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A Prostate-specific Antigen (PSA)-activated Vinblastine Prodrug Selectively Kills PSA-secreting Cells *in Vivo*

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Abstract

Currently, there is no therapy for men with androgen-refractory prostate cancer that substantially extends survival. This report characterizes by *in vitro* and *in vivo* techniques a new chemotherapeutic that is composed of desacetyl-vinblastine covalently linked to a peptide that contains a peptide bond that can be hydrolyzed by prostate-specific antigen (PSA). This compound (referred to as vinblastine-conjugate) is minimally toxic to cells in culture which do not express PSA. In the presence of PSA, the peptide moiety is hydrolyzed, generating several highly toxic metabolites that contain vinblastine. Animals bearing PSA-positive human prostate tumors that were treated with the vinblastine-conjugate experienced a >99% reduction in PSA serum level. In contrast, animals bearing PSA-positive human prostate tumors treated with the cytotoxic metabolites derived from the PSA hydrolysis of the vinblastine-conjugate showed a nonsignificant change in both PSA and tumor weight values. The cell killing activity of the vinblastine-conjugate is PSA dependent because animals bearing non-PSA-producing human tumor xenografts had a nonsignificant increase in tumor weight after vinblastine-conjugate treatment. Exploratory efficacy/toxicity studies in LNCaP tumor-bearing nude mice were conducted with animals treated for 5 consecutive days with various doses of either the vinblastine-conjugate or a PSA-generated toxic metabolite (desacetyl-vinblastine). The desacetyl-vinblastine treatment resulted in 10–70% mortality with a very slight effect on tumor growth. In contrast, vinblastine-conjugate treatments resulted in no mortality, good to excellent antitumor efficacy, very slight to slight peripheral neuropathy and myelopathy, and slight to severe testicular degeneration. Similar treatment of beagle dogs with the vinblastine-conjugate showed even less toxicity. These data support the use of the PSA-hydrolyzable vinblastine-conjugate as an experimental therapy for prostate cancer in man.

Introduction

At present, there is no therapy for men with androgen-refractory prostate cancer that substantially extends survival, although cytotoxic chemotherapy is used to palliate disease-induced pain. Recently, we, as well as others, published a strategy designed to selectively kill prostate cancer cells using the cytotoxic chemotherapeutic doxorubicin (1–3). This strategy takes advantage of the tissue-restricted production of the kallikrein protease PSA[2] by prostate cancer cells. Some other human tissues are known to synthesize small amounts of PSA (4–6) that for our purposes are insignificant. In the prostate gland, the mature form of PSA is enzymatically inactive because of the high concentration of zinc ion (7). In addition, the proteolytic activity of secreted PSA is substantially reduced in the systemic circulation because of the formation of a covalent complex between PSA and the plasma protease inhibitors, α_1-antichymotrypsin and α_2-macroglobulin (8–10). Thus, secreted PSA is only enzymatically active in the microenvironment that surrounds prostate cells. The use of a prodrug, which is activated by PSA, should therefore preferentially target PSA-secreting cells.

We constructed a proteolytic cleavage map for PSAs' physiological substrate human semenogelin I (11, 12) by digestion of semenogelin I, produced using recombinant techniques in bacteria, with PSA. The peptide bond between Gln and Ser at positions 349 and 350 in semenogelin I was the most readily cleaved peptide bond in this substrate. Systematic modifications of the amino acid residues surrounding this site, after a procedure described previously (13), led to the design of a peptide of eight amino acid residues that was rapidly hydrolyzed by PSA when this peptide was covalently linked to the 4 position of desacetyl-vinblastine.[3] This peptide-desacetyl-vinblastine-conjugate was evaluated in cell culture and animal models of human prostate cancer, as well as in safety studies using mice and dogs.

Doxorubicin, like other DNA-damaging drugs, is more active in cells expressing the wild-type p53 protein (14). In contrast, the cytotoxicity induced by the microtubule-active

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[2] The abbreviations used are: PSA, prostate-specific antigen; MTD, maximally tolerated dose; MC, mass spectrometry; NHME, normal primary human mammary epithelial cell; NHBE, normal primary human bronchial epithelial cell; HPLC, high-performance liquid chromatography; AUC, area under the curve.

[3] V. M. Garsky, personal communication.

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drug vinblastine is p53 independent (14). Because metastatic prostate disease almost always contains p53 mutations, the use of vinblastine or other microtubule-targeted drugs should be more efficacious than doxorubicin in the treatment of advanced metastatic prostate cancer.

Recently, a randomized trial in which conventional dose chemotherapy was compared with high-dose chemotherapy plus hematopoietic stem cell rescue in women with metastatic breast cancer was completed (15). This study concluded that high-dose chemotherapy (about four times the dose intensity of conventional dose chemotherapy) does not improve survival in this patient population. This result suggests that it may be necessary to deliver an increase in dose intensity of >4-fold of cytotoxic therapy to observe a survival benefit for metastatic prostate cancer patients.

Our animal xenograft studies reported herein show the vinblastine-conjugate to be more efficacious than the doxorubicin-conjugate (1) at reducing serum PSA levels and inhibiting tumor growth. Defining the MTD as 70% of the dose that causes grade 4 neutropenia, we were able to administer a 15-fold molar excess of desacetyl-vinblastine in the form of the vinblastine-conjugate to beagle dogs, a species that is a good predictor of vinblastine toxicity in humans (16, 17).

Materials and Methods

Cell Culture and Nude Mouse Experiments. Cell lines were obtained from the American Type Culture Collection (Rockville, MD). The serially passaged human prostate tumor explant CWR22 was obtained from T. Pretlow (Case Western Reserve University School of Medicine). NHMEs and NHBEs were obtained from CAMBREX (East Rutherford, NJ).

Determination of the MTD of various compounds in animals and the cytotoxic activity of these compounds against LNCaP and CWR22 tumor xenografts was performed as described previously (1). Nude mice implanted with the Colo 320 xenograft were inoculated s.c. in the left flank with 0.5 ml of a 60% Matrigel solution containing 0.8 million cells that had been trypsinized and resuspended in serum-free α-MEM. Histological examination of animal tissues was done as reported previously (1).

