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Preparation and Characterization of Monoclonal Antibody Conjugates of the Calicheamicins: A Novel and Potent Family of Antitumor Antibiotics

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ABSTRACT

The calicheamicin family of antitumor antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Their potency suggested that the calicheamicins would be excellent candidates for targeted delivery and a hydrazide prepared from the most potent and abundant of the naturally occurring derivative, $\gamma_1{}^I$, was linked to oxidized sugars on CT-M-01, an internalizing anti-polyepithelial mucin antibody. The conjugates retained the immunoreactivity of the unmodified antibody and were specifically cytotoxic toward antigen positive tumor cells *in vitro* and *in vivo*. Hydrazide analogues of less potent calicheamicin derivatives were also prepared and conjugated to CT-M-01. Comparison of the therapeutic efficacy of the conjugates against the MX-1 xenograft tumor implanted s.c. in nude mice showed that conjugates of derivatives missing the rhamnose, a sugar residue that is part of the DNA binding region of the drug, were not as promising as antitumor therapies. However, conjugates of two derivatives, $\alpha_3{}^I$ and *N*-acetyl-$\gamma_1{}^I$, in which the rhamnose residue is present but the amino sugar residue of the parent drug is either missing or modified, significantly inhibited tumor growth over a 4-fold dose range and produced long-term tumor-free survivors. Sterically hindering methyl groups adjacent to the disulfide in the linker further increased the therapeutic window of these potent conjugates.

INTRODUCTION

Over the past decade, many MoAb[2] conjugates of radioisotopes, protein toxins, and cytotoxic drugs have been prepared and tested in model systems. Radiolabeled MoAbs have proven to be effective imaging agents in the clinic and are showing some promising therapeutic results for the treatment of lymphomas and leukemias (1–4). Protein toxin-MoAb conjugates (immunotoxins) are likewise progressing in clinical trials, particularly for the treatment of lymphomas (5). In contrast, clinical progress with cytotoxic drug-MoAb conjugates has been less promising, despite encouraging preclinical data (6–8). An important factor limiting the success of drug-MoAb conjugates is the relatively low potency of standard chemotherapeutics, which is further reduced by their conjugation to MoAbs (9). With most clinically used anticancer drugs, a large number of drug molecules must be taken up by each cell to achieve cell death. Poor tumor penetration, low antigen expression, and antigenic heterogeneity limit the number of MoAb-targeted drug molecules that can reach each cell (10), and with many standard drugs, that number is too low to produce clinically significant antitumor effects. In contrast, the protein toxins such as ricin, which kill cells in a catalytic manner, have been used to prepare immunotoxins (protein-MoAb conjugates) that have produced highly significant antitumor effects *in vivo*. However, the inherent immunogenicity of the protein toxins themselves compromises the clinical utility of immunotoxins in all except severely immunocompromised patients (11–13).

Recently, several groups have concentrated their targeting research on low molecular weight cytotoxics with potencies intermediate between the protein toxins and anticancer agents such as vincristine or Adriamycin. Preclinical evaluations of conjugates of highly potent low molecular weight cytotoxics such as the trichothecenes (14) and maytansines (15), both of which are significantly more potent than vincristine, have been reported. In our own work, we have used a new family of particularly potent anti-tumor antibiotics, the calicheamicins, which were originally identified by their impressive potency in a screen for DNA damaging agents (16). The antibiotics, isolated from a broth extract of a soil microorganism *Micromonospora echinospora calichensis,* were termed the calicheamicins and later were identified as members of a new class of potent enediyne containing antibiotics which includes the esperamicins, dynemycin, and neocarzinostatin (17). The binding of the most potent of the calicheamicins, $\gamma_1{}^I$, in the minor groove of DNA and the resulting sequence-specific DNA cleavage has been described (18, 19). The calicheamicins are relatively small molecules with molecular weights in the range of M_r 1500. The small size combined with a unique mechanism of action and extreme potency suggested that the calicheamicins might be good candidates for MoAb targeting.

