Pharmacokinetics and Biodistribution of the Antitumor Immunoconjugate, Cantuzumab Mertansine (huC242-DM1), and Its Two Components in Mice

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ABSTRACT

The humanized monoclonal antibody maytansinoid conjugate, cantuzumab mertansine (huC242-DM1) that contains on average three to four linked drug molecules per antibody molecule was evaluated in CD-1 mice for its pharmacokinetic behavior and tissue distribution, and the results were compared with those of the free antibody huC242. The pharmacokinetics in blood were similar for 125I-labeled conjugate and antibody with terminal half-lives of 154 and 156 h, respectively. Pharmacokinetic analysis using an enzyme-linked immunosorbent assay (ELISA) method, which measures intact conjugate in plasma samples revealed a faster clearance for the conjugate corresponding to a half-life of 42.2 h. This faster clearance is explained as the result of clearance from circulation and concomitant clearance of drug from circulating conjugate through linker cleavage. An antibody-specific ELISA allowed the determination of the clearance rate of the antibody component from circulation. The drug clearance rate from circulating conjugate was then calculated as the difference between the clearance of the conjugate and the clearance of the antibody component and found to be about three times that of the antibody component. The above results were confirmed with a conjugate, huC242-[3H]DM1, where the linked DM1 drugs carried a stable tritium label. Tissue distribution studies with 125I-labeled conjugate and antibody showed antibody-like behavior for the conjugate; the antibody of the conjugate did not distribute or bind significantly to any solid tissue.

The effectiveness of chemotherapeutic agents is limited by their intrinsic toxicity to most, if not all, tissues. One approach to ameliorate this problem is to change the in vivo distribution of the agent. Conceptually, conjugation of such agents to monoclonal antibodies that bind selectively to tumor tissues should not only alter the distribution in vivo through the large changes in the physicochemical characteristics of the agents but also through the selective retention of the conjugate by target tumor tissue. Antibody conjugates of the cytotoxic agent mertansine (DM1, a maytansinoid) have demonstrated exceptional antitumor activity in preclinical tumor models (Chari et al., 1992; Liu et al., 1996) and are currently in clinical evaluation for the treatment of several types of tumors; in particular, cantuzumab mertansine (huC242-DM1) is evaluated in patients with colorectal, pancreatic, and nonsmall cell lung cancer (Tolcher et al., 2003).

Cantuzumab mertansine is composed of the humanized monoclonal IgG1 antibody, huC242, and the maytansinoid drug mertansine or DM1. HuC242 is a resurfaced, humanized version (Roguska et al., 1994) of the murine monoclonal antibody C242, which had been generated against the human colon cancer cell line COLO 205 (Baeckström et al., 1991) and reacts with the tumor selective carbohydrate epitope CanAg present on the membrane-associated mucine MUC1. CanAg is strongly expressed in most cancers of the colon, pancreas, and stomach and on a large percentage of nonsmall lung cancers and is only detected weakly on some normal tissues of the gastrointestinal tract (Tolcher et al., 2003). Mertansine is a semisynthetic analog of the natural compound maytansine (Kupchan et al., 1972; see Fig. 1B) and is a potent antimicrotubule agent. On average, three to four DM1 molecules are linked to an antibody molecule via a short linker that contains a hindered disulfide bond and that forms an amide bond with an ε-amino group of a lysine residue of the antibody (see Fig. 1A). Mertansine is a small molecular weight cytotoxic agent (Mr = 737.5 Da), and the conjugation of four DM1 molecules to huC242 (Mr = 147 kDa) contributes

ABBREVIATIONS: huC242-DM1, cantuzumab mertansine; ELISA, enzyme-linked immunosorbent assay; AUC_{tot}, area under the time-concentration curve; CL, clearance; $V_{ss}$, volume of distribution at steady state; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; BSA, bovine serum albumin; %ID/g, percentage of the total injected dose per gram; R, organ-to-body weight ratio; ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).
only about 2% to the molecular mass of cantuzumab mertansine. Accordingly, the biochemical characteristics of the conjugate huC242-DM1, such as its chromatographic behavior and the solubility in aqueous media, are similar to those of the antibody huC242. The questions asked with preclinical in vivo studies are therefore, whether the antibody drug conjugate demonstrates pharmacokinetic and tissue distribution characteristics similar to those of the antibody. The question is not a trivial one since the antibody has been altered through the covalent linking of three to four lipophilic drug molecules, and animals have efficient mechanisms for the recognition and removal of altered or damaged proteins from circulation (Wright and Morrison, 1994; Seternes et al., 2002).

Here we report the pharmacokinetics and biodistribution in mice for cantuzumab mertansine and compare them with those of its components, the humanized antibody, cantuzumab (huC242) and the chemically stable DM1 analog maytansine (Kupchan et al., 1972), which lacks the free sulfhydryl group of mertansine (see Fig. 1B). The pharmacokinetic parameters were obtained with 125I-labeled samples, radioactivity measurements, and nonlabeled samples using specific ELISA methods. The drug release from the conjugate was further studied with radiolabeled conjugate where a 3H label had been introduced only into the drug component [3H]mertansine ([3H]DM1). The tissue distribution of cantuzumab mertansine was also studied in mice and compared with that of the unconjugated ("naked") antibody to evaluate whether the conjugation of three to four hydrophobic drug molecules influences the in vivo distribution of the IgG1 antibody.

**Materials and Methods**

**Animals.** Female CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) or Taconic Farms, Inc. (Germantown, NY) at 7 weeks old and were housed in lexan resin cages. All animals were allowed to acclimate for 7 days before any experimental procedure was performed. The animals showed no signs of disease or illness upon arrival or before administration of the test materials. The temperature of the animal rooms was maintained at 20–25°C and the humidity between 50 and 75% with a minimum of eight air changes per hour. Animals were kept on a 12-h light/dark cycle and had free access to food (Prolab 3000 obtained from Old Mother Hubbard, Lowell, MA) and water at all times. All animal studies
were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health.