Statistical significance is reported as a $P \leq 0.05$. *P*s were determined using the Comparing Multiple Groups guidance system created by the Biometrics Research Department of Merck Research Laboratories.

The synthesis and properties of the vinblastine-conjugate will be reported at a later date.

Stability of Vinblastine-Conjugate and Doxorubicin-Conjugate in Human and Murine Plasma and Human Blood. Freshly drawn human or mouse blood was anticoagulated and the cellular fraction removed by centrifugation to yield fresh plasma. In the case of whole blood experiments, the cellular fraction was left intact during the incubations. Peptide-cytotoxin conjugate solutions were prepared in water at 0.5 mg/ml. Each conjugate (70 μl) was added to 130 μl of plasma or whole blood and incubated for various times at 37°C. Reactions were terminated by heating to 100°C for 1 min. Samples were clarified by centrifugation and then analyzed by HPLC. Values represent percentage of

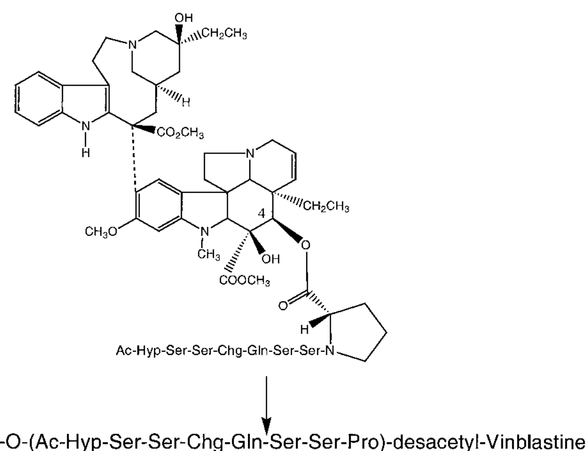

Fig. 1. Structure of the vinblastine-conjugate. *Arrow,* the peptide bond that is hydrolyzed by PSA.

reduction in peak area compared with control samples prepared in water.

Tissue Distribution of the Vinblastine-Conjugate and its Metabolites. Male nude mice weighing ~25 grams each were used for the pharmacokinetic studies of the vinblastine-conjugate and its active metabolites, 4-*O*-(Prolyl)-desacetyl-vinblastine and desacetyl-vinblastine. A multiple-dose tissue distribution study examined the concentrations of the parent drug and its metabolites in tumor and select tissues. The MTDs of vinblastine-conjugate (21.4 μmol/kg/day i.p. for 5 days) and desacetyl-vinblastine (0.26 μmol/kg/day i.p. for 5 days) were given to LNCaP tumor-bearing mice. After (24 h) the last dose of each drug tumor, muscle, liver, kidney, and brain tissues were excised and frozen in liquid nitrogen. Plasma was prepared from blood taken by cardiac puncture and frozen at −70°C until use. In the single dose distribution study, the concentration of parent drug and its active metabolites was determined in LNCaP tumor-bearing nude mice after the i.v. administration of equimolar doses of vinblastine-conjugate and desacetyl-vinblastine (9.2 μmol/kg).

The concentrations of vinblastine-conjugate and its metabolites were measured by the LC-MS/MS method. Tissues were homogenized in one volume of 10 mM ammonium acetate pH 6.0 buffer. Diluted aliquots of tissue homogenates were acidified with 1% formic acid in 20% acetonitrile/water (1/4, volume for volume) and then clarified by centrifugation. Samples underwent LC-MS/MS analysis using a Sciex API IIIplus mass spectrometer with a turbo ion spray interface used in the positive ionization mode.

Results

The Vinblastine-Conjugate Selectively Kills PSA-secreting Tumor Cells in Culture. The structure of the vinblastine-conjugate is shown in Fig. 1. PSA hydrolyzes the peptide bond between glutamine and serine generating Ser-Ser-4-*O*-(prolyl)-desacetyl-vinblastine. To demonstrate the requirement for PSA hydrolytic activity for rapid drug activation, we tested the ability of the vinblastine-conjugate and the post-PSA cleavage products for their cell killing ability in both

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Table 1 Cell-killing activity of cytotoxic compounds on human tumor cell lines and normal human primary cells

Cells were seeded into 96-well plates at 10,000 cells/well (0.1 ml/well). Various dilutions of the indicated drugs in 0.1 ml were added to the cells. The cultures were then incubated at 37°C for 72 h, at which time, 20 μl of alamar blue solution were added to each well. After an additional 7-h incubation, the plates were read at 570 and 600 nm. EC_{50}s for the cell killing are expressed as the percentage of reduction in cell numbers relative to the media controls. All studies were performed in triplicate. We analyzed other prostate tumor lines (PC3 and DuPRO), but these cells were not sensitive to vinblastine. We also analyzed a number of primary normal human prostate epithelial (NHPE) cells from CAMBREX. These NHPE cells were not sensitive to vinblastine. LNCaP, PSA-producing prostate carcinoma tumor cell line; Colo320, human colorectal adenocarcinoma cell line; T-47D, human breast ductal carcinoma cell line. ND, not determined.

Compound	EC_{50} (μM) for cell killing				
	LNCaP	Colo320	T47D	NHBE	NHME
Desacetyl-vinblastine	0.1	0.2	0.15	6	12.5
4-*O*-Prolyl-desacetyl-vinblastine	1	3	1.6	ND	ND
Vinblastine-conjugate	1.6	14	>50	>100	>100

PSA-producing and non-PSA-producing human tumor cell lines, as well as non-PSA-producing human normal primary cells. We determined the EC_{50} which represented the amount of drug required to kill 50% of the cells. Desacetyl-vinblastine and 4-*O*-(Prolyl)-desacetyl-vinblastine were potent cytotoxic agents against both transformed and normal human cells *in vitro* independent of the cells' ability to make PSA (Table 1). The vinblastine-conjugate was also a potent cytotoxic agent against human prostate cancer cells that make PSA (LNCaP) but was not cytotoxic against the human breast tumor cell line, T-47D, and the normal NHME and NHBE cells, which do not make PSA. The human colorectal adenocarcinoma cell line, Colo 320, which also does not synthesize PSA, was affected moderately by the vinblastine-conjugate. To further investigate the basis of the vinblastine-conjugate's cell killing activity against the Colo 320 cell line, the vinblastine-conjugate was incubated with Colo 320 cells in culture for 48 h. Normally, the cell kill assays use a 72-h incubation with test compounds. After this incubation, the cells were harvested and subjected to HPLC analyses to determine what cytotoxic metabolites were generated from the vinblastine-conjugate. Desacetyl-vinblastine was the only vinblastine-containing species identified (data not shown).

