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Antibody-directed enzyme prodrug therapy: pharmacokinetics and plasma levels of prodrug and drug in a phase I clinical trial

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Abstract Antibody-directed enzyme prodrug therapy (ADEPT) was administered to ten patients in a phase I clinical trial. The aim was to measure plasma levels of the prodrug 4-[(2-chloroethyl)(2-mesyloxyethyl) amino] benzoyl-L-glutamic acid (CMDA) and the bifunctional alkylating drug (CJS11) released from it by the action of tumour-localised carboxypeptidase G2 (CPG2) enzyme. New techniques were developed to extract the prodrug and drug from plasma by solid-phase adsorbtion and elution and to measure CPG2 activity in plasma and tissue. All extracts were analysed by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). CPG2 activity was found in metastatic tumour biopsies but not in normal tissue, indicating that localisation had been successful. The clearing agent SB43-gal, given at 46.5 mg/m^2, achieved the aim of clearing non-tumour-localised enzyme in the circulation, indicating that conversion of prodrug to drug could take place only at the site of localised conjugate. Plasma prodrug did not always remain above its required threshold of 3 μM for the "therapeutic window" of 120 min after dosing, but the presence of residual prodrug after the first administration of each day indicated that this could be achieved during the remaining four doses over the following 8 h. Despite considerable inter-patient prodrug plasma concentration variability, the elimination half-life of the prodrug was remarkably reproducible at 18 ± 8 min. Rapid appearance of the drug in plasma indicated that successful conversion from the prodrug had taken place, but also undesirable leakback from the site of localisation into the bloodstream. However, drug plasma levels fell rapidly by at least 50% at between 10 and 60 min with a half-life of 36 ± 14 min. Analysis of the plasma extracts by LC/MS indicated that this technique might be used to confirm qualitatively the presence of prodrug, drug and their metabolites.

Key words ADEPT · Prodrug · Carboxypeptidase G2

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Introduction

The potential to target cytotoxic drug to the tumour site offers the patient the advantage of reducing adverse side effects and normal tissue damage as a higher concentration of drug can be generated at the target site during antibody-directed enzyme prodrug therapy (ADEPT). Use of antibodies (Abs) in early attempts to target cytotoxic agents resulted in limited success due to several factors [2, 8]. In these approaches, delivery of the conjugate is limited in poorly vascularised tumours, resulting in the inability of the cytotoxic agent to reach a lethal concentration at the tumour target. Cell-surface-antigen heterogeneity further limits binding of the conjugate.

ADEPT (Fig. 1) is essentially a two-step system where the activating component is in the form of an enzyme conjugated to a monoclonal Ab. The bacterial enzyme carboxypeptidase G2 (CPG2) has been used in ADEPT protocols, conjugated to the F(ab′)$_2$ fragment of the Ab A5B7, which recognises carcinoembrionic antigen (CEA) [2,10]. The conjugate is given first and binds to the CEA-expressing cells. A galactosylated second clearing Ab (SB43-gal), raised against CPG2, can optionally be used to lower levels of conjugate in the circulation and peripheral tissues of any non-tumour-localised enzyme-Ab conjugate that could effect conversion of the prodrug at sites other than the tumour [13]. Subsequent administration of a non-toxic prodrug results in its conversion to the cytotoxic drug by the

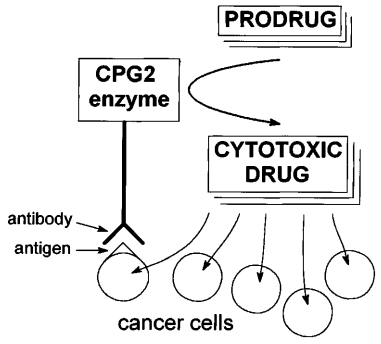

Fig. 1 Schematic representation of ADEPT. Converson of non-toxic prodrug to cytotoxic drug by the action of localised carboxypeptidase G2 (*CPG2*)

action of CPG2 at the tumour site (Fig. 2). ADEPT with CPG2 has been shown to have potential in vitro and in animal studies [2, 13, 16, 17]. Various prodrugs have been investigated for their efficacy in ADEPT with CPG2 and with other enzymes [7, 11, 12, 21, 22].

In an earlier phase I clinical trial the prodrug of choice, based on animal studies, was 4-[(2-chloroethyl)(2-mesyloxyethyl) amino] benzoyl-L-glutamic acid (CMDA, also known as CJS8; Fig. 2) [3–5, 15]. This is cleaved by CPG2 to the corresponding bifunctional alkylating drug, CJS11. Patients were grouped to receive either the prodrug alone to check for the absence of toxicity due to endogenous prodrug cleavage or full ADEPT, with variations being undertaken in the administration of the components to optimise the therapy. Plasma from some of these patients was analysed by high-performance liquid chromatography (HPLC) for prodrug and drug after administration of ADEPT, and their presence was confirmed by liquid chromatography-mass spectrometry (LC-MS) [19, 20]. The procedure used to prepare the plasma samples for HPLC was that developed by Antoniw et al. [1] for analysis of murine

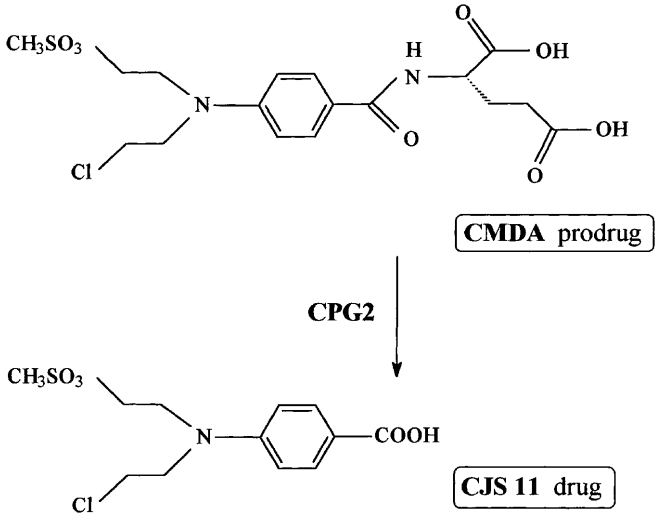

Fig. 2 Enzymic cleavage of glutamic acid from prodrug CMDA to yield the cytotoxic bifunctional alkylating agent CJS11

plasma and tissues after ADEPT administration of the prodrug 4-[*bis*(2-chloroethyl) amino] benzoyl-L-glutamic acid.

The present ADEPT clinical trial, performed by Prof. R. Begent at the Royal Free Hospital, again using the CMDA prodrug, benefits from the experience of the previous trial in the design of the regimen for administration of the three components of the therapy. Details of the dose levels of enzyme/Ab conjugate (CPG2-A5B7), clearing agent (SB43-gal) and prodrug (CMDA) given to the present patients are given below in Patients and methods. The clinical results of this trial will be reported in detail elsewhere. Collection of blood samples from patients in the previous trial was sparse. The present aim was to obtain samples more frequently from patients, the limitation being the total amount of blood that could be withdrawn, for evaluation of prodrug and drug pharmacokinetics and identification of metabolites. We also wished to verify that plasma levels of the prodrug were maintained above its Michaelis constant (K_m) of 3 μM for 120 min and to monitor circulating drug levels. The protocol required that prodrug not be given until the plasma enzyme concentration had fallen to < 0.2 U/ml. Indeed, results from the first trial showed that this value was < 0.02 U/ml after infusion of the clearing antibody [5]. In the current study, plasma samples were monitored to check that this concentration was not exceeded throughout the therapy.

