to pH 5 with 1 N hydrochloric acid. The solid which formed was collected by filtration and purified by crystallization from 50% aqueous ethanol (150 mL, v/v) to afford 60 mg (63.0%) of the product, which was shown to be identical with that obtained by method 1 by UV, ^{1}H NMR, TLC, and MS.

4-Amino-9-(β-D-ribofuranosyl)pyrrolo[2,3-*d*:5,4-*d*']dipyrimidine (27). Method 1. Raney nickel (9.0 g, net weight) was added to a suspension of **6** (1.0 g, 2.86 mmol) in water (75 mL). The reaction mixture was heated at reflux for 2 h. The Raney nickel was removed by filtration, and the filter cake was washed with hot water (150 mL) and methanol (50 mL). The filtrate was evaporated in vacuo to furnish a white solid, which was recrystallized from water (100 mL) to give a sample for analysis: yield 300 mg (33%) of **27**; mp 300 °C dec; ^{1}H NMR (Me_2SO-d_6) δ 5.26 (t, 1, $H_{2'}$, $J_{2',1'}$ = 6.0 Hz, $J_{2',3'}$ = 6.0 Hz), 6.54 (d, 1, $H_{1'}$, $J_{1',2'}$ = 6.0 Hz), 7.66 (br s, 2, NH_2), 8.47 (s, 1, H_2), 9.00 (s, 1, H_5 or H_7), 9.60 (s, 1, H_5 or H_7). Anal. Calcd for $C_{13}H_{14}N_6O_4$: C, 49.06; H, 4.40; N, 26.42. Found: C, 49.01; H, 4.57; N, 26.40.

Method 2. The nucleoside **28** (500 mg, 1.68 mmol) was mixed with formamide (15 mL, 0.38 mmol), and the reaction mixture was heated at reflux temperature. After 2 h, the reaction mixture was allowed to cool to 25 °C, and the excess formamide was evaporated at 80 °C in vacuo. The resulting residual solid was dissolved in water (80 mL), and the solution was decolorized with charcoal. The charcoal was removed by filtration, and the filtrate was allowed to stand at 5 °C for 20 h. The gel-like substance which had formed was collected by filtration and triturated with hot ethanol (20 mL). The white solid that formed was collected by filtration to afford 250 mg (50%) of **27**. This product was shown to be identical by UV, ^{1}H NMR, and TLC with the product obtained by method 1.

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Amino Acid and Dipeptide Derivatives of Daunorubicin. 1. Synthesis, Physicochemical Properties, and Lysosomal Digestion

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The synthesis of amino acid and dipeptide derivatives of daunorubicin (DNR) is described. The binding-affinity parameters for DNA of those derivatives were determined by a spectral titration method. The affinity constants of the amino acid and dipeptide derivatives are, respectively, three and ten times lower than that of DNR. The susceptibility of those derivatives toward lysosomal peptidases was studied. It was found that the Leu and the Ala-Leu derivatives are the most rapidly hydrolyzed into DNR. It is concluded that Leu-DNR and Ala-Leu-DNR could act as prodrugs of DNR, which could be activated inside or in the close vicinity of tumor cells which display a high aminopeptidase activity.

Daunorubicin (DNR), widely used in the treatment of acute leukemia,[1,2] is composed of an anthracycline aglycon linked to an amino sugar. The amino group of DNR is important for its biological activity, since it stabilizes the intercalation of the aglycon between adjacent base pairs of DNA.[3]

N-Amino acid[18] and *N*-acyl amino acid derivatives of DNR[15] have been synthesized previously in order to achieve selective biological activity of the DNR molecule on the basis of the pH difference between tumoral and normal tissues and the lower p*K* values of the derivatives formed.[15] This structure-activity approach has not taken into account the stability of the compounds toward peptidases or the tissue and subcellular distribution of the drugs and their metabolites.

