Anti-CD74 Antibody-Doxorubicin Conjugate, IMMU-110, in a Human Multiple Myeloma Xenograft and in Monkeys

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Abstract

Purpose: IMMU-110 is a drug immunoconjugate composed of doxorubicin conjugated to the humanized anti-CD74 monoclonal antibody, hLL1, at a doxorubicin/monoclonal antibody ratio of ~8:1 (mol/mol). CD74 is a rapidly internalizing molecule associated with HLA-DR, which has high expression by several tumor types. Here, we describe safety evaluations of IMMU-110 in mice and monkeys as well as efficacy studies in a xenograft model of the human multiple myeloma cell line, MC/CAR.

Experimental Design: *In vitro* binding of IMMU-110 was determined by a cell-based ELISA and cytotoxicity of IMMU-110 assayed with a tetrazolium assay. Pharmacokinetics and biodistribution of radiolabeled IMMU-110 were examined in tumor-free BALB/c mice, and the therapeutic effectiveness was evaluated in severe combined immunodeficient mice bearing MC/CAR cells. Acute toxicity of IMMU-110 was studied in CD74-positive cynomolgus monkeys (*Macaca fascicularis*).

Results: *In vitro*, IMMU-110 specifically binds to CD74 and is cytotoxic against MC/CAR cells. *In vivo*, IMMU-110 displayed a pharmacokinetic and biodistribution profile identical to that of unconjugated hLL1 monoclonal antibody, except for higher kidney uptake. Treatment with a single dose of IMMU-110 as low as 50 μg antibody/mouse (or 1.4 μg doxorubicin/mouse), 5 days post-injection of the multiple myeloma cells, resulted in cure of most mice. In mice, no host toxicity of IMMU-110 was observed at the highest protein dose tested (125 mg/kg). In cynomolgus monkeys, bone marrow toxicity was observed at 30 and 90 mg/kg doses.

Conclusions: The excellent safety and efficacy profile of IMMU-110 supports clinical testing of this immunoconjugate in the treatment of CD74-positive B-cell malignancies.

CD74 (invariant chain, Ii) is a type II transmembrane glycoprotein of 216 amino acids, which associates with the MHC class II α and β chains and directs the transport of class II molecules to lysosomal and endosomal compartments, where the invariant chain is degraded (1). The invariant chain-free MHC class II chains subsequently can bind antigenic peptides and appear on the cell surface for presentation to CD4-positive T lymphocytes. Besides having a role in the trafficking of MHC class II, CD74 is involved in B-cell maturation through activation of transcription mediated by the nuclear factor-κB p65/RelA homodimer and the coactivator $TAF_{II}105$ (2). CD74 was shown recently to bind to a cytokine, macrophage migration inhibitory factor, and to play a role in macrophage migration inhibitory factor–induced activation of signaling events, such as phosphorylation of extracellular signal-regulated kinase-1/2 and prostaglandin E_2 production (3).

CD74 is a particularly appealing target for the development of isotope, drug, and toxin immunoconjugate therapy, because it is internalized extremely rapidly into the target cells following antibody binding; CD74-positive cells internalize and catabolize ~8×10^6 molecules of anti-CD74 monoclonal antibody (mAb) per cell per day (4). Indeed, in prior studies, we have shown improved therapeutic effects in lymphoma models expressing CD74 and given anti-CD74 mAb, LL1, conjugated to diverse radionuclides, including low-energy emitters (5, 6). Prior studies with a conjugate of LL1 with doxorubicin also showed potent activity in a lymphoma model (7). In these initial studies, we showed excellent therapeutic efficacy of IMMU-110 in a xenograft model of human B-cell lymphoma, effecting cures of severe combined immunodeficient (SCID) mice given previously a lethal i.v. injection of Raji lymphoma cells, with a single 350 or 117 μg protein dose of the conjugate (7). To further develop this drug immunoconjugate into a candidate clinical product, we have generated a humanized form of anti-CD74 mAb, hLL1, by complementarity-determining region grafting, which exhibits comparable antigen binding and internalization properties to the parental murine antibody (8, 9).

Recently, we showed using immunohistochemical, flow cytometry, and PCR analyses that CD74 is expressed in a majority (19 of 22) of multiple myeloma clinical specimens

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and cell lines (10). The high prevalence of CD74 antigen expression in multiple myeloma clinical specimens, together with frequently developed resistance to anthracyclines, prompted us to perform a comprehensive preclinical evaluation of IMMU-110 conjugate in a new multiple myeloma xenograft model. Extensive safety and efficacy preclinical testing were conducted, which included *in vitro* binding, cytotoxicity, *in vivo* pharmacokinetics, tissue biodistribution, *in vivo* therapy in a SCID mouse model of a human multiple myeloma cell line, MC/CAR, and acute toxicity evaluation of IMMU-110 in nonhuman primates (cynomolgus monkeys) that express hLL1-reactive CD74. Because, in contrast to lymphomas, there is a paucity of antibody targets in multiple myeloma and yet no drug immunoconjugate has been reported to be active in this tumor type, we reasoned that this would be of particular clinical interest.

Materials and Methods

Materials. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt Cell Titer 96 Aqueous One solution cell proliferation assay was purchased from Promega (Madison, WI). The LumiGLO chemiluminescent substrate system was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Fludarabine phosphate and cyclophosphamide were obtained from Florida Infusion, Inc. (Palm Harbor, FL). Diethylenetriaminepentaacetic acid (DTPA) was purchased from Sigma Chemical (St. Louis, MO), the bifunctional chelate 2-(4-isothiocyanatobenzyl)DTPA from Macrocyclics (Dallas, TX), SMCC-hydrazide from Molecular Biosciences (Boulder, CO), human serum albumin from Alpine Biologics (Blauvelt, NY), and doxorubicin from Pharmacia (Milan, Italy). ^{111}In chloride was purchased from Perkin-Elmer Life Sciences (Boston, MA), and ^{88}Y chloride was obtained from Los Alamos National Laboratory (Los Alamos, NM). The rat anti-idiotypic antibody to hLL1, WP, was prepared at Immunomedics, Inc. (Morris Plains, NJ) as described previously (11).

