Therapeutic Effects of Monoclonal Antibody−β-Lactamase Conjugates in Combination with a Nitrogen Mustard Anticancer Prodrug in Models of Human Renal Cell Carcinoma†

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A panel of 13 renal cell carcinoma cell lines was evaluated for the expression of antigens recognized by the L6 and L49 monoclonal antibodies. All of the cell lines were strongly positive for the L6 antigen, and 9/13 bound 96.5, which, like the L49 monoclonal antibody, recognizes the p97 melanotransferrin antigen. The L6 and L49 antibodies were chemically conjugated to *Enterobacter cloacae* β-lactamase (bL), and their abilities to effect site-selective anticancer prodrug activation on two of the renal cell carcinoma cell lines (SN12P and 1934J) were evaluated in vitro and in vivo. L49−bL was 10−90-fold more potent in vitro than L6−bL for the activation of 7-(4-carboxybutanamido)cephalosporin mustard (CCM), a cephalosporin prodrug of phenylenediamine mustard (PDM). In addition, L49−bL showed higher degrees of specific SN12P and 1934J intratumoral uptake than L6−bL, even though the expression of L6 antigen was 2-fold higher than that of p97. These differences might be due to the high-affinity antigen binding of L49−bL relative to L6−bL. In vivo studies utilizing nude mice with established subcutaneous SN12P and 1934J tumor xenografts demonstrated that L49−bL/CCM combinations led to regressions and cures at well-tolerated doses, while L6−bL/CCM and the nonbinding control conjugate P1.17−bL in combination with CCM were ineffective. Conjugate localization in 1934J tumors was much lower than that observed in SN12P tumors, a finding that might acount for the higher activities of L49−bL/CCM in the latter model. These data show that the p97 antigen on renal cell carcinomas can be exploited for selective prodrug activation, even on tumors that localize very small amounts of the L49−bL conjugate.

Introduction

The applications of monoclonal antibody (mAb)−enzyme conjugates for the selective activation of anticancer prodrugs have been subject to a great deal of recent investigation (reviewed in refs 1−3). This is a two-step approach to cancer chemotherapy, the first step being the administration of a mAb−enzyme conjugate that is capable of selectively localizing into tumor masses. Once localization and clearance have taken place, the second step involves systemic treatment with an anticancer prodrug that is relatively nontoxic until it is activated by the targeted enzyme. Many mAb−enzyme/prodrug combinations have been reported,[2] and both promising preclinical[1−9] and clinical data[10,11] have been obtained.

Our work has focused on the use of mAb−β-lactamase (bL) conjugates for the activation of cephalosporin-containing prodrugs. Previously, we have demonstrated that the conjugate formed by attaching the pan-carcinoma antibody L6 to bL was capable of activating a cephalosporin-containing prodrug of doxorubicin (C-Dox), leading to in vivo antitumor activities against two different lung adenocarcinoma tumor xenografts.[5] Insight into the mechanism of activity was gained from pharmacokinetic studies showing that the combination of L6−bL and C-Dox led to significantly higher intratumoral doxorubicin concentrations than those obtained from systemic doxorubicin treatment. However, even at the maximum tolerated dose of L6−bL + C-Dox, no cures or regressions were observed. Similar results were obtained using L6−bL in combination with CCM, a cephalosporin prodrug of the alkylating agent phenylenediamine mustard.[12]

In contrast to these results, it was found that cures of established melanoma tumors were obtained in mice treated with mAb−bL conjugates against the p97 melanotransferrin antigen followed by treatment with CCM.[4,9] The conjugates used in these studies incorporated either the Fab′ fragment of the 96.5 mAb[9] or the sFv form of the L49 mAb.[4] These mAb's recognize different epitopes on the p97 antigen but otherwise appear to have quite similar properties.[4]

Although the p97 antigen is primarily associated with melanomas, it has previously been reported to be expressed on some carcinomas.[13,14] Here, we show that both the L6 and p97 antigens are present on several cell lines derived from clinical samples of human renal cell carcinoma tumors. This presented us with the opportunity to explore the effects of L49−bL + CCM in a carcinoma tumor model and to compare the relative effects of L49−bL versus L6−bL for prodrug activation. We demonstrate that L49−bL has higher affinity than L6−bL, is more potent at prodrug activation in vitro, shows superior localization characteristics, and, in

† Abbreviations: bL, β-lactamase; CCM, 7-(4-carboxybutanamido)-cephalosporin mustard; FACS, fluorescence-activated cell sorter; iv, intravenous; mAb, monoclonal antibody; MTD, maximum tolerated dose; PDM, phenylenediamine mustard; sc, subcutaneous.