A simpler method of prodrug and drug extraction from plasma was required to facilitate the processing of greater numbers of samples and to give greater accuracy and reproducibility. Lower limits of detection for prodrug and drug were necessary to enable measurement of their plasma concentrations at later collection times. The accuracy of the determinations is an important factor as it has been impossible to find a suitable internal standard for prodrug and drug extractions.

The use of mass spectrometry gives an unequivocal identification of compounds by assignment of their molecular-mass ion. A further aim of this study was therefore to obtain qualitative data to confirm the HPLC findings that plasma extracts contained prodrug and drug and to identify metabolites.

Patients and methods

Materials

The prodrug CMDA and drug 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoic acid (CJS11) were synthesised by methods described elsewhere [9, 16, 18]. The $F(ab')_2$ fragment of murine A5B7 monoclonal Ab was conjugated to the bacterial enzyme (CPG2) [10]. Galactosylated murine SB43 monoclonal Ab (SB43-gal) was prepared as described by Sharma et al. [13]. Normal human plasma was obtained from the Blood Transfusion Service (London, UK). All solvents were of HPLC grade (Romil, Loughborough, UK), and other chemicals were of the highest grade obtainable from Sigma Chemical Co. (Poole, UK) or BDH (Lutterworth, UK). Methotrexate was obtained from LaGap Pharmaceuticals Ltd. (Hants., UK).

Patients treatment

In the present ADEPT clinical trial, ten patients (six men and four women) gave fully informed consent to the administration of ADEPT after local ethics committee approval had been given. They all had histologically proven, non-resectable metastatic or locally recurrent colorectal carcinoma and fulfilled various biochemical criteria, which will be detailed in a separate clinical paper.

Day 0

A5B7-CPG2 conjugate was infused at 10 000 U/m^2 in 0.9% saline (500 ml over 2 h).

Day 1

SB43-gal clearing Ab was given as infusions of 7.5 mg/m^2 (over 6 h) followed by 15 mg/m^2 (over 18 h), each in 0.9% saline (500 ml).

Day 2

The concentration of circulating CPG2 was measured to check that it was below 0.2 U/ml. SB43-gal infusion was continued at 8 mg/m^2 in 0.9% saline (1 l over 24 h). CMDA prodrug was given over 5 min at 200 mg/m^2 in dimethylsulfoxide (DMSO; 1.5 ml) as a bolus into a fast-running infusion of 1.26% sodium bicarbonate (20 ml). The aim was to collect blood onto heparin at 5, 10, 20, 30, 60 and 120 min after prodrug administration, although this schedule was often varied for clinical reasons. Blood was centrifuged (1 000 *g*, 4 min) and the plasma was removed and stored at −20 °C. Sampling from some patients was limited for clinical reasons, and samples were occasionally taken after 2 h if subsequent dosing of CMDA was delayed. Patients usually received a further four equivalent doses of prodrug at 2-h intervals, but no further blood sample was collected.

Days 3 and 4

Patients received SB43-gal and five doses of CMDA as on day 2. Blood samples were collected after the first dose of prodrug and processed as described above.

Patients 5 and 9 (men) received a second complete cycle of ADEPT starting at 7 days after the first cycle. The blood samples from patient 4 (man) were degraded and unsuitable for analysis. Cyclosporin A was infused from day -2 at 15 mg/kg per day until the day after the final prodrug administration. It was then given orally at the same dose rate for a further 7 days.

Prodrug and drug standard solutions

CMDA prodrug (250 μ*M*) and CJS11 drug (30 μ*M*) were dissolved in DMSO. Dilutions of each were made into acetonitrile. These stock solutions allowed the required concentrations for calibration standards to be achieved by spiking of a maximum of 20 μl acetonitrile into 200 μl plasma. DMSO solutions were stored at 4 °C and diluted as required. All prodrug and drug solutions were prepared and dispensed using glassware and syringes or glass capillary micro-pipettes to avoid the risk of their reaction with plastic. Prodrug and drug were extracted from spiked plasma to give standard calibration curves. Prodrug concentrations gave the equivalent of 4–100 or 10–250 μ*M* in plasma and the drug concentrations give 0.25–15 μ*M* in plasma.

Extraction of prodrug and drug from plasma

IST Isolute SPE purification cartridges [100 mg C8(EC) sorbent bed; Jones Chromatography, Hengoed, Mid Glamorgan, UK] were fitted to combined holders/delivery needles in a manifold. This formed the lid of a tank evacuated by a low-pressure water pump creating 3-psi suction. Cartridges were wetted with methanol (1 ml) and then conditioned with 0.5 *M* acetic acid (500 μl). The integral taps of the delivery needles were closed and the cartridges were loaded with either patient plasma (200 μl), 1.26% sodium bicarbonate (200 μl) or normal human plasma (200 μl) into which was spiked the required volume of standard prodrug or drug solution. Next, 0.5 *M* acetic acid (800 μl) was added to the spiked plasma and this mixture was allowed to flow onto the bed. Cartridges were washed with 1 ml deionised water, and prodrug or drug was eluted with elution solution (80% acetonitrile in 1% acetic acid; 500 μl) directly into autosampler vials for HPLC analysis as described below.

HPLC estimation of prodrug and drug

HPLC separation was achieved using an automated Thermo Separation Products (TSP) SpectraSYSTEM (Stone, UK) under full computer control of PC1000 software via an SN4000 module. A vacuum membrane degasser conditioned the mobile phases, which were mixed and delivered by a proportioning pump (P4000). Samples were injected via an autosampler (AS3000) using the PushLoop mode. Separation was carried out on a Spherisorb C-18 fully end-capped column (5 μm, 15 cm × 4.6 mm; Hichrom Ltd., Reading, UK) protected by a repackable guard column containing Whatman pellicular C-18 material (Jones Chromatography, Hengoed, Mid Glamorgan, UK). Adequate separation of prodrug and drug could be achieved only by the use of gradient elution from 30–70% acetonitrile in water (+ 1% acetic acid) over a period of 10 min. The final concentration was maintained for 5 min, followed by a return to the starting conditions over a further 5 min. The flow rate was constant throughout at 1 ml/min. Eluted prodrug and drug were deleted at 305 nm by a high-resolution forward optical scanning detector (Spectra FOCUS). Standard calibration curves were constructed automatically by the software for prodrug and drug. These were used to estimate the concentrations in the patient plasma samples. When necessary, plasma samples were diluted with normal human plasma before extraction to bring prodrug and drug values onto the standard curves.

HPLC-ESI-MS verification of prodrug and drug in patient plasma extracts

Extracts from patient plasma were further analysed qualitatively by LC-MS to confirm the presence of prodrug, drug and any metabolites. The HPLC system consisted of a Waters 600 MS controller (Millipore Ltd., Watford, UK). Separation was achieved on a Spherisorb C-18 column as described above using an isocratic mobile phase of 25% acetonitrile in 0.1% acetic acid at a flow rate of 1 ml/min; 50% of the eluent was directed into the mass spectrometer. A Finnigan TSQ 700 mass spectrometer fitted with a Finnigan MAT electrospray ionisation source (San Jose, USA) was used for detection of the analytes. The heated capillary and high voltage were operated at 220 °C and 4.5 kV, respectively. The scan range was m/z 200–700 at 4 s/scan in the positive mode. Data acquisition was controlled by a DEC 2100 station with Finnigan ICIS and ICL software.

