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Biodistribution of an antibody-enzyme conjugate for antibody-directed enzyme prodrug therapy in nude mice bearing a human colon adenocarcinoma xenograft

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Abstract The enzyme carboxypeptidase G2 (CPG2) can be targeted to tumors by antibodies and used to activate prodrugs in a treatment called antibody-directed enzyme prodrug therapy (ADEPT). Different doses of CPG2 conjugated to the anti-CEA antibody A5B7 were administered i.v. to nude mice bearing the LS174T human colon adenocarcinoma xenograft, and the biodistribution of conjugate activity 48 and 72 h later was determined using a novel high-performance liquid chromatography (HPLC) method. Conjugate doses of 2,500 and 625 U/kg gave tumor enzyme levels of 0.5–0.6 U/g. Lower doses of 300 and 150 U/kg gave tumor enzyme levels of 0.1–0.3 U/g. Intriguingly, the best tumor:blood ratio of conjugate activity at both 48 and 72 h was achieved after administration of the 625-U/kg dose, not the 2,500-U/kg dose. After 48 h this ratio was 3.8, whereas after 72 h the value was 5.5. This conjugate dose also gave the greatest tumor:tissue ratios in all other tissues examined. After 72 h the tumor:colon ratio was 105, whereas the tumor:kidney ratio was 36. In ADEPT, to obtain maximal tumor damage to LS174T xenografts in nude mice with minimal systemic toxicity using the A5B7-CPG2 conjugate, prodrug should therefore be administered at least 72 h after a conjugate dose of 625 U/kg.

Key words Biodistribution · Carboxypeptidase G2 · ADEPT · Mice · Xenograft

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Abbreviations *ADEPT* Antibody-directed enzyme prodrug therapy · *Ab* antibody *CJS11* 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoic acid ·*CMDA* 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoyl-L-glutamic acid · *DAMPA* 2,4-diamino-N^{10}-methylpteroic acid · *CEA* carcinoembryonic antigen · *MTX* methotrexate *PBS* phosphate-buffered saline · *DMSO* dimethyl sulfoxide · *TFA* trifluoroacetic acid · *MeOH* methanol

Introduction

One of the major problems with current cancer therapies is the lack of specificity of the treatment, leading to unwanted side effects in normal tissue such as the gut lining and bone marrow [6]. Thus, current research is focused on the development of more specific methods for the delivery of toxic compounds to cancer cells. One such approach involves the use of antibodies (Ab). These can be raised against a variety of human tumor-associated antigens and then conjugated to a toxic moiety; such conjugates have included drugs (e.g. methotrexate) [16], toxins (e.g. ricin A chain) [10, 15, 32] and radioisotopes (e.g. ^{131}I) [6]. However, there are problems associated with these approaches. For example, Abs may remain in circulation for long periods, resulting in non-specific retention in normal tissues and thus leading to non-specific tissue damage. This is especially true for radioimmunoconjugates, where systemic exposure to the radioisotope may greatly limit the dose of radiation that can be given [21]. A second problem is one of heterogeneity in the expression of the tumor-associated antigen. Most tumors will contain a proportion of cells showing little or no expression of the target antigen [7, 22]. As a consequence, their destruction will depend upon bystander effects; these, in turn, are hampered by the ability of a conjugate to extravasate and diffuse through the tumor [8, 12–14].

Thus, to overcome these problems an alternative approach has been described [2, 20, 24]. In this approach

a non-toxic enzyme is conjugated to the Ab and allowed to localize to the tumor. Once this conjugate has been cleared from the circulation and normal tissues, a non-toxic prodrug is administered systemically. The prodrug is designed such that only the exogenously delivered enzyme can convert it into a toxic drug, which can then kill the tumor cells. This two-step system has been named antibody-directed enzyme prodrug therapy (ADEPT) and is illustrated in Fig. 1. The toxic drug generated by ADEPT is a small molecule that can readily diffuse within the tumor mass and, hence, reach both antigen-positive and antigen-negative tumor cells. Systemic toxicity should be avoided, as the drug is generated only at the site of the tumor by the pre-localized enzyme.

