New Antitumor Monoclonal Antibody–Vinca Conjugates LY203725 and Related Compounds: Design, Preparation, and Representative in Vivo Activity[1]

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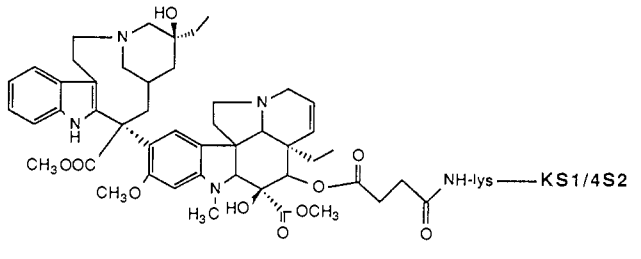
A method has been developed to allow the direct coupling of the cytotoxic vinca alkaloid 4-desacetylvinblastine-3-carbohydrazide (DAVLB hydrazide) to a variety of murine monoclonal antibodies directed against human solid tumors. Periodate oxidation of carbohydrate residues on the antibodies, followed by reaction with DAVLB hydrazide in aqueous acid affords, in most cases, conjugates with conjugation ratios of 4–6 vincas per antibody in high yield without significantly impairing antigen binding or solubility. The outcome of the conjugation reaction is highly dependent on the concentration of, and time of exposure of the protein to, the oxidant. These conjugates exhibit potent antitumor activity in vivo against a number of human solid tumor-nude mouse xenografts, with efficacy and safety increased over unconjugated DAVLB hydrazide. This antitumor activity is also superior to that of similarly prepared but nontarget tumor binding antibody–DAVLB hydrazide conjugates. MoAb–DAVLB hydrazide conjugates release DAVLB hydrazide in solution in a temperature- and pH-dependent manner. Hydrolytic release of unmodified DAVLB hydrazide from tumor-localized MoAb–DAVLB hydrazide conjugates in vivo may be an important factor in their antitumor activity.

Monoclonal antibody–drug conjugates and monoclonal antibody–toxin conjugates have gained considerable attention as potentially useful tools for the treatment of human cancers.[2-4] The promise of these new therapeutics is the prediction that the monoclonal antibody (MoAb), selected to specifically bind a particular tumor-associated antigen, will preferentially concentrate the attached oncolytic agents or toxins at or within a tumor mass. By targeting the drug to the tumor site, the conjugate should boost tumor cell killing by the drug or toxin, while diminishing unwanted side effects.

Numerous conjugates of antitumor MoAbs with a variety of cytotoxic agents, including adriamycin, vindesine, methotrexate, radionuclides, and the protein toxin ricin, have already been reported.[5,6] In the majority of these examples, the drug is coupled to the antibody lysine amino groups via amide, alkyl, or imine carbon–nitrogen bonds, while toxins such as ricin are best attached by way of heterobifunctional disulfide reagents. Several of these conjugates have been reported to inhibit the growth of tumor cells in vitro and in vivo. For example, KS1/4S2–DAVLB (LY256787, 1)[7] is a conjugate comprised of the adenocarcinoma-reactive MoAb KS1/4S2[8] coupled through its lysine amino groups to the 4-OH of the cytotoxic vinca alkaloid 4-desacetylvinblastine[9] (DAVLB, **7a**) via a succinate bridge.[10] This conjugate exhibited significant antitumor effects in vivo against human lung and colorectal adenocarcinoma xenografts in Nu/Nu (nude) mice.



1 KS1/4 - DAVLB (LY256787)

We were interested in the design and development of unique, second generation anticancer MoAb–drug conjugates. Our chemical objectives were to (1) choose from an established family of cytotoxic vinca alkaloids[11] a drug candidate bearing conjugatable functional groups, (2) explore an alternative site and method of attachment of the chosen drug to the MoAb, and (3) preserve the MoAb's antigen binding and specificity while retaining solubility. Through such a construction we hoped to achieve the biological objectives of (1) selective in vivo delivery of a potent antitumor agent to target tumor masses and (2) a significant increase in the safety and efficacy of conjugated drug over unconjugated drug. These goals appear to have been realized with KS1/4S2–4-desacetylvinblastine-3-carbohydrazide conjugate (LY203725, **9**) and related conjugates (MoAb–DAVLB hydrazide, **2**). This paper de-

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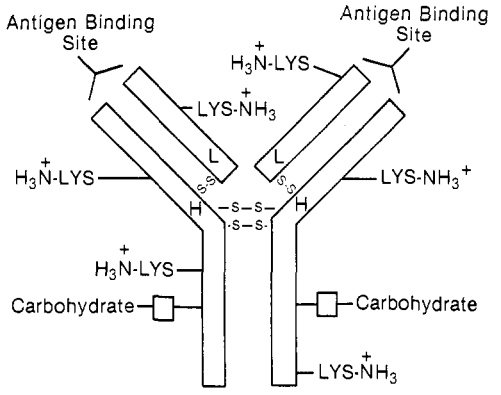
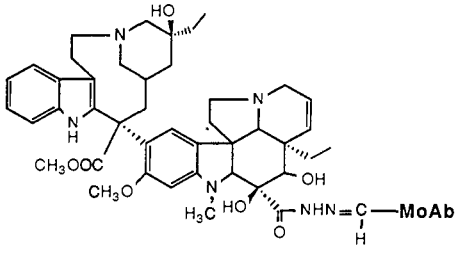


Figure 1. 2-D representation of an immunoglobulin G (IgG) molecule.

scribes the design, synthesis, and representative biological activity[12] of these new conjugates.