We have synthesized the *N*-amino acid and dipeptide derivatives of DNR listed in Table I as prodrugs of DNR. It was hoped to achieve a selective biological activity if these latent forms of DNR could be activated more selectively inside or in the close vicinity of those tumor cells which have been shown to display high aminopeptidase activity.[4-7]

In this publication, we describe the synthesis of these derivatives, their interaction in vitro with DNA, as well as their susceptibility toward lysosomal peptidases. In the following paper in this issue, we will report their uptake, metabolism, and in vitro and in vivo antitumoral activities using the murine L1210 leukemic cells as the experimental system.

Chemistry. The synthesis of the amino acid and dipeptide derivatives of DNR was performed in such a way as to avoid drastic deprotecting steps and racemization of the amino acids. In the case of hydrophilic amino acids, the amino function was blocked by a triphenylmethyl group according to Stekalatos et al.[8] and the carboxylic function was activated by esterification with *N*-hydroxysuccinimide according to Anderson et al.[9] The bulky, hydrophobic amino acids were linked with good yields by the *N*-carboxyanhydride method of Hirshmann.[10]

The authenticity and purity of the peptide derivatives were checked by elemental analysis, thin-layer chromatography, absorption, and infrared spectroscopy. Each

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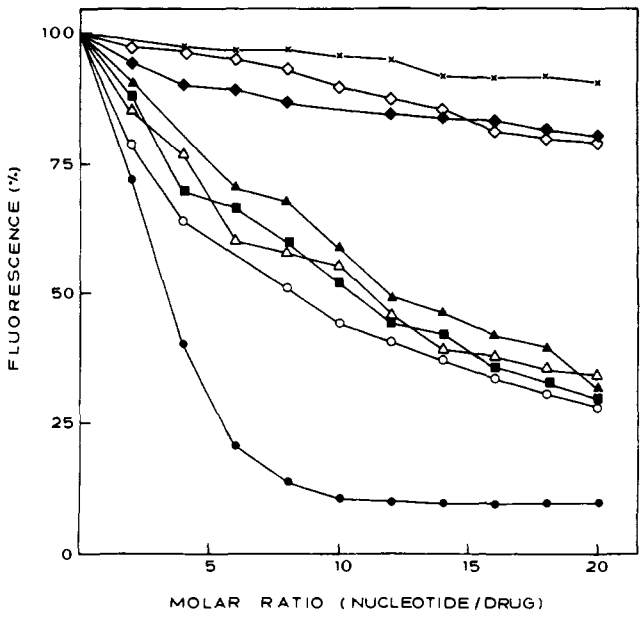

Figure 1. Effect of increasing concentrations of herring sperm DNA on the fluorescence of the anthracycline drugs. Drugs were at a final concentration of 0.35 μM, the concentration of herring sperm DNA ranging from 0 to 7 μM (molar ratio from 0 to 20): (●–●) DNR; (○–○) Gly-DNR; (■–■) Val-DNR; (▲–▲) Ala-DNR; (△–△) Leu-DNR; (◊–◊) Ala-Leu-DNR; (♦–♦) Leu-Ala-DNR; (x–x) Leu-Leu-DNR.

