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Antitumor Xenograft Activity with a Conjugate of a *Vinca* Derivative and the Squamous Carcinoma-reactive Monoclonal Antibody PF1/D

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ABSTRACT

The human squamous carcinoma-reactive murine monoclonal antibody PF1/D was used to derive a conjugate with the *Vinca* derivative 4-desacetylvinblastine-3-carboxyhydrazide (PF1/D-DAVLBHYD). This immunoconjugate was shown to be largely aggregate free and there was no loss of immunoreactivity postconjugation. When tested *in vivo* in a 3-day-established human squamous carcinoma nude mouse xenograft model, the PF1/D-DAVLBHYD conjugate eliminated tumor growth with three injections (days 3, 6, and 9) at 2 mg/kg *Vinca* content. Significant tumor suppression was also observed with 0.5 mg/kg conjugate doses. In contrast, free PF1/D antibody had minimal antitumor activity and no activity was seen with identical doses of a control non-tumor-binding IgG-DAVLBHYD conjugate. Together, these data demonstrate the specificity of the PF1/D-DAVLBHYD antitumor effects.

INTRODUCTION

Approximately 144,000 new cases of lung cancer occur annually in the United States (1), of which up to 50% are classified as squamous carcinomas (2). With only a 13% 5-year survival rate (2), alternative therapeutic approaches are clearly needed. The use of monoclonal antibodies to target therapeutic agents to lung and other solid tumors has recently gained considerable attention. This approach offers the promise of selective antibody-directed increase in drug concentration at the tumor site, with resultant increased efficacy. One strategy, reviewed by Frankel *et al.* (3), is to conjugate monoclonal antibodies to toxins or toxic polypeptides. An alternative is to use less toxic, more conventional oncolytic drugs for conjugation [for reviews see Reisfeld and Cherish (4), and Ghose *et al.* (5)]. *Vinca* alkaloids and their derivatives fall into the latter category. The *Vinca* derivative DAVLB[2] has been shown by Rowland *et al.* (6) to have therapeutic promise as an immunoconjugate when coupled with the antibodies 96.5 (melanoma reactive), 791T/36 (osteosarcoma reactive) or 11.285.14 (carcinoembryonic antigen reactive) and tested in appropriate nude mouse xenograft models. Bumol *et al.* (7) recently reported significant human tumor nude mouse xenograft suppression with the adenocarcinoma-reactive antibody KS1/4 as a DAVLB conjugate.

Another candidate for drug conjugation is PF1/D, a human squamous carcinoma-reactive murine monoclonal antibody recently reported by Fernsten *et al.* (8). This antibody has the desirable characteristic of being tumor cell surface reactive, while having limited normal tissue reactivity.

In the present report, we describe PF1/D antibody conjugates with the *Vinca* derivative DAVLBHYD (9). The PF1/D-DAVLBHYD conjugate was tested for efficacy *in vivo* by using human squamous carcinoma nude mouse xenografts. Relative to a non-target-binding IgG-DAVLBHYD conjugate, PF1/D-DAVLBHYD showed significant tumor growth inhibition at conjugate doses lower than have previously been reported for DAVLB conjugates (6, 7).

MATERIALS AND METHODS

Monoclonal Antibody. The hybridoma cell line PF1/D (8) was generated with the P3X63Ag8 myeloma fusion partner and secretes an active antibody of IgG3 isotype. The line was grown as ascites in pristane-primed BALB/c mice, yielding 7 mg/ml immunoglobulin. Following 5-μm filtration, antibody was affinity purified on a Protein A Sepharose column (Pharmacia, Uppsala, Sweden). The wash buffer was 0.01 M sodium phosphate, pH 8.0, and elution was carried out with a step gradient with 0.1 M sodium phosphate buffer, pH 3.5. Eluted fractions were immediately neutralized with 1 M Trizma, buffer (Sigma Chemical Co., St. Louis, MO) pH 7.4, and dialyzed against PBS. Antibody preparations were 0.2-μm sterile filtered and stored at 4°C until used. A non-target cell-binding IgG1 control monoclonal antibody was kindly provided by Dr. A. L. Baker, Lilly Research Laboratories, Indianapolis, IN.

