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A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug–carrier conjugate: *In vitro* and *in vivo* studies

(cancer chemotherapy/anthracyclines/lysosomes/peptidic spacer arm/L1210 leukemia)

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ABSTRACT **Daunorubicin (DNR) has been conjugated to succinylated serum albumin by an amide bond joining the amino group of the drug and a carboxyl side chain of the protein either directly or with the intercalation of a peptide spacer arm varying from one to four amino acids. During *in vitro* incubation with lysosomal hydrolases, intact DNR could be released extensively only from conjugates prepared with a tri- or tetrapeptide spacer arm. These latter conjugates remained very stable in the presence of serum. When tested *in vivo* against the intraperitoneal form of L1210 leukemia, the conjugates in which DNR was linked to serum albumin directly or via one amino acid were completely inactive but the conjugate with a dipeptide spacer arm was not more active than free DNR. In parallel with the *in vitro* studies, the best therapeutic results were obtained with the conjugates formed with tri- and tetrapeptidic spacer arms; they were much more active than DNR, inducing a high percentage of long-term survivors. Thus, use of a tri- or tetrapeptide spacer arm is essential to obtain DNR–protein conjugates that remain stable in serum and from which DNR can be released through the action of lysosomal hydrolases. The *in vivo* results suggest, moreover, that these conjugates are endocytosed by L1210 cells and that DNR is released intracellularly after digestion by lysosomal enzymes. This conjugation method can be applied to other drugs possessing a free amino group and to various potential carriers, such as antibodies, polypeptide hormones, and glycoproteins, that have amino or carboxyl side chains.**

During the past decade, the use of carriers for the selective targeting of anti-tumor drugs has been advocated with increasing frequency and has led to numerous reports on the association of drugs such as anthracyclines, methotrexate, bleomycin, chlorambucil, and 1-β-D-arabinofuranosyl cytosine (cytosine arabinoside) with carriers such as DNA (1, 2), liposomes (3, 4), immunoglobulins (5, 6), hormones (7, 8), and other proteins (9, 10) or polypeptides (11).

However, insufficient attention has been paid to the nature of the link between the drug and the carrier. For a drug–carrier conjugate to be effective, the link between drug and carrier must remain stable in the bloodstream and withstand the action of serine hydrolases. On the other hand, unless the drug is able to act in conjugated form at the cell surface, it has to be released from the carrier after interaction of the conjugate with the target cell, and its mode of release must be such as to allow the drug to reach its biochemical target—usually situated intracellularly—and to interact effectively with it. Because the most general fate of molecules bound by surface receptors is to be interiorized by endocytosis and conveyed to the lysosomes for digestion, an obvious way of ensuring appropriate release of the drug is to rely on lysosomal hydrolysis. This approach is evidently limited to drugs that are not inactivated in the lysosomes and that can reach their biochemical target from the lysosomal compartment. The principles governing this "lysosomotropic" chemotherapy have been developed in greater detail elsewhere (12, 13).

We have developed and tested, both *in vitro* and *in vivo*, a bond meeting the above requirements between daunorubicin (DNR) and bovine serum albumin. DNR was chosen because, like doxorubicin (adriamycin), it is a potent drug having, on its daunosamine moiety, a primary amino group suitable for an amide type linkage (Fig. 1) and because we know from previous work that it has the properties of lysosome resistance (1) and transmembrane diffusibility (14) needed for effective action after intralysosomal release. Albumin was selected as a model carrier because of its protein nature and ready availability. Proteins and polypeptides, (for instance, antibodies, hormones, glycoproteins, and lectins), are good candidates as carriers for antitumoral drugs.

We describe in this paper how a suitable albumin–DNR conjugate can be prepared, provided that an oligopeptidic spacer arm is intercalated between the drug and the carrier. This conjugation method was tested *in vitro* by measuring the release of DNR in the presence of serum and lysosomal hydrolases and *in vivo* by evaluating the chemotherapeutic activity of the conjugate on the L1210 murine leukemia.

