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In vivo depletion of O6-alkylguanine-DNA-alkyltransferase in lymphocytes and melanoma of patients treated with CB10-277, a new DTIC analogue

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Summary. There is increasing evidence to suggest that alkylation of guanine residues in DNA at the O6 position is the critical cytotoxic event following treatment with dacarbazine (DTIC) and related drugs and that endogenous O6-alkylguanine-DNA alkyltransferase (ATase) gene expression may be a major factor in resistance to such agents. 1-*p*-Carboxyl-3,3-dimethylphenyltriazene (CB10-277) was recently selected for clinical evaluation as a DTIC analogue with improved solubility, stability and (possibly) metabolic activation. Serial ATase levels were measured in peripheral blood lymphocytes of nine patients and in biopsied melanoma samples of two patients undergoing treatment with 24-h continuous infusion of CB10-277 (12 g/m^2). Wide individual variations in pre-treatment levels as well as in the post-treatment depletion of lymphocyte ATase were seen. Progressive depletion of lymphocyte ATase was seen during continuous infusion of CB10-277 in all patients. Complete suppression of lymphocyte ATase activity occurred in two patients whose pre-treatment ATase levels were low. Immediately following completion of the CB10-277 infusion, the median ATase activity was 17% of pre-treatment levels (range, 0–67%). At 24 h after the end of the infusion, no recovery of lymphocyte ATase activity was observed in six patients, but significant recovery to 50%, 100% and 102% of pre-treatment activity occurred in the other three. In three patients who returned for subsequent cycles of chemotherapy at 4 weeks after the first dose, pre-treatment ATase levels showed a 3- to 4-fold increase relative to the original pre-treatment values. A significant correlation was found between the extent of ATase depletion and the initial lymphocyte ATase levels ($r = 0.725$, $P < 0.05$). Haematological toxicity developed in two patients and was associated with low pre-treatment ATase activity. Depletion of tumour ATase activity was noted in these patients, with residual activity amounting to 8% and 11% of pre-treatment levels, respectively, in the biopsied melanoma tissues. These results indicate extensive metabolism of CB10-277 to a methylating agent capable of mediating alkylation of DNA and subsequent depletion of lymphocyte and tumour ATase levels and further indicate that the effects on lymphocytes may reflect effects on the target tumour.

Introduction

The effective treatment of metastatic melanoma is disappointing; dacarbazine (DTIC) remains the standard treatment and in a recent cumulative review of 1133 patients with metastatic melanoma, DTIC produced a 21% response rate [2]. Although DTIC has been reported to have good activity in murine tumour models in vivo, one possible reason for its disappointing clinical activity is that following DTIC administration, plasma levels of the active monomethyl metabolite MTIC [5-(3-methyl-1-triazeno)imidazole-4-carboxamide] are much lower in rats and humans than in mice [34]. CB10-277 (Fig. 1) has been shown to have marked activity against experimental murine tumours [10] and melanoma xenografts [16] and is significantly more effective than DTIC in inhibiting the growth of the Walker tumour in the rat [35]. Like other dialkyltriazenes, CB10-277 requires activation by oxidative *N*-demethylation, and the overall production of the putative active monomethyl metabolite in rats was 15-fold that of DTIC, suggesting that species-dependent activation is less likely to be a problem in humans [35]. Thus, due to its structural similarities to DTIC, its superior in vitro stability and solubility and its possibly improved metabolic activation, CB10-277 was selected for clinical evaluation as a DTIC analogue and is currently undergoing phase II trial in metastatic melanoma under the auspices of the

Abbreviations: ATase, O6-alkylguanine-DNA alkyltransferase; CB10-277, 1-*p*-carboxyl-3,3-dimethylphenyltriazene; DTIC, dacarbazine, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; MTIC, 5-(3-methyl-1-triazeno)imidazole-4-carboxamide

