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Immunoconjugates Containing Novel Maytansinoids: Promising Anticancer Drugs

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ABSTRACT

The potential of immunoconjugates of cytotoxic drugs for the treatment of cancer has not yet been realized owing to the difficulty of delivering therapeutic concentrations of these drugs to the target cells. In an effort to overcome this problem we have synthesized maytansinoids that have 100- to 1000-fold higher cytotoxic potency than clinically used anticancer drugs. These maytansinoids are linked to antibodies via disulfide bonds, which ensures the release of fully active drug inside the cells. The conjugates show high antigen-specific cytotoxicity for cultured human cancer cells (50% inhibiting concentration, 10 to 40 pM), low systemic toxicity in mice, and good pharmacokinetic behavior.

INTRODUCTION

Numerous attempts to target tumors with conventional antineoplastic drugs conjugated to monoclonal antibodies have met with limited success. Most frequently, such antibody-drug conjugates are insufficiently cytotoxic, *e.g.*, the conjugates are even less potent than the nonconjugated drugs (1–3). In contrast, a number of antibody conjugates with single chain ribosome-inactivating protein toxins or with isolated A-chains of two chain ribosome-inactivating protein toxins, such as ricin, have been prepared (4, 5) that are at least 10^3-fold more cytotoxic than conjugates between antibodies and antineoplastic drugs (1–3). Conjugates between antibodies and protein toxins are also much more potent than the corresponding nonconjugated toxins.

One reason for the different efficacy of these two classes of immunoconjugates may lie in the different modes of action of their toxic moieties. Protein toxins act catalytically, and it has been demonstrated that a single toxin molecule in the cytoplasm of a cell can cause the death of that cell (6, 7). In contrast, anticancer drugs currently in clinical use, such as doxorubicin, methotrexate, and the *Vinca* alkaloids, act stoichiometrically, and relatively much higher intracellular concentrations are needed to achieve comparable cytotoxicities. Intracellular concentrations of drug necessary to kill the target cells are difficult to achieve with antibody-drug conjugates for the following reasons: (*a*) a majority of commonly used anticancer drugs are only moderately cytotoxic; (*b*) antigen targets are present on cell surfaces often in only limited numbers (8); (*c*) the internalization processes for antigen-antibody complexes are frequently inefficient; and (*d*) most linkers that have been used for the conjugation of drugs to antibodies (1–3, 9–14) do not efficiently release active drug inside the cell.

We describe here an approach that seeks to overcome these problems by replacing the current anticancer drug candidates with compounds of 100- to 1000-fold higher cytotoxicity, and by conjugating these compounds with antibodies via disulfide-containing linkers that can be cleaved inside the cell to release active drug.

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MATERIALS AND METHODS

Maytansine and Maytansinoids. Maytansine (1) is a product of Takeda Chemical Industries, Ltd. (Japan). Ansamitocin P-3, a precursor that was used to synthesize maytansinoid 2 (Fig. 2) was supplied by Takeda Chemical Industries of Japan. Synthesis of maytansinoid 2 was performed as follows. Maytansine or ansamitocin P-3 was reduced with lithium aluminum hydride to give the same C-3 alcohol maytansinol as previously described (15). Maytansinol was esterified with *N*-methyl-*N*-(methyldithiopropanoyl)-L-alanine in the presence of dicyclohexylcarbodiimide and zinc chloride to give maytansinoid 2, which was purified by column chromatography on silica gel eluting with 6% methanol in chloroform. This synthesis will be described in detail elsewhere.[2]

Antibodies. Murine monoclonal antibody A7 that binds to an antigen expressed on human colon cancer cell lines (16) was a generous gift of Dr. Takahashi (Kyoto University, Kyoto, Japan). The production of the murine monoclonal antibody TA.1 that binds to the HER-2/*neu* oncogene protein has been described elsewhere (17). Murine monoclonal antibodies 5E9 (anti-human transferrin receptor) and anti-B4 (anti-CD19) were purified by methods described previously (8, 18).

