Amino Acid and Dipeptide Derivatives of Daunorubicin. 2. Cellular Pharmacology and Antitumor Activity on L1210 Leukemic Cells in Vitro and in Vivo

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Received April 30, 1980

The accumulation of amino acid and dipeptide derivatives of DNR has been studied in vitro on L1210 cells. Only Leu-DNR reaches accumulation levels close to DNR, while Val-DNR, Ile-DNR, and Leu-Leu-DNR reach intermediate values. Intracellular DNR was found when the L1210 cells were incubated in the presence of DNR, Leu-Leu-DNR, Leu-Ala-DNR, and Leu-DNR. The cytostatic activity of the derivatives in vitro on L1210 cells cannot be correlated with their uptake or conversion into DNR. At equitoxic doses given iv on the iv inoculated form of L1210 leukemia, all the derivatives are less active than DNR. When given iv on the sc inoculated L1210 leukemia, Leu-DNR, Ala-Leu-DNR, and Leu-Leu-DNR are much more active than DNR with a striking increase in ILS and reduction of tumor progression. The superiority of those compounds could be due to their greater hydrophobicity and to their hydrolysis in situ by enzymes secreted by tumor cells or present on the tumor cells surface.

N-Amino acid and dipeptide derivatives of daunorubicin (DNR) have been synthesized as potential prodrugs of this widely used antitumor antibiotic.[1] It was assumed that such prodrugs could have different pharmacokinetic properties and could regenerate DNR inside the lysosomes of tumor cells in which drugs like DNR and doxorubicin have been shown to accumulate.[2] These prodrugs could also be activated more or less selectively in the extracellular space of tumoral tissues which contain high levels of aminopeptidases and lysosomal acid proteases.[3-5]

In the first paper of this series,[6] we have shown that *N*-amino acid and dipeptide derivatives of DNR can be synthesized in good yields (up to 95%) by classical methods of protein chemistry. The derivatives possessing the L-Leu-DNR core released high amounts of DNR during an incubation with lysosomal enzymes. We have also reported previously that *N*-L-Leu-DNR is four times less toxic than DNR and possesses, at equitoxic doses, a strikingly increased therapeutic activity when compared to DNR on the subcutaneously inoculated L1210 leukemia.[7]

In the present study, we have investigated the influence of linking amino acids to DNR upon its uptake, intracellular metabolism, and cytotoxicity toward murine L1210 leukemia in vitro and in vivo.

Results

The time course of total drug (parent compound and metabolites) uptake by cultured L1210 cells is illustrated in Figure 1. The intracellular level of DNR and its metabolites reaches nearly a plateau value of 45 nmol/mg of cell protein after 6 h, and none of the derivatives is taken up as fast or to as great an extent. The accumulation levels of the derivatives vary with the nature and the number of amino acids linked to DNR. Compounds well accumulated at 6 h are Leu-DNR, Val-DNR, Ile-DNR, and Leu-Leu-DNR, with respective levels of 32, 25, 19, and 18 nmol/mg of cell protein. Levels as low as 3, 2, and 1 nmol/mg of protein are obtained with Ala-DNR, Gly-DNR, and Gly-Leu-DNR, respectively. The Leu-DNR uptake by L1210 cells in the first 30 min was not influenced by the presence in the medium of free L-leucine, even when the amino acid was present in a 50-fold excess.

The metabolism of DNR and its derivatives in cultivated L1210 cells is detailed in Table I. The main metabolite of DNR is its 13-hydroxy derivative, daunorubicinol (DOL), which accounts for less than 10% of the total cell anthracycline content. In the case of the amino acid derivatives, DNR is only found with Leu-DNR and Gly-DNR, although the intracellular levels of Ile-DNR and Val-DNR are much higher. With the dipeptide derivatives, DNR is observed intracellularly with Leu-Leu-DNR and Leu-Ala-DNR and perhaps with Ala-Leu-DNR.