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contrast to L6−bL, leads to cures of established renal cell carcinomna xenografts when combined with CCM.

Materials and Methods

Cell Lines and Reagents. The renal cell carcinoma lines were provided by Dr. Robert E. Vessella (University of Washington), the only exception being SN12P, which was obtained from Dr. Isaiah J. Fidler (University of Texas). The mAb's 96.5 (IgG2a)[13,14] and L49 (IgG1)[4] bind to distinct epitopes on p97, a human melanoma-associated antigen. L6 (IgG2a) is a pan-carcinoma antibody,[15] and P1.17 (IgG2a) was used as a nonbinding control. The Fab′ fragments of the mAb's were linked to bL via a thioether linkage as previously described.[16] Briefly, dithiothreitol was used to reduce the Fab'_2 fragments, and the resulting protein was combined with bL that had been modified with maleimides. Purification was achieved in two chromatographic steps involving first phenylboronic acid affinity chromatography followed by gel filtration. As before,[16] the purified conjugates were at least 90% monomeric by SDS−PAGE and were fully functional with respect to both the mAb binding and enzyme activities. PDM and CCM were synthesized as previously described.[12]

Expression of the L6 and p97 Antigens. 1. Fluorescence-Activated Cell Sorter (FACS) Analysis. Fluorescein conjugates of whole L49, 96.5, L6, and P1.17 were used to determine antigen expression on the renal cell carcinoma cells. For these studies, the cells were obtained either from monolayer cultures by detachment with phosphate-buffered saline containing EDTA or from tumor xenografts, which were homogenized and passed through a metal screen and a 70-μm mesh filter to obtain single-cell suspensions. The cells were incubated with varying concentrations of the mAb−fluorescein conjugates in cell culture medium, washed, and subjected to FACS analysis.

2. Scatchard Analysis. The L49− and L6−bL conjugates were radiolabeled with ^{125}I using Iodogen and Na^{125}I to specific activities of 4.2 and 1.7 μCi/μg of mAb component, respectively. The conjugates were then added in triplicate or quadruplicate to 96-well plates and serially diluted 2-fold starting at 25 nM for L49−bL and 200 nM for L6−bL. To some of the rows, unlabeled Fab'_2 mAb fragments were added at 10 μM in order to measure nonspecific binding of the unlabeled conjugates. SN12P (200 000 cells/well) or 1934J (150 000 cells/well) were added, the plates were incubated at 4 °C for at least 1 h, and the cell suspensions were then added to tubes containing 150 μL of a 1:1 solution of dioctyl phthalate and dibutyl phthalate. After centrifugation at 4 °C, the tubes were immersed in a dry ice/methanol bath and sliced in sections that contained the supernatant (unbound material) and the cell pellet (bound material). The sections were counted with a γ-counter. Specific binding was determined by subtracting the counts obtained in the cell pellets of samples that were treated with the unlabeled Fab'_2 fragments prior to conjugate treatment from the counts obtained with labeled conjugate only.

Histological Analysis. Nude mice with either SN12P or 1934J subcutaneous tumor xenografts were sacrificed, and the tumors were resected and embedded with OCT (Miles Inc., Elkhart, IN). The tumors were then frozen in liquid nitrogen and stored at −70 °C. Sections (10 μm) of SN12P and 1934J tumor xenografts were placed on glass slides and fixed with acetone. The slides were then treated with either L49−bL, L6−bL, or P1.17−bL, washed, and treated with a rabbit anti-bL antibody that was conjugated to biotin.[17] After a second wash, antigen expression was visualized with streptavidin−horseradish peroxidase/hydrogen peroxide (Vector Industries, Burlingame, CA) in combination with diaminobenzidine.

