Antibody-Targeted Chemotherapy with the Calicheamicin Conjugate hu3S193-*N*-Acetyl γ Calicheamicin Dimethyl Hydrazide Targets Lewis^y and Eliminates Lewis^y-Positive Human Carcinoma Cells and Xenografts

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ABSTRACT

Purpose: **Linking a cytotoxic anticancer drug to an antibody that recognizes a tumor-associated antigen can improve the therapeutic index of the drug. We asked whether a conjugate of the cytotoxic antibiotic *N*-acetyl γ calicheamicin dimethyl hydrazide (CalichDMH) and an antibody recognizing Lewis^y (Le^y) antigen could eliminate carcinomas that express Le^y. Because Le^y is highly expressed on carcinomas of colon, breast, lung, ovary, and prostate, a CalichDMH conjugate targeting Le^y could provide a treatment option for various cancers.**

Experimental Design: **The humanized anti-Le^y antibody hu3S193 was conjugated to CalichDMH via the bifunctional AcBut linker. Selectivity and avidity of the conjugate (hu3S193-CalichDMH) for Le^y-BSA or Le^{y+} cells was tested by BIAcore or flow cytometry. Cytotoxicity of hu3S193-CalichDMH was compared with toxicity of a control conjugate on monolayers of Le^{y+} and Le^{y-} carcinoma cells. Inhibition of tumor growth by hu3S193-CalichDMH was assessed on three types of s.c. xenografts.**

Results: **Hu3S193-CalichDMH had similar selectivity as hu3S193. The conjugate had lower affinity for Le^y-BSA but not for Le^{y+} cells. When tested on monolayers of human Le^{y+} carcinoma cells, hu3S193-CalichDMH was more cytotoxic than a control conjugate. This difference in efficacy was not noted on Le^{y-} cells. Efficacy of hu3S193-CalichDMH depended on the expression of Le^y and on the sensitivity of the cells to CalichDMH. *In vivo*, hu3S193-CalichDMH inhibited growth of xenografted human gastric (N87), colon (LOVO), and prostate carcinomas (LNCaP). When used against N87 xenografts, hu3S193-CalichDMH arrested tumor growth for at least 100 days.**

Conclusion: **Hu3S193-CalichDMH can specifically eliminate Le^{y+} tumors. These results support development of this conjugate for treatment of carcinomas.**

INTRODUCTION

The use of cytotoxic chemotherapy has improved the survival of patients suffering from various types of cancers. When used against select neoplastic diseases such as acute lymphocytic leukemia in young people (1) and Hodgkin's lymphomas (2), mixtures of cytotoxic drugs can induce complete cures. Unfortunately, chemotherapy does not cause complete remissions in the majority of cancers. Multiple reasons can explain this relative lack of efficacy (reviewed in Refs. 3–5). Among these, the low therapeutic index of most chemotherapeutics is a conspicuous target for pharmaceutical improvement. The low therapeutic index reflects the narrow margin between the efficacious and toxic dose of a drug. Evidently, this narrow margin prevents the administration of sufficiently high doses necessary to eradicate a tumor.

One strategy to circumvent this problem is the use of a so-called "magic bullet." The magic bullet was proposed in 1906 by Ehrlich (6) and consists of a therapeutic agent linked to an organ-specific carrier. Applied to cancer therapy, one can use a cytotoxic compound that is chemically linked to an antibody. This antibody should ideally recognize a tumor-associated antigen (TAA) that is exclusively expressed at the surface of tumor cells. This strategy allows the delivery of the cytotoxic agent to the tumor site while minimizing the exposure of normal tissues.

Thus far, few therapeutic agents that work by this principle are approved by the United States Food and Drug Administration. Mylotarg (7), Ontak (8), Bexxar (9), and Zevalin (10) are examples of TAA-targeted therapeutics. Ontak is a fusion protein of truncated interleukin 2 and truncated diphtheria toxin and is used against advanced-stage cutaneous T-cell lymphoma. Bexxar and Zevalin are radioimmunotherapeutics that target a radioisotope (^{131}I and ^{90}Y, respectively) to CD20 and are indicated to treat non-Hodgkin's lymphoma. Mylotarg (gemtuzumab ozogamicin) is currently approved for the treatment of acute myeloid leukemia in elderly patients. This drug (11) consists of an antibody against CD33 that is bound to a disulfide analog of the semi-synthetic *N*-acetyl γ calicheamicin by an acid-hydrolysable AcBut bifunctional linker [4-(4′-acetylphenoxy)butanoic acid]. *N*-acetyl γ calicheamicin-dimethyl hydrazide is hereafter abbreviated as CalichDMH.

Calicheamicins are potent cytotoxic antibiotics. They contain an enediyne "warhead" (Fig. 1) that is activated by reduc-

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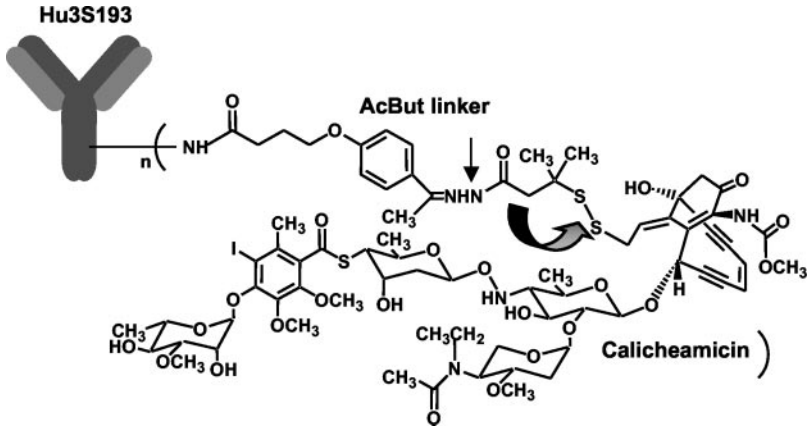


Fig. 1 Chemical structure of hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (CalichDMH). Calicheamicin was bound to lysine residues of hu3S193 by an acid-hydrolyzable linker (*AcBut*). Release of CalichDMH from the antibody occurs at the hydrazone bond (*straight arrow*). Activation of the enediyne "warhead" is induced by reduction of the -S-S-bond (*curved arrow*). Conjugates with an average of 2 or 3 molecules of CalichDMH per antibody were used for the presented experiments.

tion of the -S-S- bond causing breaks in double-stranded DNA (12).

Several lines of experimental evidence reinforce the idea that using antibodies that recognize TAAs different from CD33 could expand the application range of the magic bullet approach. Multiple conjugates of antibodies and chemotherapeutic agents (immunoconjugates) have a proven ability to inhibit growth of a host of xenografted tumors. Some examples of targeted TAAs are Her-2/*neu* (13, 14), prostate stem cell antigen (15), mucine type glycoproteins (16), epidermal growth factor receptor (17), carcinoembryonic antigen (18), CD22 (19), and Lewis^y (Le^y; 20). To achieve a cytotoxic effect, antibodies against these surface antigens were conjugated to pseudomonas exotoxin (14), maytansin (15, 16), calicheamicin (19), Rnase (17), *Vinca* alkaloids (18), or doxorubicin (20).

Ideally, the TAA should be highly expressed by tumor cells and not by normal tissue. Le^y is homogeneously expressed at high density on the surface of various tumor cells of epithelial origin. The Le^y oligosaccharide is found on 60–90% of breast, colon, gastric, pancreas, lung, and ovary carcinomas (21, 22). Its presence in normal tissues is limited to the surface of the epithelium of the esophagus, gastric mucosa, proximal small intestine, acinar cells of the pancreas, ciliated epithelium of the trachea, and the bronchus and type II pneumocytes (23). However, because Le^y is located on the epithelial surface of these tissues, the antigen should have restricted accessibility to administered antibodies (23). In addition, the fact that Le^{y-} antibody complexes can be internalized by tumor cells (22, 24) further underscores the feasibility of using this TAA as a target for antibody-targeted chemotherapy. To target Le^y, an antibody with exclusive specificity to the antigen is ideally required. During the past decades, several antibodies that recognize Le^y have been generated. Most of these, however, show cross-reactivity with Le^x and type2 H-antigen structures (25). 3S193 is a murine monoclonal antibody with exceptional specificity for Le^y (26). The humanized version of this antibody, hu3S193, has been generated by CDR grafting (22). Hu3S193 not only retains the specificity of 3S193 for Le^y but has also gained in the capability to mediate complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) (22). This antibody targets Le^y-expressing xenografts in nude mice as demonstrated by biodistribution studies with hu3S193 labeled with ^{125}I, ^{111}In, or ^{90}Y (27).