2 MoAb - DAVLB HYDRAZIDE

Conjugate Design: A. MoAb Site of Drug Attachment. IgG immunoglobulins are symmetrical, bivalent glycoproteins of approximately MW 150000, composed of two identical, short, amino acid chains ("light" (L) chains) paired with two identical, longer, amino acid chains ("heavy" (H) chains).[13] The carbohydrate content of these macromolecules averages 3–5% by weight and is usually comprised of branched chains of *N*-acetylglucosamine, D-mannose, L-fucose, and D-galactose, with *N*-acetylneuraminic acid capping the nonreducing ends.[14,15] Similar carbohydrate structures have been found on monoclonal IgG's.[15] These polysaccharide chains are usually N-linked at the *N*-acetylglucosamine reducing ends to Asn residues in the C_H2 domain on the heavy chains of the IgG molecule, a site remote from the antigen binding regions (see Figure 1). However, examples of IgG's bearing carbohydrate in the antigen binding half of the protein have been found.[15]

In contrast, lysine residues are much more widely distributed throughout the antibody primary amino acid sequence and are often found in or near the antigen binding domains. It is the ϵ amino groups of these lysine residues

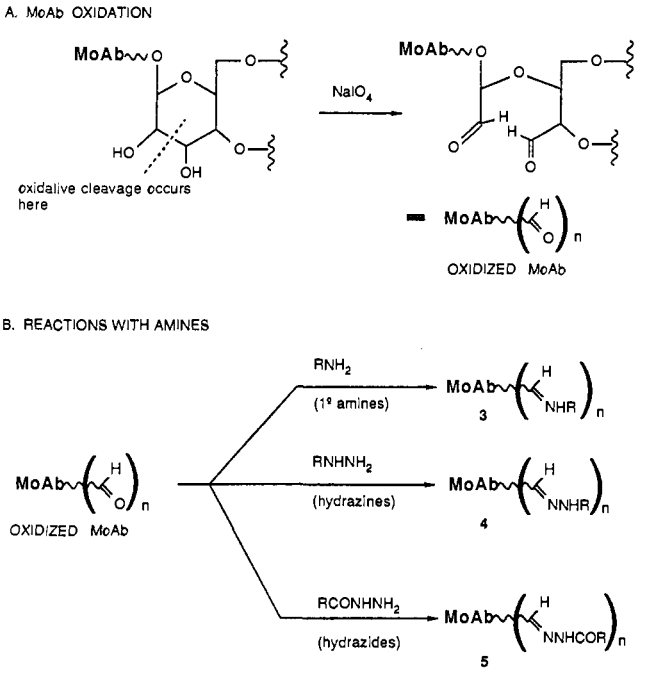


Figure 2. Periodate oxidation of MoAbs and coupling with amines.

Table I. In Vitro Biological Comparison of Several Vinca Derivatives[a]

vinca derivative[b]	P3UCLA cells[c] IC_{50}, μg/mL	CEM cells[d] IC_{50}, μg/mL
6	<0.0003[e]	0.03
7a	0.0003	ND[f]
7b	ND[f]	0.10
7c	0.003	1.4

[a] Also see ref 9a. [b] Compounds were tested as their sulfate salts. [c] IC_{50}, concentration demonstrating 50% inhibition of cell proliferation as determined by the MTT colorimetric proliferation assay (ref 33) with 48-h constant exposure of drug utilizing P3UCLA (ref 12a) human lung adenocarcinoma cell cultures. Reported IC_{50}'s are means and are within 10% of the experimental values ($N = 3$). [d] 72-h constant exposure to CCRF-CEM human leukemic lymphoblasts at 37 °C at a concentration of 2.4×10^4 cells/mL, with % inhibition of cell proliferation per given dose determined by Coulter Counter measurement (ref 34). Reported IC_{50}'s are means and are within 3% of the experimental values ($N = 2$). [e] Lowest dose tested. [f] ND = not done.

that are the traditional sites of drug attachment, as is the case with **1**. Consequently, lysine-linked antibody–drug conjugates can show significant reduction in antigen reactivity and affinity.[16,17] On the other hand, coupling drugs to the antibody carbohydrate groups could afford conjugates with little or no obstruction of antigen binding.[16,18] Furthermore, antibody hydrophilicity, and therefore solubility, should be better retained in carbohydrate-linked conjugates, as ionizable lysine ϵ amino groups would not be consumed by the coupling of the drug to the antibody.

Conjugate Design: B. Attachment of Drugs to Antibody Carbohydrate Groups. The native carbohydrate groups on an antibody are relatively inert as sites for drug attachment. However, numerous studies have

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Table II. MoAbs, Clinical Targets, and Antigen-Positive Human Tumor Cell Lines

MoAb:[a]	isotype	clinical targets	antigen-positive-human tumor cell lines[b]
KS1/4S2[c]	IgG2a	adenocarcinoma (lung, colon, breast, prostate); squamous carcinoma (lung)	P3/UCLA (lung adenocarc) HT29 (colon adenocarc)
L1KS[d]	IgG2b	all KS1/4 antigen-positive targets	same as for KS1/4S2
L2KS[d]	IgG1		
L4KS[d]	IgG2a		
9.2.27[e]	IgG2a	melanomas	M14 (melanoma)
PF4/A[f]	IgG2a	squamous carcinomas (head, neck, lung)	T222 (lung squamous carc)
PF1/B[f]	IgG3		
PF1/D[f]	IgG3		
1495.55[g]	IgG2a	CEA-bearing tumors (colon, lung, breast, and others)[h]	LS174T (colon adenocarc)
X63AG8S1[c]	IgG1	none (control myeloma IgG)	none

[a] All MoAbs listed are murine-derived. [b] For background on the origin and nature of these cell lines, see ref 12a and references therein. [c] See ref 8. [d] See ref 12d. [e] See ref 31. [f] See ref 12a and 32. [g] See ref 10. [h] CEA = carcinoembryonic antigen.

shown that glycol units on these carbohydrate moieties can be oxidatively cleaved with sodium metaperiodate ($NaIO_4$) under mild conditions, generating aldehyde sites that are suitable for coupling with amine-bearing reagents (Figure 2a).[16,19-22] Reaction of the oxidized antibody with 1° amines hypothetically provides Schiff bases **3**, while coupling with hydrazine and hydrazide reagents affords the more stable hydrazone adducts **4** and **5** (see Figure 2b).[23] The synthesis of an antimouse lymphoma antibody–daunorubicn hydrazone conjugate represents an early application of this conjugation strategy.[24] More recently, preparation of a tumor-imaging conjugate of an oxidized monoclonal antibody coupled to an amine-bearing ^{111}In-chelate has been reported.[25]

Conjugate Design: C. Choice of Drug. The cytotoxic vinca alkaloid 4-desacetylvinblastine-3-carbohydrazide (**6**) (DAVLB hydrazide),[9] a derivative of vinblastine **7b**,[26] appeared to be a good first choice as a drug candidate for building a carbohydrate-linked MoAb–vinca conjugate. It significantly inhibited the growth of human tumor cells in vitro (see Table I) and appeared superior in this activity to the related compounds desacetylvinblastine **7a**[9] the 4-hemisuccinate derivative **7c**[7] (used to prepare conjugate **1**), and vinblastine **7b**[26] (see Table I). Another attractive feature of this compound was the unique hydrazide functionality at carbon 3. This seemed well suited for coupling the potent vinca **6** directly to oxidized MoAb carbohydrate aldehyde sites via acylhydrazone bonds, as shown in structure **2**, obviating the need for any additional, and perhaps biologically deactivating, chemical bridge.

