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. ($C_{37}H_{62}N_6O_7Cl_2$) C, H, N.

***N*-[*N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-D-valylleucyl-*N*$^{\epsilon}$-(*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. ($C_{37}H_{62}N_6O_7Cl_2$) 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_2SO-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. [$C_{27}H_{46}N_6O_3Cl\cdot2(CH_3COOH)\cdot H_2O$] 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_2SO-d_6) same as for **8a**. Anal. [$C_{27}H_{46}N_6O_3Cl_2\cdot2(CF_3COOH)$] 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), 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. ($C_{32}H_{55}N_6O_{10}Cl$) 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_4OAc, 7:3) 0.39 on C_{18}-reversed phase analytical thin-layer plates; NMR (Me_2SO-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. ($C_{26}H_{41}N_6O_{10}F_6Cl$) C, H, N, Cl.

Acknowledgment. This work was supported by Grants CA 23498 and CA 12467 from the National Cancer Institute and Research Career Development Award CA 00092 to M.J.W. and by Grant 29837 from the National Cancer Institute to P.L.C. We are indebted to Drs. Jacqueline Plowman of the National Cancer Institute, Randall Johnson of Arthur D. Little Inc., and Patrick McGovern of the Upjohn Co. for their help in carrying out the in vivo testing. We also thank Margaret Bruesch and Barbara Larrain for technical assistance with the in vitro tests.

Registry No. 1, 78981-67-6; 2, 84559-70-6; 3, 74201-99-3; 4a, 3017-32-1; 4b, 66494-53-9; 5a, 78981-68-7; 5b, 84559-71-7; 6a, 74202-00-9; 6b, 84559-72-8; 7a, 84559-73-9; 7b, 84559-74-0; 8a, 84559-75-1; 8b, 84559-77-3; 9, 78981-69-8; 10, 84621-36-3; 11, 2067-58-5; 12, 42228-92-2; N^{ϵ}-Boc-D-Lys-OH, 31202-69-4; N^{ϵ}-Boc-D-Lys-OH–Cu^{2+}, 51320-80-0; N^{α}-Cbz-N^{ϵ}-Boc-D-Lys-OMe, 84559-78-4; Boc-D-Val-OH, 22838-58-0; Leu-OMe·HCl, 7517-19-3; plasminogen, 9001-91-6; plasmin, 9001-90-5; D-lysine, 923-27-3; leucine benzyl ester *p*-toluenesulfonate, 1738-77-8.

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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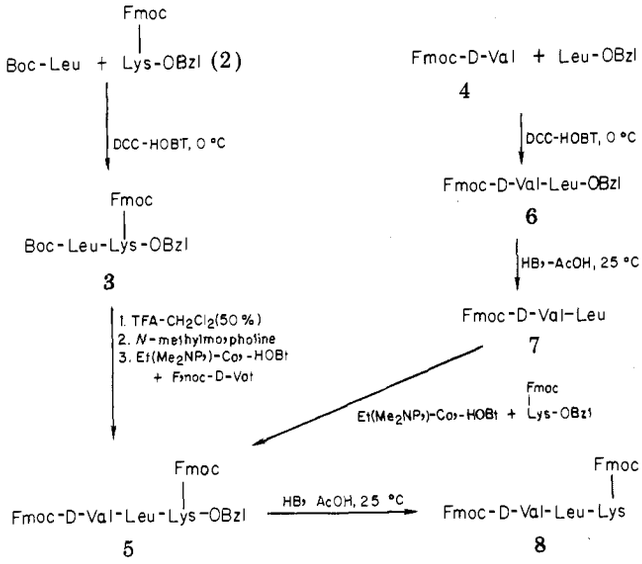
Scheme I



associated with tumors producing elevated levels of plasminogen activator provides a particularly attractive target for such an approach. First of all, high levels of plasmin activity would result from such a cascade and would be confined to the tumor milieu by ubiquitous plasmin inhibitors. Secondly, it is possible to design highly specific substrates for plasmin and similar proteolytic enzymes by incorporating a short peptide sequence into the substrate, which can then be selectively activated by a specific protease.[4,5]

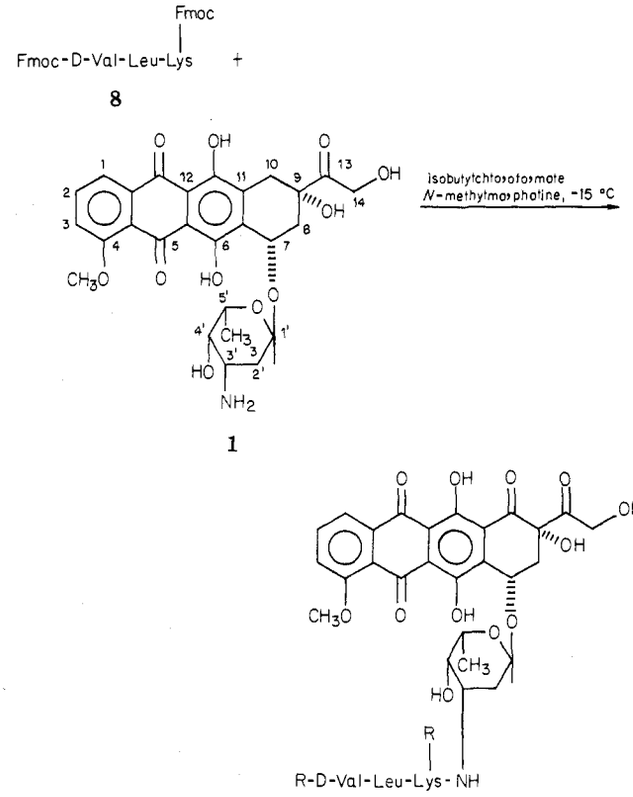
In the preceding paper,[3] showed that the peptide specifier D-Val-Leu-Lys, when attached via an amide linkage to either the antimetabolite acivicin or the alkylating agent phenylenediamine mustard, converted these drugs into prodrugs that showed improved selectivity in vitro compared to the parent drugs when tested against transformed and normal chicken embryo fibroblasts. While to date we do not have in vivo evidence that selective activation can be achieved by such an approach, it seemed worthwhile to synthesize similar prodrug derivatives of other anticancer drugs in order to explore the role that the parent drug plays in determining the success or failure of our efforts to achieve selective activation. In searching for other drugs that might be susceptible to similar modification, we focused on the drug doxorubicin (Dox) because of its great clinical importance. We expected that peptide derivatives of this drug, acylated on the amino group of daunosamine, would be far less toxic than the parent anthracycline. We report here that such peptide derivatives are more selective in vitro than the parent drugs and that in vivo they are far less toxic. However, the poor potency of the prodrugs undoubtedly would limit their utility and has prevented us from determining whether or not they show any improvement in therapeutic gain in vivo.