The ability of the vinblastine-conjugate to kill Colo 320 tumor cells that do not make PSA in culture appears to be because of the conversion of the vinblastine-conjugate to desacetyl-vinblastine. One possible mechanism for this conversion could be an attack on the ester bond between the terminal proline of the peptide moiety and the desacetyl-vinblastine by tumor cell esterases, because similar vinblastine-conjugates synthesized by us where the peptide moiety is not linked to the cytotoxic agent via an ester bond do not kill Colo 320 cells in culture (data not shown). Another possible explanation is that Colo320 cells produce a proteolytic activity that can hydrolyze the peptide moiety of the vinblastine-conjugate. There is also support for this model because studies that used the serine protease inhibitor, AEBSF, shifted the EC_{50} for the vinblastine-conjugate from 14 μM to >50 μM. These studies were not pursued further because this sensitivity of the Colo320 cells to the vinblastine-conjugate did not predict the excellent therapeutic index seen in our animal xenograft studies.

To test if the vinblastine-conjugate was more susceptible to other non-PSA proteases, we evaluated the stability of this compound in the presence of either human or murine plasma or human whole blood. The results of this experiment demonstrated that a 60-min incubation of the vinblastine-conjugate in plasma or whole blood did not alter the structure of this compound as determined by HPLC analysis.

Effect of Vinblastine-Conjugate on Tumor Xenografts in Nude Mice. Before beginning antitumor studies in mice, we determined the MTD for each drug to be analyzed. We defined the MTD as the maximum drug dose administered to nontumor-bearing mice once daily for 5 consecutive days that did not elicit any deaths. The MTD for desacetyl-vinblastine was determined to be 0.26 μmol/kg mouse body weight, and the MTD for 4-*O*-(Prolyl)-desacetyl-vinblastine was 4.6 μmol/kg. In contrast, the MTD for the vinblastine-conjugate was determined to be 21.4 μmol/kg, a value that is >8000% higher than the MTD for desacetyl-vinblastine (Table 2 and Fig. 3).

We analyzed the antitumor activity of the vinblastine-conjugate, 4-*O*-(Prolyl)-desacetyl-vinblastine, and desacetyl-vinblastine by assessing the ability of each compound to suppress the growth of human prostate cancer cell xenografts in athymic nude mice. At the end of each study, the tumors from vehicle- and drug-treated animals were excised and weighed, and the circulating serum level of tumor-produced PSA was determined. We tested two PSA-producing human prostate cancer cells: (*a*) LNCaP; and (*b*) CWR22. In one study, we administered drug treatment once daily by i.p. injection for 5 consecutive days to nude mice bearing LNCaP tumor xenografts (Table 2). The vinblastine-conjugate at doses below its MTD (15.3, 9.2, 4.9, 2.4, and 1.2 μmol/kg) gave statistically significant reductions in the average circulating serum PSA values of 99, 92, 83, 68, and 43%, respectively ($P \leq 0.05$). 4-*O*-(Prolyl)-desacetyl-vinblastine at its MTD of 4.6 μmol/kg gave a statistically nonsignificant 33% reduction in average serum PSA levels ($P = 0.4$), and desacetyl-vinblastine at its MTD of 0.26 μmol/kg also gave a statistically nonsignificant 14% reduction in average circulating serum PSA levels ($P = 0.549$). The weights of the excised tumors were also reduced by drug therapy to a similar extent in each of the treatment groups as shown for serum PSA. Tumor weights for mice treated with the vinblastine-conjugate at doses of 15.3, 9.2, and 4.9 μmol/kg were reduced by 85, 77, and 68% respectively ($P \leq 0.020$ for each). Tumor weights for mice treated with 4-*O*-(Prolyl)-desacetyl-vinblastine or desacetyl-vinblastine at the respec-

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Table 2 Efficacy of vinblastine-conjugate and its major metabolites in nude mice bearing PSA-producing and non-PSA-producing human tumor xenografts

A group of 10–12-week-old male nude mice were used for animal studies. On day 1, mice were inoculated s.c. in the left flank with the indicated cells. Later (24 h), the inoculated mice were started on the drug. Drugs were given i.p. once a day for 5 consecutive days. At the end of 5 and one-half weeks, blood was taken, and the tumors were excised. PSA and tumor weight values are expressed as a percentage of the value derived from vehicle-treated animals. LNCaP and CWR22 are PSA-producing tumors; Colo 320 is a non-PSA-producing tumor.

A. Compound	LNCaP tumor xenograft			% Tumor weight reduction (P)[b]
	MTD (μmol/kg)	Dose (μmol/kg)[a]	% PSA reduction (P)[b]	
dAc-Vin	0.26	0.26	14 (0.549)	16 (0.595)
		0.39	25 (0.460)	14 (0.686)
		0.52	70 (0.193)	57 (0.272)
4-*O*-prolyl-dAc-Vin	4.6	4.6	33 (0.400)	60 (0.226)
		5.8	19 (0.618)	2 (0.996)
		6.9	76 (0.093)	67 (0.152)
4-*O*-(Ac-HypSSChgQSSP)-dAc-Vin	21.4	1.2	43 (0.041)	53 (0.073)
		2.4	68 (0.054)	54 (0.155)
		4.9	83 (0.003)	68 (0.020)
		9.2	92 (0.001)	77 (0.009)
		15.3	99 (0.001)	85 (0.005)
		21.4	100 (0.001)	90 (0.005)
4-*O*-[Ac-HypSSChg(dQ)(dS)SP]-dAc-Vin	ND	12.3	[54 increase (0.506)]	[64 increase (0.181)]