Conjugation of Maytansinoids with Antibodies. In order to generate antibody-maytansinoid conjugates, the antibody was modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate to introduce dithiopyridyl groups, or with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate to introduce maleimido groups. May-SS-Me 2 was reduced to May-SH *3* by the following procedure. A solution of 2 (2 mM) in a mixture of ethanol (0.3 ml) and 50 mM potassium phosphate buffer (0.2 ml; pH 7.5) was treated with dithiothreitol (3 mM) at 4°C for 4 h under nitrogen. The freshly prepared May-SH 3 was purified by high-pressure liquid chromatography and characterized by high-resolution nuclear magnetic resonance analysis. The product (1.2 equivalents per pyridyldithio group or maleimido group introduced into the antibody) was mixed with the modified antibody solution in 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. The course of the reaction was followed by monitoring the increase in absorbance at 343 nm due to release of thiopyridyl group. After completion of the reaction (in approximately 40 min) the conjugate was purified by gel filtration on a Sephadex G-25 column. Modification of the antibody to a different extent with *N*-succinimidyl-3-(2-pyridyldithio)propionate gave 1 to 6 dithiopyridyl groups per antibody molecule (as assayed by liberation of thiopyridyl group using dithiothreitol), or with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate gave 1 to 6 maleimido groups (as determined by reaction with [^{14}C]cysteine). Conjugates containing 1 to 6 maytansinoid molecules per antibody molecule were prepared by this method, as calculated from absorbances at 252 and 280 nm, using extinction coefficients of $E_{280\ nm}$ = 5700 M^{-1} cm^{-1} and $E_{252\ nm}$ = 28044 M^{-1} cm^{-1} for drug and $E_{280\ nm}$ = 224000 M^{-1} cm^{-1} and $E_{252\ nm}$ = 87360 M^{-1} cm^{-1} for antibody.

Cells and Cell Culture. Human cell lines SK-BR-3 (HTB 30), Namalwa (CRL 1432), KB (CCL 17), SW-620 (CCL 227), A-498 (HTB 44), HT-29 (HTB 38), NIH:Ovcar-3 (HTB 161), and LoVo (CCL 229) were purchased from the American Type Culture Collection (Rockville, MD).

Cytotoxicity Assays. The methods for the determination of the surviving fractions of adherent and nonadherent cell cultures (by colony-forming ability and by back-extrapolation of the exponential growth curves) have been described previously (19, 20).

Pharmacological Studies. Four mice were given injections i.v. of 2 mg/kg (by antibody) of the A7-SS-May conjugate. The volume of the

[2] Manuscript in preparation.

injection samples was 150 μl. Following the injections, 75-μl samples of blood were drawn from the retroorbital plexus of the animals at various time intervals, and the pooled serum from the four animals was diluted 1:4 in PBS[3], containing 0.5% bovine serum albumin. The resulting samples (100 μl) were incubated with 1×10^6 HT-29 cells for 30 min at 0°C, washed in AB buffer, and then incubated with either FITC-labeled goat anti-mouse IgG antiserum (Sigma Chemical Co., St. Louis, MO), diluted 1:50 (v/v) with 0.5% bovine serum albumin/PBS at 4°C for 30 min, for detection of the A7 moiety, or with 0.1 μM FITC-labeled murine monoclonal anti-maytansine antibody (purified, IgG1, developed at ImmunoGen), for detection of the maytansinoid moiety. The cells were then washed with AB buffer, fixed with 1% formaldehyde in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Standard curves for the quantitation of A7 and maytansinoid were generated with A7(-SS-May)$_6$ samples dissolved in a 1:4 (v/v) mixture of murine serum and AB buffer.

Competition Binding Assays. Competition of binding between a trace amount of FITC-labeled TA.1 antibody and various concentrations of unmodified TA.1 and TA.1-May conjugates to the *neu* antigen on SK-BR-3 cells was analyzed as follows. Cells (7×10^4) were incubated with mixtures of 6 nM FITC-labeled TA.1 and different concentrations of a conjugate for 30 min at 0°C in 25 μl of AB buffer, and then washed in ice-cold AB buffer. The mean fluorescence of the cells was then measured on a flow cytometer (using a linear scale), the background (cell autofluorescence) was subtracted, and the resulting value was then compared with a control (FITC-labeled nonconjugated antibody).