A variety of enzyme-prodrug systems has been investigated. Examples include β-glucuronidase with epirubicin glucuronide [11], cytosine deaminase with 5-fluorocytosine [23, 31] and carboxypeptidase G2 (CPG2) with various mustard prodrugs [3, 27, 28]. CPG2 is isolated from *Pseudomonas* and has no mammalian homologue [26]. It has been conjugated to the $F(ab')_2$ fragments of various Abs and used in combination with the prodrug 4-[(2-chloroethy)(2-mesyloxyethyl)amino] benzoyl-L-glutamic acid (CMDA, Fig. 2A). In various xenograft systems the conjugate has been shown to activate a range of different prodrugs, resulting in tumor cell destruction; examples include choriocarcinoma [29], and breast [9] and colorectal carcinoma [25] studies. An anti-carcinoembryonic antigen (CEA) antibody, A5B7, has been conjugated to CPG2 and used in a pilot-scale clinical trial of ADEPT [4]. Repeat doses have been successfully administered to patients receiving concomitant treatment with cyclosporin. The dose of conjugate given to each patient was determined by extrapolation from experiments where nude mice carrying the LS174T tumor xenograft received 1,250–2,500 U/kg. Patients were thus given doses in the range of 5,000–20 000 U/m^2.

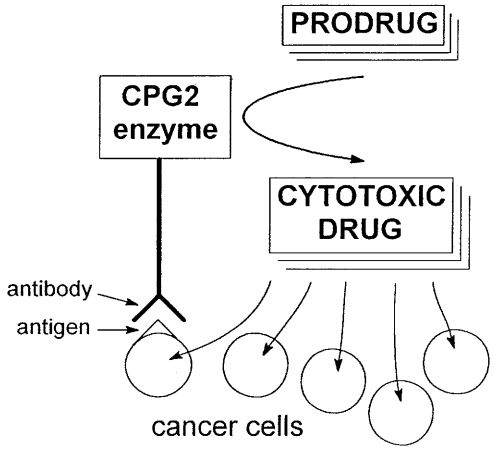


Fig. 1 Schematic representation of ADEPT. The Ab-CPG2 conjugate localizes to the tumor and the enzyme converts the subsequently delivered prodrug to a toxic drug that diffuses into the tumor and destroys tumor cells

To enable the analysis of CPG2 from very small sections of biopsy material (typically 5–10 μg) from patients, we required an extremely sensitive assay. As a model system we thus developed a novel assay that measures CPG2 activity in tumor, blood and other tissues to examine the biodistribution of CPG2 conjugate activity in nude mice bearing the LS174T xenograft. This assay is much more sensitive (approximately 20 000-fold) than the previously used spectroscopy method [19], with a detection limit of 10^{-6} U/ml reaction mixture. It is also more sensitive than a recently developed high-performance liquid chromatography (HPLC) method [5]. Previous in vivo therapy experiments have used a $[F(ab')_2]$-CPG2 conjugate dose of 2,500 U/kg [9, 25, 29] in combination with the CMDA prodrug. We describe herein that the conjugate dose providing optimal tumor targeting is 625 U/kg, rather than the highest dose of 2,500 U/kg, and that CMDA can safely be given after 48 or 72 h as the blood conjugate activity is below the threshold required for non-specific toxicity.

Materials and methods

Materials

The prodrug 4-[(2-chloroethyl)(2 mesyloxyethyl) amino] benzoyl-L-glutamic acid (CMDA) was synthesized as described previously [18]. The A5B7[$F(ab')_2$]-CPG2 conjugate was a gift from the Centre for Applied Microbiological Research (Porton Down, UK); A5B7 is a monoclonal anti-CEA antibody raised in mice that is used clinically for the radioimmunotherapy of metastatic colorectal tumors [17]. The conjugate had a CPG2 activity of 129.5 U/mg; 1 U of activity is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol methotrexate (MTX) min^{-1} ml^{-1} of reaction mixture [26]. MTX (25 mg/ml in 0.49% sodium chloride solution) was obtained from Lagap Pharmaceuticals Ltd. (Hants, UK). All chemicals and reagents were obtained from the Sigma Chemical Company (Poole, Dorset, UK) unless otherwise indicated.

Animals

Female nu/nu mice (18–22 g) were kept in a holding room maintained at 23 ± 2 °C on a 12-h light/dark cycle. They were maintained in accordance with current Home Office guidelines and received tap water and rat chow ad libitum.

Xenografts

A human CEA-producing colon adenocarcinoma cell line, LS174T [30], was used to develop a xenograft model in female nu/nu mice by s.c. cell inoculation into the flank. Subsequent passaging was accomplished by continuous s.c. implantation of 1-mm^3 xenograft fragments. When these tumors reached a weight of 0.5–1 g, the mice were randomized into control and experimental groups.

