α_v Integrin-Targeted Immunoconjugates Regress Established Human Tumors in Xenograft Models

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Abstract

Purpose: Targeted delivery of cytotoxic agents to solid tumors through cell surface antigens can potentially reduce systemic toxicity and increase the efficacy of the targeted compounds. The purpose of this study was to show the feasibility of treating solid tumors by targeting α_v integrins with antibody-maytansinoid conjugates and to test the relative *in vivo* activities of several linker-maytansinoid chemistries.

Experimental Design: CNTO 364, CNTO 365, and CNTO 366 are targeted cytotoxic agents created by conjugating the CNTO 95 anti-α_v integrin antibody with three distinct maytansinoid-linker structures. These structures were designed to have varying degrees of chemical substitution surrounding the disulfide bond linking the cytotoxic agent to the antibody. A model conjugate was shown to be specifically cytotoxic *in vitro* and highly active against established human tumor xenografts in immunocompromised rats. The *in vivo* antitumor activities of CNTO 364, CNTO 365, and CNTO 366 were compared in rat xenograft models.

Results: CNTO 365, with a linker chemistry of expected intermediate stability, was shown to be substantially more active than the other two conjugates with lesser or greater substitution around the disulfide linkage.

Conclusion: CNTO 95–maytansinoid immunoconjugates are potent antitumor agents against α_v integrin–expressing human carcinomas. These studies show for the first time the feasibility of targeting α_v integrins on solid tumors with tumor-activated prodrugs. The DM4 linker-maytansinoid configuration of CNTO 365 was substantially more active in the models tested here when compared with alternative configurations with greater or lesser chemical substitution surrounding the linker.

The α_v integrin subfamily consists of at least five members, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ (1). α_v Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ have been implicated in angiogenesis and tumor progression (2–5). Up-regulation of α_v integrins has been observed in various types of human cancer, including melanoma (6), renal (7), ovarian (8), gastric (9), breast (10), and colorectal carcinoma (11). The frequent overexpression of α_v integrins by tumors, their more limited expression by healthy tissues, and their importance in tumor growth and progression make them attractive targets for cancer therapies. CNTO 95, a fully human monoclonal antibody, inhibits α_v integrins and has *in vivo* antitumor and antiangiogenic activity (12). It is one of several α_v integrin inhibitors, including both monoclonal antibodies and small molecules, that have been tested both preclinically and clinically for the treatment of solid tumors (4).

Immunoconjugates are bifunctional molecules that combine the specificity of monoclonal antibodies to tumor antigens with the potency of cytotoxic agents (13–15). To take advantage of the α_v integrin specificity of CNTO 95, we generated the antibody-drug conjugates CNTO 364, CNTO 365, and CNTO 366. These molecules are tumor-activated prodrugs in which derivatives of the natural microbial fermentation product and extremely potent antimicrotubule agent ansamitocin P-3 (14) are linked to the antibody via disulfide bonds. CNTO 364 consists of CNTO 95 conjugated to DM1, whereas CNTO 365 and CNTO 366 contain DM4 (14, 16, 17). These moieties differ in the number of chemical substituents at important positions in the maytansinoid-antibody linker and are therefore expected to exhibit different chemical stabilities and metabolic fates in tumor cells (17, 18). Here, we describe the *in vitro* cytotoxicity of CNTO 364 and its selectivity for α_v integrin–expressing cells. We also show the superior *in vivo* antitumor effects of CNTO 364 versus its constituent components. We report the *in vivo* tumor-regressing activity of anti-α_v integrin–targeted maytansine conjugates for the first time. To date, most published studies have described DM1-conjugated antibodies. The results reported here show the differential *in vivo* efficacy of the DM1 conjugate CNTO 364 and the DM4 conjugates CNTO 365 and CNTO 366 against established α_v integrin–expressing human tumors in xenograft models.