An important feature of the hydrazone linkages of the proposed MoAb–DAVLB hydrazide conjugates **2** is that they were expected to be susceptible to acid-catalyzed hydrolysis, such as could occur during endocytosis, once the conjugate had localized at the target cancer site.[27] Significant hydrolysis might also occur at the tumor cell surface, in the absence of internalization, as the intratumoral pH of tumor tissue in several human patients has been determined to be acidic.[28] Therefore, it was predicted that DAVLB hydrazide might be released from the conjugate at the tumor site, unmodified and fully active.

Conjugate Design: D. Choice of MoAb. Monoclonal antibodies that recognize antigens on human solid tumor cells including adenocarcinomas, melanomas, and squamous carcinomas were considered highly desirable as drug targeting agents. These classes of cancers encompass most of the major types of human solid tumors such as lung, breast, colon, rectal, and skin.[29] A representative listing of the MoAbs employed in the construction of MoAb–DAVLB hydrazide conjugates described in this paper, some clinically important antigen-positive human tumor targets, and some examples of antigen-positive human tumor cells lines useful for biological testing of the MoAbs and their conjugates are shown in Table II.

Results and Discussion

Chemistry. DAVLB hydrazide (**6**) was prepared by treating vinblastine **7b**[26] with anhydrous hydrazine in absolute methanol.[9] Chromatography and recrystallization of the crude reaction product provided the desired compound in greater than 95% purity. MoAb–DAVLB hydrazide conjugates **2** were synthesized as follows: The various monoclonal antibodies examined in this study were oxidized by sodium metaperiodate in cold sodium acetate buffer and the products isolated by Sephadex size-exclusion column chromatography. Treatment of the oxidized proteins with **6** or its sulfate salt in acetate buffer provided the desired MoAb–DAVLB hydrazide conjugates. Conjugates **2** were mostly separated from unconjugated DAVLB hydrazide **6** by gravity flow Sephadex-size exclusion chromatography in phosphate buffered saline at pH 7.4. Residual unconjugated **6** (~5–10%) was removed by exhaustive dialysis; absence of free **6** in the final products was confirmed by reverse-phase HPLC. Alternatively, conjugate was purified from the crude reaction mixture by automated size exclusion and ion exchange chromatography. In this way MoAb–DAVLB hydrazide

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Table III. Summary of Conjugation Results

compd	name	% yield[a]	CR[b]	% immunoreactivity remaining[c]
9	KS1/4S2–DAVLB hydrazide	84	5.0	100
10	L1KS–DAVLB hydrazide	76	5.8	100
11	L2KS–DAVLB hydrazide	64	5.4	
12	L4KS–DAVLB hydrazide	76	4.3	100
13	9.2.27–DAVLB hydrazide	84	4.6	92
14	PF4/A–DAVLB hydrazide	86	3.9	82
15	PF1/B–DAVLB hydrazide	85	5.2	92
16	PF1/D–DAVLB hydrazide	77	4.6	109
17	14.95.55–DAVLB hydrazide	65	3.8	100
18	X63AG8S1–DAVLB hydrazide	84	5.5	0 (control)

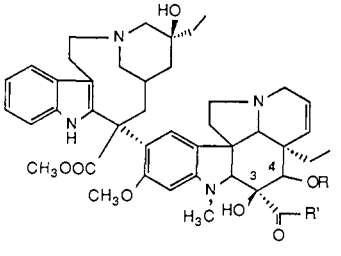
[a] Reported yields are overall protein yields after oxidation and conjugation, using method A as reported in the Experimental Section. [b] Conjugation ratio, mmol **6**/mmol MoAb; results averaged over several runs. [c] The degree of binding of conjugates to antigen-positive tumor cell lines (see Table II for cell line descriptions) was compared to that of native, conjugated parent MoAb by several different methods. Conjugates **9**, **13**, **18**: FACCS (see ref 17); conjugates **10**–**12**, **17**: RIA (see ref 13, p 72); conjugates 14–16: ELISA (see ref 35).

Table IV. Effect of Various Reaction Parameters on Conjugation Ratio[a]

study	$NaIO_4$, mM	oxidn time, min	DAVLBHYD concn, mM	CR[b]
1	0	21	5.0	0.3
	10	21	5.0	1.9
	40	21	5.0	2.0
	160	21	5.0	5.4
2	160	14	5.0	3.1
	160	21	5.0	4.3
	160	28	5.0	5.2
	160	35	5.0	6.4
	160	42	5.0	7.6
3	160	21	0.0	0.2
	160	21	1.0	3.1
	160	21	2.0	4.0
	160	21	3.0	3.9
	160	21	4.0	4.8
	160	21	5.0	4.9

[a] All data obtained for preparation of conjugate **9** (KS1/4–DAVLB hydrazide), using method A described in the Experimental Section; qualitatively similar results obtained for the preparation of all of the conjugates listed in Table I. [b] Conjugation ratio; expressed as mmol DAVLB hydrazide (**6**)/mmol KS1/4S2.

conjugates **2** containing an average of 4–6 mol of DAVLB hydrazide (**6**) per mole of antibody, as determined by dual wavelength UV spectroscopy, were routinely obtained. These results are summarized in Table III. The amount of aggregated protein in the conjugates was usually low (5–15%).