Analysis of CPG2 conjugate activity in biological samples

CPG2 cleaves MTX to give the metabolite 2,4-diamino-N^{10}methylpteroic acid (DAMPA) [1] (Fig. 2B). An indirect HPLC assay was developed to measure the concentration of DAMPA generated by CPG2. Each mouse tissue or tumor sample was washed, dried

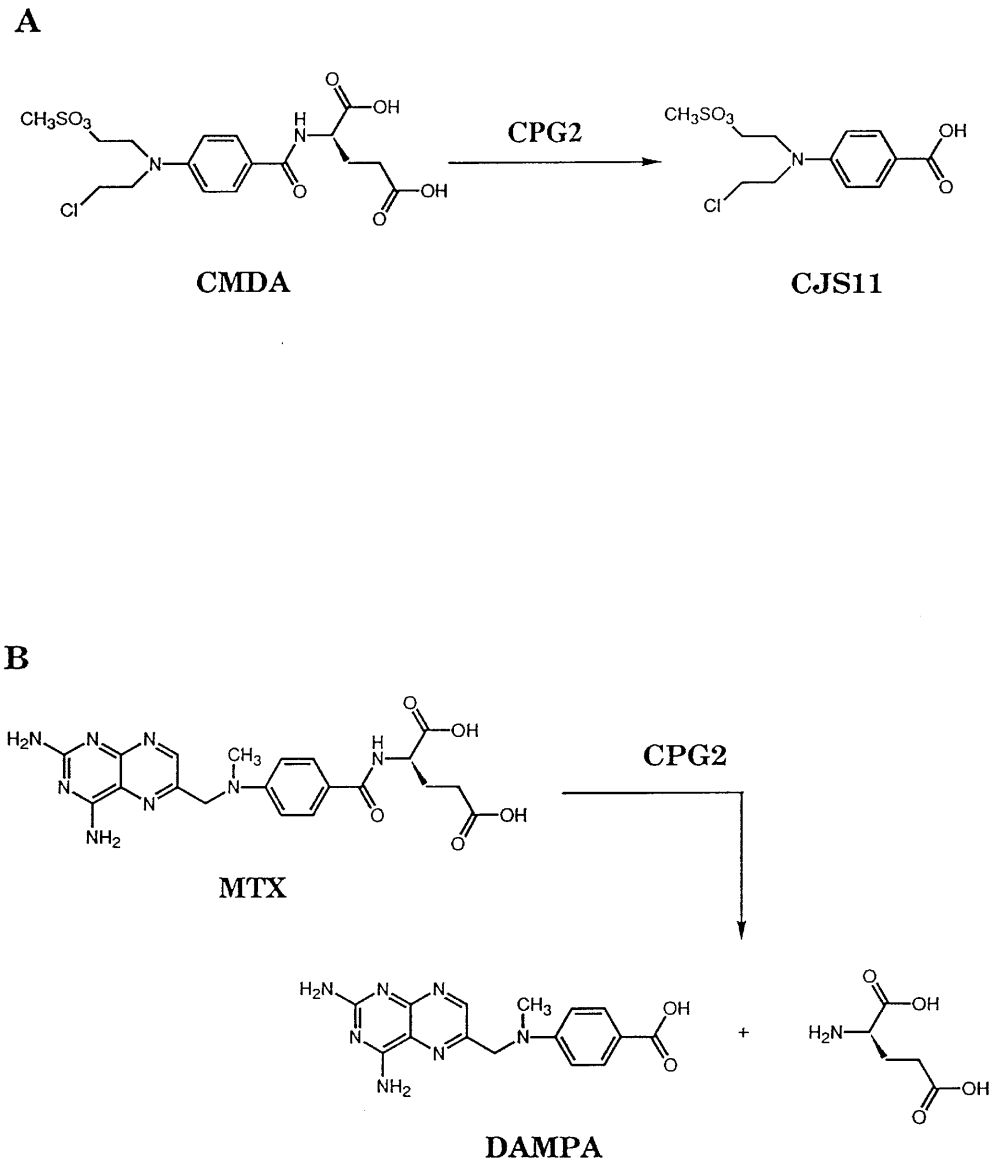


Fig. 2 **A** Structure of the prodrug CMDA and its product 4-[(2-chloroethyl)(2-(mesyloxyethyl)amino] benzoic acid (*CJS11*). **B** Structure of MTX and its metabolite DAMPA; both products are formed by CPG2-catalyzed hydrolysis