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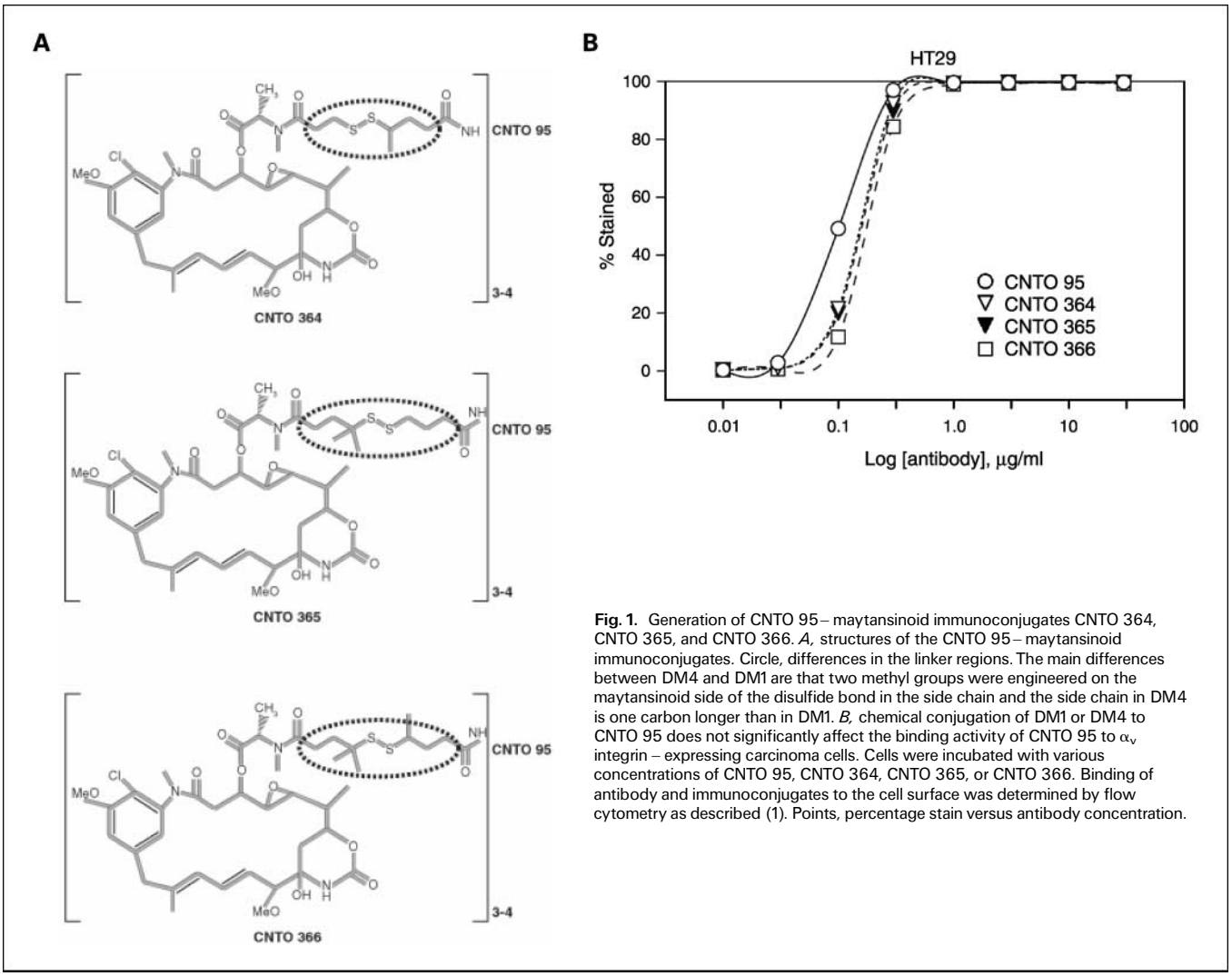

Fig. 1. Generation of CNTO 95–maytansinoid immunoconjugates CNTO 364, CNTO 365, and CNTO 366. *A,* structures of the CNTO 95–maytansinoid immunoconjugates. Circle, differences in the linker regions. The main differences between DM4 and DM1 are that two methyl groups were engineered on the maytansinoid side of the disulfide bond in the side chain and the side chain in DM4 is one carbon longer than in DM1. *B,* chemical conjugation of DM1 or DM4 to CNTO 95 does not significantly affect the binding activity of CNTO 95 to α_v integrin–expressing carcinoma cells. Cells were incubated with various concentrations of CNTO 95, CNTO 364, CNTO 365, or CNTO 366. Binding of antibody and immunoconjugates to the cell surface was determined by flow cytometry as described (1). Points, percentage stain versus antibody concentration.

Materials and Methods

Preparation of CNTO 95–DM1 and CNTO 95–DM4 immunoconjugates. The thiolated maytansine derivatives DM1 and DM4 were synthesized from the microbial fermentation product ansamitocin P3 as described (17). CNTO 95 was manufactured by Centocor R&D, Inc. (12). Antibody-drug conjugates were prepared as described previously by ImmunoGen (17). On average, 3.5 maytansinoid molecules (DM1 or DM4) were linked per antibody.

Cell lines. All cell lines were obtained from American Type Culture Collection. HT-29 human colon adenocarcinoma cells and A549 human lung carcinoma cells were cultured in αMEM (Life Technologies) supplemented with 10% fetal bovine serum. B16-F10 mouse melanoma cells were maintained in DMEM plus 10% fetal bovine serum. All cell lines were incubated at 37°C in the presence of 5% CO_2.

Flow cytometry. α_v Integrin expression on the tumor cell surface and the binding affinity of both the antibodies and immunoconjugates to the tumor cells were determined by flow cytometry as described previously (12).

