Relationships between the Formation of O\textsuperscript{6}-Methyldeoxyguanosine by 1-p-Carboxyl-3,3-dimethylphenyltriazene in DNA and O\textsuperscript{6}-Alkylguanine-DNA Alkyltransferase in Human Peripheral Leukocytes\textsuperscript{1}

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ABSTRACT

There is increasing experimental evidence to indicate that O\textsuperscript{6}-methyldeoxyguanosine (O\textsuperscript{6}-MedG) formation in DNA is a critical cytotoxic event following exposure to certain antitumor alkylating agents and that the DNA repair protein O\textsuperscript{6}-alkylguanine-DNA-alkyltransferase (ATase) can confer resistance to these agents. We recently demonstrated a wide interindividual variation in the depletion and subsequent regeneration of ATase in peripheral blood lymphocytes of patients treated with 24-h continuous infusion of 1-p-carboxyl-3,3-dimethylphenyltriazene (CB10-277) for metastatic melanoma. We have now measured the formation of O\textsuperscript{6}-MedG in the DNA of peripheral leukocytes of nine patients receiving this treatment regimen. This lesion could be detected in DNA within 1 h and a progressive increase in adduct levels occurred during the CB10-277 infusion and for 24 h after completion. Considerable interindividual variation was observed in the peak O\textsuperscript{6}-MedG levels, with values ranging from 3.0 to 23.8 nmol O\textsuperscript{6}-MedG/mol deoxyguanosine (mean, 12.3 ± 6.4 nmol O\textsuperscript{6}-MedG/mol deoxyguanosine) following the first treatment cycle, possibly as a consequence of differences in the capacity of patients to metabolize CB10-277 to a methylating agent. There was, nevertheless, a clear temporal relationship between the progressive formation of leukocyte O\textsuperscript{6}-MedG and lymphocyte ATase depletion. Repeated-measures regression showed that this was statistically significant (P < 0.001) during the CB10-277 infusion. A significant inverse correlation was also seen between pretreatment lymphocyte ATase activity and peak O\textsuperscript{6}-MedG levels in leukocyte DNA (r = —0.73) and the area under the leukocyte O\textsuperscript{6}-MedG concentration-time curve (r = —0.76). Metabolism of CB10-277 to a methylating agent could be one factor that combines with DNA repair capacity to determine clinical response, because the two responses observed in this series occurred in the two patients with the highest leukocyte O\textsuperscript{6}-MedG levels and also the lowest pretreatment ATase activity. Hematological toxicity developed in the same two patients.

INTRODUCTION

The treatment of metastatic melanoma remains unsatisfactory. Although DTIC\textsuperscript{3} is still regarded as the most effective chemotherapeutic agent and regularly produces a response rate of 20%, only 4% of patients (primarily those with soft tissue metastasis) show a complete response and responses are rare in cases with visceral metastasis (1, 2). It has been suggested that one possible reason for the relatively poor clinical activity of DTIC may be the relatively inefficient metabolic activation, compared to murine models. Thus, following equivalent doses, the plasma levels of the active monomethyl metabolite 5-(3-methyl-1-triazeno)imidazole-4-carboxamide are much lower in rats and humans than in mice (3). Another triazene, CB10-277 (Fig. 1), has been shown to have marked activity against experimental murine tumors (4) and melanoma xenografts (5) and is significantly more effective than DTIC in inhibiting the growth of the Walker tumor in rats (6). Like DTIC, CB10-277 requires activation by oxidative N-demethylation, but the overall production of the putative active monomethyl metabolite in rats was 15-fold greater than that for DTIC, suggesting that species-dependent activation is less likely to be a problem in humans (6). Thus, due to the structural similarities to DTIC, the improved in vitro stability and solubility, and the possibility of improved metabolic activation, CB10-277 was selected for clinical trial by the Cancer Research Campaign (UK) Clinical Trial Committee (7).

With respect to the mechanisms of antitumor action, there is a considerable amount of evidence to indicate that the cell-killing effects of DTIC, CB10-277, and related methylating agents are mediated mainly by the formation of O\textsuperscript{6}-MedG in DNA and that the principal mechanism of resistance is via the DNA repair protein ATase. This protein acts by the stoichiometric transfer of the methyl group from the O\textsuperscript{6}-position of guanine in alkylated DNA to a cysteine residue in the protein itself, in an autoinactivating reaction (5, 8–12). The strongest evidence for the cytotoxic effects of O\textsuperscript{6}-alkylguanine in DNA comes from experiments which show that the expression of a transfected prokaryotic or eukaryotic ATase complementary DNA in mammalian cells protects them against the toxic effects of these agents (13–17).