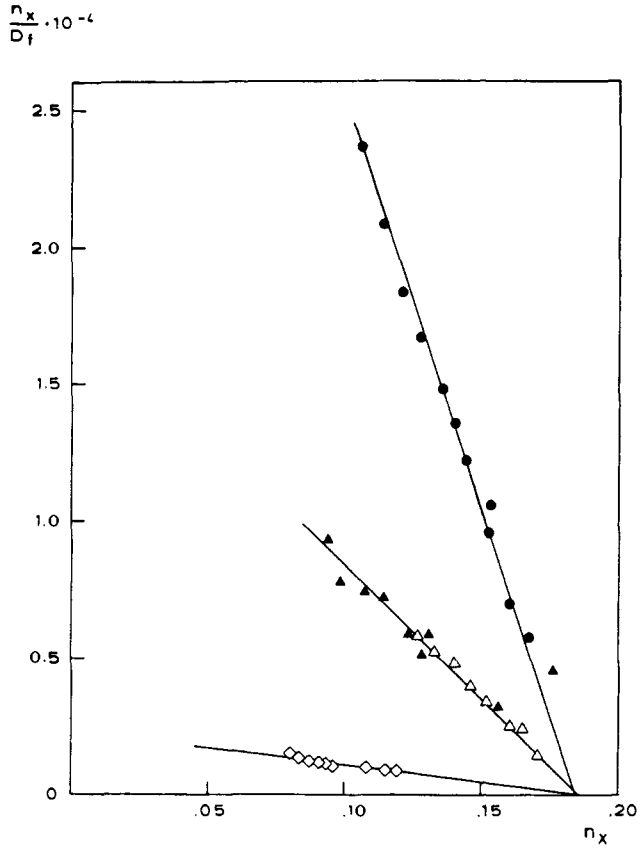

Figure 2. Scatchard plot of the binding of DNR, Leu-DNR, and Ala-Leu-DNR to herring sperm DNA in PBS at 22 °C, using the spectral titration method:[12] (●–●) DNR; (△–△) Leu-DNR; (◊–◊) Ala-Leu-DNR; (▲–▲) Ala-DNR. For details see text and Table II.

compound was found free of aglycons and DNR, as determined by thin-layer chromatography (TLC) using two eluent systems and by high-pressure liquid chromatography (LC).[11] The relative mobilities, absorption characteristics, and synthetic yields of the DNR derivatives are given in Table I.

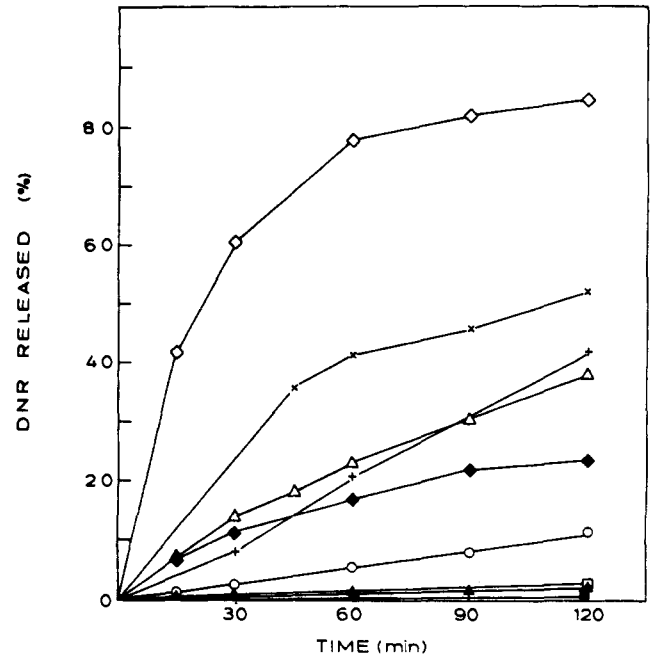

Figure 3. Digestion of the amino acid and dipeptide derivatives of DNR at pH 6.0 by a soluble fraction of purified lysosomes. Each sample incubated at 37 °C contained drug at 35 μM and lysosomal proteins at 0.5 mg/mL. After various times, aliquots were extracted and the DNR released was determined by high-pressure LC and fluorometry:[11] (△–△) Leu-DNR; (▲–▲) Ala-DNR; (□–□) Ile-DNR; (■–■) Val-DNR; (○–○) Gly-DNR; (◊–◊) Ala-Leu-DNR; (♦–♦) Leu-Ala-DNR; (x–x) Leu-Leu-DNR; (+–+) Gly-Leu-DNR.

Results

When compared to DNR, the amino acid and dipeptide derivatives are characterized by a lower molar extinction coefficient (Table I).

As illustrated in Figure 1, the quenching of the drug fluorescence induced by DNA is decreased in the case of the amino acid derivatives and nearly abolished for the dipeptide compounds. The nature of the amino acids has no significative influence.