Drug Conjugation. DAVLBHYD (9) was generously provided by G. J. Cullinan (Lilly Research Laboratories). Antibodies were conjugated to DAVLBHYD by using techniques detailed elsewhere.[3] Briefly, antibody was concentrated to approximately 10 mg/ml by vacuum dialysis in PBS and subsequently dialyzed against 0.1 M sodium acetate buffer, pH 5.6. Treatment of this solution near 0°C with 160 mM sodium metaperiodate resulted in oxidized antibody which was purified by Sephadex-G25 chromatography in 0.1 M sodium acetate buffer, pH 5.6. This material was coupled with 4-desacetylvinblastine-3-carboxyhydrazide (5 mM, at 4°C for 24 h) and purified by Sephadex G-25 chromatography in PBS, pH 7.4.

Cell Lines. P3/UCLA (lung adenocarcinoma) (10) and M21 (melanoma) (11) lines were kindly provided by Dr. T. Bumol (Lilly Research Laboratories), and were maintained in DMEM supplemented with 10% FCS plus 50 μg/ml gentamicin. The American Type Culture Collection (Rockville, MD) lines 5637 (squamous bladder carcinoma), FaDu (squamous pharyngeal carcinoma), ME180 (squamous cervical carcinoma), Du145 (prostate carcinoma), HT29 (colon adenocarcinoma), WiDr (colon adenocarcinoma), and PC3 (prostate adenocarcinoma) lines were maintained as recommended by the American Type Culture Collection. T222 (lung squamous carcinoma) (12) was maintained in DMEM plus 10% FCS plus 50 μg/ml gentamicin and was adapted to roller bottles for use in nude mouse xenograft studies.

Membrane Fluorescence. Target cells were suspended with trypsin-EDTA (Gibco, Grand Island, NY) and incubated with antibody diluted in DMEM plus 10% FCS on ice for 45 min. They were then washed twice with medium and incubated for an additional 45 min with fluorescein-labeled goat F(ab')$_2$ anti-mouse IgG (Tago, Burlingame, CA) at 20 μg/ml medium. After additional washes, the cells were either examined immediately by UV microscopy or fixed with 1% formalin in PBS and examined with an EPICS/Coulter Mark IV cell analyzer.

Chromatography. Aggregate formation was evaluated using a Superose 12 HR10/30 gel filtration column (Pharmacia) and a Pharmacia fast protein liquid chromatography system (13). Samples were applied in 0.3 ml 0.1 M Trizma buffer, pH 8.0, plus 0.1 M NaCl and eluted in the same buffer at 0.5 ml/min.

Nude Mouse Xenografts. Outbred nude mice, obtained from Charles River Breeding Laboratories (Boston, MA), were maintained in sterile laminar air flow facilities and were provided sterile water and sterile

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[2] The abbreviations used are: DAVLB, 4-desacetylvinblastine-4-hemisuccinate; DAVLBHYD, 4-desacetylvinblastine-3-carboxhydrazide; PBS, 0.01 M sodium phosphate, pH 7.4, plus 0.15 M NaCl; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

[3] B. C. Laguzza *et al.*, manuscript in preparation.

chow *ad libitum*. Mice approximately 2 months old were inoculated s.c. in the right flank with 1×10^7 tumor cells in 0.2 ml sterile PBS 24 h after receiving 350 R γ-irradiation. In efficacy studies, the animals were treated by i.v. injection in the tail vein after allowing tumor establishment for 3 days. Injections were repeated on days 6 and 9 posttumor implantation for a total of 3 doses per animal. Tumor measurements were taken in two dimensions and mass was estimated by using the formula [(length) (width^2)/2] (14). Inhibition of growth was calculated relative to growth in control animals given injections of PBS diluent alone. The control group contained 10 mice, with the test groups containing 5 mice each. The Student *t* test was used to determine the significance of differences in mean tumor masses.

RESULTS

PF1/D Cell Line Reactivity and Xenograft Model Development. The reactivity of unconjugated PF1/D with a number of human tumor cell lines and one nontransformed line was tested by using the indirect immunofluorescence technique. As is summarized on Table 1, reactivity was most evident with tumor cell lines of squamous carcinoma origin (3 of 4 positive). The prostate-derived cell line DU145 was also reactive with PF1/D. Four adenocarcinomas and one melanoma tumor cell line were negative, as was the fibroblast line FLOW2000.