Experiments during which all the derivatives were incubated for 6 h in the presence of culture medium but in the absence of cells revealed that Leu-Ala-DNR and Ala-Leu-DNR were metabolized, respectively, into Ala-DNR (70%) and Leu-DNR (20%) by the medium and serum alone. In all cases the amounts of metabolites present in the medium were very similar in the presence and absence of cells and were very close to the starting values at 0 h, except for Leu-Ala-DNR and Ala-Leu-DNR.

The cytostatic and cytotoxic effect of DNR and derivatives on cultured L1210 cells in exponential growth phase is illustrated in Figure 2. All derivatives were less active than DNR, the most active being Leu-DNR and the least active Gly-Leu-DNR.

On the basis of the weight loss observed in mice 8 days after the iv inoculation of the amino acid and dipeptide derivatives, we have estimated that their overall acute toxicity is between four and six times lower than that of DNR. Taking this into account, we have performed in vivo chemotherapeutic tests using approximately equivalent toxic doses of derivatives amounting to between four and six times those of DNR.

As documented in Table II, when L1210 cells and the drugs were administered intravenously, DNR induced, at the maximal tolerated dose, an increase in life span (ILS) of 66% with no long-term survivors; none of its derivatives produced better results. When the cells were inoculated subcutaneously (Table III) and the drugs given intravenously, DNR induced an ILS varying between 65 and 83% with about 10% of long-term survivors. It delays very significantly the development of the subcutaneous tumor. In these experimental situations, Leu-DNR, Ala-Leu-DNR, and Leu-Leu-DNR are significantly more active than DNR.

Discussion

The linking of amino acids or dipeptides to DNR induces striking modifications in the rate of cellular uptake and of accumulation levels of the anthracyclines in L1210 cells (Figure 1). These modifications depend on the nature

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0022-2623/80/1823-1171$01.00/0

Table I. Intracellular Drugs and Metabolites Found in L1210 Cells Incubated at 37 °C for 6 h in a Medium Containing DNR or Its Amino Acid (AA) and Dipeptide [$(AA)_2$] Derivatives at a Concentration of 17.7 μM

	intracellular compds, μg/mg of cell protein[a]						
compds added	DNR	DOL	AA-DNR	AA-DOL	$(AA)_2$-DNR	aglycons	total μg/mg of protein
DNR	18.9	1.9				0.3	21.1
Gly-DNR	0.2	0	0.2	0		0.8	1.2
Ala-DNR	0	0	1.2	0		0.6	1.8
Leu-DNR	0.9	0.2	16.0	0.1		0.9	18.1
Val-DNR	0	0	14.4	0.7		1.4	16.5
Ile-DNR	0	0	10.7	0		1.0	11.7
Leu-Leu-DNR	2.5	0	(7.5)	0.6	(7.5)	2.7	13.3
Ala-Leu-DNR	(0.1)	0	2.6	0	(0.1)	0.3	3.0
Leu-Ala-DNR	1.0	0	1.4	0	0	0.5	2.9
Gly-Leu-DNR	0	0	0.4	0	0.3	1.0	1.7

[a] The data in parentheses indicate that it is not possible to distinguish between these derivatives by high-pressure liquid chromatography.

