TEACHING EDITORIAL

Recent Advances with Monoclonal Antibody Drug Targeting for the Treatment of Human Cancer[1]

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I. INTRODUCTION

Drug targeting had its inception almost a century ago when the late Paul Ehrlich proposed that chemotherapeutic agents might be covalently joined to ligand substrates which had affinity for and selectivity to a target tissue such as malignant tumors. In addition, he suggested that antibodies or "magic bullets" as he described them might, in fact, be candidates for ligand substrates for drug targeting (*2*). This vision remained dormant for almost a century until the attendant technologies and support systems would be in place to begin to express this vision into a 20th century therapeutic modality.

During the past 15 years, there has been an exponential growth in the area of drug targeting as a result of the integration, interfacing, and coordination of the scientific disciplines represented by cell biology, recombinant technology, and chemistry (Figure 1). Within the area of cell biology, major advances have occurred in genetics, hybridoma technology, screening, and testing. In 1980, Benacerraf, Dausset, and Snell received the Nobel Prize in Medicine and Physiology for their pioneering efforts in elucidating the immune response gene network, that is the family of genes that dictate the ability of the mammalian immune system to respond to and process all immunogens (*3*). In the course of these important discoveries, the mouse became the representative immune system because of its prolific procreative behavior, physical size, and its brief gestation period. As a result, Snell had created the world's most sophisticated genetic library of inbred and recombinant strains of mice (*4*).

This family of mice became the instrument of knowledge that was utilized by Kohler and Milstein in their discovery of hybridoma technology. The discovery was of such magnitude that Kohler and Milstein, along with Jerne, were awarded the Nobel Prize in Medicine and Physiology in 1984, 9 years after their breakthrough (*5*, *6*). Hybridoma technology allows the fusion of a normal immunological B cell committed to making antibody with a malignant myeloma partner, thus affording a hybrid cell with the genetic information of both immortality and antibody synthesis. Each cell is thus empowered to produce unlimited amounts of a single, or monoclonal, antibody (moab) (*7*). This discovery together with automated methods of screening and testing for the selective immunoreactivity of derived moab's accelerated the growth of the drug-targeting discipline.

Recombinant DNA technology has complemented and facilitated the growth of cell biology. The ability to rapidly identify, sequence, and clone genes of an antibody has led to the elucidation of the underlying mechanisms of antibody diversity (*8*). In addition, this powerful tool has allowed the construction of chimeric and humanized monoclonal antibodies which may reduce their immunogenicity in humans, either as native moab's or in the context of drug conjugates (*9*).

Finally, chemistry has catalyzed the growth rate of both cell and molecular biology. Through chemistry, the ability to rapidly synthesize DNA, peptides, linkers, and pharmacologic agents and to characterize and purify bioconjugates has been achieved. What has become apparent is the importance of chemistry in the design and synthesis of bioconjugates; indeed, the rate-determining step for the evolution of this program has been the "new chemistry" of drugs and linkages compatible with proteins and the incorporation of the drug/linkage onto the moab.

In summary, the three disciplines interface in creating a new dimension in the expression of biotechnology that has facilitated the emergence of monoclonal antibody drug targeting in the treatment of human cancer.

Conceptually, the process of drug targeting as proposed by Paul Ehrlich is illustrated in Figure 2. Since Ehrlich's vision of targeting, many investigators in the biological fields have tried to translate his dream into a reality. In order to build a foundation of understanding for moab-based targeting of drugs, this article will review the following in turn: (1) the immune system and antibody synthesis, (2) hybridoma technology and the generation of monoclonal antibodies, (3) chemical design and synthesis of modified targeting agents for attachment to moab's, and (4) the chemistry and biological activity of moab–drug conjugates.

