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

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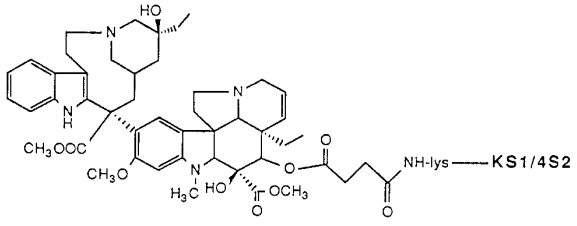
A method has been developed to allow the direct coupling of the cytotoxic vinca alkaloid 4-desacetylvinblastine-3-carbohydrazide (DAVLB hydrazide) to a variety of murine monoclonal antibodies directed against human solid tumors. Periodate oxidation of carbohydrate residues on the antibodies, followed 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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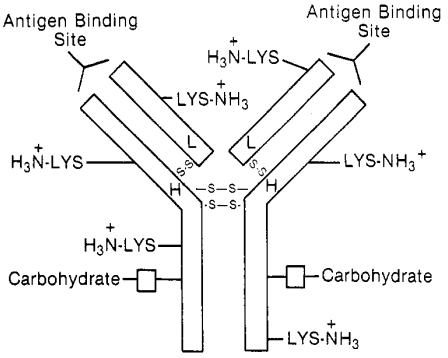
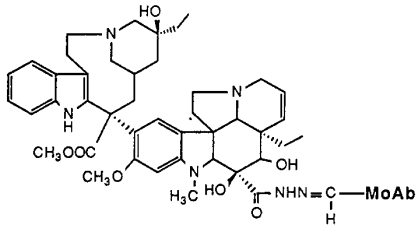


Figure 1. 2-D representation of an immunoglobulin G (IgG) molecule.

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

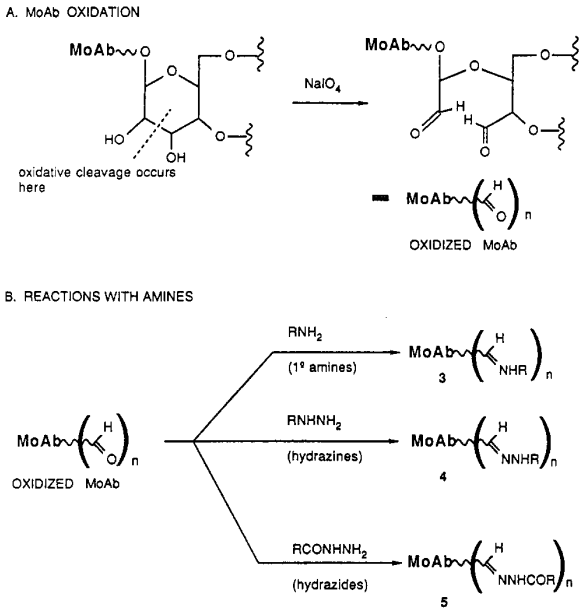


Figure 2. Periodate oxidation of MoAbs and coupling with amines.

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)

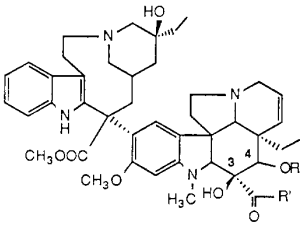
[a] Reported yields are overall protein yields after oxidation and conjugation, using method A as reported in the Experimental Section. [b] Conjugation ratio, mmol **6**/mmol MoAb; results averaged over several runs. [c] The degree of binding of conjugates to antigen-positive tumor cell lines (see Table 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

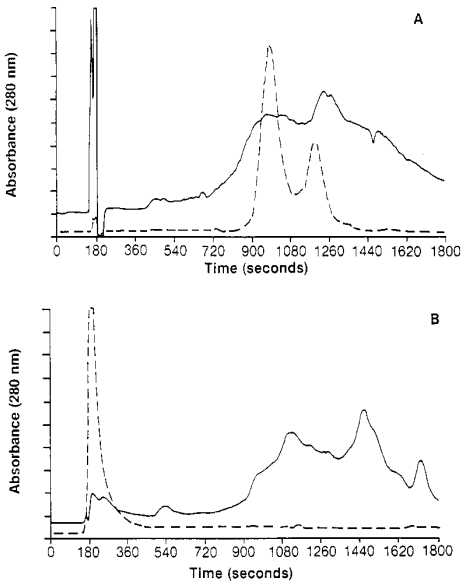


Figure 3. (a) HIC profiles of KS1/4S2–DAVLB hydrazide (**9**) vs KS1/S2 MoAb. TSK-phenyl column; 0.1 M PO_4 buffer, pH 6.5, 2–0 M NaCl gradient. Sample elution was monitored by UV at 280 nm. (—) KS1/4S2–DAVLB hydrazide (**9**); (---) KS1/4S2. Conjugate **9** was prepared by method A (Experimental Section).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

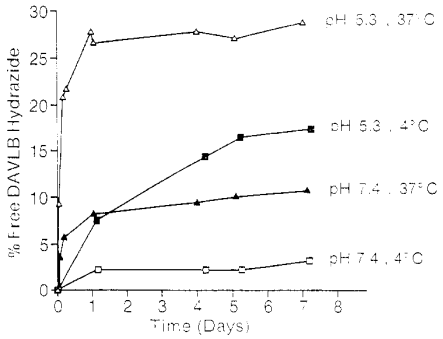


Figure 4. Release of DAVLB hydrazide (6) from KS1/4S2–DAVLB hydrazide (9). Conjugate was prepared by method B (see Experimental Section); CR = 5.3. % Free DAVLB hydrazide (6) is that % of the total amount of 6 in the conjugate sample (protein-bound + unbound) that elutes as unbound 6 on RPHPLC. This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

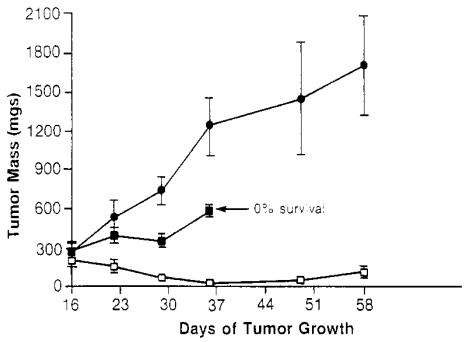


Figure 5. Effect of KS1/4S2–DAVLB hydrazide (9) vs unconjugated DAVLB hydrazide (6) on growth of established P3UCLA lung adenocarcinoma xenografts in nude mice. (—□—) 9 at 2 mg/kg (vinca content); (—■—) 6 at 2 mg/kg; (—●—) control. Each point is the mean ± the S.E. of N = 5 mice (treatment group) or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

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