STUDIES ON AMINO ACID AND PEPTIDE DERIVATIVES OF DAUNORUBICINE

Yehuda LEVIN and Ben-Ami SELA
Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Daunorubicine and adriamycin are antracycline antibiotics that inhibit DNA synthesis and have been shown to possess potent antitumor activity [1–4]. It was demonstrated that such binding is due to two types of drug–DNA complex, i.e., an intercalation involving a base-pair and chromophore interaction and an electrostatic interaction between the amino group of the sugar residue of the antibiotic and the phosphate group of DNA [5].

The therapeutic application of daunorubicine and adriamycin is, however, restricted due to their toxic side effects, such as those manifested in the heart muscle and the proliferating bone-marrow cells [6]. It was demonstrated that some chemical modifications of the drugs could bring about a significant decrease in their toxicity [7], and chemically-modified adriamycin has been reported to retain its anti-tumor activity [8].

We have taken the approach of derivatizing daunorubicine by binding peptides of various lengths to its amino group and analyzing the modified properties of the derivatized drug. The results summarized in this report have shown that the extent of binding of daunorubicine derivatives to DNA and their biological activity decreased with peptide chain length as measured by quenching of fluorescent emission and by inhibition of thymidine incorporation into drug-treated cells, respectively. Reversing the electric charge of daunorubicine or its peptidyl derivatives by succinylation strongly reduced their biological activities. When peptides were enzymatically cleaved off the derivatized drug, the biological activity was significantly regained.

Abbreviations: DR, daunorubicine; NCA, *N*-carboxyanhydride; TFA, trifluoroacetyl; DMF, dimethylformamide

2. Materials and methods

2.1. *Materials*

Commercial daunorubicin–HCl (DR) was purchased from Specia, France. Calf thymus DNA came from Worthington Biochem. Corp; protease type VI (pronase) was from Sigma; concanavalin A (con A) from Miles-Yeda; [^{3}H]thymidine, 5 Ci/mmol from Nuclear Research Center, Negev, Israel. *N*-Carboxyanhydrides (NCA) of the various amino acids, prepared by the Fuchs-Farthing method [9], were kindly provided by Mr I. Jacobson of this laboratory. The cells used in this report were Wistar male rat thymocytes prepared as in [10] and mouse lymphoma cell line EL4 (The Salk Institute, La-Jolla).

2.2. *Preparation of amino acids and peptidyl derivatives of daunorubicine*

The amino acid- and peptidyl-daunorubicine derivatives were prepared by coupling NCA of various amino acids with the free base of DR. As an example, the preparation of *N*-lysyl-daunorubicine is given below.

N-Lysyl-daunorubicine: DR (10 mg, 18 μmol) was dissolved in 2 ml dry dimethyl-formamide (DMF). The solution was shaken with solid Ag_2CO_3 and centrifuged. The supernatant, containing the free base of DR, was reacted with 5.4 mg (20 μmol) of NCA-ε-trifluoroacetyl-lysine dissolved in 0.5 ml DMF. The reaction mixture was left at 4°C in the dark for 24 h.

The DMF was evaporated in high vacuum at room temperature. The dry ε-TFA-Lys-DR obtained was dissolved in 0.1 N NaOH and left in the dark at 4°C for 2 h, in order to cleave off the TFA group, yielding *N*-Lys-DR. Traces of unreacted DR were removed by extracting with chloroform the Lys-DR solution adjusted to pH 8.5. The other amino acid derivatives of DR were obtained similarly. Homopeptides of DR were obtained by using the appropriate amounts of NCA amino acid. Heteropeptides of DR were obtained by first reacting DR with NCA of one amino acid and after 24 h the intermediate product was reacted with NCA of the second amino acid. Succinylation of DR and its peptide derivatives was achieved by adding succinic anhydride to a solution of the above compounds in sodium bicarbonate buffer, at pH 8.0, with vigorous shaking. The succinylated compounds were separated from excess succinic acid by passing through Poropak Q column [11]. Products of the derivatization procedures were characterized by thin-layer chromatography on silica gel G plates developed in chloroform:methanol:H_2O (13:6:1). The amount of amino acid or peptide bound to DR was determined in the amino acid analyzer, after total hydrolysis of the derivatives.