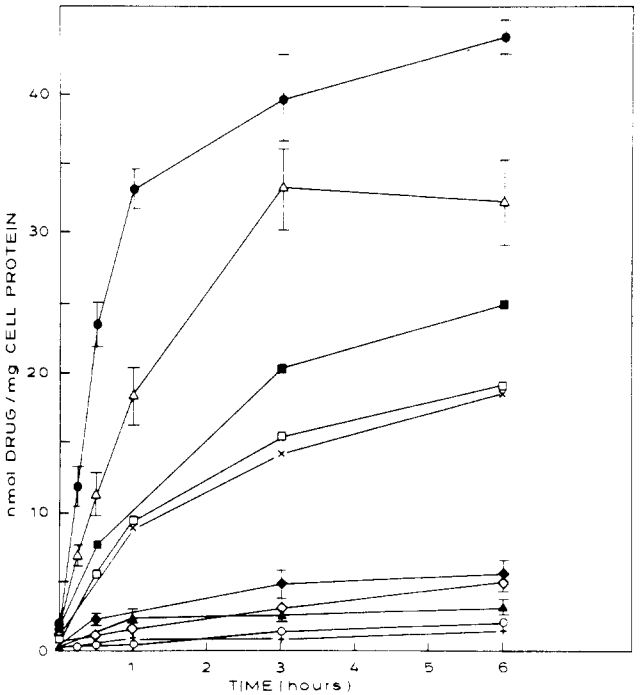

Figure 1. Uptake of daunorubicin and its amino acid and dipeptide derivatives by L1210 cells in culture. Cells were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum in the presence of the drugs at a concentration of 17.7 μM. After incubation, the cells were washed and the drugs determined by flow fluorometry after high-pressure liquid chromatography: (●) DNR; (○) Gly-DNR; (▲) Ala-DNR; (△) Leu-DNR; (□) Ile-DNR; (■) Val-DNR; (x) Leu-Leu-DNR; (◊) Ala-Leu-DNR; (♦) Leu-Ala-DNR; (+) Gly-Leu-DNR.

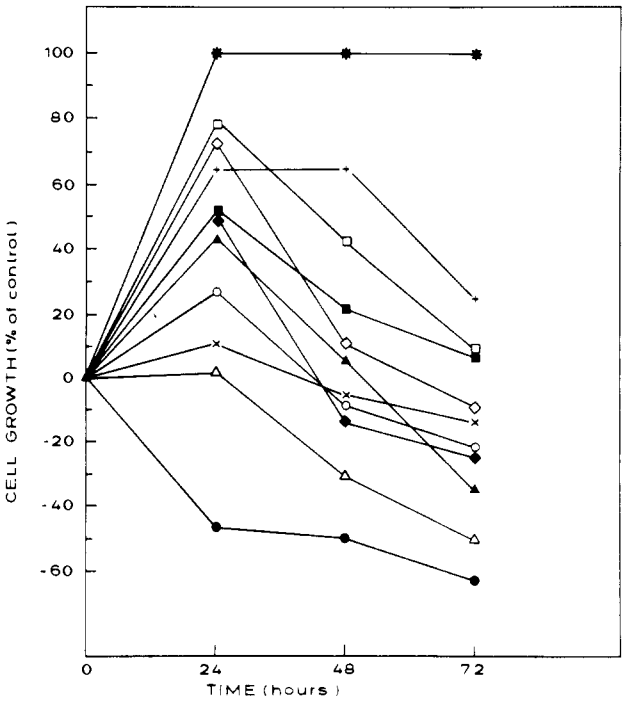

Figure 2. Cytostatic activity of DNR and its amino acid and dipeptide derivatives toward L1210 cells in culture. The L1210 cells (3×10^5 cells/mL) were incubated for 24 h before addition of the drugs at 17.7 μM to the RPMI 1640 medium containing 10% fetal calf serum. The results are expressed in percentage of growth of control cells: (●) DNR; (○) Gly-DNR; (▲) Ala-DNR; (△) Leu-DNR; (□) Ile-DNR; (■) Val-DNR; (x) Leu-Leu-DNR; (◊) Ala-Leu-DNR; (♦) Leu-Ala-DNR; (+) Gly-Leu-DNR; (*) controls.

of the linked amino acids, since only Leu-DNR reaches accumulation levels close to those of DNR while Leu-Ala-DNR, Ala-Leu-DNR, Gly-Leu-DNR, Ala-DNR, and Gly-DNR accumulate at least 10 times less than DNR after 6 h and Val-DNR, Ile-DNR, and Leu-Leu-DNR reach intermediate levels.