Estimation of CPG2 in patient plasma

The level of CPG2 in the same plasma samples used for prodrug and drug analysis was checked using methotrexate as a substrate, which undergoes a similar catalytic cleavage of the glutamic acid moiety, resulting in a UV-detectable metabolite. Incubation mixtures for the preparation of a CPG2 standard calibration line contained normal human plasma (100 μl) in 0.2 m*M* $ZnCl_2$/PBS (500 μl) and 10 m*M* methotrexate (6 μl). These were brought to 30 °C in a Techne metabolic shaking water bath (Fisons Scientific Equipment, Loughborough, UK) before the addition of various

concentrations of CPG2 (6 µl). Patients' plasma (100 µl) was pre-incubated with $ZnCl_2$/PBS (500 µl) and the reaction was started by the addition of methotrexate (6 µl). After incubation with shaking for 30 min, 200 µl was removed into 500 µl of ice-cold stop solution (MeOH + 0.1% TFA). Centrifugation (10 000 *g*, 4 min) produced a supernatant fraction, which was analysed by HPLC. The TSP HPLC system was used to measure metabolite peaks and, thus, to estimate the levels of CPG2 using an isocratic mobile phase of 70% MeOH in 60 m*M* ammonium formate containing 0.1% TFA pumped at 1 ml/min. A Spherisorb SCX cation-exchange column (15 cm × 4.6 mm; Hichrom Ltd., Reading, UK) was protected by a guard column packed with pellicular SCX material (Whatman Ltd., Maidstone, UK). Metabolite peaks were detected at 307 nm.

Pharmacokinetic calculations

The pharmacokinetics of prodrug and drug in patient plasma were calculated using the program PCNONLIN (SCI software, Kentucky, USA). Functions consisting of the sum of one, two or three exponential components were fitted to data by a least-squares method. Each set of data was analysed with one, two, and three compartments and the best fit was adopted. Non-compartmental analysis was also performed. As the infusion was very rapid (5 min) and the conversion of prodrug into drug was also rapid, the data was modeled better with i.v. models and no absorption phase.

Results

HPLC analysis

The simplified clean-up procedure prior to HPLC allowed more rapid processing than the previously used method [1]. Standard calibration curves for both prodrug and drug extracted from spiked plasma were achieved with a correlation coefficient of >0.99. The use of gradient, as opposed to isocratic, elution sharpened the peak profiles of both prodrug and drug, which were eluted with retention times of 4 and 6 min, respectively. The limits of quantification were 0.15 µ*M* for prodrug and 0.2 µ*M* for released drug, although these values were below the lowest used in the standard curves. Typical chromatograms of eluted prodrug- and drug-spiked standard plasma and the elution profile of an extracted patient's plasma sample are shown in Fig. 3.

CPG2 detection

No CPG2 was detectable in any of the patient plasma samples (lower limit of detection 5×10^{-5} U/ml plasma) at any time point following prodrug administration. Metastatic tumour biopsy samples were obtained from the livers of patients 1, 3, 5, 6 and 10; these comprised one ovarian and three liver metastases and one case of stomal recurrence. Normal liver samples came from patients 2 and 3. All were analysed for their CPG2 content by a modification of the method used for plasma. No CPG2 was detectable in either of the normal liver samples (limits of detection as for plasma), whereas CPG2 had localised in all the tumour biopsy samples (see Fig. 6). The concentrations in tumour varied between 0.1 and 0.8 U/g of tumour from five different patients.

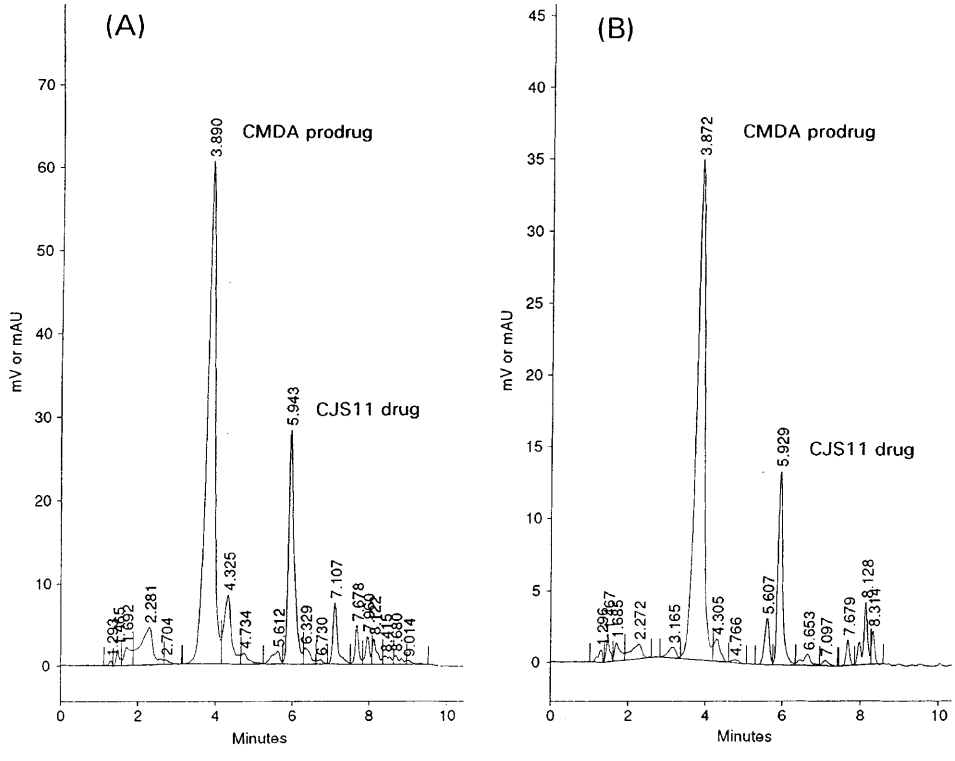

Fig. 3A,B Chromatograms generated from HPLC elution of **A** normal human plasma spiked with CMDA prodrug (60 µg/ml) and CJS11 drug (2.5 µg/ml) used as one of the standards to generate a calibration line for estimation of prodrug and drug in **B** a patient's plasma sample

Plasma prodrug and drug levels and pharmacokinetics

Prodrug was accumulating at the 3- and 5-min time points when measured, reflecting the 5-min duration of prodrug administration. For most patients, T_{max} had been reached for both prodrug and drug by the time the first blood sample was taken and all concentrations had reached maxima by 10 min except for patient 9 (cycle 2, day 3 of therapy) and patient 10 (day 2 of therapy), when levels had peaked by 20 min. The maximal plasma concentration of prodrug achieved (regardless of time) was $394 \pm 109\ \mu M$ ($n = 33$; range 45–3,308 μM). When prodrug maxima were achieved later than the earliest sampling points, drug maxima were also delayed,