To quantitate the binding of DNR and its derivatives with herring sperm DNA, we have used a spectral titration method based on the progressive hypochromic and bathochromic shifts induced by DNA in the visible absorption spectra of the drugs.[12]

The ϵ differences ($\epsilon_{475} - \epsilon_{505}$) are decreased three and six times for the amino acid and the dipeptide derivatives, respectively. The affinity constant (K_a) and the number of strong binding sites (η_{max}) of the various derivatives were estimated from the Scatchard plots based on the absorbancies measured at 475 nm for increasing DNA concentrations (Figure 2). As indicated in Table II, the affinity constants of the amino acid derivatives are decreased about three times, while those of the dipeptide compounds are reduced about ten times when compared with DNR.

In order to test the sensitivity of the amino acid and dipeptide derivatives to hydrolysis by lysosomal enzymes, they have been incubated for various periods of time at pH 4.5 and 6 in the presence of a soluble lysosomal fraction

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Table I. Comparison of the Chemical Properties and Synthetic Yields of the Anthracycline Drugs

compound	mp, °C	formula[a]	synthesis: starting amount, mmol	synthesis: method	synthesis: yield, %	chromatographic R_f of trityl intermediates: system I[b]	chromatographic R_f of trityl intermediates: system II[b]	absorption data[c]: λ_{max}, nm	absorption data[c]: ϵ_{max}	chromatographic R_f: system I[b]	chromatographic R_f: system II[b]
DNR	189 dec	$C_{27}H_{29}NO_{10}\cdot HCl$						477	9280	0.05	0.14
glycyl-DNR	206 dec	$C_{29}H_{32}N_2O_{11}\cdot HCl$	0.45	A	86	0.74	0.67	478	6320	0.04	0.05
L-alanyl-DNR	214 dec	$C_{30}H_{34}N_2O_{11}\cdot HCl$	0.49	A	95	0.82	0.90	478	6210	0.08	0.08
L-leucyl-DNR	201 dec	$C_{33}H_{40}N_2O_{11}\cdot HCl$	1.77	B	60			479	7030	0.31	0.24
L-valyl-DNR	210 dec	$C_{32}H_{38}N_2O_{11}\cdot HCl$	0.44	B	55			478	7460	0.26	0.16
L-isoleucyl-DNR	207 dec	$C_{33}H_{40}N_2O_{11}\cdot HCl$	0.44	B	43			479	6420	0.33	0.25
L-leucyl-L-leucyl-DNR	129 dec	$C_{39}H_{51}N_3O_{12}\cdot HCl$	0.44	B	49			479	8265	0.39	0.28
L-alanyl-L-leucyl-DNR	205 dec	$C_{36}H_{45}N_3O_{12}\cdot HCl$	1.00	C	63	0.76	0.70	477	8804	0.17	0.14
L-leucyl-L-alanyl-DNR	197 dec	$C_{36}H_{45}N_3O_{12}\cdot HCl$	0.33	B	63			483	8585	0.18	0.11
glycyl-L-leucyl-DNR	205 dec	$C_{35}H_{43}N_3O_{12}\cdot HCl$	0.50	A	81	0.79	0.71	481	8510	0.09	0.13

[a] Elemental analyses for C, H, N were within ±0.4% of theoretical values. [b] System I: $CHCl_3$–MeOH–H_2O (120:20:1, v/v); system II: CH_2Cl_2–MeOH–88% HCOOH–H_2O (85:15:2:1, v/v). [c] Absorption spectra were taken in 1-cm cells using 177 μM drug in PBS buffer, pH 7.4 and 25 °C.

