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, γ,y, 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, α,y and N-acetyl-γ,y, 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 MoAb2 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 antibodies, isolated from a broth extract of a soil microorganism Micromonospora echinospora calichenis, were termed the calicheamicins and later were identified as members of a new class of potent endo-enzyme containing antibiotics which includes the esperamicins, dynemycin, and neocarzinostatin (17). The binding of the most potent of the calicheamicins, γ,y, 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, 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, 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 supernatants by Centeltech, Ltd., United Kingdom. MOPC-21, a secreted murine myeloma IgG, that does not bind to any mouse antigen or human xenograft tumors, was used to prepare nonbinding isotype-matched MoAb conjugates (21, 22). Lym 2, an IgG2, 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 Technicline, Inc.

Iodination of MoAbs for use in the competitive binding assays was accomplished using the diiodinated Bolton Hunter reagent, N-succinimidyl 3-(4-hydroxy-3-125I-diodophenyl)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 125I-CT-M-01 ranged from 0.3 to 0.5 μCl/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.
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% CO₂ 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, 10^5 MX-1 cells in 0.1 ml were incubated with 0.05 ml of 4 μg/ml 125I-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 γ1i. 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 ml of [3H]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% [3H]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 the 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. Two tumors were implanted s.c. into athymic mice and test samples were injected 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 50% 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 recombination. The crude product was purified by flash silica gel chromatography eluting first with 5% ethyl acetate in chloroform and then 20% methanol in chloroform.
timated for a calicheamicin $\gamma_1$ $\beta$-mercaptopropionic acid disulfide of 4010 \text{ M}^{-1} \text{ cm}^{-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 calicheamincs 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 $\gamma_1$ hydrazide, 1248 for $\alpha_2$ hydrazide, 1091 for pseudoaglycone hydrazide, 1251 for $\alpha_1$ hydrazide, 1450 for $N$-acetyl-$\gamma_1$ hydrazide, 1436 for $\gamma_1$ dimethyl hydrazide, and 1478 for $N$-acetyl-$\gamma_1$ dimethyl hydrazide. Thus, for example, for $\gamma_1$ hydrazide conjugates, drug concentration ($\mu$g/ml) = $A_{333} / A_{333} (\text{ml/mg})$ using $\epsilon_{333}(\text{ml/mg})$ = 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 $\epsilon_{333}$ (ml/mg) = 1.43 as the standard extinction coefficient for an IgG molecule, a corrected antibody concentration (mg/ml) was calculated as:

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

These spectroscopic values proved convenient for routinely measuring drug and MoAb concentrations in the conjugates and were confirmed using radio-labeled 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 $\gamma_1$, $\alpha_2$, $\alpha_1$, $N$-acetyl-$\gamma_1$, and PSAG. The structures of these derivatives, described in detail elsewhere (26, 27), are shown in Fig. 1A. The core of the molecule shown in Fig. 1A, which includes the methyl trisulfide “trigger” which undergoes reduction to cause a molecular rearrangement of the endo-enzyme 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 $\gamma_1$, contains both the rhamnose and the aminosugar. The $\alpha_2$ analogue is missing the rhamnose, while $\alpha_1$ is missing the aminosugar and PSAG lacks both the rhamnose and aminosugars. The fifth analogue, $N$-acetyl-$\gamma_1$, was prepared by acetylation of the amino sugar of $\gamma_1$ (27). The two hydrazides ("simple" and "dimethyl") used in these studies are shown in Fig. 1B.

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 $\alpha_2$ 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 calicheamincs 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, monoclonic conjugates were prepared from each of the five hydrazide analogues. These hydrazide 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.