B. Compound	CWR 22 tumor xenograft			% Tumor weight reduction (P)[b]
	MTD (μmol/kg)	Dose (μmol/kg)[a]	% PSA reduction (P)[b]	
dAc-Vin	0.26	0.26	[14 increase (0.180)]	[19 increase (0.294)]
4-*O*-prolyl-dAc-Vin	4.6	4.6	4 (0.718)	9 (0.694)
4-*O*-(Ac-HypSSChgQSSP)-dAc-Vin	21.4	12.2	99.7 (<0.001)	89 (<0.001)

C. Compound	Colo 320 tumor xenograft			% Tumor weight reduction (P)[b]
	MTD (μmol/kg)	Dose (μmol/kg)[a]	% PSA reduction (P)[b]	
dAc-Vin	0.26	0.26	NA[c]	4 (0.880)
4-*O*-prolyl-dAc-Vin	4.6	4.6	NA	[14 increase (0.705)]
4-*O*-(Ac-HypSSChgQSSP)-dAc-Vin	21.4	12.2	NA	[21 increase (0.538)]

[a] Ten animals were treated at each dose.
[b] $P > 0.05$ indicates a nonsignificant result.
[c] NA, not applicable.

tive MTDs were insignificantly reduced by 60 and 16%, respectively ($P = 0.226$ and 0.595). We repeated these experiments using nude mice bearing another PSA-secreting human prostate tumor xenograft, CWR22 (Table 2). The vinblastine-conjugate at a dose of 12.2 μmol/kg (MTD = 21.4 μmol/kg) once per day for 5 consecutive days produced a statistically significant reduction in the average circulating serum PSA value of ~100% ($P < 0.001$) and an average tumor weight reduction of 89% ($P < 0.001$). This result suggests that the vinblastine-conjugate targets the tumor tissue because the cytotoxic metabolites of the vinblastine-conjugate gave statistically nonsignificant changes in PSA and tumor weight (Table 2).

To demonstrate the requirement for PSA for drug-induced tumor growth reduction, we used a peptide-vinblastine-conjugate that is not hydrolyzed by PSA. This conjugate (4-*O*-[Ac-HypSSChg(dQ)(dS)SP]-desacetyl-vinblastine) is similar in structure to the PSA hydrolyzable vinblastine-conjugate but contains the D-stereo isomer of glutamine and serine and was designed not to be a substrate for PSA. Incubation of this conjugate with PSA for 24 h failed to show any evidence of peptide cleavage (data not shown). To evaluate the antitumor activity of this compound, we treated LNCaP tumor-bearing nude mice i.p. once a day for 5 consecutive days. Animals treated with the PSA nonhydrolyzable vinblastine-conjugate at a dose of 12.3 μmol/kg experienced a statistically nonsignificant increase in both average serum PSA values and average tumor weight values compared with vehicle controls (Table 2): a 54% increase in PSA ($P = 0.506$) and a 64% increase in tumor weight ($P = 0.181$). In addition to using the PSA nonhydrolyzable vinblastine-conjugate as a control to demonstrate the requirement for PSA in tumor reduction, we also used the non-PSA-producing human colorectal adenocarcinoma Colo 320 cell line. In this model, animals given the vinblastine-conjugate at 12.2 μmol/kg (MTD = 21.4 μmol/kg) once per day for 5 consecutive days experienced a nonsignificant increase in tumor weight of 21% (Table 2). Other Colo 320 tumor-bearing animals that were treated at the MTD with the vinblastine-conjugate's cytotoxic metabolites, desacetyl-vinblastine and 4-*O*-(Prolyl)-desacetyl-vinblastine, experienced a nonsignificant decrease and increase in tumor weight, respectively, although Colo 320 cells in culture are killed by low concentrations of these compounds (Table 1).

Furthermore, we investigated whether the vinblastine-conjugate had an improved therapeutic index relative to desacetyl-vinblastine. To determine the relative toxicity of the vinblastine-conjugate to desacetyl-vinblastine, we compared animal weight loss and the number of animal deaths

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Table 3 Comparison of gross toxicity in LNCaP tumor-bearing nude mice treated with desacetyl-vinblastine or vinblastine-conjugate

Experimental protocols are described in the legend to Table 2. Data represent average weights of mice surviving at the end of therapy.

Vehicle Controls						
Average wgt. loss (%)[a]	14					
desacetyl-Vinblastine						
Drug doses (μmol/kg)[b]	0.26	0.39	0.52			
Average wgt. loss (%)	13	14	15			
Number mice dead[c]	1	3	7			
Vinblastine-conjugate						
Drug doses (μmol/kg)	1.2	2.4	4.9	9.2	15.3	21.4
Average wgt. loss (%)	14	10	7	5	2	7
Number mice dead	0	0	0	0	0	0

[a] Percentage of decrease from pretreatment weights.
[b] Drugs were administered once a day for 5 consecutive days.
[c] Ten mice/group at the start of study.

Table 4 Delivery of desacetyl-vinblastine to tissues after multiple dose i.p. administration of vinblastine-conjugate and desacetyl-vinblastine at their respective MTDs

Tissues were obtained at various time points over a 24-h period after the last of five daily doses. $AUC_{0-24\ h}$ values were calculated using mean concentrations (n = 2 to 3/time point).

Exposure ratio = [Desacetyl-vinblastine from vinblastine-conjugate (21.4 μmol/kg/day)]/[Desacetyl-vinblastine from desacetyl-vinblastine (0.26 μmol/kg/day)]

Tissue	dAc-vin from vin-conjugate $AUC_{0-24\ h}$ (nM/h)	dAc-vin from dAc-vin $AUC_{0-24\ h}$ (nM/h)	AUC ratio
Tumor	8781	553	16
Liver	6345	948	6.7
Kidney	29440	1429	20.6[a]
Brain	371	*[b]	~2[c]
Muscle	912	*	~5[c]
Plasma	1212	*	~6[c]

[a] May include contamination from residual urine.
[b] Insufficient concentration to calculate.
[c] Estimated using the assay quantification limit to calculate AUC in brain and muscle.

caused by treatment with the two drugs. As shown in Table 3, LNCaP tumor-bearing nude mice treated with desacetyl-vinblastine at 1, 1.5, and 2 times the MTD (0.26 μmol/kg) experienced a 13–15% body weight loss with 10–70% animal death and no significant circulating PSA or tumor weight reduction (Table 2). On the other hand, animals treated with various doses of vinblastine-conjugate at and below its MTD (21.4 μmol/kg) experienced a 2–14% body weight loss and no animal deaths with dramatic reductions in both circulating PSA and tumor weight values.