and weighed. It was then homogenized in phosphate-buffered saline (PBS) containing 0.2 m*M* $ZnCl_2$ (PBS/$ZnCl_2$) to obtain a 1% (w/v) homogenate. For human tumor biopsy analyses the homogenate concentration was lower due to the small quantity of sample available. An aliquot (600 µl) of homogenate was incubated in a shaking water bath (30 °C, 30 min) with MTX solution (10 m*M* MTX in DMSO, 6 µl). The reaction was terminated by mixing of an aliquot (200 µl) of reaction mixture with the "stop" solution [ice-cold MeOH + 0.1% (v/v) TFA, 500 µl). The mixture was centrifuged (4,000 *g*, 4 min) and the supernatant fraction, removed. Blood was treated in a similar manner except that an aliquot (25 µl) was mixed with PBS/$ZnCl_2$ (575 µl).

Indirect CPG2 analysis was performed by HPLC using a P4000 quaternary pump, an AS 3000 autosampler and a SpectroFOCUS high-resolution scanning detector set to 307 nm (Thermo-Separation Products, Stone, Staffs, UK). The metabolite DAMPA was eluted isocratically from a 150 × 4.6-mm Spherisorb S5SCX column (Hichrom Ltd., Berks., UK) using a mobile phase of methanol/0.06 *M* ammonium formate/TFA (70%/29.9%/0.1%, v/v) at a flow rate of 1 ml/min. The CPG2 activity in each tissue was quantitated by comparison of the DAMPA peak area with a previously determined standard curve of DAMPA peak area versus enzyme activity. Each standard curve was constructed using the corresponding organ taken from untreated mice. These standard curves showed some inter-organ variation due to the nature of the homogenate and the subsequent ease of extraction of the DAMPA but were reproducible, with $r \geq 0.98$.

Distribution of CPG2 conjugate activity

Groups of three to seven tumor-bearing nude mice were given varying i.v. doses of the A5B7[$F(ab')_2$]-CPG2 conjugate as follows: group 1,2500 U/kg; group 2, 625 U/kg; group 3, 300 U/kg and group 4,150 U/kg.

Statistical analysis

Where appropriate, statistical significance was evaluated using Student's two-tailed unpaired *t*-test; a *P* value of ≤ 0.05 was considered significant. After 48 and 72 h the mice in each group were culled and tumor, blood and major organs (liver, kidneys, spleen, lung and colon) were removed for analysis of enzyme activity as described above.

Results

In this study, groups of nude mice bearing the LS174T human colon adenocarcinoma xenograft were given varying doses of the A5B7[F(ab′)$_2$]-CPG2 conjugate. The conjugate activity in the tumor, blood and various tissues at 48 and 72 h after each conjugate dose was determined indirectly by HPLC, and the results are illustrated in Fig. 3. After all but the lowest dose and at both time points examined, conjugate activity was higher in the tumor than in the blood or remaining tissues. When conjugate was administered at 150 U/kg the activity at the tumor was 0.1–0.2 U/g tumor (Fig. 3A,E); when the dose was 300 U/kg this activity increased to 0.2–0.3 U/g tumor (Fig. 3B,F). However, regardless of whether the conjugate was given at doses of 625 or 2,500 U/kg, tumor activity reached similar values of 0.5–0.6 U/g (Fig. 3C,G,D,H). Conjugate activity in the remaining tissues was greater at 48 h than at 72 h after all of the doses used. This activity was particularly marked after the highest dose (2,500 U/kg) and decreased thereafter in a dose-dependent manner.

The ratio of conjugate activity detected in the tumor to that found in the blood after each conjugate dose and at both time points is illustrated in Fig. 4. A conjugate dose of 150 U/kg gave tumor:blood ratios of 1.3 and 1.2 after 48 and 72 h, respectively, whereas a dose of 300 U/kg gave values of 2.3 and 1.7, respectively. Conjugate doses of 625 and 2,500 U/kg produced higher ratio values; at 625 U/kg the value was 3.8 after 48 h and 5.5 after 72 h; this difference was found to be just significant ($P = 0.05$). The corresponding values recorded for a dose of 2,500 U/kg were 3.5 after 48 h and 3.7 after 72 h.

The ratio of conjugate activity detected in the tumor to that found in each of the remaining tissues at 48 and 72 h after each conjugate dose is illustrated in Fig. 5. The tumor:tissue ratio obtained after a dose of 625 U/kg increased with increasing time from 48 to 72 h due to conjugate clearance from the normal tissues and conjugate retention by the tumor.

