Plasmin-Activated Prodrugs for Cancer Chemotherapy. 1. Synthesis and Biological Activity of Peptidylacivicin and Peptidylphenylenediamine Mustard

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Many tumors contain elevated levels of plasminogen activator and thus produce elevated levels of the protease plasmin in the milieu of the tumor. We have hypothesized, therefore, that it should be possible to prepare peptidyl prodrug derivatives of anticancer drugs that would be locally activated by tumor-associated plasmin. As an initial test of this hypothesis, we synthesized the peptidyl prodrugs of the anticancer drugs ($\alpha S,5S$)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin, AT-125) and N,N-bis(2-chloroethyl)-p-phenylenediamine (phenylenediamine mustard) by mixed anhydride coupling of the parent drug with the protected tripeptide, Boc-D-Val-Leu-Lys(Boc)-OH, followed by deprotection with trifluoroacetic acid. The prodrugs showed an increased selective in vitro cytotoxicity for Rous sarcoma virus transformed chicken embryo fibroblasts (which produce elevated levels of plasminogen activator) compared to nontransformed fibroblasts (which produce low levels of plasminogen activator). In the presence of the plasmin inhibitor, p-nitrophenyl p'-guanidinobenzoate at 2 μg/mL, the selectivity of the phenylenediamine mustard prodrug was reduced, but there was no effect on the cytotoxicity of the free drug. Furthermore, the prodrug analogue D-valylleucyl-D-lysylphenylenediamine mustard (in which L-Lys has been replaced by D-Lys) was inactive. Finally, the prodrug derivative of acivicin did not display selective toxicity for transformed cells when the cells were cultured in plasminogen-free medium. These results suggest that plasmin hydrolysis is necessary for the activation of the prodrugs. The prodrugs were tested in vivo for antitumor activity. The prodrug of acivicin, like acivicin itself, was inactive against the B16 melanoma, a murine tumor that produces high levels of plasminogen activator. This prodrug was active against the M5076 carcinoma, a tumor that displays only moderate levels of plasminogen activator; however, despite the fact that the prodrug was 2- to 3-fold less toxic on a molar basis than acivicin, there was no evidence of an increased therapeutic index. The prodrug of phenylenediamine mustard was also slightly less toxic than the parent drug, but again there was no evidence for an improved therapeutic index against the B16 tumor.

A major problem in cancer chemotherapy today is the severe host toxicity exhibited by most of the anticancer drugs due to their poor selectivity toward cancer cells. One approach to improving the selectivity of such agents involves the synthesis of prodrugs that are themselves inactive but are designed to be activated selectively in the vicinity of the tumor by a specific tumor-associated enzyme.

Considerable evidence exists that many transformed cells display a high level of proteolytic activity,[1] due to the presence of membrane-bound serine proteinases, plasminogen activators. Although plasminogen activators are found in normal cells, substantially increased levels are found in both virally and chemically transformed cells.[1,2] Several studies recently have also demonstrated increased amounts of plasminogen activators in human neoplasms.[3] Plasminogen activators act on the serum zymogen plasminogen to give rise to another proteolytic enzyme, plasmin, and as a result of this cascade phenomenon, a small amount of plasminogen activator could be responsible for the generation of large amounts of plasmin, which might lead to a high level of proteolytic activity in the vicinity of the tumor.

Recently, we have made an attempt to exploit this feature of malignant cells through the use of specific prodrugs of cancer chemotherapeutic agents, and we have reported that two anticancer drugs with distinct modes of action, viz., ($\alpha S,5S$)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin, AT-125) and N,N-bis(2-chloroethyl)-p-phenylenediamine (phenylenediamine mustard), derivatized with the tripeptide D-Val-Leu-Lys, show an improved cytotoxicity in cultures of Rous sarcoma virus transformed chick embryo fibroblasts compared to normal fibroblasts.[4] The increased selective cytotoxicity exhibited by these agents was presumed to arise from the selective hydrolysis of the prodrugs in the cultures of the transformed cells, due to the elevated levels of plasmin produced by the
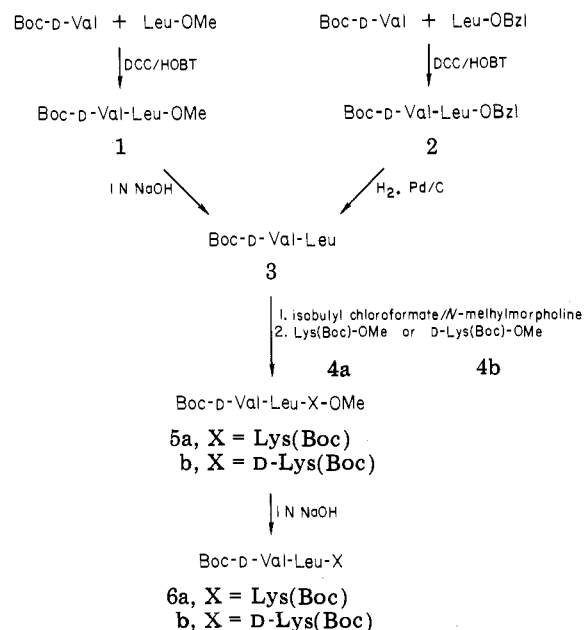
increased amounts of plasminogen activator in these cultures. In this report, we describe the synthesis of these prodrugs and present definitive evidence that the selective cytotoxicity we have observed in cell culture is due, in fact, to the selective activation of the prodrugs by plasmin. We