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

<table>
<thead>
<tr>
<th>Derivative</th>
<th>OD (µg/kg)</th>
<th>MTD (µg/kg)</th>
<th>OD (µg/kg)</th>
<th>MTD (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma_1$</td>
<td>5 (123)</td>
<td>1.25 (86)</td>
<td>5 (123)</td>
<td>1.25 (86)</td>
</tr>
<tr>
<td>$\alpha_2$</td>
<td>10 (150)</td>
<td>1.25 (75)</td>
<td>10 (150)</td>
<td>1.25 (75)</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>40 (109)</td>
<td>1.25 (73)</td>
<td>40 (109)</td>
<td>1.25 (73)</td>
</tr>
<tr>
<td>$N$-Acetyl-$\gamma_1$</td>
<td>40 (123)</td>
<td>2.0 (79)</td>
<td>40 (123)</td>
<td>2.0 (79)</td>
</tr>
<tr>
<td>PSAG</td>
<td>160 (73)</td>
<td>40 (60)</td>
<td>Not tested</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentages.*
conjugates produced a dose related inhibition of tumor growth (Fig. 3). The lowest dose tested (6.3 μg/kg × 3) was nonlethal but minimal. Animals treated by day 21 (data not shown), while at lower doses the conjugate was lethal to all doses given 4 days apart). The top dose (25 μg/kg) was lethal to all s.c. in nude mice. The conjugate was administered at 3 dose levels (as (data not shown).

Fig. 1A. A comparison of the antitumor activity of a "simple" 7,1 and a TR >4 (Table 2). As shown in Fig. 5, A and B, both of these conjugates had dramatic antitumor activity, inhibiting the growth of the MX-1 tumor at nonlethal doses and producing long-term tumor-free survivors (>100 days) at the 100-μg/kg dose. The parent drug derivatives included in the tests as controls were inactive at nonlethal doses.

In Vivo Activity of CT-M-01 Conjugates of Calicheamicin Variants. Hydrazide conjugates of the four other calicheamicins described above were conjugated to CT-M-01 and tested in vivo against the MX-1 tumor. As summarized in Table 2, structural variations in the drug had a profound effect on the therapeutic efficacy of their conjugates which did not correspond with the activity of the drugs as single agents (Table 1). For example, the CT-M-01 conjugate of α2, the analogue missing the rhamnose from the putative DNA binding region of the drug and next in potency to 7,1 against P388, had a TR <1 and showed no antitumor effects at nonlethal doses (Fig. 4) and the conjugates were inactive on antigen-negative xenograft tumors.

In contrast, conjugates of α3 and N-acetyl-7,1, analogues which contain the rhamnose but are modified at the amino sugar, were highly efficacious over a 4-fold dose range, showing an improved therapeutic window compared with 7,1 and a TR >4 (Table 2). As shown in Fig. 5, A and B, both of these conjugates had dramatic antitumor activity, inhibiting the growth of the MX-1 tumor at nonlethal doses and producing long-term tumor-free survivors (>100 days) at the 100-μg/kg dose. The parent drug derivatives included in the tests as controls were inactive at nonlethal doses.

The fifth variant of calicheamicin conjugated to CT-M-01 was PSAG, missing both the rhamnose and the amino sugar. PSAG is significantly less potent than the other derivatives against P388 leukemia. As shown in Table 2, this conjugate was ineffective and non-lethal even at doses 28-fold higher than the MTD for the 7,1 conjugate, indicating that the delivery of the "warhead" of calicheamicin on a MoAb was not sufficient to retain activity. Because of this low potency, further investigations of PSAG conjugates were not pursued.

Effects of Variations in the Hydrazone Linkage on in Vivo Activity. Changes in the linker region of the calicheamicin hydrazide conjugates were also examined as a means of increasing the TR of the calicheamicin conjugates. Methyl groups that add steric bulk adjacent to the disulfide were introduced into the hydrazide linker as shown in Fig. 1B. A comparison of the antitumor activity of a "simple" 7,1 hydrazide (7,1-hyd) and a 7,1-DM-hyd conjugate was made on the MX-1 tumor in vivo. As summarized in Table 2, the 7,1-DM-hyd conjugate had strong antitumor activity at 6 μg/kg, a dose which is ineffective with the simple 7,1-hyd conjugate (Fig. 3). In addition, no lethality was seen with the 7,1-DM-hyd conjugate at the 12.5 μg/kg dose.
doses from 6.25 to 25 μg/kg of drug × 3 doses, delivered over a total of 0.6 to 12.5 μg of control × 3 doses. The γ1, hydrazide itself included in the test was lethal at doses >0.5 μg/kg, while the MTD for the γ1-hydrazide was <6.25 μg/kg. The antitumor response of a CT-M-01 mixture with γ1-hydrazide is shown. The conjugate was lethal at 25 μg/kg (data not included) and produced a significant antitumor response at the 12.5 μg/kg dose. Right, the number of animals alive in each treatment group at day 35. In all test groups, n = 6; in the control group, n = 10; bars, ± SEM for each data point.