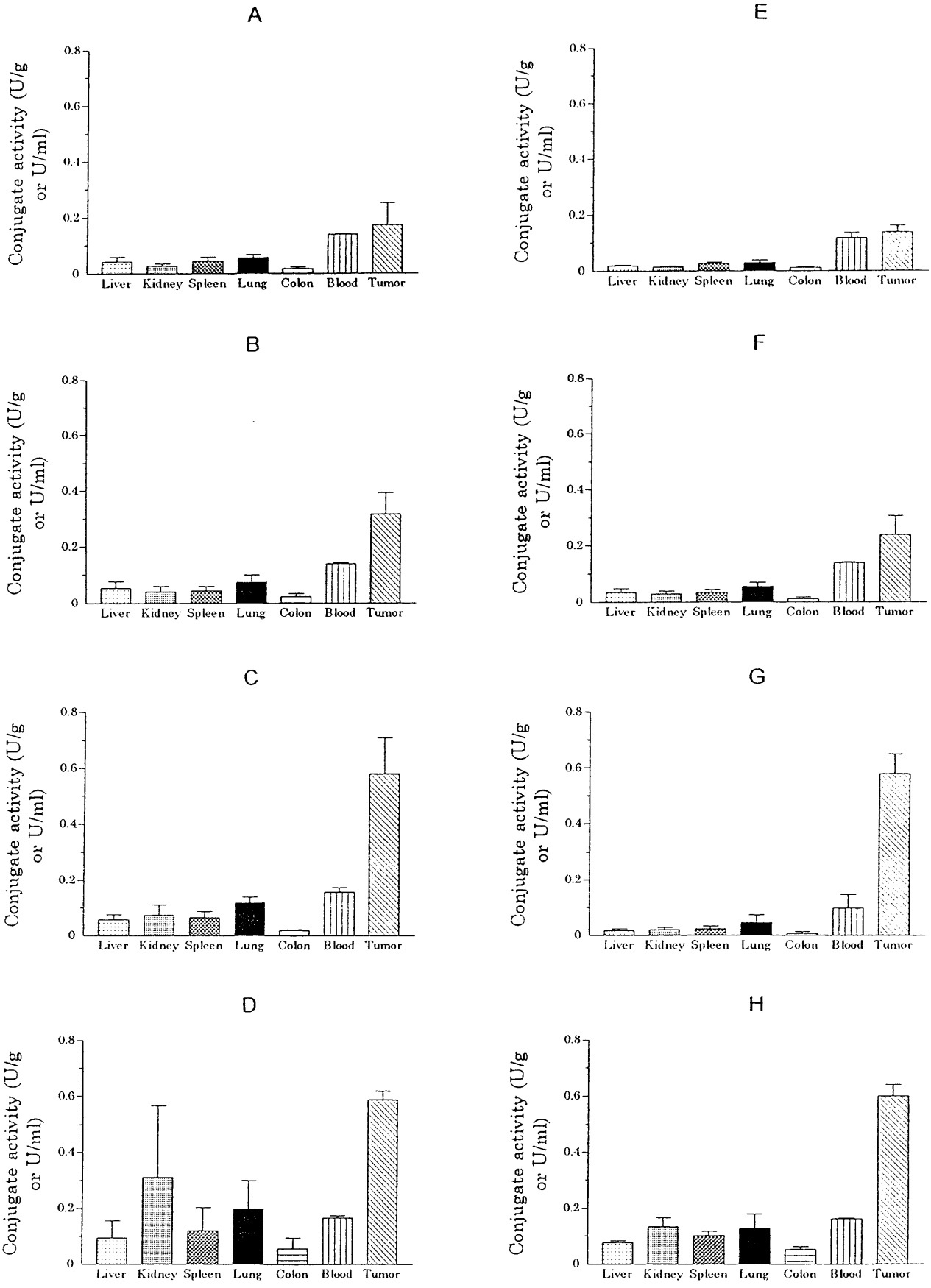
Discussion

Cancer treatment with ADEPT involves two distinct steps: the targeting of an Ab-enzyme conjugate to the tumor, followed by the administration of a non-toxic prodrug that the tumor-bound enzyme can cleave to produce a cytotoxic drug. Clearly, the efficacy of the second step depends on that of the first. The establishment of a high concentration of conjugate at the tumor will enhance the potential for production of the cytotoxic drug and, hence, potentiate tumor destruction. To investigate conjugate activity in the tumor, blood and non-tumor tissues we developed an extremely sensitive assay to measure this activity. MTX has, in common with the prodrug CMDA, a glutamate moiety (Fig. 2). Removal of this by CPG2-catalysed hydrolysis produces a metabolite, DAMPA, the measurement of which can be used to determine indirectly conjugate activity. Previously, DAMPA has been detected by the change in ultraviolet absorbance at 320 nm effected by the action of CPG2 on MTX; this has given useful results but is not a sensitive technique, with the limit of detection being 0.02 U/ml [19]. The HPLC method described herein is on the order of 20 000 times more sensitive. The lowest standard used for each standard curve, 10^{-4} U/ml, gave a reproducible DAMPA peak of 40 000 absorbance units. However, the HPLC system used in our study can detect peak areas of ~400 absorbance units and can therefore be used to detect peak areas produced by an enzyme activity of 10^{-6} U/ml.

Tissue distribution of conjugate activity can now be measured accurately to very low levels and after much smaller doses of conjugate. Furthermore, conjugate activity in biopsy and plasma samples taken from patients undergoing ADEPT can now be determined by this method, giving a much more accurate picture of ADEPT in the patient. The distribution of the conjugate has been determined following radioiodination of the conjugate [4]. Although this shows Ab distribution, it does not indicate whether the Ab has remained conjugated to the enzyme or whether the enzyme has remained active. The assay reported herein determines distribution by active enzyme activity, which is more informative and more relevant for ADEPT.