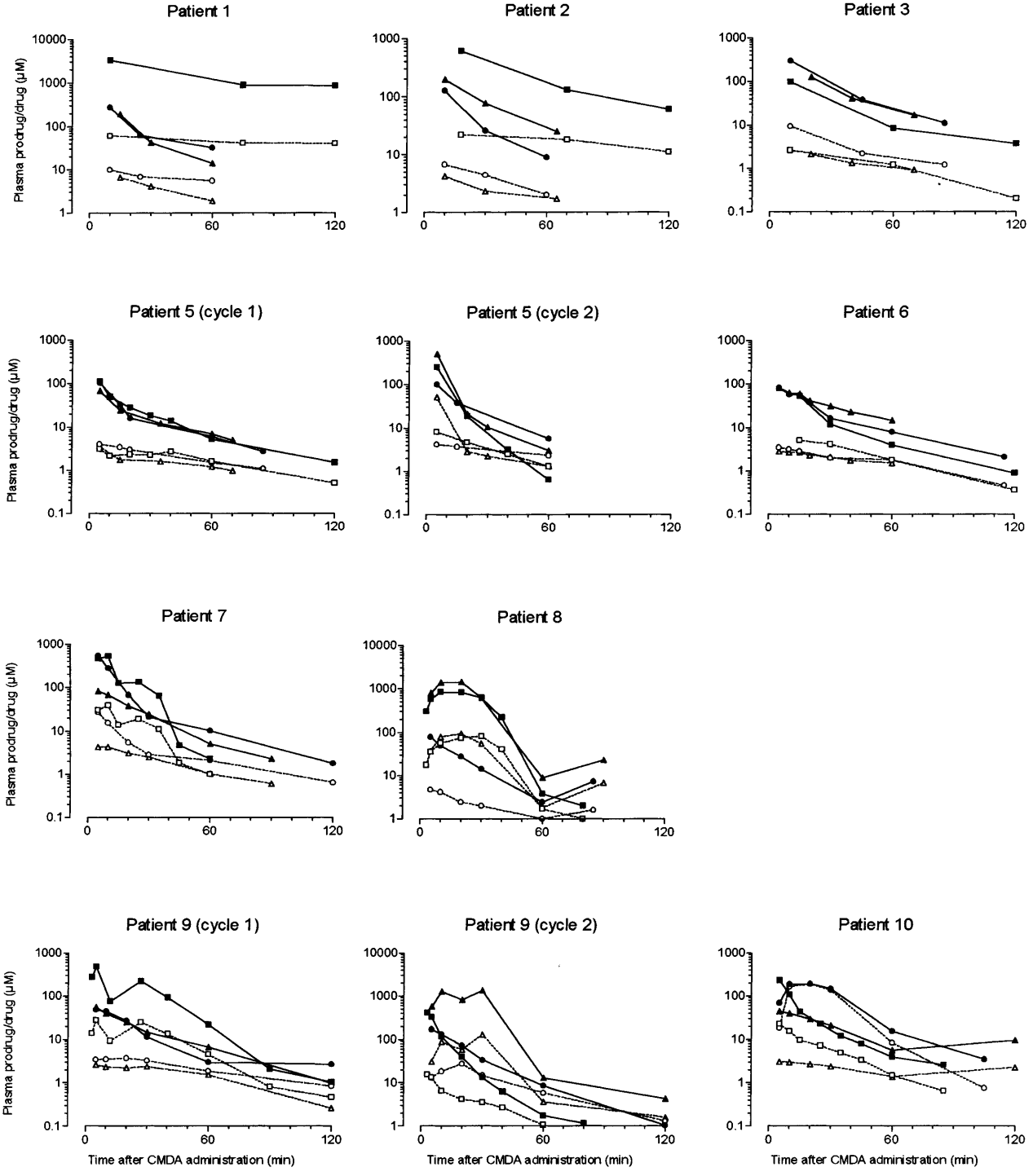

Fig. 4 Profiles of prodrug and drug in the plasma of all patients. *Black symbols* (■, ●, ▲) represent prodrug on days 1, 2 and 3, respectively, of CMDA administration. *White symbols* (□, ○, △) represent drug on the same days

reaching their highest levels at or within 10 min of those recorded for the prodrug. Released drug reached levels of 26 ± 7 μ*M* (*n* ± 33; range 2–189 μ*M*).

Prodrug and drug plasma concentration profiles from all patients from their 3 days of therapy are shown in Fig. 4, except for patient 4, whose plasma samples were degraded and unusable. All are plotted to 120 min, or less if sampling had stopped, and show the variation from patient to patient and between the days of a patient's treatment. Blood samples were only occasionally taken later than this if the next prodrug administration was delayed for clinical reasons. On their 1st day of therapy, blood samples were taken at 4 h after prodrug administration from three patients. For patient 5 (cycle 1), plasma prodrug and drug concentrations were not detectable and 0.19 μ*M*, respectively. For patient 6 they were 0.15 μ*M* and not detectable and for patient 9 (cycle 1) they were 1.29 and 0.5 μ*M*, respectively. Other minimal plasma concentrations can be seen in the plots for up to 120 min in Fig. 4. There is no apparent clinical explanation for the increase from the minimal plasma levels recorded for patient 8 on days 2 and 3 of therapy. The desirable prodrug concentration of 3 μ*M* was maintained until 120 min in this first prodrug administration of the day for each day of therapy for patients 1, 2, 3 and 10. It was also achieved by patient 6 on day 3 and by patient 9 (cycle 2) on day 3. For all other patients the level of prodrug either measured or extrapolated had fallen to < 3 μ*M* at 120 min. For all days of treatment in both cycles for patient 5, values had fallen below this level by 60 min (Fig. 4).

The variation of plasma prodrug concentrations at 10, 60, and 120 min, when measured, is shown in Fig. 5A and that for the drug is illustrated in Fig. 5B. The highest concentrations recorded at each time point are those from patient 1 and the next highest recorded at 60 and 120 min are from patient 2, in both cases on their 1st day of treatment. There was no apparent clinical explanation for these very high and sustained levels. The mean values recorded at 120 min are higher than those noted at 60 min as the fewer sampling points at this time were taken from patients who had achieved higher plasma concentrations. The SD reflects the range of values measured. However, the main groupings of points do show plasma levels continuing to fall at 120 min. For all except 6 of the total 33 sets of data analysed (3 days of therapy for each of a total of 11 cycles), drug values had fallen by more than 50% at between 10 and 60 min and some of the highest initial levels had dropped to below 5% over this period. Despite this rapid reduction in drug levels, there is a concomitant decrease in plasma prodrug levels, resulting in the ratio of prodrug to drug falling slightly from 23 ± 10 (*n* = 33; range 9–42) to 20 ± 20 (*n* = 33; range 7–38). By 120 min, when samples were taken, the ratio had decreased to 8 ± 6 (*n* = 33; range 2–18), indicating a relative rise in the drug concentration in plasma. Inter-patient variations of prodrug/drug ratios at 10 min are shown in Table 1, to correspond with the plots in Fig. 5.