We recently demonstrated a wide interindividual variation in the depletion of lymphocyte ATase during a 24-h continuous infusion of CB10-277 (18). These findings can be attributed to the CB10-277-mediated formation of O\textsuperscript{6}-MedG in lymphocyte DNA, and the subsequent repair of this adduct by ATase is manifested as a depletion of ATase activity in lymphocyte extracts. The relationship between the ability to activate CB10-277, which is reflected in the formation of O\textsuperscript{6}-MedG in DNA, and the wide range of clinical responses seen among patients with metastatic melanoma receiving this drug has not been explored. The aims of the present study are therefore to examine whether there is a correlation between O\textsuperscript{6}-MedG accumulation in leukocyte DNA and lymphocyte ATase activity and also to assess whether there is any relationship between O\textsuperscript{6}-MedG formation and clinical response.

MATERIALS AND METHODS

Patients and Blood Samples. CB10-277 (sodium salt, M, 215) was supplied as a lyophilized, pyrogen- and preservative-free powder (in 1000-mg vials) by the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). All nine patients had metastatic melanoma, and details of the individuals studied as reported previously (18) are shown in Table 1. Each patient received CB10-277 (12 mg/m\textsuperscript{2}) by continuous i.v. infusion over 24 h and the treatment was repeated every 4 weeks. Serial blood samples (20 ml) were collected at various times during the infusion and for 24 h after completion of the first cycle of CB10-277. Blood samples were dispensed into two universal containers (10 ml each) containing 0.5 ml of 0.5% EDTA, pH 8.0. One container was stored at —20°C prior to DNA extraction and radioimmunoassay for O\textsuperscript{6}-MedG levels (see below). The second container was kept at

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3 The abbreviations used are: DTIC (dacarbazine), 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; ATase, O\textsuperscript{6}-alkylguanine-DNA alkyltransferase; AUC, area under the concentration-time curve; CB10-277, 1-p-carboxyl-3,3-dimethylphenyltriazene; O\textsuperscript{6}-MedG, O\textsuperscript{6}-methyldeoxyguanosine.

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was extracted to permit two separate analyses. The value for O\textsuperscript{6}-MedG in the
orated under a gentle stream of nitrogen. The yield of DNA was normally
was removed from the final aqueous phase by shaldng with 1.5 ml of chloro
acetate, ethanol, ethanol:ether (1:1, v:v), and ether. Residual ether was evap
pH 8.0, was added and after shaking at room temperature for 10 mm the
alkylation (>0.1 \textmu mol O\textsuperscript{6}-MedG/\textmu mol) the reproducibility was ± 10%. The
the reproducibility of the analyses was ±35%, whereas at higher levels of
the difference, however, was not significant (P > 0.1). At levels of
levels were shown in Table 1. A clinical response was achieved in two
these values ranged from 83 to 707 \textmu mol/molh (see Table 1).
The response data for CB10-277 given as a 24-h infusion, peak leukocyte O\textsuperscript{6}-MedG levels achieved, and leukocyte O\textsuperscript{6}-MedG AUC values are shown in Table 1. A clinical response was achieved in two patients (C. M. and J. R.) and in both cases the highest leukocyte O\textsuperscript{6}-MedG levels (i.e., peak values and AUC values) and lowest pretreatment lymphocyte ATase levels were seen, in comparison with the remaining seven patients, who had stable or progressive disease after the first treatment cycle (see Table 1). Hematological toxicity also developed in the same two patients (18). Both leukopenia (2 of 3 grade 3 and 1 of 3 grade 4; World Health Organization scale) (22) and thrombocytopenia (2 of 3 grade 4; World Health Organization scale) occurred during the 3 evaluable courses. Following a reduction of the CB10-277 dose by 50%, no further hematological toxicity developed; this observation was also associated with less extensive ATase depletion, as reported previously (18).

