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Research Paper

Toxicological Protein Biomarker Analysis—An Investigative One-week Single Dose Intravenous Infusion Toxicity and Toxicokinetic Study in Cynomolgus Monkeys using an Antibody–cytotoxic Conjugate against Ovarian Cancer

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Introduction. Antibody—cytotoxic conjugates are complex novel therapeutic agents whose toxicological properties are not presently well understood. The objective of this study was to identify toxicological markers in serum that correlate with MLN8866 (an antibody—cytotoxic conjugate) exposure and related pathological events in monkeys.
Materials and methods. Cynomolgus monkeys were treated once with 5, 15, or 30 mg/kg MLN8866 via a 20 min intravenous infusion. MLN8866 exposure (Cmax and AUC0–4 day) was determined by quantifying MLN8866 levels in serum.
Results. The increase in MLN8866 exposure was approximately dose proportional. Two acute phase proteins in serum (serum amyloid A and haptoglobin) were correlated with MLN8866 exposure and toxicological outcomes (e.g., erythropoiesis and leucopoiesis).

KEY WORDS: biologics; biomarker; predictive toxicity; toxicokinetics; toxicology.

INTRODUCTION

Immunoconjugates (Antibody Drug Conjugates, ADCs) may improve the therapeutic index of cytotoxic drugs by using the interaction of the antibody with tumor-specific epitopes to specifically deliver a cytotoxin to cancer cells, thereby reducing toxicity to normal tissues. The success of an ADC to specifically deliver a cytotoxin to a cancer cell depends on many factors, including the tissue distribution of the epitope against which the antibody is targeted and the stability of the linkage between the cytotoxin and antibody. The number of ADCs in development has rapidly increased over the past several years (1–7). However, only one ADC (Gemtuzumab Ozogamicin for acute myeloid leukemia) has been approved to date by the US FDA (8,9).

MLN8866 is an immunoconjugate composed of a maytansine cytotoxic analog (CT) and a fully humanized monoclonal IgG1 antibody (mAb8866) joined via a disulfide bridge. The mAb8866 antigen, B7-H4, is a cell surface glycoprotein that regulates T cell immunity (10,11). The B7-H4 protein is highly expressed in ovarian and lung cancer cells but not expressed in normal human tissues including ovary, lung, spleen, thymus, placenta, liver, skeletal muscle, kidney, pancreas, prostate, testis, and small intestine (12,13). MLN8866 binding activity has been demonstrated in ES2 human ovarian cells transfected with the mAb8866 antigen. After binding to the target antigen, MLN8866 is internalized by the cancer cell and the cytotoxin is released by reduction of the disulfide bond. The released cytotoxin binds to tubulin and causes inhibition of microtubule polymerization and the subsequent inhibition of mitosis.

Biomarker profiling has become extensively used to identify changes in biochemical expression patterns related to drug toxicity or adverse events (14–19). Two-dimensional fluorescence protein gel electrophoresis, mass spectrometry and bioinformatics are combined to monitor protein profiles of tissue, plasma/sera, urine and other bio-fluids during drug treatment. Comparative analysis of protein profiles can reveal different types of biomarkers, including markers of drug toxicity or adverse events. Biomarkers demonstrating a quantitative relationship with drug exposure and traditional toxicological endpoints (i.e. histological changes, hematology parameters etc.) may be useful for assessment of novel therapeutics.

The objective of this preclinical development study was to identify toxicological biomarkers related to MLN8866 exposure and toxicity in cynomolgus monkeys. Cynomolgus monkeys were chosen as a model for preclinical development to enable IND repeat dose GLP studies. The pharmacokinetics of MLN8866 was determined using an ELISA assay. Patho-

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logic evaluation was performed to evaluate MLN8866 toxicity. Protein profiling of monkey sera was used to identify changes in protein levels correlated with immunoconjugate exposure and adverse events. The primary structure of MLN8866 was also characterized to determine sites of cytotoxin conjugation. The cytotoxic conjugated sites to MLN8866 protein structure were identified to help evaluate biological binding activities and pharmacotoxicity.

MATERIALS AND METHODS

Chemicals

CyDye DIGE Fluor labeling reagents, *N*,*N*-dimethyl formamide (DMF), dithiothreitol (DTT), urea, thiourea, pharmalyte (pH 3–10), glycerol, tris-(hydroxymethyl)-aminomethane (TRIS), glycine and lysine were purchased from Amersham BioSciences (Piscataway, NJ). Ethanol, methanol, acetic acid, water, agarose, bromophenol blue and 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma-Aldrich (St Louis, MO). Alpha-cyano-4-hydroxycinnamic acid MALDI matrix was purchased from Applied BioSystems (Foster City, CA). The 2-D lysis buffer contained 7M urea, 2M thiourea, 4% CHAPS, and 30 mM TRIS, pH 8.5. The 2 × 2-D sample buffer contained 8M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalyte and trace amounts of bromophenol blue. Equilibration buffer 1 was 50 mM TRIS, pH 8.8 containing 6M urea, 30% glycerol, 2% SDS, trace amounts of bromophenol blue and 10 mg/ml DTT. Equilibration buffer 2 was 50 mM TRIS, pH 8.8, containing 6M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml DTT. The SDS-gel running buffer was 50 mM Tris–HCl, pH 8.3, 192 mM glycine and 0.1% SDS. Rehydration solution contained 7M urea, 2M thiourea, 2% CHAPS, 2% pharmalyte and 2% DTT.