Preparation of 123I-Labeled Samples. The antibody huC242 and the conjugate huC242-DM1 were radiolabeled with 123I using the Bolton-Hunter reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) and purified over a Dowex-1 column. Radiolabeling analysis of a SDS-polyacrylamide gel run under nonreducing conditions demonstrated that the radioactivity in both samples was associated with a single band at a position for intact antibody. Furthermore, 98.4% and 99.0% of the radioactivity was precipitable with trichloroacetic acid in the conjugate and in the antibody sample, respectively. Both samples were formulated in phosphate-buffered saline (PBS) at a concentration of 1.01 mg/ml. The specific radioactivity of the 123I antibody and the 123I conjugate were 5.09 × 10^6 and 2.73 × 10^6 cpm/mg, respectively. The ratio of DM1 molecules linked per antibody molecule in the conjugate was 4.07 DM1/huC242.

Synthesis of [3H]DM1 and Preparation of huC242-[3H]DM1. [3H]Ansamitocin P-3 was synthesized by the method similar to that previously described (Sawada et al., 1993) for [14C]ansamitocin P-3, by substituting [3H]-methyl iodide for [14C]-methyl iodide. Reductive cleavage of the ester group of [3H]ansamitocin P-3 with lithium trimethylsilylammonium hydride in tetrahydrofuran at −40°C provided [3H]maytansinol. Maytansinol was acylated with N-methyl-N-3-(methylthio)-1-oxopropyl)-1-alanine in the presence of dicyclohexylcarbodiimide and zinc chloride in dichloromethane as described previously (Kawai et al., 1984). The disulfide bond in the resulting maytansinoid was reduced by treatment with diithiothreitol, and the resulting [3H]DM1 was purified by high-performance liquid chromatography using a C18 column in the reverse phase mode eluting with a gradient of water-acetonitrile. The preparation of huC242-[3H]DM1 followed the method described previously by us (Chari et al., 1992; Liu et al., 1996) and yielded a conjugate with 3.0 drug molecules linked per antibody molecule and a specific radioactivity for the conjugate of 1.72 × 10^6 cpm/μmol.

Pharmacokinetic Studies. Female CD-1 mice were grouped randomly based on their body weights. The test materials were administered intravenously via a lateral tail vein using a 27 gauge, 0.5-inch long needle. Blood samples were withdrawn from anesthetized animals [inhalation of isoflurane (Aerrane); Baxter Healthcare Co., Deerfield, IL] with glass capillary tubes from the retro-orbital blood plexus. Radioactivity of samples was determined with a LKB 1272 Clini gamma counter (Amersham Biosciences, Uppsala, Sweden) for 123I and a RackBeta 1209 Liquid Scintillation Counter (Amersham Biosciences) for 3H. Pharmacokinetic data analysis was performed using the standard algorithms of the noncompartmental analysis program WinNonlin Standard, version 3.1 (201 for plasma, serum, and blood cell lysate data and 211 for urine data; Pharsight, Mountain View, CA) (Gabrielsson and Weiner, 1997). The program calculates the area under the time-concentration curve (AUC(tot)) using the linear trapezoidal rule with an end correction. It assumes a noncompartmental model and estimates the clearance (CL) as dose (D) divided by AUC(tot) and the volume of distribution at steady state (Vss) as the product of CL and mean residence time (MRT). The kinetic rate constant, k12, was obtained from the slope of the linear part of the time-log concentration curve. The half-life t1/2 was calculated with the relationship t1/2 = ln 2/k12 for first order processes. Cmax is the highest concentration measured in vivo in the compartment indexed in Table 123I-huC242 Antibody and 123I-huC242-DM1 Conjugate. Eighteen CD-1 mice were separated randomly into two sets: one set of nine mice (body weight 23.4 ± 0.7 g, mean ± S.D.) was used for assessing the blood clearance of 123I-huC242 antibody, and each mouse received 0.1 ml of a solution of 123I-huC242 corresponding to 0.101 mg (0.69 nmol) of antibody and 5.14 × 10^6 cpm (specific radioactivity, 7.45 × 10^5 cpm/nmol). Mice in the second set (body weight 24.3 ± 0.7 g, mean ± S.D.) received the equivalent amount of 123I-huC242-DM1 conjugate (0.101 mg) in the same volume (0.1 ml) of PBS and a radioactivity amount of 2.76 × 10^6 cpm per mouse (specific radioactivity: 4.0 × 10^6 cpm/nmol, 4.07 DM1/antibody). At 2 and 30 min, 2, 11.5, and 20 h, and 2, 3, 5, 2, and 7 days after administration of the test articles, 75 μl of blood was collected from heparinized capillary tubes from three mice in each set, therefore ensuring that each animal was bled only three times to generate data for the nine time points indicated. One milliliter of a 12.5% aqueous solution of trichloracetic acid (TCA, v/v) was added to 50 μl of mouse blood sample. The samples were kept on ice for 30 min and then centrifuged to separate the supernatant from the pellet containing the precipitated protein. The pellet and 100 μl of the supernatant were counted separately for radioactivity.