**Fig. 4.** Antitumor effects of a CT-M-01 conjugate of α2-hydrazide and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated Q4D × 3, i.p. with the conjugate and appropriate controls over a range of doses from 6.25 to 25 μg/kg of drug × 3 doses, delivered on a total of 0.6 to 12.5 μg of CT-M-01/animal. The α2, hydrazide itself included in the test was lethal at doses >0.5 μg/kg, while the MTD for the α2-hydrazide was <6.25 μg/kg. The antitumor response of a CT-M-01 mixture with α2-hydrazide is shown. The conjugate was lethal at 25 μg/kg (data not included) and produced a significant antitumor response at the 12.5 μg/kg dose. Right, the number of animals alive in each treatment group at day 35. In all test groups, n = 6; in the control group, n = 10; bars, ± SEM for each data point.

**DISCUSSION**

In this article we present data that demonstrate the therapeutic potential of MoAb conjugates prepared from a novel class of antitumor antibiotics, the calicheamicins. Despite the dose-limiting toxicities seen with the parent calicheamicins, modifications have been made in both the drug and the linker to produce constructs with a significant therapeutic window. These conjugates are antigen specific and have produced dose-dependent inhibition of tumor growth with- out lethality in all antigen-positive xenograft tumors tested. The antibody used in these studies was CT-M-01, an internalizing anti-PEM MoAb which binds to a mucin antigen abundant on a number of solid tumors including breast, non-small cell lung, ovarian and colon carcinomas. Our results with CT-M-01 conjugates as well as our previous preliminary reports suggest that internalizing antibodies act as effective surrogate carriers for potent drugs such as the calicheamicins allowing them to bypass the normal mechanisms of nonsel ective drug uptake, thus rendering them less toxic (28, 31, 32).

Our efforts to optimize the targeting of the calicheamicins involved the evaluation of conjugates prepared from a number of structural analogues of the drug. Using information available on the relative potencies of various calicheamicin analogues in the P388 leukemia model, we tested a number of structural analogues of the drug, including the parent calicheamicin, modified calicheamicins, and various calicheamicin analogues with different linkages and substituents. The results of these studies are summarized in Table 1, which shows the antitumor activity of different calicheamicin derivatives conjugated with a variety of tumor-selective, internalizing MoAbs are continuing.

**Fig. 3.** Antitumor effects of a CT-M-01 conjugate of γ1-hydrazide and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated Q4D × 3, i.p. with the conjugate and appropriate controls over a range of doses from 6.25 to 25 μg/kg of drug × 3 doses, delivered on a total of 0.6 to 12.5 μg of CT-M-01/animal. The γ1, hydrazide itself included in the test was lethal at doses >0.5 μg/kg, while the MTD for the γ1-hydrazide was <6.25 μg/kg. The antitumor response of a CT-M-01 mixture with γ1-hydrazide is shown. The conjugate was lethal at 25 μg/kg (data not included) and produced a significant antitumor response at the 12.5 μg/kg dose. Right, the number of animals alive in each treatment group at day 35. In all test groups, n = 6; in the control group, n = 10; bars, ± SEM for each data point.