Table II. Apparent Binding Affinity of the Anthracycline Drugs to Herring Sperm DNA at 25 °C in PBS Buffer, pH 7.4

compound	binding affinity: $K_a \times 10^{-6}$, M^{-1}	binding affinity: n_{max}
DNR	0.31	0.16
Gly-DNR	0.13	0.16
Ala-DNR	0.09	0.19
Leu-DNR	0.13	0.20
Val-DNR	0.11	0.18
Ile-DNR	0.11	0.19
Ala-Leu-DNR	0.02	0.17
Leu-Ala-DNR	0.04	0.12
Gly-Leu-DNR	0.03	0.14

purified from rat liver.[13] The kinetics of hydrolysis at pH 6 of these derivatives into DNR, as determined by high-pressure LC, are illustrated in Figure 3. Similar results were obtained at pH 4.5 and, with the exception of very small amounts of aglycon (less than 1%), no other transformation products, such as 13-hydroxy derivatives, could be detected. After 2 h of incubation, 35% of Leu-DNR was transformed into DNR, while only 10% of Gly-DNR, 2.5% of Ile-DNR and Ala-DNR, and trace amounts of Val-DNR were hydrolyzed into DNR. After 60 min, 80% of Ala-Leu-DNR was already recovered as DNR, while the other dipeptide derivatives were hydrolyzed more slowly.

The hydrolysis kinetics of Leu-Ala-DNR, Ala-Leu-DNR, and Gly-Leu-DNR into Ala-DNR, Leu-DNR, and DNR, illustrated in Figure 4, indicate that the transformation of the dipeptide derivatives into DNR and amino acid derivatives occurs at different relative rates and that a maximum of 25% of Leu-Ala-DNR can be converted into DNR. A similar experiment could not be performed with Leu-Leu-DNR because of the very poor separation obtained by high-pressure LC between Leu-DNR and Leu-Leu-DNR.

The hydrolysis of Leu-DNR and Ala-Leu-DNR into DNR as a function of pH is illustrated in Figure 5. In both cases the maximum hydrolytic activity is characterized by a broad spectrum from pH 4.5 to 6.0.

Discussion

As illustrated by the quenching of drug fluorescence and determined by the spectrophotometric assay of the binding constant, the affinity of the DNR derivatives for DNA is significantly reduced. At a nucleotide/drug ratio of 20, the fluorescence of the amino acid derivatives and of the dipeptide compounds are reduced to about 30 and 90%, respectively, as compared to 10% for DNR. These results are comparable to those obtained according to a similar technic by Levin and Sela[14] with Ala-DNR and Ala-Ala-DNR. The apparent affinity constant for DNA of the amino acid-DNR compounds is about three times lower than that of DNR, and a similar observation was made by Gabbay et al.[15] for Gly-DNR. As could be expected from the DNA–anthracycline interaction model proposed by Pigram[3] and Gabbay,[15] the affinity constants of the dipeptide derivatives for DNA are reduced even more and are about tenfold lower than that of DNR. The intercalation of the aglycon moiety between the nucleotide base pairs and/or the interaction of the drug amine function

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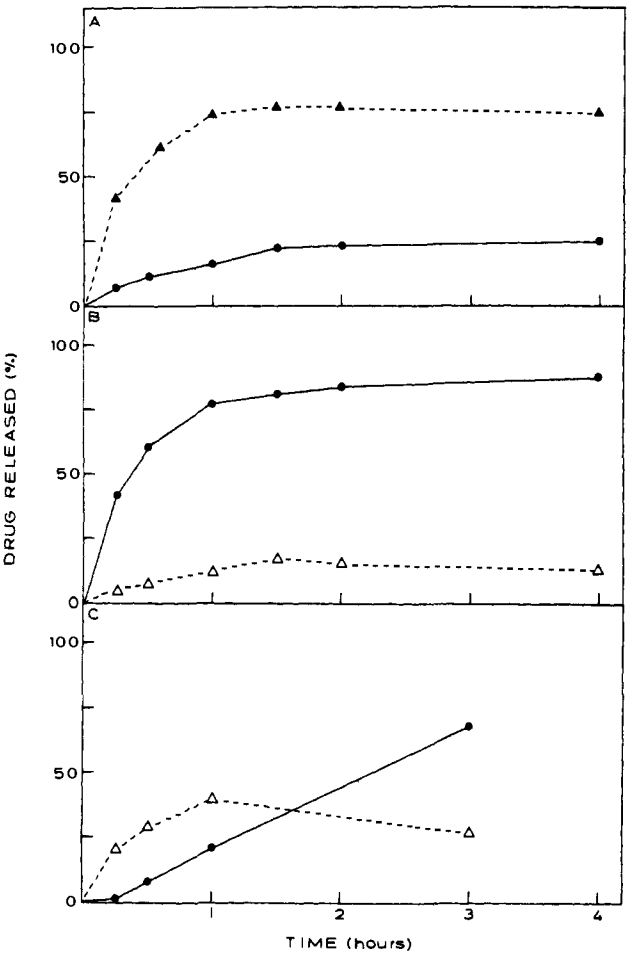