II. THE IMMUNE SYSTEM AND ANTIBODY SYNTHESIS

The cast of characters and the sequence of events that facilitate the activation of the immune response are illustrated in Figure 3. The macrophage or presenting cell takes up the antigen and presents it on its surface in the context of class II immune response gene products (*10*). This, in turn, determines the ability of the immune system to respond to the given antigen. Those clones of T cells (thymus-derived cells) designated as helpers (T_h) which express receptors for the antigen in the context of the class II self-determinant become activated through the synthesis and secretion of IL-1 (interleukin 1) (*10*). This cytokine, in turn, activates those clones of T_h cells to produce and express receptors for IL-2 (interleukin 2), a T cell growth factor which supports the growth of the autocrine T cell network. This highly sophisticated central pathway facilitates and supports the growth of both the humoral response represented by B cells (bone marrow derived cells committed to producing antibody) and the cell-mediated response represented by T cell mediated delayed type hypersensitivity (T_{DTH}) (*10*). The central pathway sustains the cell-mediated response by producing factors which support the growth and differentiation of the T cell mediating DTH, thus moving the process to the end stage effector function. Similarly, the central pathway activates the B cell response via the production

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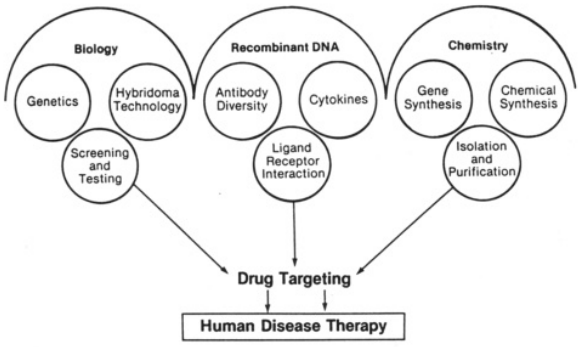


Figure 1.

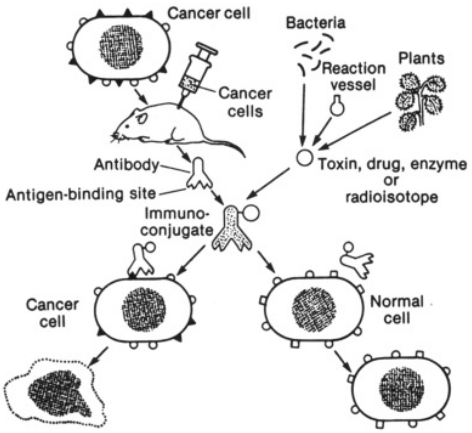


Figure 2. Immunoconjugate-mediated site-directed therapy. Reproduced with the permission of R. John Collier and Donald A. Kaplan [(1984) *Sci. Am.* **251** (1), 56].

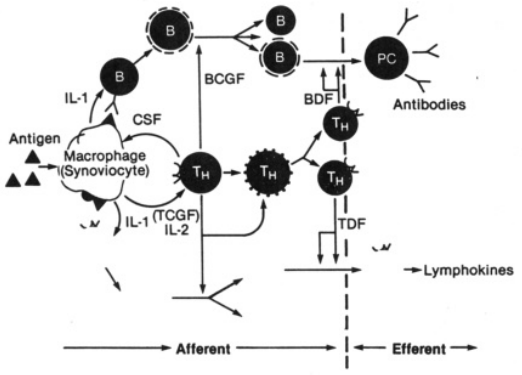


Figure 3.

of growth and differentiating factors. As a result, the B cell recognizing the antigenic epitope matures to the end stage effector cell, the plasma B cell (PC), which secretes the antibody specific for the antigen at the incipient stage (*10*).

In addition to the utility of antibody in maintaining the survival of the organism, the antibody was recognized as an attractive candidate for ligand targeting. If one had the capability to intercept a specific plasma B cell clone and produce unlimited sources of moab, the moab's potential for recognizing and binding, selectively, to a given epitope, would make it the "universal ligand" in targeting. As a result of the Kohler/Milstein hybridoma breakthrough, the ability to select for and produce unlimited quantities of moab became a reality and thus fueled the research in the targeting program (*5*, *7*).

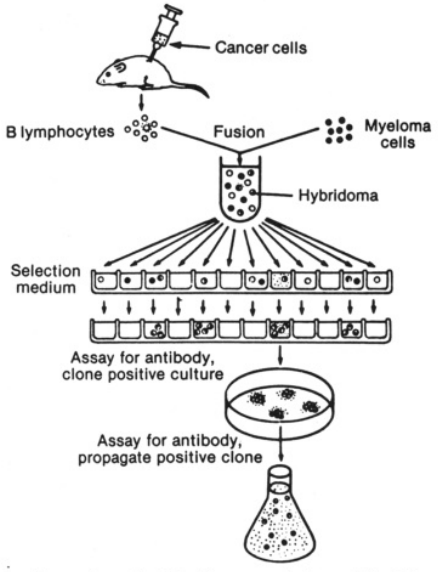


Figure 4. Reproduced with the permission of R. John Collier and Donald A. Kaplan [(1984) *Sci. Am.* **251** (1), 56].