We have generated a calicheamicin conjugate of hu3S193 (hu3S193-CalichDMH) by an identical linker technology as was used to produce Mylotarg. The efficacy of hu3S193-CalichDMH was evaluated *in vitro* and *in vivo*. The presented data demonstrate the selective antitumor efficacy of hu3S193-CalichDMH for Le^{y+} tumor cells and xenografts.

MATERIALS AND METHODS

Cells and Culturing Conditions. DLD-1 (CCL-221), HCT8S11, HCT8S11/R1, and LOVO (CCL-229) are colon carcinoma cell lines that display Le^y antigen on the cell membrane. NCI-H157 (CRL-5802), NCI-H358 (CRL-5807), and A549 (CCL-159) are lung carcinoma cell lines. Of these three cell lines, NCI-H358 displayed detectable levels of Le^y on the cell surface. Both gastric carcinomas, N87 (CRL-5822) and AGS (CRL-1739), express Le^y. A431 (CRL-1555) is a cervical carcinoma cell line. The cell line used in the present studies does not express Le^y. MDA-MB435 (Le^{y-}) and MDA-MB-361 (Le^{y+}) were used as models of breast carcinoma cells. PC3-MM2 (Le^{y-}) and LNCaP (Le^{y+}; CRL-1740) were derived from prostate carcinomas. All of the cell lines except HCT8S11, HCT8S11/R1 (28), MDA-MB435, and PC3-MM2 were purchased from the American Type Culture Collection. Cell lines obtained from American Type Culture Collection were maintained in culture medium as specified in the American Type Culture Collection catalogue. HCT8S11and HCT8S11/R1 (28) were a gift from Dr. Marc Mareel (University Hospital, Ghent, Belgium). These cells were grown in RPMI 1640 supplemented with 10% v/v fetal bovine serum, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 units/ml penicillin. MDA-MB435 and PC3-MM2 were obtained from Dr. I. Fidler (MD Anderson Cancer Center, Houston, TX). These cells were cultured in MEM supplemented with 10% v/v fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 0.2 mM nonessential amino acids, 2% MEM vitamin solution, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Antibodies and Conjugates. Hu3S193 is a humanized anti-Le^y antibody (IgG1) produced in NSO cells (Ludwig Insti-

tute for Cancer Research). Hu3S193 is derived from the mouse monoclonal antibody 3S193. HuS193 was engineered so that only the complementary determining regions are from murine origin. Rituxan (IDEC Pharmaceuticals Corporation and Genentech, San Diego and San Francisco, CA) is a chimeric antibody that combines the murine heavy and light chain variable regions with the human IgG1-κ constant regions. The antibody recognizes the B-lymphocyte marker CD20. For fluorescence-activated cell sorter (FACS) analysis, human IgG (huIgG; Zymed, San Francisco, CA) and FITC-labeled goat anti-huIgG (FITC/α-huIgG; Zymed) were used as control antibody and as secondary antibody, respectively.

Mylotarg (CMA-676) is a calicheamicin conjugate produced by Wyeth. We used a batch with an average of 35 μg calicheamicin conjugated to 1 mg antibody (3.6:1; M:M).

Rituxan and hu3S193 were conjugated to CalichDMH as follows. The antibody at a protein concentration of ~10 mg/ml was adjusted to pH 8.0–8.5 with a high molarity non-nucleophilic buffer (1 M HEPES). Next, an excipient (sodium octanoate) that prevents protein aggregation was added to a final concentration of 0.1–0.2 M. Finally, 5% of the protein mass of activated calicheamicin derivative (*N*-acetyl γ calicheamicin DMH AcBut and *N*-hydroxysuccinimide ester; see Ref. 11) was added as a concentrated solution (10–20 mg/ml) in an organic solvent (ethanol or dimethylformamide). This reaction mixture was then incubated at 25–35°C for 1–2 h. Progress of the reaction was monitored by size-exclusion chromatography-high-performance liquid chromatography. After completion of the reaction, the conjugate was separated from aggregated antibody and free calicheamicin on a preparative size-exclusion chromatography column. The amount of CalichDMH per antibody for conjugate preparations that were used ranged between 22 and 47 μg/mg (2.2:1 and 4.8:1 M:M) and between 17 and 30 μg/mg (1.7:1 and 3:1 M:M) for hu3S193-CalichDMH and Rituxan-CalichDMH, respectively.

Plasmon Resonance Analysis (BIAcore). The Lewis-BSA conjugates (*i.e.*, H type I-, H type II-, Sialyl Le^a-, Sialyl Le^x-, Sulfo Le^a-, Sulfo Le^x-, Le^a-, Le^b-, Le^x-, and Le^y-BSA) were purchased from Alberta Research Council (Edmonton, Alberta, Canada). The antigen/BSA loading was between 20 and 42 mol antigen/mol of BSA. Each antigen was immobilized to the surface of a CM5 biosensor chip at a density of 4000–9000 resonance units/flow cell. The chip was activated by the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl/N-hydroxysuccinimide at a flow rate of 5 μl/min for 6 min, followed by the addition of the Lewis-BSA antigens at 5 μl/min for 6 min at a concentration of 50 μg/ml in 10 mM sodium acetate buffer (pH 4.5). The Sulfo-Lewis and Sialyl-Lewis-BSA conjugates were coupled at pH 4.0. Surplus binding sites were blocked with 1 M ethanolamine-HCl (pH 8.5) at 5 μl/min for 6 min. Binding specificity analysis was performed in HEPES-buffered saline (HBS-EP, 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 50 ppm polysorbate 20) at a flow rate of 20 μl/min. Hu3S193 was injected for 3 min at 6.67 nM or 50 nM. The amount of antibody that remained bound after a 30-s wash with HBS-EP buffer was measured. The antigenic surface was regenerated by 10 mM NaOH and 200 mM NaC1 for 1 min at 20 μl/min to re-establish a baseline.

For kinetic analysis, antibody was used in concentrations of 1–16 nM. The density of Le^y-BSA was 9000 RU. Association and dissociation were measured in HBS-EP buffer for 3 and 15 min at 30 μl/min.

FACS Analysis. Aliquots of 10^5 cells were suspended in 100 μl PBS supplemented with 1% v/v BSA. The cells were then incubated at 4°C for 30 min in various concentrations of primary antibody, hu3S193 or huS193-CalichDMH as specified in the result section. Binding of the primary antibody to the cells was revealed by FITC/α-huIgG. Results were expressed as either mean channel fluorescence (MCF) or relative mean channel fluorescence (reMCF). The MCF represents the average fluorescent intensity of a cell population after binding of the primary antibody and the consecutive staining with a fluorescent-labeled secondary antibody. The reMCF is the MCF obtained after probing a cell population with hu3S193 divided by the MCF after probing with huIgG.

Determination of the ED_{50} of hu3S193 *in Vitro*. A vital dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium] staining was used to determine the number of surviving cells after exposure to various treatments. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2*H*-tetrazolium (nonradioactive cell proliferation assay kit) was purchased from Promega (Madison, WI) and used according to the manufacturer's specifications. For each cell line a calibration curve (cell number *versus* absorbance after 2 h) was established to estimate an appropriate initial seeding density. Cells were then seeded in 96-multiwell dishes at a density of 750-5000 cells/well dependent on the cell type. Immediately after seeding, the cells were exposed to various concentrations [0, 0.01, 0.05, 0.1, 1, 10, 100, and 500 ng calicheamicin equivalents (cal.eq)/ml] of CMA-676, hu3S193-CalichDMH, CalichDMH, or PBS. After determination of the number of cells surviving 96 h of drug exposure, the ED_{50} was calculated based on the logistic regression parameters derived from the dose-response curves. The ED_{50} was defined as the concentration of drug (CalichDMH) that caused a 50% reduction of the cell number after 96-h exposure to the drug. It should be noted that a cal.eq is the concentration of CalichDMH given either as a pure substance or as a conjugate. Dependent on the amount of CalichDMH bound to the antibody (antibody-drug loading), identical cal.eq for different conjugates can imply different protein concentrations.