Dose Preparation

MLN8866 dosing solutions (5, 15, 30 mg/kg) were prepared in an aqueous solution of 20 mM histidine, 3% sucrose, 3% mannitol, pH 5.5 (control/vehicle) immediately prior to use.

Animals and Experimental Design

The in-life portion of the study was conducted at Charles River Laboratories, Sparks, NV. The treatment of animals was performed in accordance with Charles River's Institutional Animal Care and Use Committee (IACUC) guidelines, the regulations outlined in the US FDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals (ILAR publication, 1996, National Press). A total of eight female Cynomolgus monkeys (2.9–5.4 years old) were randomly assigned into control and treatment groups based on body weight ($N = 2$/group). Monkeys were dosed once via a 20 min intravenous infusion at dose of 0 (vehicle), 5, 15 or 30 mg/kg MLN8866 and evaluated over a 7-day period. The day of dosing was designated Day 1.

Clinical and Pathologic Evaluation

The animals were evaluated for changes in clinical signs, including food consumption (daily); body weight; body temperature; heart rate and respiration rate (predose and at the end of infusion); serum chemistry, hematology, and coagulation parameters. One Group 4 animal died on Day 6; one animal each from Groups 3 and 4 were euthanized moribund or for humane reasons on Day 6; and remaining surviving animals were euthanized on Day 8. At termination/death, a full necropsy was conducted on all animals, and over 40 tissues per animal were collected, preserved and processed, and then examined microscopically.

Blood Sample Collection

Whole blood samples were collected by venipuncture from an available vein other than the site of injection at predose (Day 0) and at 1, 2, 4, 8, 24, 48 (Day 2), 96 (Day 4), 120 (Day 6), and 168 (Day 8) hour(s) post end infusion MLN8866. Serum was obtained by centrifuging (1,200 × g) the whole blood at 4°C. All serum samples were stored at −80°C until used for analysis.

Quantitation of MLN8866 in Serum

The concentration of MLN8866 (mAb8866-CT) in the serum samples were determined using an ELISA assay. The ELISA assay was developed in house and did not distinguish between the varying levels of conjugation of cytotoxin to the antibody. Therefore, it measured the total concentration of antibody with at least one cytotoxin conjugated to MLN8866. The MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) was used to identify levels of cytotoxic (CT) conjugation in CT–antibody conjugate (4).

For the ELISA assay, microtiter plates were coated with an antibody that binds to the maytansine analog cytotoxin. MLN8866 in the serum samples was captured by the anti-cytotoxin antibody and detected with a mouse anti-human IgG horseradish peroxidase (HRP). Following the addition of the colorimetric substrate tetramethylbenzidine, the optical density of the incubation was determined at the 650 nm wavelength.

The concentration of MLN8866 was determined relative to a standard curve of known concentrations of MLN8866 spiked in serum. A 4-parameter logistic curve-fitting algorithm was used to prepare the standard curve (Molecular Devices Corp, Sunnyvale, CA). Seven calibration standards (2, 4, 8, 16, 32, 64, 96, and 128 ng/mL) and three quality control (QC) solutions (6.25 ng/mL [Low QC], 25 ng/mL [Medium QC], and 100 ng/mL [High QC]) were used to prepare the standard curve. Acceptable calibration curves consisted of seven non-zero concentrations in duplicate over the target range, plus a blank sample. The lower limit of quantification (LLOQ) and upper limit of quantitation (ULOQ) of the assay were 4.7 ng/mL and 180 ng/mL, respectively. Test serum samples from monkeys were diluted from 1:1,000 to 50,000 in assay buffer (1% bovine serum albumin in Dulbecco's phosphate-buffered saline) prior to quantitation. Preliminary experiments were conducted to

assure that serum dilution did not influence the MLN8866 quantitation results.

Toxicokinetic Data Analysis

Toxicokinetic data analysis of the individual serum concentration data was performed using WinNonlin®, Version 5.0 (Pharsight Corp., Mountain View, CA). Kinetic parameters were estimated using a noncompartmental model (Model 202; constant IV infusion). The AUC_{0-4day} was calculated using the linear trapezoidal rule. Concentrations of MLN8866 that were below the limit of quantitation (BLQ) were set to 0.00 ng/mL. A 20-min infusion time was used. The ratios of C_{max} to dose and AUC_{0-4day} to dose were calculated in WinNonlin®.

Serum Protein Profile

Serum samples were vortexed and then centrifuged at 8,765 × g for 30 min. The supernatant was collected. 2-D protein lysis buffer (5 μL) was added to 1 μL of supernatant. Samples were labeled with fluorescent CyDyes for protein labeling. The CyDyes (Amersham BioSciences) was added to each sample (1 μL, 0.2 nmol/μL in DMF). Samples were mixed thoroughly by vortexing and placed on ice for 30 min in the dark. The labeling reaction was stopped by addition of lysine (1 μL, 10 mM). Samples were mixed well and left on ice for 15 min in the dark. Three samples (labeled with different dyes) were combined. The sample mixture was diluted with 2 × 2-D sample buffer.