**Table 2: Comparison of efficacy of calicheamicin-CT-M-01 hydrazide conjugates**

<table>
<thead>
<tr>
<th>Sugar Derivative</th>
<th>Rhamnose</th>
<th>Amino</th>
<th>MED * (μg/kg × 3)</th>
<th>MTD * (μg/kg × 3)</th>
<th>TR MTD/MED</th>
<th>% Control</th>
<th>Tumor-free survivors at day 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ1, hydrazide</td>
<td>+</td>
<td>+</td>
<td>12.5</td>
<td>12.5</td>
<td>1</td>
<td>13.6</td>
<td>0 of 6</td>
</tr>
<tr>
<td>α2, hydrazide</td>
<td>-</td>
<td>-</td>
<td>30.0</td>
<td>30.0</td>
<td>&lt;1</td>
<td>43.6</td>
<td>0 of 6</td>
</tr>
<tr>
<td>α3, hydrazide</td>
<td>+</td>
<td>+</td>
<td>&lt;25.0</td>
<td>&gt;100.0</td>
<td>&gt;4</td>
<td>0.0</td>
<td>0 of 6</td>
</tr>
<tr>
<td>N-Ac-γ1, DM-hyd</td>
<td>+</td>
<td>Modified</td>
<td>&lt;25.0</td>
<td>&gt;100.0</td>
<td>&gt;4</td>
<td>0.0</td>
<td>0 of 6</td>
</tr>
<tr>
<td>PSAG-hyd</td>
<td>-</td>
<td>-</td>
<td>&gt;350.0</td>
<td>&gt;350.0</td>
<td>&lt;1</td>
<td>78.7</td>
<td>0 of 6</td>
</tr>
<tr>
<td>γ1, DM-hyd</td>
<td>+</td>
<td>+</td>
<td>6.0</td>
<td>&gt;12.5</td>
<td>2</td>
<td>0.0</td>
<td>6 of 6</td>
</tr>
<tr>
<td>N-Ac-γ1-hydrazide</td>
<td>&lt;50.0</td>
<td>300.0</td>
<td>&gt;6</td>
<td>0.0</td>
<td>&lt;6</td>
<td>0.0</td>
<td>6 of 6</td>
</tr>
<tr>
<td>MOCP-21 (nonspecific)</td>
<td>&gt;300.0</td>
<td>300.0</td>
<td>&lt;1</td>
<td>63.0*</td>
<td></td>
<td>c</td>
<td></td>
</tr>
</tbody>
</table>

* Measured at 35 days post-tumor implantation; n = 6 per test group; n = 10 in the control group.

a Tumor-free survivors for >49 days, at which time the animals were sacrificed.

b Measured on day 28, no tumor-free animals on day 28.

c Measured on day 28, no tumor-free animals on day 28.

d Measured at 35 days post-tumor implantation; n = 6 per test group; n = 10 in the control group.

e Measured on day 28, no tumor-free animals on day 28.

Unpublished results.
model and the MTD of the various analogues in normal mice, we explored structural features of the drug essential for its activity. The calicheamicins bind in the minor groove of DNA and make double-stranded cuts with a specificity for the TCCT/AGGA tetramer (19). We have shown that conjugates of γ1 make equivalent DNA cuts in target cells (28), suggesting that DNA cleavage is the mode of action of the drug, even after conjugation.

Among the four calicheamicin analogues compared with the parent γ1 in this study, two analogues, α2i and PSAG, were missing the rhamnose at the end of the DNA binding region and in neither case were these agents effective as conjugates in vivo. Conjugates prepared with α2i and N-acetyl-γ1i derivatives, in which the DNA binding region is intact, yet the amino sugar has been eliminated or modified, showed a clear therapeutic advantage over the γ1i, α2i, and PSAG conjugates. Although the function of the amino sugar itself in the calicheamicin structure has not been established, it has been speculated to play a role as a carrier to transport the drug into cells or across membranes. The α2i and N-acetyl-γ1i DM hydrazide conjugates may owe their favorable profile to the fact that the DNA binding region of the drug has remained intact while the internalizing antibody can serve as a surrogate for the amino sugar, bringing the potent drug into the target cells. Our data suggest that an intact DNA binding region of calicheamicin is required for optimal antitumor activity, although others have reported the potent antitumor effects of certain enediyne molecules missing a DNA binding region (33).

Triggering of the enediyne is the key event in the activation of calicheamicins. Although the intracellular compartment and rate of triggering of the conjugated drug has not yet been established, the evidence so far accumulated suggests that activation through disulfide cleavage occurs after the conjugate has been internalized. Studies are in progress using radiolabeled drug and conjugates to follow the intracellular trafficking of the conjugated and unconjugated forms of the calicheamicins to explore differences in intracellular processing of the conjugated and unconjugated drug.