Results

Chemical Synthesis. Because of the sensitivity of doxorubicin (1) toward acidic conditions, the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) protecting group[6] was used in the preparation of the peptidyl derivatives (**10** and **17**). The synthesis of the Fmoc-protected tripeptide **8** was accomplished by the route shown in Scheme I. Boc-Leu-N^{ϵ}-Fmoc-Lys-OBzl (**3**), obtained in 68% by coupling Boc-Leu and N^{ϵ}-Fmoc-Lys-OBzl (**2**) with dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBT), was deprotected with trifluoroacetic acid, and the free amine thus obtained was coupled with Fmoc-D-Val (**4**) in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide [(Et(Me$_2$NPr)-Car] hydrochloride and HOBT, forming **5** in 82% yield. In an alternative route to **5**, Fmoc-D-Val (**4**) was coupled with the leucine benzyl ester by using DCC and HOBT to give **6** in 55% yield. Removal of the benzyl ester from **6** with 27% HBr–AcOH at 25 °C gave the dipeptide acid **7** in 98% yield. This acid was then coupled with N^{ϵ}-Fmoc-Lys benzyl ester (**2**) via the Et(Me$_2$NPr)-Car and HOBT procedure, yielding the protected tripeptide **5** in 52% yield. The tripeptide acid Fmoc-D-Val-Leu-N^{ϵ}-Fmoc-Lys (**8**) was obtained in 95% yield from **5**, after removal of the benzyl ester with 27% HBr–AcOH at 25 °C.

To prepare the peptidyl doxorubicin, we converted the peptidyl acid (**8**) into a mixed anhydride with isobutyl chloroformate and *N*-methylmorpholine and then allowed it to react with the free amine of doxorubicin (**1**), yielding the corresponding peptidyl derivative **9** in 69% yield (Scheme II). Attempted removal of the Fmoc groups from **9** by using piperidine[7] or morpholine[8] under various conditions resulted in incomplete deprotection. However, the Fmoc protecting groups were smoothly removed by treatment of **9** with anhydrous liquid ammonia for 3 h at

Scheme II



9, R = Fmoc
10, R = H (D-Val-Leu-Lys-Dox)
11, R = Tfa

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Table I. Cytotoxicity[a] of Doxorubicin (1) and D-Val-Leu-Lys-Dox (10) in Cultures of Normal or Rous Sarcoma Virus Transformed Chicken Embryo Fibroblasts

time of treatment	drug	cells	ED_{50},[b] μM	selectivity index[c]	improvement[d]
5 h	1	normal	0.9	0.36	5.8×
		transformed	2.5		
	10	normal	54	2.1	
		transformed	26		
24 h	1	normal	0.10	0.56	6.8×
		transformed	0.18		
	10	normal	3.0	3.8	
		transformed	0.8		

[a] Cytoxicity measured as inhibition of [^{3}H]thymidine incorporation into DNA, as described in ref 3a. [b] ED_{50} = concentration giving 50% inhibition of [^{3}H]thymidine incorporation. [c] Selectivity index = ED_{50} for normal cells/ED_{50} for transformed cells. [d] Improvement = selectivity index for prodrug/selectivity index for parent drug.

−33 °C. The deprotected product, 3′-(D-Val-Leu-Lys)-Dox (**10**) was isolated as the dihydrochloride salt.

In an earlier attempt to produce 3′-(D-Val-Leu-Lys)-Dox (**10**), we utilized the peptide derivative Tfa-D-Val-Leu-N^{ϵ}-Tfa-Lys in which the amino groups were protected with the base labile trifluoroacetate group.[9] This tripeptide was coupled with doxorubicin (1) by the isobutyl chloroformate and *N*-methylmorpholine-mediated mixed anhydride procedure, giving **11** in 48% yield. Attempts to remove the Tfa protecting groups from **11** with 3 equiv of tetramethylguanidine[10] or 0.1 N NaOH at 25 °C for 12 h produced partially deprotected material that still contained one Tfa group.[11] More prolonged treatment of **11** under the above conditions produced a complex mixture of products resulting from the degradation of **11**. Similar results were obtained with sodium methoxide. Aqueous NH_3 also failed to deprotect **11** and formed a blue-colored product that was later identified as the 5-imino derivative of **11**.[12]

The synthesis of 3′-*N*-(Boc-Val-Leu-Lys)-Dox (**17**), a lipophilic analogue of **10**, was accomplished as outlined in Scheme III. The dipeptide ester Boc-Val-Leu-OBzl (**12**) was synthesized in 88% yield according to the method describing for the corresponding D-valine analogue. Catalytic hydrogenation of **12** over Pd/C produced the dipeptide acid **13** (98% yield), which was then reacted with *N*-hydroxysuccinimide and DCC at 0 °C to give the corresponding active ester[13] (86% yield). Treatment of **14** with the sodium salt of N^{ϵ}-Fmoc-Lys at 25 °C gave the protected tripeptide acid **15** in 67% yield. Attempted use of any tertiary ammonium salt of N^{ϵ}-Fmoc-Lys in the above reaction led to substantial reduction in the yield of **15**. This protected tripeptide acid (**15**) was then coupled with the doxorubicin, via the mixed-anhydride procedure described for **9**, forming **16** in 58% yield. Treatment of **16** with anhydrous liquid NH_3, as described for **10**, produced 3′-*N*-(Boc-Val-Leu-Lys)-Dox (**17**) (89%), isolated as the monohydrochloride.