Efficacy of hu3S193-CalichDMH *in Vivo*. S.c. tumors of N87, LOVO, and LNCaP were grown in athymic nude mice (Charles River, Wilmington, MA). Female mice of 1.5–3 months old were injected with 5×10^6 N87 or 10^7 LOVO cells per mouse. LNCaP cells were injected in male nude mice that were 3 months old. To grow tumors, N87 as well as LNCaP cells had to be mixed (1:1, v/v) with Matrigel (Collaborative Biomedical Products, Belford, MA) before injection (total volume = 200 μl). Two perpendicular diameters of the tumor were measured at least once a week by calipers. The tumor volume was calculated according to the formula of Attia and Weiss (29): $A^2 \times B \times 0.4$. A and B are symbols for the smaller and the larger tumor diameter, respectively. Before treatment, mice with a s.c. tumor of at least 100 mm^3 were selected. Independent of the time period that various tumors needed to reach this volume, we arbitrarily assigned the time of first treatment with conjugate or control as day 1. The treatment schedules, dose, and number

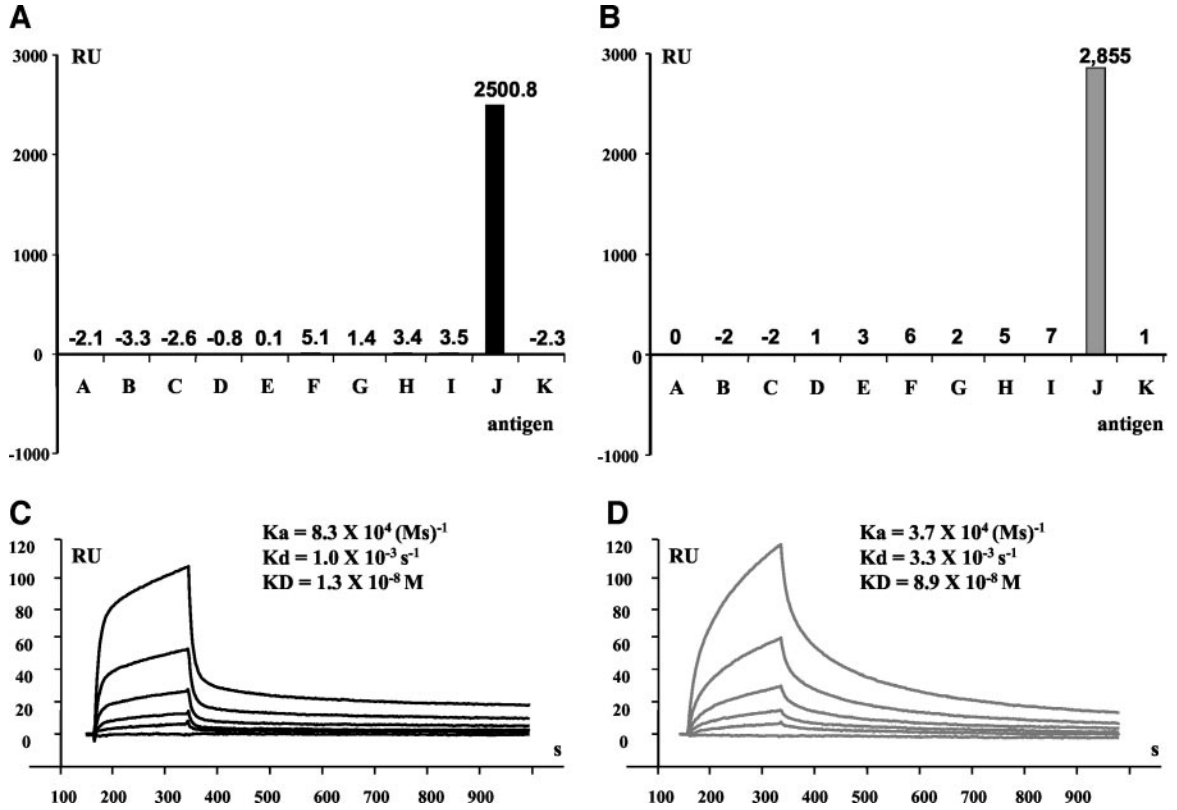


Fig. 2 Comparison of binding specificity and kinetics of hu3S193 and huS193-*N*-acetyl γ calicheamicin dimethyl hydrazide (CalichDMH) by plasmon resonance analysis. Blood group antigen-BSA conjugates were coupled to carboxy-methyl dextran coated chips. The chips were irrigated with 50 nM hu3S193 (*A*) or hu3S193-CalichDMH (*B*) in HBS-EP buffer at a rate of 20 μl/min for 180 s. The amount of antibody that remained bound after 30 s of washing was plotted. The binding is expressed in resonance units (resonance units, *Y*-axes in *A* and *B*). The binding substrates are indicated on the *X*-axes (*A* = H type I, *B* = H type II, *C* = sialyl-Le^a, *D* = sialyl-Le^x, *E* = sulfo-Lewis (Le)^a, F = sulfo-Le^x, *G* = Le^a, *H* = Le^b, *I* = Le^x, *J* = Le^y, *K* = BSA). *C* and *D* show the kinetics of the hu3S193 (*C*) and hu3S193-CalichDMH (*D*) interactions with Le^y-BSA. Antibody as well as conjugate (32, 16, 8, 4, 2, and 1 nM) was allowed to interact with Le^y-BSA (density 9000 resonance units) for 180 s. The *curves* in *C* and *D* represent the amount (resonance units, *RU*) of antibody (*C*) or conjugate (*D*) bound to Le^y-BSA as a function of time.

of mice per group are specified in the result section and in the figure legends. Statistical analysis involved a 2-tailed Student's *t* test. The analysis was performed on the various samples at ~30 days after initiation of treatment. This time point was chosen to allow comparison of all of the groups before mice had to be killed because of too large a tumor burden.

All of the experimental procedures involving mice were approved by the Wyeth Pearl River Animal Care and Use Committee according to established guidelines.

RESULTS

Specificity and Kinetics of the Binding of hu3S193 and hu3S193-CalichDMH to Le^y. To ascertain that conjugation of CalichDMH to hu3S193 did not compromise the binding to Le^y, the antibody and conjugate were subjected to plasmon resonance analysis (BIAcore). As shown in Fig. 2, hu3S193 (Fig. 2*A*), as well as hu3S193-CalichDMH (Fig. 2*B*), only recognized Le^y-BSA and none of the after oligosaccharide antigens, H type I, H type II, sialyl-Le^a, sialyl-Le^x, sulfo-Le^x, Le^a, Le^b, or Le^x.

The kinetics of the binding of hu3S193-CalichDMH differed from those of hu3S193. The k_d of hu3S193-CalichDMH was 7-fold higher than the k_d of hu3S193. This reduced affinity of the conjugate resulted from a combined 2.2-fold decrease of the k_a (Fig. 2*C*) and a 3.3-fold increase of the k_d (Fig. 2*D*) value.

Binding of hu3S193 and hu3S193-CalichDMH to N87 Cells *in Vitro*. The kinetic parameters determined by BIAcore rely on the binding of the antibody or conjugate to the artificial Le^y-BSA substrate. This substrate may not fully mimic the natural configuration of Le^y epitopes on the surface of tumor cells. To verify whether conjugation affected the binding of hu3S193 to Le^{y+} cells the amounts of hu3S193 and hu3S193-CalichDMH that bound to N87 cells were compared by flow cytometry (FACS). As shown in Fig. 3, the MCF obtained after exposing N87 to various concentrations of either hu3S193 or hu3S193-CalichDMH was similar.

