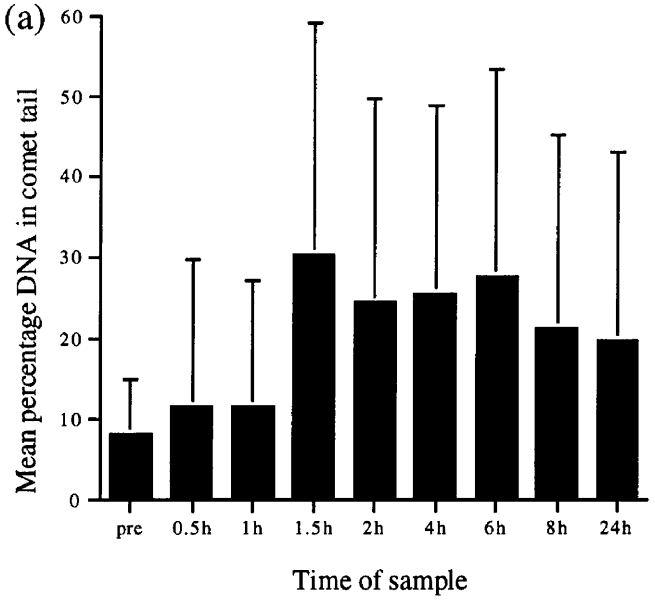


Fig. 1 In vitro assay to determine DNA strand breaks using the alkaline comet assay in PBLs following treatment with increasing concentrations (range 0–1000 μg/ml) of temozolamide (Y-*axis* DNA damage expressed as percentage DNA in the tail, *bars* SD; X-*axis* concentration of temozolamide)



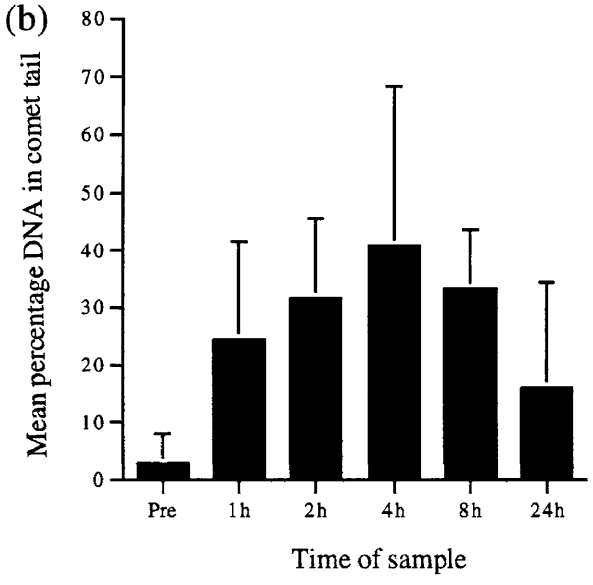


Fig. 2 **a** In vitro time-course of PBLs treated with temozolamide 100 μg/ml. Aliquots of PBLs were taken prior to drug exposure and at 0.5, 1, 2, 4, 6, 8 and 24 h. There was substantial variation in strand breaks in individual cells reflected in a wide standard deviation. **b** Time-course of DNA damage in PBLs from melanoma patients following cycle 1 of dacarbazine therapy assessed by the alkaline comet assay. Data are presented as the means from eight patients studied (Y-*axis* DNA damage expressed as percentage DNA in the tail, *bars* SD; X-*axis* sample time following drug exposure)

quently PBLs were only collected at 0, 4 and 24 h from a further 31 patients on cycle 1 of treatment (dacarbazine alone) and 18 patients on cycle 2 (dacarbazine and tamoxifen). There was a low level of DNA damage in PBLs prior to dacarbazine (median ± SD 0 ± 4.59, interquartile range 0–2.05). Following chemotherapy there was a peak in mean percentage DNA in the tail at 4 h (median ± SD 26.6 ± 17.41%, interquartile range 14.75–40.5%; signed rank's test, $P < 0.001$) with gradual, but incomplete repair 24 h after treatment (median ± SD 10.6 ± 9.66%, interquartile range 5.0–16.9%; signed rank's test, $P < 0.001$). There was considerable interpatient variation in the level of DNA damage sustained after administration of dacarbazine (Fig. 3). The mean percentage DNA in the tail at 4 h was strongly correlated with the DNA damage at 24 h ($r = +0.833$, $P < 0.001$; Fig. 4a). There was no significant difference between cycles 1 and 2 for paired data ($n = 18$) in pretreatment, 4-h and 24-h samples (signed rank's test, $P > 0.1$ in all cases).