Pharmacokinetic Studies with huC242-DM1 Conjugate Using ELISA Methods. Antibody- and conjugate-specific ELISA techniques were used to measure the concentrations of antibody and conjugate in the same samples of plasma from CD-1 mice administered with the conjugate. Each of nine CD-1 mice (body weight 23.4 ± 1.1 g, mean ± S.D.) received 0.32 ml of a huC242-DM1 solution in PBS containing 0.234 mg of conjugate (30 mg/kg conjugate containing 160 μg/kg linked DM1; 3.2 DM1 molecules linked per antibody molecule). At 2 and 30 min, 1, 4, and 8 h, and 1, 2, 3, 5, and 8 days after administration of huC242-DM1, 75 μl of blood was collected from three mice into heparinized capillary tubes. This assured that not more than two blood samples were withdrawn from an animal on the same day. The plasma (35 μl) was separated by centrifugation and stored at −80°C until used to measure huC242-DM1 and huC242 concentrations by ELISAs. The ELISA methods were adopted from those reported previously by us (Shah et al., 1993). The concentration of intact conjugate was determined as follows: plates were coated with (100 μl/well) a 1 μg/ml solution of murine antimaytansinoid monoclonal antibody (ImmuNoGen, Inc., Cambridge, MA) in PBS. The plates were incubated overnight and then blocked with 1% casein in Tris-buffered saline (TBS) (Sigma-Aldrich, St. Louis, MO). A set of huC242-DM1 standards in the concentration range of 3.9 to 1000 ng/ml was prepared in PBS containing 2% mouse serum along with appropriate dilutions of the test plasma samples. Fifty microliters of standard sample or internal control sample diluted to fall into the linear range of the standard curve (15.6–250 ng/ml) were added to the plates and then incubated at 37°C for 30 min. Reagents were removed and the plates were washed and blotted dry. Horseradish peroxidase-labeled donkey anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was diluted to 1:5000 in casein–blocking buffer. A volume of 100 μl/well was added to the plates, which were then incubated at room temperature for 1 h. Unbound secondary antibody was washed away repeatedly and the plates were coated with (100 μl/well) a 0.5 μg/ml solution of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) in PBS overnight at 4°C. The plates were incubated overnight and then blocked with 1% casein in Tris-buffered saline (TBS) (Sigma-Aldrich, St. Louis, MO) in 0.1 M citrate buffer, pH 4.0, with 0.03% hydrogen peroxide. The plates were read at 405 nm in a BioTek plate reader (Bio-Tek Instruments, Winooski, VT), and the data were analyzed using the four-parameter curve-fitting program, KC4 (Bio-Tek Instruments). The acceptance criteria for assay plates included standards, which fell in the linear portion of the standard curve, a coefficient of variability (%CV) of ≤10%, and readings for internal controls that fell within ±10% of their expected values. For each sample dilution, the mean of the duplicates was used to determine the concentration of huC242-DM1. The lower limit of quantification was about 15.6 ng/ml in the 2% serum matrix.

The ELISA for the determination of the concentration of huC242 antibody component of the conjugate was as follows: plates were coated with (100 μl/well) a 0.5 μg/ml solution of goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc.) in PBS overnight at 4°C. The plates were blocked with 1% casein in TBS at 37°C for 1 h. Appropriate dilutions of the plasma samples were made in 2% mouse serum. Fifty microliters of each sample dilution were added in duplicate to the plates and then incubated at 37°C for 1 h. A standard curve of huC242-DM1 in the concentration range of 3.13 to 800 ng/ml was run in triplicate, and a linear range of 12.5 to 200 ng/ml was established. The plates were washed repeatedly and then horseradish peroxidase-labeled donkey anti-human IgG was added to the
plates at a 1:5000 dilution (100 µl/well). After incubation at room temperature for 1 h, the plates were washed repeatedly and then developed using ABTS (Zymed Laboratories) in 0.1 M citrate buffer, pH 4.0, with 0.03% H₂O₂ for 15 min at room temperature. The light absorption at 405 nm was measured on a BioTek plate reader. For each sample dilution the mean of the duplicates was used to determine the concentration of the huC242 component. The lower limit of the assay in plasma was about 10 ng/ml in a 2% serum matrix. The acceptance criteria for the ELISA data were the same as described above.

[¹²¹I]DM1 Drug Release in Vivo from huC242-¹²¹I]DM1 Conjugate. Conjugate comprising unlabeled antibody and tritium-labeled maytansinoid DM1 (huC242-¹²¹I]DM1) was administered i.v. to nine mice (body weight 26.2 ± 0.9 g, mean ± S.D.). Each mouse received 0.25 ml of a solution that contained 0.103 mg (0.7 nmol) of antibody linked to 2.1 nmol [¹²¹I]DM1 (3.0 [¹²¹I]DM1 drug molecules per molecule of huC242). The radioactivity of the administered solution was determined by mixing 50 µl of the solution with 50 µl of mouse serum and then with 15 µl of scintillant. Under these conditions of counting, more quenching was observed, and the dose of radioactivity administered in 0.25 ml was 8520 cpm/mouse (the counting was for 1 h), and the specific radioactivity of [¹²¹I]DM1 was calculated to be 4057 cpm/nmol. After 2 and 30 min, 2, 6, 12, 24, 48, and 72 h, blood (120 µl) was collected from three mice in nonheparinized capillaries tubes assuring that animals were bled maximally three times. Serum (50 µl) was withdrawn from the tubes to determine the radioactivity, and approximately 10 µl of serum from each sample was stored at −80°C until it was used to measure the concentration of huC242 antibody component by ELISA as described above.

Pharmacokinetic Study with Maytansine. Maytansine obtained from the National Cancer Institute (Bethesda, MD) was stored at −80°C in ethanol at a concentration of 500 µg/ml as assayed by absorbance at 252 nm using an ε₉₂₉ of 28,044 M⁻¹·cm⁻¹. Before dosing, the solution was diluted 20-fold with PBS (pH 6.5). Fifteen CD-1 mice (body weight 26.2 ± 1.5 g, mean ± S.D.) were each injected with 2.62 µg of maytansine (100 µg/kg) in a volume of 105 µl and were randomly divided into five groups (n = 3). Blood (150 µl) was withdrawn into heparinized capillaries tubes twice from animals in each group, therefore allowing for 10 different collection time points which were 0.5, 1, 2, 5, 10, and 30 min and 1, 2, 4, and 8 h after administration of the test samples. Samples were centrifuged, and 70 µl of plasma was withdrawn. The rest of the samples, which contained the cell pellets, were resuspended in 320 µl of water and placed on ice for 5 min to generate a blood cell lysate. The plasma and cell lysate samples were stored at −80°C until used in ELISAs to measure maytansine concentration. While it may be assumed that 5 µl of plasma was left with the blood cell fraction (Jacoby and Fox, 1984), the measurements made on the cell lysate were not subject to any correction. Urine was also sampled at 5, 10, and 30 min and 1, 2, 4, and 8 h by stimulating urination through bladder massaging. Each time the bladder of the mice was emptied 20 min before urine collection.