Taken together, the results from FACS and BIAcore analysis indicated that conjugation of CalichDMH to hu3S193 did not affect the specificity for Le^y-positive cells (data not shown) or for Le^y-BSA (Fig. 2). The altered kinetic parameters of hu3S193-CalichDMH as compared with hu3S193 did not cor-

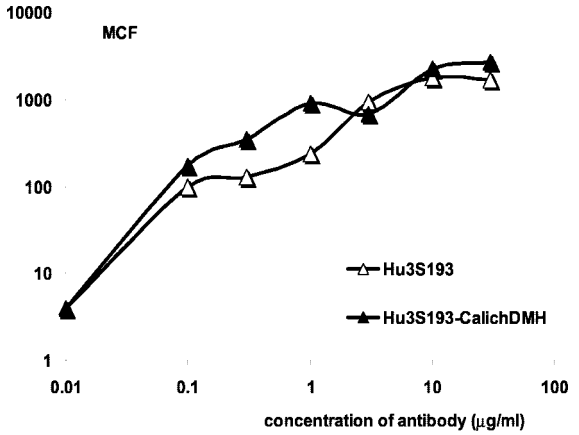


Fig. 3 Comparison of the binding of hu3S193 and hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (*CalichDMH*) by fluorescence-activated cell sorter analysis. N87 cells were incubated with various concentrations (*X*-axis) of hu3S193 or hu3S193-CalichDMH. The amount of bound conjugate or antibody was expressed as mean channel fluorescence (*MCF*, *Y*-axis). The MCF represents the average fluorescent intensity of a cell population after binding of the primary antibody and the consecutive staining with a fluorescent-labeled secondary antibody.

relate with different amounts of conjugate or antibody that could bind to N87 cells.

Hu3S193 Binds to Various Cultured Carcinoma Cells. The presence of Le^y on a series of human tumor cell lines was evaluated by FACS analysis. The MCF values in Table 1 are the average fluorescent intensity of cell populations after binding with the primary antibodies (huIgG and hu3S193) and consecutive staining with a fluorescent-labeled secondary antibody. HuIgG is a negative control. The MCF is proportional to the number of bound primary antibody molecules. The majority (8 of 13) of the investigated cell lines expressed Le^y as seen by at least a 10-fold (= relative MCF, reMCF) increase of the MCF after hu3S193 binding over the MCF of the negative control. Except for the two lung carcinomas, examples of cell lines with high expression of Le^y were found in each histiotypic tumor category. All of the tumor cells of colorectal and gastric origin were Le^y-positive.

Hu3S193-CalichDMH Specifically Inhibits Growth of Le^y-Expressing Carcinoma Cells *in Vitro*. The efficacy of hu3S193-CalichDMH was compared *in vitro* to that of CalichDMH (free drug) and CMA-676 (control conjugate). CMA-676 is an AcBut-linked conjugate of an antibody against CD33 and CalichDMH. CD33 is a leukocyte differentiation antigen expressed by multipotential hematopoietic stem cells and acute myeloid leukemic cells. Therefore, expression of this antigen by the carcinoma cell lines outlined in Table 1 was considered unlikely. Indeed, FACS analysis showed that the amount of CMA-676 bound to these cell lines was similar to the amount of control $huIgG_1$ demonstrating the lack of CD33 expression. The highest binding of CMA-676 was determined in PC3MM2 cells (reMCF = 3). Therefore, CMA-676 treatment controls for the efficacy of released CalichDMH without antigen targeting of the conjugate. This could result from the hydrolytic release of CalichDMH caused by acidification of the culture medium by the cells or possibly by lysosomal hydrolysis after internalization of the conjugate by pinocytosis. Free hu3S193 antibody did not affect the growth of LOVO, N87, or AGS when used in concentrations ranging from 1×10^{-4} to 6.9 μg protein/ml. This range of protein concentrations was equivalent to the amounts of antibody given as a conjugate. The results of a representative experiment are shown in Table 2. The table shows the ED_{50} of the three treatments (hu3S193-CalichDMH, CalichDMH, and CMA-676) on cell lines with various amounts of Le^y (see reMCF values). The ED_{50} indicates the dose (ng/ml) at which 50% of the cell culture survives after exposure to CalichDMH or to conjugates for 96 h. The ED_{50} of hu3S193-CalichDMH was consistently lower in Le^y-positive cells (reMCF >10) than the ED_{50} of CMA-676. The data presented in Fig. 4 support this conclusion. Panels *A* and *B* of Fig. 4 show the ED_{50} values (pooled from nine experiments) of CMA-676 and hu3S193-Cal-ichDMH on AGS (Le^y-positive cells) and PC3MM2 (Le^y-negative cells). Interexperimental variation of the ED_{50} of both conjugates was observed. However, the ED_{50} range of hu3S193-CalichDMH was consistently lower than that of CMA-676 when the efficacy of the conjugates on the Le^{y+} AGS cells was tested (Fig. 4*A*). In contrast, these ranges were superimposed when the efficacy of both conjugates was determined on the Le^{y-} PC3MM2 cells (Fig. 4*B*). Fig. 4*C* shows that this result was unlikely caused by the selection of the two cell lines. Fig. 4*C* illustrates that a comparison of the ED_{50}s of CMA-676 and hu3S193-CalichDMH in parallel experiments using Le^{y+} cells showed on average a lower ED_{50} for hu3S193-CalichDMH than for CMA-676 (fold CMA-676 < 1). This finding was independent of the origin of the cell line, its sensitivity to calicheamicin, and its relative amount of Le^y. The parameter fold CMA-676 was >1 when various Le^{y-} cells were used. Various hu3S193-CalichDMH conjugate preparations

Table 1 Expression of Le^y on cultured human carcinoma cells as evaluated by the increased mean channel fluorescence after probing with hu3S193 as compared to probing with a control antibody (huIgG)

Cell line	Antibody	huIgG	hu3S193
Colon carcinoma			
DLD-1		2.7	1167.2
HCT8S11		4.1	2221.5
HCT8S11/R1		2.8	1162.0
LOVO		4.7	62.3
Lung carcinoma			
A549		2.8	5.3
H157		2.4	3.4
Gastric carcinoma			
AGS		2.9	1062.8
N87		4.0	771.1
Prostate carcinoma			
LNCaP		4.8	49.7
PC3		4.6	40.6
PC3-MM2		3.1	18.5
Breast carcinoma			
MDA-MB361		9.4	297.9
MDA-MB435		3.1	3.2

Le, Lewis.

Table 2 ED_{50} values *in vitro* after treatment of carcinoma cells with calichDMH,[a] CMA-676, or hu3S193-calichDMH

	Cell lines							
	Le^{y+}							Le^{y-}
	AGS	HCT8S11	N87	LOVO	LNCaP	HCT8S11/R1	NCI-H358	PC3-MM2
Le^y (reMCF[b])	100	92	57	37	33	31	13	1.7
Treatment								
CalichDMH	<0.01[c]	30	12	2.2	3.3	33	3	6
CMA-676	5	>500	222	60	4	>500	120	20
Hu3S193-calichDMH	0.32	20	90	32	<0.01	23	90	50
Fold CMA-676[d]	0.06	<0.04	0.41	0.53	<0.03	<0.05	0.75	2.50

[a] CalichDMH, *N*-acetyl γ calicheamicin dimethyl hydrazide; Le, Lewis.
[b] reMCF, relative MCF, *i.e.* MCF after probing with hu3S193 divided by the MCF after probing with control hulgG.
[c] The number reflects the ED_{50} in ng/ml calicheamicin equivalents.
[d] Fraction determined by the formula: ED_{50}[hu3S193-calich.DMH]/ED_{50}[CMA-676].

were used for these experiments (22 and 47 μg CalichDMH/mg protein) indicating that the observations were independent of this variable. Taken together, the results illustrate the selective cytotoxicity of hu3S193-CalichDMH due to targeting CalichDMH to Le^y.