2.3. *Binding to DNA*

To a 2 ml sample of DR or its derivatives at 5×10^{-6} M in PBS were added 20 μl aliquots of DNA solution (1 mg/ml). The material was excited at 485 nm and the fluorescent emission at 580 nm was measured in an Aminco-Keirs spectrophosphorimeter, according to [12].

2.4. *Fluorescent microscopy*

Rat thymocytes (10^7 cells/tube) were incubated with DR or with its derivatives for 20 min at 37°C and were then washed twice with cold PBS. The cells were examined with a Zeiss microscope equipped with epifluorescent illumination and filter setting for rhodamine fluorescence.

2.5. *Drug activity*

The activity of DR and its derivatives was assayed by the inhibition of thymidine incorporation into the tested cells. Aliquots of 100 μl Eagle's medium supplemented with 5% fetal calf serum containing 0.5×10^6 EL4 cells were dispersed in wells of a microtiter plate and various amounts of DR or its derivatives were added to the cells for overnight incubation in a humidified incubator in 5% CO_2 at 37°C. [^{3}H]Thymidine was then added (1 μCi/well) and after incubation for additional 2 h the cells were filtered and their radioactivity counted. DR and its derivatives were also used to measure the inhibition of thymidine incorporation into mouse thymocytes stimulated by the con. A. The cells were cultured in Falcon tubes (3×10^6 cells/tube) with 3 μg/ml con A for 48 h. The tested drugs were then added for 2 h, followed by a 2 h pulse of [^{3}H]thymidine, before counting the incorporation of the radioactive precursor into acid-insoluble material deposited on GF/C filters.

3. Results and discussion

The extent of binding of DR and its derivatives to DNA was determined by measuring the quenching of fluorescent emission of the tested drugs as in [12]. As can be seen from fig.1, the extent of binding of Ala-

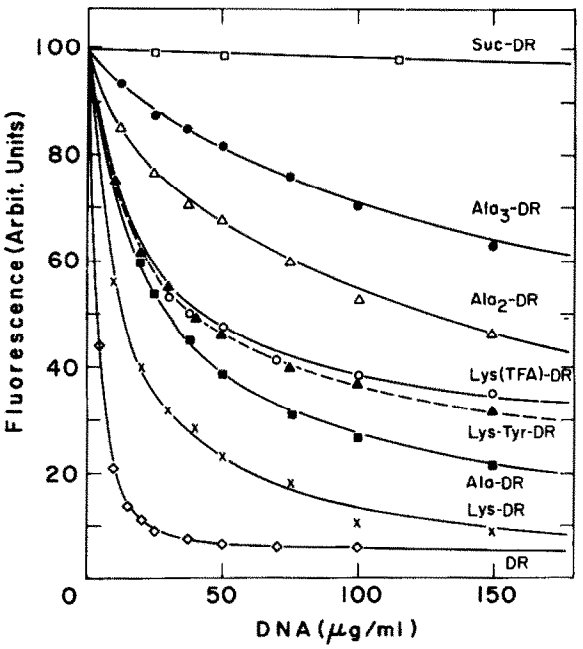

Fig.1. Fluorescent emission of daunorubicine and its peptidyl derivatives (5×10^{-6} M) against DNA concentrations: excitation, 485 nm; emission, 580 nm.

DR is lower than that of the underivatized drug. The binding decreases with length of the alanyl chain attached to DR. This effect of peptide length on the extent of binding is also seen when comparing Lys-DR to Lys-Tyr-DR (fig.1). Lys-DR shows a significantly stronger binding to DNA than Ala-DR. This enhanced binding is probably due to the additional basic ε-amino group of the lysine, that could strengthen the binding between the amino moiety of DR and the phosphate residue of DNA. The enhancing effect of the extra amino group on binding to DNA, is further demonstrated when comparing the binding of ε-TFA-Lys-DR to that of Lys-DR. Succinylation of the DR or its peptide derivatives results in an almost complete loss of their ability to bind to DNA.