Figure 4. Digestion of the dipeptide derivatives of DNR at pH 6.0 by the soluble fraction of purified lysosome. Each sample incubated at 37 °C contained drug at 35 μM and lysosomal proteins at 0.5 mg/mL. After various times, aliquots were extracted and DNR as well as amino acid derivatives released were determined by high-pressure LC and fluorometry. A: (●–●) DNR; (▲--▲) Ala-DNR, released from Leu-Ala-DNR. B: (●–●) DNR; (△--△) Leu-DNR, released from Ala-Leu-DNR. C: (●–●) DNR; (△--△) Leu-DNR, released from Gly-Leu-DNR.

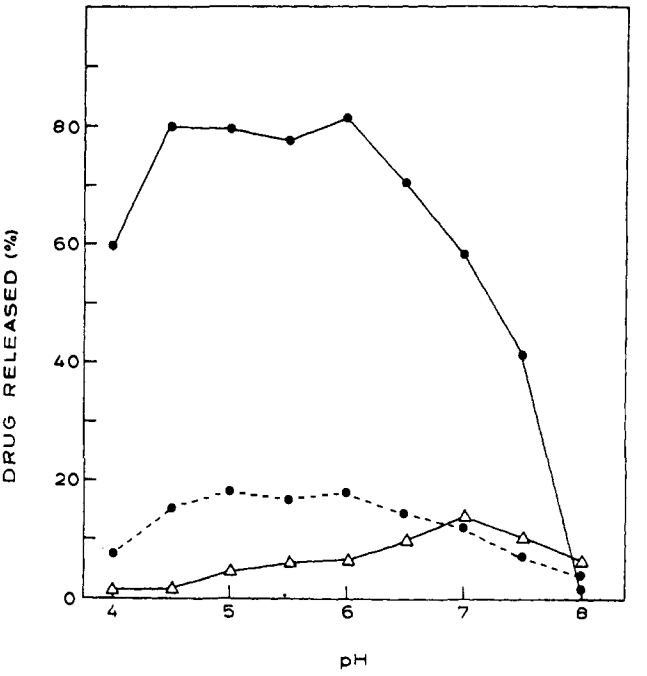

Figure 5. Effect of pH on the digestion of L-alanyl-L-leucyl-DNR and L-leucyl-DNR by a soluble fraction of purified lysosomes. The concentration of acetate (pH 3 to 5.5) or phosphate (pH 6 to 8) buffers in each sample was 0.1 M and they contained 5 mM cysteine. Each sample incubated at 37 °C contained 35 μM drug and 0.5 mg of lysosomal proteins per milliliter. After various times, aliquots were extracted and DNR released was determined by high-pressure LC and fluorometry: (●–●) DNR; (△–△) Leu-DNR released from Ala-Leu-DNR; (●--●) DNR released from Leu-DNR.

with the DNA phosphate groups should indeed be increasingly hindered by the shift of the amino group position resulting from its substitution by one and two amino acids.

Our objective is to develop DNR derivatives that could release DNR through the action of amino peptidases or lysosomal hydrolases. Among the amino acid compounds, only Leu-DNR and, to a lesser extent, Gly-DNR seem to fulfill this condition. The hydrolysis of Leu-DNR by lysosomal enzymes is at its highest between pH 5 and 6 (Figure 5).