In vitro *cell proliferation assays.* Cells were plated at a density of 4,000 per well in 96-well plates, and allowed to recover overnight (16 h) in full culture medium. The medium was removed by aspiration. CNTO 364 or control compounds were added in full culture medium at concentrations indicated in the figure legends. After 24 h incubation with CNTO 95 or CNTO 364, the medium was replaced with fresh compound-free culture medium. Cells were incubated for an additional 72 h. Cell proliferation and survival was determined using the ATPLite assay (Perkin-Elmer Life Sciences) according to the manufacturer's instruction.

Animals and tumor cell inoculation. Female Rowett nude rats (4-6 weeks of age) from Harlan Laboratory were used in these studies. Food and water were supplied *ad libitum*. The animals were maintained in a facility approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture. The protocol was reviewed and approved by the Centocor Institutional Animal Care and Use Committee. The animals were injected with HT-29 or A549 cells s.c. in the rear flank and randomized to treatment groups when the mean tumor volume was ~250 mm^3. CNTO 364, CNTO 365, or the control compounds were administered i.v. on various schedules as described in the text starting on the day of randomization. Body weight was recorded twice weekly.

Tumor assessment and statistical method. Tumor volume was recorded twice weekly. Tumors were measured with electronic vernier calipers in two dimensions (length and width) in millimeters (mm). Tumor volume (mm^3) was calculated using the formula $V = (\text{length} \times \text{width} \times \text{width}) / 2$. Any tumor volume <13 mm^3 for more than three

consecutive measurements or the absence of a visible tumor at termination of the study was considered a complete regression.

Repeated-measures ANOVA with Dunnett's test or unpaired *t* test using GraphPad Prism 4 software (GraphPad Software) with a 95% confidence interval was used to analyze the data.

Results

Chemical structures and binding properties of anti-α_v integrin immunoconjugates. The chemical linkages between CNTO 95 and the maytansine derivatives are disulfide bonds. Alternative maytansinoid derivatives (DM1, DM4) and linkers have been developed to achieve sterically hindered disulfide bonds that differ in their resistance to cleavage, which can impact compound properties *in vivo* (17). In these studies, we examined the properties of several immunoconjugates with varying degrees of substitution surrounding the disulfide bond. These included the CNTO 95–maytansinoid immunoconjugates CNTO 364 (CNTO 95–SPP-DM1), CNTO 365 (CNTO 95–SPDB-DM4), and CNTO 366 (CNTO 95–SPP-DM4). The structures of the linkers and cytotoxic molecules present in each of these conjugates are shown in Fig. 1A.

Antigen-binding activity of CNTO 95 was retained after chemical conjugation with the cytotoxic moieties. We evaluated whether chemical conjugation to maytansine derivatives affected the antigen-binding activity of CNTO 95 to tumor cells by flow cytometry. As shown in Fig. 1B, each of the immunoconjugates and unconjugated CNTO 95 displayed very similar binding affinities for α_v integrin–bearing HT-29 human colon carcinoma cells. Saturating concentrations of each molecule stained virtually 100% of these cells. The relative EC_{50} values were 0.10 μg/mL for CNTO 95, 0.14 μg/mL for CNTO 364, 0.15 μg/mL for CNTO 365, and 0.18 μg/mL for CNTO 366. These results show that the conjugation process did not have a deleterious effect on cell binding of the antibody and that the three immunoconjugates have essentially identical cell-binding properties.

***CNTO 364 was selectively cytotoxic to α_v integrin–expressing human carcinoma cells* in vitro.** We assessed the ability of CNTO 364 to selectively kill antigen-bearing tumor cells. We first confirmed the presence or absence of the target α_v integrin on the surface of the test cells. Cell surface α_v integrin expression on A549 human lung carcinoma, HT-29 human colon carcinoma, and mouse B16-F10 melanoma cells was detected by flow cytometry. The cells were labeled with monoclonal antibody 1953Z, a mouse monoclonal antibody recognizing the human integrin α_v subunit, or with CNTO 95. The binding profiles of CNTO 95 and mAb1953Z on both A549 and HT-29 cells were essentially the same, as shown in Fig. 2A. B16-F10 mouse melanoma cells express mouse α_v integrins (data not shown), but do not specifically bind to either CNTO 95 or mAb1953Z. These data are consistent with