The antigen-positive cell lines were tested for growth in 350 R-irradiated nude mice. As Table 1 shows, the lines FADU and T222 grew as xenografts with 100% tumor take. These lines also had the advantage of producing tumors of uniform size and shape. T222 was selected for subsequent studies because of its good growth characteristics in roller bottles, allowing for convenient scale-up for *in vivo* studies

Drug Conjugation and Immunochemical Analysis. The conjugation procedure consistently resulted in an average of 4-6 mol of *Vinca* hydrazide per mol of antibody as determined by dual wavelength UV analysis of the conjugate at 270 and 279 nm. Yields of conjugate from free antibody were typically greater than 80%. Fig. 1 shows that essentially 100% of the antigen-binding capacity of the antibody was maintained as measured by flow cytometry with an antigen-positive target. Fig. 2 demonstrates that both the free antibody and conjugate contained relatively few aggregates as determined by Superose 12 chromatography. The leading shoulder associated with the conjugate preparation (Fig. 2*B*) is characteristic of aggregates and represents about 10% of the total material.

***In Vivo* Efficacy.** Palpable tumor masses were present on all animals at day 3, when therapy was initiated. The animals received three i.v. injections on days 3, 6, and 9. The PF1/D-DAVLBHYD conjugate eliminated tumor growth when administered at 2 mg *Vinca* content/kg (Fig. 3*A*), with all five animals in this group being tumor free. These mice were observed for a total of 8 weeks postimplantation and they remained tumor free throughout that period. The 0.5-mg/kg group showed significant tumor suppression, which dropped to insignificant levels in the 0.12-mg/kg group. A non-tumor-binding IgG-

Table 1 *Cell line membrane reactivity with PF1/D and xenograft growth*

Membrane reactivity with PF1/D antibody was determined as described in "Materials and Methods." Reactive lines were injected s.c. into 350 R-irradiated nude mice (1×10^7 cells/mouse) and evaluated weekly for tumor growth for up to 8 weeks. The number of tumor takes over the total number of mice given injections is indicated.

Type and origin	Designation	PF1/D reactivity	Xenograft growth
Squamous carcinomas			
Lung	T222	+	5/5
Bladder	5637	+	0/5
Pharynx	FaDu	+	5/5
Cervix	ME180	−	
Adenocarcinomas			
Lung	P3/UCLA	−	
Colon	HT29	−	
	WiDr	−	
Prostate	PC3	−	
	DU145	+	3/5
Melanoma	M21	−	
Normal fibroblast	FLOW2000	−	

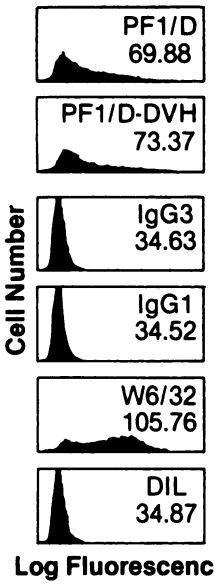

Fig. 1. Immunoreactivity of PF1/D-DAVLBHYD conjugate. Viable T222 cells were reacted with the indicated reagents at 5 μg/ml/5×10^5 cells: PF1/D, free monoclonal antibody prior to conjugation; PF1/D-DVH, postconjugation with DAVLBHYD; IgG3, myeloma control; IgG1, myeloma control; W6/32, HLA-reactive control; DIL, PBS background control. Mean fluorescence intensity values are indicated.