In this article, we describe the structure-activity profile of a series of calicheamicin analogues conjugated to the anti-PEM MoAb, CT-M-01, which binds to an internalizing antigen present on a number of solid tumor types including breast, ovarian, colon, and non-small cell lung carcinomas. The goals of these studies were 3-fold: (*a*) to determine the feasibility of preparing calicheamicin conjugates with antitumor activity; (*b*) to investigate the correlation between the potency of structural analogues of the calicheamicins and their therapeutic potential as conjugates; and (*c*) to test linker variations in an effort to optimize the therapeutic window for the conjugates.

MATERIALS AND METHODS

Monoclonal Antibodies. CT-M-01, also known as 7F11C7, is an internalizing IgG_1 that recognizes the PEM antigen, located preferentially on the cell surface of human cancerous epithelial cells. Originally developed to recognize the human milk fat globule membrane of breast carcinomas, CT-M-01 binds with high affinity to a broad spectrum of solid tumors and is internalized into its target cells after binding (20). The murine CT-M-01 and MOPC-21 used in these studies were produced and purified from tissue culture supernatant by Celltech, Ltd., United Kingdom. MOPC-21, a secreted murine myeloma IgG_1 that does not bind to any mouse antigen or human xenograft tumors, was used to prepare nonbinding isotype-matched MoAb conjugates (21, 22). Lym 1, an IgG_1 reactive with human B-lymphocytes, but not with solid tumor lines, was used as a negative control in the *in vitro* binding studies (23) and was produced and purified from tissue culture supernatants by Techniclone, Inc.

Iodination of MoAbs for use in the competitive binding assays was accomplished using the diiodinated Bolton Hunter reagent, *N*-succinimidyl 3-(4-hydroxy-3-[^{125}I]diiodophenyl)propionate, purchased from New England Nuclear. The MoAb was iodinated at pH 7.4 in phosphate buffer at a protein concentration of 1.25 mg/ml, using 5 mCi of Bolton-Hunter reagent for each 2.5 mg of protein iodinated. The labeled MoAb was purified by gel filtration chromatography and exhaustive dialysis. The specific activity of the ^{125}I-CT-M-01 ranged from 0.3 to 0.5 μCi/μg protein and the labeling did not interfere with the antigen binding of the MoAb.

Cells and Culture Methods. An MX-1 human breast carcinoma cell line, established in this laboratory, was used as the experimental target for CT-M-01

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[1] To whom requests for reprints should be addressed.

[2] The abbreviations used are: MoAb, monoclonal antibody; PEM, polyepithelial mucin; PBS, phosphate buffered saline; MTD, maximum tolerated dose; MED, minimum effective dose; TR, therapeutic ratio (= MTD/MED); DMF, dimethyl formamide; OD, optimal dose; DM, dimethyl; PSAG, pseudoaglycone; $\gamma_1{}^I$-DM-hyd, $\gamma_1{}^I$ dimethyl hydrazone; $\gamma_1{}^I$-hyd, $\gamma_1{}^I$ hydrazone; t, triplet.

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in vitro (24). The tissue culture line was derived as a clonal isolate in agarose medium from a tumor transplant of human breast carcinoma (MX-1) growing in athymic mice. MX-1 cells were propagated in RPMI 1640 containing 5% fetal calf serum, 5 μg/ml insulin and transferrin, 5 ng/ml selenium, and 50 μg/ml gentamicin. Cultures were maintained in a humidified, 5% CO_2 incubator at 36°C and were subcultured once each week by scraping the loosely attached cells from the culture flasks. The MX-1 cells bind approximately 200,000 molecules of CT-M-01/cell *in vitro*, approximately 25% of which is internalized within 4 h.[3] The XC rat sarcoma cell line was obtained from the Naval Biomedical Laboratory, Oakland, CA. These cells were propagated as monolayers and subcultured following dispersal with 0.25% trypsin. For *in vitro* cytotoxicity tests, streptomycin, 50 μg/ml and penicillin, 50 units/ml, were incorporated into the medium.

Immunoreactivity of the MoAbs and conjugates was measured by a direct radioimmunoassay comparing the competitive binding of the test sample with that of iodinated CT-M-01. For each assay, 10^6 MX-1 cells in 0.1 ml were incubated with 0.05 ml of 4 μg/ml ^{125}I-CT-M-01 (specific activity, ~0.3–0.5 μCi/μg) and 0.05 ml of serial 4-fold dilutions of the test samples, the highest concentration being 200 μg/ml. After a 1-h incubation, the cells were washed 3 times with Dulbecco's PBS and transferred to fresh tubes and counted. Binding inhibition curves were plotted and 50% inhibitory concentration values for each conjugate were compared with that of the unmodified control antibody as a relative measure of retention of immunoreactivity.