Scheme I

(1) Quigley, J. in "Surfaces of Normal and Malignant Cells"; Hynes, R. O., Ed.; Wiley: Chichester, U.K., 1979; p 247.
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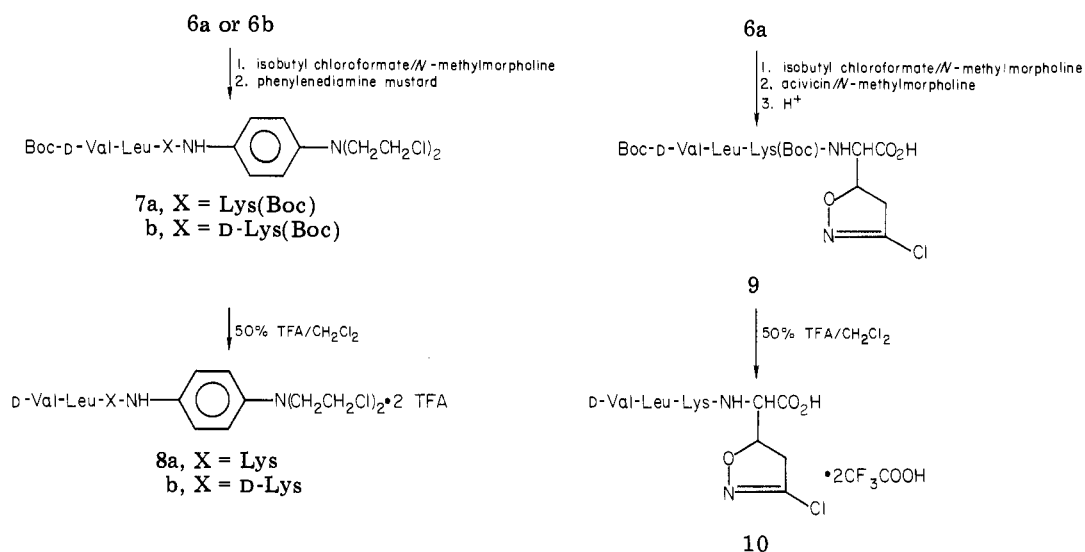
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Scheme II

	acivicin	acivicin prodrug	phenylene-diamine mustard	phenylene-diamine mustard prodrug
normal cells	8	210	14	70
transformed cells	7	40	12	10

^{a} Cytotoxicity data are given as the ED_{50}, the dose (μM) producing 50% inhibition.[4] The acivicin and its prodrug were tested for their ability to inhibit [^{3}H]thymidine incorporation into DNA. The mustard and its prodrug were tested for their ability to inhibit cell growth.
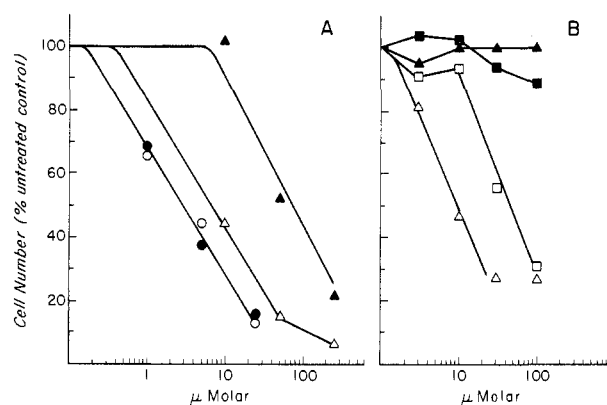

Figure 1. D-Valylleucyllysylphenylenediamine mustard requires proteolytic activation for selective cytotoxicity. (A) Protection of transformed cells from the action of D-valylleucyllysyl-phenylenediamine mustard by p-nitrophenyl p'-guanidino-benzoate. Chicken embryo cells transformed by Rous sarcoma virus were treated with the protease inhibitor at 2 μg/mL for 0.5 h prior to the addition of the drugs. Cytotoxicity was measured as previously described:[4] (○) phenylenediamine mustard; (●) phenylenediamine mustard plus p-nitrophenyl p'-guanidino-benzoate; (△) D-valylleucyllysylphenylenediamine mustard; (▲) D-valylleucyllysylphenylenediamine mustard plus p-nitrophenyl p'-guanidinobenzoate. (B) D-Valylleucyl-D-lysylphenylenediamine mustard is not cytotoxic: (△) D-valylleucyllysylphenylenediamine mustard, transformed cells; (□) D-valylleucyllysylphenylenedi-amine mustard, normal cells; (▲) D-valylleucyl-D-lysyl-phenylenediamine mustard, transformed cells; (■) D-valyl-leucyl-D-lysylphenylenediamine mustard, normal cells.