Animals, cell line, and antibodies. Seven- to 8-week-old female BALB/c homozygous or SCID (C.B-17) mice were purchased from Taconic Farms, Inc. (Germantown, NY). All animal studies were approved by the Center for Molecular Medicine and Immunology's Institutional Animal Care and Use Committee and conducted in compliance with Association of Assessment and Accreditation of Laboratory Animal Care, U.S. Department of Agriculture, and Department of Health and Human Services regulations. The tolerability study in cynomolgus monkeys (*Macaca fascicularis*; 2-5 kg) was done at the Charles River Laboratories, Inc., Sierra Division (Sparks, NV). Treatment of the animals was in accordance with regulations outlined in the U.S. Department of Agriculture Animal Welfare Act (9 CFR, Parts 1-3) and the conditions specified in the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research publication, National Academy Press, 1996).

A CD38-positive human multiple myeloma cell line, MC/CAR (ATCC CRL 8083), was purchased from American Type Culture Collection (Manassas, VA) and cultured in suspension in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20% (v/v) fetal bovine serum, penicillin G (100 units/mL), and streptomycin sulfate (50 μg/mL). Flow cytometry analysis showed that the cells were positive for CD138 (syndecan-1), a prognostic marker for multiple myeloma (data not shown), and 92% of the cells were positive for hLL1 staining. MC/CAR cells produce, but do not secrete, immunoglobulin (IgG1). For experimentation, only cells in the exponential phase of cell growth were used. Daudi and Raji Burkitt's B lymphoma cells were also obtained from American Type Culture Collection and grown as suspension cultures in RPMI 1640 with 10% (v/v) fetal bovine serum, penicillin G (100 units/mL), and streptomycin sulfate (50 μg/mL). hLL1 and a negative isotype-matched control, humanized IgG1 antibody, hRS7 (mAb against epithelial glycoprotein-1), were manufactured at Immunomedics as described previously (9, 12).

Preparation of doxorubicin immunoconjugates. Doxorubicin conjugates of mAbs (hLL1 or hRS7) were prepared in a similar manner as published already (7). Briefly, doxorubicin was reacted with an equimolar amount of the commercially available cross-linker SMCC-hydrazide (4-[*N*-maleimidomethyl] cyclohexane-1 carboxylhydrazide). The doxorubicin-SMCC intermediate was isolated by repeated extraction of starting materials from the reaction mixture with acetonitrile followed by diethyl ether washing and drying to obtain a red powder. The mAb was reacted with a 40-fold molar excess of DTT to reduce disulfide bonds of the IgG, predominately the interchain disulfide bonds. The reduced antibody was separated from low molecular weight materials by diafiltration, and the number of available free thiol groups on the antibody was determined by the Ellman reaction (13), resulting in an average of eight free thiol groups per molecule of IgG. The doxorubicin-SMCC was dissolved in DMSO and reacted with mAb at 4-fold molar excess to the number of available thiol groups. Residual doxorubicin derivatives bound by hydrophobic bonding with protein hydrophobic groups were removed by passage through a column of BioBeads (Bio-Rad, Hercules, CA). The efficacy of purification was shown by purifying hLL1 that was reacted with doxorubicin in the absence of the SMCC linker. The antibody eluted from the column with no detectable absorbance at 496 nm, indicating that BioBeads are effective at removing noncovalently bound doxorubicin.

The amount of doxorubicin linked to the mAb was determined by UV-visible absorbance of the conjugate at 280 and 496 nm, indicating the concentration of the antibody and doxorubicin, respectively. The doxorubicin conjugates of mAbs were shown to be homogeneous by size-exclusion high-performance liquid chromatography when compared with unsubstituted mAb. The amount of free doxorubicin (doxorubicin not covalently bound to antibody) present in the final formulation was determined to be <7% by solid-phase extraction followed by high-performance liquid chromatography (14).

In vitro *cell binding.* Cell binding of IMMU-110 to MC/CAR cells was determined by an indirect cell surface binding ELISA. Briefly, cells were placed in 96-well plates (2×10^5 per well) and subsequently incubated with serial dilutions of IMMU-110, naked hLL1, nonspecific negative control mAb-drug conjugate (hRS7-doxorubicin), or nonspecific mAb (hRS7) on ice for 1.5 hours. After incubation, the plates were washed thrice with PBS and further incubated with a secondary antibody, horseradish peroxidase goat anti-human IgG Fc specific (The Jackson Laboratory, West Grove, PA), for an additional 1 hour at 4°C. At the end of the incubation, plates were washed with PBS as before, and the bound antibody was detected with a luminescent horseradish peroxidase substrate (LumiGLO). The plates were read in an Envision plate reader (Perkin-Elmer Life Sciences), using a luminescence protocol.

In vitro *cytotoxicity.* The *in vitro* cytotoxicity of IMMU-110 was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt dye reduction assay. Briefly, MC/CAR, Daudi, or Raji cells were placed in 96-well plates (2×10^5 per well) and treated with IMMU-110, hRS7-doxorubicin, free doxorubicin, or naked hLL1 for 4 hours at 37°C. Cells were then washed twice with PBS before replacing with fresh medium and incubated for an additional 44 hours at 37°C. At the end of the incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt dye was added, and following an additional 2 to 3 hours at 37°C, the plates were read in an Envision plate reader at 490 nm.