Biological Testing. In Figure 1 we show a comparison of the cytotoxicity of D-Val-Leu-Lys-Dox and Dox against

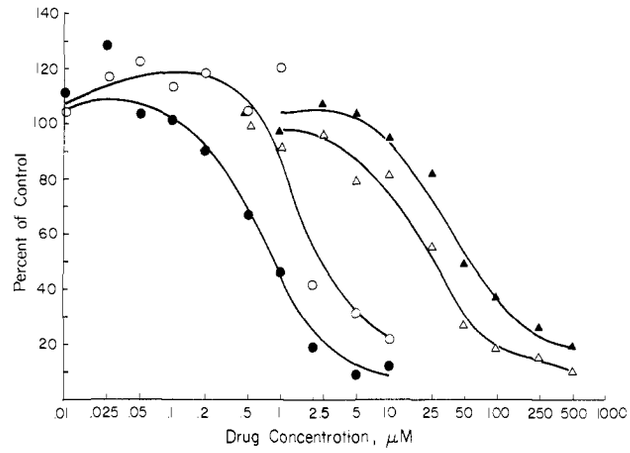
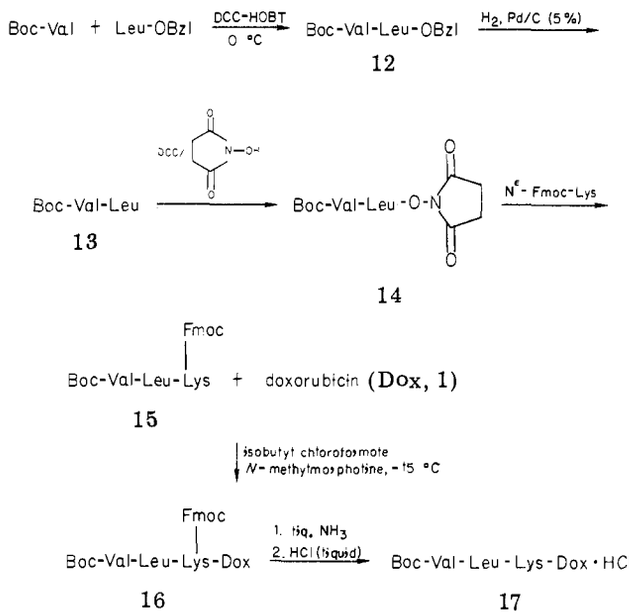


Figure 1. Cytotoxicity of doxorubicin and D-Val-Leu-Lys-Dox in cultures of normal or Rous sarcoma virus-transformed chicken embryo fibroblasts. Cells were treated with drug or prodrug for 5 h before determination of the rate of [^{3}H]thymidine incorporation into DNA, as described in ref 3a: circles, doxorubicin; triangles, D-Val-Leu-Lys-Dox; filled symbols, normal cells, open symbols, transformed cells.

Scheme III



normal and Rous virus transformed chicken embryo fibroblasts. In this experiment the cells were exposed to the drugs for 5 h. In Figure 2 we show the results of a similar test in which the cells were exposed to the drugs for 24 h. It is interesting to note that in both cases Dox was slightly more toxic to normal than to transformed cells, while the

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(11) The elemental analysis of the product obtained after treatment of **11** with base suggested the presence of one Tfa group in the molecule. Moreover, the product was found not to be a substrate for plasmin or trypsin. This further suggested that the Tfa group present in the product was probabply on the ε-amino group of Lys in the peptide.
(12) Recently, an analogous method has been used for the synthesis of 5-iminodaunorubicin (a blue-colored product): See Tong, G. L.; Henry, D. W.; Acton, E. M. *J. Med. Chem.* **1979**, *22*, 36.
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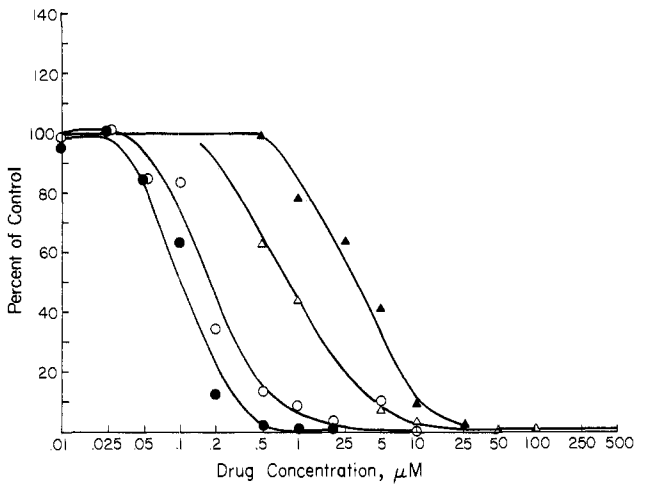

Figure 2. Cytotoxicity of doxorubicin and D-Val-Leu-Lys-Dox in cultures of normal or Rous sarcoma virus transformed chicken embryo fibroblasts. Cells were treated with drug or prodrug for 24 h before determination of the rate of [^{3}H]thymidine incorporation into DNA, as described in ref 3a: circles, doxorubicin; triangles, D-Val-Leu-Lys-Dox; filled symbols, normal cells; open symbols, transformed cells.

reverse was true for the prodrug. The ED_{50} for inhibition of DNA synthesis by the two drugs is displayed in Table I. Increasing the duration of drug treatment lowered the ED_{50} proportionately. However, the relative toxicity of the drugs for normal vs. transformed cells was not significantly affected by the duration of treatment. This is reflected in the fact that the degree of selective killing of transformed cells in culture (which we term the "selectivity index", in analogy with the term "therapeutic index" used in vivo studies) was similar at both treatment times. The results indicate at both times the prodrug showed about a 6-fold overall improvement in selective cytotoxicity to transformed as compared to normal cells relative to Dox. This degree of improvement in selective cytotoxicity is similar to the improvement we have seen with other drugs that we have converted to plasmin-activatable prodrugs. However, this improved selectivity of the Dox prodrug has only been obtained at the cost of a greatly decreased potency of the prodrug, which reflects the fact that, in contrast to our earlier prodrugs of the alkylating agent phenylenediamine mustard or the antimetabolite acivicin (AT-125),[3a] the Dox prodrug is a very poor plasmin substrate. Even at 24 h, only a few percent of the prodrug has been converted to Dox itself, as judged either by cytotoxicity (Figure 2) or thin-layer chromatography (data not shown).