Results

Synthesis. Two tripeptide specifiers, D-Val-Leu-Lys and D-Val-Leu-D-Lys, were synthesized in suitably pro-tected forms as outlined in Scheme I. Boc-D-valine was condensed with leucine methyl ester by using N,N-di-cyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT)[5] to give the dipeptide methyl ester 1 (91%), which gave the corresponding dipeptide acid, Boc-D-Val-Leu (3) after saponification (72%). An alternative method for the preparation of 3 involved the synthesis of Boc-D-Val-Leu-OBzl (2), followed by catalytic hydrogenolysis over Pd/C to give 3 (89% overall). The dipeptide 2 was coupled either with N^ϵ-Boc-Lys-OMe (4a) or the corresponding D-lysine derivative (4b) via the isobutyl chloroformate and N-methylmorpholine-mediated mixed anhydride proce-dure,[6] to give good yields of the fully protected tripeptides 5a and 5b, respectively, after purification by silica gel column chromatography. Saponification of 5a and 5b furnished Boc-D-Val-Leu-N^ϵ-Boc-Lys (6a) and Boc-D-Val-Leu-N^ϵ-Boc-D-Lys (6b) (86% and 79%, respectively).

Syntheses of prodrugs of phenylenediamine mustard[7] (11) and acivicin (12) were accomplished as described in Scheme II. The protected tripeptide 6a was converted into a mixed anhydride with the aid of isobutyl chloro-formate and N-methylmorpholine and then allowed to react with either the free amine of phenylenediamine mustard (11) or the N-methylmorpholinium salt of acivicin (12) to yield the corresponding peptidyl derivatives 7a and 9. The peptidyl derivative 7b was also synthesized in a similar manner from 6b and 11. Removal of the Boc-protecting groups in each case was accomplished by treatment of protected derivatives with 50% trifluoroacetic acid–methylene chloride for 30 min at 25 °C, yielding the final prodrugs 8a, 8b, and 10 as their trifluoroacetate salts.

Biological Results. Neither acivicin nor phenylene-diamine mustard displayed significantly selective cyto-

also discuss the activity of these compounds in preliminary in vivo tests in experimental animal tumor systems.

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Table II. Inhibition of DNA Synthesis[a] by Acivicin (Aciv) and D-Valylleucyllysylacivicin

	ED_{50}, μM	
	aciv	D-Val-Leu-Lys-aciv
normal cells	6	250
transformed cells	5	70
normal cells, plasminogen free	4	300
transformed cells, plasminogen free	9	700

[a] Measured as inhibition of [³H]thymidine incorporation into acid-precipitable material.[4]

toxicity for malignant cells.[4] However, conversion of these agents to peptidyl prodrugs rendered them 5–10 times more cytotoxic for transformed cells than for the corresponding normal cells[4] (Table I). To determine whether this selective cytotoxicity was dependent on the activation of the prodrugs by the plasmin found in the transformed cell cultures, we tested the cytotoxicity of the peptidyl mustard and the free mustard in transformed cultures that were treated with the potent plasmin inhibitor *p*-nitrophenyl *p*-guanidinobenzoate (Figure 1A). It is apparent that the plasmin inhibitor had no effect on the activity of the free mustard but that it decreased the activity of the prodrug by a factor of 10. We also prepared a prodrug derivative in which the L-lysine residue of the peptide moiety was replaced by D-lysine. This substitution should block the activation of the prodrug by plasmin. The data in Figure 1B demonstrate that the substitution of D-lysine for L-lysine resulted in a drug that was inactive over the concentration range tested and showed no selectivity for transformed cells. Finally, we tested the activity of the peptidyl derivative of acivicin in medium from which the plasminogen had been removed by affinity chromatography (Table II). In the absence of plasminogen, the peptidylacivicin prodrug displayed a greatly decreased potency and no selectivity for transformed cells. Taken together, these data strongly suggest that our prodrugs are selectively cytotoxic to transformed cells because they are selectively activated by the plasmin in the transformed cell cultures.

In seeking to extend these results to the in vivo situation, we naturally wanted to choose a tumor test system that produced high levels of plasminogen activator and was also sensitive to the parent drugs. The standard transplantable murine tumors offer the obvious advantages that only a relatively small amount of drug is required for testing, and a great deal of work has been done in quantitating drug effectiveness in these systems. Accordingly, we screened a variety of transplantable mouse tumors for plasminogen activator activity and found that the B16 melanoma, which fortunately is quite sensitive to phenylenediamine mustard, had the highest level of activity of those tumors surveyed (manuscript submitted). The M5076 ovarian carcinoma had considerably less activity, but because it is the only transplantable mouse solid tumor in which acivicin shows much activity, we decided to test the acivicin prodrug against this tumor as well.

The results of a test of phenylenediamine mustard and the peptidylphenylenediamine mustard injected intraperitoneally against the intraperitoneal B16 tumor are presented in Table III. The peptidyl prodrug, while clearly active, did not demonstrate an improved selectivity in vivo compared to phenylenediamine mustard itself. A repeat of this test gave essentially similar data. We also tested the peptidylacivicin prodrug injected intraperitoneally against the subcutaneous B16 tumor, but neither the parent drug nor the prodrug derivative showed sig-

Table III. Comparison of Phenylenediamine Mustard and D-Valylleucyllysylphenylenediamine Mustard on Mice Bearing the Intraperitoneal B16 Melanoma

drug	dose per injection[a]		toxic deaths	% T/C[b]
	mg/kg	μmol/ kg		
phenylenediamine mustard	8	29.6	6/10	
	4	14.8	0/10	
	2	7.4	0/10	
	1	3.7	0/10	145
	0.5	1.85	2/10	127
	0.25	0.09	0/10	114
D-valylleucyllysyl- phenylenediamine mustard	23.8	29.7	0/10	
	11.90	14.9	0/10	
	5.95	7.4	0/10	159
	2.98	3.7	0/10	134
	1.49	1.86	1/10	109
	0.74	0.09	0/10	105

[a] Intraperitoneal daily dose days 1–9. [b] Survival of treated/survival of control animals. Blank values indicate % T/C < 100, presumably reflecting delayed toxicity of the drugs.