In Vitro Cytotoxicity. Cells were plated out in 96-well plates (4000 cells/well for SN12P cells, 10 000 cells/well for 1934J cells) in tissue culture medium containing 10% fetal bovine serum and were allowed to adhere for 24 h at 37 °C. Varying concentrations of L49−bL, L6−bL, or P1.17−bL were added in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, and incubation was continued for 30 min at 4 °C. Saturation of cell-surface antigens was achieved by incubating some of the cells with L49 (0.1 mg/mL) or L6 (1 mg/mL) prior to conjugate exposure. After conjugate exposure, the cells were washed with RPMI medium containing 10% fetal bovine serum, treated with CCM (3 μM) for 1 h, and washed. The cells were pulsed 20 h later with [^{3}H]thymidine (1 μCi/well) for 4 h (SN12P cells) or 6 h (1934J cells). The cells were harvested and counted with a β-counter.

In Vivo Experiments. 1. General Procedures. All experiments were performed in athymic nude mice (Harlan Sprague−Dawley) bearing subcutaneous SN12P or 1934J tumor xenografts and were initiated when the mice were 8−11 weeks old. Both models were established by injecting cells subcutaneously, followed by in vivo passaging. Tumors in passages 3−7 were used in the experiments described. Conjugates, drug, and prodrug at various concentrations were injected intravenously such that the total injection volume was 10 mL/kg of body weight. CCM was administered in a solution of 119 mM sodium bicarbonate containing 10% DMSO, and PDM was administered in physiological saline. The bL conjugates were diluted in 1 mg/mL bovine serum albumin in phosphate-buffered saline prior to administration. In all experiments, the dose of injected conjugate was 0.8 mg of mAb component/kg of body weight.

2. Conjugate Localization. Localization studies in mice (3−5/group) were performed with radiolabeled mAb−bL conjugates according to a previously described procedure.[15] L49, L6, and P1.17 conjugates of bL were labeled with ^{125}I using Iodogen/Na^{125}I to respective specific activities of 1.6, 1.4, and 1.4 mCi/mg of mAb for the experiment with SN12P and to 4.2, 2.3, and 2.1 mCi/mg of mAb for the experiment with 1934J. The conjugates were diluted to 4×10^7 cpm/mL with 1 mg/mL bovine serum albumin in phosphate-buffered saline and unlabeled mAb−bL conjugates to a final concentration of 0.08 mg of mAb/mL. At various time points after conjugate administration, blood samples were collected. The animals were sacrificed at 72 h, and tissues were excised, weighed, and counted with a γ-counter.

3. In Vivo Therapy and Toxicity Experiments. Mice (5−6/group) with established growing tumors were treated with bL conjugates, followed 72 h later by CCM. Treatment with mAb−bL + CCM was carried out on a weekly schedule for a total of 3 rounds. The animals were monitored daily for general health and weekly for weight and tumor growth. Tumor volumes were calculated using the formula (longest length × perpendicular width^2)/2. A cure was defined as an established tumor that, after treatment, was not palpable for at least 10-tumor-volume doubling times. MTDs led to less than 20% sustained (≥2 weeks) weight loss, led to no treatment-related deaths, and were within 50% of the dose where such events took place.

Results

p97 and L6 Antigen Expression on Renal Cell Carcinoma Cell Lines. Previous studies have shown that the L6 mAb binds to a 24-kDa surface protein present on lung, breast, colorectal, and ovarian carcinomas.[14,18] 96.5[9,13] and L49[4] are mAb's that bind with high affinity to distinct epitopes on the p97 melanotransferrin antigen, which is primarily associated with melanoma but is also found on lung, breast, ovarian, and renal cell carcinomas.[13,17] The presence of the L6 and p97 antigens on a panel of 13 renal cell carcinoma cell lines was determined by fluorescence-activated cell sorter analysis, in which the binding of fluorescein conjugates of L6 and 96.5 was compared to that of the nonbinding control P1.17−fluorescein. As shown in Table 1, L6 showed significant levels of binding to all of the renal cell carcinoma cell lines, while 96.5 binding was mixed. With the exception of the SN12P cell line, the specific binding of L6 was higher than that of 96.5.