2-D Fluorescence Protein Gel Electrophoresis

Protein electrophoresis was used for analysis of differences in protein abundance between samples. Proteins were resolved using isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. An Ettan IPGphor II Isoelectric Focusing System (Amersham BioSciences) with immobilized pH gradient (IPG) gel strips (Amersham Biosciences) was used for IEF. Samples (250 μL) were loaded into 13 cm IPG strips. Mineral oil (1mL) was added on top of each strip. IEF was run at 20°C in the dark for 22 h. After IEF, the IPG strips were incubated in equilibration buffer 1 for 15 min with slow shaking. The strips were rinsed in equilibration buffer 2 for 10 min with slow shaking. The strips were rinsed in gel running buffer and transferred to the gel. Gels were sealed with 0.5% (w/v) agarose solution (in gel running buffer). SDS-PAGE was run using a SE 600 Ruby vertical electrophoresis system (Amersham Biosciences) and 13.5% SDS-PAGE gels at 15°C for 4 h. A Typhoon 9400 Variable Mode Imager with Typhoon TRIO Software (Amersham BioSciences) was used to scan the gels. Image Quant Software and DeCyder 2-D Differential Analysis Software version 6.0 (Amersham BioSciences) were used for quantitation and statistical analysis.

Protein Identification

An Ettan Spot Picker (Amersham BioSciences) robotic system was used to pick protein spots from the 2-D gels. Gel slices were washed in a 200 μL ammonium buffer (50:50 acetonitrile/50 mM ammonium bicarbonate), rinsed with 400 μL water, soaked in acetonitrile for 10 min, dried out, rehydrated in trypsin solution (15 μL, 10 μg/mL trypsin in 25 mM ammonium bicarbonate and 3% acetonitrile), and then incubated for 15 min at 37°C with additional 25 mM ammonium bicarbonate. Protein gel slices were incubated at 37°C for 24 h in an Eppendorf Thermomixer R (Eppendorf North America, Westbury, NY) and analyzed by MALDI-TOF/TOF MS.

Antibody–cytotoxin Protein Sequence Analyses

The mAb8866 and mAb8866-CT (MLN8866) in samples were reduced with dithiothreitol (DTT) at 90°C for 10 min, alkylated with iodoacetamide (IAA) in the dark at room temperature for 20 min, and then digested with trypsin at pH 8.5 at 37°C overnight. The disulfide bonds between CT and mAb8866 as well as the interchain disulfide bridges between the antibody heavy chains and light chains were reduced by the DDT to produce several free sulfhydryl groups. The reactive sulfhydryl groups of CT and mAb8866 were then alkylated using IAA to form stable thioether linkages. The enzymatic reaction was terminated by adding 0.1% trifluoroacetic acid (TFA).

MALDI-TOF/TOF MS (Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry) was used to identify levels of CT conjugation in MLN8866 conjugates. 0.5 μL of digested mAb8866 or MLN8866 was spotted directly onto a MALDI plate. 0.5 μL of α-cyano-4-hydroxycinnamic acid (5 mg/mL in 50% ACN/0.1%TFA) was added to the spot and air dried. The dried samples were re-suspended with 3 μL of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 50%ACN/0.1%TFA) and 0.5 μL of resuspended solution was spotted onto a MALDI plate. MALDI TOF/TOF MS data were collected using 4700 Explorer 2.0 (Applied Biosystems, Foster City, CA). Data Explorer 4.5 (Applied Biosystems, Foster City, CA) was used to analyze mass spectra.

The samples were ionized with a 337 nm laser, and 1000 laser shots (scans) were used to produce each spectrum. The instrument was operated in reflector mode with delayed extraction and an accelerating voltage of 20 kV from the ion source. Mascot (Matrix Science, Inc., Boston, MA) was used to identify proteins from NCBInr protein databases based on mass spectrometry tryptic peptide m/z base peaks.

RESULTS

Mortality

Administration of greater than 15 mg/kg MLN8866 resulted in pre-terminal death and/or a clinical condition necessitating euthanasia. One monkey administered a high-dose (30 mg/kg) MLN8866 died before the end of the study on Day 6. Two monkeys, one administered 30 mg/kg and the other 15 mg/kg, were euthanized on Day 6 for humane reasons. The remaining five monkeys were euthanized at the end of the study on Day 8.

Clinical and Pathological Evaluation

Intravenous infusion of MLN8866 was well tolerated in the low dose (5 mg/kg) group. No major adverse effects were observed. Administration of MLN8866 at mid (15 mg/kg) and high (30 mg/kg) dose levels resulted in changes in hematology

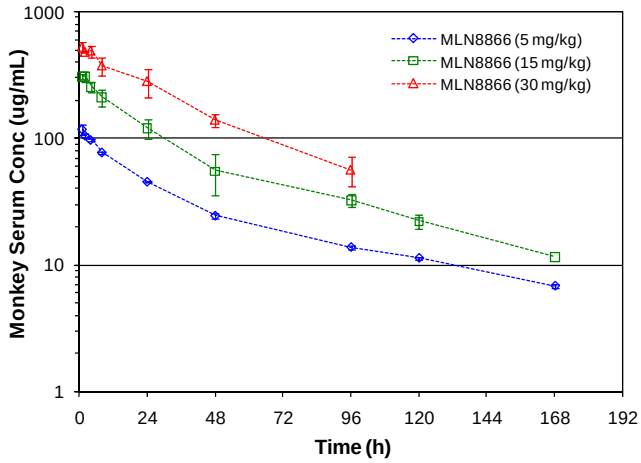

Fig. 1. The mean serum concentrations of MLN8866 *versus* time for the different dose levels (5, 15 and 30 mg/kg MLN8866) administered to Cynomolgus monkeys.