Urinary 3-methyladenine excretion

Timed urine collections were completed from 35 patients in cycle 1 and from 18 patients in cycle 2. There was a low background level of 3-MeA prior to chemotherapy (median 2.40 nmol/h, interquartile range 1.0–4.5 nmol/h) consistent with previously reported results on environmental and dietary exposure [36]. Following treatment with dacarbazine there was a significant increase in 3-MeA excretion within 4 h (median 18.5 nmol/h, interquartile range 7.60–44.5 nmol/h; signed rank's test, $P < 0.001$) peaking during the 4–10-h collection time period (median 33 nmol/h, interquartile range 20.4–48.65 nmol/h; signed rank's test, $P < 0.001$). There was a decrease in 3-MeA excretion by 24 h although the rate of excretion at this time-point remained elevated compared to baseline (median 12.1 nmol/h, interquartile range 5.35–23.48 nmol/h; signed rank's test, $P < 0.001$;

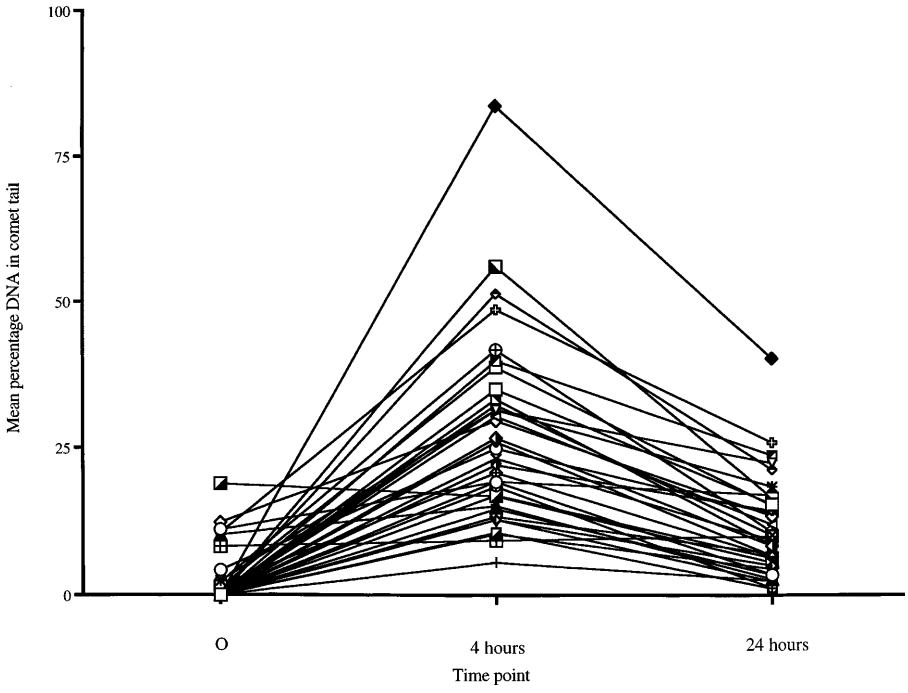


Fig. 3 Interindividual variations in DNA strand breaks in PBLs evaluated by the alkaline comet assay following dacarbazine treatment in vivo (cycle 1, $n = 37$). Median pretreatment 0 (interquartile range 0–2.05), 4 h after treatment 26.6 (interquartile range 14.75–40.5), 24 h after treatment 10.6 (interquartile range 5.0–16.9) (Y-*axis* DNA damage expressed as percentage DNA in the tail; X-*axis* PBLs taken pretreatment, and 4 and 24 h after dacarbazine treatment)

Fig. 5). There was no significant difference in 3-MeA excretion between cycles 1 and 2 at each time-point (signed rank's test, $P > 0.1$ in all cases).

Peak urinary 3-MeA excretion (4–10-h collection) was positively correlated with PBL DNA strand breaks measured by the comet assay at both 4 h ($r = +0.39$, $P = 0.036$) and at 24 h ($r = +0.46$, $P = 0.01$; Fig. 4b). There was no significant correlation between the comet assay and urinary 3-MeA excretion at other time-points ($P > 0.1$ in all cases).