Tissue Distribution of Vinblastine-Conjugate and Its Metabolites. We sought to determine whether the administration of vinblastine-conjugate would lead to desacetyl-vinblastine accumulating preferentially in PSA-secreting tissues. We followed a procedure described previously (18). Dosing (i.p.) at the MTD of vinblastine-conjugate and desacetyl-vinblastine (21.4 and 0.26 μmol/kg, respectively) once per day for 5 consecutive days was carried out in separate groups of LNCaP tumor-bearing nude mice. Plasma and tissue were obtained at various time points over a 24-h period after the last of the five daily doses. We then determined the concentrations of desacetyl-vinblastine in tumor, liver, kidney, brain, muscle, and plasma tissues. The concentration of desacetyl-vinblastine in tumor tissue was greater at all time points in animals given vinblastine-conjugate. The peak concentration in tumor tissue was increased 2200%, and the $AUC_{0-24\ h}$ value for desacetyl-vinblastine in tumor tissue was increased 1600% in the vinblastine-conjugate-treated mice as compared with mice treated with desacetyl-vinblastine (Table 4). Among evaluated tissues, this ratio was only larger in kidney tissue where the result may be confounded because of the presence of residual urine in the kidney tissue sample. Histological examination of renal tissue showed no drug-induced toxicity (see below).

Furthermore, separate groups of LNCaP tumor-bearing nude mice were dosed i.v. with either a equimolar amount of vinblastine-conjugate or desacetyl-vinblastine (9.2 μmol/kg; Table 5). With the exception of plasma, the $AUC_{0-24\ h}$ ratio of desacetyl-vinblastine derived from vinblastine-conjugate to desacetyl-vinblastine given as desacetyl-vinblastine was largest in tumor tissue. The data show that animals given the vinblastine-conjugate had ~3-fold more desacetyl-vinblastine in their LNCaP tumor tissue than other evaluated tissues.

Histological Evaluation of Mouse and Dog Tissues. We histologically examined 34 different tissues taken from LNCaP tumor-bearing athymic nude mice 5.5 weeks after the first injection of vinblastine-conjugate or desacetyl-vinblastine given once daily for 5 consecutive days. Mice were treated with vinblastine-conjugate at doses of 2.4, 4.9, 9.2, 15.3, and 21.4 μmol/kg and desacetyl-vinblastine at 0.26 μmol/kg. Five mice were evaluated at each dose. A subset of these data describing all tissues where histological changes

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Table 5 Delivery of desacetyl-vinblastine to tissues after i.v. administration of single equimolar doses of vinblastine-conjugate and desacetyl-vinblastine

Tissues were obtained at various time points over a 24-h period after the tail vein administration of the indicated drug dose. $AUC_{0-24\ h}$ values were calculated using a mean concentration of $n = 3$/time point. In this experiment, vinblastine-conjugate was given at less than half of its MTD, whereas desacetyl-vinblastine-treated animals were given a lethal dose of 35 times the MTD.

	Exposure ratio = [Desacetyl-vinblastine from vinblastine-conjugate (9.2 μmol/kg)]/[Desacetyl-vinblastine (9.2 μmol/kg)]		
Tissue	dAc-vin from vin-conjugate $AUC_{0-24\ h}$ (nM/h)	dAc-vin from dAc-vin $AUC_{0-24\ h}$ (nM/h)	AUC ratio
Tumor	2510	8740	0.29
Liver	3650	38750	0.094
Kidney	8090	98450	0.082
Brain	*[a]	288	
Muscle	*	2140	
Plasma	2340	2680	0.87

[a] Insufficient concentration to calculate.

Table 6 Exploratory toxicity study in LNCaP tumor-bearing nude mice

An exploratory efficacy study was conducted to compare the vinblastine-conjugate with desacetyl-vinblastine. The vinblastine-conjugate and desacetyl-vinblastine were tested at their MTDs of 21.4 and 0.26 μmol/kg, respectively. These dosages were given as single i.p. injections once a day for 5 consecutive days to adult male nude mice that had been inoculated s.c. with 17 million LNCaP cells before treatment. Animals were necropsied 4 weeks after the last drug dose. Histological effects were observed only in the tissues indicated below. Grade: 0, none; 1, very slight; 2, slight; 3, moderate; 4, marked; 5, severe.

	Animal no.	Terminal body weight (grams)	Tumor weight (grams)	Serum PSA (μg/ml)	Tumor cellularity (grade)	Testicular degeneration (grade)	Myelopathy (grade)	Neuropathy (grade)
Control vehicle	1	27.1	0.98	127.66	4	1	0	0
	2	26.4	1.01	154.86	4	1	0	0
	3	26.95	0.79	213.97	5	2	0	0
	4	29.2	1.26	216.96	4	0	0	0
	5	26.2	1.87	204.36	5	1	0	0
Average		27.17	1.18	183.56	4.4	1	0	0
Desacetyl-vinblastine (0.26 μmol/kg)	6	30.3	0.79	205.96	4	0	0	0
	7	27.4	0.58	86.24	4	0	0	0
	8	27.1	0.91	175.6	4	0	0	0
	9	31.2	1.92	319.97	4	0	0	0
	10	28	0.96	82.09	4	0	0	0
Average		28.8	1.03	173.97	4	0	0	0
Vinblastine-conjugate (21.4 μmol/kg)	11	28.18	0.09	0	1	4	0	1
	12	29.25	0.1	0	1	4	1	2
	13	29.19	0.1	0	1	3	1	2
	14	28.29	0.14	1.53	2	5	0	1
	15	27.88	0.08	0	1	5	1	2
Average		28.56	0.1	0.31	1.2	4.2	0.6	1.6