The intracellular accumulation of anthracyclines results from their interaction with nuclear DNA and from their trapping in the lysosomes.[2] These drugs seem to permeate freely through the cell membrane, and there are experimental arguments in favor of an active extrusion mechanism.[8-10] The intracellular accumulation of the amino acid and dipeptide derivatives will be influenced by the following factors: (1) by the fact that, as shown in the preceding paper in this issue,[6] the affinity for DNA of the amino acid and dipeptide compounds is decreased about three- and tenfold, respectively; (2) by the fact that the pK_a of the derivatives is decreased by 1 pH unit for Leu-DNR (unpublished results) and Gly-DNR[11] and that, as a consequence, their level of intralysosomal accumulation could be reduced about tenfold;[12] (3) by the metabolism and conversion of the derivatives into DNR; and (4) by the hydrophobicity and molecular weight of the derivatives

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Table II. Chemotherapeutic Activity of DNR and Its Amino Acid and Dipeptide Derivatives on the Intravenously Inoculated L1210 Leukemia[a]

drug	dose, (mg/kg)/day	increase in life span, %	no. of survivors on day 30/total no. of mice	wt change on day 8, %
DNR	10	42	0/18	−1.1
	11	66	0/37	−3.2
	12	123	6/34	−8.4
Gly-DNR	44	21	0/10	−6.7
	48	26	0/10	−5.0
Ala-DNR	50	14	0/7	−4.4
Leu-DNR	40	30	0/8	−3.2
	44	39	0/7	−1.0
	48	48	0/10	−2.1
Val-DNR	48	5	0/8	+3.3
Leu-Leu-DNR	48	13	0/7	−2.5
Ala-Leu-DNR	42	25	0/8	−3.4
Gly-Leu-DNR	60	15	0/7	−3.4
	66	18	0/10	−6.0

[a] Female DBA_2 mice were inoculated iv on day 0 with 10^4 leukemic cells. Drugs were administered iv on days 1 and 2.

which will influence their rate of diffusion through the cell membranes and their affinity for the active outwards transport mechanism. By measuring the octanol–water distribution we can classify the derivatives as follows with decreasing hydrophobicity (expressed as the $\log_n P_0$): Leu-Leu-DNR (4.44); Leu-Ala-DNR (3.33); Leu-DNR (3.27); Ala-Leu-DNR (2.94); Ala-DNR (2.44); DNR (1.72); Gly-DNR (1.42). The hydrophobicity of Ile-DNR and Val-DNR can be estimated to be very close to that of Leu-DNR.

The relatively high accumulation levels of Leu-DNR, Val-DNR, Ile-DNR, and Leu-Leu-DNR are most likely explained by their high hydrophobicity, although they are not metabolized extensively into DNR after 6 h. Their high permeation rate through the cell membrane could compensate for a decreased affinity for DNA and lysosomes. Leu-Ala-DNR, which is also very hydrophobic, is no exception, since it is very rapidly hydrolyzed in the culture medium into Ala-DNR. The high uptake levels of Leu-DNR are not due to a transport mechanism recognizing the leucyl moiety of the drug, since its uptake is not affected by the presence in the culture medium of a 50-fold excess of free L-leucine.

The activity of the DNR derivatives on L1210 cells in vitro cannot be correlated with their levels of intracellular accumulation or with their intracellular conversion into DNR. Indeed, Val-DNR and Ile-DNR, which accumulate more than all other derivatives with the exception of Leu-DNR, are along with Gly-Leu-DNR the least and the most slowly active (Figure 2). These three compounds are, on the other hand, not converted intracellularly into DNR after 6 h of incubation. This is also the case for Ala-DNR which is, however, more active. We cannot exclude at this stage the fact that the amino acid derivatives are active as such when incubated at high concentrations and for long periods in vitro with cells. This latter hypothesis is substantiated by the fact that D-Leu-DNR, which cannot be hydrolyzed into DNR, is also active in cells in vitro, while