Toxicity and response

A total of 122 courses of chemotherapy were administered to 39 patients (20 adjuvant, 19 metastatic). Of those patients having adjuvant treatment, 18 completed four cycles (one discontinued after one cycle due to toxicity and

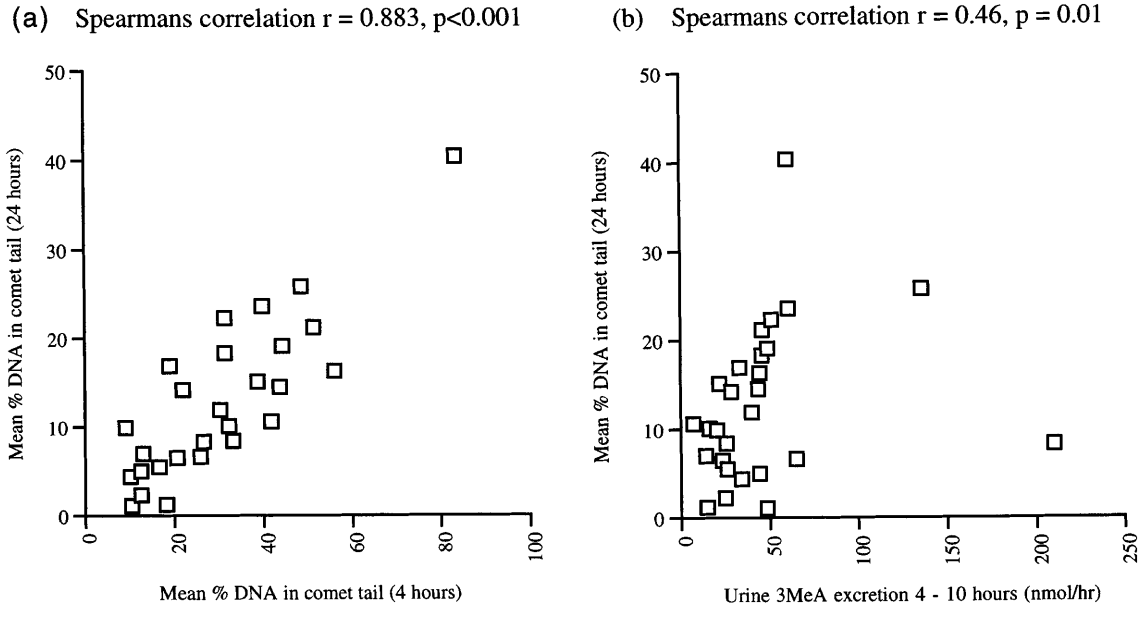


Fig. 4 **a** Correlation between mean percentage DNA in the tail in PBLs, measured by the comet assay, at 4 and 24 h after treatment with dacarbazine (Y-*axis* 24-h comet assay, X-*axis* 4-h comet assay). **b** Correlation between mean percentage DNA in the tail in PBLs measured by the comet assay 24 h after treatment and peak urinary 3-MeA excretion (4–10-h collection) (Y-*axis* 24-h comet assay, X-*axis* urinary 3-MeA, nmol/h)