Pharmacokinetics and biodistribution. The pharmacokinetics and biodistribution of radiolabeled hLL1 or IMMU-110 were evaluated in naive (tumor-free) BALB/c mice. The benzyl-DTPA conjugate of hLL1 was prepared using 2-(4-isothiocyanatobenzyl)DTPA as described previously for similar humanized antibodies (15). Matrix-assisted laser desorption/ionization time-of-flight analysis of the benzyl-DTPA-hLL1

conjugate indicated an average of 0.98 benzyl-DTPA per unit of hLL1. The doxorubicin conjugate of benzyl-DTPA-hLL1 was made in an identical manner to the doxorubicin conjugate of hLL1 and was analyzed to contain ~8 molecules of doxorubicin per unit of benzyl-DTPA-hLL1 by UV-visible absorption dual wavelength analyses at 280 and 496 nm. Benzyl-DTPA-hLL1-doxorubicin was radiolabeled with buffered ^{111}In (2.5 mCi/mg) for 30 minutes at room temperature, analyzed by instant TLC on silica gel impregnated glass fiber sheets (Gelman Sciences, Ann Arbor, MI) developed in 10 mmol/L EDTA, and showed an incorporation of 95.9% ^{111}In into the conjugate. It was purified on two successive G-50 spin columns to yield an injectable conjugate that was found to be 99.9% and 99.5% pure by instant TLC and size-exclusion high-performance liquid chromatography analyses, respectively. Benzyl-DTPA-hLL1 was radiolabeled with ^{88}Y at ~0.1 mCi/mg for 30 minutes at room temperature. After two successive spin column purifications, instant TLC and size-exclusion high-performance liquid chromatography indicated 99.0% and 98.9% level purity of ^{88}Y-benzyl-DTPA-hLL1, respectively. Naive (tumor-free) BALB/c mice were injected i.v. with a mixture of 0.001 mCi ^{88}Y-DTPA-hLL1 and 0.02 mCi ^{111}In-DTPA-IMMU-110 supplemented with unlabeled DTPA conjugates of hLL1 or IMMU-110, so that each animal received a total dose of 10 μg each of hLL1 and IMMU-110. At selected times after dosing (1, 2, 4, 16, 48, 72, and 168 hours), groups of five mice were anesthetized and a blood sample was withdrawn by cardiac puncture. Major tissues were removed, weighed, and placed in containers. Blood samples and tissues were counted in a calibrated gamma counter, Minaxi λ Auto-Gamma 5000 series gamma counter (Packard Instrument Co., Downers Grove, IL) for ^{111}In (channels 120-480) and ^{88}Y (channels 600-2,000). A crossover curve was generated to correct for the back scatter of ^{88}Y energy into the ^{111}In counting window.

In vivo *survival experiments.* SCID female mice weighing 18 to 22 g (8-10 mice per group) were pretreated with a chemotherapeutic cocktail of fludarabine phosphate (0.4 mg/mouse) and cyclophosphamide (2 mg/mouse) to abrogate any innate immunity in the mice 3 days before an i.v. injection of 1×10^7 MC/CAR cells. IMMU-110 and other therapeutics were injected i.v. 5 days after tumor cell inoculation. Mice were monitored daily and euthanized when they developed hind leg paralysis or lost 20% of their pretreatment weight.

Tolerability of IMMU-110 in severe combined immunodeficient mice. Groups of SCID mice were dosed with IMMU-110 ranging from a protein dose of 350 μg/mouse (17.5 mg/kg) to 2.5 mg/mouse (125 mg/kg). This translates to a doxorubicin equivalent dose ranging from 10 to 70 μg/mouse. Mice were monitored for visible signs of toxicity and body weight loss. The maximum tolerated dose (MTD) was defined as that at which no deaths occurred, and body weight loss was ≤20% of pretreatment animal weight. On termination of the study (60 days), animals were sacrificed and tissues from selected mice were harvested and subjected to histopathologic analysis.

Tolerability of IMMU-110 in cynomolgus monkeys. A single-dose pilot tolerability study was done to characterize the acute toxicity of IMMU-110 following i.v. infusion to cynomolgus monkeys. A total of 10 monkeys were assigned to five treatment groups (vehicle and IMMU-110 at doses of 3, 10, 30, and 90 mg/kg; n = 2 per group). This translates to a doxorubicin equivalent dose ranging from 0.09 to 2.6 mg/kg. All animals were dosed once as a 1-hour i.v. infusion. The animals were evaluated for changes in clinical observations, body weight, ophthalmic and electrocardiographic variables, and clinical pathology indices [i.e., serum chemistry, hematology, coagulation, and cardiovascular variables (troponins T and I)]. All animals were euthanized following 14 days of observations. At termination, a full necropsy was conducted on all animals, and tissues were examined microscopically.

Data analysis. *In vitro* binding data were analyzed by nonlinear regression to determine the equilibrium dissociation constant (K_d). For *in vitro* cytotoxicity studies, dose-response curves were generated from the mean of triplicate determinations, and IC_{50} values were obtained using the GraphPad Prism software (Advanced Graphics Software, Encinitas, CA). Pharmacokinetic data were analyzed using the standard algorithms of noncompartmental analysis program WinNonlin version 4.1 (Pharsight, Mountain View, CA). In the biodistribution study, comparisons were made between the tissues of mice injected with ^{88}Y-DTPA-hLL1 and ^{111}In-DTPA-IMMU-110 using Student's t test after determination of equality of variance using the f test and a Grubbs' Critical Z test for any outliers. Survival studies were analyzed using Kaplan-Meier plots (log-rank analysis) with GraphPad Prism software. Differences were considered significant at $P < 0.05$.

Results

In vitro cell binding experiments were done to determine the targeting effectiveness of IMMU-110 to CD74-positive MC/CAR cells. IMMU-110 had significantly higher binding to MC/CAR cells than the nonspecific drug conjugate, hRS7-doxorubicin, at all concentrations (Fig. 1). The binding of IMMU-110 was comparable with that of naked hLL1. Both IMMU-110 and naked hLL1-bound CD74 with subnanomolar affinity (K_d of hLL1 = 0.5 ± 0.02 nmol/L versus K_d of IMMU-110 = 0.8 ± 0.2 nmol/L). The binding of hLL1 or IMMU-110 seems to saturate at a concentration of 1 μg/mL. The results indicate that coupling of doxorubicin to hLL1 at the ratio of ~8 molecules of doxorubicin per molecule of antibody did not interfere with the specificity and avidity of hLL1 to CD74-positive MC/CAR cells.