The conjugate assay was used in the present study to determine the optimal dose of conjugate for ADEPT and to examine the clearance of conjugate activity from the blood and non-tumor tissues. In the process, an optimal scheduling for prodrug administration was determined. Nude mice bearing the LS174T human colon adenocarcinoma xenograft received varying i.v. doses of conjugate: 2,500, 625, 300 or 150 U/kg. The mice were culled after 48 and 72 h, and conjugate activity in the tumor, blood and major non-tumor tissues was determined (Fig. 3). Although the enzyme was conjugated to a F(ab′)$_2$ monoclonal antibody fragment, enzyme activity could be measured in all tissues examined at both 48 and 72 h after all doses. This activity is important, as any non-specific toxicity may be due to hydrolysis of the prodrug to the toxic drug at sites other than the tumor. Activity in non-tumor tissues was greatest at both time points after a dose of 2,500 U/kg. This activity was

Fig. 3A–H Biodistribution of A5B7[F(ab′)$_2$]-CPG2 conjugate activity in nude mice. Groups of 3–7 mice received varying i.v. doses of conjugate: 150 U/kg (**A,E**), 300 U/kg (**B,F**), 625 U/kg (**C,G**) or 2,500 U/kg (**D,H**). At 48 (**A–D**) and 72 h (**E–H**) the animals were killed and their tissues, excised and enzyme conjugate activity was determined indirectly by HPLC. Data represent mean values. *Bars* SD


A
B
C
D
E
F
G
H
Conjugate activity (U/g or U/ml)
0
0.2
0.4
0.6
0.8
Liver
Kidney
Spleen
Lung
Colon
Blood
Tumor

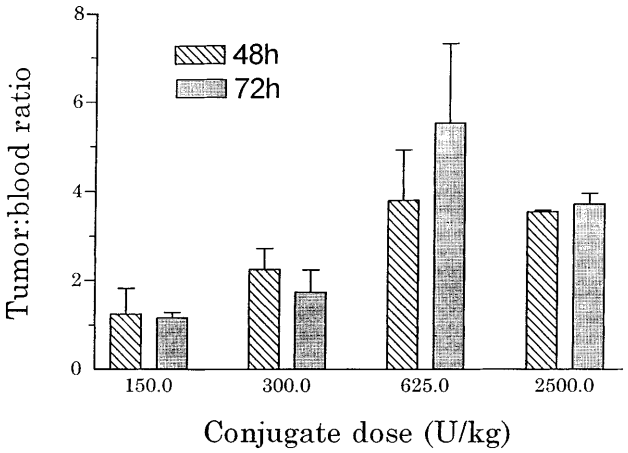


Fig. 4 Tumor:blood ratios determined for nude mice bearing the LS174T human colon adenocarcinoma xenograft at 48 and 72 h after administration of varying i.v. doses of A5B7[F(ab′)$_2$]-CPG2 conjugate. Data represent mean values. *Bars* SD