Table IV. Comparison of Acivicin with D-Valylleucyllysylacivicin in Mice Bearing Early M5076 Ovarian Carcinoma

drug	dose per injection[a]		toxic deaths	% T/C[b]
	mg/kg	μmol/ kg		
acivicin	16	89.6	10/10	
	8	44.8	10/10	
	4	22.4	2/10	143
	2	11.2	0/10	141
	1	5.6	0/10	125
D-valylleucyllysyl- acivicin	51.2	89.6	6/10	
	25.6	44.8	0/10	143
	12.8	22.4	0/9	125
	6.4	11.2	0/10	116
	3.2	5.6	1/10	112
	1.6	2.8	0/10	105

[a] Intraperitoneal daily dose (days 1–10) for early tumor group and daily (days 13–22) for advanced tumor group. [b] Survival of treated/survival of control animals. Median survival of control group was 28 days for the early tumor and 13 days from the initiation of drug treatment for the advanced tumor group.

Table V. Comparison of Acivicin with D-Valylleucyllysylacivicin in Mice Bearing Advanced Subcutaneous M5076 Ovarian Carcinoma

drug	dose per injection[a]		toxic deaths	% T/C[b]
	mg/kg	μmol/ kg		
acivicin	16	89.6	7/10	258
	8	44.8	4/10	258
	4	22.5	0/10	253
	2	11.2	0/10	235
	1	5.6	0/10	169
D-valylleucyllysyl- acivicin	51.2	89.6	4/10	223
	25.6	44.8	0/10	250
	12.8	22.4	0/10	200
	6.4	11.2	0/10	208
	3.2	5.6	0/10	208
	1.6	2.8	0/10	108

[a,b] See corresponding footnotes in Table IV.

nificant activity (data not shown). We therefore tested acivicin prodrug against the early and advanced M5076

carcinoma. Both compounds were significantly active in this test system (Tables IV and V), but there is no evidence that in vivo the prodrug offers any improvement over the parent compound.

Discussion

The goal of developing a selectively activated antitumor drug remains one of the most difficult in pharmacology. The severe toxicity of current drugs, however, continues to stimulate research toward this goal. Since many tumors produce high levels of the protease plasminogen activator[1] and thus are likely to possess high levels of plasmin in their milieu, we have hypothesized that plasmin-activated cytotoxic agents should preferentially kill malignant cells.[4] Indeed, in this paper we present evidence that such agents are selectively cytotoxic for malignant cells in culture and that their cytotoxic action is dependent on activation by plasmin.

However, attempts to demonstrate selective toxicity with our agents in vivo have thus far met with little success. Quite recently an excellent mathematical analysis of the problems associated with site-specific delivery of prodrugs in vivo was published by Stella and Himmelstein.[10] Their analysis clearly showed that the pharmacological properties of the drug, e.g., clearance rates, lipophilicity, etc., are apt to be as important determinants of site-specific prodrug delivery as are the detailed kinetics of drug activation. In the absence of detailed pharmacological studies of drug and prodrug distribution, it is difficult to state with certainty the reasons for the failure of our drugs to show substantially improved selectivity in vivo, but some general points are worth discussing.

Because degradation of peptides in the blood is usually only a minor process in vivo, degradation in the tissues being more important,[12] and because the tests in which we have measured increased selectivity of our drugs in vitro are carried out in the presence of serum, we did not expect that degradation of our drugs in serum would be a major route of drug metabolism. In order to check this point, we incubated the prodrug D-valylleucyllysylphenylenediamine mustard in heparin-treated rat plasma and measured the release of phenylenediamine mustard at 37 °C using the colorimetric assay described by Dalton and Hebborn.[13] We found less than 4% degradation in 1 h using a final plasma concentration of 50%. Dilution of the plasma to a final concentration of 10% produced 8% degradation in 1 h, thus suggesting the presence of plasma inhibitors of drug degradation (unpublished results). Since the chloroethyl group in the prodrug hydrolyze in water to nontoxic degradation products with a half-life of approximately 30 min at 37 °C (unpublished results), it does not seem likely that release of phenylenediamine mustard from the prodrug by enzymatic hydrolysis in blood could be a significant route of metabolic breakdown in vivo. Of course, this result does not imply that nonspecific activation of the prodrug might not occur at other extratumor sites. For example, some nonspecific liver esterases are capable of degrading aromatic amides.[11]

An additional difficulty that might have prevented us from observing improved selectivity in vivo concerns the design of the in vivo test itself. Because phenylenediamine mustard is apparently only active when injected ip against an ip tumor, we were compelled to use this route of injection in our in vivo test. Although we did see good activity by this route, it may be that such injection of both prodrug and drug directly into the vicinity of the tumor made it unlikely that we would observe selective activation, since in the control experiment the drug was already being delivered into the tumor site.

The failure of the acivicin prodrug to show improved selectivity in vivo may also be due to degradation at extratumor sites. Although aminopeptidases are unable to degrade peptides whose amino terminus is in the D configuration as here, it is quite possible that ubiquitous carboxypeptidases could release acivicin from the prodrug. An additional difficulty with the acivicin prodrug may be that the test system we were finally compelled to use, the M5076 carcinoma, is only weakly active fibrinolytically (manuscript submitted).