Hu3S193-CalichDMH Selectively Inhibits Growth of Xenografts in Nude Mice. The efficacy *in vivo* of Hu3S193-CalichDMH was tested on s.c. xenografts from gastric (N87; Fig. 5), prostate (LNCaP; Fig. 6), and colon (LOVO; Figs. 7 and 8) carcinomas. Unless indicated otherwise, three doses of each conjugate and control were given i.p. with an interval of 4 days (Q4Dx3). At 1, 2, and 4 μg cal.eq/dose/mouse, hu3S193-CalichDMH significantly inhibited the tumor growth of N87 xenografts (Fig. 5). A complete response rate of 100, 60, and 10% was also observed at 4, 2, and 1 μg cal.eq/dose/mouse, respectively. The term complete response indicates that the size of the xenograft decreases and never exceeds the initial average tumor volume during 100 days after initiation of treatment. To identify the target specificity of hu3S193, CMA-676 and Rituxan-CalichDMH were used as negative controls. CMA-676 as well as Rituxan-CalichDMH were selected for this purpose, because FACS analyses showed that the antigens recognized by CMA-676 (CD33) or Rituxan (CD20) were either completely absent from (CD33) or present in trace amounts (CD20) on the surface

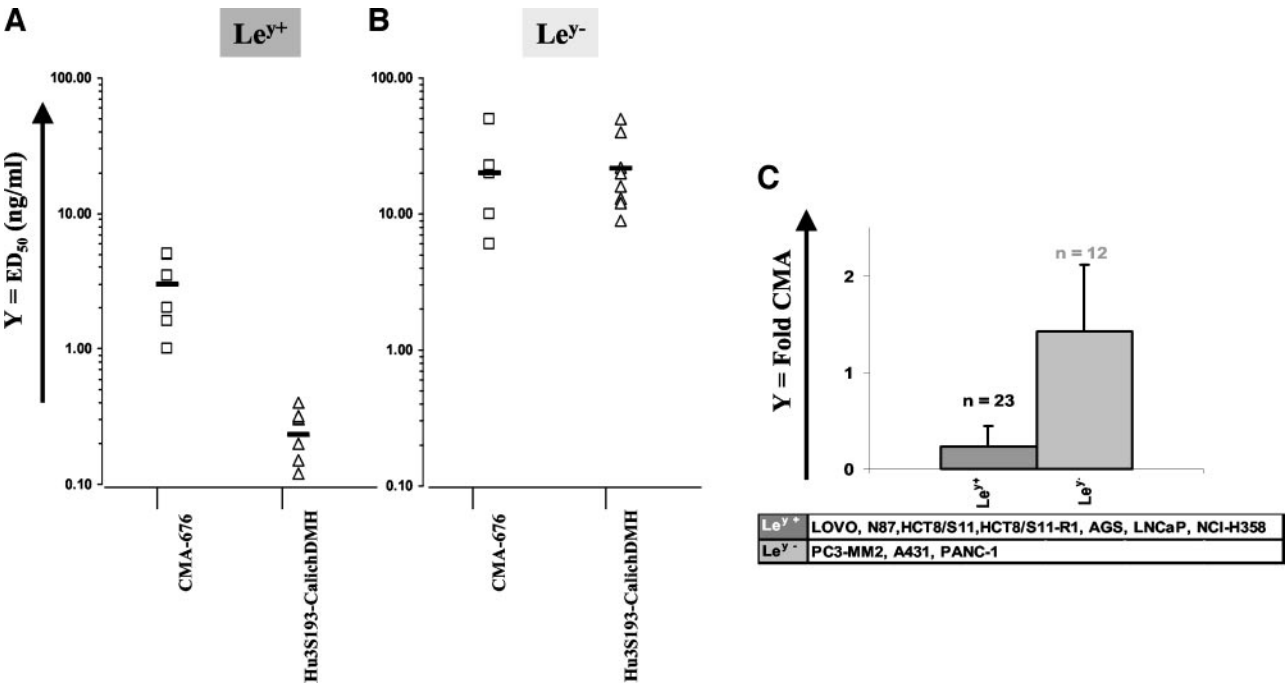
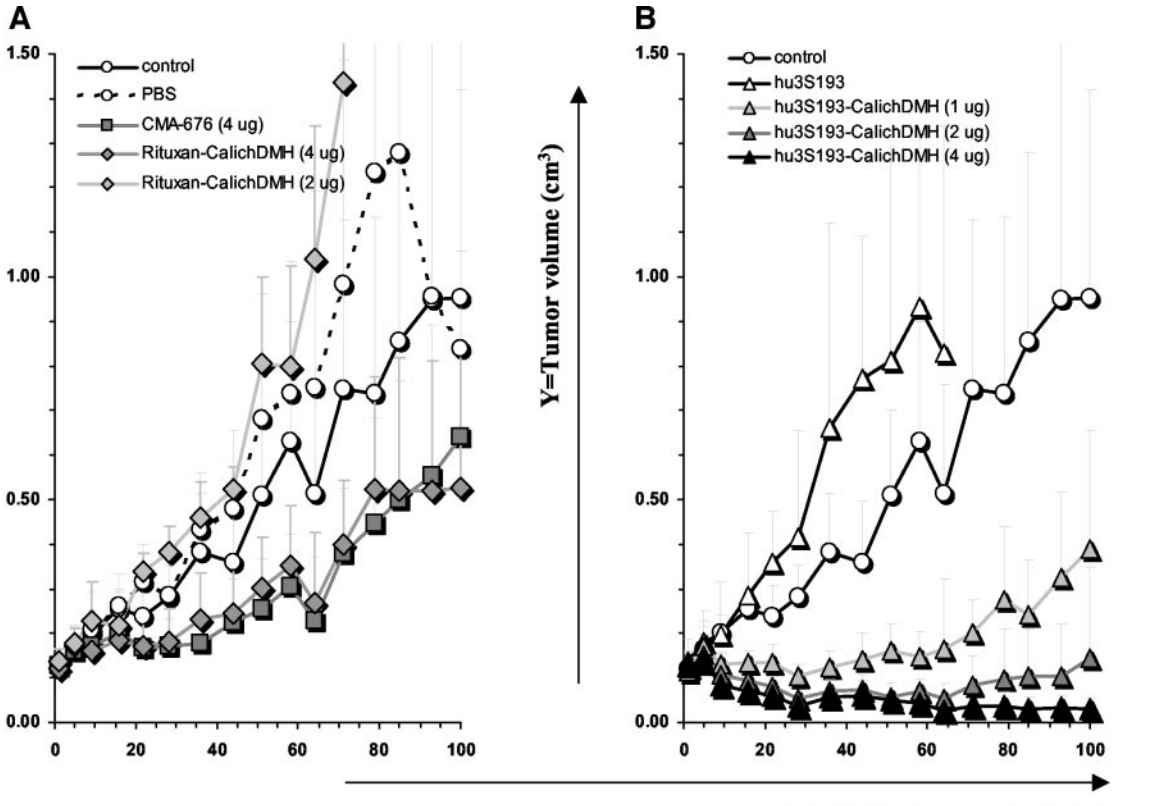


Fig. 4 Selective efficacy of hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (CalichDMH) on Lewis $(Le)^{y+}$ cells. ED_{50} values of hu3S193-CalichDMH and CMA-676 were pooled from nine experiments. The ED_{50} values of hu3S193-CalichDMH (−) and CMA-676 (○) were compared on Le^{y+} cells (AGS, *A*) and on Le^{y-} cells (PC3MM2, *B*). Notice that the average ED_{50} (⌐) of hu3S193-CalichDMH is lower than the average ED_{50} of CMA on Le^{y+} but not on Le^{y-} cells. For a group of 10 cell lines, the ED_{50} values of hu3S193-CalichDMH were also evaluated against those of CMA-676, used as an internal control in each experiment (fold CMA-676). In Part *C*, *n* = number of independent ED_{50} determinations.