III. HYBRIDOMA TECHNOLOGY AND THE GENERATION OF MONOCLONAL ANTIBODIES

The process of producing a monoclonal antibody is illustrated in Figure 4. The human cancer tissue is presented as an antigen to the mammalian immune system. The mouse is the system of choice because hybridoma technology was developed within the context of mouse genetics (*7*). The immune system processes the cancer tissue and begins to make antibody as has been described. Monitoring to determine the reactivity against the cancer tissue is done by assaying serum against original cancer tissue (*7*). Once the animal is making polyclonal antibody against the target, plasma B cells are obtained by excising the spleen, harvesting the B cells, and fusing them with malignant myeloma cells (*5*, *7*). The cells are then propagated in a medium in which only fused hybridoma cells can survive (e.g., a medium such as hypoxanthine/aminopterin/thymidine, which selects only for a survival pathway of fused hybridoma cells). The moab's derived from the surviving hybridoma cells are screened in a high-speed automated selection process against malignant and normal tissue. Those moab's that have good immunoreactivity against malignant tissue and minimal reactivity against normal tissue are selected and further evaluated as targeting ligands in the context of drug conjugates (*5*, *7*).

Pursuant to understanding the chemistry of designing and developing conjugates, it is important to review the structure and the attendant biochemical characteristics of the antibody. A representation of an IgG class of antibody is illustrated in Figure 5 (*11*). The antibody is composed of identical heavy chains denoted by the subscript H which are joined by two disulfide linkages located in the "hinge region" of the antibody. Two identical light chains denoted by the subscript L are joined to the heavy chains by disulphide bonds connecting the constant portion of the light chain (C_L) to the heavy chain first constant region (C_H1). The amino terminus of each chain is located at the variable portions of both the heavy and light chains, V_H and V_L, respectively. The C terminus of the light chain is located at the C_L domain and that of the heavy chain is at the C_H3 domain (*11*). The immunoreactivity of the moab is controlled by the variable

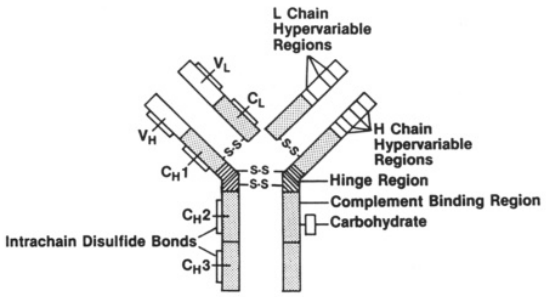


Figure 5.

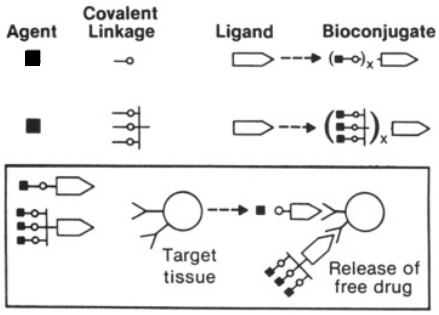


Figure 6. Design and synthesis of agent–linkage–ligand conjugate: (1) Agent–linkage–ligand construction expresses inherent biological activity or facilitates its release at the target site. (2) Agent and/or linkage amenable to stoichiometric determination within the context of the bioconjugate.

domains and is comprised of three peptide sequences in the hypervariable region of the light chain and four sequences in the heavy chain (*11*). The species characteristics of the moab are expressed both in the framework region of the variable domain as well as specific sequences of the C_H and C_L regions. In addition, classes of moab's such as IgM and IgE differ from IgA, IgD, and IgG by the addition of C_H4 domain at the C terminus (*12*). Finally, within species, classes and subclasses are characterized by subtle differences in C region sequences. The complement binding receptor and an N-glycosylated carbohydrate is located within the C_H2 domain. This fortuitous location of the sugar substrate provides a unique functionality for regioselectively incorporating drugs outside of the antigen-binding region. The importance of this linkage will be discussed later.