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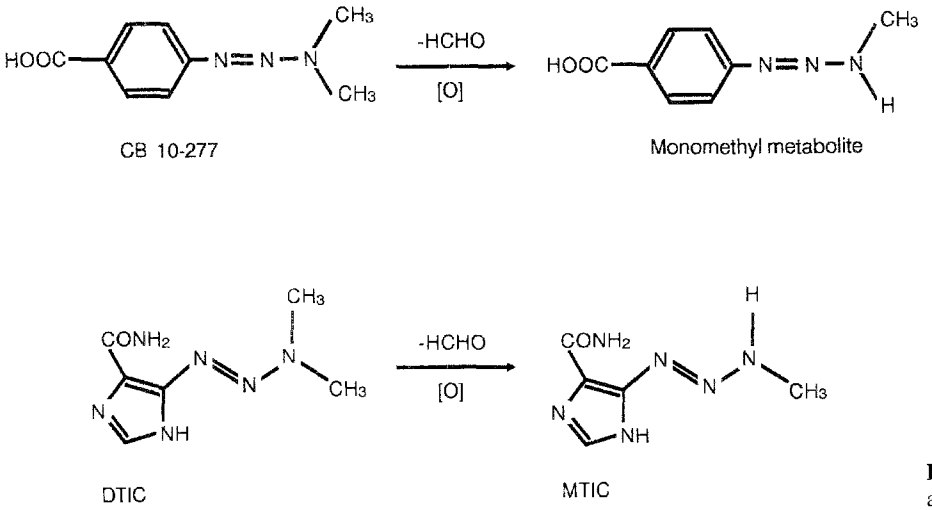

Fig. 1. Structures of CB10-277 and DTIC and their monomethyl metabolites

Cancer Research Campaign (UK) Clinical Trial Committee.

The mechanism of the antitumour activity of DTIC remains unclear, but there is evidence to suggest that the metabolite MTIC methylates DNA, producing, among 12 other lesions, O^6-methylguanine [11]. It has been shown in experimental models that resistance to DTIC and other methylating agents involves the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase (ATase), which transfers the methyl group from the O^6 position of guanine to an internal cysteine residue in an auto-inactivating stoichiometric reaction. Thus, ATase-deficient cell lines are more sensitive to killing by these alkylating agents [8, 11, 20, 21, 27, 32]. Further evidence for the importance of ATase comes from in vitro studies in which depletion of endogenous ATase by prior exposure to either O^6-methyl- or -benzylguanine or non-toxic doses of simple methylating agents [13, 15, 18, 19, 38, 39] rendered the cells much more sensitive to subsequent treatment with methylating and chloroethylating agents. In addition, melanoma xenografts with high ATase activity are more resistant to DTIC than are xenografts with low activity [16]. The strongest evidence for the cytoprotective role of ATase comes from experiments in which ATase-deficient cultured mammalian cells that had been transfected with cloned pro- or eukaryotic ATase genes and expressed them at high levels were more resistant to the toxic effects of such alkylating agents [3, 7, 12, 14, 23, 36, 37].

Determination of the pre-treatment levels and the depletion rates of ATase in peripheral lymphocytes and tumour tissue following treatment with alkylating agents should provide therapy-relevant information; if ATase depletion is seen in tumour tissue following the administration of CB10-277 or related drugs, one can assume that DNA methylation and, hence, metabolic activation has occurred. In addition, if complete loss of ATase activity is demonstrated, an excess of the toxic O^6-methylguanine lesions and, hence, killing of cells might be expected. Residual ATase activity may indicate resistance or the presence of resistant cells in the tumour population. Indeed, in HL-60 cells the methylating agents need to inactivate ATase before cytotoxicity is observed; in contrast, the chloroethylating agents induce inactivation of ATase only after the cells have been incubated with concentrations 7–12 times higher than the cytotoxic dose [18]. Unlike the methylating agents, the toxic event following treatment with the chloroethylating nitrosoureas is the formation of DNA interstrand cross-links. Thus, ATase measurements may allow us to improve drug dosage and delivery schedules or may indicate useful combinations with the nitrosoureas in which drug resistance is also mediated by ATase.

The present study was based on our recent observations of progressive depletion of ATase activity in peripheral blood lymphocytes of patients with metastatic melanoma treated with sequential DTIC and fotemustine [24]. We examined the extent and the kinetics of ATase depletion and regeneration in peripheral blood lymphocytes of nine patients and in biopsied tumour materials of two patients with metastatic melanoma treated with 24-h continuous infusion of CB10-277.