While the exact identity of the enzymes responsible for the apparent nonspecific activation of our drugs in vivo remains undetermined, one obvious candidate is plasmin itself. Since it is clear that fibrinolysis is not a unique property of tumor cells, one might expect a wide distribution of fibrinolytic activity in vivo such that any fibrinolytic activity due to the tumor itself would be negligible. However, numerous studies have shown that cancer patients and tumor-bearing animals often have elevated levels of fibrin degradation products in their plasma,[14] arguing that in vivo the tumors *are* a major source of fibrinolytic activity. We have confirmed these observations for the case of mice carrying the B16 tumor (manuscript submitted). Thus, a prodrug that was a perfect mimic of a fibrin clot quite possibly would be degraded preferentially at the site of a tumor. In future work we plan to explore still other prodrugs that we hope will be resistant to degradation by either amino- or carboxypeptidases and that will in addition have greater inherent stability than the phenylenediamine mustard prodrug. Pharmacological studies of these drugs will hopefully provide insight into how to translate in vitro selectivity into the far more difficult in vivo situation.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were determined on a Rudolph Autopol polarimeter. Elemental analyses were performed by the Microanalytical Service Laboratory of the School of Chemical Sciences, University of Illinois. Proton nuclear magnetic resonance spectra were recorded on Varian Associates spectrometers, Models HR-220 and EM-390, and are expressed as δ units (parts per million) relative to tetramethylsilane as internal standard. The ^{1}H NMR data are presented in the following form: δ value of signal (peak multiplicity, coupling constant, integrated number of protons, nature of protons). Mass spectra were recorded on a Varian Associates MAT 731 spectrometer.

Analytical thin-layer chromatography was carried out with Merck silica gel 60 F-254 plastic-backed plates, unless otherwise specified. Spots were visualized by UV, iodine vapor, or ninhydrin spray. Column chromatography was performed on a medium-pressure liquid chromatograph (MPLC) system, designed and built in our laboratory.[8] The essential components are a Milton-Roy Series D pump, an ISCO Model UA-5 ultraviolet detector, an Isco fraction collector, and silica gel 60 prepacked glass column (Lobar Lichoprep Si60 column) from E. Merck. The following TLC solvents were used: A, *n*-BuOH–acetic acid–water (5:1:1); B, methylene chloride–ethyl acetate (1:1), C, methylene chloride–2-propanol (8:2); D, 2-butanone–acetone–water (65:25:25).

All the amino acids used were of the L configuration unless otherwise specified, and the derivatives were purchased from

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Penninsula Laboratories (San Carlos, CA), Bachem Fine Chemicals (Torrance, CA), and Sigma Chemical Co. (St. Louis, MO). N^{ϵ}-tert-Butoxycarbonyllysine methyl ester was synthesized as described.[9] Acivicin (NSC-163501, AT-125) was a gift from B. Naff (National Cancer Institute) and the Upjohn Co. Phenylenediamine mustard was prepared as described in the literature.[7] Sera and media were from GIBCO. Dog plasmin was a gift from W. Mangel, Department of Biochemistry at this University. Cell culture and in vitro cytotoxicity testing were performed as described.[4]

General Procedures. Method A. Coupling Reaction with Dicyclohexylcarbodiimide/1-Hydroxybenzotriazole (HOBT). The amino acid ester hydrochloride or *p*-toluenesulfonate (10 mmol) was dissolved in dry DMF (15 mL) and neutralized with triethylamine (1.39 mL, 10 mmol) at 0 °C. Boc-protected amino acid (10 mmol) and HOBT·H_2O (10 mmol) were added, followed by the slow addition of a solution of DCC (11 mmol) in methylene chloride (25 mL). The reaction mixture was allowed to stir at 0 °C for 4 h and overnight at room temperature. The urea formed was removed by filtration, and the filtrate was evaporated to an oily residue. The residue was dissolved in ethyl acetate (150 mL) and washed successively with 10% citric acid, water, saturated $NaHCO_3$, and water. After the extract was dried over $MgSO_4$ and the solvent evaporated, the crude product was obtained as a solid. The final purification was achieved by recrystallization from the appropriate solvent.

Method B. Coupling Reaction with Isobutyl Chloroformate/N-Methylmorpholine (Mixed Anhydride Method). A suitably protected peptide carboxylic acid (1 mmol) was dissolved in dry THF (5 mL), and to this were added at -10 to -15 °C N-methylmorpholine (1 mmol) and isobutyl chloroformate (1 mmol). The mixture was stirred at that temperature for 15 min, followed by the addition of a solution of the amine component in DMF unless specified otherwise. Stirring was continued at that temperature for another 2 h and at room temperature overnight. Solvent was evaporated under reduced pressure, and the crude product was dissolved in ethyl acetate (50 mL) and washed with 10% citric acid or cold 1 N HCl, water, saturated $NaHCO_3$, and water. The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure to yield the crude product as a foam in most of the cases. The final purification was achieved by recrystallization or by silica gel 60 column chromatography (MPLC).