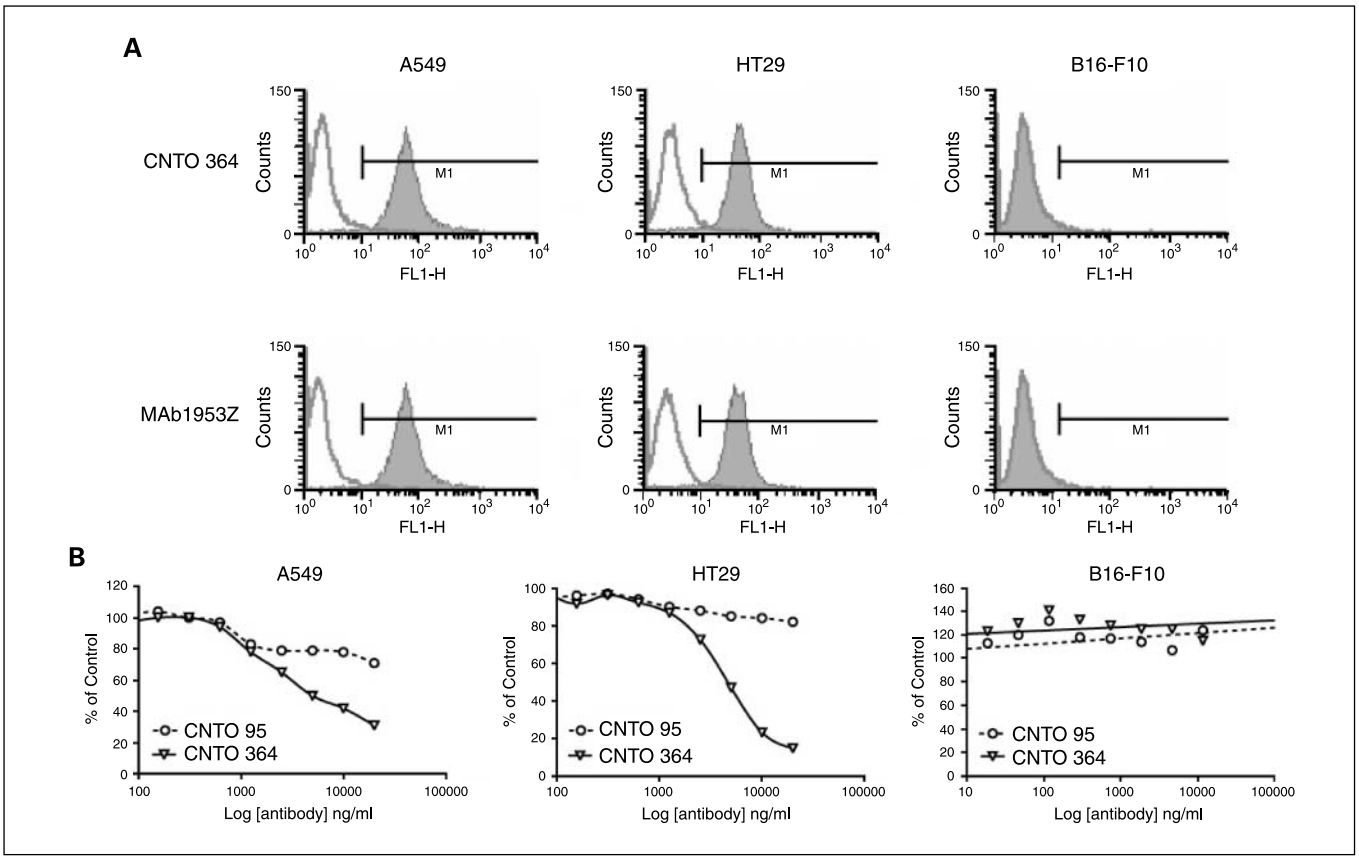

Fig. 2. *In vitro* characterization of CNTO 364 immunoconjugate. *A,* α_v integrin expression on A549, HT-29, and B16-F10 cells. Flow cytometry was done as described (1). *B,* CNTO 364 inhibits A549 and HT-29 cell proliferation but not that of B16-F10 mouse melanoma cells. Effects of CNTO 364 on cell proliferation were evaluated by the ATPLite assay as described in Materials and Methods. Cell survival was expressed as percentage of control (without treatment).