6 R = H, R' = $NHNH_2$
7a R = H, R' = OCH_3
7b R = $COCH_3$, R' = OCH_3
7c R = $CO(CH_2)_2COOH$, R' = OCH_3

In developing the oxidation-coupling procedure described above, several reaction parameters were explored. The ability to conjugate DAVLB hydrazide (**6**) to the MoAbs was clearly dependent on the extent of prior oxidation of the protein. Conjugation ratio (mmol vinca/mmol MoAb) increased with increasing periodate concentration and time of exposure of the MoAb to the oxidant (Table IV, studies 1 and 2). The temperature of the oxidation step also significantly influenced the outcome of the vinca coupling reaction (data not shown). The degree of coupling of DAVLB hydrazide (**6**) to oxidized MoAb was also dependent on the concentration of vinca

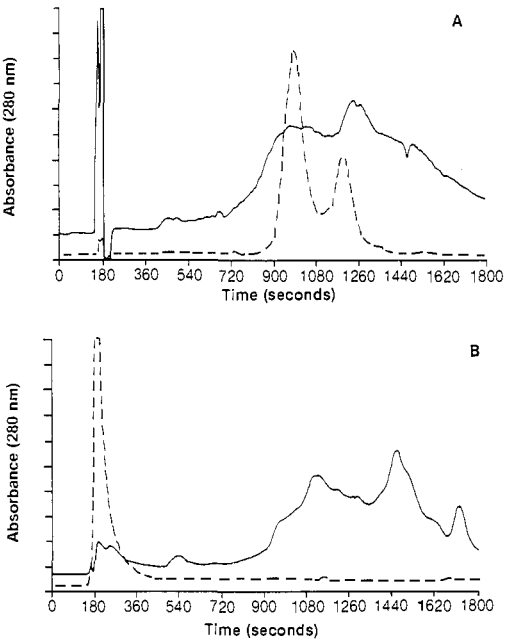


Figure 3. (a) HIC profiles of KS1/4S2–DAVLB hydrazide (**9**) vs KS1/S2 MoAb. TSK-phenyl column; 0.1 M PO_4 buffer, pH 6.5, 2–0 M NaCl gradient. Sample elution was monitored by UV at 280 nm. (—) KS1/4S2–DAVLB hydrazide (**9**); (---) KS1/4S2. Conjugate **9** was prepared by method A (Experimental Section). (b) RPC profiles of KS1/4S2–DAVLB hydrazide (**9**) vs KS1/4S2–DAVLB (**1**). TSK phenyl column; 0.1 M PO_4, pH 6.5, 0–30% CH_3CN gradient. Elution was monitored by UV at 280 nm. (---) KS1/4S2–DAVLB hydrazide (**9**); (—) KS1/4S2–DAVLB (**1**). Conjugate **9** was prepared by method A (see Experimental Section).

employed (Table IV, study 3) but appeared insensitive to pH in the range of pH 4–6 (not shown).

The hydrophilicities of the DAVLB hydrazide conjugates in this study, relative to their parent MoAbs, were determined by Hydrophobic Interaction (HIC) and Reverse Phase chromatography (RPC). Figure 3a compares the HIC elution profiles of KS1/4S2–DAVLB hydrazide conjugate (**9**) to KS1/4S2. These data show that conjugate **9** is only slightly less hydrophilic than the unmodified MoAb. In contrast, the much more hydrophobic, lysine-linked conjugate KS1/4S2–DAVLB **1** does not elute under these conditions (not shown). Figure 3b shows the results of a comparison of the RPC elution profiles of conjugate **9** and conjugate **1** using a stronger eluting solvent. In this system, **1** elutes slowly from the column, even in the presence of the organic cosolvent CH_3CN. In marked contrast, **9**, being much more hydrophilic, elutes near the void volume under these conditions, as does the parent

Table V. In Vivo Efficacy of MoAb-DAVLB Hydrazide Conjugates in Human Tumor-Nude Mouse Xenografts: Tumor Initiation Model

compd	name	human tumor xenografts:[a] ED_{50} (mg/kg)[b]				
		P3UCLA	HT29	LS174	T222	M14
6	DAVLB hydrazide	1.0	1.0	1.0	2.0	0.5
9	KS1/4S2-DAVLB hydrazide	<0.0625[c]	<0.25[c]	NT[d]	NT	>2.0[e]
10	L1KS-DAVLB hydrazide	0.05	0.0625	0.15	NT	NT
13	9.2.27-DAVLB hydrazide	NT	NT	NT	NT	<0.0625[c]
16	PF1/D-DAVLB hydrazide	NT	NT	NT	0.3	NT
17	14.95.55-DAVLB hydrazide	0.6	NT	0.15	NT	NT
18	X63AG8S1-DAVLB hydrazide	>1.0	3.0	NT	>2.0[e]	NT

[a] See Table II for descriptions of cell lines and corresponding MoAb reactivity. [b] Dose of DAVLB hydrazide (6), given alone or as a conjugate, required to cause 50% suppression of tumor growth as compared to controls, 28 days post tumor implantation. % suppression determined as the ratio of average tumor mass of the treatment group (N = 5) to that of the control group (N = 10). The total amount of conjugate (vinca + MoAb) administered to achieve the ED_{50} dose of conjugated 6 is approximately 30–40 times the value shown. See Experimental Section for dosing protocols and details. Also see ref 7 and 12. [c] Lowest dose tested. [d] NT = not tested. [e] Highest dose tested.

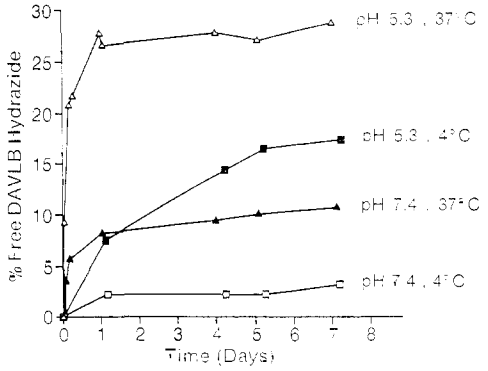


Figure 4. Release of DAVLB hydrazide (6) from KS1/4S2-DAVLB hydrazide (9). Conjugate was prepared by method B (see Experimental Section); CR = 5.3. % Free DAVLB hydrazide (6) is that % of the total amount of 6 in the conjugate sample (protein-bound + unbound) that elutes as unbound 6 on RPHPLC. This material represented 84–95% of all eluting substances combined.