Despite the poor potency of the prodrug, we decided to test the drug in vivo against the murine B16 melanoma, a tumor that we know to be fibrinolytically active (manuscript in preparation), because we felt that if the prodrug had a sufficiently long serum halftime, there might be sufficient time to activate a significant amount of the prodrug—hopefully at the tumor. The results are shown in Table II. As can be seen, the prodrug did show significant antitumor activity in this system but only at the highest doses tested. Approximately 40-fold the molar concentration of Dox is needed to produce a similar T/C. Because of this poor potency, we do not know if the T/C obtained at this maximal dose of the prodrug is the highest that could be obtained in this system.

The poor in vivo potency we saw with the prodrug suggested that it did not remain in the body long enough to be significantly activated. This was confirmed in a preliminary experiment in which we measured the excretion of the prodrug into the bile of Sprague–Dawley rats. More than 80% of the injected dose as measured by total fluorescence was eliminated in 5 h (data not shown). Furthermore, when the mice treated with 9 mg/kg of Dox, which died on day 17, were dissected, a marked distention of the small intestine was noted. This is characteristic of the response to toxic doses of doxorubicin. In contrast, no evidence of such toxicity was seen in mice that died on the same day even at the highest doses of prodrug used (equivalent to 75 mg/kg of Dox. This again suggests there was a very little activation of the prodrug in vivo.

Table II. In Vivo Antitumor Activity of Doxorubicin (1) and Peptidyl Prodrugs (10 and 17) against the B16 Melanoma

drug	dose,[a] mg/kg	% T/C[b]
1	1	111
	3	137
	6	158
	9	90
	12	68
10	5	105
	15	103
	30	97
	45	105
	60	113
	75	121
17	5	105
	15	100
	30	110
	60	100

[a] Dose is given as doxorubicin equivalents injected intraperitoneally on days 5, 9, and 13 after intraperitoneal tumor implantation. [b] Median survival of treated/control animals. Decreased survival at higher doses is attributed to drug toxicity.

We wondered if a less polar prodrug might show better activity and, therefore, investigated the prodrug 3′-(Boc-L-Val-Leu-Lys)-Dox in which the D-Val amino acid of the previous drug was replaced by a protected L-amino acid. This prodrug also showed a roughly 6-fold improvement in selective cytotoxicity in vitro, again with poor potency (data not shown), and when tested in vivo showed no real activity at up to 60 mg of Dox equiv/kg (Table II). (On a day 1 schedule, significant activity was seen with T/C = 135 at 21.3 mg/kg, but Dox itself at 4 mg/kg showed T/C = 289 on this schedule.)

Discussion

As noted above, the ability of the doxorubicin prodrugs to demonstrate improved in vivo selectivity could not be fairly tested because of the poor potency of the prodrug. It is interesting that this low potency was predicted quite accurately by the in vitro tests against the chicken embryo fibroblasts. While one can hardly be certain that some alteration of the peptide would not convert the prodrug into a considerably better plasmin substrate, this does not seem very likely. Almost certainly the presence of the large doxorubicin moiety at the critical bond that must be hydrolyzed for drug activation is a severe impediment to the hydrolysis by endopeptidases such as plasmin. On the other hand, these results do not imply that other amino acid or peptidyl derivatives of doxorubicin might not offer advantages to the parent molecule, and, indeed, Trouet and co-workers[14] have reported some promising in vivo results with compounds such as leucyldaunorubicin. This

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compound, however, is probably hydrolyzed to daunorubicin by aminopeptidases in vivo—a possibility that could not occur with our drugs due to either the D configuration of the terminal valine group or the presence of the *t*-Boc protecting group. Levin and Sela[15] also reported some derivatives of daunomycin with improved chemotherapeutic activity, but in most cases their drugs also did not have blocked amino termini; so again, liberation of the parent drug by ubiquitous aminopeptidases seems likely. The improvement seen with these drugs, while obviously desirable, may well be due to changes in the overall pharmacokinetics of the drugs rather than due to any particular drug targeting.

One possible way to increase the potency of our prodrugs might be to insert a spacer linkage between the peptide and the drug moiety so as to facilitate peptide hydrolysis. The spacer might consist of amino acids that could subsequently be hydrolyzed by lysosomal proteases[16] or low pH[17] as have recently been described, or alternatively, the spacer might be of the type we have referred to as a self-immolative connector,[10] which is designed to release the active drug spontaneously following enzymatic hydrolysis of the bond between the peptide and the connector.

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 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 in the field-desorption (FD) mode.

Analytically 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.

All the amino acids used were of the L configuration unless otherwise specified, and the derivatives were purchased from Penninsula Laboratories (San Carlos, CA), Bachem Fine Chemicals (Torrance, CA), and Sigma Chemical Co. (St. Louis, MO). Doxorubicin was a gift from Drs. Lednicer (Adria Laboratories) and F. Arcamone (Farmitalia). Sera and media were from GIBCO. Dog plasmin was a gift from W. Mangel, Department of Biochemistry, University of Illinois. Cell culture and in vitro cytotoxicity testing were performed as described.[3a] Fmoc-D-Val was prepared according to procedures used to synthesize Fmoc-L-Val.[18]

N^{ϵ}-Fmoc-Lys Benzyl Ester *p*-Toluenesulfonate (2). N^{α}-Boc-N^{ϵ}-Fmoc-Lys (3.65 g, 7.79 mmol) was dissolved in a 1:1 solution of trifluoroacetic acid in methylene chloride (36 mL), and the mixture was stirred at room temperature for 30 min. The solvent was removed by evaporation in vacuo, and the residue was purified by trituration with dry ether. After filtering and drying over P_2O_5 and NaOH, the N^{ϵ}-Fmoc-Lys trifluoroacetate salt was obtained as a white solid (3.35 g), mp 120–125 °C dec.