Method C. Saponification of Peptide Esters in Aqueous Acetone. To a solution of peptide ester (1.1 mmol) in a mixture of acetone (6 mL) and DMF (1 mL) was added 1 N NaOH (1.5 mL) and the mixture was stirred at room temperature for 1 h. Solvent was removed under reduced pressure without warming. The residue was dissolved in water (20 mL) and extracted with ether, and the aqueous phase was acidified with solid citric acid in the cold. The oily product that separated was extracted in ethyl acetate, washed with water, dried ($MgSO_4$), and concentrated to yield the peptide acid.

Method D. Removal of the *tert*-Butoxycarbonyl Protecting Group. A solution of protected peptide derivative (1 mmol) in 50% trifluoroacetic acid–methylene chloride reagent (5 mL) was stirred at room temperature for 30 min. The excess reagent was removed by evaporation under reduced pressure; the residue was triturated several times with dry ether, and the solid product obtained was finally dried overnight in vacuo over P_2O_5 and NaOH pellets. In most of the cases, the products, thus obtained, needed no further purification.

N^{ϵ}-(*tert*-Butoxycarbonyl)-D-lysine Methyl Ester Hydrochloride (4b). The title compound was prepared from D-lysine following a similar sequence of reactions as described[9] for the corresponding L-lysine analogue. N^{ϵ}-Boc-D-Lys-OH (mp 249–251 °C dec) was obtained in 68% yield from D-lysine, via N^{ϵ}-Boc-D-Lys-OH–Cu^{2+} complex (mp 253–254 °C). The above compound was then converted into N^{α}-Cbz-N^{ϵ}-Boc-D-Lys-OMe by sequential treatment with benzyl chloroformate and diazomethane: yield 73% (oil); NMR ($CDCl_3$) δ 1.45 (s, 9 H, *t*-Bu), 1.4–1.9 (m, 6 H, methylenes), 2.94 (q, J = 6.2 Hz, 2 H, methylene), 3.62 (s, 3 H, methyl), 4.1 (m, 1 H, methine), 4.7 (m, 1 H, NH), 5.0 (s, 2 H, benzylic CH_2), 5.5 (m, 1 H, NH), 7.3 (s, 5 H, aromatic). The above protected compound (20 mmol) was hydrogenated in methanol (100 mL) over 5% Pd/C (0.2 g), in the presence of 1 equiv of HCl, for 40 min at room temperature and atmospheric pressure to give

the title compound 4b: yield 96% (after crystallization from methanol–ether); mp 165–167 °C; R_f (A) 0.35. Anal. ($C_{12}H_{25}$-N_2O_4Cl) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucine Methyl Ester (1). This compound was prepared by coupling Boc-D-Val-OH with Leu-OMe·HCl according to *method A*: yield 91% (recrystallized from pentane); mp 89–90 °C; R_f (B) 0.75; NMR ($CDCl_3$) δ 0.94 (q, J = 6.2 Hz, 12 H, isopropyl), 1.45 (s, 9 H, *tert*-butyl), 1.62 (q, J = 5.9 Hz, 2 H, methylene) 1.9–2.1 (m, 2 H, methine), 3.7 (s, 3 H, methyl), 3.95 (q, J = 6.6 Hz, 1 H, CH), 4.4–4.7 (m, 1 H, CH), 5.04 (d, J = 8.2 Hz, 1 H, NH), 6.55 (d, J = 8.2 Hz, 1 H, NH); mass spectra (FD), *m/e* 331 (MH⁺), 330, 287, 275, 258. Anal. (C_{17}-$H_{32}N_2O_5$) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucine Benzyl Ester (2). The title compound was prepared from Boc-D-Val-OH and leucine benzyl ester *p*-toluenesulfonate by *method A*: yield 91% (after recrystallization from petroleum ether); mp 99–100 °C; R_f (B) 0.83; NMR ($CDCl_3$) δ 0.94 (q, J = 6.2 Hz, 12 H, isopropyl), 1.45 (s, 9 H, *tert*-butyl), 1.62 (q, J = 5.9 Hz, methylene) 2.0–2.3 (m, 2 H, CH), 3.95 (q, J = 6.8 Hz, 1 H, CH), 4.5–4.8 (m, 1 H, CH), 5.02 (d, J = 8.0 Hz, 1 H, NH), 5.15 (s, 2 H, benzylic), 6.5 (d, J = 8.2 Hz, 1 H, NH), 7.32 (s, 5 H, aromatic). Anal. ($C_{23}H_{36}N_2O_5$) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucine (3). A solution of 2 (10 mmol) in methanol (60 mL) was hydrogenated over 5% PdC (0.1 g) for 1 h under atmospheric pressure. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo and recrystallized from ether–hexane to afford the title compound: yield 98%; mp 143–144 °C; R_f (B) 0.35; NMR ($CDCl_3$) δ 0.94 (q, J = 6.2 Hz, 12 H, isopropyl), 1.45 (s, 9 H, *tert*-butyl), 1.65 (q, J = 5.9 Hz, 2 H, methylene), 1.95–2.25 (m, 2 H, CH), 4.0–4.3 (m,1 H, CH), 4.4–4.8 (m, 1 H, CH), 5.25–5.65 (m, 1 H, NH), 6.95 (m, 1 H, NH), 9.6–9.9 (m, 1 H, carboxy). Anal. ($C_{16}H_{30}N_2O_5$) C, H, N. Compound 3 was also prepared from 1 according to *method C* in 72% yield (after purification).