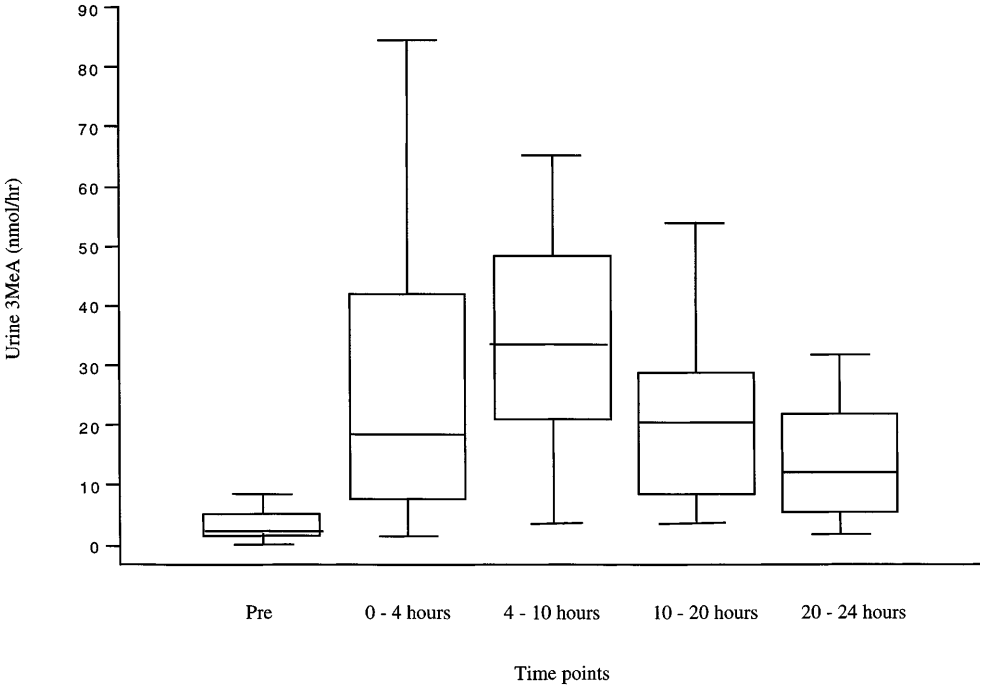


Fig. 5 Box whisker plot of urinary 3-MeA excretion in patients with malignant melanoma following cycle 1 of dacarbazine. Total urine volume excreted during each time period was collected and measured. Aliquots from each collection were analysed for 3-MeA and the results expressed as median rate of excretion (nmol/h) from all patients studied. Pretreatment median 2.4 nmol/h (interquartile range 1.0–4.5), 0–4-h median 18.5 nmol/h (interquartile range 7.6–44.5), 4–10-h median 33 nmol/h (interquartile range 20.4–48.65), 10–20-h median 20.25 (interquartile range 8.4–29.1), 20–24-h median 12.1 (interquartile range 5.35–12.1). The *box* shows median 3-MeA and is marked by the first and third quartiles. Whiskers extend to 1.5 times the interquartile range

one relapsed after three cycles). The median number of courses for the 19 patients with metastatic disease was two with only three patients completing six courses of treatment. Of the 39 patients treated, nine had treatment delayed for 1 week due to neutropenia or thrombocytopenia and two required a 25% dose reduction. Four patients discontinued treatment after one cycle (two with toxicity and two with disease progression).

The predominant toxicities were vomiting (three patients grade III, one grade IV), neutropenia (three grade III, two grade IV), thrombocytopenia (two grade III, none grade IV), stomatitis and fatigue (Table 2).

All 19 patients with metastatic disease were evaluable for response. There were no complete or partial responses. Five patients (26%) had documented disease stabilization for at least 3 months after completion of treatment and 14 had progressive disease.

Correlation of DNA damage with toxicity

In view of the lack of clinical responses in this study individual patient toxicity from chemotherapy was evaluated against DNA damage measured by both the comet assay and by urinary 3-MeA excretion.

Emesis and neutropenia were the most frequently observed toxicites from dacarbazine. Mean percentage DNA in the tail measured by the comet assay at 4 h and 24 h after treatment on cycle 1 was evaluated against observed toxicity for that cycle of chemotherapy with patients analysed in two groups – no toxicity or toxicity (WHO grade I to IV). Median percentage DNA in the tail at 24 h was significantly greater in patients with emesis (Wilcoxon rank sum, $P = 0.03$) compared to those with no emesis. This did not reach statistical significance at 4 h (Table 3). Comparison of neutropenia with the comet assay at 4 h and 24 h showed a higher mean percentage DNA in the tail for patients with toxicity, although this did not reach statistical significance (Wilcoxon rank sum, $P > 0.1$ in each case).

There was no significant difference in peak urinary 3-MeA excretion between patients with no toxicity compared to those with toxicity (vomiting or neutropenia) on cycle 1 (Wilcoxon rank sum, $P > 0.1$).