Ala-Leu-DNR seems to be the derivative which is the most sensitive toward an enzymatic hydrolysis, since 85% of it is transformed into DNR after 2 h of incubation in the presence of lysosomal hydrolases (Figure 3). From the data of Figure 4 one can estimate that the release of DNR from Ala-Leu-DNR is mainly the result of a dipeptidyl aminopeptidase, the optimum pH of which is reached between 4 and 6 (Figure 4). Leu-Leu-DNR and Gly-Leu-DNR are hydrolyzed at a much lower rate. Gly-Leu-DNR seems to be first hydrolyzed into Leu-DNR and then subsequently into DNR (Figure 4). The very low rate and degree of digestion of Leu-Ala-DNR into DNR seem to be the result of a competition between a leucine aminopeptidase-like enzyme and a dipeptidyl aminopeptidase. The higher activity of the former enzymatic activity results in the release of 75% of Ala-DNR which, as shown before (Figure 3), is resistant to the action of lysosomal enzymes.

The Leu, Ala-Leu, Gly-Leu, and Leu-Leu derivatives can, moreover, be considered as prodrugs of DNR.[16] Their chemotherapeutic properties could be significantly different from those of DNR if their pharmacokinetic properties are different and if they can be activated inside the lysosomes of tumoral cells in which drugs such as DNR and doxorubicin are known to accumulate.[17] One other interesting feature of these prodrugs is that they might be activated more or less selectively in the extracellular space of tumoral tissues which have been shown to contain a high level of lysosomal enzymes and acid proteases, such as cathepsin B and D.[4-6]

The in vitro and in vivo activities of these amino acids and dipeptide derivatives are described in the following paper in this issue.

Experimental Section

The 0.1 M borate buffer (pH 10.2) has the following composition: boric acid, 6.18 g; KCl, 7.46 g; 1 N NaOH, 88 mL for 1 L of distilled water. Extractions were repeated until the aqueous layer was free of drug, as estimated by the red color; extracts were dried over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure (25 mm) below 45 °C. Melting points were determined on a Mettler apparatus. Thin-layer chromatography on 1 × 7 cm silica gel 60 covered plastic sheets (E. Merck,

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Darmstadt, Germany) was used for identification purposes and as a purity test. The following systems were used: I, chloroform–methanol–water (120:20:1, v/v); II, methylene chloride–methanol–88% formic acid–water (85:15:2:1, v/v). Analytical high-pressure liquid chromatography was performed on E. Merck prepacked silica gel 60 columns and using a Gilson Spectro-glo fluorometer as detector, as described previously.[11,20] The *N*-amino acid[18] and *N*-dipeptide derivatives of DNR were prepared by either of the three following methods.

Method A. The *N*-hydroxysuccinimide ester of the *N*-trityl amino acids were prepared by condensing the *N*-trityl amino acids[8] with *N*-hydroxysuccinimide in dimethoxyethane (DME) following the procedure of Andersen et al.[9]

To a solution of 0.45 mmol of drug in 8 mL of dimethylformamide (DMF), 0.51 mmol of the *N*-hydroxysuccinimide ester of *N*-trityl amino acid and 0.45 mmol of triethylamine (Et_3N) were added.

The solution was stirred at room temperature for 24 h, the solvent was evaporated, and the residue was dissolved in 5 mL of chloroform–methanol (99:1, v/v) and filtered on a column filled with 15 g of silica gel 60 from E. Merck (70–230 mesh). The pooled fractions corresponding to the *N*-trityl aminoacyl derivatives of the drugs were evaporated, and deprotection was carried out in 10 mL of 75% acetic acid during 1 h. The solution was cooled to 0 °C and neutralized by NH_4OH. The precipitate was washed with distilled water, and the filtrate was extracted with chloroform and dried overnight on anhydrous Na_2SO_4. The solvent was evaporated, 20 mL of distilled water was added, and the chlorhydrate was formed by the addition of an equivalent of 1 N HCl. After freeze–drying, the chlorhydrate was obtained as a red powder. The chromatographic R_f of the trityl intermediates are given in Table I for both TLC systems used.