The above lysine derivative was mixed with dry benzyl alcohol (20 mL), *p*-toluenesulfonic acid (1.4 g), and benzene (30 mL) and refluxed for 24 h, with the removal of water by azeotropic distillation. At the end of this period, the reaction mixture was chilled, and dry ether (200 mL) was added to precipitate the crude product. After standing at 0 °C for 2 days, the product was collected by filtration: yield 3.8 g (78%) (after crystallization from methanol–dry ether); mp 104–105 °C; R_f (2-butanone–acetone–water, 8:1:1) 0.39; NMR (Me_2SO-d_6) δ 1.3 (m, 4 H, methylenes), 1.8 (m, 2 H, methylene), 2.32 (s, 3 H, CH_3), 2.95 (m, 2 H, methylene), 3.32 (m, 1 H, CH), 4.08 (m, 1 H, CH), 4.25 (s, 2 H, CH_2), 5.20 (s, 2 H, CH_2, benzylic), 7.0–7.95 (br m, 14 H, aromatic and NH), 8.32 (br s, 3 H, NH_3^+). Anal. ($C_{35}H_{38}N_2SO_7$) C, H, N.

Boc-Leu-Lys(Fmoc) Benzyl Ester (3). To a solution of **2** (2.5 g, 3.96 mmol), *N*-methylmorphine (0.44 mL, 3.96 mmol), Boc-Leu (0.92 g, 3.96 mmol), and HOBT·H_2O (0.67 g, 4.35 mmol) in dry DMF (15 mL) was added dropwise a solution of DDC (0.90 g, 4.35 mmol) in dry methylene chloride (15 mL) at 0 °C under stirring. After the addition was complete, the reaction mixture was stirred for 5 h at 0 °C and at 25 °C for an additional 12 h. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate, washed with saturated $NaHCO_3$, water, 10% citric acid, and water. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The crude product was purified by silica gel column chromatography with methylene chloride–ethyl acetate (10:1): yield 1.8 g (68%) (after crystallization from MeOH); mp 159–160 °C; R_f (methylene chloride–ethyl acetate, 2:1) 0.63; NMR ($CDCl_3$) δ 0.90 (d, J = 6 Hz, 6 H, isopropyl), 1.42 (s, 9 H, *tert*-butyl), 1.12–1.92 (br m, 8 H, methylenes), 3.12 (q, J = 7 Hz, 2 H, methylene), 4.10–4.80 (br m, 5 H, methylene and methines), 5.05 (m, 1 H, NH), 5.15 (s, 2 H, benzylic CH_2), 6.78 (m, 1 H, NH), 7.33 (s, 5 H, phenyl), 7.20–7.80 (m, 8 H, aromatic); mass spectra (FD), m/e 647 (M^+). Anal. ($C_{37}H_{49}N_3O_7$) C, H, N.

N^{α}-Fmoc-D-Val-Leu-N^{ϵ}-Fmoc-Lys Benzyl Ester (5). Method A. The protected dipeptide acid Fmoc-D-Val-Leu (**7**; 0.35 g, 0.77 mmol) and N^{ϵ}-Fmoc-Lys-OBzl·TsOH (**2**; 0.49 g, 0.77 mmol), *N*-methylmorpholine (0.085 mL, 0.77 mmol), and HOBT·H_2O (0.12 g, 0.77 mmol) were dissolved in dry DMF (12 mL) and cooled to 0 °C. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (1.63 g, 0.85 mmol) was added to 0 °C, and the mixture was stirred at that temperature for 4 h and 25 °C for an additional 10 h. The solvent was removed in vacuo, the residue was triturated with water and filtered, and the filtrate was dried. The crude product was finally crystallized from methanol: yield 0.36 g (52%); mp 218–220 °C; R_f (methylene chloride–ethyl acetate, 3:1) 0.63.

Method B. The dipeptide Boc-Leu-Lys(Fmoc)-OBzl (**3**; 1.40 g, 2.09 mmol) was dissolved in 50% trifluoroacetic acid in methylene chloride (20 mL) and stirred at 25 °C for 30 min. The solvent was removed in vacuo, and the residue was triturated with dry ether, yielding the solid product: R_f (methylene–chloride–methanol, 9:1) 0.31. This dipeptide amine trifluoroacetate salts was dissolved in dry DMF (15 mL), neutralized with *N*-methylmorpholine (0.23 mL, 2.09 mmol) at 0 °C, and mixed with Fmoc-D-Val (**4**; 0.71 g, 2.09 mmol) and HOBT·H_2O (0.35 g, 2.29 mmol), followed by the addition of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (0.44 g, 2.30 mmol) at the same temperature. The reaction mixture was stirred at that temperature for 4 h and at 25 °C for 6 h. Workup and crystallization afforded 1.5 g of the tripeptide: yield 82%; mp 217–218 °C; NMR (Me_2SO-d_6) δ 0.90 (q, J = 6.3 Hz, 12 H, isopropyl), 1.25–1.95 (m, 10 H, methylene and CH), 2.10 (m, 1 H, CH), 2.95 (m, 2 H, methylene), 3.85 (m, 1 H, CH), 4.23 (m, 8 H, methylenes, CH and NH), 5.15 (s, 2 H, benzylic CH_2), 6.32 (m, 1 H, NH), 6.80 (m, 1 H, NH), 7.37 (s, 5 H, phenyl), 7.35–7.89 (br m, 17 H, aromatic and NH); mass spectra (FD), m/e 892 (M^+). Anal. ($C_{54}H_{60}N_4O_8$) C, H, N.