Patients and methods

Drugs and treatment of patients. CB10-277 (sodium salt, MW 215) was supplied as a lyophilised, pyrogen- and preservative-free powder in 1,000-mg vials by the Developmental Therapeutics Programme, National Cancer Institute (Bethesda, Md., USA). All nine patients had metastatic melanoma and the clinical characteristics are shown in Table 1. Patients received CB10-277 (12 g/m^2) in a 24-h i.v. infusion and treatment was repeated every 28 days. Serial blood samples were collected at various times during the infusion and for 24 h after completion of the first course of CB10-277. Serial blood samples were also taken from two patients who returned for subsequent treatment, whereby the CB10-277 dose was halved (6 g/m^2) because of haematological toxicity. Blood samples were drawn into a 20-ml universal container containing 0.5 ml 0.5% ethylenediaminetetraacetic acid (EDTA) and were stored at 4° C before the isolation of lymphocytes. Tumour biopsies were performed on two patients with metastatic s.c. melanoma nodules before and after CB10-277 treatment; because of the possibility of tissue heterogeneity in ATase expression, biopsy specimens were taken from two adjacent metastatic nodules. Signed informed consent was obtained according to the guidelines of the South Manchester Health Authority Ethical Committee and the Royal College of Physicians, London. The phase II trial of CB10-277 was carried out under the auspices of the Cancer Research Campaign (UK) Clinical Trials Committee.

Table 1. Patients' characteristics and response

Patient/ figure	Age (years)/ sex (M/F)	Metastatic sites	Response	ATase (fmol/mg ± SD)	
				Initial	Nadir
1/Fig. 2a	37/F	Lung/nodes	PD[a]	15 ± 0.4	BD
2/Fig. 2b	54/M	Lung/brain	MR	45 ± 1.1	BD
3/Fig. 2c	20/F	Lung/soft tissue	PD	250 ± 9.5	43 ± 1.0
4/Fig. 2d	50/M	Lung/nodes	PD	206 ± 4.5	24 ± 0.8
5/Fig. 2e	66/F	Lung/nodes	PD	163 ± 0.8	6 ± 0.1
6/Fig. 2f	66/F	Liver	PD	86 ± 2.4	15 ± 0.6
7/Fig. 2g	31/F	Lung/nodes	PD[b]	158 ± 10.0	36 ± 0.3
8/Fig. 2h	65/M	Liver	PD	262 ± 0.9	80 ± 5.0
9/Fig. 2i	53/F	Brain	PD	141 ± 4.7	63 ± 2.5

MR, Mixed response; PD, progressive disease; BD, below detection levels

[a] This patient showed a partial response at chemotherapy cycle 4 but relapsed after cycle 6

[b] This patient showed a partial response at chemotherapy cycle 3 but relapsed after cycle 6

Tissue and ATase extract preparation. Peripheral blood lymphocytes (mononuclear cell fraction) were isolated by centrifugation on Ficoll (Pharmacia, Uppsala, Sweden) [5], washed with phosphate-buffered saline, centrifuged again into a pellet and stored at –20° C. Apparently normal tissue was removed from the melanoma nodules, which were then stored at –20° C. The lymphocyte pellets and melanoma nodules (200–400 mg) were sonicated (10 s at 10 μm peak distance followed by cooling in ice and then resonication for 10 s at 18 μm) in 1 ml buffer I (50 mM TRIS-HCl, 3 mM dithiothreitol, 1 mM EDTA; pH 8.3). Phenylmethysulphonyl fluoride (8.7 mg/ml in ethanol) was added to a final concentration of 87 μg/ml immediately after the second sonication. Sonicates were centrifuged at 16,000 rpm in a microcentrifuge for 10 min at 4° C and supernatants were transferred to fresh tubes in ice and assayed for ATase activity. Protein concentration was measured by the Bradford method using bovine serum albumin as the standard [6].