parameters associated with impaired hematopoiesis and bone marrow toxicity. Relative to pre-study values, indicators of circulating erythrocyte mass (red blood cell counts, hemoglobin concentration and hematocrit) decreased to a greater extent in mid dose animals compared to the control and low dose groups (Day 6 or 8). This finding was not observed in the high dose group. The effect could have been masked by the marked dehydration observed in these animals. (Note: hematology analysis was not available for the high dose animal found dead on Day 6). A normal regenerative response in reticulocytes was observed in control and low dose animals. There was no regenerative response in reticulocytes observed in the mid and high dose animals (Day 6 or 8). These observations indicated impaired erythropoiesis in the bone marrow. Neutrophil counts, and to a lesser extent monocyte, eosinophil and basophil counts decreased in mid and high dose animals (Day 6 or 8) relative to pre-study values suggesting impaired leukopoiesis in bone marrow. A decrease was also observed for the control and low dose groups although to a much lesser degree. These findings were consistent with the severe erythroid and myeloid cell depletion observed on histologic examination of the bone marrow in high dose animals.

Changes in the serum chemistry of mid and high dose animals related to MLN8866 administration included observations on Day 2 of increased alkaline phosphatase consistent with minimal to mild hypertrophy and/or hyperplasia of biliary and gallbladder epithelium. An increase in aspartate aminotransferase was also observed. The increase in aspartate aminotransferase was likely related to single cell necrosis and/or apoptosis within intestine, tissues with a squamous epithelial lining, and glandular tissues. Decreased albumin on Days 6 or 8 was also considered test article related. Increased alanine aminotransferase in one high dose animal on Day 2 was of uncertain relationship to the test article, but the finding suggests possible mild hepatocellular injury.

Toxicokinetics

MLN8866 (antibody-conjugate, mAb8866-CT) was detected in the serum of all treated monkeys through Day 8 or until death/euthanasia on Day 6. The mean serum concentrations of MLN8866 *versus* time for the different dose levels (5, 15 and 15 mg/kg MLN8866) are shown in Fig. 1. The Mean TK parameters for MLN8866 are presented in Table I. As shown in Fig. 2, the increase in MLN8866 C_{max} was slightly less than dose proportional. The mean observed clearance (CL) of MLN8866 ranged between 1.2 to 1.5 mL/h/kg and was independent of dose level (5–30 mg/kg) (P = 0.17). The mean observed volume distribution (V_{ss}) was also independent of dose level. The V_{ss} ranged between 60.5 and 63.5 mL/kg.

Serum Protein Profile and Identification

The expression of two serum proteins (at about 9 and 40 kDa) increased with MLN8866 dose level as shown in Fig. 4. The proteins were identified as serum amyloid A protein (SAA) and haptoglobin using MALDI-TOF/TOF MS, Mascot, and NCBInr databases. The relative fold- increase in protein level (Day 4-to-Day 0 ratio) was determined at each dose level based on 2D protein gel quantitation. The fold-increase of SAA in serum was approximately 3.1, 30.9, and 74.2 for doses of 5, 15, and 30 mg/kg MLN8866, respectively. The fold-increase of haptoglobin in serum was approximately 2.6, 4.2, and 9.2 at doses of 5, 15, and 30 mg/kg MLN8866, respectively. The fold-increase in SAA and haptoglobin levels were

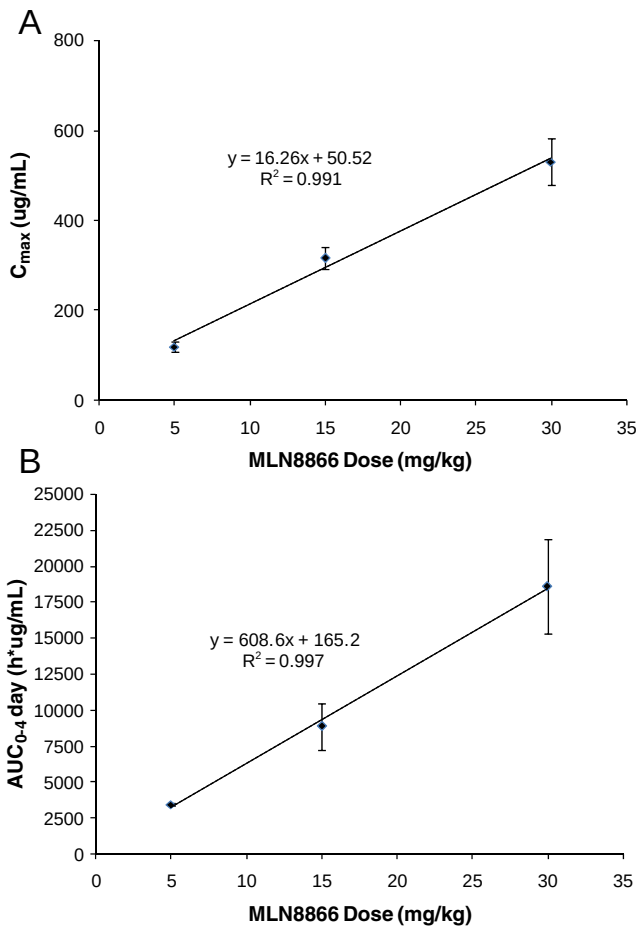

Fig. 2. The relationship of MLN8866 C_{max} (**A**) and $AUC_{0-4\ day}$ (**B**) to dose level.