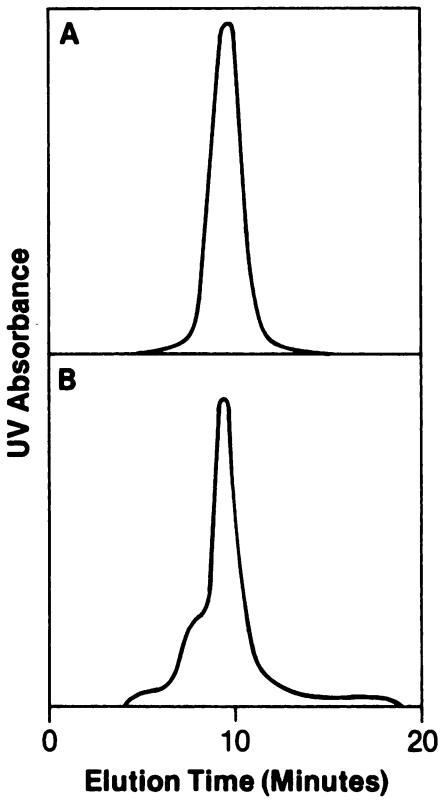

Fig. 2. Superose 12 gel filtration chromatography. *A*, free PF1/C prior to conjugation; *B*, postconjugation.