ATase assay. ATase assay was carried out as previously described [24], with slight modifications. Varying amounts of cell extract were incubated with [^{3}H]-methylnitrosourea-methylated calf-thymus substrate DNA (specific activity, 19 Ci/mmol) at 37° C for 2 h in a total volume of 500 μl of a 1-mg/ml solution of bovine serum albumin in buffer I. After incubation, bovine serum albumin (100 μl of a 10-mg/ml solution in buffer I) and perchloric acid (200 μl of a 4-M solution) were added in rapid succession. A further 2 ml 1 M perchloric acid was added and the mixture was heated at 75° C for 40 min. Samples were clarified by centrifugation, and the precipitates were washed with 4 ml 1 M perchloric acid before being resuspended in 300 μl 0.01 M sodium hydroxide and then dissolved in 3 ml aqueous scintillation fluid (Ecoscint A; National Diagnostics). Counting efficiency was approximately 28%. Specific activity measurements were based on a minimum of three points on the linear part of the curve. ATase activity was expressed as femtomoles of methyl transferred to protein per milligram of total protein in the extract.

Results

ATase depletion and recovery in peripheral lymphocytes

Pre-treatment lymphocyte ATase levels ranged from 15 to 262 fmol/mg protein (median, 158 fmol/mg total protein; Table 1). In all nine patients, progressive depletion of ATase activity was seen during the 24-h continuous infusion of CB10-277. In two patients whose pre-treatment ATase values were 15 and 45 fmol/mg protein, respectively, suppression of ATase was to below detectable levels at 2 and 16 h, respectively (Figs. 2a, b). In the remaining seven patients (Figs. 2c–i), progressive depletion of ATase activity occurred, the median residual ATase activity amounting to 17% of the pre-treatment value at the end of the CB10-277 infusion. No recovery of ATase activity was seen in six patients for up to 24 h after completion of the CB10-277 treatment (Figs. 2a–f) but significant recovery to 50%, 100% and 102% of pre-treatment levels occurred in the other three (Figs. 2g–i). A significant correlation was found between the extent of ATase depletion and the initial lymphocyte ATase levels ($r = 0.725$, $P < 0.05$; Fig. 3). In two individuals (patients 1 and 2, Table 1), serial ATase assays were repeated for subsequent therapy, whereby the CB10-277 dose was halved to 6 g/m^2 because of the development of haematological toxicity. In comparison with therapy cycle 1 (no residual activity), a significantly less extensive ATase depletion was seen at the end of the CB10-277 infusion, with residual ATase activity of amounting to 73% and 85% of the pre-treatment levels, respectively (Fig. 4).

A 3- to 4-fold increase in the pre-treatment lymphocyte ATase activity was seen in three individuals (patients 1, 6 and 9) who returned 4 weeks later for subsequent chemotherapy (Fig. 5). No increase was seen in the other six patients. One of these subjects (patient 6) died rapidly of progressive disease, and another (patient 9) also had documented progressive disease and was subsequently treated with radiotherapy.

ATase activity in tumour biopsies

Pre-treatment ATase levels were 65 and 117 fmol/mg protein, respectively, in two melanoma biopsy samples. These values were within the range of ATase activity detected in our earlier melanoma biopsies [28]. Following CB10-277 treatment, residual ATase activity amounted to 7 and 9 fmol/mg protein, respectively, in further biopsies. The tumour ATase activity appeared to fall in parallel with lymphocyte ATase levels during CB10-277 treatment (Fig. 6). Although it is based on data from only two patients, this is the first clinical demonstration of ATase depletion in tumour tissue following chemotherapy.

Haematological toxicity and clinical response

An interesting finding was the development of haematological toxicity in the two individuals (patients 1 and 2) with the lowest pre-treatment ATase levels (15 and 45 fmol/mg protein, respectively). Both leucopenia (2/3 grade 4, 1/3 grade 4, WHO scale) and thrombocytopenia (2/3 grade 4, WHO scale) occurred during 3 evaluable courses. Following a reduction of the CB10-277 dose by 50%, no further haematological toxicity was documented, but this observation was also associated with less extensive ATase depletion (Fig. 4). In the remaining seven patients whose pre-treatment ATase levels exceeded 50 fmol/mg protein (Figs. 2c–i), no evidence of haematological toxicity was seen. It is noteworthy that in