IV. THE CHEMICAL DESIGN AND SYNTHESIS OF MODIFIED TARGETING AGENTS FOR ATTACHMENT TO MOAB'S

In designing the bioconjugate, it is important to distinguish the integrity of the three components of a bioconjugate: the agent to be delivered, the covalent linkage, and the moab. The linkage must not diminish the biological activity of the modified agent nor compromise the moab's ability to target. Two general strategies for conjugating an agent to a moab are represented in Figure 6. In the first strategy, the modified agent is reacted directly with functional groups on the surface of the moab. In this process, the loading of the drug would be determined by the number of available attachment sites on the moab. Alternatively, one can engraft the agent onto a matrix substrate and then react this unit with the moab. In the second process, the amount of agent delivered can be increased without having to increase the number of covalent bonds to the moab (*13*). Regardless of which strategy is chosen, the chemistry of bioconjugate construction must be guided by the following tenets: (1) the attachment of agent to moab must be achieved without altering its immunoreactivity, (2) the conjugate must be constructed with a chemical linkage that will either allow the retention of the biologic properties of the agent or facilitate the release of the free drug at the target site, (3) the design of the agent/linkage chemistry should be compatible with covalent attachment to either the intact moab or its fragments, (4) the synthesis of the conjugate should be done in a manner that would minimize immunogenicity, and (5) the construction of the conjugate must be amenable to large-scale production (Figure 7).

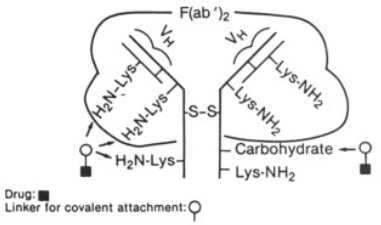


Figure 7. Moab sites of agent attachment and chemical methods for moab agent modification. Goals: (1) attach agent to moab without altering serological activity, (2) construct conjugate with retention of biological properties of agent, (3) construct conjugate with linkage that facilitates release of free drug at target site, (4) design of agent/linkage compatible with covalent attachment to $F(ab')_2$ fragment, (5) construct conjugate with minimal immunogenicity, and (6) synthesis of conjugate amenable to large-scale production.

The moab is attractive as a targeting ligand because (1) it has many potential sites of drug attachment and (2) it can maintain immunoreactivity even as subfragments. Represented in Figure 7 are the potential sites of covalent attachment on the intact moab and the proteolytic fragment, $F(ab')_2$ (*14*). The most accessible sites for drug attachment on the polypeptide chains are the ϵ amino groups of the lysine residues (approximately 90 lysines in a moab) and the carbohydrate moiety of the C_H2 domain (*15*). One can imagine oxidizing the carbohydrate to generate aldehyde functions from the *vic*-diols, which can react with various drug functionality such as hydrazides (*16*, *17*). Drugs can be incorporated at the lysine residue through the construction of stable amide linkages. In addition, the lysine residues are important sites for drug attachment in the $F(ab')_2$ fragments since these have no carbohydrate.

Having reviewed the required characteristics of the bioconjugate and the sites of covalent attachment to the moab, it is appropriate to identify the representative chemical linkages that have been utilized in drug targeting. The succinate linkage has been employed in joining desacetylvinblastine to the moab via an amide bond to the lysine amine (*18*, *19*). This, of course, discourages release of the free drug from the conjugate (see Figure 8, entry 1).

The sulfhydryl-bearing A chains of the toxins ricin, diphtheria toxin, and abrin have been joined to the moab via disulfide linkages. For example, the moab has been reacted with the *N*-hydroxysuccinimide ester of 4-(2-pyridyldithio)butyric acid to introduce several latent thiols onto moab lysines. This, in turn, is reacted with the sulfhydryl-containing toxin to yield the moab conjugate as the disulfide. The lability of the disulfide bond and its resultant short half-life has encouraged the construction of hindered disulfides such as the α-methyl butyrate (see Figure 8, entry 2, R = CH_3). This minor change in the linkage enhanced the circulation half-life of the correspondingly linked moab–ricin A significantly (*20*, *21*).