An ELISA method was used to determine the concentration of maytansine in whole blood, the plasma, and the blood cell lysate. Plates were coated with (100 µl/well) a solution of 0.1 µg/ml DM1 that had been conjugated to bovine serum albumin (BSA-DM1, Immunogen, Inc.). Following an overnight incubation at 4°C, the wells were blocked with a 0.5% BSA solution in TBS (pH 7.5) for 1 h at room temperature. Dilution samples (50 µl) of a maytansine standard solution prepared with normal mouse plasma and test samples (50 µl) were mixed in separate plates with 50 µl of a 0.1 nM solution of biotinylated anti-maytansine antibody (Immunogen, Inc.). Then 50 µl of the mixtures were transferred to the BSA-DM1-coated plate. After incubation at 37°C for 30 min, the plate was washed with 0.1% Tween/TBS and then treated with 100 µl/well of a 1:2000 dilution of streptavidin-horseradish peroxidase solution (Jackson ImmunoResearch Laboratories Inc.). The assay plate was washed extensively and then developed using ABTS (Zymed Laboratories) dissolved in 0.1 M citrate buffer, pH 4.0, containing 0.03% hydrogen peroxide as described above.

Tissue Distributions of [¹²⁵I]-huC242 Antibody and [¹²⁵I]-huC242-DM1 Conjugate in Mice. Thirty-six female CD-1 mice were separated into two sets. One set of 18 mice (body weight 27.8 ± 2.6 g, mean ± S.D.) was used for measuring tissue distribution of [¹²⁵I]-huC242 and a second set (body weight 27.8 ± 2.7 g, mean ± S.D.) for [¹²⁵I]-huC242-DM1. All animals were dosed at 1.0 mg/kg of antibody. Therefore, each mouse in the first set received 0.2 ml of antibody solution containing 27.8 µg of [¹²⁵I]-huC242 with 2.6 × 10⁶ cpm of radioactivity. Mice in the second set received 0.2 ml of conjugate containing 27.8 µg of [¹²⁵I]-huC242-DM1 with 2.0 × 10⁶ cpm and 1.18 µg of linked DM1 (average, 3.2 DM1 molecules linked per antibody molecule). At 2 and 6 h and 1, 2, 4, and 8 days after administration of the test articles, three mice from each set were euthanized. Immediately thereafter, the blood was flushed from the animals by injecting PBS into the left (3 ml) and right (2 ml) ventricle of the heart and draining the liquid through incisions in the vena cava and the left atrium, respectively. The organs or tissues, blood, heart, lungs, spleen, kidneys, liver, stomach, small intestine, colon, uterus, muscle, fat, thyroid gland, brain, and tail were collected, weighed, and assayed for radioactivity. The percentage of the total injected dose per gram of a given organ (%ID/g) was then calculated. Values for the %ID per whole organ were calculated with the formula: %ID per organ = %ID/mg × the animal weight × R, with R being the organ to body weight ratio. Rat values were experimentally determined for most organs by isolating and weighing the organs from five individual untreated animals (for values see Table 2). R × 100 values indicated the percentage of the total dose of the antibody and conjugate, respectively. The calculated values are the published values of 8.0 (Morton et al., 1993), 40 (for rats, Tang et al., 2002), and 25 (Pellomountner et al., 1998), respectively.