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^{ϵ}-(*tert*-butoxycarbonyl)lysine Methyl Ester (5a). The above compound was prepared by coupling 3 with N^{ϵ}-(*tert*-butoxycarbonyl)lysine methyl ester (4a) according to *method B*: mp 147–148 °C; yield 73% (after recrystallization from ethyl acetate–ether); R_f (B) 0.28; [α]$_D$ (*c* 1, MeOH) -39.7°; NMR ($CDCl_3$) δ 0.92 (q, J = 6.2 Hz, 12 H, isopropyl), 1.45 (s, 18 H, *tert*-butyl), 1.05–2.54 (m, 10 H, methylene, CH), 3.1 (m, 2 H, CH_2), 3.70 (s, 3 H, methyl), 4.05 (m, 1 H, CH), 4.57 (m, 2 H, α-CH), 5.24 (m, 1 H, NH), 6.2 (m, 2 H, NH), 6.95 (m, 1 H, NH); mass spectra (FD), *m/e* 573 (MH⁺), 516, 508. Anal. ($C_{28}H_{52}N_4O_8$) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^{ϵ}-(*tert*-butoxycarbonyl)-D-lysine Methyl Ester (5b). The title compound was synthesized from 3 and 4b by using *method B*: yield 68% (after recrystallization from ethyl acetate–ether); mp 144–145 °C; R_f (B) 0.29; NMR ($CDCl_3$) same as for 5a. Anal. ($C_{28}H_{52}N_4O_8$) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^{ϵ}-(*tert*-butoxycarbonyl)lysine (6a). The title compound was prepared from 5a using *method C*: yield 86% (amorphous solid); R_f (C) 0.71; [α]$_D$ (*c* 1, MeOH) -27.9°; NMR ($CDCl_3$) δ 0.94 (q, J = 6.2 Hz, 12 H, isopropyl), 1.10–2.45 (m, 28 H, *tert*-butyl, methylenes and CH), 3.05 (m, 2 H, methylene), 3.80 (m, 1 H, CH), 4.1 (m, 1 H, CH), 4.5 (m, 1 H, CH), 5.15 (m, 1 H, NH), 5.8 (m, 1 H, NH), 6.2 (m, 1 H, NH), 6.9 (m, 1 H, NH), 9.85 (m, 1 H, COOH). Anal. ($C_{27}H_{50}N_4O_8$) C, H, N.

N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^{ϵ}-(*tert*-butoxycarbonyl)-D-lysine (6b). The title compound was obtained from 5b according to the *method C*: yield 79%; mp 95–100 °C (amorphous solid); NMR ($CDCl_3$) same as for 6a. Anal. (C_{27}-$H_{50}N_4O_8$) C, H, N.

N-[N^{α}-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^{ϵ}-(*tert*-butoxycarbonyl)lysyl]-N',N'-bis(2-chloroethyl)-*p*-phenylenediamine (7a). To a solution of 6a (1.2 g, 2.15 mmol) in dry THF (9 mL) were added N-methylmorpholine (0.24 mL, 2.15 mmol) and isobutyl chloroformate (0.28 mL, 2.15 mmol) at -15 °C under nitrogen. After 15 min of stirring, a solution of phenylenediamine mustard (0.9 g, 3.33 mmol) and N-methylmorpholine (0.37 mL, 3.33 mmol) in a mixture of DMF (7 mL) and deaerated water (3 mL) was added. The reaction mixture was then stirred at that temperature for an additional 2 h and

at room temperature overnight. The solvent was evaporated under reduced pressure, and the residue was dissolved in ethyl acetate (100 mL), washed with saturated NaHCO$_3$, water, and cold 1 N HCl, and dried (MgSO$_4$). Upon removal of solvent, the crude product was obtained as a foam (pink), which was decolorized and crystallized from ethyl acetate–ether to give 1.3 g of a white solid (75%): mp 164–165 °C; R_f (B) 0.29; NMR (CDCl$_3$) δ 0.95 (q, J = 6.2 Hz, 12 H, isopropyl), 1.15–2.3 (m, 28 H, *tert*-butyl, methylene, CH), 2.95–3.15 (m, 2 H, methylene), 3.6 (t, J = 6 Hz, 8 H, methylenes), 4.25–5.2 (m, 4 H, α-CH and NH), 6.6 (m, 2 H, NH), 7.1 (m, 1 H, NH), 7.4 (AA′BB′ pattern, J = 8.5 Hz, 4 H, aromatic); mass spectra (FD), m/e 744 (MH$^+$). Anal. (C$_{37}$H$_{62}$N$_6$O$_7$Cl$_2$) C, H, N.

N-[N^α-(*tert*-Butoxycarbonyl)-D-valylleucyl-N^ϵ-(*tert*-butoxycarbonyl)-D-lysyl]-N',N'-bis(2-chloroethyl)-p-phenylenediamine (7b). The title compound was prepared from 6b and phenylenediamine mustard according to the procedure described for 7a: yield 66% (crystallized from ethyl acetate–ether); mp 115–117 °C; NMR (CDCl$_3$) same as for 7a. Anal. (C$_{37}$H$_{62}$N$_6$O$_7$Cl$_2$) C, H, N.