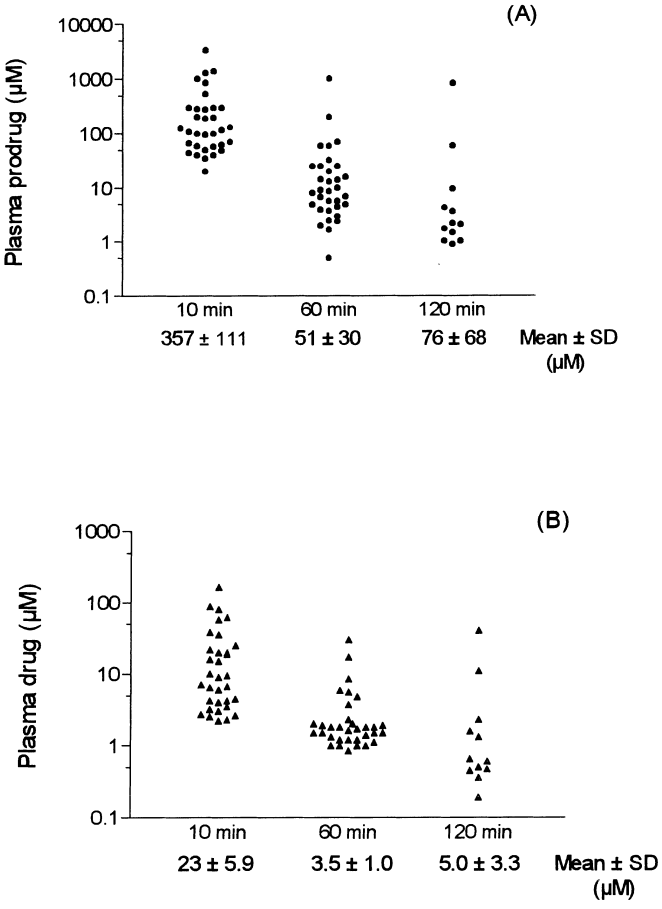

Fig. 5A,B Distribution of all measured plasma **A** prodrug and **B** drug concentrations at 10, 60 and 120 min

Prodrug and drug were reported as simple clearance values from the first clinical trial [5], whereas Antoniw et al. [1] used a two-compartment model for both prodrug and drug for analysis of experiments in mice. In the present trial, no pharmacokinetic calculation was made for patients 1, 2 or 3 as their blood sampling had been limited and not enough data points were available for the calculation of terminal half-lives. C_0 was not calculated for any patient, as the time taken for administration of the prodrug would never expose the bloodstream

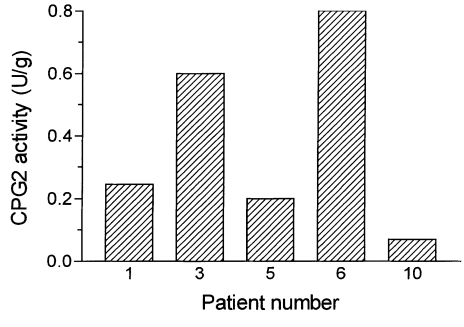

Fig. 6 CPG2 activity measured in metastatic tumour biopsy samples taken from the patients indicated

Table 1 Ratios of plasma prodrug to drug calculated for each patient at 10 min after the administration of CMDA prodrug and ratios of pharmacokinetic parameters $t_{1/2}$, AUC 60, AUC INF

Patient ID		Ratio at 10 min (prodrug/drug)	$t_{1/2}$ (drug/prodrug)	AUC 60 (prodrug/drug)	AUC INF (prodrug/drug)
1	Day 1	54			
	2	28			
	3	n/a			
2	Day 1	n/a			
	2	18			
	3	49			
3	Day 1	33			
	2	30			
	3	n/a			
5(1)	Day 1	25	6	11	6
	2	17	2	9	6
	3	12	n/a	11	n/a
5(2)	Day 1	14	3	11	8
	2	14	n/a	10	n/a
	3	10	2	9	8
6	Day 1	n/a	n/a	6	n/a
	2	19	n/a	11	n/a
	3	21	2	17	10
7	Day 1	14	1	10	9
	2	17	2	14	12
	3	17	2	12	10
8	Day 1	15	n/a	n/a	n/a
	2	13	3	10	4
	3	17	n/a	n/a	n/a
9(1)	Day 1	10	1	10	9
	2	11	1	6	4
	3	20	n/a	9	n/a
9(2)	Day 1	17	1	13	11
	2	7	2	4	3
	3	14	n/a	n/a	n/a
10	Day 1	7	1	5	5
	2	1	n/a	1	n/a
	3	13	5	10	2

n/a Not applicable: blood sampling started later than 10 min (plasma ratios) or one of the values not calculated (pharmacokinetic parameters)

to a concentration as high as would be extrapolated. Although most blood sampling was complete by 120 min, when further prodrug administration was delayed, data from later time points were included for modeling. Data were entered into models assuming an i.v. bolus route as discussed in Patients and methods.

Table 2 gives the parameters calculated when prodrug data for patients 5–10 were analysed with one- and two-compartment and non-compartmental models. Ideally, the half-life of drug (and prodrug) should be calculated from blood samples obtained at time points extending beyond those obtained in the present study. However, the necessity to redose the prodrug at 2-h intervals precluded the collection of blood samples at later times except in the three instances mentioned above. The elimination half-life and AUCs calculated to 60 min and extrapolated to infinity were calculated from a non-compartmental model, except for the incomplete values recorded for the three patients indicated. Elimination half-lives and clearance volumes were calculated in a one-compartment model. Of the total 24 days of therapy administered to the six patients (two patients received two cycles), 14 were reliably modeled with a correlation coefficient of >0.9 and standard errors of $< 50\%$. The others were unreliable or could not be modeled, as indicated. A two-compartmental model was reliable for 5 days of therapy data from patients 5, 6, 9, 10 as indicated. The $t_{1/2}$ (β, elimination) and clearance values are included with those calculated from the one-compartment model for purposes of comparison. Values for $t_{1/2}\alpha$ were calculated from these data but are meaningless when the 5-min period taken for administration of the prodrug is taken into consideration.

Drug data from patients 5–10 were entered into the same models as the prodrug data and the calculated parameters are shown in Table 3. In the non-compartmental model it was not possible to calculate the elimination half-life for 9 of the total 24 sets of therapy data. Reliable analyses were also less complete in the one-compartment model, although five sets of data had standard errors of $< 50\%$ but correlation coefficients of 0.68–0.90 [patient 7, day 1; patient 8, days 2 and 3;

Table 2 Pharmacokinetic parameters calculated for pro-drug in 1- and 2-compartment and non-compartmental models (*Nr* Not reliable, *Nm* Not modeled by software)

Patient ID (cycle)	Non-compartmental model			1-Compartment model (2-compartment)		
	$t_{1/2}$ (min)	AUC 60 (μ*M* min)	AUC INF (μ*M* min)	$t_{1/2}$ (min)	CL (ml/min)	V_D (ml)
5 (1) day 1	18	1,379	1,681	18 (16)	193 (117)	5,013
2	26	1,243	1,450	18 n/r	219 n/r	n/m
3	29	1,055	1,322	17	265	6,501
5 (2) day 1	9	2,263	2,271	9 (9)	107 (103)	1,390
2	n/m	1,687	n/m	7	230	2,323
3	16	4,234	4,304	14 n/r	68 n/r	n/m
6 day 1	28	728	911	25	182	6,566
2	24	1,424	1,776	22 (53)	149 (145)	4,730
3	30	1,913	2,541	19	127	3,482
7 day 1	15	6,794	6,842	9	31	403
2	21	4,688	5,106	19 n/r	39 n/r	n/m
3	18	1,639	1,809	14	149	3,010
8 day 1	22	n/m	n/m	n/m	n/m	n/m
2	56	1,177	1,907	23	148	4,912
3	n/m	n/m	n/m	9 n/r	7 n/r	n/m
9 (1) day 1	60	8,136	8,656	26 n/r	34 n/r	n/m
2	49	993	1,356	n/m	n/m	n/m
3	28	1,073	1,442	25 (39)	231 (226)	8,333
9 (2) day 1	16	3,074	3,129	12	79	1,378
2	18	2,948	3,267	12	97	1,680
3	n/m	n/m	n/m	26 n/r	9 n/r	n/m
10 day 1	30	1,920	2,220	n/m (29)	n/m (103)	n/m
2	21	6,682	7,217	40 n/r	38 n/r	n/m
3	108	1,212	3,172	21	240	7,273