In Vitro Cytotoxicity Assays. To evaluate cytotoxicity in drug or conjugate samples, viable cells (10^5/0.2 ml) were aliquoted into 15-ml test tubes which contained 0.2 ml of the sample to be tested at the appropriate concentration. Concentrations were all normalized to microgram equivalents of calicheamicin γ_1^I. Tubes were vortexed and incubated at 37°C for 7 min, and the pellets washed 3 times with 8 ml of medium. One ml of medium was added to each pellet, the cells were vortexed, and 0.2 ml was removed and placed in a well of a 96-well plate (2×10^4 cells). These cells were incubated for 3 days, at which time 0.1 ml of supernatant was removed and replaced with 0.2 mCi of [^{3}H]thymidine in 0.1 ml of fresh medium. Incubation was resumed for an additional 24 h at which point the cells were harvested and counted. Growth inhibition curves of each drug or conjugate were plotted and the 50% inhibitory concentration value (concentration of drug equivalents needed for 50% [^{3}H]-thymidine uptake inhibition) of each sample was determined.

In Vivo Tests for Anti-tumor Activity. Drug and drug hydrazides were tested for antitumor activity against lymphocytic leukemia P388 in mice according to the protocol described by Geran *et al.* (25). As expected, the MoAb conjugates which do not recognize the murine tumors were inactive in this system and were evaluated instead for antitumor effects against human breast xenograft tumors implanted in athymic mice by procedures previously described (26). The two breast carcinomas studied were the ductal cell MX-1 and undifferentiated MX-2, both obtained as xenograft transplants from the Division of Cancer Treatment and the Division of Cancer Prevention of the National Cancer Institute. Tumors were implanted s.c. into athymic mice and test samples were inoculated i.p. or i.v. at several dose levels, every 4 days for a total of 3 doses, starting 2–3 days after tumor implantation. Each test group contained 6 mice and in each test a control group of 10 mice were given injections of a volume of PBS, pH 7.4 (the conjugate vehicle), equivalent to the volume of the highest conjugate dose (usually 0.5 ml). Tumor mass was determined by measuring the tumor diameter once weekly for 35–49 days post-tumor implantation. Significant antitumor activity was defined as a sustained 58% inhibition of mean tumor mass compared with untreated controls in groups with greater than 65% survivors. A TR was defined as the MTD/MED and used as a measure of the therapeutic window for the conjugates or drugs tested.

Preparation of Thiol Hydrazides. 3-Mercaptopropionyl hydrazide used in the preparation of the "simple" hydrazide conjugates was prepared as follows: 9.2 ml of methyl 3-mercaptopropionate (83 mmol) were added dropwise over 2 h to 5.4 ml (3 eq) of anhydrous hydrazine in 100 ml of refluxing tetrahydrofuran under argon. After an additional 2 h at reflux, the reaction mixture was cooled and the solvent was removed in a vacuum. The excess hydrazine was removed by addition of toluene and reconcentration. The crude product was purified by flash silica gel chromatography eluting first with 5% ethyl acetate in chloroform and then 20% methanol in chloroform.

3-Mercapto-3-methylbutyryl hydrazide used in the preparation of the "dimethyl" hydrazides was prepared as follows: 9 ml (1.3 eq) of thiolacetic acid was added to 10 g of 3,3-dimethyl acrylic acid. This mixture was heated at reflux under argon for 6 h. The excess thiolacetic acid was removed under aspirator vacuum and the resultant oil was dissolved in 100 ml absolute ethanol containing 200 μl of concentrated sulfuric acid. This reaction was refluxed for 24 h before adding 16 ml of hydrazine and then for an additional 24 h under argon. The reaction mixture was concentrated and the residue was dissolved in a mixture of brine and saturated sodium bicarbonate. The product was extracted with several volumes of chloroform. The combined chloroform layers were dried with magnesium sulfate, filtered, and reduced in volume to an oil. This oil was purified by flash chromatography on silica gel with a methanol-chloroform gradient and then crystallized from chloroform-hexane to give 3-mercapto-3-methylbutyryl hydrazide as a low-melting solid.