occurred, in animals treated at the MTD for both drugs, is presented in Table 6. Treatment with desacetyl-vinblastine at its MTD resulted in a very slight effect on suppressing tumor growth with no detectable long-term effects on nontumor tissue. This result is consistent with the drug metabolism data shown in Table 4 where tumor tissue in animals treated at the MTD for desacetyl-vinblastine contained 16 times less desacetyl-vinblastine than did tumor tissue taken from animals treated with the vinblastine-conjugate at its MTD. Under the conditions of this experiment, the tumor tissue in animals treated with desacetyl-vinblastine is not exposed to sufficient cytotoxic agent to cause a significant histological effect. The vinblastine-conjugate treatments gave good to excellent antitumor efficacy, very slight myelopathy, and very slight to slight peripheral neuropathy at the highest dose and slight to severe testicular degeneration over a drug concentration range from 4.9 to 21.4 μmol/kg. No long-term effects were seen in any other tissue. Acute dose-limiting toxicity for the vinblastine-conjugate and desacetyl-vinblastine is because of bone marrow suppression (data not shown).

An exploratory toxicity study in beagle dogs was completed with i.v. infusions of desacetyl-vinblastine and vinblastine-conjugate. Two males were given 0.6 μmol/kg vinblastine-conjugate and two males were given 0.04 μmol/kg desacetyl-vinblastine by i.v. infusion of 30 ml over 30 min once a day for 5 consecutive days (MTD for both drugs). The vinblastine-conjugate-treated dogs had a transient decrease in leukocyte counts during the treatment period with subsequent recovery during the next 20-day observation period. Microscopic examination of 40 tissues taken at the end of the observation period indicated a very slight sciatic neuropathy, a very slight skeletal muscle degeneration and/or cellular infiltration, and a very slight testicular degeneration (1 dog). These changes were not seen in

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the dogs given 0.04 μmol/kg desacetyl-vinblastine. However, the changes seen in the dogs given vinblastine-conjugate in this study are consistent with the pathology induced by vinblastine in other laboratory animals. The treatment-related changes in the nerves, skeletal muscles, and testes induced with this dose of vinblastine-conjugate were considered minimal and reversible.

Comparison of Vinblastine-Conjugate to Doxorubicin-Conjugate in Multiple Species. We reported recently the synthesis of a compound composed of a PSA hydrolyzable peptide covalently linked to the glycosidic amine of doxorubicin (1, 13). This compound is ~10-fold less toxic than conventional doxorubicin on a molar basis when administered i.p. to LNCaP tumor-bearing nude mice using death as the end point for dose-limiting toxicity. As reported herein, the vinblastine-conjugate is >80-fold less toxic than conventional desacetyl-vinblastine in this animal model (Table 2 and Fig. 3). To compare antitumor potencies of these two conjugates, we performed a nude mouse tumor xenograft experiment in which both compounds were tested. We assayed both conjugates at approximately one-ninth of their MTD values (MTD is defined as the maximum dose achievable with no animal deaths). The results are shown in Fig. 2. Animals dosed with the doxorubicin-conjugate at 3.6 μmol/kg had a statistically nonsignificant increase in both circulating serum PSA and tumor weight values of 19 ($P = 0.6$) and 21% ($P = 0.5$), respectively. Animals dosed with the vinblastine-conjugate at 2.4 μmol/kg had a statistically significant reduction in both circulating PSA and tumor weight values of 64 ($P = 0.03$) and 47% ($P = 0.05$), respectively (10 animals in each treatment group).

We have also determined the MTD of both conjugates in beagle dogs. In this species, 70% of the dose that caused grade 4 neutropenia was used as the MTD. On a molar basis, we were able to administer i.v. 3-fold more doxorubicin-conjugate than conventional doxorubicin and 15-fold more vinblastine-conjugate than desacetyl-vinblastine (Fig. 3). Drugs were administered i.v. over 30 min once a day for 5 consecutive days.

Discussion

The vinblastine-conjugate is composed of an eight amino acid peptide containing a PSA hydrolytic site that is covalently linked to desacetyl-vinblastine. This agent is intended as a therapy for the treatment of hormone-refractory prostate cancer. The vinblastine-conjugate is relatively innocuous and requires proteolytic hydrolysis of its peptide component to become cytotoxic. This proteolytic activation is preferentially carried out by the prostate tissue-restricted protease PSA (insignificant amounts of PSA, for our purposes, are synthesized by other tissues; Refs. 4–6). High intracellular zinc ion concentrations in prostate cells and plasma-localized protease inhibitors restrict the proteolytic activity of PSA to the extracellular microenvironment of PSA-secreting cells (7–10). In theory, the vinblastine-conjugate should circulate freely in the body and be preferentially activated at sites of prostate cancer tissue by PSA. Thus, the vinblastine-conjugate should have better antitumor activity

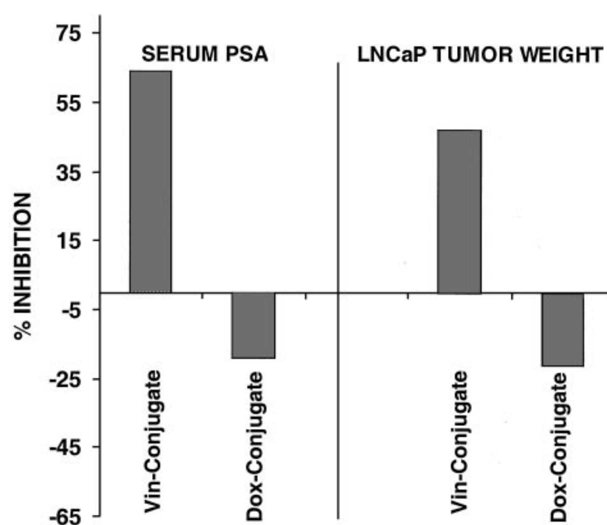

Fig. 2. Inhibition of circulating serum human PSA and tumor weights in nude mice bearing LNCaP tumor xenografts treated with the vinblastine-conjugate or the doxorubicin-conjugate (1). Each conjugate was administered at approximately one-ninth the MTD to a group of 10 animals. The vinblastine-conjugate is more efficacious than the doxorubicin-conjugate when the drugs are administered at equivalent toxicities. No effect is seen with the doxorubicin-conjugate, because at this dose, the drug does not elicit an antitumor response. The percentage of change is from vehicle-treated animals. The changes reported for the doxorubicin-conjugate are not statistically significant. The changes reported for the vinblastine-conjugate are significant at the $P = 0.05$ level.

with less toxicity than conventional vinblastine against prostate cancers.