Table I. The Mean Toxicokinetic Parameters of MLN8866 in Cynomolgus Monkeys

Dose (mg/kg)	C_{max} (μg/mL)	$AUC_{04\ day}$ (h*μg/mL)	C_{max}/dose (μg/mL/mg/kg)	$AUC_{04\ day}$/dose (h*μg/mL/mg/kg)	CL (mL/h/kg)	Vss (mL/kg)	*N* (# of animals)
5	119±11	3,470±106	23.7	693	1.2	60.5	2
15	316±24	8,860±1,620	21.1	590	1.5	63.5	2
30 [a]	530±52	18,600±3,250	17.7	620	1.4	60.9	2

[a] All animals administered the 30 mg/kg dose were euthanized prior to Day 8.

correlated with MLN8866 exposure (C_{max} and $AUC_{0-4\ day}$) as shown in Figs. 5 and 6.

Antibody–cytotoxin Protein Analyses

The cytotoxic conjugated sites to MLN8866 protein structure were identified to help evaluate biological binding activities and pharmacotoxicity. Tryptic peptides were prepared and analyzed using MALDI-TOF MS. The MALDI MS tryptic peptide profiles of mAb8866 (monoclonal antibody) and MLN8866 (immunoconjugate) were compared (Fig. 3). Compared to three tryptic peptides of mAb8866 (*m/z* 1901.5, 1810, 644), three peptides of MLN8866 (*m/z* 2060.5, *m/z* 1969, *m/z* 803) had an additional mass of 159 Daltons (i.e., the mass of IAA thioether linker). The disulfide bonds between CT and mAb8866 were reduced by the DDT to produce several free sulfhydryl groups. The reactive sulfhydryl groups (i.e., the sites of CT linked to mAb8866 via disulfide bonds) of tryptic mAb8866 peptides were alkylated using IAA to form stable thioether linkages.

An example of peptide sequencing is shown in Fig. 3 (for the tryptic peptides at *m/z* 1901.5 from mAb8866 and *m/z* 2060.5 from MLN8866 or CT-mAb8866). The MLN8866 tryptic peptides EIVMTQSPATLSVSPGER (*m/z* 2060.5) and VEIK (*m/z* 803) from mAb8866 light chains were identified as CT conjugated peptides. The MALDI-TOF MS/MS structural fingerprints showed that CT was conjugated to the linker of the glutamic acid N-terminal of EIVMTQSPATLSVSPGER and the lysine residue of VEIK in the mAb8866 light chains, respectively. An additional CT was linked to glycine of the peptide GVQLVESGGGLVQPGGSLR (*m/z* 1969) in the mAb8866 heavy chain. Each MLN8866 monoclonal antibody (mAb8866) was conjugated with zero to six cytotoxic molecules, resulting in a heterogeneous mixture of MLN8866 isoforms.

DISCUSSION

This study demonstrates that treatment of monkeys with the antibody–cytotoxic conjugate MLN8866 (5, 15, 30 mg/kg) results in a dose-dependent increase in serum amyloid A (SAA) and haptoglobin levels in serum. As shown in Fig. 4, SAA levels were significantly increased in all animals 48 h (Day 2) following post end infusion MLN8866. The levels of SAA remained high over the next 2 days in mid (15 mg/kg) and high (30 mg/kg) dose animals. Compared to pre-dose levels, the levels of SAA on Day 4 were increased 3.1, 30.9 and 74.2 fold for doses of 5, 15, and