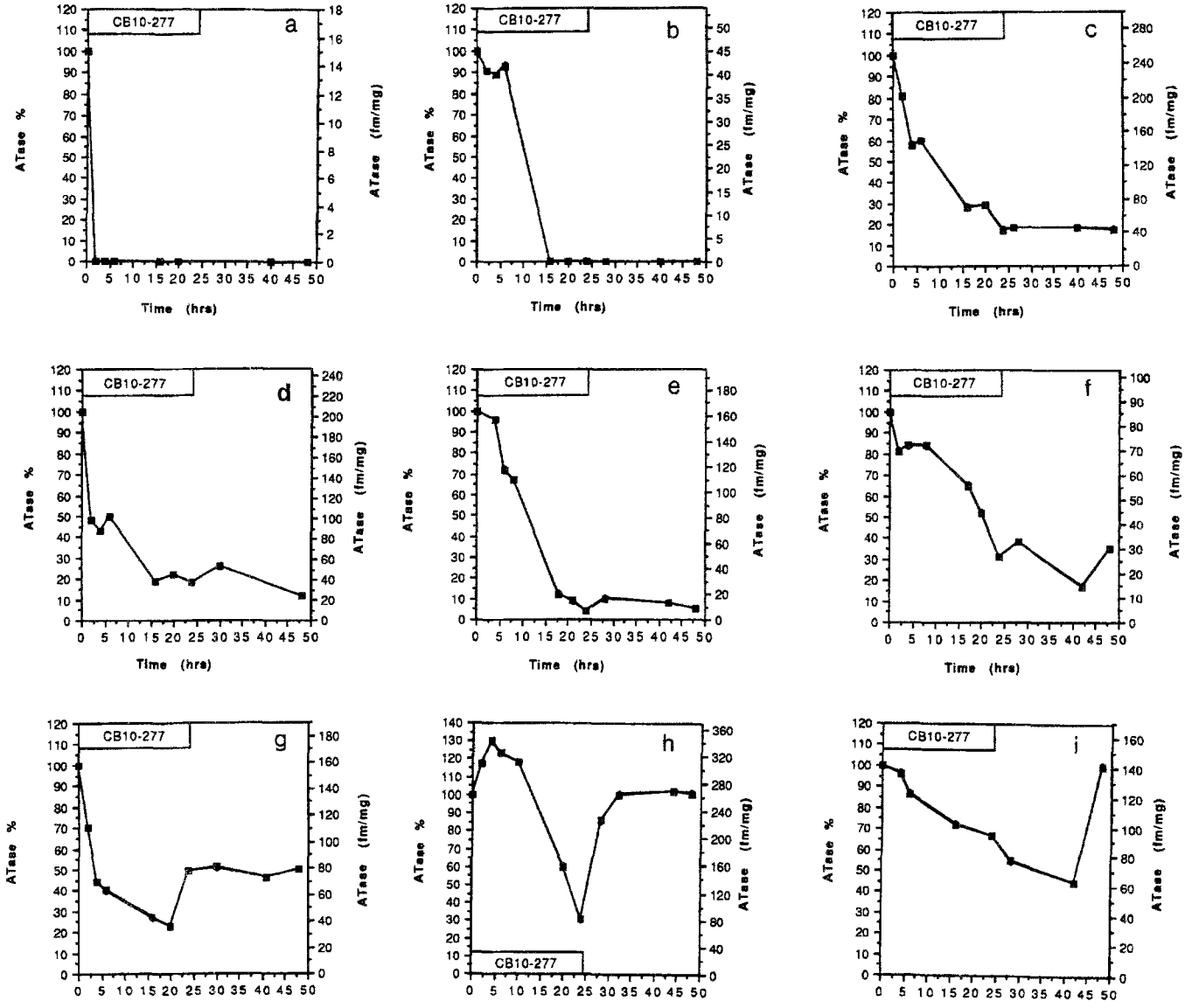

Fig. 2 a–i. ATase activity expressed as a percentage of pre-treatment activity and actual levels (fm/mg protein) measured at various times during and after CB10-277 continuous infusion (*box*). Data points represent mean values for 3 estimations, which agreed within ± 10%

the phase I CB10-277 study, haematological toxicity developed following 4 of 8 courses given at a dose of 12 g/m^2 whereas no haematological toxicity was observed when patients received a lower dose of CB10-277 (4.7–8.0 g/m^2) in 31 evaluable courses [30].