MATERIALS AND METHODS

Amino Acid and Peptide Derivatives of DNR. DNR·HCl was obtained from Rhône-Poulenc, S.A. (France). *N*-L-Leucyl-DNR (Leu-DNR) was synthesized by reaction of the *N*-carboxyanhydride derivative of L-leucine with DNR as described (15). *N*-L-alanyl-L-leucyl-DNR (Ala-Leu-DNR) was prepared by reaction of Leu-DNR with the *N*-trityl alaninate of *N*-hydroxysuccinimide (16, 17). *N*-L-leucyl-L-alanyl-L-leucyl-DNR (Leu-Ala-Leu-DNR) and *N*-L-alanyl-L-leucyl-L-alanyl-L-leucyl-DNR (Ala-Leu-Ala-Leu-DNR) were synthesized as described for Ala-Leu-DNR by successive condensation of Ala-Leu-DNR and Leu-Ala-Leu-DNR with the appropriate amino acid.

In an alternative procedure, the tri- and tetrapeptides were first synthesized by the solid-phase method of Merrifield (18) and subsequently linked to DNR in the presence of dicyclohexylcarbodiimide and *N*-hydroxysuccinimide.

Conjugation of DNR and Derivatives to Bovine Serum Albumin. The protein carrier was first succinylated. Bovine serum

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Abbreviations: DNR, daunorubicin; HPLC, high-pressure liquid chromatography; ECD, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl.

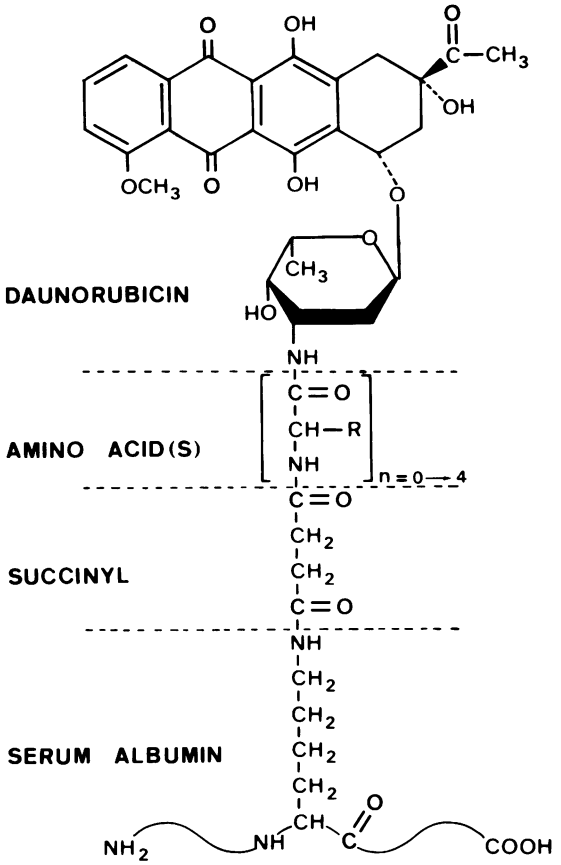

FIG. 1. Structure of the daunorubicin–albumin conjugates. Daunorubicin was linked to succinylated serum albumin either directly ($n = 0$) or via an oligopeptidic spacer arm composed of one to four amino acids ($n = 1$–4).

albumin (Armour, Eastbourne, England) was dissolved in water at 100 mg/ml, and the pH was adjusted to 7.5 with 0.5 M NaOH. Succinic anhydride (0.68 mmol; Aldrich, Beerse, Belgium) was then added stepwise while the pH was maintained at 7.5 with 0.5 M NaOH. Another 0.68 mmol of succinic anhydride was added subsequently, and the preparation was extensively dialyzed against phosphate-buffered saline (NaCl, 137 mM; KCl, 3 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM), sterilized by filtration on Millipore GS filters (0.22 μm), and kept at 4°C. The yield varied between 88% and 95% as determined from the number of remaining free amino groups measured with trinitrobenzenesulfonate (19).

For the conjugation step, 20 μmol of DNR or of its derivatives were added to 50 mg of succinylated albumin (5 ml of solution at 10 mg/ml). Then, 7.5 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (ECD) (Sigma) were added, and the solution was kept in the dark at 4°C without stirring. Another 3.75 mg of ECD was then added, and the solution was kept overnight at 25°C.

The drug–protein conjugate was separated from the remaining free drug and reagents by filtration on Bio-Gel P-100 (100–200 mesh; Bio-Rad). The elution profiles of proteins and anthracyclines were monitored by measuring absorbance at 280 and 475 nm, respectively. Free drug adsorbed on the protein was eliminated by adsorption chromatography on Porapak Q (Waters Associates). The Porapak used was first suspended in ethanol for 15 min and then washed extensively with H_2O and phosphate-buffered saline, which served also as eluant (20).