Table 2 Worst toxicity (all cycles)

	WHO grade					Total evaluable
	0	I	II	III	IV	
Vomiting	24	5	5	3	1	38
Neutropenia	24	5	4	3	2	38
Thrombocytopenia	33	2	1	2	0	38
Anaemia	32	4	1	1	0	38
Mucositis	32	6	4	0	0	38
Fatigue	27	10	1	0	0	38
Diarrhoea	37	0	1	0	0	38
Neuropathy (motor)	31	6	0	1	0	38

Discussion

This study demonstrated that the alkaline comet assay and measurement of urinary 3-MeA by immunoaffinity ELISA are sensitive techniques for evaluating and monitoring DNA damage and repair in vivo following cytotoxic chemotherapy with dacarbazine. Initial validation of the comet assay confirmed that PBLs, both with and without DNA damage, were stable when stored on ice for up to 4 h and at −70 °C. The second phase of our study validated the comet assay in vitro. Above a minimum threshold, PBLs had increasing DNA damage

Table 3 Mann-Whitney *U*-test for difference between the mean percentage DNA in the tail in PBLs assessed by the comet assay 24 h after cycle 1 of dacarbazine treatment for patients with no toxicity and those with WHO grade I, II, III or IV toxicity for the most frequently encountered toxicities, vomiting and neutropenia

	Number of patients		50th centile (%)		95% CI	
	Vomiting	Neutropenia	Vomiting	Neutropenia	Vomiting	Neutropenia
No toxicity	26	29	8.8	9.2	5.8–13.6	6.0–14.8
With toxicity	10	7	14.8	14.2	9.9–34.9	8.0–37.0
P-value			0.03	0.124		

with increasing concentrations of temozolamide. This is consistent with the results of other studies that show that the amount of DNA in the comet tail, measured as the percentage of DNA in the tail or tail moment, is dependent upon the dose of the DNA-damaging agent administered [18].

In vitro exposure of PBLs to temozolamide for 24 h resulted in an increase in DNA damage peaking at 90 min (Fig. 2a). This is consistent with degradation of the drug to the active metabolite MTIC and methylation of the DNA leading to strand breaks during base excision repair. There was substantial variation in the amount of DNA damage between individual cells. A reduction in DNA damage was seen at later time-points indicating repair of the bases. However, the later time-points may have included apoptotic cells and therefore in vitro the DNA damage detected by the comet assay may not just have been measuring strand breaks caused by base excision repair. In the clinical samples apoptotic cells are lost during processing of the PBLs over Lymphoprep and therefore the level of DNA damage measured by the comet assay following treatment with a methylating agent may more accurately reflect strand breaks caused as a result of base excision repair.

The main purposes for evaluating the comet assay were to determine patient variability in DNA damage and, indirectly, base excision repair, after exposure to cytotoxic drugs in vivo, and to compare this with the measurement of DNA adducts by urinary 3-MeA excretion. This could be important for designing individual patient treatment schedules. This study used PBLs, because of accessibility, as an indirect indicator for monitoring DNA damage in vivo although it would be preferable to evaluate the technique in tumour samples from either biopsies or fine needle aspirates. We initially determined the time-course for DNA damage in PBLs following intravenous dacarbazine therapy in eight patients (Fig. 2b). Prior to treatment there was little or no damage detectable with a gradual rise peaking 4 h post-treatment. There was a reduction in DNA damage at 8 h with persisting damage seen 24 h after chemotherapy. This time-course of DNA damage is consistent with the short plasma half-life of dacarbazine (plasma $T_{1/2} = 35$ min) [19] and its rapid activation to MTIC in the liver.

Urinary 3-MeA excretion followed a similar time-course to strand breaks measured by the comet assay. There was a low background level of excretion in all patients prior to treatment which is primarily due to the natural occurrence of 3-MeA in food [36]. After dacarbazine administration, urinary 3-MeA excretion increased within 4 h, peaking during the 4–10-h collection time period. The kinetics of repair and excretion of 3-MeA in humans is rapid ($T_{1/2} \cong 4$ h) [36] and this time-course is therefore consistent with peak DNA methylation occurring within 4 h of exposure to dacarbazine with 3-MeA being rapidly excised by AAG and repaired by the base excision repair pathway. 3-MeA is the major methylation product of deoxyadenosine in double helical DNA. This contrasts with methylation of adenosine in RNA or nucleotide pools which occurs almost exclusively at the 1-position of adenine [39]. There was a weak positive correlation between DNA strand breaks measured at 4 and 24 h with peak 3-MeA excretion at 4–10 h. The comet assay measures strand breaks formed during DNA repair by the base excision repair pathway. 3-MeA is only one of the cytotoxic methyl lesions repaired by this pathway which may be the reason why the correlation between the assays was not stronger. In addition urinary 3-MeA measures the total body elimination capacity for 3-MeA from all pathways whilst in this study we just looked at strand breaks in one cell type. This may suggest variability in the extent, and time-course of repair in different normal and tumour tissues. It is interesting to note that only a very small part of the administered dose of dacarbazine becomes bound to DNA. The total amount of 3-MeA excreted in the 24 h immediately after administration, and noting that the peak of excretion of this adduct occurs within 24 h [36], corresponds to 0.01 to 0.1% of the administered dose of dacarbazine.