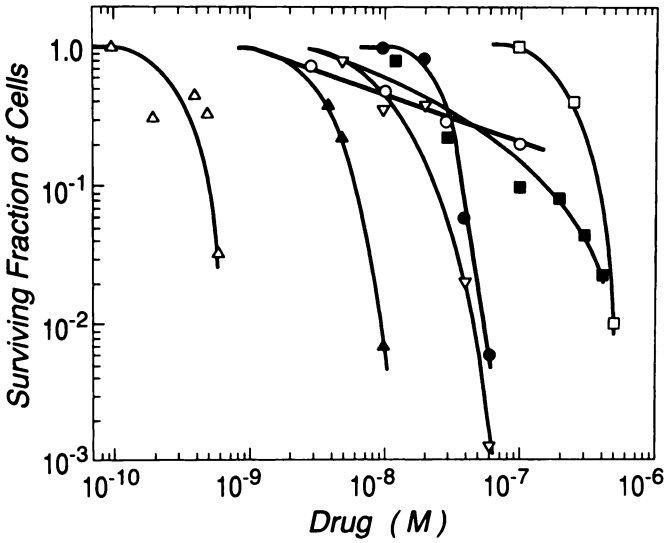

Fig. 1. Comparison of the cytotoxicity of various anticancer drugs for cultured human cells. The Burkitt's lymphoma cell line Namalwa was exposed to different drugs for 24 h at 37°C and the surviving fractions of cells were determined by a direct cytotoxicity assay. The symbols represent maytansine (△), actinomycin D (▲), colchicine (▽), daunomycin (●), vinblastine (○), methotrexate (■), and mitomycin C (□).

RESULTS AND DISCUSSION

We have chosen a highly cytotoxic drug, maytansine, for the preparation of immunoconjugates. Maytansine kills cells by interfering with the formation of microtubules and depolymerization of already formed microtubules (21). *In vitro* screening revealed that maytansine (1) (21, 22) is about 100- to 1000-fold more toxic for a range of human cancer cell lines than are most other anticancer drugs. For example, maytansine is highly cytotoxic for Namalwa cells (Fig. 1), with an IC_{50} of 4×10^{-10} M. Despite this high potency, maytansine was ineffective in human clinical trials (23) because of its high systemic toxicity, which resulted in a low therapeutic index. We reasoned that the nonspecific toxicity of maytansine could be lowered and the therapeutic index increased by the targeted delivery of this drug in conjugated form. In order to exploit the cytotoxic potential of maytansine in the conjugate, however, it is necessary to release the drug at the target cell in fully active form.

Two types of cleavable linkers have been widely used in the preparation of antibody-drug conjugates: acid-labile linkers (24–28) and peptidase-sensitive linkers (29). However, with few exceptions (30, 31), the cytotoxic potency of the resulting conjugates for cultured cell lines is very low, much lower than the cytotoxicity of the unconjugated drug, suggesting that internalization and release of drug molecules from these conjugates is inefficient. In addition, release into the medium of even a small fraction of a potent drug from a relatively nonpotent conjugate abolishes any hope of a specific cytotoxic effect. Internalization processes are poorly understood and difficult to influence, but the release of drugs may be improved by changing the design of the linker between the drug candidate and the antibody molecule.

On the basis of the promising results that have been obtained with antibody-toxin conjugates, where the conjugating linkage is a disulfide bond (5), we asked whether disulfide linkers would produce antibody-maytansinoid conjugates of comparable potency. Methotrexate linked to poly-D-lysine is the only reported drug conjugate where cleavage of a disulfide bond between the toxic and the targeting moiety releases the active drug, but this is only moderately cytotoxic (32). To obtain a highly cytotoxic drug that has a thiol "handle," we have synthesized a new maytansinoid, 2 (Fig. 2). This novel compound 2 contains a methyl disulfide group and is 3- to 10-fold more cytotoxic than native maytansine (Table 1). The corresponding thiol-containing derivative 3 is as cytotoxic ($IC_{50} = 1 \times 10^{-11}$ M for SK-BR-3 cells after a 24-h exposure) as the disulfide-containing derivative 2.