N^{α}-Fmoc-D-Val-Leu Benzyl Ester (6). To a solution of leucine benzyl ester *p*-toluenesulfonate (0.393 g, 1 mmol) and *N*-methylmorpholine (0.109 mL, 1 mmol) in dry DMF (6 mL) were added Fmoc-D-Val (**4**; 0.34 g, 1 mmol) and HOBT·H_2O (0.3 g, 2 mmol). The reaction mixture was cooled to 0 °C, and a solution of DCC (0.23 g, 1.1 mmol) in dry DMF (4 mL) was added under stirring. Stirring was continued at 0 °C for 4 h and at 25 °C for 10 h. Dicyclohexylurea was removed by filtration, and the filtrate

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was concentrated in vacuo. The oily residue was dissolved in EtOAc, washed successively with saturated $NaHCO_3$, water, cold 1 N HCl, and water, dried ($MgSO_4$), and evaporated in vacuo. The crude product was crystallized from 50% ether–hexane: yield 0.31 g (55%); mp 134–36 °C; R_f (CH_2Cl_2–EtOAc, 9:1) 0.53; NMR ($CDCl_3$) δ 0.95 (q, J = 6.3 Hz, 12 H, isopropyl), 1.45 (m, 3 H, methylene and CH), 2.15 (m, 1 H, CH), 4.35 (m, 4 H, Fmoc CH_2 and α-CH), 4.71 (m, 1 H, Fmoc, CH), 5.18 (s, 2 H, benzylic CH_2), 5.40 (m, 1 H, NH), 6.45 (m, 1 H, NH), 7.35 (s, 5 H, phenyl), 7.25–7.85 (m, 8 H, aromatic); mass spectra (FD), m/e 543 [$(MH)^+$]. Anal. ($C_{33}H_{38}N_2O_5$) C, H, N.

N^{α}-Fmoc-D-Val-Leu (7). The protected dipeptide **6** (0.543 g, 1 mmol) was dissolved in 27% HBr in acetic acid (5 mL) and stirred at room temperature for 3 h. The reaction mixture was then poured into a beaker containing crushed ice and stirred vigorously. The white precipitate that formed was collected by filtration, washed with water, ether, and dried in vacuo over P_2O_5. Recrystallization from ether afforded the dipeptide acid in 98% yield: mp 137–139 °C; NMR ($CDCl_3$) δ 0.92 (q, J = 6.2 Hz, 12 H), 1.62 (m, 3 H), 2.15 (m, 1 H), 4.35 (m, 4 H), 4.75 (m, 1 H), 5.7 (m, 1 H), 6.8 (m, 1 H), 7.42–4.9 (m, 8 H, aromatic); mass spectra (FD), m/e 453 [$(MH)^+$], 452 (M^+). Anal. ($C_{26}H_{32}N_2O_5$) C, H, N.

N^{α}-D-Fmoc-D-Val-Leu-N^{ϵ}-Fmoc-Lys (8). Fmoc-D-Val-Leu-Lys(Fmoc)-OBzl (**5**; 1.5 g, 1.68 mmol) was dissolved in a solution of 27% HBr in acetic acid (10 mL), and the solution was stirred at 25 °C for 2.5 h. After workup, as described for **7**, the tripeptide acid was obtained as a white solid: yield 1.28 g (95%); mp 217–219 °C; NMR (Me_2SO-d_6) δ 0.85 (q, J = 6.4 Hz, 12 H, isopropyl), 1.2–2.0 (m, 10 H, methylenes and CH), 2.08 (m, 1 H, CH), 2.94 (m, 2 H, methylene), 3.88 (m, 1 H, CH), 4.25 (m, 8 H, methylenes, CH and NH), 7.30 (t, J = 7.1 Hz, 4 H, aromatic), 7.40 (t, J = 7.3 Hz, 4 H, aromatic), 7.51 (m, 2 H, NH), 7.71 (m, 4 H, aromatic), 7.87 (d, J = 7.4 Hz, 4 H, aromatic), 8.2 (m, 1 H, carboxy); mass spectra (FD), m/e 803 [$(MH)^+$]. Anal. ($C_{47}H_{54}N_4O_8$) C, H, N.

3′-N-(N^{α}-Fmoc-D-Val-Leu-N^{ϵ}-Fmoc-Lys)-Dox (9). To a solution of **8** (0.6 g, 0.75 mmol) in dry DMF (6 mL) were added N-methylmorpholine (0.082 mL, 0.75 mmol) and isobutyl chloroformate (0.096 mL, 0.75 mmol). The mixture was stirred for 5 min, and a solution of doxorubicin hydrochloride (0.44 g, 0.75 mmol) and N-methylmorpholine (0.082 mL, 0.75 mmol) in dry DMF (6 mL) was added at that temperature. After stirring for 2 h at −15 °C, the reaction mixture was allowed to warm up to room temperature and stirred at that temperature for 12 h, protected from light. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate, washed successively with water, 5% citric acid, and water, dried ($MgSO_4$), and concentrated in vacuo. The product was crystallized from ethyl acetate in the cold, giving 0.68 g (69%) of the product: mp 177–179 °C; NMR ($CDCl_3$) (selected peaks only) δ 0.95 (m, 15 H, isopropyl and methyl), 3.95 (s, 3 H, OCH_3), 4.76 (s, 2 H, 10-H_2), 7.20–7.9 (m, 21 H, aromatic and NH); mass specra (FD), m/e 1328 [$(MH)^+$]. Anal. ($C_{74}H_{81}N_5O_{18}$) C, H, N.

3′-N-(D-Val-Leu-Lys)-Dox·2HCl (10). To dry distilled liquid NH_3 (100 mL) was added a solution of **9** (102.4 mg) in dry THF (5 mL) and the deep-blue mixture was stirred (protected from light) at the relux temperature of liquid NH_3 for 3 h. The ammonia was evaporated under a stream of nitrogen, and the residue was triturated with dry ether and filtered, giving the product as red solid. This was then dissolved in methanol (5 mL) and filtered. To the filtrate was added a solution of HCl in dioxane (6.5 N) (24 μL), and the resulting solution was concentrated. The product was finally precipitated by the addition of dry ether and filtered, and the filtrate was dried over P_2O_5 and KOH pellets to give 69 mg (93%) of **10**: mp 190–196 °C dec; R_f (methylene chloride–methanol, 1:1) 0.54; NMR (Me_2SO-d_6) (selected peaks only) δ 0.94 (m, 15 H, isopropyl and methyl), 4.02 (s, 3 H, OCH_3), 4.76 (s, 2 H, 10-H_2), 7.38 (m, 1 H, H-2, aromatic), 7.72 (m, 1 H, H-4, aromatic), 7.91 (d, J = 7.8 Hz, 1 H, H-1, aromatic); mass spectra (FD), m/e 884 [$(M - 2HCl)^+$] 858. Anal. ($C_{44}H_{63}N_5O_{14}Cl_2{\cdot}4H_2O$) C, H, N.