Relationship between Lymphocyte ATase Depletion and Leukocyte O\textsuperscript{6}-MedG Levels. The data were analyzed using repeated-measures regression with pretreatment ATase as a constant covariate, O\textsuperscript{6}-MedG as a time-varying covariate, and post-treatment ATase as the dependent variate. An inverse temporal relationship was seen between the progressive formation of O\textsuperscript{6}-MedG and ATase depletion (see Fig. 2) and this relationship was statistically significant

Figure 1. Structures of CB10-277 and DTIC and their monomethyl metabolites. MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Patient & Age/gender & Metastatic sites & Response\textsuperscript{a} & Initial ATase (fmol/mg)\textsuperscript{b} & Peak O\textsuperscript{6}-MedG (\textmu mol deoxyguanosine) & O\textsuperscript{6}-MedG AUC (\textmu mol/molh) \\
\hline
C. M.    & 37/F      & Lung/nodes   & PR\textsuperscript{e}   & 15 ± 0.4               & 23.8              & 707.0          \\
J. R.    & 54/M      & Lung/brain   & PR\textsuperscript{e}   & 45 ± 1.1              & 18.7              & 531.2          \\
G. M.    & 20/F      & Lung/soft tissue & PD               & 250 ± 9.5              & 9.46              & 270.6          \\
F. F.    & 50/M      & Lung/nodes   & NC                   & 206 ± 4.5              & 14.3              & 361.2          \\
M. U.    & 66/F      & Lung/nodes   & PD                   & 163 ± 0.8              & 10.1              & 298.4          \\
M. C.    & 66/F      & Liver        & PD                   & 86 ± 2.4               & 15.6              & 490.6          \\
J. H.    & 31/F      & Lung/nodes   & NC                   & 158 ± 10.0             & 9.25              & 122.2\textsuperscript{d} \\
E. D.    & 65/M      & Brain        & PD                   & 262 ± 0.9              & 6.74              & 197.4          \\
J. K.    & 53/M      & Brain        & PD                   & 141 ± 4.7              & 2.98              & 83.1           \\
\hline
\end{tabular}
\caption{Characteristics and responses of patients}
\end{table}

\textsuperscript{a} Response was assessed after the first CB10-277 treatment; PR, partial response; PD, progressive disease; NC, no change.
\textsuperscript{b} Mean ± SD of 3 determinations (from Ref. 18).
\textsuperscript{c} Hematological toxicity.
\textsuperscript{d} AUC at 30 h, therefore excluded from Spearman correlation calculation in Fig. 4.

Statistical Methods. Using the BMDP (version 7) statistical software program, ATase activity during and after CB10-277 infusion was regressed against O\textsuperscript{6}-MedG levels at each time point and also against pretreatment ATase, using repeated-measures regression (21). Pretreatment ATase activity was also correlated with peak O\textsuperscript{6}-MedG level and area under the O\textsuperscript{6}-MedG concentration-time curve, using Spearman correlation coefficients.

RESULTS

O\textsuperscript{6}-MedG Formation in Leukocyte DNA. O\textsuperscript{6}-MedG could be detected in peripheral blood leukocyte DNA shortly after CB10-277 infusion, and there was generally a progressive increase in O\textsuperscript{6}-MedG levels during the 24-h treatment period (Fig. 2). After the end of the infusion, the rate of increase of O\textsuperscript{6}-MedG slowed and generally there appeared to be no net induction over the next 24 h. In two patients (E. D. and M. C.) an early leveling off of O\textsuperscript{6}-MedG levels was seen (Fig. 2), and it is noteworthy that the two suffered from severe liver and brain metastases, respectively. Considerable interindividual variation was observed in the maximal levels of O\textsuperscript{6}-MedG, with values ranging from 2.98 to 23.8 \textmu mol O\textsuperscript{6}-MedG/mol deoxyguanosine (mean, 12.3 ± 6.40 \textmu mol O\textsuperscript{6}-MedG/mol deoxyguanosine), a difference of approximately 10-fold, following the first treatment cycle. When values were expressed as the O\textsuperscript{6}-MedG AUC, which gives an integrated measure of the formation and persistence of O\textsuperscript{6}-MedG, a similar large interindividual variation was also seen with the AUCs; these values ranged from 83 to 707 \textmu mol/molh (see Table 1).