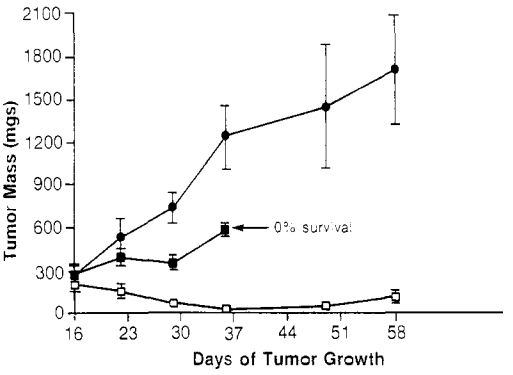


Figure 5. Effect of KS1/4S2-DAVLB hydrazide (9) vs unconjugated DAVLB hydrazide (6) on growth of established P3UCLA lung adenocarcinoma xenografts in nude mice. (—□—) 9 at 2 mg/kg (vinca content); (—■—) 6 at 2 mg/kg; (—●—) control. Each point is the mean ± the S.E. of N = 5 mice (treatment group) or N = 10 mice (control group).

MoAb KS1/4S2 (not shown). These findings are consistent with the fact that concentrated (>10 mg/mL), homogeneous solutions of conjugate 9 are more easily prepared than for 1.

The potential for MoAb-DAVLB hydrazide conjugates to release free DAVLB hydrazide (6) by hydrolysis was evaluated by subjecting several of the conjugates listed in Table III to storage in buffers at different pH's and temperatures. The amount of free DAVLB hydrazide (6) released over time under each set of conditions was then measured by Reverse Phase HPLC analysis. Figure 4 shows data obtained from a 7-day stability study of KS1/4S2-DAVLB hydrazide (9); qualitatively similar results were obtained with conjugates 12 and 13. While little free DAVLB hydrazide (6) was formed in the sample of conjugate 9 stored at physiological pH and 4 °C, release of 6 was much more significant at acidic pH. At physiological temperature (37 °C), release of 6 was accelerated and enhanced at both pH's, with the greatest release by far occurring at pH 5.2. In all samples, the majority of release occurred in the first 24 h, except in the case of the sample stored at pH 5.2 and 4 °C, which continued to exhibit slow, sustained release of 6 from the conjugate over the entire time course of the study.

Biological Results: A. Conjugate Immunoreactivity. The degree of antibody immunoreactivity remaining post conjugation was determined by measuring the binding of each conjugate to an antigen-positive target tumor cell and comparing the result to that obtained with the parent unconjugated MoAb. Results for several conjugates are summarized in Table III. All of the conjugates listed in Table III showed excellent retention of antibody target cell binding. For example, by indirect fluorescence techniques, KS1/4S2-DAVLB hydrazide 9 gave a mean of 99.4% (S.E. = 5.549; N = 16) retention of immunoreactivity of the parent KS1/4S2 MoAb (see Table III).

Biological Results: B. In Vivo Antitumor Activity.[12] The DAVLB hydrazide conjugates were evaluated in vivo for their ability to suppress growth of antigen-bearing, solid human tumors subcutaneously implanted in nude (Nu/Nu) mice. Table V gives, for each MoAb-DAVLB hydrazide conjugate listed, the minimum effective dose (mg/kg) of DAVLB hydrazide (6), given as a conjugate, required to achieve 50% suppression of growth of each type of tumor xenograft indicated. In this "Tumor Initiation" model,[7] in which the tumor load prior to conjugate administration was relatively small, all of the hydrazide conjugates caused significant growth suppression of their respective tumor targets. This antitumor activity of each conjugate was superior to that of parent, unconjugated antibody (data not shown), unconjugated DAVLB hydrazide (6), and to control, nontumor binding DAVLB hydrazide conjugate 18 (note: conjugate 9 served as a control for conjugate 13 against M14 xenograft; a control conjugate was not run for conjugate 17 against LS174 xenograft). For example, KS1/4S2-DAVLB hydrazide (9), had an ED_{50} (<0.0625mg/kg) at least 16 times less than that of free DAVLB hydrazide (6) (1.0 mg/kg) and control conjugate 18 (>1.0mg/kg) against P3UCLA lung adenocarcinoma xenografts. The therapeutic index (lethal dose/ED_{50}) of this conjugate (TI = 64), based on DAVLB hydrazide (6) content, was also significantly increased over that of free 6 (TI = 4). Interestingly, the in vitro activity of these conjugates was often slightly less than that of unconjugated DAVLB hydrazide (e.g., IC50 = 3.4 ng/mL

for L1KS-DAVLB hydrazide (10) vs 28 ng/mL for unconjugated hydrazide; [^{3}H]leucine uptake, P3/UCLA cells; J. J. Starling, unpublished results).

The MoAb-DAVLB hydrazide conjugates were also effective against well-established tumors. Figure 5 displays the in vivo activity of conjugate 9 against 16-day established P3UCLA human lung adenocarcinoma xenografts. At the dose indicated (mg/kg vinca content of 9), not only was tumor growth suppressed by conjugate administration but actual regression of >90% of the original tumor mass occurred with no apparent toxicity. In contrast, an equivalent vinca dose of unconjugated DAVLB hydrazide (6) only plateaued growth, with no regressions observed. Furthermore, this dose of unconjugated 6 was lethal to the entire treatment group, prior to completion of the experiment. DAVLB hydrazide conjugates of nontumor binding MoAbs as well as KS1/4S2 alone were inactive in this model (not shown).[12b]

Discussion. The chemistry used to construct the MoAb-DAVLB hydrazide conjugates described in this paper is reproducible and applicable to a wide variety of MoAbs of varying isotype. Overall protein recoveries are high, and acceptable conjugation ratios are consistently obtained. Although the conjugation conditions prescribed in this paper generally do not provide the maximum conjugation ratio possible per given MoAb, they do assure a reasonable balance of yield, minimal aggregate content, conjugation ratio, product solubility, and retention of immunoreactivity.