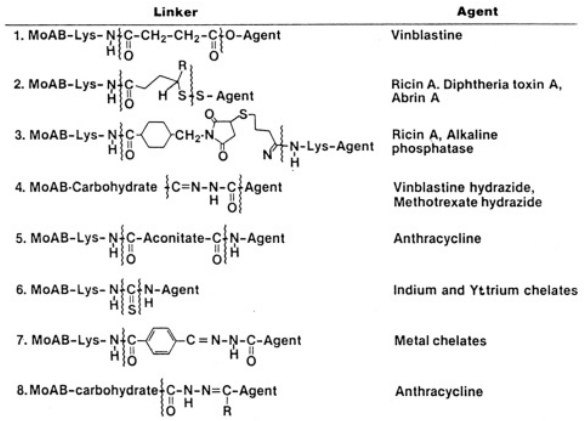


Figure 8. Chemical linkages for covalent attachment of agent to ligand.

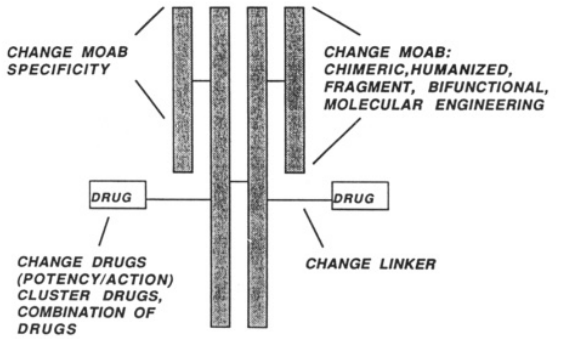


Figure 9. Structure–activity relationship: modification for moab–drug conjugates.

An even more stable sulfhydryl-based moab/agent linkage has been achieved through formation of a thio–ether bond. The substrates ricin and alkaline phosphatase have been linked to moab in the following two-step procedure: (1) the lysine residue of either the substrate or the moab has been reacted with thiolane hydrochloride to give the 4-sulfhydrylbutyrimidate derivative and (2) sulfhydrylbutyrimidate has been joined to the complementary protein that bears a 4-(methylenemaleimido)cyclohexylcarboxamide via a conjugate addition of sulfur to maleimide (see Figure 8, entry 3) (*22*). The application of this linkage in the targeting of alkaline phosphatase for prodrug activation will be described in a later discussion (*22*).

Investigators at Cytogen and Lilly Research Laboratories have reported the oxidation of moab–carbohydrate and use of the resultant aldehydes for linkage of drug hydrazides (*16*, *17*). Cytogen scientists have prepared the methotrexate hydrazone conjugate and Lilly Research investigators have synthesized the vinblastine hydrazone conjugate (see Figure 8, entry 4). The vinblastine hydrazone–moab conjugate will be described later in the review as an example of designing and developing conjugates in a structure–activity relationship based on human clinical feedback.

The anthracyclines, exemplified by adriamycin and daunomycin, are a family of oncolytics that have been very challenging as candidates for conjugation. The need for the release of the free drug in order to express its DNA-binding activity has required the development of labile linkages compatible with the functionality of the anthracyclines. Reisfeld and others have constructed the acid-labile aconitate amide linkage through the lysine amine of the moab and the amine group of the glycoside of the anthracycline (see Figure 8, entry 5) (*23*).

The importance of chemical linkers is most effectively highlighted in the targeting of radionuclides for both imaging and therapy. Historically, ^{125}I and ^{131}I have been incorporated onto moab by iodination of the tyrosine residues (*24*). As imaging and therapy conjugates, the clinical data thus far have not been encouraging because of the rapid dehydrohalogenation of iodine. More recently, Meares has helped pioneer the construction of indium and yttrium conjugates through the development of nuclide chelate linkages (*25*). Investigators from Hybritech have utilized this technology in advancing the use of indium in imaging of solid tumors and then using the matched yttrium conjugate for therapy (see Figure 8, entry 6) (*26*). Most recently, investigators at NeoRx have reported a radionuclide matched pair of technitium and rhenium for imaging and therapy, respectively. Thus, dosimetry data collected from the indium or technitium moab conjugate has allowed the therapy with the corresponding yttrium or rhenium moab conjugate. This has resulted in the first clinical response of a human solid tumor with a rhenium conjugate (*27*).