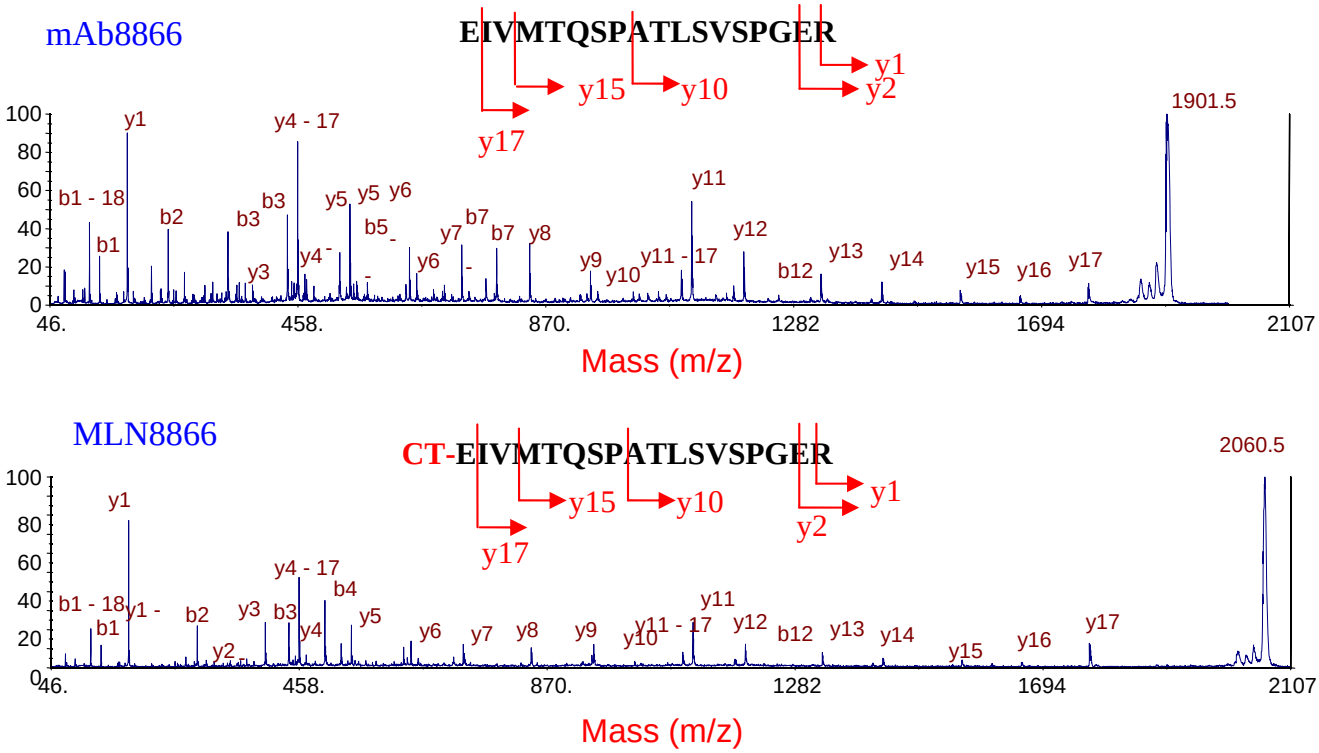

Fig. 3. Comparison of the MALDI-TOF MS spectra of the tryptic peptides ELVMTQSPATLSVSPGER (*m/z* 1901.5) from the unconjugated antibody (mAb8866) and CT-ELVMTQSPATLSVSPGER (*m/z* 2060.5) from the antibody–cytotoxic conjugate (MLN8866).

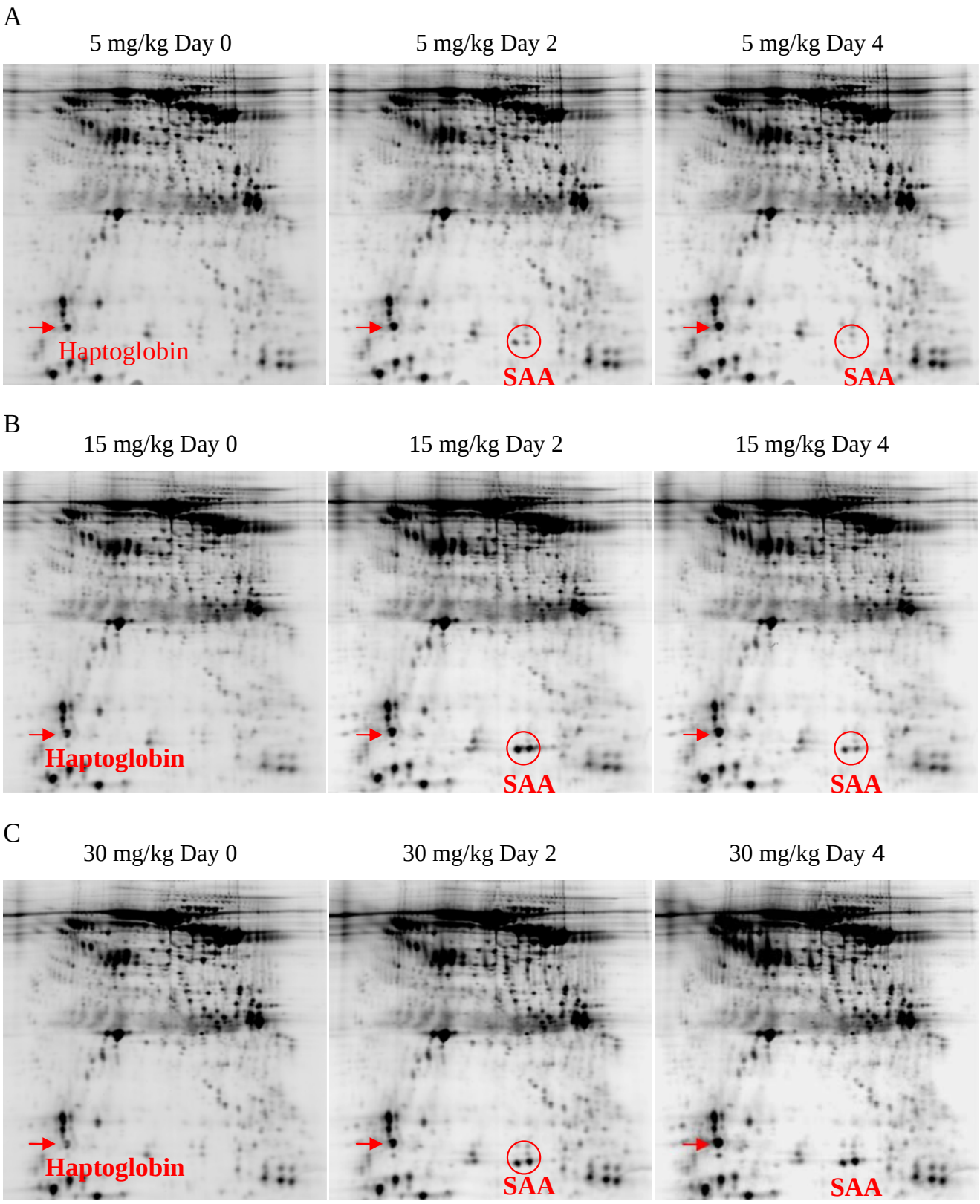

Fig. 4. Serum protein profiles of monkeys treated with 5 mg/kg (**A**), 15 mg/kg (**B**) or 30 mg/kg (**C**) MLN8866 at predose (Day 0) and on Days 2 and 4.