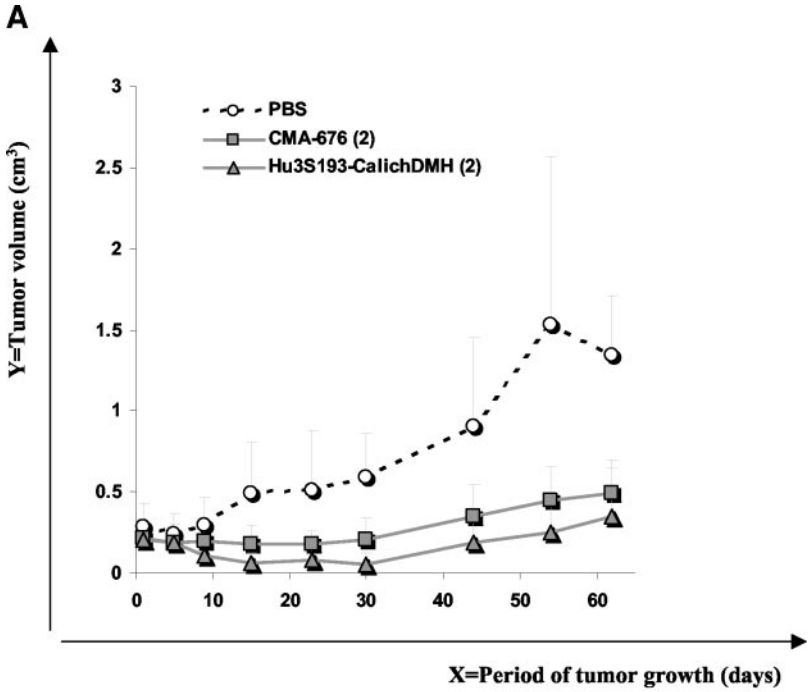
C

PBS n = 10	Control n = 8	Hu3S193 n = 5	Hu3S193-CalichDMH (4) n = 8	Hu3S193-CalichDMH (2) n = 8	Hu3S193-CalichDMH (1) n = 7	Rituxan-CalichDMH (4) n = 7	Rituxan-CalichDMH (2) n = 4	CMA-676 (4) n = 10	
1.000	0.950	0.294	<0.001	<0.001	<0.001	0.019	0.070	0.003	PBS
	1.000	0.284	<0.001	<0.001	<0.001	0.014	0.032	0.002	Control
		1.000	0.024	0.027	0.042	0.093	0.774	0.081	Hu3S193
			1.000	0.193	<0.001	<0.001	<0.001	<0.001	Hu3S193-CalichDMH (4)
				1.000	0.001	0.001	<0.001	<0.001	Hu3S193-CalichDMH (2)
					1.000	0.019	<0.001	0.016	Hu3S193-CalichDMH (1)
						1.000	<0.001	0.693	Rituxan-CalichDMH (4)
							1.000	<0.001	Rituxan-CalichDMH (2)
								1.000	CMA-676 (4)

Fig. 5 Inhibition of N87 tumor growth by hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (*CalichDMH*). Mice bearing N87 [Lewis $(Le)^{y+}$, $CD33^{-}$, and $CD20^{-}$] xenografts of 100 mm^3 were treated with control conjugates (CMA, rituxan-CalichDMH), PBS, hu3S193, or hu3S193-CalichDMH. Mice in each group received three doses i.p. The amount of each dose in calicheamicin equivalents is specified in the legend. Conjugates and controls were injected at days 1, 5, and 9. *A* shows the efficacy of control conjugates. *B* illustrates the effects of hu3S193 and of its CalichDMH conjugate. *Bars,* SD of the average tumor volume at each time point. Differences in tumor size among the treated groups of tumor bearing mice have been probed by a 2-tailed Student's t test. The *P*-values at day 28 are shown in *C*. *n* = number of mice per group.

of N87 cells. These CalichDMH conjugates controlled for the carrier function of immunoglobulins (IgG_4, CMA-676 and IgG1, Rituxan) and the hydrolytic release of CalichDMH. As shown in Fig. 5, these conjugates inhibited tumor growth to a lesser extent than hu3S193-CalichDMH at equivalent or lower doses. Moreover, 0% complete response rates were observed after treatment with control conjugates. Hu3S193, when administered at a dose and regimen equivalent to the protein amount (120 μg) given with 4 μg cal.eq. hu3S193-CalichDMH had no effect. Previous experiments showed that administration of CalichDMH at doses equivalent to hu3S193-CalichDMH did not inhibit any of the tumor models tested thus far (data not shown). Therefore, administration of CalichDMH has been omitted as a control in the current studies.

The capacity of hu3S193-CalichDMH to inhibit tumor growth was also demonstrated in a prostate (LNCaP) and a colon carcinoma (LOVO) model. Fig. 6 shows that hu3S193-CalichDMH inhibits LNCaP growth at 2 μg/cal.eq./dose/mouse. However, the limited number of mice ($n = 5$) used in this experiment did not allow for statistical distinction of this



B

PBS n = 5	Hu3S193-CalichDMH (2) n = 5	CMA-676 (2) n = 6	
1.000	0.008	0.030	PBS
	1.000	0.062	Hu3S193-CalichDMH (2)
		1.000	CMA-676 (2)

Fig. 6 Inhibition of LNCaP (prostate carcinoma) tumor growth by hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (*CalichDMH*). Tumor-bearing mice were treated with hu3S193-CalichDMH, PBS, or the control conjugate CMA. The number between brackets in the legend (*A*) indicates the amount (μg) of CalichDMH per dose per mouse. Differences in tumor size among the treated groups have been probed by a 2-tailed Student's t test (*B*). The *P*-values at day 30 are reported. n = number of mice.

effect from that of CMA-676 (Fig. 6*B*). Hu3S193 inhibited growth of LOVO− xenografts to a lesser extent than observed with N87− xenografts. Control conjugates (Rituxan-CalichDMH or CMA-676) caused a negligible tumor inhibition (Fig. 7). The inhibition caused by hu3S193-CalichDMH was more prolonged than that of the control conjugates. For example differences between, on the one hand, the tumor size after treatment with Rituxan-CalichDMH at doses of 4 and 2 μg cal.eq./mouse (Q4Dx3) and, on the other hand, the tumor size after treatment with PBS were only significant for 16 days ($P < 0.05$). In contrast, treatment with hu3S193-CalichDMH at doses of 4, 2 and 1 μg cal.eq/mouse (Q4Dx3) resulted in statistical differences from the PBS treatment for 43, 22, and 16 days respectively.

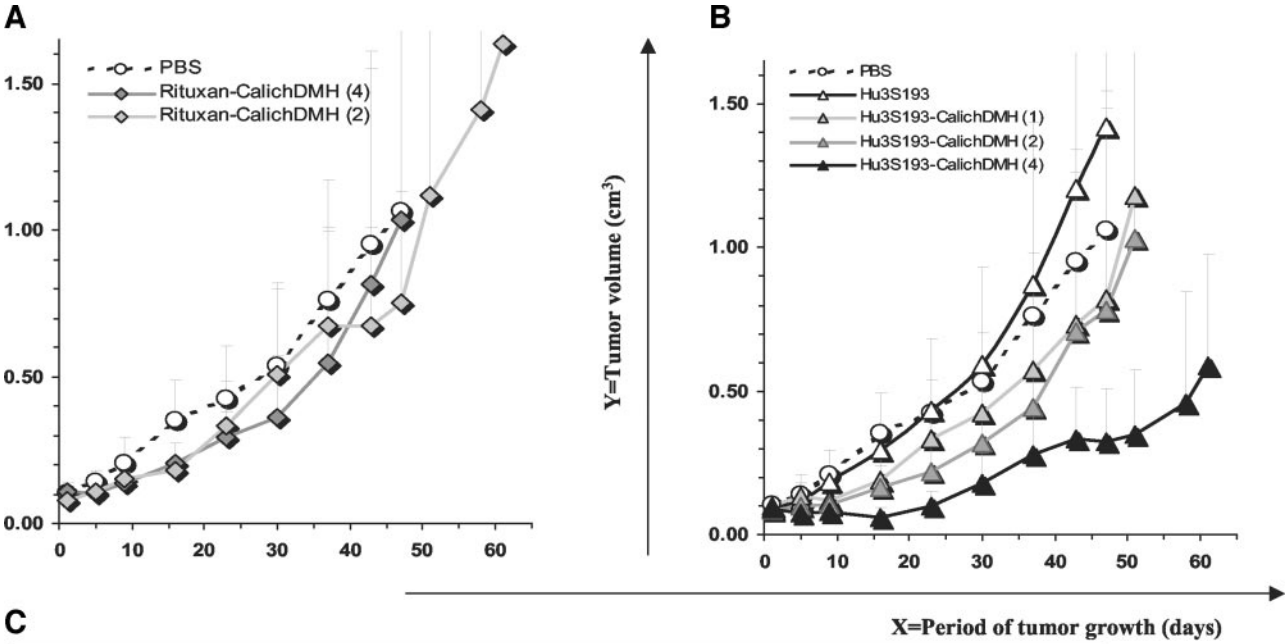
Furthermore, administering a second regimen of hu3S193-CalichDMH (Fig. 7*B*) or a fourth dose (Fig. 8*B*) improved the efficacy. The fact that increasing the total amount of CalichDMH per mouse from 12 to 16 μg did not result in lethality also indicated that 4 μg CalichDMH (Q4Dx3) of hu3S193-CalichDMH was at most 75% of the maximum nonlethal dose. This was consistent with previous determinations of the maximum nonlethal dose (18 μg cal.eq) of a CalichDMH conjugate to an anti-CD22 antibody (19). This antibody was bound to CalichDMH by the same AcBut linker as used in hu3S193-CalichDMH.