Offord and Rose at the University of Geneva as well as King at Rockefeller have reported the use of carboxybenzaldehyde as a linker for incorporating a ketone or aldehyde functionality onto the lysine amine of the protein (see Figure 8, entry 7) (*28*, *29*). Offord and Rose have reported the construction of hydrazone conjugates via this linkage (*29*).

A complementary approach for modifying the carbohydrate aldehyde linkage has been one in which a hydrazide has been incorporated onto the moab for reaction with drugs containing carbonyl functions. Investigators at Cytogen have built in adipic dihydrazide linkage to join the oxidized carbohydrate of the moab to the ketone of an anthracycline via hydrazone linkages (*30*). Barton et al. at Lilly have utilized a reductive amination of the moab carbohydrate aldehyde with glutamic hydrazide and subsequently constructed a releasable attachment to an anthracycline via its ketone (see Figure 8, entry 8) (*31*).

As one begins to correlate the biological with the chemical components of a moab drug conjugate, it is clear that developing a medicinal chemistry structure–activity relationship (SAR) becomes a multidimensional challenge (illustrated in Figure 9) (*32*). As has been emphasized in earlier portions of this review, there are a variety of opportunities for independent structural modifications of the conjugate that are expressed in the framework of the moab, the linker, and the drug.

The quality of targeting of the conjugate to selective tissue can be achieved by changing either the specificity of the moab or simply its affinity (i.e., its on/off rate). If a human antibody response to the murine moab drug conjugate becomes a problem, the constant regions and the framework portion of the variable region can be replaced by human sequences through molecular engineering (*8*). In addition, if the intact moab with its attendant effector function domains such as complement binding present an innocent tissue bystander liability, fragments of the moab such as $F(ab')_2$ that no longer carry these domains can be synthesized and conjugated with drug (see Figure 9) (*14*).

The chemical linker is the heart of the conjugate. It determines the ability of the drug to express its activity either as an integral part of the conjugate or allows its release at a rate that is dictated by the chemistry. The

choice of a releasable or nonreleasable linkage as it impacts on the drug toxicity will be discussed specifically with respect to the moab–vinca conjugate in the next portion of this review.

V. THE CHEMISTRY AND BIOLOGICAL ACTIVITY OF MOAB–DRUG CONJUGATES

The oncolytic drug can be selected on the basis of a combination of its clinical effectiveness, its mode of action, and its potency against the tumor target. It is most important to understand that the parameters of biodistribution and local drug concentration may dramatically change a drug's profile as a conjugate compared to its unmodified form. As discussed before, the SAR that unfolds for a targeted drug is one that is multidimensional and dependent on a variety of structural changes, i.e., moab, linker, and drug. The ability to enhance the quality of the conjugate depends on the transmission of information from the clinic to the laboratory and applying this information to the preclinical model (see Figure 9) (*32*).

As an example of the design, the development, and the evaluation of a drug conjugate, I would like to describe a program that I have participated in, one that is generically representative of the drug-targeting efforts occurring at the many academic and industrial institutions throughout the world today. In this example, the importance of human clinical feedback is emphasized for adjusting the SAR and enhancing the efficacy of the drug conjugate.