Results

Pharmacokinetic Studies with Radiolabeled Conjugate [¹²⁵I]-huC242-DM1 and Radiolabeled Antibody [¹²⁵I]-huC242. Radiolabeled conjugate and antibody with similar specific radioactivities, 2.73 × 10⁷ cpm/mg and 5.09 × 10⁷ cpm/mg, respectively, were prepared with the help of Bolton-Hunter reagent. CD-1 mice were injected intravenously with the same amount (4.16 mg/kg) of either [¹²⁵I]-labeled antibody or [¹²⁵I]-labeled conjugate that had, on the average, 4.07 molecules of DM1 linked per antibody molecule. Blood samples were withdrawn at different time points over a period of 7 days to assay the level of [¹²⁵I] radioactivity in the whole blood. The data are shown in Fig. 2, where the blood concentrations of antibody and conjugate, respectively, as calculated from the radioactivity measurements, are plotted against the time of sampling in hours after administration. Each data point is the mean value from three samples from three individual animals. We also demonstrated (data not shown) that at each time point about 99% of the radioactivity was associated with protein (precipitable by trichloroacetic acid). The two clearance curves for radiolabeled antibody and conjugate shown in Fig. 2 are superimposable within the precision of the experiments and show the standard biphasic characteristics of an initial rapid α or distribution phase lasting about 10 h and a slower β or disposition phase. Therefore, all data points beyond 10 h (11.5–168 h) were used for the calculation of the kinetic parameters of the β phase. The rate constants, kᵣᵣ, for the clearance of the antibody and the conjugate were derived from the slopes of the curves in Fig. 2 and found to be equal within the precision of the experiment, i.e., 4.45 × 10⁻³ h⁻¹ for the antibody and 4.5 × 10⁻³ h⁻¹ for the conjugate. These
dioactivity, therefore, can only describe the behavior of the antibody component and no information is obtained for the drug component. Since the half-life of the active conjugate may be the product of clearance from circulation and the loss of linked drug from the conjugate, we performed pharmacokinetic studies with unlabeled conjugate using ELISA methods that assay the intact conjugate and the antibody component separately. Mice were injected intravenously with 10 mg/kg of conjugate bearing 3.2 covalently linked DM1 molecules per antibody molecule. At different time points thereafter, over a period of 8 days, blood samples were withdrawn (from three animals at each time point), and plasma was prepared. The conjugate concentration in the plasma samples was measured with an ELISA, which captured the conjugate with an antimyotansinoid antibody (anti-DM1) and then detected it with a horseradish peroxidase-conjugated donkey anti-human IgG immunoglobulin. Since the standard curve for the ELISA was prepared with the conjugate that was administered to the animals and that contains 3.2 covalently linked DM1 molecules, the results will represent equivalent amounts of administered conjugate with a DM1 to antibody ratio of 3.2. In the same plasma, the antibody component of the conjugate was measured with an ELISA that used a goat anti-human IgG immunoglobulin in the capture step and the same horseradish peroxidase-labeled donkey anti-human IgG immunoglobulin as above in the detection step. The measured plasma concentrations of conjugate and antibody component were plotted against sampling time in hours after administration, and the resulting clearance curves are shown in Fig. 3. Data points are mean values from three samples obtained from three different animals. Both curves display the expected biphasic behavior, although the conjugate plasma clearance curve has a steeper slope for the disposition phase. The pharmacokinetic parameters derived from this data set are given in Table 1. Although only the AUC\textsubscript{tot} for the conjugate clearance curve can be determined with sufficient accuracy and the value for the antibody component is only an estimate (AUC\textsubscript{192,∞} is 36.6% of AUC\textsubscript{tot}), both sets of data are listed. The AUC\textsubscript{tot} for the conjugate curve was decreased to about a quarter of that for the antibody component (7.01 versus 27.68 h mg\(^{-1}\) ml\(^{-1}\)) and CL, increased accordingly from 0.36 to 1.43 ml h\(^{-1}\) kg\(^{-1}\), whereas the clearance unrelated pharmacokinetic parameters, such as the \(C_{\text{max}}\) and \(V_{\text{ss}}\), are similar for both curves (see Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Test Article (Compartment)</th>
<th>Measured Entity</th>
<th>Protein</th>
<th>Linked DM1</th>
<th>(t_{1/2}(\beta))</th>
<th>(k_\beta) (Period)</th>
<th>AUC\textsubscript{tot}</th>
<th>CL</th>
<th>(V_{\text{ss}})</th>
<th>(C_{\text{max}})</th>
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<tr>
<td>[(^{125}\text{I})]huC242-DM1 (blood)</td>
<td>[(^{125}\text{I})]huC242</td>
<td>4.16 0.08</td>
<td>153.7</td>
<td>4.5 \times 10^{-3} (10–168 h)</td>
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<td>0.51</td>
<td>115.7</td>
<td>74.8</td>
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<td>0.51</td>
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<td>2.9 \times 10^{-2} (6–72 h)</td>
<td>1.99</td>
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<td>146.1</td>
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</table>

**Pharmacokinetic Studies with huC242-DM1 Conjugate Using ELISA Methods.** The \(^{125}\text{I}\) label was introduced into the conjugate by labeling the antibody component with the Bolton-Hunter reagent; the above measurements of radioactivity, therefore, can only describe the behavior of the antibody component and no information is obtained for the drug component. Since the half-life of the active conjugate may be the product of clearance from circulation and the loss of linked drug from the conjugate, we performed pharmacokinetic studies with unlabeled conjugate using ELISA methods that assay the intact conjugate and the antibody component separately. Mice were injected intravenously with 10 mg/kg of conjugate bearing 3.2 covalently linked DM1 molecules per antibody molecule. At different time points thereafter, over a period of 8 days, blood samples were withdrawn (from three animals at each time point), and plasma was prepared. The conjugate concentration in the plasma samples was measured with an ELISA, which captured the conjugate with an antimyotansinoid antibody (anti-DM1) and then detected it with a horseradish peroxidase-conjugated donkey anti-human IgG immunoglobulin. Since the standard curve for the ELISA was prepared with the conjugate that was administered to the animals and that contains 3.2 covalently linked DM1 molecules, the results will represent equivalent amounts of administered conjugate with a DM1 to antibody ratio of 3.2. In the same plasma, the antibody component of the conjugate was measured with an ELISA that used a goat anti-human IgG immunoglobulin in the capture step and the same horseradish peroxidase-labeled donkey anti-human IgG immunoglobulin as above in the detection step. The measured plasma concentrations of conjugate and antibody component were plotted against sampling time in hours after administration, and the resulting clearance curves are shown in Fig. 3. Data points are mean values from three samples obtained from three different animals. Both curves display the expected biphasic behavior, although the conjugate plasma clearance curve has a steeper slope for the disposition phase. The pharmacokinetic parameters derived from this data set are given in Table 1. Although only the AUC\textsubscript{tot} for the conjugate clearance curve can be determined with sufficient accuracy and the value for the antibody component is only an estimate (AUC\textsubscript{192,∞} is 36.6% of AUC\textsubscript{tot}), both sets of data are listed. The AUC\textsubscript{tot} for the conjugate curve was decreased to about a quarter of that for the antibody component (7.01 versus 27.68 h mg\(^{-1}\) ml\(^{-1}\)) and CL, increased accordingly from 0.36 to 1.43 ml h\(^{-1}\) kg\(^{-1}\), whereas the clearance unrelated pharmacokinetic parameters, such as the \(C_{\text{max}}\) and \(V_{\text{ss}}\), are similar for both curves (see Table 1).
During the \( t_\beta \) or disposition phase, both curves demonstrate first-order kinetics. The rate constants \( k \) were calculated through linear regression analysis using all data points from 8 to 192 h and found to be \( 5.7 \times 10^{-3} \) h\(^{-1} \) for the antibody component and \( 1.64 \times 10^{-2} \) h\(^{-1} \) for the conjugate, which correspond to half-lives \( t_{1/2}(\beta) \) of 122.6 h for the antibody and 42.2 h for the conjugate. These results, together with the data from the clearance study with \(^{125}\text{I}\)-labeled samples, indicate that the number of linked drug molecules per antibody molecule diminishes during the time it takes to clear the antibody component from circulation of mice. The conjugate clearance curve should therefore be labeled an apparent clearance curve, since it is the combination of elimination from circulation and the clearance of linked DM1 from circulating conjugate. Since clearance rates are additive, the apparent clearance of conjugate (\( CL_{\text{con}} = 1.43 \) ml h\(^{-1}\) kg\(^{-1}\)) can be presented as the sum of the clearance of total antibody (\( CL_{\text{AB}} = 0.36 \) ml h\(^{-1}\) kg\(^{-1}\)) and the clearance of DM1 from circulating conjugate (\( CL_{\text{DM1}} = 1.07 \) ml h\(^{-1}\) kg\(^{-1}\)). This indicates that the loss of DM1 drug from circulating conjugate is about three times as fast as the clearance of the antibody component from circulation.