The vinblastine-conjugate is a selectively potent cytotoxic agent against PSA-secreting cells in culture (Table 1). In nude mouse xenograft studies using the PSA-secreting human LNCaP prostate cancer cell line, the vinblastine-conjugate had much better antitumor activity than its cytotoxic metabolites desacetyl-vinblastine and 4-*O*-(Prolyl)-desacetyl-vinblastine, as measured by reduced circulating serum PSA levels and tumor weights (Table 2). We obtained similar results using a second transplantable PSA-secreting human prostate cancer tumor, CWR22. In control studies, treatment of a non-PSA-producing human tumor xenograft, Colo 320, with vinblastine-conjugate showed a statistically nonsignificant tumor-promoting activity. Additionally, we treated LNCaP tumor-bearing nude mice with a related conjugate that is not a PSA substrate. Animals treated with this related conjugate also experienced a statistically nonsignificant increase in their tumor burden (Table 2). Furthermore, we did dose titration studies using desacetyl-vinblastine and the vinblastine-conjugate to treat PSA-secreting LNCaP tumors in nude mice (Table 3). The vinblastine-conjugate did not show signs of gross toxicity at its maximally effective treatment doses as evidenced by minimal changes in mouse body weights and no animal deaths after therapy as compared with tumor-bearing animals treated with the vinblastine-conjugate metabolite, desacetyl-vinblastine.

Drug localization studies demonstrate that the greater efficacy of the vinblastine-conjugate was at least partially because of specific targeting of this compound to PSA-secret-

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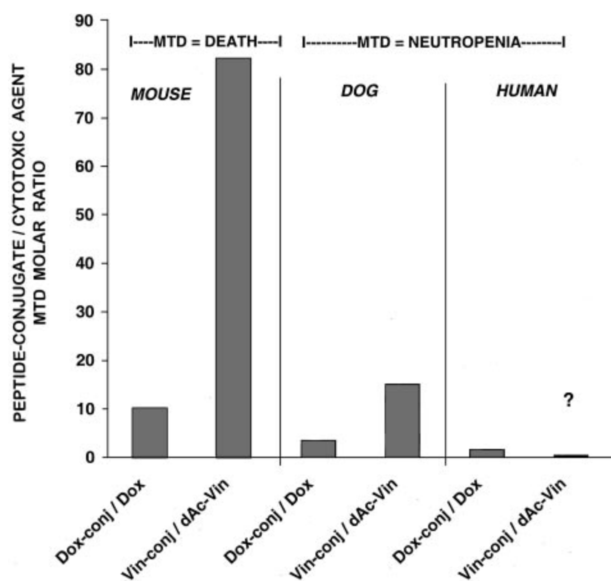

Fig. 3. Ratios of the MTD of native doxorubicin and doxorubicin-conjugate and native desacetyl-vinblastine and vinblastine-conjugate in mouse, dog, and human. The MTD in the mouse is defined as the highest dose of drug that did not elicit death. The MTD in dog and human is defined as 70% of the dose that caused grade 4 neutropenia. The mouse data are derived from multiple experiments where a minimum of 10 animals/experiment was treated with each drug. The dog data are derived from one experiment where each drug was administered to 2 animals. The MTD for the doxorubicin-conjugate in man on a once-every-3-week schedule is 225 mg/m^2 (this is equivalent to 90 mg/m^2 doxorubicin; Ref. 19). The historical MTD of doxorubicin in man on a once-every-3-week schedule is 60 mg/m^2.

ing tissues (Tables 4 and 5). When equimolar doses of vinblastine-conjugate and desacetyl-vinblastine were administered (in this experiment, vinblastine-conjugate was given at less than half of its MTD, whereas desacetyl-vinblastine-treated animals were given a lethal dose of 35 times the MTD) to tumor-bearing animals, the highest level of desacetyl-vinblastine in the vinblastine-conjugate-treated animals, with the exception of plasma, was found in tumor tissue.

Comparison of the preclinical studies of our previously described PSA-activated doxorubicin-conjugate (1) with the preclinical studies of the vinblastine-conjugate described herein suggest that the vinblastine-conjugate may have a superior therapeutic index. When both of these PSA hydrolyzable cytotoxic-conjugates were tested head to head in the LNCaP tumor xenograft model at the same fraction of their MTDs, the vinblastine-conjugate induced both serum PSA and tumor weight reduction, whereas the doxorubicin-conjugate did not (Fig. 2). In addition, in mice, comparing the highest dose of each drug that did not cause any animal deaths, we were able to administer, on a molar basis, >800% more of the vinblastine-conjugate (Fig. 3). The vinblastine-conjugate was also less toxic in beagle dogs using neutropenia as the dose-limiting toxicity (Fig. 3). We were able to administer to these animals ~500%, on a molar basis, more vinblastine-conjugate than doxorubicin-conjugate. We have over the past year completed Phase I clinical studies using the PSA hydrolyzable peptide doxorubicin-conjugate (1). The historical MTD value for doxorubicin in humans is 60 mg/m^2 on a once-every-3-week schedule. As detailed in Ref. 19, we determined the MTD of the doxorubicin-conjugate in humans to be 225 mg/m^2. Doxorubicin is 40% of the mass of the doxorubicin-conjugate; therefore, we administered the equivalent of 90 mg/m^2 or an MTD increase of 50%. The preclinical mouse and dog animal data show that we can deliver more desacetyl-vinblastine than doxorubicin on a molar basis when both drugs are administered as peptide-conjugates (~500%-fold more in dogs). The neutropenic dose-limiting acute toxicity of vinblastine in humans is nearly identical to the dose-limiting acute toxicity of this compound in dogs (0.07 mpk *versus* 0.08 mpk, respectively; Refs. 16 and 17). This knowledge, along with the information supplied by the above preclinical studies, suggests that we may be able to administer on a molar basis 8–10-fold more desacetyl-vinblastine in the form of the conjugate than desacetyl-vinblastine to man.