DISCUSSION

The immunoconjugate hu3S193-CalichDMH causes selective tumor growth inhibition in carcinoma models *in vitro* and *in vivo*.

In vitro, the efficacy of hu3S193-CalichDMH is qualitatively dependent on the expression of Le^y and on the sensitivity of the tumor cells to calicheamicin. Quantitatively, the efficacy is neither directly proportional to the sensitivity of tumor cells to calicheamicin nor to the amount of Le^y expressed on the cell surface. Furthermore, hu3S193-CalichDMH is selectively cytotoxic when compared with CMA-676. The ED_{50} of free drug remained, in the majority of cases, lower than the ED_{50} of hu3S193-CalichDMH. This finding is in contrast with results obtained with CMA-676 or CMC-544 (19). CMC-544 is a CD22-targeted immunoconjugate of a humanized IgG4 anti-CD22 monoclonal antibody, g5/44, that is linked to CalichDMH with the same acid-labile linker as used for CMA-676 and hu3S193-CalichDMH. CMA-676 and CMC-544 were more toxic *in vitro* than CalichDMH for CD33- or CD22-expressing cells, respectively.

One can envision several explanations for the difference in selectivity of hu3S193-CalichDMH on the one hand and that of CMA-676 and CMC-544 on the other hand. CMC-544 and CMA-676 have a higher affinity for their respective



C

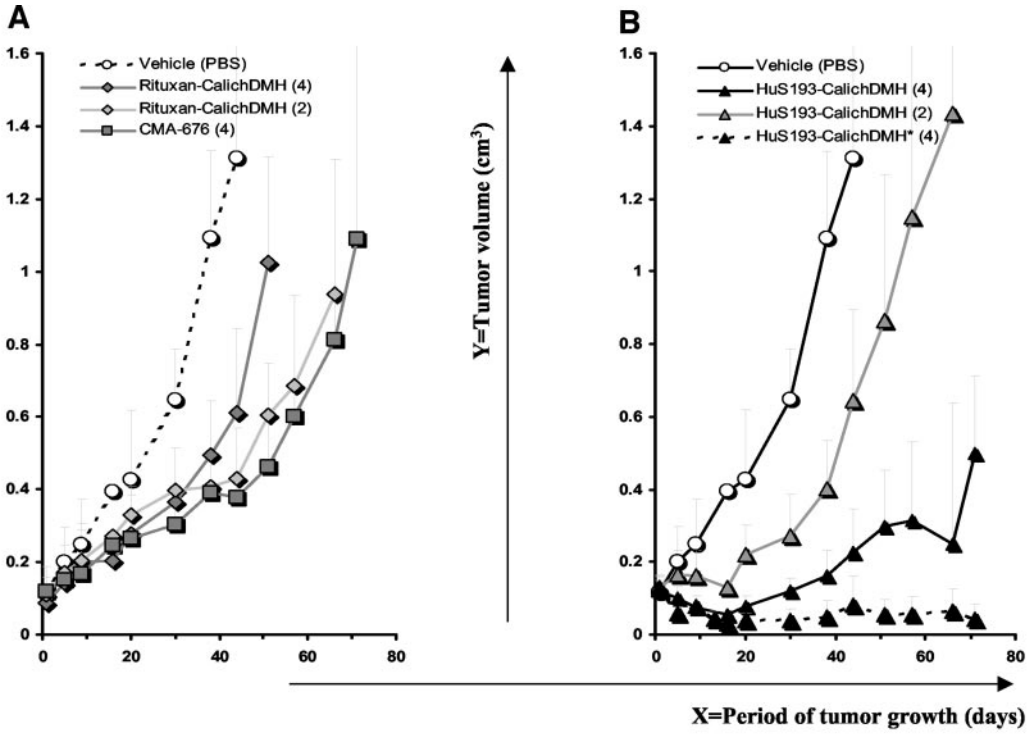
PBS n = 10	Hu3S193-CalichDMH (4) n = 6	Hu3S193-CalichDMH (2) n = 6	Hu3S193-CalichDMH (1) n = 9	Hu3S193 n = 7	Rituxan-CalichDMH (4) n = 7	Rituxan-CalichDMH (2) n = 5	
1.000	0.002	0.100	0.378	0.713	0.173	0.865	PBS
	1.000	0.142	0.040	0.019	0.078	0.077	Hu3S193-CalichDMH (4)
		1.000	0.438	0.108	0.683	0.233	Hu3S193-CalichDMH (2)
			1.000	0.296	0.652	0.602	Hu3S193-CalichDMH (1)
				1.000	0.158	0.681	Hu3S193
					1.000	0.350	Rituxan-CalichDMH (4)
						1.000	Rituxan-CalichDMH (2)

Fig. 7 Inhibition of LOVO (colon carcinoma) tumor growth by hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (*CalichDMH*). Mice bearing LOVO xenografts of 100 mm^3 were treated with control conjugates (rituxan-CalichDMH, *A*), PBS (*A* and *B*), hu3S193 (*B*), or hu3S193-CalichDMH (*B*). Except for the group treated with hu3S193-CalichDMH at 4 μg/dose, mice in each group received three doses i.p. The amount (μg) of each dose in calicheamicin equivalents is specified in the legend. Conjugates and controls were injected at days 1, 5 and 9. The group designated hu3S193-CalichDMH, received an additional regiment of three doses at day 43, 47, and 51. The number of mice per group (*n*) is reported in *C*. Differences in tumor size at day 30 were probed for statistical significance by a 2-tailed Student's t test.

antigens than hu3S193 has for Le^y. Whereas the low affinity may negatively influence the efficacy of a conjugate *in vitro*, the internalization rate of Le^y can also be a determining factor for the selectivity of hu3S193. The Le^y antigen is cytophysiologically different from CD33 and CD22 in a sense that internalization of Le^y depends on the internalization of the glycoprotein or glycosphingolipid that carries the antigen. Hence, although Le^y internalization has been reported for various cell types (22, 24), it is difficult to imagine that different cell types would have similar internalization rates for Le^y. A different internalization rate could also explain why two cell lines with comparable sensitivity to CalichDMH and expression of Le^y (*e.g.*, LNCaP and LOVO; Table 2) still have different sensitivity to hu3S193-CalichDMH. Le^y expression on the cell surface is also variable. The average MCF of eight independent measurements on N87 cells was 4160 with a SD of 46%. Similar variability was observed previously in cultures of H3396 breast carcinoma and ascribed to variation in culture density (30). How the aforementioned variables of Le^y internalization and expression influence the efficacy of hu3S193-CalichDMH needs to be investigated to potentially establish predictive parameters for the efficacy of hu3S193-CalichDMH. Nonetheless, our experiments *in vitro* demonstrated that these variables could only account for quantitative interexperimental variation of the ED_{50} of hu3S193-CalichDMH. Qualitatively, the presence of Le^y on the cell surface correlated consistently with a higher efficacy of hu3S193-CalichDMH than that of CMA-676.