\begin{align*}
\text{O\textsuperscript{6}-MedG Analysis.} & \quad \text{The procedure used for the determination of O\textsuperscript{6}-MedG in DNA by radioimmunoassay has been described in detail elsewhere (19, 20). The results are expressed as \textmu mol O\textsuperscript{6}-MedG/mol of the parent base (deoxyguanosine) and the lower limit of detection was \approx 0.04 \textmu mol O\textsuperscript{6}-MedG/mol deoxyguanosine. From the nine sets of blood samples taken, enough DNA was extracted to permit two separate analyses. The value for O\textsuperscript{6}-MedG in the second analysis of these samples was within \pm 15% of the values of the first analysis. The difference, however, was not significant (P > 0.1). At levels of methylation approaching the limit of detection (\textapprox 0.1 \textmu mol O\textsuperscript{6}-MedG/mol), the reproducibility of the analyses was \pm 35%, whereas at higher levels of alkylation (>0.1 \textmu mol O\textsuperscript{6}-MedG/mol) the reproducibility was \pm 10%. The mean values of the two sets of data are used in these results.}
\end{align*}
Fig. 2. Relationship between leukocyte $O^6$-MedG levels (○) and lymphocyte ATase activity (●) in nine patients treated with CB10-277. Letters in the top corner of each panel refer to patients listed in Table 1.

Fig. 3. Relationship between pretreatment ATase activity and leukocyte peak $O^6$-MedG levels. $R$, correlation coefficient. Letters by each point refer to patients listed in Table 1.

Fig. 4. Relationship between pretreatment ATase activity and leukocyte $O^6$-MedG AUC versus time. $R$, correlation coefficient. Letters by each point refer to patients listed in Table 1.

**DISCUSSION**

This study reports the formation of $O^6$-MedG in leukocyte DNA of patients receiving a 24-h continuous infusion of CB10-277 chemotherapyna and indicates that CB10-277 is metabolized to a methylating agent (6, 23) in amounts sufficient to react with leukocyte DNA, generating $O^6$-MedG. There was an approximately 10-fold variation in the maximum levels of $O^6$-MedG formed in the nine patients studied, and this may have been a consequence of differences in the capacities for CB10-277 activation, uptake of the monomethyl metabolite, methylating agent detoxification (e.g., via glutathione), and/or ATase-mediated repair of $O^6$-MedG in DNA. It is tempting to speculate that these could be among the factors that determine clinical response.

Two of the nine patients studied (C. M. and J. R.; see Table 1) responded after the first course of treatment, and in both cases the peaks of $O^6$-MedG formation and $O^6$-MedG AUC values were higher than those seen in the other seven patients, in whom the disease either remained stable or progressed. Peak $O^6$-MedG levels were, however, ($P < 0.001$). The fall in ATase levels was statistically significant ($P < 0.001$) during the 24-h infusion of CB10-277 but not subsequently. Overall there was a significant change of ATase with time ($P < 0.001$) and it was also evident that the pretreatment ATase activity influenced ATase levels during and after CB10-277 infusion ($P < 0.001$).

Using Spearman correlation coefficients, an inverse correlation was also seen between pretreatment lymphocyte ATase levels and the level of $O^6$-MedG formed in total peripheral leukocyte DNA, expressed either as peak levels ($r = -0.73$; Fig. 3) or as the leukocyte $O^6$-MedG AUC values ($r = -0.76$; Fig. 4).
only slightly higher than those for the two patients in whom the disease progressed (M. C.) or no change was reported (F. F.). Clearly, the extent to which the present results can be extrapolated to clinical response will depend on whether \(O^\alpha\)-MedG levels in peripheral leukocyte DNA correlate with those of target tumor DNA. Studies in rats, however, showed that for certain agents DNA methylation occurred to broadly similar levels in several different tissues, including leukocyte DNA, following administration of methylating agents, even those requiring metabolic activation (24–26). Measurements of adduct formation in circulating leukocytes may therefore potentially provide a sensitive means of predicting tumor exposure to alkylating agents (24, 25). In the phase I CB10-277 study, responses occurred in four of 11 evaluable melanoma patients when CB10-277 was given as a short infusion (7).