Table 1. 96.5 and L6 Binding to Renal Cell Carcinoma Cell Lines Relative to the Nonbinding P1.17 Control

	binding ratios[a]	
cell line	96.5[b]	L6[c]
SN12P	35[c]	15.7
1072F	2.5	56.6
769P	3.0[c]	40.2
786O	1.3	10.3
Pastor	9.1	18.1
TK 204	2.2	10.3
TK 164	6.3[c]	34.9
Caki-1	1.9	9.7
1934J	1.8	9.9
ACHN	1.6	17.4
SK−RC-6	0.2[c]	7.1
SK−RC-7	0.3[c]	3.2
A704	0.5[c]	9.0

[a] Cells were incubated with mAb−fluorescein conjugates, washed, and subjected to fluorescence-activated cell sorter analysis. Binding ratios represent the mean channel fluorescence ratios of 96.5−fluorescein and L6−fluorescein to P1.17−fluorescein. [b] 0.3 μg of mAb component/mL unless otherwise indicated. [c] 30 μg of mAb component/mL.

Table 2. Scatchard Analysis of L49−bL and L6−bL Antigen Binding[a]

cell line	conjugate	affinity (nM)	antigens/cell
SN12P	L49−bL	2.9	2.9×10^5
SN12P	L6−bL	150	3.4×10^5
1934J	L49−bL	2.9	6.6×10^4
1934J	L6−bL	190	4.9×10^5

[a] ^{125}I-Labeled L49−bL and L6−bL conjugates were combined with SN12P or 1934J cells. Antigen affinity and density were determined by Scatchard analysis of the amount of bound and free material.

On some cell lines, it was only possible to detect specific 96.5 binding by performing the assay at low mAb concentrations. Using a binding ratio of 1.3 as a cutoff, 9/13 of the renal cell carcinoma cell lines tested positive for the p97 antigen. We selected to further study the SN12P and 1934J cell lines, since they exhibited vastly different levels of p97 expression but bound L6 quite well.

In Vitro Activities. Conjugates of bL were prepared by combining sulfhydryl-containing L6 and L49 Fab′ fragments with maleimide-substituted enzyme. As previously reported,[5,9,16] conjugates formed in this manner retained the antigen-binding and enzymatic activities of the component proteins from which they were derived. Scatchard analysis of the binding of radiolabeled L6−bL and L49−bL to SN12P and 1934J cells was used to determine antigen density and binding affinity (Table 2). Consistent with the fluorescence-activated cell sorter data shown in Table 1, SN12P cells had higher levels of the p97 antigen compared to the 1934J cell line. In addition, it was found that the antigen density for L6 exceeded that of p97 on both of the renal carcinoma cell lines. The affinity constant for L49−bL binding was 2.9 nM, which is comparable to that previously reported for whole 96.5 antibody binding to p97 on melanoma tumor cell lines.[9]

Previous studies have demonstrated that the prodrug CCM (Scheme 1) is significantly less cytotoxic than the corresponding drug PDM and that CCM activation could be induced with mAb−bL conjugates.[9,12] Cytotoxicity experiments were designed to extend these findings to include renal carcinoma and to compare the relative