The inhibitory activity of DR and its peptide derivatives on thymidine incorporation into tumor cells is shown in table 1. The activity of the alanyl derivatives decreases with the number of alanyl residues in the drug, and no inhibitory activity whatsoever is found with poly(DL-Ala)-DR where the number of alanyl residues is over 5. Succinylation of DR or of its peptide derivatives strongly reduces the inhibitory activity of these drugs on thymidine incorporation (table 1). Very similar results not shown in table 1 were obtained by testing DR and its derivatives on con A-stimulated mouse lymphocytes. The inhibitory activity of the peptide derivatives of DR on thymidine incorporation correlates in most cases with the extent of binding of these drugs to DNA as measured by quenching of fluorescent emission. Similar observations were made when cells were incubated with the drugs and the pattern of staining was examined by fluorescent microscopy. Whereas DR showed a very strong nuclear staining, Leu-DR and Ala-DR stained the nucleus very faintly and Ala_2-DR or Ala_3-DR concentrated on the periphery of the nucleus without staining the inside. All succinylated derivatives tested as well as the long chain compound poly(DL-Ala)-DR, did not show any staining of the cells.

However, a complete correlation was not always found between the extent of binding of the derivatized DR to DNA as measured by fluorescent emission and its inhibitory activity on thymidine incorporation. Thus, Lys-DR binds to DNA in vitro much stronger than TFA-Lys-DR, although both derivatives inhibit thymidine incorporation to the same extent (table 1).

Table 1
Inhibition of [^{3}H]thymidine incorporation by daunorubicine and its derivatives[a]

Substance	% Inhibition of [^{3}H]thymidine incorporation Concentration of drug	
	10^{-5} (M)	5×10^{-5} (M)
Daunorubicine	99.7	99.8
Ala-DR	97	98
Ala_2-DR	82	99
Ala_3-DR	73	94
DL-Ala_5-DR	0	50
Leu-DR	99.5	99.7
ε-TFA-Lys-DR	98.5	99.7
Lys-DR	96	99.8
Lys_2-DR	42	99.5
α Suc-Lys-DR	10	42
Suc-DR	2	32
Lys-Tyr-DR	30	99.3
Lys-Phe-DR	99.5	99.9
Suc Ala_2 Phe DR	55	73
Suc Ala_2 Phe DR + pronase	94.5	99.6
Suc-Phe_2-DR	18	47
Suc-Phe_2-DR + pronase	92	96

[a] Based on values of [^{3}H]thymidine incorporation into EL4 lymphoma cells as described under section 2. Results are averaged from 3 independent experiments

The fact that the binding of DR derivatives to DNA cannot always be correlated with their overall inhibitory effect on nuclear metabolism, may be explained by the reported binding of DR to the other cellular components as phospholipids [13] and mitochondria [14].

The peptidyl derivatives of DR which are poor inhibitors of cell growth, can regain part or most of their activity by cleaving off the peptides by proteolysis. Thus succinyl-Ala_2-Phe-DR which has a very low activity is potentiated when treated with pronase (0.1 mg/ml for 2 h) (table 1). These derivatives might therefore serve as slow releasing drugs if they will be reactivated by proteolytic activity in their milieu following administration to the body.

All of the amino acid and peptide derivatives were found to be less toxic to C57Bl/6 mice than the native DR molecule, and the succinylated derivatives were not lethal when injected in doses of at least 500 mg/kg compared with a lethal dose of 5–8 mg/kg

recorded for the native DR. Thus the high toxic threshold of the derivatives allows their administration at much higher doses. In vivo experiments currently conducted in our laboratory have indeed confirmed that some of the amino acid derivatives of daunorubicine are far more efficient than the native drug in prolonging the life span of tumor-bearing mice, and in some cases completely curing them, with reduced toxic side effects, (Y.L., B.-A.S., in preparation). The preparation and study of other amino acid derivatives of daunorubicine may provide a new group of chemotherapeutic agents with superior anti-tumor activity.

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