The drug/protein molar ratio was computed from the absorbance of the solution at 475 nm ($E^{1\%}_{1\,cm} = 165$ assumed for DNR) and from the protein content as measured by the method of Lowry *et al.* (21). The conjugates were finally sterilized by filtration on Millipore GS filter (0.22 μm) and stored in the dark at 4°C.

Digestion of the Drug–Protein Conjugates by Lysosomal Enzymes. Drug–protein conjugates at a final anthracycline concentration of 17.8 μM were incubated at 37°C in the presence of a purified lysosomal fraction (0.5 mg of protein per ml) in 0.1 M citrate, pH 5.5/5 mM cysteine. The lysosomes were isolated from the livers of rats treated with Triton WR-1339 (22). At various times the amount of intact DNR released was determined by high-pressure liquid chromatography (HPLC) on 100-μl aliquots as described (23).

Incubation in the Presence of Serum. Drug–protein conjugates were incubated at 37°C at a final anthracycline concentration of 17.8 μM in the presence of 95% calf serum. Aliquots were analyzed for release of intact DNR by HPLC as above.

Chemotherapeutic Tests. Female DBA_2 mice (Charles River, France) were inoculated intraperitoneally with 10^4 L1210 leukemic cells on day 0 and with the drugs on days 1 and 2. Mice were weighed daily, and the weight change on day 8 was taken as an index of overall toxicity. The percentage increase in life-span and the number of long-term survivors on day 30 were used as chemotherapeutic indices.

RESULTS

DNR, Leu-DNR, Ala-Leu-DNR, Leu-Ala-Leu-DNR, and Ala-Leu-Ala-Leu-DNR were conjugated to succinylated albumin (Fig. 1) and the sensitivities of the conjugates to serum and lysosomal hydrolases *in vitro* and their chemotherapeutic activities *in vivo* were studied in parallel.

Conjugation of DNR and Its Derivatives to Succinylated Serum Albumin. Irrespective of whether DNR or its peptide derivatives were used, a conjugation yield in anthracycline varying between 56% and 76% was observed. The use of succinylated albumin decreased the formation of albumin polymers, and chromatography of the conjugates on Sepharose 6B (Pharmacia, Fine Chemicals, Uppsala, Sweden) indicated that more than 70% of the conjugates behaved like monomeric albumin and less than 5% was excluded owing to a molecular weight higher than 1,000,000.

The number of anthracycline molecules (DNR or peptide derivatives) linked per molecule of albumin varied between 10 and 21. Analysis by HPLC after chloroform/methanol extraction (23) of the conjugates treated by Porapak chromatography showed that a maximum of 5% of the DNR or derivative bound to albumin was not covalently linked and could be removed by extraction in organic solvents. Without Porapak chromatography, this proportion could be as much as 20% or more, in spite of the Bio-Gel filtration step.

Digestion by Lysosomal Enzymes. Fig. 2 illustrates the release of free DNR observed during incubation of the various DNR conjugates with purified lysosomal enzymes. No DNR was released from albumin–DNR, and very little was released from albumin–Leu-DNR or albumin–Ala-Leu-DNR. The rate of DNR release increased markedly when the peptide spacer arm was lengthened to three or four amino acids. About 60% of the bound drug was released as free DNR from albumin–Leu-Ala-Leu-DNR and 75%, from albumin–Ala-Leu-Ala-Leu-DNR after 10 hr of incubation.

A pH optimum of 5.5 was observed for the enzymatic release of DNR from albumin–Ala-Leu-Ala-Leu-DNR in the presence of lysosomal hydrolases.

Stability in Presence of Serum. Albumin–Leu-Ala-Leu-DNR and albumin–Ala-Leu-Ala-Leu-DNR were stable in pres-