The response data for CB10-277 given as a 24-h infusion are shown in Table 1. In one mixed responder, a response occurred in the lung but the disease progressed in the brain (patient 2). This result may not be surprising, as it has been demonstrated that negligible DNA alkylation occurs in the rodent brain as compared with other organs following DTIC administration [29]. There were two early responses, but the disease progressed after the last cycle of chemotherapy (patients 1 and 7). Interestingly, two responders had low pre-treatment lymphocyte ATase levels (patients 1 and 2). No response was seen in the other six patients treated with CB10-277. In the phase I CB10-277 study, 4 responses were seen in 11 evaluable patients when CB10-277 was given as a short infusion [30].

Discussion

The present studies demonstrate that CB10-277 can lead to inactivation of ATase in human peripheral lymphocytes. This indicates that like DTIC, CB10-277 is metabolised, presumably predominantly in the liver [30, 35], to a methylating agent, which is produced in sufficient amounts to react with peripheral blood lymphocytes, generating O^6-methylguanine. The O^6-methylguanine lesions repaired by lymphocyte ATase, causing an apparent depletion of

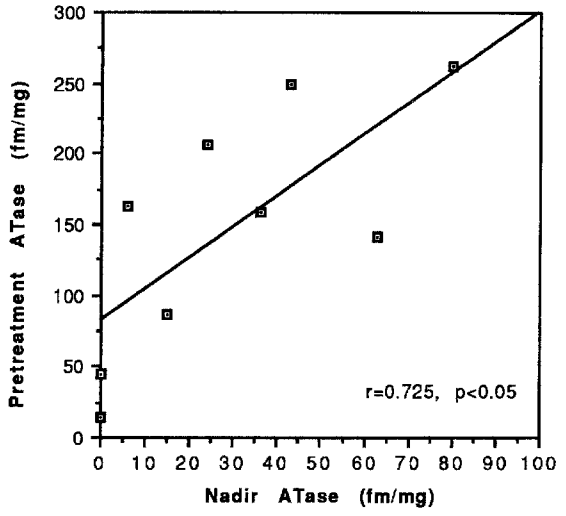

Fig. 3. Correlation between pre-treatment ATase activity and nadir ATase activity

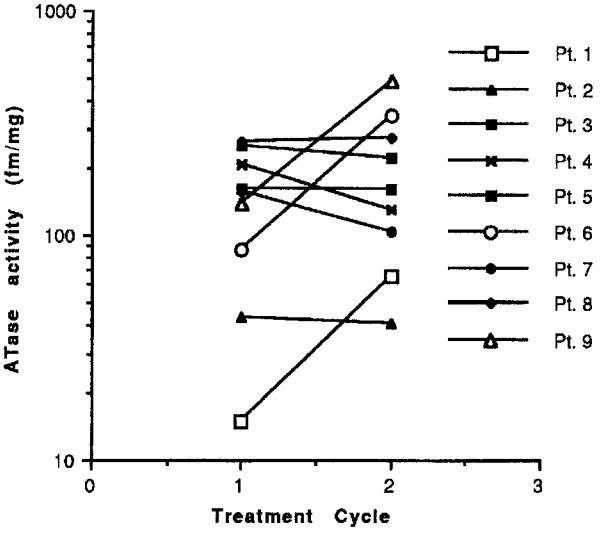

Fig. 5. Pre-treatment lymphocyte ATase activity (fm/mg protein) measured in patients (*Pt.*) 1–9 at the first and second cycles of CB10-277 treatment

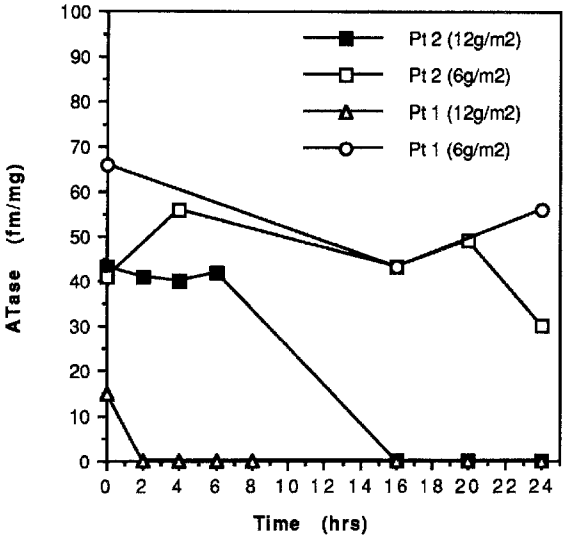

Fig. 4. Comparison of the kinetics of lymphocyte ATase (fm/mg protein) depletion during different cycles in 2 patients (*Pt.*) who received both 12 and 6 g/m^2 CB10-277