Method B. The *N*-carboxyanhydrides of amino acids were synthesized according to the procedure of Hirschmann et al.[10] To an ice-cold solution of the drug in 200 mL of 0.1 M borate buffer, pH 10.2, vigorously stirred under nitrogen, a solution of the *N*-carboxyanhydride of the amino acid in an excess of 15% in 10 mL of acetone at −10 °C was added. After 5 min, the pH was brought to 3.5 by 6 N H_2SO_4 for 15 min and the solution neutralized by 1 N NaOH. The product was extracted as the free base with 450 mL of chloroform in portions. The extracts were dried over Na_2SO_4, filtered, and evaporated. Quantitative separation of the product from aglycon and side products was affected by silica gel column chromatography (60 g of silica gel 60 from E. Merck, 70–230 mesh) using chloroform–methanol (95:5 v/v). The solvent from the pooled fractions corresponding to the free base was evaporated. Upon addition of 20 mL of distilled water, the chlorhydrate was formed by the careful addition of a stoichiometric amount of 1 N HCl and freeze–drying of the solution.

Method C. The *N*-trityl-L-alanyl-L-leucyl-DNR was synthesized following method A. Deprotection was carried out by adding 20 mL of 75% acetic acid and stirring during 1 h at 20 °C. Thereafter the solution was cooled in an ice bath and neutralized by concentrated NH_4OH using a pH meter. The product was extracted by chloroform and dried overnight on Na_2SO_4. After evaporation of the solvent and chlorhydrate formation by addition of an equivalent of 1 N HCl, the triphenylcarbinol was filtered and the filtrate freeze–dried.

Interaction with DNA. A. Binding Studies. The spectral titration method used to determine the binding affinity parameters has been published previously.[12] To 2.0 mL of a drug solution at 200 μg/mL PBS at pH 7.4 (PBS: NaCl, 137 mM; KCl, 3 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM) were added 0.05 to 2.00 mL of herring sperm DNA (highly polymerized, type VII, Sigma, St. Louis) at 2.34 mg/mL 0.9% NaCl, and the volume was completed to 4.0 mL by PBS. The absorbancies at 475 nm for each solution were read on a Gilford spectrophotometer (Gilford, Ohio), and the free and bound drug concentrations were estimated from those values.[12] The spectral titration data were analyzed by the Scatchard technique according to the following equation: $n_x = n_{max} - (1/K_a)(N_x/D_f)$, where n_x is the number of moles of drug bound per mole of DNA nucleotide, n_{max} represents the maximal binding, K_a is the affinity constant for the drug–DNA complex, and D_f the concentration of unbound drug. A plot of n_x/D_f vs. n_x gives the values of n_{max} and K_a (see Table II).

B. Quenching of Fluorescence. Increasing amounts of a DNA solution (2.34 mg/mL) were added to tubes containing drugs (3.5 μM in PBS), such as to obtain DNA nucleotide/drug molar ratios ranging from 0 to 20, assuming a mean nucleotide molecular weight of 327.[19] The fluorescence of the solutions was measured on a Zeiss PMQ II spectrofluorometer using 480 and 560 nm as excitation and emission wavelengths. The results are expressed (Figure 1) as percent of the fluorescence obtained in the absence of DNA.

"In Vitro" Digestion by Lysosomal Extracts. A soluble fraction of purified rat liver lysosomes[13] at a protein concentration of 3 mg/mL was used. To 0.3 mL of 0.5 M buffer at the desired pH were added 0.25 mL of the lysosomal enzyme preparation, 0.5 mL of a solution of 15 mM cysteine, and 0.45 mL of the drugs to obtain a final drug concentration of 35 μM. Controls were made without the enzymatic preparation. After various times of incubation, an aliquot of 0.1 mL was taken, and the drugs were extracted and analyzed by the high-pressure liquid chromatography method described previously.[11,20]

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