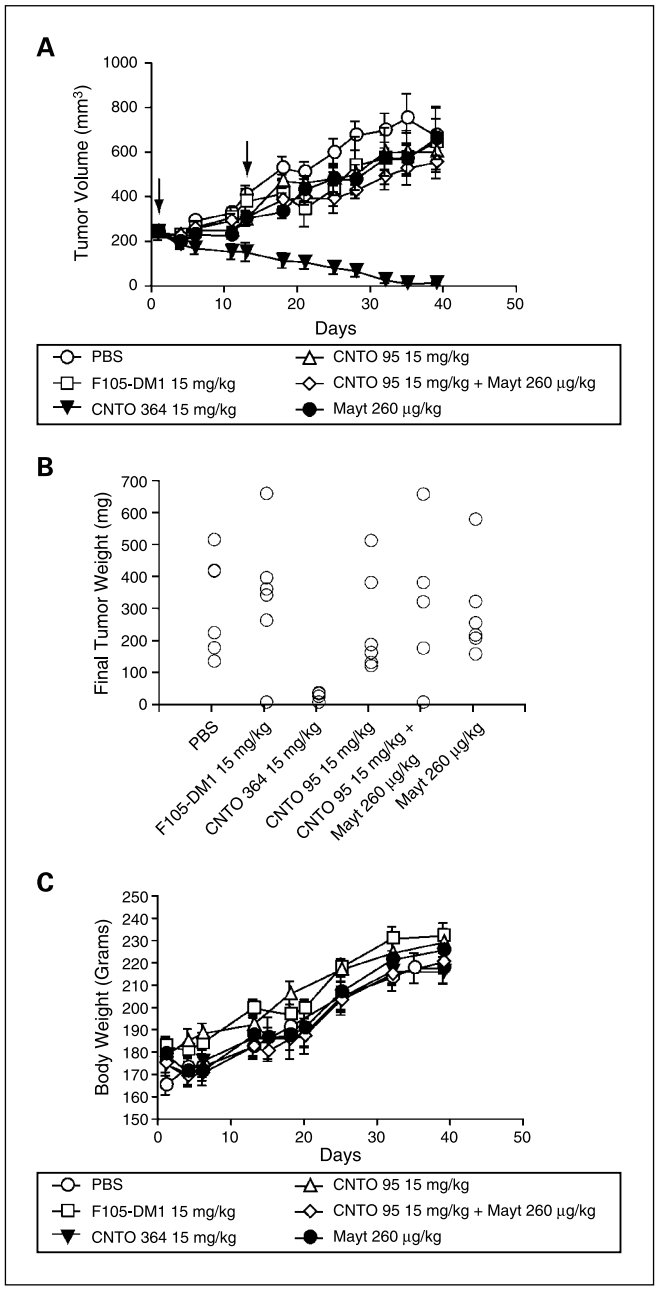

Fig. 3. CNTO 364 regressed established A549 human lung carcinoma tumors in female nude rats. Nude rats were s.c. inoculated with A549 human lung carcinoma cells. Animals were stratified to six groups ($n = 6$) and treatment was initiated on a biweekly dosing schedule when the mean tumor volume reached 250 mm^3. *A,* arrows, dosing times. Points, mean tumor volumes; bars, SE. *B,* final tumor weight distribution for each group. *C,* changes in body weight were plotted versus time. Points, mean ($n = 6$); bars, SE. Arrows, day of treatment.

our previous observation that CNTO 95 does not bind to mouse α_v integrins (12). B16-F10 cells were therefore used as a control to evaluate the *in vitro* specificity of the cytotoxic effects of CNTO 364.

The effects of CNTO 364 on survival of CNTO 95 binding cells and nonbinding cells were tested using an ATPLite assay. This assay quantifies the total number of live cells and reflects changes due to the effects of both cell proliferation and cell death. Treatment of A549 cells and HT-29 cells, which express human α_v integrins and bind CNTO 95, reduced the cell number in both a time-dependent (data not shown) and dose-dependent manner, as shown in Fig. 2B. In contrast, CNTO 364 did not show any apparent cytotoxic effects on the B16-F10 cells. CNTO 95 alone had no significant effect on the growth of either A549 or HT-29 cells. These data show that the cytotoxic effects of CNTO 364 were due to the targeted effects of the conjugated cytotoxic molecules rather than the integrin binding effects of the constituent CNTO 95, and that specific cellular binding of the conjugate was necessary for cell killing in this assay.

CNTO 364 regressed established s.c. A549 human lung tumor xenografts. The *in vivo* antitumor efficacy of these novel α_v integrin–targeted immunoconjugates was tested using human tumor xenograft models. For these studies, nude rats were chosen as hosts for the human tumors because CNTO 95 binds to both rat and human α_v integrins (12). CNTO 364 was therefore expected to target both the human α_v integrins in the tumor xenograft and the rat α_v integrins, which are expressed in