Table 1 gives the 4-hour IC_{50} values of IMMU-110, hRS7-doxorubicin, and free doxorubicin against the multiple myeloma cell line (MC/CAR) and other CD74-positive non-Hodgkin's lymphoma cell lines (Daudi and Raji). IMMU-110 displayed a 20-fold higher cytotoxicity than the nonspecific control doxorubicin conjugate (hRS7-doxorubicin) against MC/CAR cells. In Raji and Daudi cells, IMMU-110 was highly potent (IC_{50} of 1.5 and 0.8 μmol/L, respectively), whereas hRS7-doxorubicin did not show significant cell killing at the highest concentration tested (21 μmol/L). Naked hLL1 did not have significant cytotoxicity at the concentrations tested in all

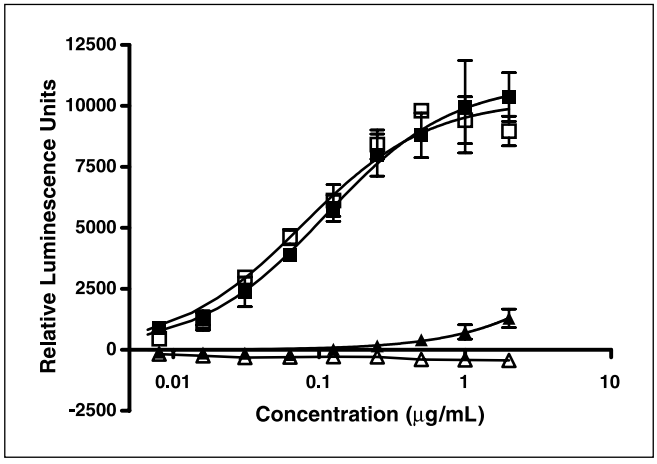

Fig. 1. *In vitro* cell binding of IMMU-110 to MC/CAR cells as a function of concentration. MC/CAR cells were incubated with increasing concentrations of naked hLL1 (□), IMMU-110 (■), nonspecific control antibody hRS7 (△), or nonspecific control mAb-drug conjugate, hRS7-doxorubicin (▲), for 1.5 hours on ice. After incubation, plates were washed thrice with PBS and further incubated with a secondary antibody, horseradish peroxidase goat anti-human IgG Fc specific, for an additional 1 hour at 4°C. Bound antibodies or drug conjugates were detected using a chemiluminescent horseradish peroxidase substrate. *Points,* average of three replicates from one representative experiment; *bars,* SD.

Table 1. *In vitro* cytotoxicity of IMMU-110 against MC/CAR cells

	IC_{50}* (μmol/L, doxorubicin equivalent)		
	Raji	Daudi	MC/CAR
IMMU-110 (hLL1-doxorubicin)	1.5	0.8	0.9 ± 0.7
hRS7-doxorubicin	>21	>21	18.3 ± 0.08
hLL1 (μmol/L antibody)	>3	>3	>3
Free doxorubicin	0.8	1.1	0.08 ± 0.02
Specificity ratio	>14	>26	20

*Drug concentration responsible for 50% growth inhibition in 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt dye reduction assay, calculated with data from three to five separate experiments.

the three cell lines. In CD74-negative multiple myeloma cell lines (ARD and OPM-6), IMMU-110 and nonspecific mAb-doxorubicin conjugate had similar IC_{50} values, both being much less toxic than free doxorubicin, presumably because the drug does not get incorporated (data not shown).

The pharmacokinetics and biodistribution of radiolabeled hLL1 and IMMU-110 were determined in tumor-free BALB/c mice. hLL1 and IMMU-110 were conjugated with the radiometal chelator, benzyl-DTPA, and radiometallic isotopes were attached to the benzyl-DTPA mAb. The benzyl-DTPA conjugates of both hLL1 and IMMU-110 were radiolabeled with ^{88}Y and ^{111}In, respectively, in high yield, with a low percentage of unbound material and no aggregated proteins. Both ^{111}In and ^{88}Y conjugates retained 100% immunoreactivity as determined by size-exclusion high-performance liquid chromatography analysis after mixing with the hLL1 anti-idiotypic mAb, WP (data not shown). Further, *in vitro* binding assays on MC/CAR cells suggested that the avidity of DTPA conjugates of both naked hLL1 and IMMU-110 was similar to that of the naked hLL1 (K_d of hLL1 = 0.9 ± 0.2 nmol/L versus K_d of hLL1-DTPA = 0.9 ± 0.1 nmol/L versus K_d of IMMU-110-DTPA = 1.3 ± 0.2 nmol/L; data not shown). In tumor-free BALB/c mice, radiolabeled IMMU-110 had a pharmacokinetic profile similar to that of radiolabeled mAb hLL1 (Fig. 2). Both hLL1 mAb and IMMU-110 had a biphasic clearance from the circulation characterized by an initial rapid redistribution (α) lasting ~5 hours and a later slower clearance (β) phase. Data points beyond 5 hours were used to compute the terminal half-life ($t_{1/2\ \beta}$) of hLL1 (136 hours) and IMMU-110 (121 hours). IMMU-110 had a mean residence time similar to that of hLL1 (222 hours for IMMU-110 versus 210 hours for hLL1). The rate of clearance of both IMMU-110 and hLL1 was 0.015 mL/h. Thus, the coupling of doxorubicin to hLL1 did not significantly alter the pharmacokinetic profile of hLL1 mAb. At 1-hour postinjection, there was significantly higher IMMU-110 versus hLL1 in the blood ($P < 0.05$; Table 2); however, at all the remaining time points, there were no significant differences. In biodistribution studies, at early time points, there were some instances of significant differences between the two reagents in a few of the tissues. However, there were no trends, except for the kidneys, which were consistently higher for IMMU-110 uptake ($P < 0.05$).