In vivo, hu3S193-CalichDMH inhibited tumor growth in three separate models. Hu3S193-CalichDMH caused regression and growth arrest of gastric carcinoma xenografts (N87) for at least 100 days. Prostate carcinoma xenografts (LNCaP) ceased to grow after administration of hu3S193-CalichDMH, and inhibition of tumor growth was obtained with colon carcinoma xenografts (LOVO). In the LOVO model, the efficacy of hu3S193-CalichDMH was improved by increasing the amount of the conjugate without exceeding the minimum lethal dose. Concerning the experiments *in vivo*, two points deserve additional discussion.

In the first place, CalichDMH-conjugates (CMA-676,



C

PBS n = 7	Hu3S193-CalichDMH (4) n = 9	Hu3S193-CalichDMH (2) n = 8	Hu3S193-CalichDMH* (4) n = 4	Rituxan-CalichDMH (4) n = 7	Rituxan-CalichDMH (2) n = 7	CMA-676 (4) n = 5	
1.000	0.001	0.002	0.000	0.022	0.115	0.014	PBS
	1.000	0.009	0.053	0.001	0.058	0.002	Hu3S193-CalichDMH (4)
		1.000	0.001	0.221	0.356	0.665	Hu3S193-CalichDMH (2)
			1.000	0.001	0.022	0.004	Hu3S193-CalichDMH* (4)
				1.000	0.821	0.453	Rituxan-CalichDMH (4)
					1.000	0.543	Rituxan-CalichDMH (2)
						1.000	CMA-676 (4)

Fig. 8 Comparison of tumor growth inhibition by hu3S193-*N*-acetyl γ calicheamicin dimethyl hydrazide (*CalichDMH*) administered at 4 μg Q4Dx3 or Q4Dx4. Mice bearing LOVO xenografts of 100 mm^3 were treated with control conjugates: Rituxan-CalichDMH and CMA (*A*), PBS (*A* and *B*), or hu3S193-CalichDMH (*B*). Mice in each group received three or four doses i.p. The amount (μg) of each dose in calicheamicin equivalents is specified in the legend. Conjugates and controls were injected at days 1, 5, and 9. The group designated hu3S193-CailchDMH∗, received an additional dose at day 13. The number of mice per group (*n*) and *P*-values of a 2-tailed Student's t test are indicated in *C*.

Rituxan-CalichDMH) that targeted antigens absent in the tumor cells exerted significant growth-inhibitory effects against LNCaP and N87 tumors. This growth inhibition is not simply caused by free CalichDMH in the blood circulation, because 4 μg of CalichDMH (Q4Dx3) did not reduce tumor growth in either model (data not shown). Requirement of conjugation of CalichDMH to an antibody suggests the necessity of a carrier to concentrate the drug in the tumor. Such carrier function of an antibody has been defined as "passive targeting" (31). The therapeutic efficacy by passive targeting is not an artifact caused by the use of Matrigel, which was used to improve tumor take. Matrigel is capable of binding immunoglobulins and could, therefore, retain the conjugate in the tumor. However, tumor xenografts (*e.g.*, A431 cervix carcinoma) that were established without Matrigel were also subject to growth inhibition by passive targeting.[3] *Vice versa*, passive targeting was not observed in non-Hodgkin's lymphoma models that required the use of Matrigel to grow as xenografts (19). This refuted the notion that passive targeting would be a phenomenon exclusively and obligatorily associated with the use of Matrigel. Thus far, little is known about the underlying pathophysiological mechanism of passive targeting. Takakura *et al.* (31) reported that IgG was a suitable carrier for passive tumor targeting. This was evidenced by accumulation of radiolabeled IgG in xenografts. We obtained similar results using I^{131}-labeled p67.6 (anti-CD33).[3] In anal-

[3] Unpublished observations.

ogy with the enhanced permeability and retention effect that is responsible for polymer-drug targeting (32), the proposed mechanism of antibody accumulation in the tumor was based on the enhanced vascular permeability of tumor tissue (33). This would enable the tumor to siphon off macromolecules with a large molecular weight and a negative charge. Extending this reasoning, CalichDMH conjugated to IgG with an acid hydrolyzable linker could be released by the acidic interstitial medium of the tumor. Free CalichDMH, in turn, becomes capable of penetrating and destroying tumor cells. The fact that therapeutic efficacy caused by passive targeting was frequently observed with calicheamicin-antibody conjugates can be due to the high potency of calicheamicin. Passive targeting has rarely been seen with antibodies that carry less potent cytotoxic agents (34). Our experience with calicheamicin conjugates demonstrated that the sensitivity of the tumor cells *in vitro* was directly proportional to the growth inhibition *in vivo* caused by passive targeting. The antitumor efficacy of passive targeting was readily distinguishable from antigen-specific targeted chemotherapy. The latter caused longer lasting antitumor effects and was usually more effective at lower doses. Regardless of the mechanism of passive targeting, it is clear that targeting mechanisms other than antigen-antibody recognition had to play a role in the antitumor efficacy of immunoconjugates in certain xenografts. Nonetheless, the fact that passive targeting remains a tumor-specific phenomenon only adds to the therapeutic benefit of antibody-targeted chemotherapy. Currently, there is no definite evidence for therapeutic benefit in man by passive targeting of CalichDMH conjugates. However, the possibility that mechanisms other than targeting a TAA may contribute to the efficacy of these conjugates was substantiated recently by the observation that Mylotarg can be effective in patients with CD33-negative leukemia (35).

Secondly, the efficacy of hu3S193-CalichDMH was variable for the different xenograft models. Our data indicated that this variability was partly due to a combination of Le^y expression and sensitivity of the tumors to calicheamicin. The efficacy of hu3S193-CalichDMH on LOVO- xenografts improved by increasing the number of doses of hu3S193-CalichDMH, indicating that an efficacious therapeutic regimen of the conjugate may vary depending on the targeted tumor. As mentioned earlier, the carrier glycoproteins of Le^y could play a role in the internalization of hu3S193. In other words, the membrane glycoprotein make-up of individual tumors could indicate the sensitivity of tumors to hu3S193-CalichDMH. Evaluation of this hypothesis can provide valuable information for the clinical application of hu3S193-CalichDMH, and will, therefore, be the subject of additional studies.

The limitations of efficacy in xenograft models in predicting clinical outcome are generally acknowledged. In this regard, a conjugate of an anti-Le^y antibody (BR96) and doxorubicin yielded efficacy in xenograft models similar to hu3S193-CalichDMH (36). The doxorubicin conjugate encountered dose-limiting toxicity in clinical trials (37). Relative lack of efficacy of this conjugate at the tolerated dose necessitated its discontinuation from additional development as single therapy for metastatic breast cancer. Hu3S193-CalichDMH has several characteristics that may help circumvent the problems encountered with the doxorubicin conjugate. In radioimaging studies, hu3S193 reveals tumor lesions but does not penetrate to the epithelium of the gastrointestinal track (Le^{y+}; Ref. 38). This finding renders it unlikely that hu3S193-CalichDMH would cause targeted gastrointestinal toxicity. In addition, because CalichDMH is at least 10-fold more potent than doxorubicin, the amount of conjugate necessary to obtain clinical efficacy may be considerably lower for hu3S193-CalichDMH than for BR96-dox. The need for lower amounts of hu3S193-CalichDMH could reduce the toxicity potentially caused by effector functions of the antibody (CDC and ADCC).

In conclusion, selectivity and efficacy of hu3S193-CalichDMH against Le^{y+} tumors supports additional evaluation of this conjugate for clinical application.

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