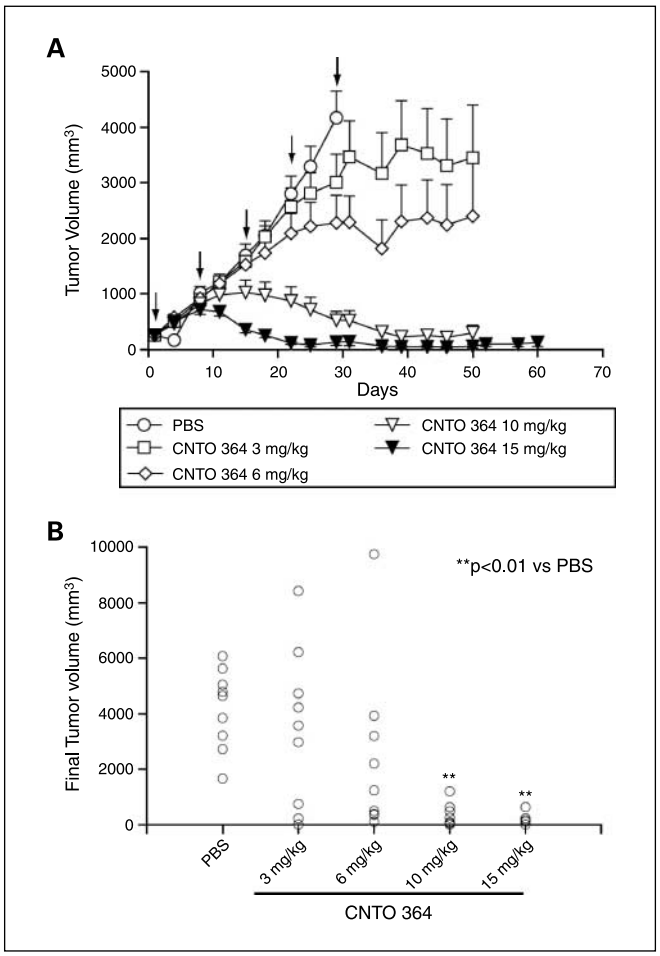

Fig. 4. CNTO 364 dose dependently inhibited the growth of advanced HT-29 human colon carcinoma xenografts in female athymic rats. Nude rats were s.c. inoculated with HT-29 cells. When the mean tumor volume reached 250 mm^3, animals were stratified into five groups of nine rats based on tumor volume. Arrows, dosing times. Treatment with CNTO 364 was started on the grouping day (day 1). Animals in the 3, 6, and 10 mg/kg groups received five weekly i.v. injections; the 15 mg/kg group was dosed on days 1, 8, and 29. *A,* points, mean tumor volumes ($n = 9$); bars, SE. *B,* final mean tumor volume. **, $P < 0.01$ versus PBS control group. Arrows, day of treatment.

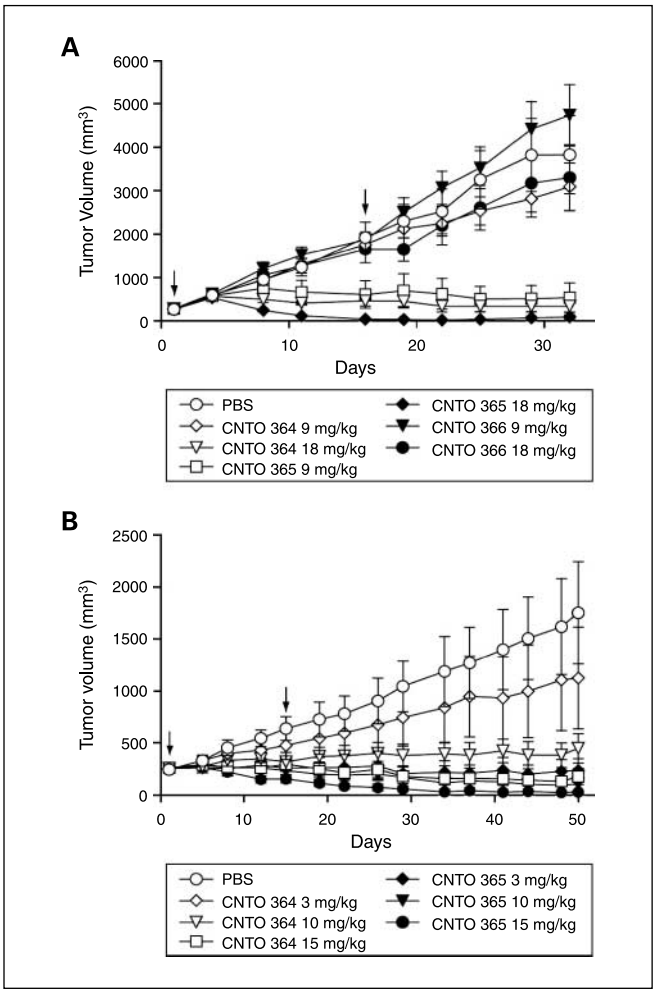

Fig. 5. Comparison of antitumor activity of CNTO 364, CNTO 365, and CNTO 366. *A,* nude rats bearing established HT-29 human colon tumor xenografts (mean tumor volume 250 mm^3) were stratified into seven groups ($n = 6$). Animals received i.v. injections of PBS (vehicle control), CNTO 364, CNTO 365, or CNTO 366 at 9 and 18 mg/kg on days 1 and 15 (*arrows*). *B,* nude rats were inoculated with A549 human lung carcinoma cells. When average tumor volume reached 250 mm^3, animals were grouped ($n = 6$) and i.v. dosed with PBS, CNTO 364, or CNTO 365 at 3, 10, and 15 mg/kg on days 1, 15, and 29. Points, mean; bars, SE. Arrows, day of treatment.

normal rat tissues and in the blood vessels within the tumor. It is important to note that although the affinity of CNTO 95 and CNTO 364 for rat α_v integrins is ~40-fold lower than for human integrins (12), substantial binding to rat integrins is predicted at the dose levels tested in these studies. Using the rat, it was possible to collect information on both the antitumor efficacy and the tolerability of the CNTO 364 immunoconjugate.