The therapeutic efficacy of IMMU-110 was evaluated in SCID mice bearing MC/CAR cells. We have shown previously that treatment with a single dose of 350 μg protein of IMMU-110 cured 100% of animals in a non-Hodgkin's lymphoma (Raji) xenograft model (7). Therefore, in the first therapy experiment, animals injected with MC/CAR cells were treated with 350 μg protein dose of IMMU-110 5 days postinjection of cells. Treatment with IMMU-110 resulted in curing (i.e., no evidence of tumor on gross pathologic examination) 70% of animals and significantly improving the life span of the remaining 30% of animals over those treated with saline ($P < 0.0001$; Fig. 3). The therapeutic efficacy of IMMU-110 was significantly better than that of naked hLL1 ($P < 0.05$), the mixture of naked hLL1 and free doxorubicin ($P < 0.0005$), free doxorubicin ($P < 0.0001$), or the nonspecific antibody-doxorubicin conjugate, hRS7-doxorubicin ($P < 0.0001$). Treatment with naked hLL1 resulted in curing 30% of animals and significantly improving the life span of the remaining 70% of animals over those treated with saline ($P < 0.005$). Treatment with free doxorubicin at either 10 μg (doxorubicin equivalent dose to 350 μg IMMU-110) or at the MTD of 60 μg/mouse (data not shown) did not improve the life span of mice over those treated with saline ($P > 0.05$). In addition, mice receiving nonspecific mAb-drug conjugate, hRS7-doxorubicin, at a dose of 350 μg/mouse, did not show a significantly improved life span over mice receiving saline ($P > 0.05$).

A second therapeutic study was designed to establish the dose-response relationship of IMMU-110 in the MC/CAR xenograft model (Table 3). SCID mice were injected i.v. with 1×10^7 MC/CAR cells and treated with a single bolus i.v. injection of various doses of IMMU-110 (50, 125, 250, 500, 1,000, and 2,000 μg protein doses of IMMU-110). These translate to 1.5, 3.8, 7.5, 15, 30, and 60 μg doxorubicin equivalent doses, respectively. As controls, groups of mice were treated with equivalent doses of a mixture of naked hLL1 and

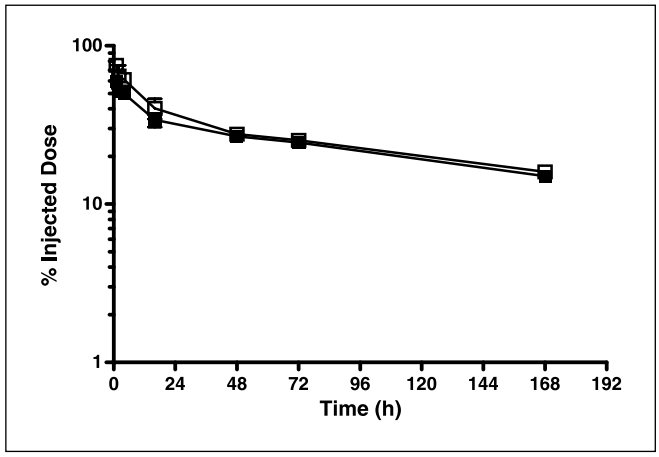

Fig. 2. Blood clearance of hLL1 versus IMMU-110 in naive BALB/c mice. Naked hLL1 and hLL1-doxorubicin (IMMU-110) were conjugated with benzyl-DTPA. Benzyl-DPTA-hLL1 was radiolabeled with ^{88}Y and benzyl-DTPA-hLL1-doxorubicin was radiolabeled with ^{111}In. Naive BALB/c mice were coinjected i.v. with ^{88}Y-benzyl-DTPA-hLL1 (□) and ^{111}In-benzyl-DTPA-IMMU-110 (■). At selected times after dosing, mice were bled by cardiac puncture and a blood sample was counted for radioactivity. *Points,* mean of injected dose in blood ($n = 3$); *bars,* SD.

Table 2. Tissue biodistribution of ^{88}Y-DTPA-hLL1 versus ^{111}In-DTPA-IMMU-110