Table 3 Pharmacokinetic parameters calculated for drug in 1- and 2-compartment and non-compartmental models (*Nr* Not reliable, *Nm* Not modeled by software)

Patient ID (cycle)	Non-compartmental model			1-Compartment model (2-compartment)
	$t_{1/2}$ (min)	AUC 60 (μ*M* min)	AUC INF (μ*M* min)	$t_{1/2}$ (min)
5 (1) Day 1	104	127	263	n/m
2	53	141	255	42
3	n/m	95	n/m	n/m
5 (2) Day 1	28	206	273	26
2	n/m	177	n/m	65
3	37	480	549	18 n/r (38)
6 Day 1	n/m	159	n/m	27
2	n/m	126	n/m	41
3	66	114	255	56
7 Day 1	17	702	727	11
2	36	329	444	26 n/r (72)
3	30	140	190	28
8 Day 1	n/m	n/m	n/m	31 n/r
2	148	122	432	42
3	n/m	2,753	n/m	32
9 (1) Day 1	42	832	974	35
2	52	165	309	59
3	n/m	116	n/m	n/m
9 (2) day 1	20	240	275	21
2	28	834	1,103	43
3	n/m	4,028	n/m	48 n/r
10 Day 1	30	380	467	28 n/r (23)
2	n/m	6,049	n/m	37 n/r
3	500	126	1,651	202 n/r

patient 9, (cycle 1) day 1 and (cycle 2) day 2]. Data from three patients [patient 5, (cycle 2) day 3; patient 7, day 2; and patient 10, day 1] were better represented by a two-compartment model as opposed to a one-compartment model. Comparisons of the prodrug-to-drug elimination half-life and AUC at 60 min and infinity are shown in Table 1.

LC-MS analyses

Various extracts of patient plasma prepared for HPLC were also subjected to LC-MS. The aim was to provide an unequivocable assignation to the peaks of prodrug and drug. Mono- and bis-hydrolysis products of the prodrug and drug have previously been identified [19], but the possibility of further metabolites had not been investigated.

The ion chromatograms resulting from the analysis of a plasma sample from patient 5, assigning the prodrug, the drug and their mono-hydrolysis products by their mass ions, are shown in Fig. 7. A trace of acetyl hydrolysed prodrug with an Mr of 414 (retention time 5.6 min) was also found in another patient (data not shown). Mass spectra recorded for the prodrug, the drug and their hydrolysis products are shown in Fig. 8. A carbonium ion with m/z = 304, derived from the prodrug, is seen in the prodrug (m/z = 451) mass spectrum. The hydrolysed form of this ion (m/z = 226) is present in the corresponding hydrolysed prodrug spectrum (m/z = 373). The protonated molecular ion of the drug is confirmed as m/z = 322 and its hydrolysed form, as m/z = 244.

Discussion

The primary aim of ADEPT is to deliver higher doses of an active drug directly to the tumour, thus sparing the normal tissues from toxicity. This can be achieved only if the Ab-enzyme conjugate remains localised at the tumour site and is not available to prodrug at non-tumour sites. The prodrug is ideally given as soon as possible after the conjugate to allow the longest possible "time window" for multiple dosing before immunogenicity becomes a problem. This requires complete clearance of enzyme conjugate from non-tumour sites as quickly as possible. Recent modeling studies have confirmed these criteria as having the greatest influence on the resulting therapeutic index [6].

The schedule established by the first clinical trial confirmed that the use of SB43-gal as a clearing agent removed circulating CPG2 [14]. However, it was also recognised that CPG2 levels below those of the lower limits of the spectrophotmetric assay used were capable of activating prodrug to the toxic drug [20]. The dose of Ab-enzyme conjugate in this second trial was 10 000 U/m^2. In the previous trial the earlier patients had received 20,000 U/m^2 and the later patients, 10 000 or 5,000 U/m^2. The dose of SB43-gal infused over 4 days in the present trial was 46.5 mg/m^2 as compared with 240 mg/m^2 for the earlier patients and 50 mg/m^2 for the later patients in the previous trial. The HPLC assay developed for analysis of CPG2 in tissue and plasma has a detection limit 2,000× lower than that of the spectrophotometric assay previously used [5]. The use of this method to estimate CPG2 in plasma confirmed that the lower level of SB43-gal Ab currently used was efficient at clearing the circulating CPG2 conjugate prior to prodrug administration, leaving no detectable enzyme in the bloodstream. The CPG2 analyses from the metastatic tumour and normal liver samples show clearly that the enzyme-Ab conjugate localises at the tumour and not in the normal liver (Fig. 6). However, no correlation could be made between these levels and the plasma prodrug levels of the patients from whom the biopsy samples had been taken.

The prodrug was scheduled for administration at five doses per day given 2 h apart for 3 days. Theoretically this would allow the concentration of prodrug to be maintained at greater than its K_m of 3 μM for at least 2 h after the prodrug dose to enable maximal conversion to drug at a K_{cat} rate of ~700 mol mol^{-1} s^{-1} [11]. During the first clinical trial [3, 5], groups of patients were given CMDA prodrug alone without prior conjugate administration. Peak concentrations of 33–111 μM were achieved and modeled to a single compartment with a first-order kinetic rate, and the biological half-life was calculated as 25–35 min [3] and 29.4 ± 4.8 min [5]. In the same trial, since plasma from only one patient receiving full ADEPT was studied in detail, no inter-patient comparison was possible. In that study, prodrug concentrations reached values of 30–80 μM at between 5 and 10 min, similar to those achieved after prodrug alone, but the data from only 1 day were plotted to 60 min when the level was 11 μM, with other days' data terminating at 30 min. Prodrug was maintained at higher levels on successive treatment days, but extrapolation would indicate 60-min values of < 3 μM on all but the last day of therapy.