Calicheamicin Analogues. The γ_1^I, α_2^I, and α_3^I calicheamicins were all isolated from the fermentation broth of *M. echinospora calichensis* as described previously (27). The structural elucidation of these compounds, the isolation of the pseudoaglycone derivative, and the synthesis of *N*-acetyl calicheamicin γ_1^I from calicheamicin γ_1^I have been described in detail as well (28).

Hydrazide derivatives of the calicheamicins were prepared by displacement at the methyltrisulfide moiety of the analogues with the mercaptohydrazides described above. Preparation of the calicheamicin γ_1^I hydrazide typifies this procedure: 70 mg (0.051 mmol) of calicheamicin γ_1^I in 100 ml of acetonitrile at −15°C was added to 13.2 mg (2 eq) of 3-mercaptopropionyl hydrazide in 1 ml of acetonitrile. The reaction was warmed to 4°C for 24 h, and then the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on Merck silica gel (packed with carbon tetrachloride) using a gradient of 5–15% methanol in chloroform to give 55 mg of a yellowish glass. This material can be used directly or dissolved into chloroform containing a trace of methanol and precipitated by being poured into a rapidly stirring 1:1 mixture of ether-hexane to give a white powder which can be stored indefinitely at −15°C. The high-performance liquid chromatography retention time for this compound is 5.0 min with 41% acetonitrile/0.1 M aqueous NH_4OAc (Zorbax ODS C-18, 4.6 mm x 25 cm column; 2 ml/min) (for calicheamicin γ_1^I retention time is 5.5 min with 56% acetonitrile). Mass spectrum (FAB) is 1408 (M + H^+), 1430 (M + Na^+); and with added acetone, 1448 (M + 40 + H^+, acetone hydrazone). The nuclear magnetic resonance (300 mHz, $CD_3OD/CDCl_3$) of this compound is virtually identical to the published spectrum for calicheamicin γ_1^I (26) except for the following: absence of a CH_3-methyl trisulfide-resonance and a new resonance at 2.97 ppm (2H, t, $J_{a,b}$ = 7Hz, CH_2), 2.56 (2H, t, $J_{a,b}$ = 7Hz, CH_2).

Conjugation of Calicheamicin Hydrazides to MoAbs. The method used for the preparation of MoAb conjugates involved reactions with carbohydrate-derived aldehydes, as previously described (29). The conjugation of all the hydrazide and dimethyl hydrazide derivatives followed a similar procedure as outlined below. MoAb, at a concentration of 5 mg/ml in 50 mM sodium acetate buffer, pH 5.5, was oxidized at 4°C for 30 min with 12.5 mM sodium periodate (prepared freshly as a 100 mM stock in the acetate buffer). The oxidized antibody was desalted by gel filtration (PD-10 columns) and then reacted at 25°C with a 30-fold molar excess of calicheamicin γ_1^I hydrazide in DMF as described below. A typical conjugation reaction mixture contained approximately 3 mg/ml protein and 1 mg/ml drug hydrazide in a mixed solvent consisting of 50 mM sodium acetate buffer, pH 5.5, containing 15% DMF. After 3 h, the reaction was quenched with 5% of the reaction volume of 0.2 M $NaCNBH_3$ and 1% (v/v) of the reaction volume of 1 M acethydrazide for 1 h. The $NaCNBH_3$ reduction step was included in all the experiments in this article. However, subsequent studies indicated under the conditions used in this study little, if any, reduction of the hydrazone moiety occurred and conjugates made with and without reduction showed no differences, including in biological properties. Acethydrazide does function in blocking unreacted free aldehyde groups and minimizing aggregation and therefore is routinely included in all aldehyde-based conjugation reactions. To terminate the conjugation reactions, conjugates were passed through a desalting column equilibrated with 50 mM phosphate buffer, 100 mM NaCl, at pH 6.5, and exhaustively dialyzed against the same buffer. Monomeric conjugates were separated from dimers and higher order aggregates by S-200 gel exclusion chromatography.