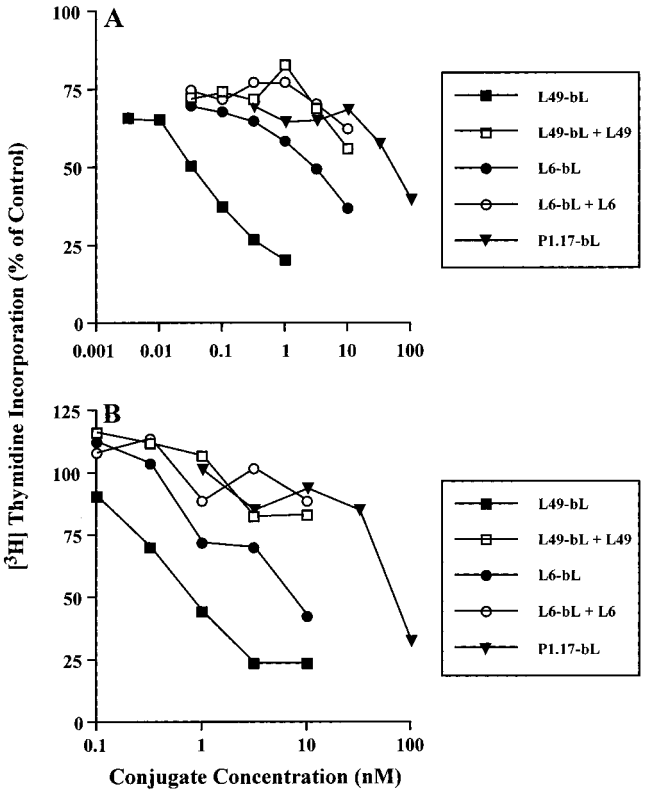
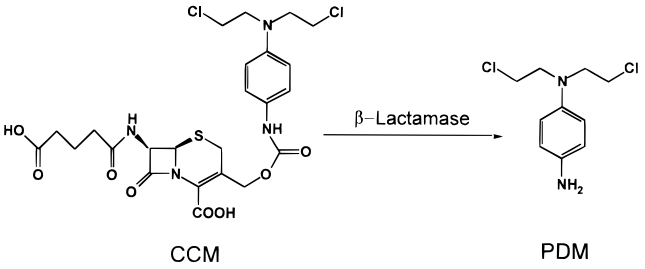


Figure 1. In vitro cytotoxicity of CCM (3 μM) on (A) SN12P and (B) 1934J renal cell carcinoma cell lines. The cells were treated with varying concentrations of the conjugates, washed, and then exposed to CCM for 1 h. After further incubation, cytotoxicity was quantified by measuring [^{3}H]thymidine incorporation relative to untreated control cells. To demonstrate that prodrug activation was immunologically specific, cells were either treated with the nonbinding control conjugate P1.17−bL prior to CCM exposure or saturated with unconjugated mAb's (0.1 mg/mL L49, 1 mg/mL L6) prior to conjugate treatment.

Scheme 1. Enzymatic Conversion of CCM (Prodrug) to PDM (Drug)



abilities of L49−bL and L6−bL for prodrug activation. SN12P and 1934J cells were exposed to various mAb−bL concentrations, unbound material was washed off, and CCM was added at a fixed concentration of 3 μM, which has previously been shown to have little cytotoxic activity in the absence of bL.[9,12] Both conjugates induced prodrug activation (Figure 1). Interestingly, L49−bL displayed significantly more potent prodrug activation activity than did L6−bL on both cell lines, although the relative antigen expression for the L49 mAb was lower than that of L6 (Table 2). Also noteworthy was the finding that the potency of L49−bL correlated with p97 antigen expression on the two cell lines, since higher concentrations of L49−bL were needed to effect CCM activation on 1934J cells (Figure

1B) compared to SN12P cells (Figure 1A). Thus, both high-affinity binding and the level of antigen expression appear to influence the degree to which CCM is activated.

Two independent methods established that prodrug activation was immunologically specific. These involved saturating cells with unconjugated L49 or L6 prior to conjugate exposure and exposing cells to the nonbinding control conjugate P1.17−bL prior to CCM treatment. Both of these procedures led to greatly reduced levels of prodrug activation. The in vitro results suggest that L49−bL might be therapeutically superior to L6−bL for treating SN12P and 1934J renal cell carcinoma cell lines since it binds with higher affinity and less is needed for effective prodrug activation.