\textbf{[\(^{3}\text{H}\)]DM1 Drug Release in Vivo from huC242-[\(^{3}\text{H}\)]DM1 Conjugate.} To be able to directly measure the release of covalently linked DM1 drug from the conjugate in circulation, we prepared a conjugate of unlabeled huC242 antibody and tritiated \([^{3}\text{H}]\)DM1 drug and performed pharmacokinetic studies with the huC242-[\(^{3}\text{H}\)]DM1 conjugate. Animals were injected intravenously with \( 3.93 \) mg/kg conjugate (\( 8520 \) cpm per mouse, scintillation counting time was 1 h) that had \( 3.2 \) \([^{3}\text{H}]\)DM1 molecules linked per antibody molecule. Blood samples were withdrawn at several time points until 72 h after the intravenous administration of the conjugate. At later times there was no longer sufficient radioactivity present in the serum for accurate measurements. Serum samples were prepared and assayed for radioactivity by scintillation counting and for the huC242 component by the ELISA method described above. The clearance curve for the radioactivity and the ELISA clearance curve for the antibody component of the conjugate are shown in Fig. 4A and the calculated parameters are listed in Table 1. The curves show that the radioactive drug is cleared faster from circulation than the antibody component of the conjugate (\( CL_{\text{con}} = 3.59 \) ml h\(^{-1}\) kg\(^{-1}\) versus \( CL_{\text{AB}} = 0.61 \) ml h\(^{-1}\) kg\(^{-1}\)). With the assumption that released DM1 drug has a short serum half-life and therefore never achieves significant serum levels (see pharmacokinetic data below), the radioactiv-
ity measured in serum represents the amount of DM1 covalently bound in the huC242-[3H]DM1 conjugate. Thus the radioactivity clearance (CL[3H]) is a composite of the clearance of the conjugate from circulation as measured by the CL[3H] and the clearance of DM1 from circulating conjugate. The latter calculates as 2.98 ml h⁻¹ kg⁻¹ and is therefore nearly five times as fast as the clearance of the antibody component. The kinetic parameters for the β phase were calculated using the data points from 6 to 72 h (see Table 1). First-order rate constants kβ were found to be 2.9 × 10⁻² h⁻¹ for the radioactivity and 6.9 × 10⁻³ h⁻¹ for the antibody component, which correspond to half-lives t1/2(β) for the clearance of the radioactivity and the antibody component of 23.9 and 99.8 h, respectively.

With the help of the specific molar radioactivity of [3H]DM1, the number of linked DM1 molecules per antibody molecule was calculated for each sampling point. When the logarithm of the number of linked drugs was plotted versus sampling time (see Fig. 4B) the set of data points could be described best with a linear relation (square of correlation coefficient: r² = 0.869). This indicates that the loss of linked drug molecules from the conjugate is a first-order or pseudo first-order process.

Pharmacokinetic Studies with Maytansine. For the interpretation of the results above we had assumed a very rapid clearance from circulation of released DM1 drugs. Therefore, for experimental verification we set out to study the pharmacokinetics of maytansine, a very close chemical analog of DM1 that lacks the unstable sulfhydryl group (see Fig. 1B). Mice were injected intravenously with a nontoxic dose of maytansine (0.1 mg/kg) and blood samples were collected at different time points. The samples were processed to allow the determination of the maytansine concentration in whole blood, plasma, and blood cell lysate using an ELISA method. The data yielded three clearance curves (see Fig. 5) that are all characterized by an initial distribution phase with a rapid and large decline in the concentration of maytansine over about 30 min, which is followed by a slower rate of disappearance during the disposition phase with a first-order rate constant kβ of about 0.3 h⁻¹. One minute after the injection, only about 3.5% of the injected dose was still present in the plasma, and after 30 min the content had declined to 0.2%, thus validating our assumption that after the administration of huC242-[3H]DM1 conjugate the measurable amount of [3H]DM1 in plasma is nearly exclusively found in the conjugate.

The clearance curves also show that a remarkably higher concentration (about 7-fold) of maytansine is present in the blood cell compartment than in the plasma during the test period of 8 h. This suggests that maytansine associates with cells due to its known lipophilic character, and its clearance from plasma is in equilibrium with the blood cell compartment. As a consequence, the concentration of drug in the whole blood compartment is larger than that in plasma with about 11.4% of the injected dose remaining in the blood 1 min after administration and about 2% remaining 30 min postinjection. The pharmacokinetic parameters derived from the curves (see Table 1) are also typical for a lipophilic drug. Particularly the Vss is manyfold larger than the whole blood compartment, indicating that maytansine is extensively bound to tissues.