Table III. Chemotherapeutic Activity of DNR and Its Amino Acid and Dipeptide Derivatives on Subcutaneously Implanted L1210 Leukemia[a]

drug	dose, (mg/kg)/day	increase in life span, %	no. of survivors on day 30/ total no. of mice	mean tumor diameter, mm		wt change, %, on day 8	no. of expts
				on day 8	on day 12		
controls		0	0/141	7.5 ± 1.4		+1.7	18
DNR	10	65 ± 17	0/58	0.3 ± 0.2	4.1 ± 0.8	−4.3 ± 2.7	7
	11	67 ± 14	3/37	0.4 ± 0.6	2.9 ± 0.9	−5.9 ± 1.8	4
	12	83 ± 12	3/36	0	2.0 ± 1.2	−6.3 ± 3.5	4
Gly-DNR	44	40	0/8	2.4	7.5	−6.2	1
	53	94	0/10	0	0.6	−9.3	1
	60	17	0/9	0	0	−16.1	1
Ala-DNR	60	37 ± 1	0/18	1.3 ± 0.1	3.2 ± 1.2	−5.9 ± 1.8	2
	66	61	0/9	0	4.2	−8.5	1
Leu-DNR	40	166 ± 100	7/17	0.2 ± 0.2	1.3 ± 0.6	−1.3 ± 0.7	2
	44	215 ± 20	14/25	0	0.1 ± 0.2	−3.3 ± 0.6	3
	46	>225	7/9	0	0	−8.5	1
Val-DNR	48	17	0/10	1.8	7.8	−2.2	1
Ile-DNR	44	41	0/9	0	2.1	−2.5	1
Leu-Leu-DNR	53	65	2/10	0	0	+0.4	1
	60	210 ± 47	17/27	0	0.2 ± 0.3	−2.8 ± 1.0	3
	66	153	4/9	0	0	−7.4	1
Ala-Leu-DNR	53	168 ± 89	12/34	0	0.7 ± 0.8	−5.0 ± 1.4	4
	60	193 ± 64	19/35	0	0.3 ± 0.4	−8.2 ± 5.5	4
	66	73	1/10	0	0	−23.4	1
Leu-Ala-DNR	60	48	0/7	1.2	3.6	−11.8	1
	66	89	0/10	0	0.9	−14.3	1
Gly-Leu-DNR	53	40	0/8	2.4	3.6	+4.3	1
	60	38	0/9	1.2	3.4	−1.5	1
	66	79	2/10	0.2	2.7	−5.7	1

[a] Female DBA_2 mice were inoculated sc on day 0 with 10^5 L1210 cells harvested from a 6- to 8-day-old ascitic form of L1210 leukemia. Drugs were administered iv on days 1 and 2. Results of several experiments are expressed as the mean plus or minus the standard deviation.

completely inactive in vivo.[7]

When tested at more or less equitoxic doses, in vivo via the iv route, in the iv inoculated form of L1210 leukemia (Table II), all the derivatives tested showed a much lower antitumoral effect than their parent compound DNR. The death of the mice prior to day 30 was not due to the toxicity of the drugs but resulted from the generalized spread of the leukemic cells. These results correlate more or less with the results obtained with L1210 cells in vitro and suggest that the same factors which play a role in the in vitro activity of the compounds are at stake in vivo when L1210 cells are inoculated intravenously and multiply at first mainly in organs like the liver and the spleen.

The results obtained in mice inoculated subcutaneously with L1210 cells are much more interesting. In this experimental system there are two therapeutic criteria, since one determines first the increase in survival time and must take into account that the animals die from a secondary spread of leukemic cells from the subcutaneous inoculation site and that one follows secondly the development of the subcutaneous tumor. When considering the first of these criteria in Table III, it is obvious that equitoxic doses of Leu-DNR and Ala-Leu-DNR are much more active than DNR and are immediately followed in efficiency by Leu-Leu-DNR, while all other derivatives are more or less as active as DNR. If one considers the second criterium, namely, the tumor size on day 12, Leu-Leu-DNR is the most active, followed by Ala-Leu-DNR and Leu-DNR. Val-DNR, Ala-DNR, and Gly-Leu-DNR are as active as DNR, while Leu-Ala-DNR, and Gly-DNR show some improvement over DNR at higher doses. Except for Ala-Leu-DNR, the death of the mice occurring prior to day 30 were not the result of the toxicity of the drugs. Of the 87 mice surviving more than 30 days, only 6 developed tumors, mainly in the Leu-Leu-DNR series (4 mice over 21).