We therefore prepared antibody conjugates of the maytansi-

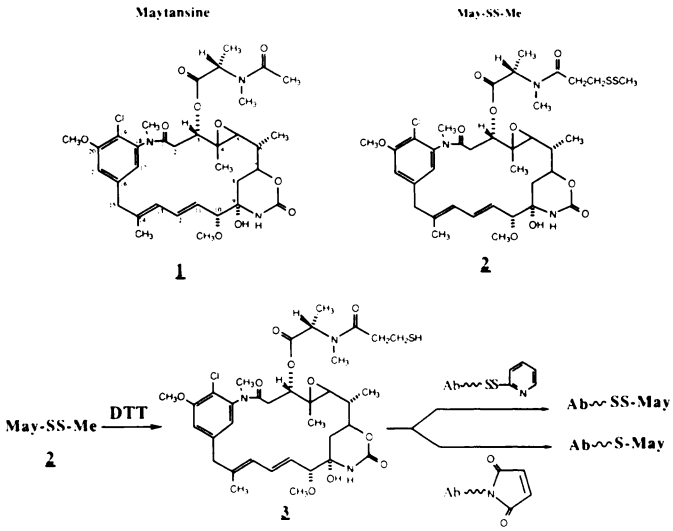

Fig. 2. Maytansinoids and their conjugation to antibodies. In order to generate antibody-drug conjugates the antibody was modified with SPDP [*N*-succinimidyl-3-(2-pyridyldithio)-propionate] to introduce dithio-pyridyl groups, or with SMCC [succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate] to introduce maleimido groups. May-SS-Me 2 was reduced to May-SH 3 (see "Materials and Methods") and reacted with the modified antibodies.

[3] The abbreviations used are: PBS, phosphate-buffered saline; AB buffer, minimum essential medium modified for suspension cultures supplemented with 2.5% pooled human serum of AB type; FITC, fluorescein isothiocyanate; IC_{50}, the inhibiting concentration of a drug that leaves a surviving fraction of 0.5 *i.e.*, kills 50% of cells.

Table 1 *Comparison of in vitro cytotoxicity of maytansine and May-SS-Me (2)*[a]

Cell line	IC_{50} (M)	
	Maytansine	May-SS-Me (2)
KB (human epidermoid cancer)	3.4×10^{-11}	1.1×10^{-11}
SK-BR-3 (human breast cancer)	1.1×10^{-10}	1.1×10^{-11} (1.0×10^{-13})[b]
SW-620 (human colon cancer)	1.0×10^{-10}	9.0×10^{-12}
A-498 (human renal cancer)	$>10^{-10}$	1.0×10^{-10}
NIH:Ovcar-3 (human ovarian cancer)	1.0×10^{-10}	5.0×10^{-11}
HT-29 (human colon cancer)	ND[c]	2.5×10^{-11}
LoVo (human colon cancer)	ND	4.0×10^{-11}

[a] Cytotoxicity of these drugs at different concentrations has been measured after a 24-h exposure of cells to the drugs, using a clonogenic assay, and logarithm of surviving fractions *versus* drug concentration plots have been generated similar to the plots shown in Fig. 3*c*. Values for IC_{50} were determined from these plots.
[b] After a 72-h exposure.
[c] ND, not done.

noid 3 and the murine monoclonal antibody TA.1 (Fig. 2), using linkers containing either a disulfide bond or a noncleavable thioether bond. The TA.1 antibody binds to the HER-2/*neu* oncogene protein (also known as c-*erb*-2) that is expressed at high levels on human breast tumor cells (17). Conjugates with different molar ratios of covalently bound drug to antibody (ranging from one to six molecules of drug per molecule of antibody) have been prepared. In a competition binding assay, it was shown that the specific affinity of the antibody moiety is fully preserved in all conjugates (Fig. 3*a*).

The specific cytotoxicity of TA.1(-SS-May)$_4$ was tested *in vitro* on the human breast cancer cell line SK-BR-3, which expresses 3×10^5 *neu* surface antigens per cell. Exposure of the target cells for 24 h to various concentrations of the drug conjugate showed a concentration-dependent cytotoxic effect, with an IC_{50} of 1.6×10^{-11} M (Fig. 3*b*), demonstrating that the drug conjugate achieved a degree of cytotoxicity similar to that of the free drug. The cytotoxic effect of TA.1(-SS-May)$_4$ could be abolished by an excess of nonconjugated TA.1 antibody, indicating that the killing of cells depends upon the specific antibody-antigen interaction. In addition, this conjugate was at least 1000-fold less cytotoxic towards *neu*-negative KB cells (Fig. 3*b*).