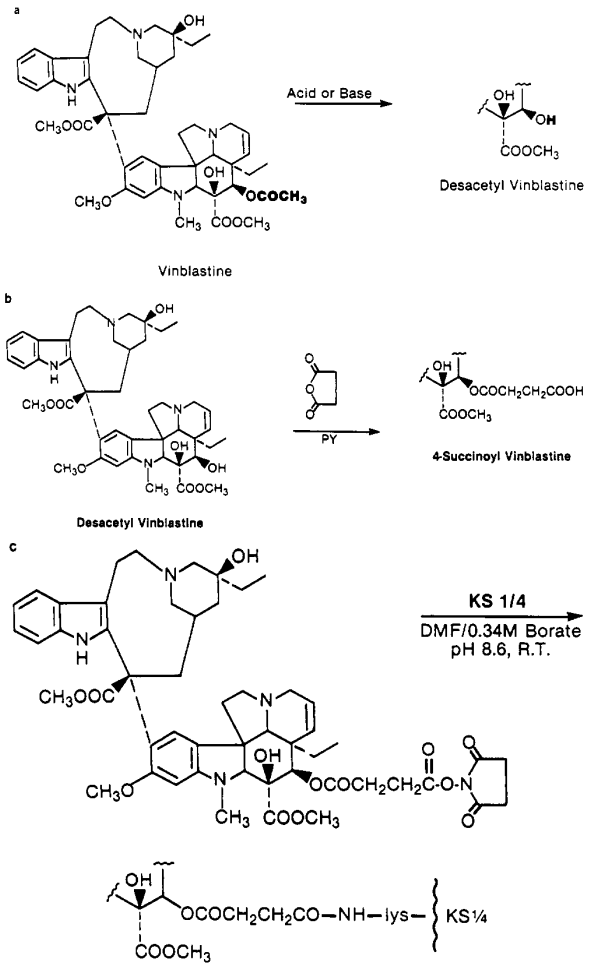
Our investigators at Lilly chose a vinca alkaloid for the designing of a moab–drug conjugate, partly because of our vast prior experience in chemical modification of the vincas and partly because of its biological potency for treatment of human cancer (*33*). The vinca substrate for conjugation was prepared by the reaction of vinblastine with acid or base effecting the deesterification of the 4-position of the vindoline component. This, in turn, was reacted with succinic anhydride to afford the 4-succinate of desacetylvinblastine (DAVLB), a substrate bearing the nonreleasing linker, succinate (see Scheme I, parts a and b) (*18*, *19*). The choice of the succinate linker was made, initially, in order to evaluate the inherent activity of the vinca in the context of the conjugate, and to minimize the liability of free vinca.

The moab selected for targeting was identified as KS1/4, a murine moab developed by Walker in Reisfeld's laboratory at Scripps (*34*). The moab KS1/4 recognizes a tumor/epithelial associated antigen (40 KD) found in high epitope density on human adenocarcinomas (*35*). The primary target of this moab is lung and colorectal adenocarcinoma (*36*).

Pursuant to attaching the vinca to KS1/4, the DAVLB hemisuccinate was converted to its *N*-hydroxysuccinimide active ester. This, in turn, was reacted with KS1/4 in aqueous borate buffer in pH 8.6 at room temperature to afford, after a series of chromatographies, a 50% yield of the conjugate (see Scheme I, parts a–c) (*16*, *30*, *45*). Stoichiometric evaluation of the conjugate by ultraviolet spectrophotometry indicated a conjugation ratio of 4–6 drugs/moab (*36*). This chemical process and the corresponding biochemical purification proved to be reliable, reproducible, and amenable to large-scale production (*36*). The analytical profile that was developed for monitoring the quality of KS1/4–DAVLB and all our other drug conjugates is illustrated in Figure 10.

The difficulty with selecting a relevant in vivo animal model for evaluating the biological potential of a new moab–drug conjugate is that the targeting substrate recognizes "human epitopes". Consequently, a model had

Scheme I



to be created which would accommodate the pharmacology of the drug as part of a conjugate as well as the ability of the conjugate to target the human tumor tissue. The in vivo experimental system which has been widely utilized in evaluating drug conjugates has been the "athymic nude mouse" xenograft model. The inbred athymic mouse does not express a thymus; consequently, it is immunologically impotent and unable to reject tissue grafts such as malignant tumors from another species (*38*). The in vivo nude mouse human xenograft models that have been selected for our moab–drug conjugate evaluations are described in Figure 11 (*32*).

The traditional path for tracking antitumor potency of standard oncolytics has been in an in vitro potency assay measuring the ability of the agent to inhibit tumor growth. With respect to the drug conjugate KS1/4–DAVLB, which was constructed with a nonreleasing linker to minimize free drug, the in vitro assay measured the potency of the vinca in the context of the conjugate. Predictably, the in vitro assay showed the conjugate to be of a low potency, one whose IC_{50} was about 200-fold lower than that of vinblastine (Figure 12) (*18b*, *39*). Clearly, the utility of targeting a low-potency conjugate could only be evaluated in an in vivo system, one that would allow the dramatic change in biodistribution to be expressed in tumor inhibition or regression.

In an in vivo nude mouse tumor xenograft model, which measures the effect of an agent against a P3UCLA human

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