Tissue	1 h	2 h	4 h	16 h	48 h	72 h	168 h
^{111}In-IMMU-110 (%ID/g)*							
Heart	8.3 ± 1.9	10.5 ± 0.7	11 ± 2.3	7.2 ± 1.2	5.4 ± 0.5	5.0 ± 0.7	2.8 ± 0.3
Liver	12 ± 1.8	13 ± 1	12 ± 1.3	6.9 ± 1.0	7.3 ± 0.8	6.9 ± 0.4	5.1 ± 0.6
Spleen	13 ± 2.4	15 ± 2.1	15 ± 2.8	11 ± 1.6	11 ± 1.0	8.4 ± 2.0	8.7 ± 0.8
Kidney	13 ± 2.9	14 ± 2.6	14 ± 1.9	9.4 ± 1.2	8.3 ± 0.8	9.1 ± 1.1	7.6 ± 0.6
Lung	11 ± 4.7	9.7 ± 0.9	11 ± 1.2	6.9 ± 1.8	6.0 ± 1.1	5.5 ± 1.1	3.3 ± 0.7
Stomach	1.5 ± 0.4	1.8 ± 0.5	2.4 ± 0.3	0.9 ± 0.2	1.5 ± 0.3	1.5 ± 0.4	1.1 ± 0.2
Small intestine	2.7 ± 0.5	3.4 ± 0.6	4.3 ± 0.8	2.3 ± 0.3	2.1 ± 0.2	2.0 ± 0.1	1.3 ± 0.1
Large intestine	1.2 ± 0.3	2.2 ± 0.3	3.0 ± 0.4	2.2 ± 0.4	2.1 ± 0.3	1.8 ± 0.3	1.3 ± 0.2
Muscle	0.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.4	1.7 ± 0.4	1.8 ± 0.2	1.5 ± 0.2	1.0 ± 0.2
Bone	4.1 ± 1.3	4.1 ± 1.7	4.9 ± 0.8	5.4 ± 0.9	5.9 ± 1.3	7.8 ± 1.4	9.9 ± 2.5
W. bone	2.6 ± 1.0	2.8 ± 0.7	3.5 ± 1.4	3.9 ± 0.6	5.5 ± 2.8	8.3 ± 1.6	8.1 ± 1.4
Bladder	1.6 ± 0.5	1.5 ± 0.5	4.3 ± 1.7	6.3 ± 1.6	5.1 ± 2.5	6.2 ± 1.2	3.4 ± 1.9
Skin	1.6 ± 0.3	2.6 ± 0.4	3.1 ± 0.4	4.6 ± 0.6	4.9 ± 0.5	4.4 ± 0.3	3.0 ± 0.3
Blood	48 ± 5.3	45 ± 2.4	42 ± 3.3	26 ± 3.9	21 ± 1.9	19 ± 1.5	12 ± 1.3
^{88}Y-hLL1 (%ID/g)*							
Heart	5.8 ± 1.3	7.5 ± 0.6	8.2 ± 1.7	5.4 ± 1	4.9 ± 0.4	4.6 ± 0.6	3.3± 0.3
Liver	12 ± 2.2	12 ± 1.3	11 ± 1.3	5.7 ± 0.8	5.8 ± 0.6	6.2 ± 0.4	5.9 ± 0.7
Spleen	11 ± 2.5	12 ± 1.8	12 ± 2.4	7.8 ± 1.9	9.1 ± 0.7	7.5 ± 1.8	11 ± 1.1
Kidney	8.4 ± 1.9	9.0 ± 1.9	9.1 ± 1.5	5.6 ± 1.1	5.0 ± 0.5	5.9 ± 0.9	5.9 ± 0.6
Lung	8 ± 3.2	6.8 ± 0.8	8.1 ± 1.7	5.1 ± 1.5	5.1 ± 1.0	4.9 ± 1.2	3.6 ± 0.8
Stomach	1.1 ± 0.3	1.3 ± 0.4	1.8 ± 0.2	0.7 ± 0.2	1.3 ± 0.2	1.4 ± 0.4	1.3 ± 0.2
Small intestine	2.0 ± 0.4	2.7 ± 0.6	3.5 ± 0.7	1.8 ± 0.3	1.9 ± 0.2	1.8 ± 0.1	1.5 ± 0.1
Large intestine	0.8 ± 0.2	1.6 ± 0.3	2.3 ± 0.4	1.6 ± 0.3	1.7 ± 0.3	1.5 ± 0.3	1.4 ± 0.2
Muscle	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.3	1.3 ± 0.3	1.5 ± 0.2	1.4 ± 0.2	1.1 ± 0.3
Bone	3.0 ± 1.1	3.0± 1.3	3.4 ± 0.6	3.9 ± 0.5	4.7 ± 1.2	6.6 ± 1.4	9.7 ± 2.0
W. bone	1.9 ± 0.7	1.9 ± 0.4	2.4 ± 0.9	2.7 ± 0.4	4.6 ± 2.3	6.8 ± 1.2	7.7 ± 1.1
Bladder	1.6 ± 0.5	1.3 ± 0.4	3.6 ± 1.6	5.4 ± 1.2	5.4 ± 0.8	5.3 ± 1.1	3.8 ± 2.3
Skin	1.2 ± 0.2	1.9 ± 0.3	2.3 ± 0.3	3.6 ± 0.5	4.4 ± 0.4	4.0 ± 0.3	3.5 ± 0.4
Blood	60 ± 7.6	57 ± 11	42 ± 3.3	31 ± 6.1	22 ± 2.4	20 ± 1.5	12 ± 1.6

*Mean ± SD of % injected dose per gram in various organs (n = 5).

free doxorubicin. In the group of animals treated with various doses of IMMU-110, the cure rate was in the range of 75% to 100% (Table 3), so that a clear dose-response could not be established because of the high potency of the lowest doses given; mice treated with as little as a 50 μg protein dose of IMMU-110 cured 88% of the animals. All the groups of mice treated with the mixture of naked hLL1 and free doxorubicin also had significantly improved life span compared with mice injected with saline or the nonspecific drug conjugate ($P < 0.005$), although the efficacy was significantly less than that of the IMMU-110 conjugate ($P < 0.05$). Mice injected with a 2,000 μg protein dose of nonspecific control mAb-doxorubicin conjugate, hRS7-doxorubicin, did not have a significantly improved life span over mice injected with saline ($P > 0.05$). In another study done in SCID mice bearing Burkitt's lymphoma (Daudi cells), 100% of animals were cured with a single i.v. 350 μg protein dose of IMMU-110 (data not shown).

In tolerability studies done in SCID mice, those injected with IMMU-110 did not exhibit any signs of toxicity, such as weight loss or changes in body appearance, even at the highest possible (volume limitation) single-dose tested, 2.5 mg protein dose of IMMU-110/mouse (125 mg/kg protein dose or 3.6 mg/kg doxorubicin dose). Thus, the MTD was not reached in this single-dose study. All major organs of the body, including heart, liver, lung, spleen, small intestine, large intestine, and reproductive organs, were histopathologically normal.

To determine if cynomolgus monkeys were a relevant model for preclinical toxicology studies, cross-reactivity studies of hLL1 with tissues from healthy human, cynomolgus, and rhesus monkey tissues were done. The staining pattern of hLL1 for rhesus and cynomolgus monkey tissues was observed to be similar with that for human tissues (data not shown). hLL1 specifically stained CD74-positive cells in lymph nodes but did not cross-react with fibroblasts. hLL1 reacted with potentially CD74-expressing cells (lymphocytes and dendritic cells) in all tissues examined and hematopoietic precursors in bone marrow and mononuclear cells in the spleen. Some endothelial cell staining also was observed in the heart, liver, kidney, lymph node, and spleen. Acute safety studies in CD74-positive cynomolgus monkeys revealed no IMMU-110-related morbidity or mortality at any dose. The clinical observations, physical examinations, body weight, ophthalmic examination, and electrocardiograms were normal. Clinical pathology indices, including serum chemistry, hematology, coagulation variables,