N^{α}-Boc-Val-Leu-Benzyl Ester (12). The title compound was synthesized by coupling Boc-Val (5 g, 23 mmol) with leucine benzyl ester p-toluenesulfonate (9.04 g, 23 mmol) via the DCC–HOBT method, as described for the corresponding Fmoc-D-Val analogue (**6**): yield 8.5 g (88%) (an oil); NMR ($CDCl_3$) δ 0.94 (q, J = 6.2 Hz, 12 H), 1.45 (s, 9 H, *tert*-butyl), 1.62 (q, J = 6 Hz, 2 H, methylene), 2.18 (m, 2 H, CH), 3.95 (q, J = 6.6 Hz, 1 H, CH), 4.5–4.8 (m, 1 H, CH), 5.02 (m, 1 H, NH), 5.15 (s, 2 H, CH_2 benzylic), 6.5 (m, 1 H, NH), 7.35 (s, 5 H, phenyl).

N^{α}-Boc-Val-Leu (13). The dipeptide ester **12** (5 g, 11.9 mmol) was hydrogenolyzed over Pd/C (5%) (0.2 g), in methanol (50 mL), for 45 min. The product was isolated, after removal of the catalyst by filtration, in 98% yield (3.85 g): mp 103–104 °C; R_f (methylene chloride–ether, 1:1), 0.45; NMR ($CDCl_3$) δ 0.92 (q, J = 6.2 Hz, 12 H, isopropyl), 1.45 (s, 9 H, *tert*-butyl), 1.65 (q, 5.9 Hz, 2 H, methylene), 2.10 (m, 1 H, CH), 4.15 (m, 1 H, CH), 4.6 (m, 1 H, CH), 5.25–5.65 (br m, 1 H, NH), 6.85 (m, 1 H, NH), 9.8 (br s, 1 H, carboxy). Anal. ($C_{16}H_{30}N_2O_5$) C, H, N.

N^{α}-Boc-Val-Leu N-Hydroxysuccinimide Ester (14). To a solution of **13** (3.66 g, 11 mmol) and N-hydroxysuccinimide (1.47 g, 12.8 mmol) in dry DMF (10 mL) was added dropwise a solution of DCC (2.64 g, 12.81 mmol) in dry methylene chloride (20 mL) at 0 °C. Upon completion of the addition, the reaction mixture was stirred at 0 °C for 6 h and allowed to stand at that temperature overnight. Dicyclohexylurea was removed by filtration, and the filtrate was concentrated in vacuo. The oily residue thus obtained was dissolved in ethyl acetate, washed with water, and dried ($MgSO_4$). Upon removal of the solvent, the product was obtained as a foam (7 g), which was crystallized from methylene chloride–hexane: yield 4.1 g (86%); mp 154–155 °C; R_f (methylene chloride–ether, 1:1) 0.74; NMR ($CDCl_3$) δ 0.95 (q, J = 6.6 Hz, 12 H), 1.45 (s, 9 H), 1.78 (dd, J = 9.8 and 3 Hz, 2 H), 2.2 (m, 2 H), 2.82 (s, 4 H, succinimide methylenes), 3.98 (q, J = 6.8 Hz, 1 H), 5.0 (m, 2 H), 6.65 (m, 1 H). Anal. ($C_{20}H_{33}N_3O_7$) C, H, N.

N^{α}-Boc-Val-Leu-N^{ϵ}-Fmoc-Lys (15). To the suspension of N^{ϵ}-Fmoc-Lys·F_3OAc (2.0 g, 4.82 mmol) in water (15 mL) containing $NaHCO_3$ (0.86 g, 9.65 mmol) was added a solution of **14** (2.0 g, 4.84 mmol) in dioxane (25 mL) at room temperature. After stirring for 2 h at that temperature, the reaction mixture became clear. Stirring was continued at room temperature for 24 h. A thick white precipitate formed during this period. The reaction mixture was diluted with water, chilled, and filtered. The crude product, after drying, was crystallized from ethyl acetate to give 2.2 g of **15** (76%): mp 170–172 °C; NMR (Me_2SO-d_6) δ 0.90 (q, J = 6.8 Hz, 12 H, isopropyl), 1.39 (s, 9 H, *tert*-butyl), 1.2–1.85 (br m, 10 H), 2.0 (m, 1 H, CH), 3.02 (m, 2 H), 3.80 (m, 1 H, NH)., 4.15–4.55 (m, 4 H), 6.55 (m, 1 H, NH), 7.20 (m, 1 H, NH), 7.30 (t, J = 7.2 and 7.0 Hz, 4 H, aromatic), 7.39 (t, J = 7.2 and 7.0 Hz, 4 H, aromatic, 7.66 (d, J = 7.2 Hz, 4 H, aromatic), 7.81 (d, J = 7.1 Hz, 4 H), 7.93 (m, 1 H, NH); mass spectra (FD), m/e 681 [$(MH)^+$]. Anal. ($C_{37}H_{52}N_4O_8$) C, H, N.

3′-N-(N^{α}-Boc-Val-Leu-N^{ϵ}-Fmoc-Lys)-Dox (16). A solution of **15** (0.2 g, 0.3 mmol) in dry DMF (5 mL) containing N-methylmorpholine (0.033 mL, 0.3 mmol) was added isobutyl chloroformate (0.039 mL, 0.3 mmol) at 15 °C. After the reaction mixture was stirred for 2 min at that temperature, a solution of doxorubicin hydrochloride (0.18 g, 0.3 mmol) and N-methylmorpholine (0.033 mL, 0.3 mmol) in dry DMF (3 mL) was added. The mixture was stirred at −15 °C for 4 h and at 25 °C for an additional 4 h. The solvent was removed in vacuo, the residue was triturated with 5% citric acid and filtered, and the filtrate was washed with water and dried. The crude product was finally crystallized from ethyl acetate: yield 0.21 g (58%); mp 171–175 °C; NMR ($CDCl_3$, 360 MHz) (selected peaks only) δ 0.91 (m, 15 H, isopropyl and CH_3), 1.42 (s, 9 H, *tert*-butyl), 1.25–2.0 (br m, 15 H, methylenes and methines), 4.02 (s, 3 H, OCH_3), 4.76 (s, 2 H, CH_2, 10-H_2), 7.35 (m, 6 H, aromatic), 7.56 (m, 2H, aromatic), 7.75 (m, 3 H, aromatic); mass spectra (FD), m/e 1206 [$(MH)^+$]. Anal. ($C_{64}H_{79}N_5O_{18}$) C, H, N.