Although more experimental data are needed in order to explain the superiority of Leu-Leu-DNR, Leu-DNR, and Ala-Leu-DNR against the subcutaneous form of L1210 leukemia, we would like to point out that these three compounds are amongst the most hydrophobic DNR derivatives which are very significantly hydrolyzed in vitro by lysosomal hydrolases.[6] We think that the superiority of these derivatives is due to the fact that they easily reach the subcutaneous tissues because of their greater hydrophobicity and to the fact that they are hydrolyzed into DNR by enzymes secreted by the tumor cells. The existence of such a secretion and of the presence of proteases on the tumor cell surface has been described in several experimental tumor systems.[4,5,13-15]

The therapeutic usefulness of these compounds, such as Leu-DNR, becomes even more obvious, since we have shown that they accumulate much less than DNR in the heart muscle[16] and are much less cardiotoxic at overall equitoxic doses in the chronic cardiotoxic test performed in the rabbit.[17]

Experimental Section

Daunorubicin (DNR) was provided as the hydrochloride by Rhône-Poulenc (Paris, France). The neutral *N*-amino acid and *N*-dipeptide derivatives of DNR were synthesized as described in the preceding paper in this issue.[6]

Murine L1210 leukemic cells were obtained in ascitic fluid from Drs. C. Gosse and J. Morizet at Villejuif (France). These cells were propagated since 1972 in our laboratory by ip inoculation into DBA_2 mice. For in vitro experiments, ascitic L1210 cells were obtained from DBA_2 mice and cultivated in a spinner flask[18] using RPMI 1640 medium (Eurobio, Paris, France), supplemented by 10% fetal calf serum.[19]

For drug uptake and cytotoxicity experiments, 6×10^6 and 9×10^5 cultured L1210 cells, respectively, were incubated at 37 °C into 3 mL of medium to which the drugs were added to a final concentration of 17.7 μM. After various times the cells were centrifuged and washed with PBS. The quantitative determination of the drugs and their metabolites was performed by high-pressure liquid chromatography after extraction as described previously.[20,21]

For the cytostatic assay, 9×10^5 cultured L1210 cells were incubated at 37 °C into 3 mL of medium to which compounds were added 24 h later (day 0) to a final concentration of 17.7 μM. On days 1, 2, and 3 after addition of the drugs, the cells were centrifuged at 4 °C for 10 min at 1200 rpm (IEC centrifuge, rotor no. 253), washed three times with phosphate-buffered saline, and finally resuspended in 0.5 M NaOH. Cellular proteins were determined by the method of Lowry.[22] The results are expressed as a percent of the growth of control cells, calculated as the difference in milligrams of cell protein for treated cells on day *n* (1, 2, or 3) minus the value for the untreated cells on day 0 divided by the difference of milligrams of cell protein for untreated cells at day *n* minus the value on day 0.

The chemotherapeutic activity of the drugs was evaluated in L1210 murine leukemia. Female DBA_2 mice were inoculated on day 0 either intravenously with 10^4 L1210 cells or subcutaneously with 10^5 L1210 cells harvested from a 6- to 8-day-old ascitic form of L1210 leukemia. The drugs were given iv on days 1 and 2 after the inoculation of cells. The chemotherapeutic results were expressed in terms of increase in life span (ILS) and of animal surviving 30 days.

When the L1210 leukemia was inoculated sc, the progression of the tumor was followed by daily estimation of the average tumor diameter with calipers. The maximal tolerated dose was considered as the highest dose inducing no mortality or weight losses smaller than 5% on day 8.[23] The use of weight losses as an index of general toxicity is well established and currently used by the NCI.[24]

Acknowledgment. This work was supported by the Caisse Générale d'Epargne et de Retraite, Brussels (Belgium), and by the Rhône-Poulenc, S.A., Paris (France). We also thank A. Zenebergh for her help with L1210 cultures. We are indebted to Mrs. M. Debroux-Dechambre, C. de Ville de Goyet, B. Hennau, and E. Verstraeten for their skillful technical assistance.

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