Conjugate Localization. SN12P and 1934J cells were established in vivo by injecting cells subcutaneously into nude mice and serially passaging the resulting tumors. Histological analyses of tumor cross sections revealed that both the p97 and L6 antigens were expressed and that expression was homogeneous and strong (data not shown). A more quantitative measurement of antigen expression was obtained by FACS analysis of single-cell suspensions derived from representative tumors. This revealed that in both tumor models, the L6 antigen was expressed at higher levels than p97. The L6-to-p97 antigen ratios for SN12P and 1934J cells derived from the in vivo passaged tumors were 2.0 and 1.9, respectively. These ratios were independent of passage number. Thus, both antigens are expressed on tumor xenografts, but at ratios that differ from those measured in vitro (Table 1).

Studies were then undertaken to establish the extent of mAb−bL localization in the sc tumor xenografts. Radiolabeled L49−bL and L6−bL (0.8 mg/kg) were injected iv into tumor-bearing mice, and the amount of radioactivity in the tumors, blood, and several other organs was determined 72 h later. L49−bL and L6−bL localized in SN12P tumors in concentrations that were greater than in any of the other tissues tested (Figure 2A). Localization was dependent on antigen binding, since the nonspecific control conjugate, P1.17−bL, did not display preferential intratumoral retention. In this model, intratumoral uptake of L49−bL (0.35% injected dose/g) exceeded that of L6−bL (0.22% injected dose/g), even though the antigen for L6 is expressed at a 2-fold higher concentration than p97. A much smaller degree of conjugate localization was found in 1934J tumors (Figure 2B). At 72 h postconjugate administration, L49−bL demonstrated very modest tumor/normal tissue ratios, while the intratumoral concentration of L6−bL was not significantly different from that measured in the spleen, liver, and kidneys.

In Vivo Therapeutic Efficacy. The antitumor activities of the mAb−bL conjugates in combination with CCM were determined in mice with established SN12P or 1934J sc tumor xenografts. Treatment of SN12P-bearing animals was initiated 22 days posttumor implant, at which time the tumors were approximately 220 mm^3. The mAb−bL conjugates were injected iv at 0.8 mg of mAb component/kg of body weight, followed 3 days later by iv injection of CCM. This dosing regimen was carried out a total of three times on a weekly basis.

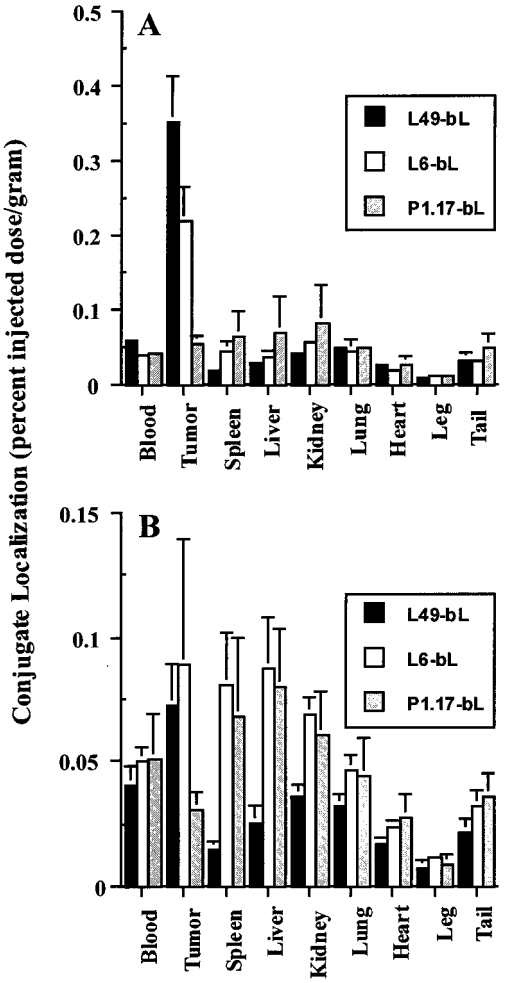


Figure 2. Distribution of ^{125}I-labeled mAb−bL conjugates (0.8 mg/kg) in mice with (A) SN12P and (B) 1934J sc tumor xenografts. The amount of radioactivity in the indicated tissues was determined 72 h postconjugate treatment.