Finally, we found no evidence of toxicities unrelated to vinblastine in any tissue from mice or dogs treated with the vinblastine-conjugate. In conclusion, these experiments demonstrate that the PSA hydrolyzable vinblastine-conjugate is a more effective antitumor agent than desacetyl-vinblastine, producing much less nontumor toxicity and eliminating treatment-related mortality. These findings support the use of the vinblastine-conjugate as an experimental agent for the treatment of hormone-refractory prostate cancer in man.

References

1. DeFeo-Jones, D., Garsky, V. M., Wong, B. K., Feng, D-M. Bolyar, T., Haskell, K., Kiefer, D. M., Leander, K., McAvoy, E., Lumma, P., Wai, J., Senderak, E. T., Motzel, S. L., Keenan, K., Van Zwieten, M., Lin, J. H., Freidinger, R., Huff, J., Oliff, A., and Jones, R. E. A peptide-doxorubicin 'prodrug' activated by prostate-specific antigen selectively kills prostate tumor cells positive for prostate-specific antigen *in vivo*. Nat. Med., *6:* 1248–1252, 2000.

2. Denmeade, S. R., Nagy, A., Gao, J., Lilja, H., Schally, A. V., and Isaacs, J. T. Enzymatic activation of a doxorubicin-peptide prodrug by prostate-specific antigen. Cancer Res., *58:* 2537–2540, 1998.

3. Khan, S. R., and Denmeade, S. R. *In vivo* activity of a psa-activated doxorubicin prodrug against psa-producing human prostate cancer xenografts. Prostate, *45:* 80–83, 2000.

4. Diamandis, E., Yu, H., and Sutherland, D. J. A. Detection of prostate-specific antigen immunoreactivity in breast tumors. Breast Cancer Res. Treat., *32:* 301–310, 1994.

5. Yu, H., Diamandis, E., and Sutherland, D. J. A. Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin. Biochem., *27:* 75–79, 1994.

6. Levesque, M., Yu, H., D'Costa. M., and Diamandis, E. Prostate-specific antigen expression by various tumors. J. Clin. Lab. Anal., *9:* 123–128, 1995.

7. Malm, J., Hellman, J., Hogg, P., and Lilja, H. Enzymatic action of prostate-specific antigen (PSA or hK3): substrate specificity and regulation by Zn(2+), a tight-binding inhibitor. Prostate, *45:* 132–139, 2000.

8. Christensson, A., Laurell, C., and Lilja, H. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. Eur. J. Biochem., *194:* 755–763, 1990.

9. Leinonen, H. J., Zhang, W., and Stenman, U. Complex formation between PSA isoenzymes and protease inhibitors. J. Urol., *155:* 1099–1103, 1996.

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10. Otto, A., Bar, I., and Birkenmeir, B. Prostate-specific antigen forms complexes with human α2-macroglobulin receptor/ldl receptor-related protein. J. Urol., *159:* 297–303, 1998.

11. Lilja, H. A kalikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J. Clin. Investig., *76:* 1899–1903, 1985.

12. Lilja, H., Oldbring, J., Ramevik, G., and Laurell, C-B. Seminal vesicle secreted proteins and their reactions during gelation and liquefication of human semen. J. Clin. Investig., *80:* 281–285, 1987.

13. Garsky, V. M., Lumma, P. K., Feng, D-M. Wai, J., Ramjit, H. G., Sardana, M. K., Oliff, A. O., Jones, R. E., DeFeo-Jones, D., and Freidinger, R. M. The synthesis of a prodrug of doxorubicin designed to provide reduced systemic toxicity and greater target efficacy. J. Med. Chem., *44:* 4216–4224, 2001.

14. O'Connor, P. M., Jackman, J., Bae, I., Myers, T. G., Fan, S., Mutoh, M., Scudiero, D. A., Monks, A., Sausville, E. A., Weinstein, J. N., Friend, S., Fornace, A. J., and Kohn, K. V. Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute Anticancer Drug Screen and correlations with growth-inhibitory potency of 123 anticancer agents. Cancer Res., *57:* 4285–4300, 1997.

15. Stadtmauer, E. A., O'Neill, A. Goldstein, L. J., Crilley, P. A., Mangan, K. F., Ingle, J. N., Brodsly, I., Martino, S., Lazarus, H. M., Erban, J. K., Sickles, C., and Glick, J. H. Conventional-dose chemotherapy compared with high-dose chemotherapy plus autologuous hematopoietic stem-cell transplantation for metastatic breast cancer. N. Eng. J. Med., *342:* 1069–1076, 2000.

16. Physicans Desk Reference, Medical Economics Company, Inc., 2001.

17. Carter, S. K. Principles of cancer chemotherapy. *In*: G. H. Theilen and B. R. Madewell (eds.), Veterinary Cancer Medicine, pp. 167–182. Philadelphia: Lea & Febiger, 1987.

18. Wong, B. K., DeFeo-Jones, D., Jones, R. E., Garsky, V. M., Feng, D-M. Oliff, A., Chiba, M., Ellis, J. D., and Lin, J. H. PSA-specific and non-psa-specific conversion of a psa-targeted peptide conjugate of doxorubicin to its active metabolites. Drug Metab. Dispos., *29:* 313–318, 2001.

19. DiPaola, R. S., Rinehart, J., Neminaitis, J., Ciardella, M., Goodin, S., Adams, N., Williams, A., Schwartz, M., Winchell, G., Wichersham, M., Deutsch, P., Dula, D., Gleason, D., and Yao, S. A phase I and II trial of a psa activated peptide-doxorubicin conjugate, with and without prednisone, in patients with hormone refractory prostate cancer. Am. Soc. Clin. Oncol., Abs 727, 2001.

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