3′-N-(N^{α}-Boc-Val-Leu-Lys)-Dox·HCl (17). The title compound was prepared from **16**, according to the method described for **10**, in 89% yield: mp 196–198 °C dec; NMR (Me_2SO-d_6) δ 0.90 (m, 15 H, isopropyl and methyl), 1.43 (s, 9 H, *tert*-butyl), 4.03 (s, 3 H, OCH_3), 4.76 (s, 2 H, 10-H_2), 7.41 (d, J = 9 Hz, 1 H, H-2 aromatic), 7.76 (m, 1 H, H-3, aromatic), 7.96 (d, J = 7.6 Hz, 1 H, H-1 aromatic); mass spectra (FD), m/e 984 [$(MH)^+$]. Anal. ($C_{49}H_{70}N_5O_{16}Cl$) C, H, N.

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Registry No. 1·HCl, 25316-40-9; **2**, 84624-14-6; **3**, 84624-15-7; **3**·TFA, 84624-16-8; **4**, 84624-17-9; **5**, 84624-18-0; **6**, 84624-19-1; **7**, 84624-20-4; **8**, 84624-21-5; **9**, 84624-22-6; **10**, 84624-23-7; **10** (free base), 84680-48-8; **12**, 24210-16-0; **13**, 27506-15-6; **14**, 84642-33-1; **15**, 84624-24-8; **16**, 84624-25-9; **17**, 84624-26-0; **17** (free base), 84680-49-9; N^{α}-Boc-N^{ϵ}-Fmoc-Lys, 84624-27-1; N^{ϵ}-Fmoc-Lys·TFA, 84624-29-3; Boc-Leu, 13139-15-6; Boc-Val, 13734-41-3; leucine benzyl ester *p*-toluenesulfonate, 1738-77-8; *N*-hydroxysuccinimide, 6066-82-6; plasmin, 9001-90-5.

Cardioselectivity of β-Adrenoceptor Blocking Agents. 2. Role of the Amino Group Substituent

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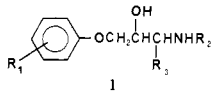
A series of 1-(aralkylamino)-3-(aryloxy)propan-2-ols were synthesized, and their apparent dissociation constants (K_{app}) were determined by using rat ventricular muscle (RVM) and rat lung membrane (RLM) preparations. Analysis of the binding studies suggests the existence of different modes of binding dependent on the presence or absence of the 4-substituent in the aryloxy ring and the nature of that ring. Without 4-substitution only one compound (**4**), bearing the 2-(2-methoxyphenoxy)ethyl substituent on the amino group, shows high cardioselectivity. Introduction of the 4-acylamido substituent into the phenoxy ring renders all compounds cardioselective. The cardioselective influence of 4-substitution is diminished or eliminated when the phenoxy ring is replaced by naphth-1-yloxy.

In our earlier paper[1] we defined cardioselectivity of β-adrenoceptor blocking agents at the molecular level as having a higher affinity to the β_1 than to the β_2 adrenoceptor. That definition, based on the physiological studies of the β adrenoceptors by Lands et al.,[2] requires the measurement of the apparent dissociation constants of the investigated blockers. Various researchers use different physiological testing methods, making structure–activity relationship studies difficult to compare. The use of an isolated receptor system allows a more exact look at the structural requirements for binding and the differences between the classes of receptors by eliminating obscuring factors such as blood clearance, metabolism, and distribution, which are encountered in in vivo studies.

The existing literature points to molecular alterations in the 1-amino-3-(aryloxy)propan-2-ols (I) that may lead to cardioselectivity. Those alterations are (1) 4-substitution (R_1) in the aryloxy group with a rigid substituent of at least three atoms in size;[3] (2) placement of an aralkyl,[4] aryloxyalkyl,[5] or alkyloxyalkyl[5] on the 1-amino group (R_2); or (3) stereospecific alkyl substitution on carbon 1 (R_3) in the propan-2-ol moiety.[6,7] The assumption that all three above-mentioned alterations do not change the site of binding with the receptors should permit "fine tuning", leading to a compound that incorporates the optimal groups in one molecule of superior cardioselectivity.



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To check a part of that assumption and to complement the existing knowledge of the structural differences between the receptors, we describe herein the synthesis of 15 1-(aralkyl)- or 1-[[(aryloxy)alkyl]amino]-3-(aryloxy)-propan-2-ols (Table I) and report the apparent dissociation constants of 26 β-adrenoceptors blockers (Table II).

Chemistry. As illustrated in Scheme I, the phenol substrates II (purchased or synthesized by well-known methods) were converted to epoxide intermediates III by using the conditions described by Shtacher.[8] The epoxides were purified by crystallization from ethyl acetate or column chromatography on silica gel with 10% MeOH and CH_2Cl_2. The reaction of the epoxides III with an excess of amine (1.4-fold) in boiling methanol gave the desired product IV. The purification of the products often required repeated preparative LC of their free bases and recrystallization of their salts with oxalic acid. The synthesized compounds are listed in Table I.

Pharmacology. The apparent dissociation constants (K_{app}, μM) of the β-adrenoceptor blockers used in this study were determined by the use of a competitive binding assay with (−)-[^{3}H]dihydroalprenolol. The assay and the preparation of rat lung membranes (RLM) have been described in detail previously.[9,10] We used a different procedure for the preparation of rat ventricular muscle receptor-rich membrane fragments (RVM) than in our previous reports;[1,10] the RVM was prepared by the method of Baker and Potter[11] (see Experimental Section). K_{app} values represent the average of at least six individual measurements on three different days with receptor fractions prepared freshly for that day. Apparent affinities of several previously reported compounds were noticed to increase. Such changes upon altering the method of tissue preparation are not uncommon.[12,13] The method of Baker and Potter eliminates the contractile proteins that account for a large degree of nonreceptor binding of the β blockers. Such nonreceptor binding can increase the K_{app} value by

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