The conjugation ratio achieved for a particular MoAb is highly dependent on exposure of the protein to oxidant. This is consistent with our postulate that DAVLB hydrazide (6) is condensing with periodate-generated aldehyde sites on the carbohydrate chains of the MoAb. Further evidence that aldehyde sites are present following oxidation is provided by the ready reaction of the oxidized antibody with the classical aldehyde determination reagent, 2,4-dinitrophenylhydrazine, and by the incorporation of tritium when exposed to [^{3}H]sodium borohydride[30] (data not shown). Other experiments are underway to further elucidate the exact chemical nature of these conjugates.

Most MoAbs conjugated by this method showed little alteration in antigen binding, as predicted. However, this was not the case for all MoAbs that were evaluated. Although it conjugated well (CR = 6.5, 78% yield), one MoAb's ability to bind to antigen-positive tumor cells was essentially abolished by the conjugation procedure. Even very mild oxidation of this MoAb resulted in complete loss of antigen binding (J. J. Starling and B. C. Laguzza, unpublished results). Thus, oxidation of and coupling amine-bearing reagents to MoAb carbohydrate sites does not guarantee per se that the antibody will retain its antigen recognition and binding characteristics, contrary to popular expectations.[16,18]

One of the anticipated advantages of a MoAb-DAVLB hydrazide conjugate was the potential for acid-catalyzed release of unmodified, fully active DAVLB hydrazide (6) from the conjugate, once the conjugate had localized at the target tumor site. Our data shows that in solution, free DAVLB hydrazide can be released from the MoAb-DAVLB hydrazide conjugates and that the release is significantly more effective at acid pH. Even at physiological pH, release can occur, albeit at a much slower rate. In light of these data and recent observations that the intratumoral pH of human tumor tissue appears to be acidic,[28] it seems very likely that in vivo, free DAVLB hydrazide will be hydrolytically cleaved from the tumor-localized conjugate, whether it is internalized by the tumor cell or not. The potential for extracellular release of DAVLB hydrazide could prove crucial in those cases where the cells within a target tumor do not all express the target antigen. These antigen-negative cells would still be susceptible to extracellular DAVLB hydrazide released by conjugate localized on neighboring, antigen-positive cells. Experiments to study target cell processing of these conjugates, as well as their in vivo metabolism, are now in progress.

All of the MoAb-DAVLB hydrazide conjugates tested in vivo showed potent antitumor effects against their respective human solid tumor targets in nude mice. These effects were superior to free DAVLB hydrazide (6), parent monoclonal antibody and to control, nontarget tumor binding MoAb-DAVLB hydrazide conjugates. The margin of safety for conjugated DAVLB hydrazide (6) was also significantly greater than that for unconjugated 6. These results, taken together, demonstrate that the antitumor activity of these conjugates is due to an antigen-mediated, MoAb-drug targeting event and cannot be explained by invoking a nonspecific, protein carrier-mediated drug delivery phenomenon. The potency of this class of MoAb-drug conjugates is further demonstrated by the ability of KS1/4S2-DAVLB hydrazide 9 to significantly regress large, well-established tumors in vivo without apparent toxicity. This effect could not be achieved by unconjugated 6, even at maximum tolerated doses. Similar findings have been achieved with most of the MoAb-DAVLB hydrazide conjugates was have tested. Thus, the efficacy, as well as potency and safety, of DAVLB hydrazide (6) is enhanced by conjugation to periodate-oxidized MoAbs.

Summary

DAVLB hydrazide (6) has been readily conjugated to a variety of periodate-oxidized MoAbs selective for antigen-bearing, human tumor cells. Conjugation ratios of 4–6 vincas/MoAb are reproducibly obtained in good yield and are controlled by a variety of reaction parameters. These conjugates retain much of the original MoAbs' antigen binding capabilities and exhibit significant antitumor effects in vivo against human tumor xenografts in nude mice. The antitumor effects of the conjugates are superior to unconjugated DAVLB hydrazide (6), unconjugated MoAbs, and control nontumor binding DAVLB hydrazide oxidized MoAb conjugates. MoAb-DAVLB hydrazide conjugates 2 appear to have an improved therapeutic index over unconjugated DAVLB hydrazide (6).

Experimental Section

General. UV spectra were recorded on a Beckman Model DU-7 spectrophotometer. HPLC analyses were performed on a system comprised of a Rheodyne 7125 injector, LDC ConstraMetric III pump, 150-mm IBM C-8 reverse phase column, and LDC SpectoMonitor III variable wavelength UV detector; signal output was recorded and integrated by a Hitachi D-2000 Chromato-Integrator. Gravity flow Sephadex size-exclusion chromatography was performed on Pharmacia Sephadex G-25 (med mesh) packed into open glass columns coupled to an ISCO Model V^4 UV absorbance detector. Conjugates were concentrated in a Bio-Molecular Dynamics Micro ProDiCon negative pressure protein dialysis chamber equipped with ProDiMem PA-30 membranes. Buffers were prepared with deionized, distilled water processed through a Millipore Milli-Q reagent water system. pH 7.4 phosphate buffer

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was prepared with Gibco Dulbecco's phosphate buffered saline. All other solvents and chemicals were reagent grade. Automated chromatography was performed with a Pharmacia LCC 500 Controller and P-500 liquid chromatography pumps coupled to a UV2 absorbance detector. TSK Phenyl, Sephadex G-25M, Q-Sepharose Fast Flow and Prepacked 10/30 HR Superose 12 columns are all products of Pharmacia Fine Chemicals.