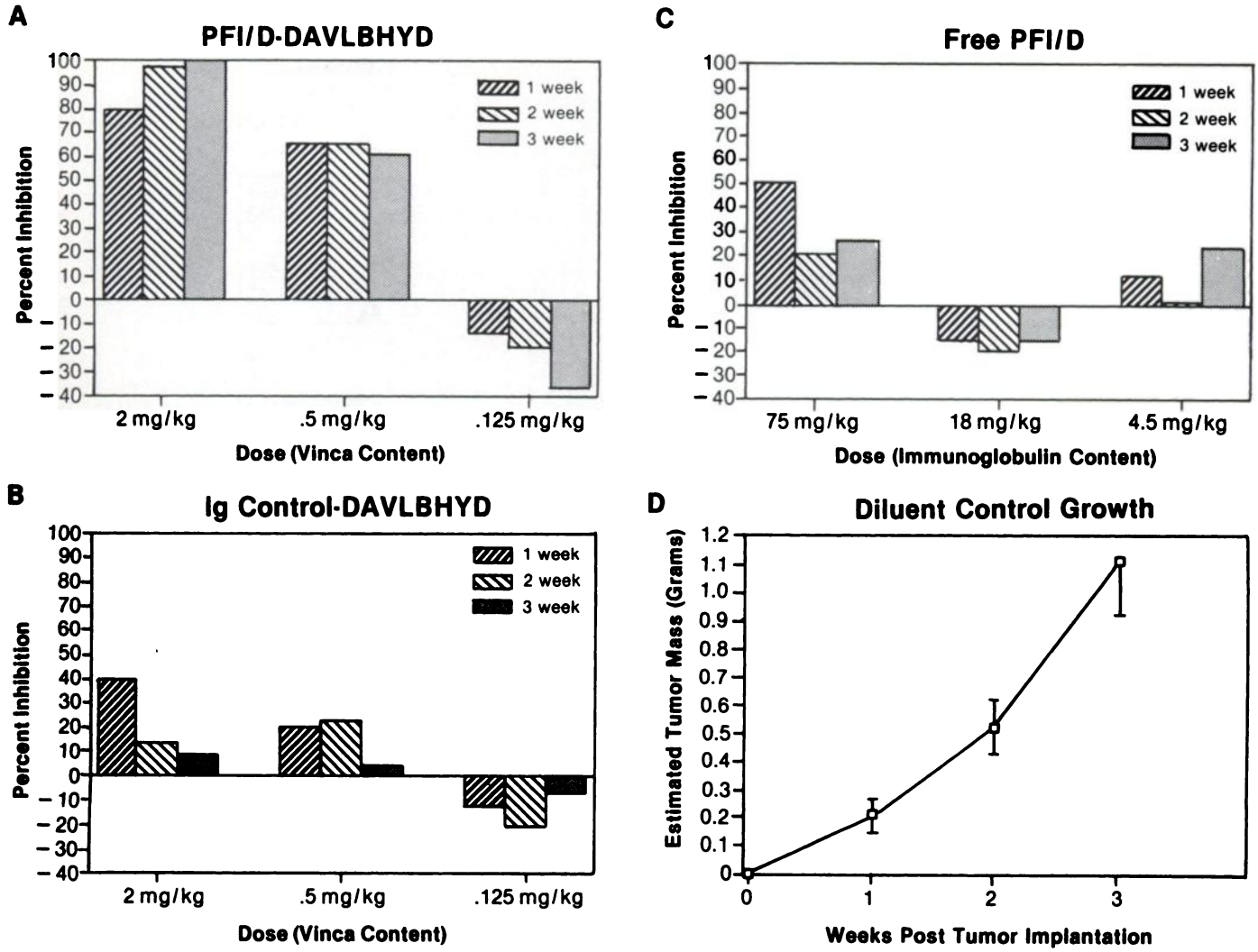

Fig. 3. PF1/D-DAVLBHYD suppression of T222 nude mouse xenografts. Tumor cells (1×10^7) were injected s.c. in the flank on day 0 and the mice were treated as indicated on days 3, 6, and 9. Tumor masses were estimated at 1, 2, and 3 weeks postimplantation. *A*, PF1/D-DAVLBHYD drug conjugate. Student's *t* test analysis of the data indicated that the 2-mg/kg and 0.5-mg/kg dose groups were significantly smaller than the PBS diluent controls at all time points [2 mg/kg: ($P < 0.01$); 0.5 mg/kg ($P < 0.05$)]. Other groups did not show statistically significant differences. *B*, non-tumor-binding Ig-DAVLBHYD conjugate control. The differences in tumor mass relative to the control group were not statistically significant. *C*, free PF1/D monoclonal antibody. Doses of free antibody equivalent to the protein content of the immunoconjugate doses depicted in *A* were administered. The high dose group (75 mg/kg) was significantly smaller than the saline controls at the 1-week measurement only ($P < 0.05$). Other groups and other time points did not show significant differences. *D*, PBS diluent control growth. Estimated tumor masses of the control group are indicated.

DAVLBHYD conjugate given in an identical protocol showed insignificant tumor suppression at all dose levels (Fig. 3*B*), demonstrating the antigen-dependent nature of the PF1/D-DAVLBHYD effect. Free PF1/D antibody given at a protein dose equivalent to the protein in the conjugate-treated animals showed significant tumor suppression in the 75-mg/kg dose at the time of the first measurement (Fig. 3*C*). This effect was reduced to insignificant levels by the time of the second and third measurements, in clear contrast to the effects seen with the conjugate. One group of animals (not plotted) received free DAVLBHYD at 2 mg/kg which resulted in tumor suppression insignificantly different from controls (−15% at 3 weeks post-implantation). Note that during the course of this experiment the tumors in the diluent control groups ranged up to approximately 1 g (Fig. 3*D*).

DISCUSSION

These studies support the feasibility of using monoclonal antibodies to target drugs to tumors. The squamous carcinoma-reactive antibody PF1/D was successfully conjugated to the *Vinca* derivative DAVLBHYD, with good yield. More importantly, the PF1/D-DAVLBHYD conjugate was able to eliminate 3-day-established human tumor xenografts by using a three-dose protocol. It is noteworthy that the mice which were tumor free at the 3-week time point remained tumor free for an additional 5-week period, at which point the experiment was terminated.

The PF1/D-DAVLBHYD tumor-suppressive effect was clearly antigen mediated, since treatment with a non-tumor-binding IgG-DAVLBHYD control conjugate was ineffective at suppressing tumor growth. The role of the drug moiety of the conjugate was demonstrated by the lack of prolonged activity of unconjugated PF1/D. In the experiments described here, free DAVLBHYD at 2 mg/kg produced no significant tumor suppression. In other experiments we have observed variable effects with this dose of free drug, with tumor suppression ranging up to 40%. Such effects are associated with toxicity, which has not been observed with PF1/D-DAVLBHYD conjugates. Free DAVLBHYD at 1 or 0.5 mg/kg has no suppres-

sive effect in this model,[4] unlike PF1/D-DAVLBHYD conjugates at identical dose levels.