Patients in the present study reached maximal prodrug concentrations at times similar to those reported for the first trial, although the mean plasma concentration was somewhat higher (Figs. 4, 5). This is mainly due to patients 1 and 2 (men) having unexplained prolonged high prodrug levels on their 1st day of therapy and patient 8 (woman) having initially high prodrug levels on the 1st and 3rd days. This considerable inter-patient variation is to be expected, given the advanced state of their disease. The few later prodrug analyses at time points of up to 4 h show prodrug levels falling to low levels, but prodrug is normally redosed at 120 min. This suggests that the residual prodrug, even if present below the desired level of 3 μ at 120 min, could accumulate, with the four subsequent prodrug administrations of each day resulting in the prodrug concentration being maintained above 3 μM for the remaining 8 h of therapy. The delayed maximal prodrug plasma concentrations

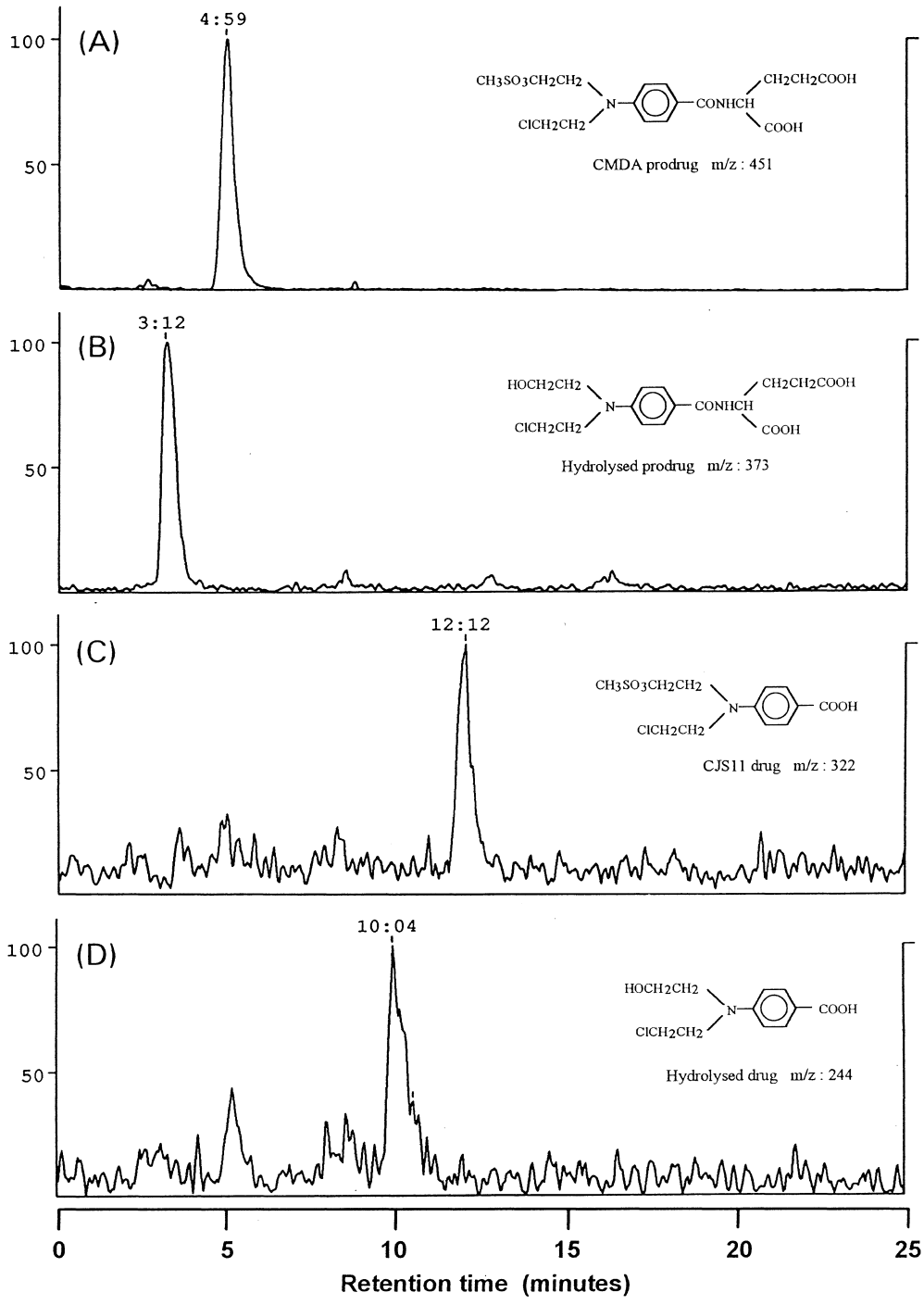

Fig. 7A–D Ion Chromatograms obtained from the LC-MS analysis of an extract of a plasma sample from patient 5, showing the presence of **A** the prodrug, **C** the drug and **B, D** their respective hydrolysed metabolites

observed in patient 8 (days 1 and 3 of therapy), patient 9 (cycle 2, day 3) and patient 10 (day 2) could have been due to slow localisation of the prodrug. However, as generation of the drug was rapid, this is unlikely to have been the case. Again, there is no overall correlation between patients or between the 3 days of their therapy.

The extracts prepared for HPLC by the newly developed method reported herein are amenable to LC-MS analysis. Prodrug was detectable in most of the extracts from plasma samples for up to 60 min but drug was observed only in the earlier (higher concentration) samples. The metabolites formed by hydrolysis of the mesyl group for both compounds and acetyl hydrolysed prodrug were also identified. All these compounds produced protonated molecular ions (Fig. 7), and the presence of the carbonium ions of both prodrug and drug (Fig. 8) was also noted. Thus, LC-MS is a powerful too for the identification of metabolites and confirmation of the prodrug/drug system.