Periodate-Oxidized Monoclonal Antibody. The MoAbs listed in Table II were oxidized as follows for KS1/4S2: To a rapidly stirring solution of 470 mg (3.13×10^{-3} mmol) of monoclonal antibody KS1/4S2[8] in 47 mL of 0.1 M NaOAc buffer at pH 5.6 at ~0–2 °C (ice bath cooling) was added, in one portion, granular sodium metaperiodate (1.6 g, 7.5 mmol). The resultant slurry was stirred in the dark with continued ice cooling for 21 min. The reaction mixture was then quenched by the rapid addition of 3.0 mL (35.5 mmol) of a 12.5 M aqueous solution of ethylene glycol. Stirring was continued in the cold and dark, and the resultant slightly cloudy mixture was centrifuged (2000*g*, 10 min, room temperature). The clear, colorless supernatant was separated from a small white pellet and immediately chromatographed in two equal portions over Sephadex G-25 (medium mesh, 90 g, 2.5 × 100 cm bed) in 0.1 M NaOAc buffer at pH 5.6. Elution was monitored at 280 nm. The fractions containing oxidized KS1/4S2 were collected and combined to give 445 mg (94% yield) of oxidized protein at 5.8 mg/mL as determined by UV spectroscopy at 280 and 270 nm [ϵ_{280} and ϵ_{270} (phosphate buffer, ph 7.4) = 2.14×10^5 M^{-1}·cm and 1.8×10^5 M^{-1}·cm, respectively]. The material was further characterized by a slight but reproducible bathochromic shift in the observed UV_{max} for KS1/4S2 from 279.5 nm to 279 nm and a slight increase in the 270/280 ratio from 0.84 to ~0.87.

MoAb–4-Desacetylvinblastine-3-carbohydrazide Conjugates 2. Method A. The synthesis of 4-desacetylvinblastine-3-carbohydrazide (**6**) has already been reported.[9a] The MoAb–DAVLB hydrazide conjugates listed in Table III were prepared as follows for KS1/4S2-DAVLB hydrazide conjugate (**9**). To an ice-cold solution of freshly prepared oxidized KS1/4S2 (442 mg, 2.95×10^{-3} mmol) in 160 mL of 0.1 M sodium acetate buffer at pH 5.6 was added dropwise, with moderate stirring, 12.9 mL of a freshly prepared solution of 4-desacetylvinblastine-3-carbohydrazide (**6**) (693 mg, 0.901 mmol) in DMF. The resultant solution was stirred under a nitrogen atmosphere at 4 °C for 24 h in the dark. The then slightly cloudy crude reaction mixture was centrifuged (2000g, 20 min, room temperature) and the clear, colorless supernatant was immediately chromatographed in two equal portions over Sephadex G-25 (medium mesh, 90 g, 2.5 × 100 cm bed) in 0.01 M phosphate buffer containing 0.15 M NaCl at pH 7.4. Elution was monitored at 280 nm. The desired conjugate eluted in the protein fraction followed immediately by unconjugated DAVLB hydrazide. The isolated protein fractions were combined and then concentrated and dialyzed in vacuo against pH 7.4 phosphate buffered saline at 4 °C. Sterile filtration through a Millex-GV 0.22-μm filter provided 390 mg of KS1/4S2-4-desacetylvinblastine-3-carbohydrazide conjugate **9** at 10 mg/mL with a conjugation ratio of 5.1 as determined by dual wavelength UV analysis (see Determination of Conjugation Ratio below). Overall yield of **9**, based on protein recovery (380 mg), was 81% from KS1/4S2. Analytical size exclusion chromatography (Superose 12 gel, buffer) indicated an aggregate content of 10–15%. The final product was stored at 4 °C.

MoAb–4-Desacetylvinblastine-3-carbohydrazide Conjugates 2. Method B. KS1/4–DAVLB hydrazide (835 mL) crude conjugation reaction mixture, prepared as in method A, was charged onto a 14 × 41 cm column of Sephadex G-25M at a flow rate of 50 mL/min. The column had been previously equilibrated in, and elution was effected with, 50 mM triethanoamine buffer at pH 8, containing 10% acetonitrile. Elution was monitored at 280 nm. The solvent-exchanged conjugate, now separated from unreacted DAVLB hydrazide (**6**), was loaded onto a 4.4 × 36 cm column of Q-Sepharose Fast Fow, previously equilibrated in 50 mM triethanolamine buffer, pH 8. Flow rate was maintained at 5 mL/min. Following sample application, the column was washed with 200 mL of the equilibration buffer containing 90 mM sodium chloride. The column was eluted with a 6000-mL linear gradient of sodium chloride, from 90 mM to 240 mM, in the triethanolamine buffer. Elution was monitored at 280 nm. Fractions were collected during gradient elution of the column and monitored for protein aggregate content by analytical size-exclusion chromatography on an HR 10/30 Superose 12 column (100 mM sodium phosphate buffer, pH 7, containing 15% acetonitrile). Fractions with an aggregate content of less than 12%, eluting between 130 mM and 150 mM sodium chloride in the gradient, were pooled, concentrated 6-fold, and dialyzed into phosphate buffered saline, pH 7.4. Final product exhibited a conjugation ratio of 5.3, a concentration of 6.7 mg/mL, and an aggregate content of 6%. Yield (from KS1/4S2) = 42%; free DAVLB hydrazide (**6**) < 2% by reverse-phase HPLC.

Determination of Conjugation Ratio. MoAb–DAVLB hydrazide conjugates **2** at 0.5 mg/mL in phosphate buffer was analyzed by UV at 280 and 270 nm. Conjugation ratio was determined by measuring the total absorbance at these two wavelengths for the conjugate sample and then solving two simultaneous Beer's law equations for the vinca and antibody concentrations in the sample by using the ϵ values mentioned above for the antibody and $\epsilon_{280} = 1.08 \times 10^4$ M^{-1}·cm and $\epsilon_{270} = 1.23 \times 10^4$ M^{-1}·cm for the vinca in pH 7.4 phosphate buffered saline. The conjugation ratio is defined as the quotient of the vinca concentration divided by the MoAb concentration and is in units of mmol DAVLB hydrazide (**6**)/mmol MoAb.

Reverse-Phase Analytical HPLC Technique. A 1.0 mg/mL solution (0.5 mL) of MoAb–DAVLB hydrazide conjugate **2** in 0.1 M phosphate buffered saline, pH 7.4, was injected onto an IBM C-8 reverse-phase HPLC column and eluted with a mobile phase of 62:38 MeOH/pH 7.4 0.1 M aqueous phosphoric acid 1.5% in diethylamine at a flow rate of 1.0 mL/min. Elution was monitored by UV at 270 nm. In this system, free DAVLB hydrazide (**6**) eluted as a moderately broad peak at ~9.0 min, as confirmed by a reference sample. The amount of free **6** in the conjugate test sample was calculated from the HPLC peak area.