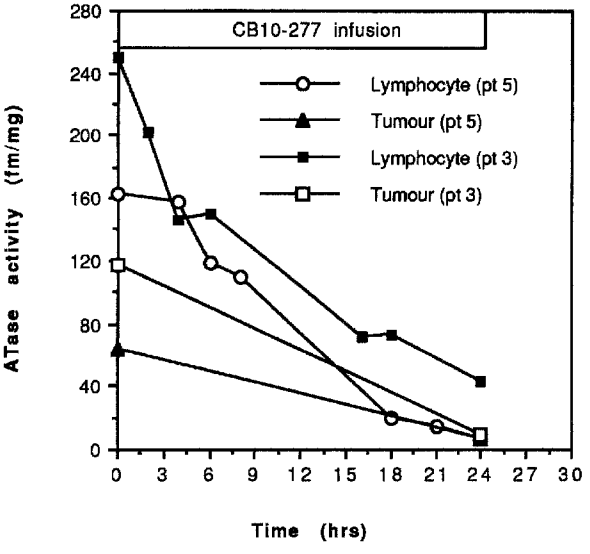

Fig. 6. ATase activity (fm/mg protein) measured in lymphocytes and biopsied melanoma tissues of 2 patients (*Pts.* 3 and 5; Table 1). Biopsies were performed before and immediately after the CB10-277 infusion

ATase activity in lymphocyte extracts. Similar to our earlier findings in patients treated with DTIC given in a single bolus dose [24] the present trial revealed variations in pre-treatment lymphocyte ATase activity as well as in the rate of depletion and recovery of lymphocyte ATase activity in patients given CB10-277 in a 24-h infusion. Complete suppression of lymphocyte ATase activity was seen in two patients whose pre-treatment ATase levels were low. Progressive depletion of lymphocyte ATase activity was seen in the remaining seven patients during CB10-277 infusion. At 24 and 48 h following the initiation of CB10-277 treatment, median lymphocyte ATase activity amounted to 17% of pre-treatment levels. Following completion of the CB10-277 infusion, no recovery of ATase activity was observed in six patients, but recovery to 50%, 100% and 102% of pre-treatment activity had occurred by 24 h in the other three patients, respectively. These variations presumably reflect a combination of individual differences in CB10-277 metabolism and in ATase gene transcription and translation rates.

We also found that low pre-treatment lymphocyte ATase activity was associated with the development of grade 4 haematological toxicity in two patients whose ATase activity fell to undetectable levels at 2 and 16 h after the start of CB10-277 therapy. A major problem in cancer chemotherapy is the prediction of significant and sometimes life-threatening haematological toxicity. Our finding suggests that screening for pre-treatment ATase activity in peripheral lymphocytes may be one way of predicting and, hence, preventing such toxicity when CB10-277 or related

drugs are used. Indeed, we have previously shown that ATase-deficient murine haematopoietic stem cells transfected with an *Escherichia coli* ATase gene are highly resistant to the toxic effects of methylating and chloroethylating agents, which strongly suggests that ATase provides protection against the haematological effects of these agents [22]. In addition, a significant correlation has been demonstrated between ATase levels in peripheral lymphocytes and those in bone marrow myeloid precursors [17]. In the present trial, no further haematological activity developed when the dose of CB10-277 was halved in the abovementioned two patients, and this finding was also associated with incomplete ATase depletion. In phase I studies of CB10-277, no evidence of haematological toxicity was encountered when lower dose of CB10-277 (<12 g/m^2) were used, and this observation was associated with a lower level of monomethyl metabolites [30]. These findings together with our ATase depletion data make it tempting to speculate that less DNA alkylation may have occurred in the bone marrow after treatment with the lower dose. Therefore, dose reduction following the identification of patients with low lymphocyte ATase levels may be a strategy for the prevention of haematological toxicity following chemotherapy. No haematological toxicity was seen in the remaining seven patients receiving CB10-277 (12 g/m^2) whose pre-treatment ATase levels exceeded 50 fmol/mg protein, despite the observation of substantial ATase depletion at 24 and 48 h following CB10-277 administration. A more extensive clinical study is now needed to explore the relationship between pre-treatment lymphocyte ATase activity and bone marrow toxicity in patients treated with CB10-277 and related chemotherapeutic agents.