particularly marked in the kidney and lung. At 48 h the mean activity value detected in the kidney was 0.31 U/g, whereas that found in the lung was 0.20 U/g. At 72 h the corresponding values were 0.13 U/g for each tissue. It appears that by 48 h after injection the conjugate has cleared from the blood to a similar extent for all doses, and there is a steady, if slight, dose-dependent increase in enzyme levels in normal tissues, where clearance is slower. Within the tumor, where there is specific binding of the conjugate, there is a proportionally greater dose-dependent rise in activity as compared with normal tissues until the level of antigen saturation appears to be reached (625 U/kg). The relatively large rise in activity noted for several of the normal tissues as the dose was increased from 625 to 2,500 U/kg probably reflects the problem of clearing such a large concentration of antibody (0.5 mg) from the body.

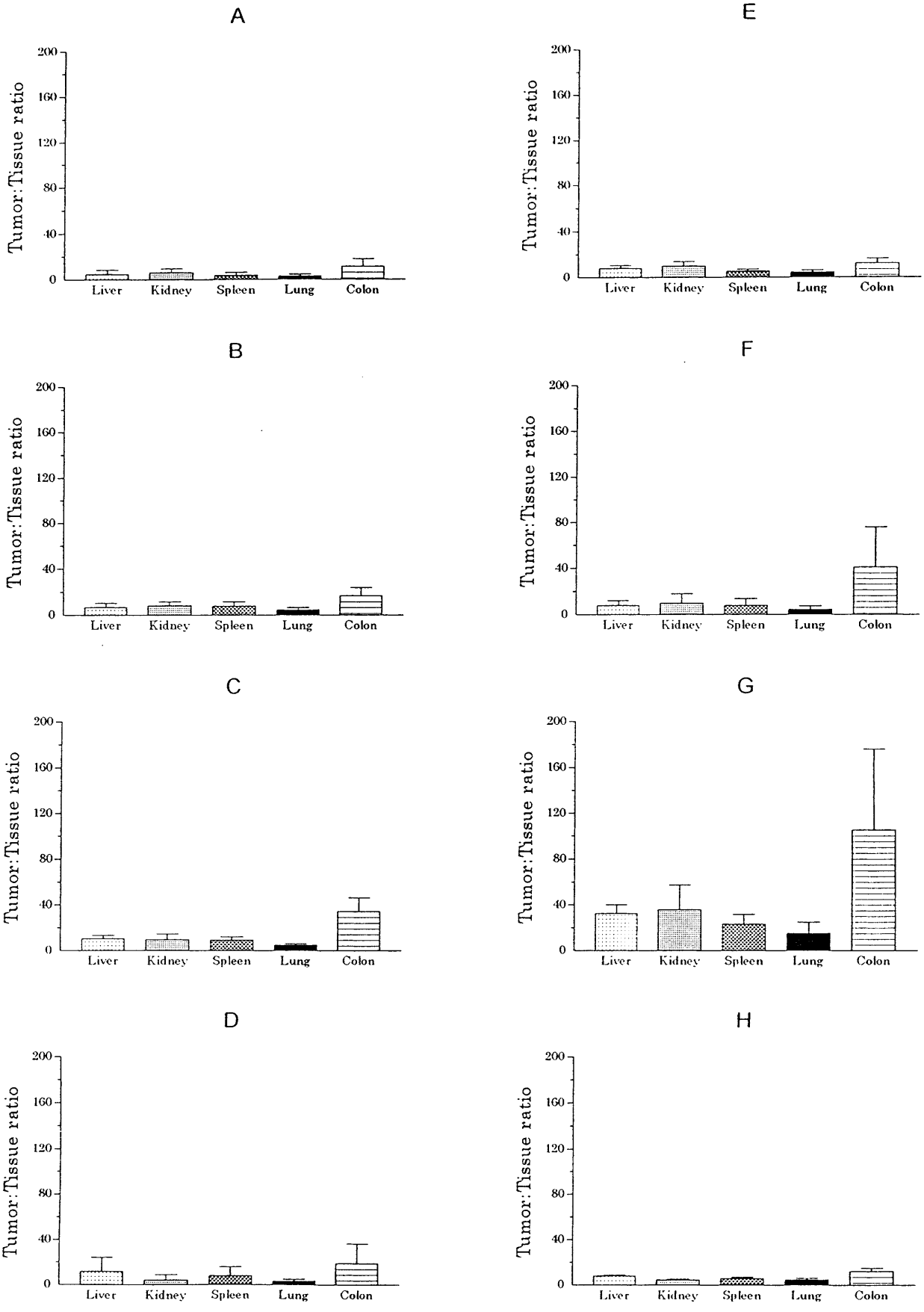
In the tumor the largest conjugate dose of 2,500 U/kg was chosen because it had been shown to cause tumor cell destruction when used in conjunction with the prodrug CMDA [25]. However, we found that the conjugate activity of the tumor at 48 and 72 h after a dose of 2,500 or 625 U/kg was the same. Intriguingly, the administration of 4-fold lower amounts of conjugate to the animal results in the same conjugate activity in the tumor. This is possibly due to saturation of the antigenic sites in the tumor by the Ab of the conjugate at the 625 U/kg dose. The LS174T tumor has a total CEA content ranging from 25 to 150 μg/0.5 g tumor as calculated from radioimmunoassay studies (G.M. Boxer, personal communication). When a 25-g mouse is treated with 625 U/kg, the total dose is approximately 16 U. The specific activity of the conjugate is 129.5 U/mg; thus, the protein dose is 120 μg. Only a very small percentage of the CEA will be available for antibody targeting, as the conjugate does not diffuse far from the blood vessel. It is therefore feasible that an antibody dose of 120 μg will saturate the available antigen-binding sites, even when the tumor dose is 4% of the injected dose/g. Thus, the highest conjugate dose of 2,500 U/kg would lead to higher circulating concentrations of conjugate without yielding a concomitant improvement in tumor concentrations.