DAVLB Hydrazide (6) Release Studies. Solutions of ion exchange purified conjugate **9** (method B) at a final concentration of 3.0 mg/mL in pH 7.4 phosphate buffered saline were prepared. For studies to be performed under acidic conditions, the solutions were further acidified by dropwise addition of 20% (v/v) aqueous acetic acid in the cold. The solutions were then stored at 37 °C (water bath) or 4 °C (refrigerator). At the time points indicated in Figure 4, aliquots were removed, diluted to 1.0 mg/mL, and analyzed for unconjugated **6** by reverse-phase HPLC as described above.

In Vivo Tumor Inhibition Studies: A. Tumor Initiation Model.[7] All conjugates were analyzed by reverse-phase HPLC prior to testing (see above). Conjugate samples containing >5% free DAVLB hydrazide (**6**) were first redialized against phosphate-buffered saline. Unconjugated DAVLB hydrazide (**6**) was administered as its sulfate salt in phosphate-buffered saline. Outbred nude mice (Charles River Breeding Laboratories, Boston, MA) approximately 2-months old were injected subcutaneously in the flank with 1×10^7 tumor cells in 0.2 mL of sterile phosphate buffered saline on day 0. For P3UCLA, HT29, LS174, and M14 xenografts, the animals were dosed by iv injection in the tail vein with either conjugate, free DAVLB hydrazide, or diluent on days 2, 5, and 8. Tumor measurement was on day 28. For the T222 xenograft system, the animals received 350R γ-irradiation 24 h prior to tumor innoculatión on day 0. They were then dosed on days 3, 6, and 9, with tumor measurement on day 28. In all cases, palpable tumor masses were present prior to dosing. Tumor masses were estimated from tumor volume measurements made with electronic digital calipers interfaced with a computer utilizing the formula mass = [(length) × (width^2)/2].[7] The percent inhibition of tumor growth by conjugate or free DAVLB hydrazide was calculated relative to control animals treated with phosphate-buffered saline diluent alone by taking the ratio of the average tumor mass of the treatment group (N = 5) to that of the control group (N = 10).

B. KS1/4S2-DAVLB Hydrazide (9) vs DAVLB Hydrazide (6) against P3UCLA Xenografts. Established Tumor Model.[7] Nude mice were subcutaneously implanted with tumor cells on day 0 as above in A. Conjugate **9**, free DAVLB hydrazide (**6**) sulfate salt (treatment group, N = 5), or phosphate-buffered saline diluent (control animals, N = 10) were administered once weekly iv for 3 weeks beginning on day 16. Tumor masses were determined as in A once weekly beginning on day 16. Standard

error of the means are reported where appropriate.

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Structure–Activity Studies of Trichothecenes: Cytotoxicity of Analogues and Reaction Products Derived from T-2 Toxin and Neosolaniol

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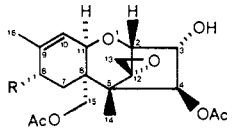
Forty-two analogues and reaction products derived from T-2 toxin or neosolaniol were assayed for their cytotoxicity to cultured mouse lymphoma cells. Structure–activity relationships confirmed the stereospecific nature of the cytotoxic action of T-2. Cytotoxicity was particularly susceptible to changes at C3, C4, C9, and C10 but was relatively unaffected by changes at C8, which appears to represent a region of steric tolerance in the interaction of T-2 with a cellular constituent. The most potent compounds were T-2, diacetoxyscirpenol, and a series of C8 ester analogues 11 and 31–35.

Trichothecene mycotoxins are toxic secondary metabolites produced by various species of fungi such as *Fusarium*, *Trichoderma*, *Trichothecium*, *Myrothecium*, *Stachybotrys*, *Cephalosporium*, and *Verticimonosporium*.[1] Chemically they are sesquiterpenoids, usually containing a relatively unreactive epoxide moiety. Trichothecenes, particularly those produced by species of *Fusarium* such as deoxynivalenol and T-2 toxin (1), have been implicated as the causative agents of various mycotoxicoses occurring in farm animals and man.[2] At the cellular level trichothecenes are potent inhibitors of protein synthesis in eukaryotic cells,[3] and one of the more potent naturally occurring metabolites, diacetoxyscirpenol (2) (DAS, anguidine), has undergone clinical trials as an anticancer agent.[4]



1 R = $-OCCH_2CH(CH_3)_2$

2 R = H

Structure–activity relationships of trichothecenes are therefore of interest with regard to the possible development of novel anticancer agents and to the etiology of mycotoxicoses occurring in animals and man. The first major structure–activity study was reported by Grove and Mortimer[5] following the isolation and characterization of diacetoxyscirpenol. Eighteen analogues and rearrangement products derived from diacetoxyscirpenol and nivalenol were assayed for their cytotoxicity toward HeLa cells. The main conclusion was that destruction of the epoxide moiety and rearrangement of the ring system virtually eliminated cytotoxicity. Further structure–activity studies, predominantly using trichothecenes isolated from laboratory cultures, have been reported from several laboratories.[6-11] More recently approximately 60 synthetic analogues derived from diacetoxyscirpenol were evaluated for their antitumor properties in mice.[12] Jarvis and co-workers have reported extensive investigations of the more complex macrocyclic trichothecenes.[13] All of these studies indicate a stereospecific interaction of trichothecenes with a receptor, presumed to be located on the ribosome. In this present paper we report additional structure–activity studies of synthetic trichothecenes, derived by modification of one of the most potent of the naturally occurring trichothecenes, T-2 toxin, or from its partial hydrolysis product neosolaniol (3). Some of the compounds reported herein were synthesized systematically as part of the structure–activity study; others were products of chemical investigations of T-2 toxin.

Chemistry

The natural metabolites T-2 toxin and neosolaniol were used as starting materials for this work. Analogues modified at C3, C4, or C15 are shown in Table I, those modified at C8 are shown in Table II, and those modified at C9–C10 are shown in Table III. New derivatives were characterized principally by NMR and mass spectroscopy. NMR as-

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