The IgG-DAVLBHYD control conjugate used in these studies was of the IgG1 isotype. Its primary purpose was to control for effects of altered drug biodistribution as opposed to controlling for isotype-dependent effects such as complement fixation and antibody-dependent cellular cytotoxicity. Note that free antibody had minimal antitumor activity and therefore probably did not participate in such reactions. Nevertheless, it could perhaps be argued that an IgG3 control conjugate would have more closely matched the PF1/D antibody. Unfortunately, this could not be accomplished. There are few non-tumor-binding IgG3 control antibodies available in the quantities necessary to carry out studies such as these. Of those we have evaluated to date, poor solubility has led to low yields with significant levels of aggregation.[4] This profile is in contrast to what we observe with PF1/D conjugates, and such a preparation might be expected to have an altered biodistribution relative to the active conjugate. In contrast, the IgG1 used here conjugates in much the same manner as PF1/D, resulting in similar molecular profiles. Also, the tumor reactivity profiles of the IgG1 and an IgG3 myeloma protein are identical (Fig. 1). Together this information leads us to be comfortable in using the data generated with an IgG1-DAVLBHYD control to support our conclusions.

The ineffectiveness of free antibody may seem to be in contrast to the suggestion by Fernsten *et al.* (8) that PF1/D mediates antibody-dependent cellular cytotoxicity. It should be noted, however, that the present studies were carried out in irradiated nude mice, which represent severely immunodepressed hosts. Antibody-dependent cell-mediated cytotoxicity may not have been possible under these conditions. Participation of the PF1/D-DAVLBHYD conjugate in antibody-dependent cell-mediated cytotoxicity or other cytolytic mechanisms may be a welcome addition in a clinical setting where a functional immune response remains. On the other hand, it is conceivable that modification of antibody by drug conjugation will mask sites important for interaction with effector mechanisms, thereby reducing their impact.

Identification of an antigen-positive human tumor which grows well as a nude mouse xenograft was important to the successful completion of these studies. Cell surface fluorescence studies confirmed the preferential reactivity of PF1/D with squamous carcinoma-derived cell lines (8). The only exception noted was the cell line Du145, which has been described as "epithelial-like" (15) and may be of similar histological origin. The antigen-positive cell lines were tested for their ability to grow as nude mouse xenografts. The most strongly reactive line, 5637 (data not shown), failed to grow as a xenograft. The lines which did grow well as xenografts (T222 and FaDu) showed considerable heterogeneity in reactivity with the antibody, resulting in broad cell analyzer peaks. This is suggestive that the PF1/D antigen is expressed by individual cells in a cyclic fashion. Nevertheless, xenografts of T222 were successfully inhibited with short term PF1/D-DAVLBHYD conjugate treatments. This leads us to two speculations. One is that the PF1/D antigen may be expressed more consistently *in vivo*, or may be cycled on and off the cell surface rapidly enough to allow all cells to interact with antibody. Alternatively, active *Vinca* moieties may be released within the tumor mass, affecting neighboring cells with low antigen copy number on their surfaces. The latter possibility seems especially encouraging, since it would open the door to treatment of naturally occurring tumors showing antigenic heterogeneity. Efforts to detail more fully the fate of the PF1/D-DAVLBHYD conjugate within the tumor mass are under way.

Successful therapy with immunoconjugates will require that a number of critical criteria can be met. Among them is the retention of antibody-binding capacity postconjugation. The methods used here result in linkage of the vinblastine derivative via the carbohydrate side chains of the antibody.[3] It has been our experience that this results in little or no reduction in immunoreactivity and is therefore presumed to be contaminated with little denatured protein. Superose 12 chromatography confirmed that the preparation was largely aggregate free. The importance of the latter point may increase as we learn more about the role of host anti-mouse immune responses in immunoconjugate recipients. Certainly it has been demonstrated in animal models that the presence of denatured protein aggregates can greatly increase immunogenicity (16, 17) and presumably result in more rapid clearance. The ability to generate immunoconjugate preparations with minimal aggregation may, therefore, prove to be significant.

DAVLBHYD has been shown to have efficacy as a conjugate of the antibody KS1/4.[5] Similar results were obtained by using the antibodies L1/KS, L2/KS, and L4/KS, which also define epitopes on the KS1/4 antigen.[6] We are in agreement that with appropriate antibody selection, this chemistry can result in excellent retention of drug activity and antigen-binding capacity. The studies presented in this report demonstrate that the immunoconjugate PF1/D-DAVLBHYD has good efficacy in a relevant xenograft model. This, combined with the tumor-selective nature of the PF1/D antibody (8), suggests that this conjugate may warrant further investigation as a potential therapeutic agent for the treatment of squamous cell carcinoma.

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