The cytotoxicity of TA.1(-SS-May)$_n$ conjugates (where n is an average number of maytansinoid molecules per antibody) could be increased by linking more drug molecules per antibody molecule, and it reached its maximum value at $n = 4$ (Table 2). Treatment of SK-BR-3 cells with TA.1(-SS-May)$_4$ for 72 h was even more effective in killing cells ($IC_{50} = 3 \times 10^{-12}$ M; 99.9% of cells were killed at 0.1 nM concentration; Fig. 3*c*). Again, the cytotoxicity of TA.1 (-SS-May)$_4$ could be abolished by an excess of nonconjugated TA.1 antibody. Also, the cytotoxicity of anti-B4(-SS-May)$_6$, a conjugate with an antibody of the same isotype but that does not bind to the target cells, was more than 300-fold lower with an IC_{50} greater than 1×10^{-9} M (Fig. 3*c*). A TA.1-May conjugate in which the drug molecules are linked via a noncleavable thioether bond was 200-fold less potent under the same conditions (Fig. 3*c*). Similar results have been obtained with maytansinoid conjugates of the A7 antibody (16) directed against human colon cancer cell lines (Fig. 3*d*), and the antibody 5E9 directed against the human transferrin receptor (data not shown).

Pharmacological studies in mice showed that, following an i.v. injection, A7(-SS-May)$_6$ is cleared slowly from the blood of mice (Fig. 4). The difference in the time course of clearance of the maytansinoid moiety of the conjugate and the antibody moiety of the conjugate indicates that the conjugate slowly dissociates. Free drug is then quickly cleared (in a separate experiment we determined that the half-life for maytansine in the circulation is less than 20 min, data not shown). An alternative explanation is that the fraction of the conjugate that contains more drug molecules per antibody is cleared faster than the rest of the conjugate. We are currently developing a more stable disulfide-containing linker.

The conjugate A7(-SS-May)$_6$ was not toxic for the animals: 8 of 8 mice survived when given a dose of 8 mg/kg (by antibody) of the maytansinoid conjugate (containing 200 μg/kg of may-

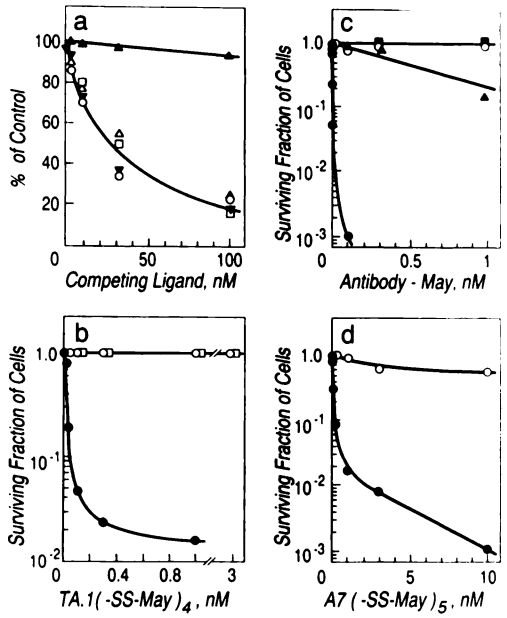

Fig. 3. Interaction of antibody-maytansinoid conjugates with cultured cells. (*a*) Competition of binding between a trace amount of FITC-labeled TA.1 antibody and unmodified TA.1 and TA.1-May conjugates to the *neu* antigen on SK-BR-3 cells (see "Materials and Methods"). Briefly, cells were incubated with mixtures of FITC-labeled TA.1 and different concentrations of an antibody or a conjugate. The mean fluorescence of the cells was then measured on a flow cytometer, the background (cell autofluorescence) was subtracted, and the resulting value was then compared with a control (FITC-labeled nonconjugated antibody). The symbols represent competition by antibody TA.1 (▼), by conjugates TA.1(-SS-May)$_n$, with $n = 2$ (○), $n = 4$ (△), $n = 6$ (□), or with a nonbinding antibody (anti-B4) of the same isotype (▲). (*b*) Cytotoxicity of TA.1(-SS-May)$_4$. Cytotoxicity was measured in the presence (○) or in the absence of 1 μM nonconjugated TA.1 (●) on antigen-positive SK-BR-3 cells and compared to the cytotoxicity measured on antigen-negative KB cells (□). Cells were exposed to the conjugate for 24 h at 37°C, and the surviving fractions of cells were then determined by using a clonogenic assay. (*c*) Cytotoxicity of TA.1(-SS-May)$_4$ in the presence (○) or in the absence of 1 μM nonconjugated TA.1 (●), TA.1(noncleavable linker-May)$_4$ (▲), and anti-B4(-SS-May)$_6$ (■) for SK-BR-3 cells after an extended (72 h) exposure. The surviving fractions of cells were determined by a clonogenic assay. (*d*) Cytotoxicity of A7(-SS-May)$_5$ for HT-29 cells. We determined with a binding experiment using a sample of radioiodinated A7 antibody that HT-29 cells express on the average 8×10^5 A7 antigens per cell. The methods used in these experiments are described in Ref. 8. Cells were incubated with the conjugate for 72 h in the presence (○) or in the absence (●) of 1 μM A7 antibody. The surviving fractions of cells were then determined by a clonogenic assay.