Nude rats bearing established A549 tumors of ~250 mm^3 were stratified to six groups and treated i.v. on days 1 and 13 with 15 mg/kg of CNTO 364, equivalent to 260 μg/kg of DM1 (maytansinoid), or one of five control therapies. An irrelevant antibody-DM1 conjugate, F105-DM1, was tested to determine if the activity of the CNTO 364 conjugate was dependent on integrin binding. In addition, because CNTO 95 itself has antiangiogenic and antitumor activity and DM1 is a potent cytotoxic agent targeting proliferating cells, treatment with unmodified CNTO 95, an unconjugated maytansinoid agent (free maytansine), and a mixture of CNTO 95 plus unconjugated maytansine were evaluated to determine the importance of conjugation to the activity of CNTO 364. CNTO 95, included here as a comparator, is active in tumor growth inhibition in some early treatment models (12), but is typically not active against aggressive established tumors such as those in this model.

As shown in Fig. 3A and B, i.v. administration of CNTO 364 at 15 mg/kg produced four complete and two partial regressions out of six animals by day 39. The non–tumor-binding F105-DM1 conjugate produced one complete regression, whereas free CNTO 95 plus free maytansine produced two of six partial regressions. PBS, free CNTO 95, or free maytansine had no effect on tumor growth. The mean tumor volume of the CNTO 364–treated group was significantly lower than those of all comparator groups ($P < 0.001$). There was no significant difference in mean tumor volume in the other treatment groups ($P > 0.05$).

These results show that the regression of the advanced A549 lung tumors was specifically caused by the CNTO 95–DM1 conjugate, CNTO 364, and not by its constituent components, alone or in combination. Using body weight as an indicator of tolerability, Fig. 3C shows that with this dosing regimen CNTO 364 was well tolerated, with only a 3% loss of body weight compared with pretreatment body weight. The irrelevant antibody conjugate F105-DM1 produced a similar loss in body weight, suggesting that this effect was not related to antigen targeting of the cytotoxic molecules in this model.

Dose-dependent growth inhibition of advanced HT-29 human colon tumor xenografts. The antitumor effects of CNTO 364 were further evaluated using the HT-29 human colon carcinoma xenograft model in nude rats. Animals were inoculated s.c. with HT-29 human colon carcinoma cells and stratified into five groups of nine animals each when the mean tumor volume reached 250 mm^3. PBS and 3, 6, or 10 mg/kg CNTO 364 were administered weekly for 5 weeks by i.v. injection, starting on the day of stratification (day 1). The 15 mg/kg dose of CNTO 364 was given on days 1, 8, and 29. As shown in Fig. 4A and B, these treatment regimens resulted in a dose-dependent inhibition of tumor growth, with tumor regressions at each dose level tested. CNTO 364 treatment at 10 mg/kg produced three complete regressions among nine animals, whereas there were four complete and four partial regressions in the 15 mg/kg group. All dose levels of CNTO 364 were well tolerated on this dosing schedule as shown by a lack of body weight loss (data not shown).

Comparison of the antitumor activities of CNTO 95–maytansinoid immunoconjugates with various stereo-hindered linkers. We compared the *in vivo* antitumor effects of CNTO 364, CNTO 365, and CNTO 366 (Fig. 1A) in nude rats bearing established HT-29 human colon carcinoma tumors of ~250 mm^3. As shown in Fig. 5A, CNTO 365 at 9 and 18 mg/kg produced the greatest tumor growth inhibition among the three CNTO 95–maytansinoid immunoconjugates ($P < 0.01$ versus PBS control). CNTO 364 at 18 mg/kg had similar efficacy to CNTO 365 at 9 mg/kg and seemed to be substantially less potent than CNTO 365. CNTO 366, the conjugate with the most highly substituted maytansinoid and linker, and therefore the one expected to have the most stable disulfide bond, was largely ineffective in this model ($P > 0.05$).

We next compared the efficacy of various doses of CNTO 364 and CNTO 365 in the A549 lung cancer xenograft model. Tumor-bearing rats received CNTO 364 or CNTO 365 at 3, 10, or 15 mg/kg. As shown in Fig. 5B, each regimen was active when compared with the saline control-treated cohort. CNTO 365 was substantially more active than CNTO 364 at each dose level tested. This was evidenced by a statistically significant greater level of tumor growth inhibition by the 3 mg/kg regimen of CNTO 365 compared with the 3 mg/kg dose of CNTO 364 ($P < 0.01$). The growth curve of the group receiving 3 mg/kg of CNTO 365 was not significantly different ($P > 0.05$) from that of the group that received 15 mg/kg of CNTO 364, suggesting a roughly 5-fold potency advantage for CNTO 365 in this model. The 10 and 15 mg/kg doses of CNTO 365 each produced three complete tumor regressions.