The drug concentrations of calicheamicin derivatives and drug loading on conjugates were determined spectroscopically. The extinction coefficient de-

[3] C. F. Beyer, unpublished results.

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temined for a calicheamicin $\gamma_1{}^I$ β-mercaptopropionic acid disulfide of 4010 M^{-1} at 333 nm (in 10% DMF in PBS), a species that represents the attached disulfide form of the drug, was used as a standard. Since none of the structural modifications of the calicheamicins described here significantly affect the chromophore of the drug, this extinction coefficient was adjusted for variations in the molecular weight of the analogues and used for all studies. The molecular weights for the hydrazide calicheamicin derivatives included in this study are: 1408 for $\gamma_1{}^I$ hydrazide, 1248 for $\alpha_2{}^I$ hydrazide, 1091 for pseudoaglycone hydrazide, 1251 for $\alpha_3{}^I$ hydrazide, 1450 for *N*-acetyl-$\gamma_1{}^I$ hydrazide, 1436 for $\gamma_1{}^I$ dimethyl hydrazide, and 1478 for *N*-acetyl-$\gamma_1{}^I$ dimethyl hydrazide. Thus, for example, for $\gamma_1{}^I$ hydrazide conjugates, drug concentration (μg/ml) = A_{333}/ϵ_{333} (ml/mg) using ϵ_{333} (ml/mg) = 2.85 (4010/1408). The contribution to protein absorbance made by the calicheamicin at 280 nm was estimated to be 3 times the absorbance value calculated for the drug at 333 nm. Using ϵ 333 (ml/mg) = 1.43 as the standard extinction coefficient for an IgG molecule, a corrected antibody concentration (mg/ml) was calculated as:

$$\frac{A_{280} - (3 \times A_{333})}{1.43}$$

These spectroscopic values proved convenient for routinely measuring drug and MoAb concentrations in the conjugates and were confirmed using radiolabeled drug and independent determinations of protein concentration using standard BioRad reagents and assay procedures.

RESULTS

Characterization of Calicheamicin Derivatives for Conjugation. The five structural analogues of calicheamicin used in this study have been previously designated $\gamma_1{}^I$, $\alpha_2{}^I$, $\alpha_3{}^I$, *N*-acetyl-$\gamma_1{}^I$, and PSAG. The structures of these derivatives, described in detail elsewhere (26, 27), are shown in Fig. 1*A*. The core of the molecule shown in Fig. 1*A*, which includes the methyl trisulfide "trigger" which undergoes reduction to cause a molecular rearrangement of the enediyne bicyclic "warhead" (the part of the molecule that generates a diradical that produces double-strand DNA breaks) and the sugar/aromatic ring "backbone" is common to all five of the analogues used in these studies. Structural variations relate to the presence or absence of the rhamnose (at R′) and/or aminosugar (at R″) as indicated. The most potent "parent" compound, calicheamicin $\gamma_1{}^I$, contains both the rhamnose and the aminosugar. The $\alpha_2{}^I$ analogue is missing the rhamnose, while $\alpha_3{}^I$ is missing the aminosugar and PSAG lacks both the rhamnose and aminosugars. The fifth analogue, *N*-acetyl-$\gamma_1{}^I$, was prepared by acetylation of the amino sugar of $\gamma_1{}^I$ (27). The two hydrazides ("simple" and "dimethyl") used in these studies are shown in Fig. 1*B*. It should be noted that the hydrazides are disulfide versions of the trisulfide "parent" compounds and were prepared from the trisulfide analogues for conjugation to MoAbs containing periodate oxidized sugars.