Table 2 *Comparison of in vitro cytotoxicity of TA.1(-SS-May)$_n$ conjugates for the SK-BR-3 cell line after a 24-h exposure*[a]

Conjugate	IC_{50} (M)
TA.1(-SS-May)$_1$	4.6×10^{-10}
TA.1(-SS-May)$_2$	2.1×10^{-10}
TA.1(-SS-May)$_4$	1.2×10^{-11}
TA.1(-SS-May)$_6$	2.0×10^{-11}

[a] Cytotoxicity of these conjugates at different concentrations has been measured after a 24-h exposure of cells to the conjugates, using a clonogenic assay, and logarithm of surviving fractions *versus* drug concentration plots have been generated similar to the plots shown in Fig. 3*c*. Values for IC_{50} were determined from these plots. n is an average number of maytansinoid molecules per antibody molecule.

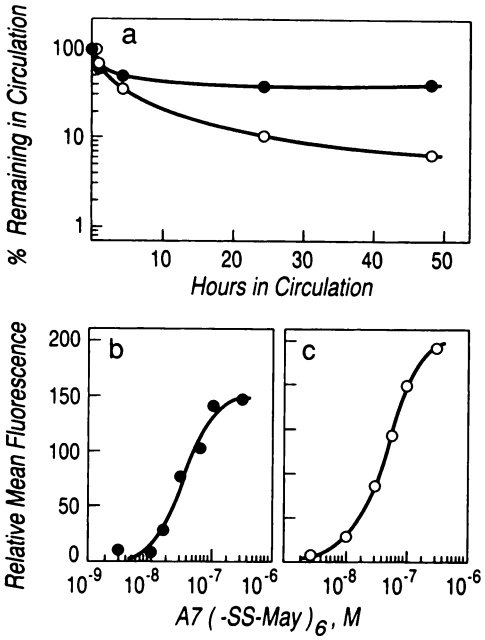

Fig. 4. Clearance and degradation of A7(-SS-May)$_6$ during circulation in CD-1 mice after i.v. injection. (*a*) Four mice were given injections of 2 mg/kg (by antibody) of conjugate; 75-μl samples of blood were drawn from the animals and then analyzed for the presence of the A7 and the maytansinoid moiety as described in "Materials and Methods." Symbols designate A7 moiety (●), and maytansinoid moiety (○). Standard curves for the quantitation of A7 (*b*), and maytansinoid (*c*) were generated with A7(-SS-May)$_6$ samples dissolved in a 1:4 (v/v) mixture of murine serum and AB buffer.

tansinoid) in a single bolus injection (the highest dose tested). These injections effected peak serum concentrations of about 0.1 mg/ml (0.6 μM). Free maytansine has a 10% lethal dose of 400 μg/kg in mice (23).

The high specific cytotoxicity of maytansinoid conjugates toward tumor cell lines in conjunction with their low systemic toxicity indicates that these potent conjugates may possess a therapeutic index sufficient for the effective treatment of human cancer.

We believe that the approach described here is not limited to maytansinoids, and that it opens the door for the clinical use of other highly cytotoxic drugs that are too toxic to be used alone. We also envision that conjugates of small drugs may be made with fragments of antibodies, further reducing the size of the resulting therapeutic agents and thereby offering the potential for better penetration of solid tumors. The development of "humanized" antibodies will offer an opportunity to produce drug conjugates that would be less immunogenic than similar conjugates of murine antibodies.

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