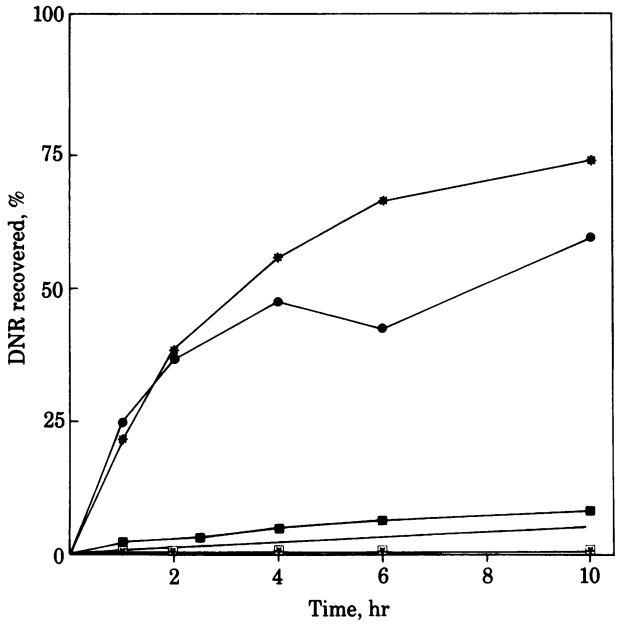

FIG. 2. Influence of length of oligopeptidic spacer arm on the release of DNR linked to succinylated serum albumin during incubation for up to 10 hr at 37°C and pH 5.5 in the presence of purified rat liver lysosomes. The release of free intact DNR was followed by HPLC and fluorometry. ⊠, From albumin–DNR; ○, albumin–Leu-DNR; ■, albumin–Ala-Leu-DNR; ●, albumin–Leu-Ala-Leu-DNR; *, albumin–Ala-Leu-Ala-Leu-DNR.

ence of serum. After 24-hr incubation in the presence of 95% calf serum, the maximal release of DNR amounted to only 2.5% of the bound drug.

Chemotherapeutic Results. The therapeutic effects of DNR and its various conjugates on the intraperitoneal form of L1210 leukemia are summarized in Table 1. DNR exerted a moderate activity, with an increase in life-span of 39% at a dose of 2 mg/kg; the 5 mg/kg dose is toxic. It induced a weight loss of more than 10% on day 8 and the death of some animals before the controls.

Albumin–DNR and albumin–Leu-DNR had no chemotherapeutic effect at 5 and 7.5 mg/kg and seemed to have little toxicity because no significant weight loss was observed on day 8 at the highest dose. The effects of albumin–Ala-Leu-DNR at 5 and 7.5 mg/kg were similar to the effect of DNR at a lower dosage; albumin–Leu-Ala-Leu-DNR and albumin–Ala-Leu-Ala-Leu-DNR had markedly higher therapeutic effects, with an average of 65% survivors on day 30. Moreover, at a dose of 7.5 mg/kg, these conjugates induced a distinctly lower weight loss than did DNR at 5 mg/kg. Therefore, they seem to be less toxic as well. Succinylated albumin had neither therapeutic nor toxic effects, even at doses higher than those used with the conjugates.

DISCUSSION

DNR has been linked covalently to succinylated serum albumin either directly or with the intercalation of a spacer arm consisting of one to four amino acids. The condensation of the aminosugar moiety of DNR and the carboxylic side chains of succinylated albumin was realized with the aid of water-soluble carbodiimide.

The direct conjugate between DNR and succinyl albumin was entirely resistant to hydrolysis by lysosomal enzymes. This could be related either to an intrinsic resistance of the succinyl–daunosamine linkage to lysosomal hydrolases or to steric hindrance by the bulky protein molecule.

Table 1. Chemotherapeutic activity of DNR–albumin conjugates

Drug	DNR/protein, mol/mol	Dose, mg/kg/day		ILS,* %	Survivors on day 30†	Weight variation, %‡
		As protein	As DNR			
DNR	—	—	2	39	5/91	+0.4
	—	—	5	6	0/52	−12.3
	—	—	7.5	7	0/8	−11.0
Albumin–DNR	14.9	40	5	−2	0/7	+6.5
	11.7	51	5	9	0/9	+2.2
	11.6	77	7.5	8	0/10	+0.9
	11.6	77	7.5	9	0/6	−0.9
Albumin–Leu-DNR	12.1	49	5	6	0/10	+3.5
	12.1	74	7.5	6	0/10	−3.4
Albumin–Ala-Leu-DNR	14.5	41	5	30	0/10	−4.7
	14.4	62	7.5	33	0/10	−1.3
Albumin–Leu-Ala-Leu-DNR	20.5	29	5	>211	8/10	+0.4
	17.0	35	5	>200	6/10	+0.5
	20.7	43	7.5	>211	8/10	−7.9
	17.1	52	7.5	>200	7/10	−1.7
Albumin–Ala-Leu-Ala-Leu-DNR	15.2	39	5	107	4/10	−3.0
	14.9	40	5	>211	7/10	−3.9
	13.8	43	5	>189	5/9	+0.9
	15.1	59	7.5	>211	6/10	−9.8
	15.1	59	7.5	>211	10/10	−2.5
	15.1	59	7.5	>200	4/8	−4.4
	13.9	64	7.5	>189	7/9	−0.4
Albumin	—	59	—	−6	0/16	+9.1
	—	89	—	−1	0/8	+2.4

L1210 cells (10^4) were injected intraperitoneally on day 0 into DBA_2 mice. Drugs were given intraperitoneally on days 1 and 2.