A novel finding in the present study was an extensive depletion of ATase activity in biopsied tumour tissue following chemotherapy. However, despite this finding, the rate of response by patients with melanoma to treatment with CB10-277 was disappointing, with one mixed response and two early responses being obtained. This suggests that sufficient ATase may have been present or effective regeneration of ATase may have occurred such that the toxic O^6-methylguanine lesions could be effectively repaired, thereby reducing the effectiveness of the chemotherapeutic agent. It might also be that ATase levels are not homogeneous throughout the melanoma nodules [25] and that non-representative samples or nodules with particularly low levels of ATase were taken for ATase assay. In this case, the ATase-deficient tumour cells may be killed, but the resistant population could survive and continue to grow. A further disadvantage of CB10-277 is that it is a monofunctional agent and it has been shown to be 300–2,400 times less cytotoxic than an equivalent dose of a bifunctional agent [4]. Since CB10-277 is capable of depleting tumour ATase activity, one possible approach to overcome drug resistance might be to use a chloroethylnitrosourea after CB10-277, since the principal mechanism of chloroethylnitrosourea resistance involves the same DNA repair enzyme, ATase, which can remove the chloroethyl group from the O^6 position and, hence, prevent the formation of cytotoxic DNA interstrand cross-links [11, 32]. In fact, using sequential DTIC and the new chloroethylating agent fotemustine, we have recently improved the clinical response rate in metastatic melanoma [1] from 21% for single-agent treatment to up to 40%, supporting the use of such drug combination and delivery schedules.

As observed in our earlier study in patients treated with DTIC [24], in the present trial we found that a small number of patients (three) showed an increase in pre-treatment lymphocyte ATase levels when they returned for subsequent CB10-277 therapy. As is the case for many chemotherapeutic agents, resistance to DTIC and MTIC develops rapidly in vivo [9] and in vitro [31], and in the latter case this arises from enhanced repair of the cytotoxic O^6-guanine lesions [21]. This resistance may be related to the DNA cytosine methylation status of the ATase gene controlling regions in surviving lymphocytes following CB10-277 treatment, as it has recently been shown that methylation of the ATase gene is correlated with ATase expression [33]. Another possibility is that expression of ATase activity in peripheral lymphocytes may be heterogeneous [25]; if alkylation-mediated killing of ATase-deficient lymphocytes occurs after CB10-277 treatment, the ATase-proficient lymphocytes will survive and continue to divide, the result being a net increase in ATase specific activity. If this cycle-dependent increase in ATase activity is reflected in the target tumour, it might indicate that tumour resistance will increase with the number of treatment schedules, a highly undesirable effect and one that should be monitored as closely as possible in future studies. There appeared to be a decrease in pre-treatment ATase levels following one cycle of DTIC therapy, which suggests that different agents might produce different longer-term responses, although this possibility needs to be investigated in much larger groups of patients [26]. With the availability of ATase cDNA probes and ATase antibodies, it might be possible to elucidate the molecular mechanism behind the development of drug resistance in tumour tissues.

Clearly, many factors are involved in the prediction of tumour sensitivity in patients. A major clinical difficulty is to obtain serial tumour biopsies during the course of chemotherapy. One approach that would include all of the variable factors discussed above would be to use peripheral lymphocytes to monitor ATase activity so as to identify the optimal chemotherapy dose, schedule and delivery to overcome drug resistance. Our findings in two patients suggest that ATase depletion in peripheral lymphocytes may correlate with that in tumour tissue following CB10-277 administration and this needs to be extended to related drugs and other tumour types.

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