There was considerable interpatient variability in the number of DNA strand breaks sustained by PBLs by 4 h (median percentage of DNA in the tail was 26.6%, interquartile range 14.75–40.5%) after administration of dacarbazine and in the extent of repair at 24 h (median percentage DNA in the tail was 10.6%, interquartile range 5.0–16.9%). These wide interindividual differences were also seen in 3-MeA excretion. The overall pattern of damage and repair for both assays was similar between patients with metastatic disease and those receiving adjuvant treatment. The large interindividual variations may reflect differences in the activation of the drug in the liver and the subsequent action and metabolism of its metabolites. Alternatively there may be variability in the capacity of patients to repair 3-MeA

lesions and in the lymphocytes' ability to repair DNA damage by the base excision repair pathway. However, the detection of 3-MeA directly in lymphocyte DNA is much more challenging due to the relatively low levels of adducts. The comet assay and urinary 3-MeA could be used in conjunction with assays for MTIC which is the major metabolite of dacarbazine. However, recent reports have highlighted the difficulty of such studies due to the instability of MTIC [3, 7].

Comparison of the comet assay and urinary 3-MeA measurements for cycles 1 and 2 of treatment showed no significant difference for paired data and no consistent intraindividual pattern between cycles. There was little or no detectable DNA damage or increase in 3-MeA in baseline samples prior to cycle 2 indicating that damage is not carried over from one cycle to the next, in contrast to previous work that has demonstrated an increase in O^6-MeG [38]. Likewise there was no detectable difference in the peak damage at 4 h or in the partial repair at 24 h suggesting that there is no persistent in vivo activation or upregulation of enzymes involved in the base excision repair pathway between cycles. Tamoxifen treatment was commenced 24 h after the first infusion of dacarbazine and therefore, in this study, was not demonstrated to have an effect on the level of DNA damage sustained. This suggests that the putative mechanism of action of tamoxifen in improving response rates in patients with malignant melanoma treated with dacarbazine is not directly related to an action on this DNA repair pathway despite the well-characterized ability of tamoxifen to form DNA adducts [20]. Further randomized clinical trials are required to elucidate whether there is a real effect.

There were no clinical responses seen in the 19 patients with metastatic melanoma, despite previous reports of response rates between 10 and 20% for dacarbazine. We were therefore not able to demonstrate any correlation between the alkaline comet assay or urinary 3-MeA and response in this series. The treatment schedule was well tolerated with few patients experiencing clinically significant toxicity. However, despite this, evaluation of the comet assay at 24 h with toxicity in both the adjuvant and metastatic patients has shown a statistically significant increase in mean percentage DNA in the tail for patients with vomiting compared to those with no toxicity (Wilcoxon rank sum, $P = 0.03$). The increased median comet assay value at 24 h for patients with neutropenia did not reach statistical significance (Table 3).

Malignant melanoma is relatively resistant to conventional cytotoxic therapy and therefore reliable predictors of response and toxicity after one cycle of treatment would be useful clinically. This study has demonstrated that the alkaline comet assay is a simple, robust and reproducible method that can be applied to the clinical situation for in vivo monitoring and that urinary 3-MeA, measured by immunoaffinity ELISA, is a noninvasive technique for assessing repair of methyl lesions following chemotherapy with dacarbazine. Studies with larger numbers of patients would be needed to confirm whether the techniques are predictive of toxicity following cytotoxic chemotherapy. Similarly a more active chemotherapy schedule, in different tumour types, would be needed to assess whether there is an association between DNA damage measured by the comet assay and clinical response. Prospective studies using pharmacodynamic end points to adjust dose should be considered.

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