* Increase in life-span relative to untreated controls.

† Number of survivors on day 30/total number of mice.

‡ Mean percentage increment in weight of the animals between days 0 and 8.

In order to overcome this problem, we intercalated an oligopeptidic spacer arm, varying from one to four amino acids, between DNR and the succinylated protein. Leucine was selected as the amino acid adjacent to DNR because, among several DNR derivatives tested, Leu-DNR is the most rapidly and extensively hydrolyzed by lysosomal enzymes (15). Ala-Leu-DNR, Leu-Ala-Leu-DNR, and Ala-Leu-Ala-Leu-DNR were chosen as intermediates because Ala-Leu-DNR was found to be the best substrate for acid hydrolases among three dipeptide DNR derivatives examined (15) and because the alternation of alanine and leucine provided tri- and tetrapeptide derivatives that are sufficiently water soluble as well as sensitive to lysosomal hydrolysis.

The approach chosen proved successful. Some degree of hydrolysis was observed even with a single amino acid in the spacer arm, but it was very slow. Only a slight improvement was obtained when a second amino acid was intercalated. However, when a third amino acid was inserted, the extent of hydrolysis by lysosomal enzymes increased from 8% to 60% in 10 hr. It reached 75% with a tetrapeptide spacer arm. These tri- and tetrapeptide conjugates remained perfectly stable in the presence of serum, as required for authentic lysosomotropic drug–carrier complexes.

The chemotherapeutic efficiency of the conjugates paralleled closely their sensitivity to lysosomal hydrolysis, even to the point of becoming significant with a dipeptide spacer arm and of increasing dramatically when the number of intercalated amino acids was increased from two to three. This does not in itself prove that the conjugates act according to the theoretical lysosomotropic model. But it certainly supports such a conclusion strongly, especially since an alternative explanation cannot readily be proposed on the basis of what is known of the processing of proteins by cells.

If it seems likely, therefore, that the therapeutic activity of the conjugates depends on lysosomal release of the drug; their relatively lower toxicity, and consequently improved therapeutic index compared to free DNR, remains to be explained. Relative cosegregation of both the target cells and the drug conjugates in the peritoneal cavity provides the simplest explanation. But it is possible that the use of succinylated albumin as carrier may have fostered selective uptake because we have found recently that succinylation of albumin enhances its endocytosis by L1210 cells *in vitro* and that conjugates of Leu-Ala-Leu DNR or Ala-Leu-Ala-Leu-DNR with nonsuccinylated albumin have only a small chemotherapeutic effect on L1210 leukemia.

Prior succinylation of the carrier protein was originally adopted to increase the yield of drug conjugation and to decrease the amount of protein polymerization in the presence of carbodiimide. Although it turned out to be advantageous in the present case, it is undesirable in a general conjugation procedure to be used with proteins selected for their ability to bind specifically to surface receptors of the target cells. Such binding properties are likely to be altered drastically by succinylation in many cases.

Whatever improvement may be made in the actual coupling procedure, it is clear that the tri- and tetrapeptide arms described in this paper allow the linking of DNR to a protein by a covalent bond that, although being stable in serum, is sensitive to lysosomal hydrolases, and that they yield conjugates active *in vivo*. These three criteria, especially the *in vivo* activity, were not entirely met in the previously published procedures for linking DNR directly to proteins by means of carbodiimide (6), glutaraldehyde (6), or periodate oxidation (24) or indirectly with a leucylarginylglucopyranosyl spacer arm (10).

Our spacer arms are likely to find many applications. In principle, they can serve to link DNR to any proteins, as well as to many other potential carrier molecules that possess, or can be fitted with an appropriate amino or carboxyl group. Conversely, other drugs besides DNR can be modified by this procedure, provided they have a free amino group or some other convenient attachment point, and have the ability to reach their intracellular target in active form if released inside lysosomes. Our results thus open exciting prospects of using antibodies, peptide hormones, glycoproteins, and other substances that can be recognized by cell-surface receptors as carriers not only for antitumoral drugs but also for other drugs—for instance, chemotherapeutic agents against intracellular parasites (25).

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