We also found that two patients developed grade 4 hematological toxicity and these two patients (C. M. and J. R.; see Table 1) achieved the highest \(O^\alpha\)-MedG levels and leukocyte \(O^\alpha\)-MedG AUC values. No further hematological toxicity developed when the dose of CB10-277 was halved, and this was associated with less extensive lymphocyte ATase depletion, as reported previously (18). In the phase I study, no hematological toxicity was encountered when a lower dose of CB10-277 (<12 g/m²) was used, and this observation was associated with lower monomethyl metabolite levels (27). These findings, together with our \(O^\alpha\)-MedG data, suggest that less DNA methylation may have occurred in the bone marrow following administration of lower doses.

A progressive increase in \(O^\alpha\)-MedG levels was seen during CB10-277 infusion and this continued, albeit at a slower rate, for 24 h after treatment in most of the patients studied. In this respect, it is interesting to note that, in addition to \(N\)-demethylation, CB10-277 is subject to extensive conjugation, primarily with glucuronic acid, and it is not unreasonable to suggest that this conjugate may allow the prolonged availability of CB10-277 to react with cellular DNA after discontinuation of drug treatment. This is in contrast to DTIC, where maximal \(O^\alpha\)-MedG levels occurred at approximately 3–5 h and low levels were detected 24 h after DTIC administration (28).

Whereas ATase levels have been determined in peripheral blood lymphocytes (18), there was clear evidence of a relationship between the accumulation of \(O^\alpha\)-MedG in leukocyte DNA and the progressive depletion of lymphocyte ATase activity during CB10-277 infusion, suggesting concomitant effects in the two populations of cells examined. Repeated-measures regression of ATase activity during and after CB10-277 treatment versus \(O^\alpha\)-MedG and pretreatment ATase levels showed that changes in ATase levels were statistically significant during the 24 h of continuous drug infusion, whereas after 24 h this was not the case; this could be a consequence of ATase recovery due to de novo synthesis in three of the patients studied (J. H., E. D., and J. K.; see Fig. 2). Possibly of greater significance was the correlation between pretreatment lymphocyte ATase levels and peak leukocyte \(O^\alpha\)-MedG levels (\(r = -0.73\)) or leukocyte \(O^\alpha\)-MedG AUC values (\(r = -0.76\)). However, we cannot dismiss the possibility that this apparent relationship is fortuitous, in view of the fact that ATase activity was measured in the lymphocyte population, whereas \(O^\alpha\)-MedG levels were measured in total WBC, which comprise 30–50% lymphocytes and 50–70% granulocytes. Further studies in which both ATase and \(O^\alpha\)-MedG levels are measured in both populations or in total WBC would help to resolve this question. Nevertheless, the two patients with the highest peak \(O^\alpha\)-MedG levels had the lowest pretreatment ATase levels and these fell to below detectable levels during the CB10-277 infusion. The observation that these same two patients showed tumor responses and had hematological toxicity suggests that pretreatment lymphocyte ATase levels might prove to be a potentially useful indicator of responses of melanoma and normal tissues to treatment with this class of agent, although it must be emphasized that this suggestion is based on two individuals and must therefore be considered very tentative. In this context, the depletion of ATase in melanoma following CB10-277 treatment is closely related to depletion in peripheral lymphocytes (18), and the latter might thus be used as an indicator of events in the tumor. High intracellular ATase levels may thus protect against the damaging effect of DNA methylation by CB10-277. Because wide differences in ATase activity have been reported in melanoma tissue (29, 30), this may result in significant differences in the accumulation of the toxic \(O^\alpha\)-MedG in target tumor tissue. It is noteworthy that other studies have demonstrated a correlation between the extent of platinum-DNA adduct formation measured in peripheral leukocyte DNA and disease response in ovarian cancer patients treated with cisplatin-based chemotherapy (31, 32).

The wide individual variations in the extent of DNA methylation achieved in peripheral leukocytes following CB10-277 administration may thus be indicative of differences in capacities for metabolic activation as well as DNA repair activity; these parameters, in conjunction with estimates of ATase activity, may be of considerable value in predicting clinical response and/or hematological toxicity on an individual basis, assuming that methylation in leukocyte DNA reflects that occurring in tumor and normal tissues.

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Note Added in Proof

The two responses described were observed after the first treatment cycle. However, disease progression occurred in the brain metastasis in patient J.R. after treatment cycle 3 and this may be due to inability of CB 10-277 to cross the blood brain barrier.

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