The therapeutic effects were compared to those of PDM at the maximum tolerated dose (4.5 mg/kg/injection).

PDM had no effect on the growth of SN12P tumors (Figure 3A,B). In contrast, pronounced antitumor activities were obtained in mice treated with L49−bL 72 h prior to CCM administration. These effects were significantly greater than those observed in the L6−bL-treated animals (Figure 3A). For example, the combination of L49−bL and CCM (180 mg/kg/injection, MTD) led to complete tumor regressions in all the animals, while none of the tumors regressed in animals that received L6−bL and CCM (240 mg/kg/injection, MTD). In a separate experiment, the antitumor activities of L49−bL and P1.17−bL were compared for therapeutic efficacy when each was combined with CCM (Figure 3B). Cures were obtained in all of the animals treated with L49−bL and CCM at either the 180 (the MTD) or 120 mg/kg/injection dose levels. Dropping the dose level further to 60 mg/kg/injection resulted in 2/5 cures and 1/5 complete regressions at the termination of the experiment. These effects were immunologically specific, since there was no significant activity associated with P1.17−bL and CCM at the MTD (240 mg CCM/kg/injection). Thus, the therapeutic activities of L49−bL in combination with CCM are pronounced,

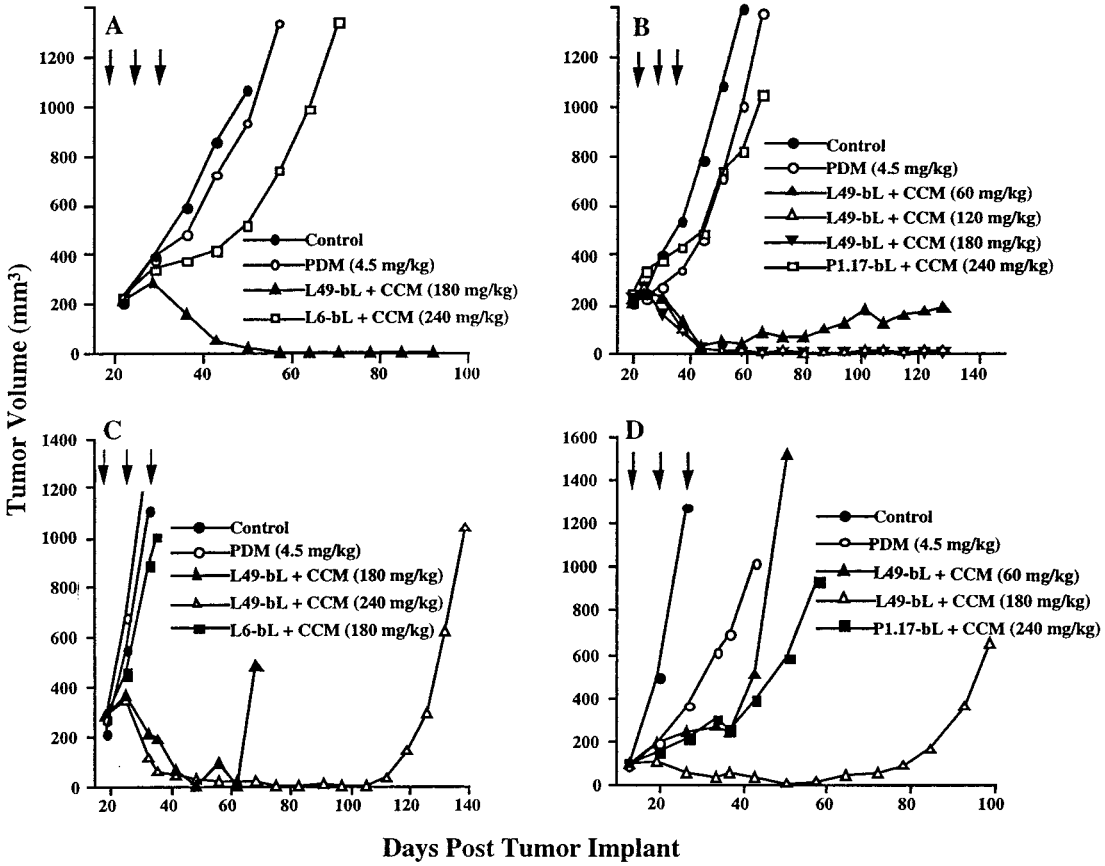


Figure 3. Therapeutic effects of mAb−bL/CCM combinations in nude mice with (A and B) SN12P and (C and D) 1934J sc tumor xenografts. Conjugates (0.8 mg/kg) were injected iv on the days indicated by the arrows, and CCM was administered 72 h later. The therapeutic effects were compared to those of PDM at the MTD.

immunologically specific, and greater than that of systemic PDM administration.

Similar experiments performed in mice with 1934J tumors also demonstrated that the combination of L49−bL with CCM was therapeutically superior to similarly treated L6−bL animals (Figure 3C). Complete regressions were obtained in mice that were treated with 240 mg/kg CCM (MTD, 5/5 complete regressions) and 180 mg/kg CCM (4/5 complete regressions) following L49−bL treatment. Eventually, all tumors at the lower dose and 3/5 at the higher dose grew out. L6−bL in combination with CCM at the MTD (180 mg/kg/injection) had very little effect on tumor growth. Additional experiments on smaller 1934J tumors established that the effects were immunologically specific and superior to those of PDM (Figure 3D). The MTDs in this study were 180 mg/kg CCM following L49−bL administration and 240 mg/kg after treatment with nonspecific conjugate, P1.17−bL. The latter combination resulted in antitumor activities that were comparable to those of L49−bL and CCM at 60 mg/kg. Thus, L49−bL in combination with CCM demonstrates therapeutic efficacy in a tumor model that has very low levels of conjugate localization.