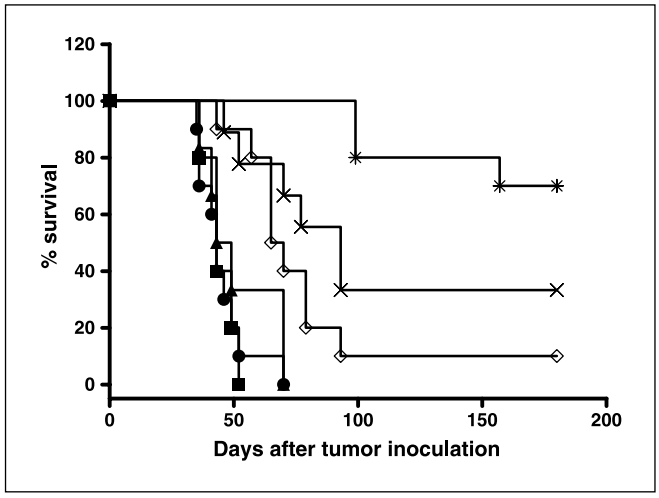

Fig. 3. Therapeutic efficacy of IMMU-110 in SCID mice injected with MC/CAR cells. SCID mice (8-10 mice per group), pretreated with fludarabine phosphate and cyclophosphamide, were inoculated i.v. with 1×10^7 MC/CAR cells. After 5 days, mice were treated with a single bolus injection of 350 μg IMMU-110 (✱). Control animals were treated with saline (■), 10 μg free doxorubicin (▲), 350 μg nonspecific drug conjugate, hRS7-doxorubicin (●), 350 μg naked hLL1 (×), or a mixture of 350 μg naked hLL1 and 10 μg free doxorubicin (◇).

and urinalysis, also were normal, except as noted below. In addition, there were no IMMU-110-related effects on the specific markers of cardiac injury, troponin T or troponin I. However, at the dose of 30 mg/kg, bone marrow hypocellularity was observed histologically, but without depletion of circulating blood cells, whereas both bone marrow and peripheral blood cell reductions were observed for those animals given the 90 mg/kg dose. Both animals treated at this dose exhibited decreased indicators of circulating RBC mass (i.e., RBC and hemoglobin concentration). By day 14, RBC and hemoglobin concentration were ~25% lower than day 1 (predose). WBCs diminished by ~25% of their predose value, including a significant drop in neutrophils. Platelet counts dropped for both animals, although the degree was highly variable. The bone marrow toxicity was considered most likely due to the cytotoxic effects of the doxorubicin component of IMMU-110. Another histopathologic finding potentially related to IMMU-110 administration was the presence of pigment in lymph nodes, particularly in animals dosed at 10, 30, and 90 mg/kg. However, because there was no evidence of tissue damage, the presence of this pigmentation was not considered to be adverse. Due to the small number of monkeys used, no statistical analysis could be done.

Discussion

This investigation shows that the IMMU-110 conjugate has considerable potential for the treatment of multiple myeloma. *In vitro* cell binding experiments established that it specifically binds to CD74-positive MC/CAR cells and that the coupling of doxorubicin to hLL1 does not alter the avidity of antibody to cells. *In vitro* cytotoxicity results established that IMMU-110 is specifically cytotoxic and highly potent against CD74-positive MC/CAR cells. Coupling of doxorubicin to hLL1 at the ratio of ~8 drug molecules per molecule of antibody did not significantly alter the pharmacokinetic profile of the antibody component in the conjugate. However, the experiments reported here only represent the pharmacokinetics and biodistribution profile of the antibody component of IMMU-110, because the radioisotope is exclusively attached to the DTPA chelate that is coupled to the hLL1 mAb. Although there were some instances of transient difference in normal tissue uptake at the earliest time points (1-4 hour postinjection), there were no consistent trends, with the exception of kidneys. It is not clear at this time why we observed higher renal uptake of IMMU-110 versus naked hLL1 in these studies. It should be noted that IMMU-110 has a isoelectric point of 0.06 pH units higher than naked hLL1, indicating that it is a molecule with greater positive charge (data not shown). In addition to differences in charge, there are also differences in the overall hydrophobicity of the protein on addition of ~8 doxorubicin groups on to the molecule. These changes in charge, structure, and hydrophobicity could contribute to altered renal clearance. Because hLL1 does not cross-react with murine CD74, it is likely that the differences observed in renal uptake of hLL1 versus IMMU-110 will not affect the toxicity profile of the immunoconjugate. Further, in both SCID mice and monkeys, no acute IMMU-110-related toxicity was observed in the kidneys on histopathologic examination, and no abnormal urinalysis variables were observed.

Excellent therapeutic results with IMMU-110 were obtained in a mouse xenograft model of multiple myeloma, which is likely due to the specific binding and extremely rapid rate of

Table 3. Dose-response effects of IMMU-110 and mixture of naked hLL1 and free doxorubicin in MC/CAR-bearing SCID mice

Group	Median survival time (d)*	Long-term survivors (survival time >180 d)
Control	38	0/8
Nonspecific mAb-doxorubicin conjugate, hRS7-doxorubicin (2,000 μg)	47	0/8
IMMU-110 (50 μg)	>180	7/8
IMMU-110 (125 μg)	>180	8/8
IMMU-110 (250 μg)	>180	8/8
IMMU-110 (500 μg)	>180	8/8
IMMU-110 (1,000 μg)	>180	6/8
IMMU-110 (2,000 μg)	>180	7/8
hLL1 (50 μg) + free doxorubicin (1.5 μg)	75	2/8
hLL1 (125 μg) + free doxorubicin (3.8 μg)	121	2/8
hLL1 (250 μg) + free doxorubicin (7.5 μg)	91	2/8
hLL1 (500 μg) + free doxorubicin (15 μg)	123	4/7
hLL1 (1,000 μg) + free doxorubicin (30 μg)	96	1/7
hLL1 (2,000 μg) + free doxorubicin (60 μg)	116	0/7

*Median survival times (n = 7-8) of SCID mice treated with a single dose of therapeutics 5 days after inoculation of MC/CAR cells.

internalization of IMMU-110. The internalization rate of hLL1 is ~100 times faster than that observed with other mAbs that are generally considered to be rapidly internalized, such as mAbs to CD19, CD22, and the transferrin receptor (4). The literature suggests that internalization is a requirement for improved therapeutic efficacy of other mAb-based therapeutics, such as immunoliposomal doxorubicin (16) or similar doxorubicin-mAb conjugates (17). We hypothesize that following internalization into the low pH compartments of the cell (lysosomes and endosomes) the hydrazone linkage between hLL1 and doxorubicin is hydrolyzed to release free doxorubicin. Free doxorubicin can survive the acid-labile environment and passively diffuse to the nucleus and other organelles of the cell to elicit antitumor activity. The CD74 antigen is most likely degraded in lysosomes. A nonspecific drug conjugate (hRS7-doxorubicin) dosed at up to 2 mg protein/mouse failed to show any therapeutic effect, thus confirming the specific targeting of IMMU-110.