The anti-tumor effects of the five parent calicheamicin derivatives, along with their respective hydrazides, were compared *in vivo* in the P388 leukemia model (Table 1). In each experiment, the test drug was administered i.p. to normal mice carrying P388 leukemia and a comparable group of nontumored animals. The lethality of the drug in the nontumored animals was used to determine MTD. In Table 1, we report the percentage of increase in life span for each derivative at two doses: the OD that gives the greatest percentage of increase in life span in the P388 animals, and the MTD, determined in the nontumored animals. Several conclusions can be made from these data. For all five derivatives, the MTD was less than the OD. For example, the greatest increase in life span resulting from treatment with any of these derivatives was 150% for the $\alpha_2{}^I$ calicheamicin, at a dose 8-fold higher than the MTD. The potency of the hydrazides was 2–8-fold less than that of the corresponding parent compounds for all analogues. From these data it is clear that although the calicheamicins are highly potent, toxicity limits their therapeutic efficacy as single agents against P388 leukemia. Similar dose-limiting toxicities also were seen for these calicheamicin analogues when they were studied as single agents in other murine tumors, such as B16 melanoma (30), and in the xenograft tumors as well (see below). To test the potential of these compounds as targeted agents, monomeric conjugates were prepared from each of the five hydrazide analogues. These hydrazone conjugates had drug loadings of 2 to 3 molecules of calicheamicin equivalents/MoAb molecule and retained greater than 85% of the immunoaffinity of the unmodified MoAb.

Activity and Specificity of CT-M-01-$\gamma_1{}^I$ Hydrazone Conjugates. Calicheamicin $\gamma_1{}^I$, the calicheamicin $\gamma_1{}^I$ hydrazide, and two conjugates, a CT-M-01-$\gamma_1{}^I$ hydrazide conjugate that binds to the MX-1 cells and a nonbinding control Lym-2-$\gamma_1{}^I$ hydrazide conjugate, were com-

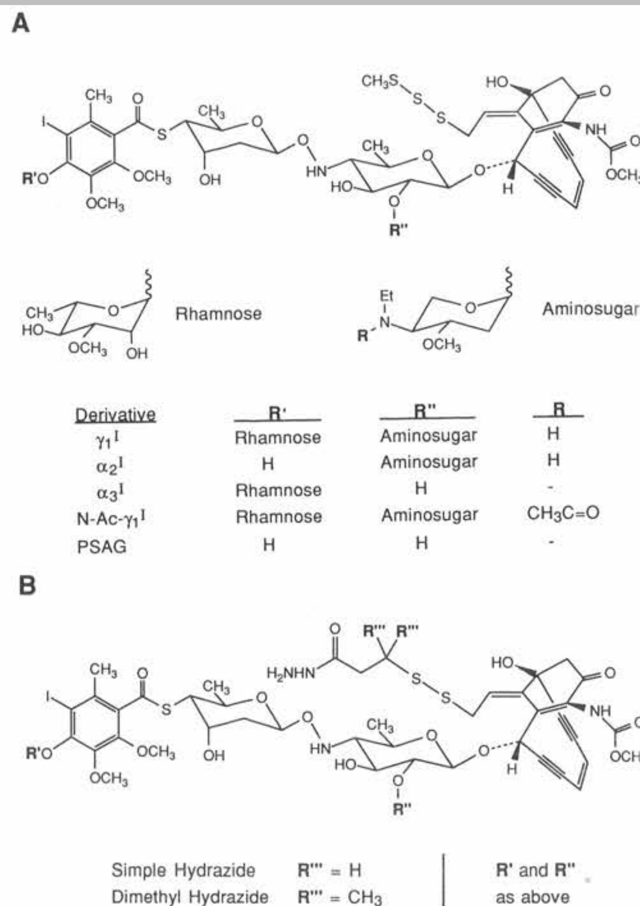

Fig. 1. In *A*, structural analogues of the calicheamicins which vary in the integrity of the rhamnose and amino sugars are outline schematically. *B*, comparable hydrazide analogues prepared from each derivative are shown.

Table 1 *Comparison of antitumor effects and lethality of calicheamicin analogues and hydrazides*

Derivative	% of increase in life span[a]			
	Parent drug		Hydrazide	
	OD (μg/kg)	MTD (μg/kg)	OD (μg/kg)	MTD (μg/kg)
$\gamma_1{}^I$	5 (123)	1.25 (86)	5 (83)	2.5 (75)
$\alpha_2{}^I$	10 (150)	1.25 (75)	10 (130)	5 (83)
$\alpha_3{}^I$	40 (109)	10 (73)	80 (73)	80 (73)
N-Acetyl-$\gamma_1{}^I$	40 (123)	20 (79)	160 (63)	160 (63)
PSAG	160 (73)	40 (60)	Not tested	

[a] Numbers in parentheses, percentages.

IMMUNOGEN 2094, pg. 5
Phigenix v. Immunogen
IPR214-00676

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