The current conjugate dose used for patient treatment has been determined by extrapolation from animal experiments [4]. Thus, extrapolating from the results obtained in the present study, it may be possible in future to treat ADEPT patients with a 4-fold lower dose of conjugate and yet effect tumor destruction.

The lower doses of conjugate (150 and 300 U/kg) gave tumor conjugate activities of 0.10–0.20 U/g at 48 h and 0.15–0.30 U/g after 72 h. Consequently, there may be a dose threshold below which the conjugate activity of the tumor decreases in a dose-dependent manner. This threshold effect is best seen by comparison of the tumor:blood conjugate activity ratios detected at 48 and 72 h after each dose (Fig. 4). After a dose of 150 U/kg the ratio at 48 and 72 h is approximately 1. This rises to approximately 2 after the 300-U/kg dose. After a dose of 625 U/kg the ratio is 3.7 at 48 h and 5.5 at 72 h, and after a dose of 2,500 U/kg it is 3.7 at both time points. Clearly, the most effective time for prodrug administration is when the ratio of conjugate activity is highest in the tumor as compared with the blood and non-tumor tissues. This timing avoids the generation of large amounts of toxic drug at sites other than the tumor. For blood this time would be 72 h after a conjugate dose of 625 U/kg. To determine the best time for the non-tumor tissues, one can consider the tumor:tissue conjugate activity ratios (Fig. 5). At both time points and after all doses, each of these ratios are greater than 1, showing the conjugate activity of the tumor to be greater than that of each normal tissue. The largest ratios occur at 72 h after a conjugate dose of 625 U/kg. For example, the values recorded for tumor:liver and tumor:kidney are approximately 40, whereas the value noted for tumor:colon is greater than 100. Thus, for the in vivo system described, the optimal time for prodrug administration is at least 72 h after conjugate administration.

In summary, we have developed a very sensitive assay for Ab-CPG2 conjugate activity. We used this assay to show that the optimal dose of A5B7[F(ab′)$_2$]-CPG2 conjugate for LS174T xenograft targeting in nude mice that protects the normal tissues is 625 U/kg and that the most favourable distribution of conjugate activity is achieved at at least 72 h after the i.v. administration of this dose.

Fig. 5 A–H Tumor:tissue ratios obtained from nude mice given varying i.v. doses of A5B7[F(ab′)$_2$]-CPG2 conjugate: 150 U/kg (**A,E**), 300 U/kg (**B,F**), 625 U/kg (**C,G**) or 2,500 U/kg (**D,H**); ratios were determined at 48 (**A–D**) and 72 h (**E–H**). Data represent mean values. *Bars* SD


A
B
C
D
E
F
G
H
Tumor:Tissue ratio
0
40
80
120
160
200
Liver
Kidney
Spleen
Lung
Colon

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