Discussion

Previously, we have shown that treatment of nude mice with an anti-p97−bL conjugate and CCM led to cures of established melanoma xenografts.[9] The p97 target antigen is widely expressed on melanomas but has been shown to be present on several carcinomas as well.[13,14] In the studies presented here, we investigated the activities of L49−bL and CCM on renal cell carcinomas, which we show display varying amounts of the p97 antigen. The two tumor models explored, SN12P and 1934J, were also positive for the L6 antigen. This provided us with the opportunity to compare the therapeutic effects obtained from conjugates that differ in both their binding and localization characteristics.

In vitro prodrug activation studies showed that L49−bL was significantly more potent than L6−bL (Figure 1). It was possible to achieve IC_{50} values on the SN12P and 1934J cell lines with 10 and 90 times less L49−bL than L6−bL, respectively. This is in spite of the fact that the L6 antigen is expressed in greater quantities on both cell lines than the p97 antigen (Table 2). The most likely explanation for the potency difference is that L49−bL binds 51−64 times more strongly to its antigen than does L6−bL (Table 2). Therefore, it is likely that under the assay conditions, fewer molecules of L6−bL may have been bound to the cells when CCM was added.

A further indication that L49−bL is superior to L6−bL for site-selective prodrug activation stems from the in vivo localization study shown in Figure 2. At 72 h postconjugate administration, 1.6 times more L49−bL than L6−bL was retained in SN12P tumors. A number of factors including affinity, conjugate pharmacokinetics and biodistribution, and antigen accessibility might contribute to this difference. Taken together, the results from the conjugate localization and in vitro prodrug

activation studies lead to the prediction that L49−bL would have greater therapeutic efficacy than L6−bL for the treatment of SN12P tumor xenografts and that the L49−bL/CCM combination would induce greater responses in SN12P tumors compared to 1934J tumors. As shown in Figure 3, both of these expectations were borne out experimentally.

The data reported here demonstrate that the p97 antigen on renal cell carcinoma xenografts represents an exploitable target for selective prodrug activation. Cures of established SN12P tumors were obtained with well-tolerated doses of L49−bL in combination with CCM. Furthermore, it was possible to induce tumor regressions in1934J tumors which localized very small amounts of the L49−bL conjugate. We are currently extending the use of L49−bL to include several other p97-positive carcinoma tumor models.

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