We also collected the urine from the animals during the experiment and assayed it for maytansinoids. In accordance with the high lipophilicity and poor water solubility of the drug, very little, about 2% of the injected dose was excreted through the urine during the 8-h test period (data not shown).

Tissue Distributions of 125I-huC242 Antibody and 125I-huC242-DM1 Conjugate in Mice. To further assess if the chemical linking of four lipophilic maytansinoid drugs to the humanized IgG1 antibody huC242 altered the in vivo behavior of the antibody, we compared the tissue distribution of the maytansinoid conjugate huC242-DM1 with that of the antibody. Two separate groups of mice were treated either with 125I-labeled antibody or 125I-labeled conjugate at a 1 mg/kg dose via tail vein injections. At 2 and 6 h and 1, 2, 4, and 8 days after the administration, three animals in each group were euthanized and exsanguinated by flushing the circulation with phosphate-buffered saline. The organs were then removed, weighed, and assayed for 125I radioactivity. The %ID/g was calculated for each animal. The mean values for the three animals at each time point were calculated and are graphically presented in Fig. 6, A and B. A comparison of the two graphs shows that no significant differences at any of the tested time points could be observed for the tissue localization of the antibody from the conjugate or the unmodified antibody. At any time point measured, both agents are largely present in the blood compartment. The observed accumulation in the tail is assumed to be an experimental artifact from the tail vein injection.

We also calculated (see Materials and Methods) the total accumulation of 125I-huC242-DM1 conjugate in each organ as a percentage of the injected dose (%ID) at the six different test time points. These values were used to calculate the total recovery of conjugate in the animal as a percentage of the injected dose as well as the fraction contained in the blood (see Table 2). The total recovery was 90.1% ID at 2 h after the injection and...
declined to 21.9% ID at 8 days after the injection, which is in good accordance with the clearance curve from serum (see Fig. 2). This shows that whole body clearance is similar to clearance from the blood compartment. Furthermore, the percentage of antibody from the conjugate in the blood of the total amount in the animal stayed constant at about 55% during the whole test period of 8 days. Together these results further confirm that the antibody of the conjugate did not distribute or bind significantly to any solid tissue and was in equilibrium between the intravascular and extravascular plasma compartments during the whole test period of 8 days. Analogous results were obtained with the antibody but are not shown.

Discussion

The first pharmacokinetic study used $^{125}$I-labeled antibody and conjugate samples and followed the disappearance of the radiolabel from the blood of injected mice. Similar biphasic clearance curves were obtained for both test articles (Fig. 2). The initial distribution phase lasted about 10 h and was followed by a disposition phase with a half-life of 153.7 to 155.8 h and an elimination rate constant $k_{el}$ of $4.5 \times 10^{-3}$ h$^{-1}$. These values agree with previously reported data for the clearance of mouse-human chimeric antibodies from the circulation of mice (Zuckier et al., 1994). We described our pharmacokinetic results as estimates because we collected data only for up to 7 days (168 h) after intravenous administration of the samples. This time period is too small to generate accurate data for samples with half-lives of 153.7 and 155.8 h. However, the test period was not prolonged because of the possibility of generating an immune response to the humanized antibody. It had been demonstrated (Zuckier et al., 1994) that immune elimination of human-mouse chimeric antibodies often began in BALB/c mice on days 6 to 8 after intravenous administration.

At the cellular level, cantuzumab mertansine exerts its anticancer activity through a multistep process, which includes binding to a MUC1 carbohydrate epitope on the cell surface through its antibody component, internalization of the conjugate-antigen complex by the cancer cell, and release of DM1 from the antibody. This allows DM1 to reach its intracellular target tubulin and to inhibit tubulin polymerization. The pharmacologic activity of the conjugate is therefore dependent on the activity of all three of its components, i.e., the binding of the antibody, the cytotoxic or antimitotic activity of the drug, and the stability and cleavability of the linker. The above-described pharmacokinetic analysis with $^{125}$I-labeled conjugate only yields information about the antibody component of the conjugate, since the Bolton-Hunter-labeling method introduces the radioisotope exclusively to the antibody.

To perform a pharmacokinetic analysis of the “whole active conjugate” we employed an ELISA that captures the conjugate on the plate with an antimaytansinoid antibody and detects it with an anti-human IgG reagent. The same serum samples were also assayed for the antibody component with an ELISA that used an anti-human IgG capturing step and the same detection step as above. As expected, the clearance curve for the antibody component was similar to that obtained with the $^{125}$I-labeled antibody and conjugate samples; however, the curve for the intact conjugate showed a faster $\beta$ elimination and shorter $\beta$ half-life of 42.2 h (Fig. 3 and Table 1). The clearance rate for the conjugate was about four times faster than that for the antibody component (Table 1), which can only be explained by the loss of drug molecules from the conjugate, since the $^{125}$I-labeled naked antibody and conjugated antibody had identical clearance parameters. The observed clearance curve for the conjugate is therefore the sum of clearance from circulation as measured by the antibody clearance and clearance of drug molecules from circulating conjugate. The data in Table 1 shows that the rate of drug clearance from conjugate, calculated as the difference in the clearance rate of the conjugate and the antibody component, is about three times the rate of clearance of the antibody component from circulation of mice.

With a second method, we sought to measure directly the clearance of conjugated drug. Thus, we used the conjugate huC242-[${}^3$H]DM1 where the DM1 drug carries a radioactive tritium label. Despite the expected release of drug from circulating conjugate, greater than 99% of any radioactivity present in the serum of animals at any sampling point could be attributed to conjugated drug since studies with a nonconjugated maytansinoid drug showed very rapid clearance of...
the specific molar radioactivity of \([3H]DM1\), we calculated the percentage of clearance of \([3H]DM1\) from both experiments, although the data from the experiment with \(huC242-[3H]DM1\) may be less accurate because of the method of measurement.