30 mg/kg MLN8866 respectively. Serum levels of haptoglobin were elevated 48 h (Day 2) following treatment with 15 and 30 mg/kg MLN8866 and remained relatively high on Day 4 after treatment. The levels of haptoglobin on Day 4 were increased 2.64, 4.22, and 9.16 times compared to pre-dose levels for treatment with 5, 15, and 30 mg/kg MLN8866, respectively. Haptoglobin levels were not significantly increased following treatment with 5 mg/kg MLN8866. No changes in serum levels of SAA and haptoglobin were observed for monkeys treated with only the vehicle dosing solution.

SAA and haptoglobin are positive acute phase response proteins whose plasma levels increase in response to inflammation. SAA consists of a family of apolipoproteins that bind to high-density lipoprotein. SAA has effects on cholesterol metabolism and causes adhesion and chemotaxis of phagocytic cells and lymphocytes (20,21). Haptoglobin is a plasma α_2-glycoprotein that binds to free haemoglobin and protects the body from oxidative tissue damage. Production of acute phase response proteins is stimulated by inflammatory cytokines released by activated monocytes/macrophages. Stimulation of SAA and haptoglobin production is mediated by interleukin-6-type cytokines and tumor necrosis factor α and synergistically enhanced by glucocorticoids (20–24).

The fold-increase (Day 4-to-Day 0 ratio) in SAA and haptoglobin levels show a quantitative relationship with MLN8866 exposure (C_{max} and $AUC_{0–4\ day}$) as shown in Figs. 5 and 6. The monkeys' exposure to MLN8866 at 15 mg/kg was about 2.5-fold higher than that at 5 mg/kg (Table I). The exposure at 30 mg/kg was approximately 5-fold higher than at 5 mg/kg. The fold-increase inSAA following mid (15 mg/kg) and high (30 mg/kg) doses MLN8866 were approximately 10-fold and 24-fold higher, respectively, than after low (5 mg/kg) dose treatment. The increase in haptoglobin following mid and high dose administration was approximately 1.6-fold and 3.5-fold higher, respectively, than low dose treatment. Further studies are needed to determine the overall characteristic patterns of change in SAA and haptoglobin levels with time over the dosing range used in this study.

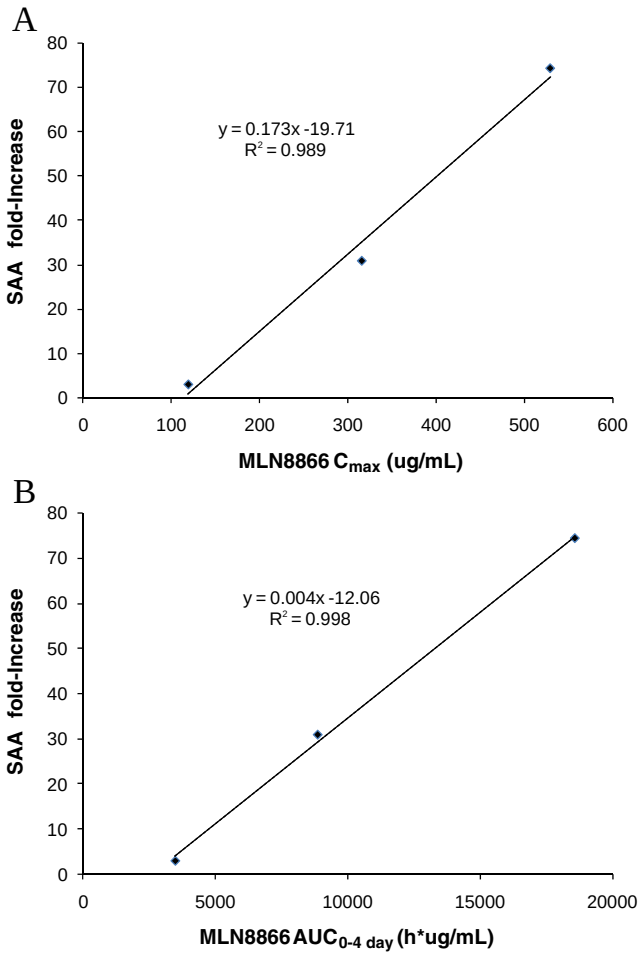

Fig. 5. The relationship of the fold-increase in serum amyloid A (SAA) in monkey serum and MLN8866 C_{max} (**A**) and $AUC_{0–4\ day}$ (**B**).