In addition to selecting an optimal form of the drug itself, we have improved the therapeutic potential of the calicheamicin conjugates by increasing the stability of the linker. There is significant literature precedent to suggest that the introduction of steric bulk adjacent to a thiol in disulfide-based linkers effects the stability of a conjugate in serum and modulates the ease with which drug is released at the tumor site (34–36). Preliminary studies in our laboratory have suggested that the stability of calicheamicin disulfides toward reduced glutathione is proportional to the steric bulk placed adjacent to the disulfide group. These results fit with the increased therapeutic potential seen for conjugates prepared with the dimethyl linker (37). The linkage between calicheamicin and the MoAb actually contains two possible sites for drug release: the hydrazide can be cleaved by acid hydrolysis, and the disulfide bond can be cleaved by reduction. We are currently evaluating the relative importance of these two release mechanisms.

In conclusion, we have demonstrated the potential of the calicheamicin conjugates by increasing the stability of the linker. There is significant literature precedent to suggest that the introduction of steric bulk adjacent to a thiol in disulfide-based linkers effects the stability of a conjugate in serum and modulates the ease with which drug is released at the tumor site (34–36). Preliminary studies in our laboratory have suggested that the stability of calicheamicin disulfides toward reduced glutathione is proportional to the steric bulk placed adjacent to the disulfide group. These results fit with the increased therapeutic potential seen for conjugates prepared with the dimethyl linker (37). The linkage between calicheamicin and the MoAb actually contains two possible sites for drug release: the hydrazide can be cleaved by acid hydrolysis, and the disulfide bond can be cleaved by reduction. We are currently evaluating the relative importance of these two release mechanisms.

In conclusion, we have demonstrated the potential of the calicheamicins for targeted delivery. Through the process of MoAb conjugation, we have converted a potent, yet toxic series of antibiotics into effective antitumor agents, with a significant therapeutic window for treating solid tumors. Studies are in progress to establish the activity profile of both CT-M-01 and other MoAb calicheamicin conjugates in a variety of preclinical models.

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Fig. 6. A comparison of the antitumor effects of CT-M-01 conjugate of N-acetyl γ1i hydrazide and N-acetyl γ1i DM hydrazide and controls on the MX-2 xenograft tumor. Athymic mice implanted with the s.c. MX-2 breast carcinoma were treated Q4D × 3, i.p., with the two conjugates at doses of 95 and 175 μg/kg of drug/dose × 3 doses. The N-acetyl-γ1i hydrazide and N-acetyl-γ1i DM hydrazide were included as controls and were not effective at nonlethal doses (data not shown). The N-acetyl-γ1i DM-hyd conjugate produced 6 of 6 tumor-free survivors at both doses and, as shown, were more effective that the "simple" N-acetyl-γ1i hyd conjugates. Right, the number of animals alive in each treatment group at day 35. In all test groups, n = 10; bars, ± SEM for each data point.

Fig. 5. Antitumor effects of a CT-M-01 conjugate of α2i, hydrazide (A) and N-acetyl γ1i (B) and controls on the MX-1 xenograft tumor. Athymic mice implanted with the s.c. MX-1 breast carcinoma were treated Q4D × 3, i.p. with the conjugate and controls over a range of doses from 25 to 100 μg/kg of drug × 3 doses. The parent α2i and N-acetyl-γ1i drugs included in their respective tests as controls were lethal at doses of 25 μg/kg × 3 and above. The conjugates in both tests produced 100% tumor-free survivors at the top dose of 100 μg/kg × 3 doses. The N-acetyl-γ1i conjugate was tested i.p. or i.v. and found to give equivalent results by either route of administration. A mixture of the N-acetyl-γ1i hydrazide plus CT-M-01 at a dose of 50 μg/kg was lethal. Right, the number of animals alive in each treatment group at day 42. In all test groups, n = 6; in the control group, n = 10; bars, ± SEM for each data point.
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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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