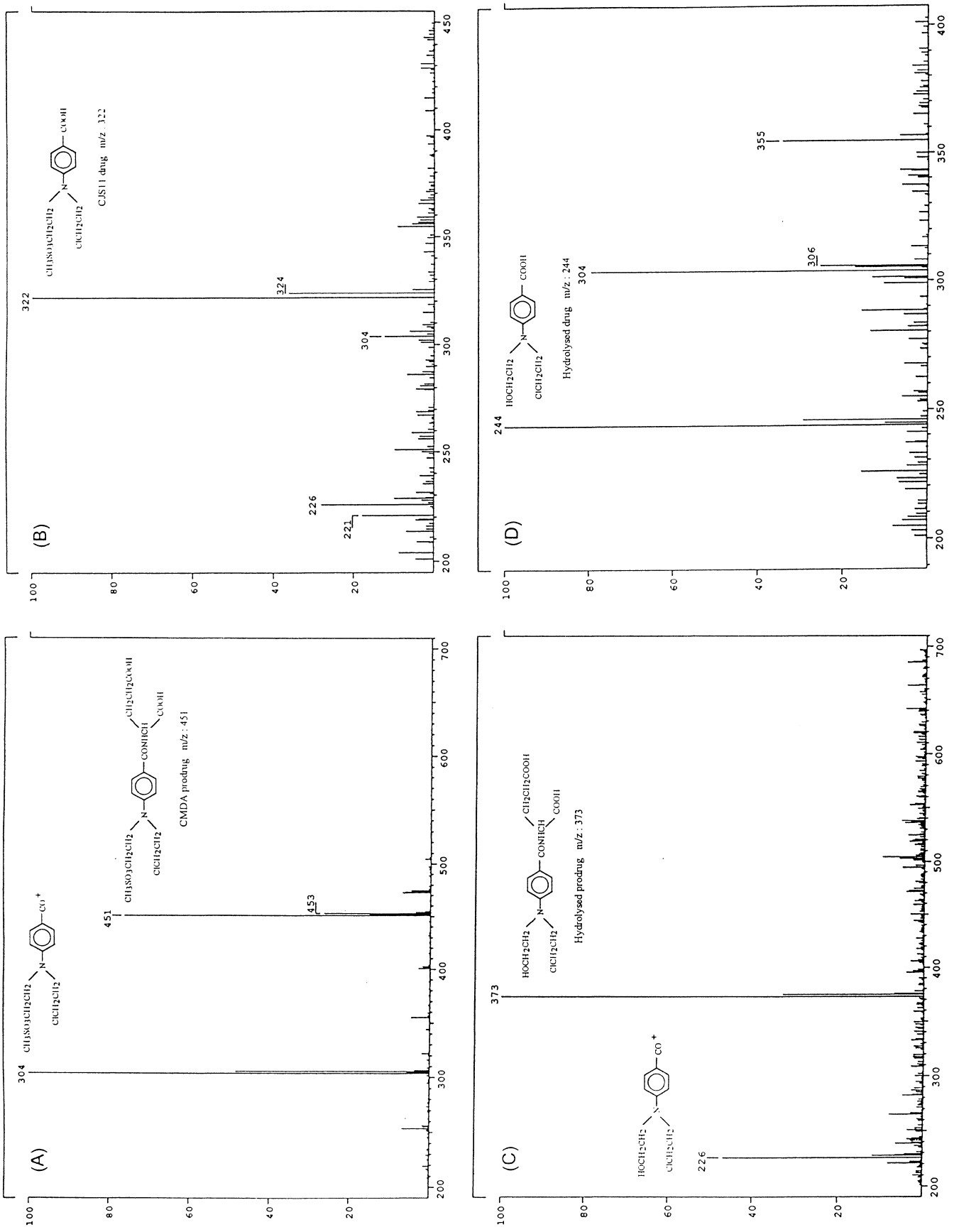

Fig. 8A–D Mass spectra of **A** the prodrug, **B** the drug and **C, D** their hydrolysis products, confirming their presence in a patient's plasma extract

The limited blood sampling and the time taken for administration of the prodrug invalidate the values calculated for $t_{1/2}\alpha$. However, as the α-phase has little effect on the AUC, clearance can be reliably calculated. The chemical half-life of the prodrug is 984 min [18], but the biological half-life of approximately 30 min, as determined in the previous trial from administration of CMDA alone, is short enough to have some effect on the clearance of prodrug from the plasma during the sampling period. However, the shorter elimination half-life of 18 min calculated from a day's therapy in the earlier trial indicates additional clearance from the plasma. Extrapolation of the other days' data from this patient would indicate a much shorter $t_{1/2}$. In the present trial the range of half-life values is wider, although values are very consistent when calculated in a non-compartmental or one-compartment model. The half-life for the β-phase was also computed in a two-compartment model for five patients. Although different models were employed and some of the data would not model, $t_{1/2}$ values are remarkably reproducible in this trial with a mean value of 18 ± 8 min ($n = 21$). The lower value obtained for the elimination as compared with the biological half-life of the prodrug, combined with the presence of the drug, confirms that conversion has taken place. The relationship between the maximal prodrug concentrations and the elimination half-life is not constant between patients but is less variable between the 3 days of therapy for an individual patient. Clearance is again variable and cannot always be reliably calculated, and a low figure such as the 31 ml/min recorded for patient 7 (day 1) could be due to the erratic plasma prodrug concentrations measured.

Drug concentrations in the bloodstream should ideally be as low as possible to exclude possible peripheral tissue toxicity, but the drug is in a more dynamic state than the prodrug. It is being continually released from the prodrug by the tumour-localised Ab-enzyme conjugate, is removed by uptake to the tumour and, possibly, other peripheral tissues and is also being excreted. For the duration that the drug has been measured in plasma, it can also undergo chemical reaction (half-life 58 min) to metabonates [19]. Plasma drug concentrations of 1.9–50 μM recorded at time points of between 5 and 10 min in the earlier trial proved similar to those noted for most patients in the present trial. In the previous trial, whereas plasma prodrug was estimated beyond 30 min only on the last day of therapy, drug levels were analysed to 90 min on each day. At 60 min, levels of 1.5–3.0 μM were similar to the concentrations found currently. The prodrug levels recorded for the one earlier patient studied were at maxima at 5 min after prodrug administration, but the drug peaked later on 2 of the 4 days.

In the present trial the drug profiles follow those of the prodrug more closely, suggesting rapid prodrug localisation and efficient and constant conversion to prodrug to drug. The exceptions in the current trial are patient 5 (cycle 2, day 1), in whom the drug level is higher than the prodrug concentration after 40 min and patient 9 (cycle 2, day 2), in whom the drug does not peak until 20 min although the prodrug concentration is maximal at 5 min. Some very high drug plasma values are listed for patients 1, 2 and 8, which might suggest that the prodrug is in the plasma rather than at the localised conjugate. However, as we demonstrated in all patients that there was no CPG2 in the plasma and yet the drug had been generated, we conclude that substantial conversion had taken place in these cases and that all plasma drug came from leak-back from the tumour (or other tissues).

For all patients whose drug profiles follow those of the prodrug (Fig. 4), the ratios of prodrug to drug listed in Table 1 are similar at 10 min for each patient on the 3 days of their therapy, regardless of the maximal plasma levels achieved. This is also true when high levels occur for patients 1, 2 and 8. The rising values after minima calculated for patients 8 (days 2 and 3) and 10 (day 3) could again be due to erratic sampling, although the drug profile follows that of the prodrug. Drug values fall by at least 50% at between 10 and 60 min on every day of treatment, but the ratios decrease with time for each patient, indicating the presence of a relatively higher concentration of drug in the plasma. However, this would also reinforce the conclusion stated above that the prodrug is rapidly taken up by the conjugate. The drug would appear to have a longer elimination half-life of 36 ± 14 min ($n = 19$) in the present patients rather than the 12- to 25-min values cited for the previous trial [3, 5].

Comparison of the elimination half-life of prodrug and drug (Table 1) emphasises the more rapid clearance of prodrug from the blood, confirming its localisation to the conjugate. A higher ratio of prodrug to drug is desirable and persistence of the drug could signify a combination of its efficient generation but slow entry into the tumour. Like the ratios for half-life, the ratio of AUC measured at 60 min and infinity does not vary much between patients (Table 1). However, the lower prodrug/drug AUC ratio measured at infinity, although extrapolated, would confirm persistence of the drug. For more accurate estimation of all parameters, scheduling of blood sampling in future trials would benefit from being extended beyond 2 h, possibly after the last prodrug administration of the day. However, this must be balanced with the clinical concern of the total volume of blood that can be withdrawn from a patient.

Scheduling of the components of ADEPT in this clinical trial has thus produced many of the desired features required for success. The results reported herein show the potential of ADEPT to deliver a high concentration of prodrug to the tumour. A prodrug concentration of 3 μM is not always maintained for 2 h, but residual prodrug is likely to accumulate to achieve that concentration with the four subsequent administrations of each day. Although circulating drug plasma levels are higher than desired and do persist, they are always below the drug's 50% inhibitory concentration (IC_{50}) of 200 μM found in vitro [18], and we have shown from

analysis of the current clinical trial that the source of the drug is leak-back and not conversion from the prodrug in the bloodstream. Unfortunately, the small biopsy samples did not allow analysis of their prodrug and drug content to confirm that the necessary concentrations had been achieved. Prodrug/drug systems with shorter drug half-lives are therefore desirable, and recognition of this aspect has led to investigations at a pre-clinical level [7, 22]. This should result in lower drug plasma concentrations, thus further reducing the problem of peripheral toxicity and improving the efficacy of the therapy.

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