N-(D-Valylleucyllysyl)-N',N'-bis(2-chloroethyl)-p-phenylenediamine Bis(trifluoroacetate) (8a). This compound was prepared from 7a according to *method D*: yield 94% (after crystallization from methanol–ether); mp 140–145 °C dec; R_f (2-butanone–acetone–water, 65:25:25) 0.24; NMR (Me$_2$SO-d_6) δ 0.94 (q, J = 6.2 Hz, 12 H), 1.32–2.15 (br m, 10 H), 2.9–3.1 (m, 2 H), 3.6 (t, J = 6 Hz, 8 H), 4.25–4.95 (m, 6 H), 5.4 (m, 4 H), 7.4 (AA′BB′ pattern, J = 8.5 Hz, 4 H); mass spectra (FD), m/e 574 [(MH)$^+$]. Anal. [C$_{27}$H$_{46}$N$_6$O$_3$Cl·2(CH$_3$COOH)·H$_2$O] C, H, N.

N-(D-valylleucyl-D-lysyl)-N,N-bis(2-chloroethyl)-p-phenylenediamine Bis(trifluoroacetate) (8b). This compound was prepared from 7b as described for 8a: yield 100%; mp 99–120 °C dec; NMR (Me$_2$SO-d_6) same as for 8a. Anal. [C$_{27}$H$_{46}$N$_6$O$_3$Cl$_2$·2(CF$_3$COOH)] C, H, N.

α-[N-(*tert*-Butoxycarbonyl)-D-valylleucyl-N-(*tert*-butoxycarbonyl)lysylamino]-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid (9). The title compound was prepared by mixed anhydride coupling of 6a with acivicin as described in *method*

B. The final product was obtained in 84% yield, after recrystallization from methylene chloride–ether; mp 184–186 °C; R_f (C); 0.59 NMR (CDCl$_3$) δ 0.9 (q, J = 6.2 Hz, 12 H, isopropyl), 1.05–2.32 (br m, 26 H, *tert*-butyl, methylenes), 2.40–2.88 (m, 2 H, CH$_2$), 3.1 (m, 2 H, CH$_2$), 3.4 (d, J = 9 Hz, 2 H, CH$_2$), 4.0 (m, 2 H), 4.4 (m, 2 H), 4.65 (m, 2 H), 5.1 (m, 2 H), 6.1 (m, 1 H), 7.78 (m, 1 H), 8.1 (m, 2 H). Mass spectra (FD), m/e 719 (MH$^+$), 686, 617, 559. Anal. (C$_{32}$H$_{55}$N$_6$O$_{10}$Cl) C, H, N.

α-[(D-Valylleucyllysyl)amino]-3-chloro-4,5-dihydro-5-isoxazoleacetic Acid Bis(trifluoroacetate) (10). The title compound was prepared in 100% yield from 9 according to *method D*: mp 154 °C dec; R_f (acetonitrile–0.1 M NH$_4$OAc, 7:3) 0.39 on C$_{18}$-reversed phase analytical thin-layer plates; NMR (Me$_2$SO-d_6) δ 0.92 (q, J = 6.2 Hz, 12 H), 1.25–2.24 (br m, 10 H), 3.1 (m, 2 H), 3.6 (d, J = 9 Hz, 2 H, CH$_2$), 4.1 (m, 2 H), 4.5 (m, 2 H), 4.8 (m, 2 H), 5.5 (m, 4 H); mass spectra, m/e 520 [(M + H)$^+$]. Anal. (C$_{26}$H$_{41}$N$_6$O$_{10}$F$_6$Cl) C, H, N, Cl.

Plasmin-Activated Prodrugs for Cancer Chemotherapy. 2. Synthesis and Biological Activity of Peptidyl Derivatives of Doxorubicin

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We have synthesized peptidyl prodrugs of doxorubicin (Dox) designed to be selective substrates of plasmin. Such prodrugs might be locally activated by the elevated levels of plasmin produced near many solid tumors under the action of tumor-associated plasminogen activators. One such prodrug, 3′-(D-Val-Leu-Lys)-Dox, was obtained via a mixed-anhydride coupling with isobutyl chloroformate between the protected peptide Fmoc-D-Val-Leu-N^ϵ-Fmoc-Lys-OH and doxorubicin, followed by removal of the Fmoc groups with anhydrous ammonia. Compared to doxorubicin, the prodrug showed about a 7-fold improved selective cytotoxicity against chicken embryo fibroblasts transformed with the Rous sarcoma virus (which produce high levels of plasminogen activator) compared to normal cells (which produce low levels of plasminogen activator). However, the prodrug was a very poor plasmin substrate, and although in vivo tests against the murine B16 melanoma showed that the prodrug was active, the maximum T/C obtained was less than that achieved by doxorubicin even at 25 times the molar concentration of prodrug. Qualitatively similar results were obtained for a far more hydrophobic prodrug, 3′-(Boc-Val-Leu-Lys)-Dox. These results demonstrate that peptidyl prodrugs of doxorubicin designed as plasmin substrates are more selective anticancer agents in vitro than doxorubicin itself but that the bulky anthracycline moiety probably prevents efficient plasmin-catalyzed conversion to the active parent drug, so that, in their present form, these drugs are not potent enough to allow a determination as to whether or not they are more selective in vivo.

Currently there is a renaissance of interest in the idea of designing potential site-specific anticancer prodrugs based on the rationale that tumors that contain a high level of some specific enzyme might convert the prodrug to the pharmacologically active drug in the vicinity of the tumor,

resulting in lower drug concentrations at sites of limiting toxicity.[1,2] We have suggested[3] that the plasmin activity

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