Free doxorubicin given at equivalent doses as present in the immunoconjugate or given at the MTD were not effective in this model possibly due to its rapid redistribution leading to low plasma drug concentrations. Treatment with naked hLL1 mAb also resulted in significant improvements in the life span of mice implanted with MC/CAR cells. *In vitro*, we have shown previously that hLL1 inhibits cell proliferation and induces apoptosis in the presence of an appropriate cross-linking agent but does not show antibody-dependent, cell-mediated cytotoxicity or complement-mediated cytotoxicity (9). The hLL1 component of the conjugate may thus be contributing to the overall activity of IMMU-110 in these studies. It should be noted, however, that the doxorubicin conjugate is significantly more effective than the naked antibody alone or the mixture of naked hLL1 and free doxorubicin in equivalent concentrations as the drug conjugate, thus confirming the improved and specific efficacy of this targeted drug immunoconjugate.

The therapeutic index, or the ratio of toxic dose to the effective dose, of any therapeutic agent is an essential criterion in considering its clinical potential. In SCID mice, IMMU-110 failed to show any toxicity, including myelotoxicity and cardiotoxicity, up to the maximum single dose tested of 2.5 mg/mouse (protein dose = 125 mg/kg; doxorubicin equivalent dose = 3.6 mg/kg), although the MTD of free doxorubicin was reached at 3 mg/kg. Therefore, conjugation of doxorubicin to hLL1 mitigated the toxicity of free doxorubicin in SCID mice. Doses higher than 2.5 mg IMMU-110 could not be tested due to limitations in the volume of material that can be injected via the i.v. route. Further, in therapy experiments, the minimum dose tested of 50 μg/mouse (equivalent to 1.5 μg doxorubicin dose/mouse) cured 88% of the animals. These data indicate that the therapeutic index of IMMU-110 in the MC/CAR mouse xenograft model is >50-fold. Thus, the comprehensive dose escalation efficacy and toxicity evaluation studies described here confirm that, in addition to non-Hodgkin's lymphoma, IMMU-110 is well tolerated and is highly active in this CD74-positive multiple myeloma xenograft model. We appreciate that these experiments could underestimate the therapeutic index of IMMU-110 in SCID mice; therefore, future experiments will aim at determining the minimum effective and the MTDs.

Lack of cross-reactivity with the rodent CD74 makes safety studies in mice less relevant. Therefore, we did preliminary tolerability studies of IMMU-110 in nonhuman primates (cynomolgus monkeys), because hLL1 reacts with monkey CD74. Although these acute toxicity studies were done in a few animals, they suggest that IMMU-110 is well tolerated up to 30 mg/kg, at which level the first signs of bone marrow toxicity appeared. This translates to a human clinical infusional dose of ~1110 mg/m^2 mAb dose or a 32 mg/m^2 doxorubicin dose. Although bone marrow toxicity was observed at 30 mg/kg, IMMU-110 had no acute cardiotoxicity and did not show any adverse effects to other major organs at doses up to 90 mg/kg. Future experiments will aim to determine the recovery rate of hematologic variables following IMMU-110 treatment, especially because significant bone marrow suppression was not observed at the doses tested.

The frequent and high-level expression of CD74 on multiple myeloma clinical specimens, together with very encouraging preclinical safety and efficacy data, suggest that this disease is an excellent target for therapy with this drug immunoconjugate. In recent clinical trials, substitution of Stealth liposomal doxorubicin for free doxorubicin in the vincristine-Adriamycin-dexamethasone regimen was shown to improve the safety profile and convenience of the induction regimen without compromising efficacy (18). In a similar fashion, IMMU-110 could replace free doxorubicin in the vincristine-Adriamycin-dexamethasone treatment schedule. In addition, LL1 has been conjugated to a liposome bearing an anticancer agent (dioleoylated derivative of the anticancer drug 3′,5′-*O*-dioleoyl-FdUrd) and shown to have improved efficacy *in vitro* (19).

A major obstacle to the successful treatment of multiple myeloma is the emergence of drug resistance. Although 60% to 80% of patients treated with melphalan and prednisone or vincristine-Adriamycin-dexamethasone achieve an objective response to the induction therapy, almost all patients eventually relapse. The tumor cells in the majority of vincristine-Adriamycin-dexamethasone–refractory patients exhibit multidrug resistance due to the overexpression of P-glycoprotein (20–23). We hypothesize that P-glycoprotein-mediated efflux could be circumvented by delivering drugs (e.g., doxorubicin) via a rapidly internalizing antibody (hLL1) to CD74-positive tumor cells, thus bypassing the activity of multidrug resistance transporters. Indeed, a recent study reported that an immunoconjugate composed of doxorubicin conjugated to an antibody against insulin-like growth factor receptor allowed bypassing of the P-glycoprotein-mediated resistance *ex vivo* and *in vivo* (24). Hence, the role of IMMU-110 in reversing P-glycoprotein-mediated resistance will be the subject of future studies.

In conclusion, we have shown that IMMU-110 is an antibody-drug conjugate with a potent efficacy and excellent safety profile in an animal model of human multiple myeloma in addition to our previously shown efficacy in a non-Hodgkin's lymphoma model. IMMU-110 also showed an acceptable safety profile up to 30 mg/kg in pilot acute safety studies in cynomolgus monkeys. Therefore, this drug immunoconjugate represents a rational choice for clinical development as a potential new therapeutic for multiple myeloma, non-Hodgkin's lymphoma, and possibly other CD74-positive neoplasms.

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