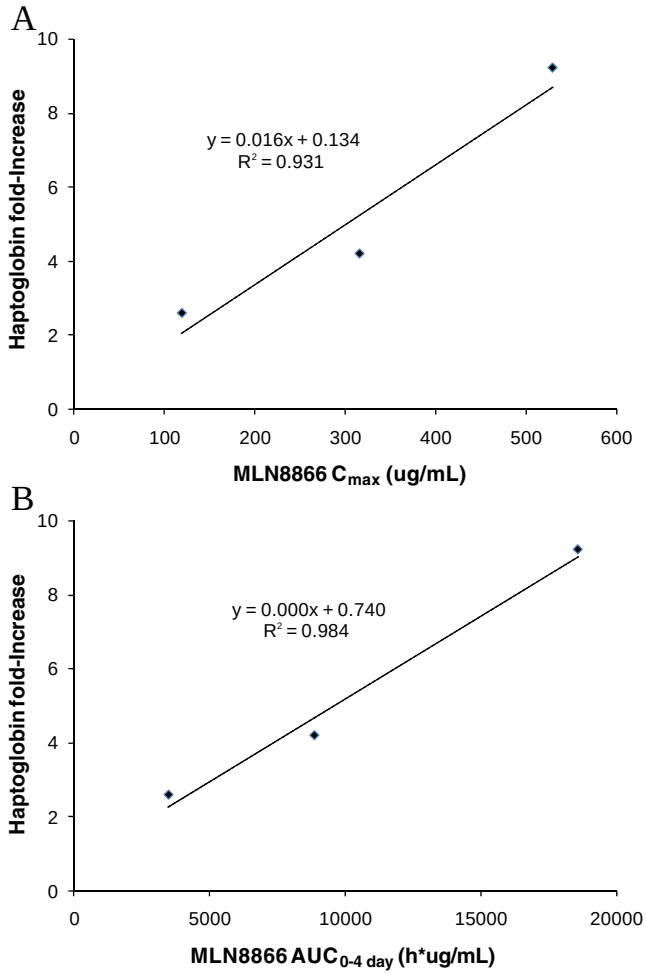

Fig. 6. The relationship of the fold-increase in haptoglobin in monkey serum and MLN8866 C_{max} (**A**) and $AUC_{0–4\ day}$ (**B**).

MLN8866 dose effects on clearance pathways may have an effect on the changes in SAA and haptoglobin levels in response to treatment. Results of this study show that MLN8866 has a low clearance (CL = 1.2 to 1.5 mL/h/kg), low volume of distribution (V_{ss} = 60.5 to 63.5 mL/kg), and a long terminal half-life ($t_{1/2}$ = ~76 h). The mean observed clearance (CL) rates of MLN8866 were 1.2, 1.5, and 1.4 mL/h/kg at doses of 5, 15, and 30 mg/kg, respectively. Additional experiments are required to determine a wider range of MLN8866 dose effects on clearance pathways.

There are two clearance pathways of biotherapeutic antibodies: target clearance (specific) and reticuloendothelial

system (RES) clearance (non-specific) (25). Specific target clearance is saturable, nonlinear, and attributed to the antigen. RES is attributed to phagocytic cells, primarily monocytes and macrophages (accumulating in lymph nodes and the spleen). RES clearance of antibodies is linear and regulated through the interaction with various Fc receptors. Studies by Yang, X.D. *et al.* (26) and Mahler, D.A. *et al.* (27) reported that high clearance at low antibody doses was antigen-mediated antibody clearance. However, at higher doses, antibody clearance was mainly non-specific by RES when the antigen binding sites was saturated. MLN8866, huC242-DM1 (28), and MLN2704 (3) are all conjugated a maytansine analog to a fully humanized monoclonal IgG1 antibody via a disulfide bridge. Systemic clearance of MLN8866 under the conditions of this study was similar to huC242-DM1 clearance (1.43 mL/h/kg) (28) but lower than MLN2704 (J591-DM1) clearance (3.38 mL/h/kg) (3). Further experiments are required to determine whether increases in marker levels were due to the effects of non-specific antibody binding (RES clearance) or the immunoconjugate itself (target clearance).

Serum levels of acute phase proteins (SAA and haptoglobin) also show a quantitative relationship with toxicity assessments. A minimal to mild hypertrophy and/or hyperplasia of biliary and gallbladder epithelium tissue, and single cell necrosis and/or apoptosis within intestine and gallbladder tissues were observed for mid (15 mg/kg) and high (30 mg/kg) dose animals (on Day 2). A normal regenerative response in reticulocytes was observed in control and 5 mg/kg animals. However, no regenerative response in reticulocytes was observed in the 15 mg/kg and 30 mg/kg monkeys (on Day 6 or 8), indicating impaired erythropoiesis in the bone marrow. Neutrophil, monocyte, eosinophil and basophil counts decreased in 15 mg/kg and 30 mg/kg monkeys (on Day 6 or 8) relative to pre-study values suggesting impaired leukopoiesis in bone marrow. These findings were consistent with the increased SAA and haptoglobin levels in 15 mg/kg and 30 mg/kg animals (Fig. 4). MLN8866 caused increased cell death in gastrointestinal and myeloid tissues based on histopathological findings and that this may have led to an acute phase response.

The site of conjugation of the toxin to the antibody is of importance since a diminished interaction between the antibody and its antigen may arise if the conjugation occurs in the complementarity determining region (CDR). Cytotoxic immunoconjugates often exist as a mixture of different isoforms, each having a defined number of toxins linked to the antibody. Conjugation of the cytotoxin to mAb MLB8866 resulted in a heterogenous mixture consisting of mAb MLN8866 conjugated to a range of numbers of cytotoxins (range = 0–6), with each "isoform" potentially having its own unique pharmacological and toxicological properties. The ELISA assay used in this study for the quantitation of MLN8866 did not distinguish between the varying levels of conjugation of the cytotoxin antibody. Additional studies are needed to evaluate the effects of different levels of cytotoxin conjugation.

CONCLUSION

In conclusion, the levels of serum amyloid A protein (SAA) and haptoglobin in monkey serum increased with dosing levels of MLN8866 (5, 15, and 30 mg/kg). The fold-increase (Day 4-to-Day 0 ratio) in SAA and haptoglobin serum levels correlated with MLN8866 exposure (C_{max} and $AUC_{0–4\ day}$) and toxicological outcomes. Histological examination showed increased cell death in gastrointestinal and myeloid tissues of mid (15 mg/kg) and high (30 mg/kg) dose animals compared to low (5 mg/kg) dose animals. These changes may have led to an acute phase response and increase in serum SAA and haptoglobin. The results of this study suggest SAA and haptoglobin may have potential as biomarkers for monitoring pathological changes associated with MLN8866 treatment in Cynomolgus monkeys.

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