Theoretically one would expect similar results for the clearance rate calculated from a shorter observation period (72 versus 192 h). An explanation for the difference may be that the ELISA method underestimated the loss of drug molecules from the conjugate.

The ELISA experiment showed a 4-fold increased clearance rate for the conjugate over that of the antibody component, whereas the increase obtained using \(huC242-[3H]DM1\) was 6-fold. Theoretically one would expect similar results from both experiments, although the data from the experiment with \(huC242-[3H]DM1\) may be less accurate because of the shorter observation period (72 versus 192 h). An explanation for the difference may be that the ELISA method underestimated the loss of drug molecules from the conjugate. The antimaytansinoid antibody was used in the capture step of the ELISA and the efficiency of capturing may not be proportional to the number of linked maytansinoid drugs per antibody molecule. One also cannot exclude the possibility that there are different rates for the cleavage of individual drugs from the antibody or that there is selective early clearance of a population of conjugate molecules with the highest ratio of linked drug molecules per antibody molecule. The conjugate is prepared by random modification of the antibody to introduce an average of three to four drug molecules per antibody molecule. This results in a mixture of conjugate species with different drug to antibody ratios according to a binominal distribution. Both mechanisms would explain the faster clearance rate calculated from a shorter observation period. Under the assumption that such heterogeneous behavior would already be detectable during a 72-h period, we subjected the \(huC242-[3H]DM1\) data to further analysis. Using the specific molar radioactivity of \([3H]DM1\), we calculated the number of linked drug molecules per antibody molecule in the \(huC242-[3H]DM1\) samples obtained from the animals and plotted the logarithm of the results versus sampling time (Fig. 4B). Analysis of the graph revealed that the set of data points obtained could be described with a linear relation (\(r^2 = 0.869\)), which indicates a first-order or pseudo first-order process for the loss of drug molecules from circulating conjugate. This eliminates the possibility of preferred removal of highly modified conjugate species and indicates that all drug links have similar cleavage rates.

The most likely mechanism for release of DM1 from conjugate is cleavage of the disulfide bond in the linker via a disulfide exchange reaction. Analysis of human plasma has shown (Mills and Lang, 1996) that there is a balance between free cysteine, cystine, and cysteine bound via a disulfide bond.

The conjugate may also happen during recycling of the conjugate from endothelial cells. Homeostasis of IgG in serum is partially maintained by the uptake into endothelial cells followed by partial recycling through the FcRn or Brambell receptor (FtRB) (Junghans, 1997). During this journey the conjugate may encounter a 0.1 to 10 mM intracellular concentration of glutathione (Meister, 1988), which would cause rapid cleavage of the drug disulfide link.

The biodistribution study with \(^{251}\) labeled conjugate and antibody samples revealed no difference between the two samples in regard to accumulation in 14 different organs and tissues at six different time points from 2 h to 8 days after intravenous administration of the test articles (Fig. 6, A and B). Furthermore, the determination of the amount of the antibody of the conjugate as a %ID in the whole body and in the blood compartment at the six different time points (Table 2) showed that at any time point about 55% of the antibody of

**Table 2**

Accumulation of \(^{125}\) -huC242-DM1 conjugate in whole organs of mice at different time points after i.v. administration expressed as %ID in the whole body and in the blood compartment.

<table>
<thead>
<tr>
<th>Organ</th>
<th>% ID (mean ± S.D.) of (^{125}) -huC242-DM1 Conjugate in Whole Organs at Different Time Points after Intravenous Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>Blood</td>
<td>50.2</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.7</td>
</tr>
<tr>
<td>Heart</td>
<td>0.52</td>
</tr>
<tr>
<td>Lung</td>
<td>0.69</td>
</tr>
<tr>
<td>Liver</td>
<td>5.23</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.33</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.82</td>
</tr>
<tr>
<td>Small intestine</td>
<td>2.97</td>
</tr>
<tr>
<td>Colon</td>
<td>1.19</td>
</tr>
<tr>
<td>Uterus</td>
<td>0.52</td>
</tr>
<tr>
<td>Fat</td>
<td>4.0</td>
</tr>
<tr>
<td>Muscle</td>
<td>4.0</td>
</tr>
<tr>
<td>Tail</td>
<td>2.9</td>
</tr>
<tr>
<td>Total</td>
<td>90.1</td>
</tr>
</tbody>
</table>

\( R \times 10^6 \) value is the organ weight as a percentage of the total body weight (organ weight/body weight \times 100) and was determined experimentally as described under Materials and Methods except for blood, muscle, and fat for which published values were used.

\(^*\) The whole organ was used to measure the radioactivity, and the %ID was calculated directly from the experimental value.
the conjugate is located in the blood compartment. This indicates similar clearance rates from the blood compartment and the whole body, which is in good agreement with published findings for mouse-human chimeric antibodies in mice (Zuckier et al., 1994). We conclude that the covalent linking of three to four maytansinoid drug molecules to the humanized C242 antibody does not change the distribution of the antibody in mice. Although at the later sampling points most if not all drug molecules have been lost from circulating conjugate, the antibody is still modified by the linker molecule.

Comparison of the pharmacokinetic parameters of maytansine and the antibody maytansinoid conjugate shows the expected large differences. The small lipophilic agent maytansine clears rapidly from plasma and binds to tissues such as blood cells (Fig. 5 and Table 1), which leads to a small plasma AUCtot value and a large volume of distribution. Through conjugation of the maytansinoid to a monoclonal antibody, the drug acquires the pharmacokinetic characteristics of an antibody with a more than 60-fold increased AUCtot (Table 1) and a volume of distribution close to the whole body, which is in good agreement with published findings for mouse-human chimeric antibodies in mice and the whole body, which is in good agreement with published findings for mouse-human chimeric antibodies in mice.

Acknowledgments

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References


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