Discussion

The novel CNTO 95–maytansinoid immunoconjugates described here combine the targeting specificity of the CNTO 95 monoclonal antibody to α_v integrins with the extraordinary potency of the maytansinoid class of cytotoxic molecules. The α_v integrins are broadly expressed at low levels in normal tissues, but evidence has shown that they are present at much higher levels in a wide variety of human tumors and during angiogenesis (1, 5). This pattern of expression makes α_v integrins attractive targets for immunoconjugate therapeutics. The high frequency of α_v overexpression in human tumors, especially in major solid tumor types, suggests that a successful therapy targeting this antigen could find widespread utility. The α_v integrins are further distinguished as targets for this approach by their overexpression in the angiogenic endothelial cells of tumors (1–5). This expression pattern may present the opportunity to bring antiangiogenic or antivascular mechanisms to bear on tumors in addition to strong direct antitumor cytotoxicity.

Here, we have shown for the first time the specificity and efficacy of α_v integrin–targeted immunoconjugates. CNTO 364 was shown to be specifically cytotoxic *in vitro* for α_v-expressing human tumor cells. Both CNTO 364 and CNTO 365 were highly effective *in vivo* against established human colon and lung tumors in rat xenograft models. Importantly, these *in vivo* effects were dose dependent and specific to the immunoconjugate and not its constituent parts, demonstrating that the effects were due to successful targeting of the cytotoxic maytansinoid molecules. Our studies lend further support to recent reports that immunoconjugates containing the more disulfide-hindered DM4 maytansine derivative can be more active *in vivo* when compared with DM1 disulfide conjugates (17). Widdison et al. (17) showed that a huC242-DM4 conjugate was substantially more active than the analogous DM1 conjugate in a COLO 205 human colon cancer xenograft model. The observed improved activity has been proposed to be due in part to an increased circulating half-life of the DM4 conjugates and consequent delivery of more of the cytotoxic molecules to the tumor site (17). This is consistent with previous work showing that increased chemical substitution of carbons adjacent to disulfides substantially increases their stability (18, 19). The more substituted linkers used in this study have been shown to be more resistant to reduction by DTT *in vitro* and to result in increased circulating half-life in mice when conjugated to another human antibody.[3] Additional factors that could contribute to differential *in vivo* activity include the rate of release and the chemical nature of the active cytotoxic species in the target cells. The cellular and molecular details of the differential *in vivo* activity observed by Widdison et al. and in our study remain unclear. The results reported here suggest that DM4 conjugates with antibodies can lead to improved antitumor activity that might ultimately translate to improved clinical efficacy with this technology. The conjugate containing the most hindered linker-maytansinoid chemistry, CNTO 366, was essentially inactive in our *in vivo* studies. The reason for this remains unclear but might be explained by the nature of the drug-linker-lysine adduct released upon metabolism in the target cell (20, 21).

The *in vivo* studies reported here were conducted in immunocompromised rats rather than mice due to the lack of reactivity of the targeting antibody in mice and its partial reactivity in rats. The ability of the conjugate to recognize its target in tumor hosting rats allowed us to test for efficacy in a context where tolerability could be assessed to some extent. We expected both certain levels of toxicity caused by the interaction of CNTO 364/365 with host tissues expressing rat α_v integrins and potentially antiangiogenic contributions to efficacy. However, due to the 40-fold lower affinity of CNTO 95 for rat integrins relative to human integrins (12), the toxicity or the antiangiogenic activity of the CNTO364/365 immunoconjugates may be underestimated. The tolerability of this class of immunoconjugate molecule will be better assessed in a nonhuman primate species, such as the cynomolgus monkey, where the affinity of the targeting antibody is much more similar to that observed for human α_v integrins (12).

A number of α_v integrin antagonists have been developed for the treatment of human cancers, including both small molecules and monoclonal antibodies (4, 22). The ligands for α_v integrins have also been used as targeting agents to deliver genes or radioisotopes to neovasculature within solid tumors (4, 22). CNTO 95–maytansinoid immunoconjugates are of particular interest due to their impressive preclinical efficacy in cancer models combined with the proven practicality of the technology for producing maytansinoid-linked antibodies for clinical testing (14, 17). In conclusion, our studies illustrate a promising approach for the treatment of human solid tumors as evidenced by the ability of these molecules to regress multiple types of established human tumor xenografts. These effects may ultimately be extended to other α_v integrin–overexpressing tumor types, including melanoma (6), ovarian (8), and breast (10) carcinoma. The possibility for efficacy due to tumor antivascular or antiangiogenic effects suggests the potential for treating a still broader array of cancers.

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[3] R. Lutz, unpublished data.

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