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Pharmacological and Therapeutic Efficacy of Rubidazone in Mice. Comparison with Daunomycin and Adriamycin*

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Abstract—*The pharmacological and therapeutic effects of rubidazone, the benzoylhydrazone chlorhydrate of daunorubicin (DNR), were compared with those of DNR and adriamycin (ADM) on an equimolar basis in an experimental animal system. The initial plasma levels of rubidazone fluorescence appeared to be higher than those of DNR and ADM but reached a similar level in* 30 min. *Tissue levels of these compounds were essentially similar except in the spleen and kidney where rubidazone and DNR levels were substantially higher than ADM. The patterns of* in vitro *uptake of rubidazone into L* 1210 *murine leukemic cells were of a mixed type as seen in DNR and ADM, namely, rapid initial uptake followed by gradual progressive increase. Decreased DNR fluorescence at later hours suggested an efflux of the compound. Rubidazone and DNR retention in L* 1210 *cells* in vivo *decreased rapidly in the initial hours and rubidazone displayed the lowest fluorescence retention of the* 3 *compounds. Therapeutic effects on mice bearing L* 1210 *leukemia were on the order of ADM > rubidazone ≧ DNR. The therapeutic effects of the* 3 *compounds were universally decreased in immunosuppressed mice bearing L* 1210 *leukemia. A slower rate of fluorescence decay from tumor cells during the first hours after drug administration appeared to be the only parameter correlated with the therapeutic superiority of ADM.*

INTRODUCTION

TWO PREPARATIONS of glycoside antibiotics of the anthracycline group, DNR and ADM, were shown to be among the most useful chemotherapeutic agents available for the treatment of malignant neoplasms in man (See recent reviews in [1–3]). The side effects of the two antibiotics are similar, including alopecia, stomatitis, nausea and vomiting and dose-limiting hematological and cardiac toxicity. After demonstration of the encouraging clinical efficacy of these compounds, further attempts were made to explore the anthracycline antibiotics through isolation of new compounds or chemical modification of existing drugs in a search for more active and less toxic compounds.

Rubidazone (RP 22050), the benzoylhydrazone chlorhydrate of DNR, is a new semisynthetic derivative (Fig. 1). Jacquillat *et al.* reported 40 (57%) complete remissions in 70 patients with previously untreated acute myelocytic leukemia and 13 (50%) in 26 patients with acute myelocytic leukemia in first relapse [4]. Clinical trial of rubidazone has been initiated in the United States [5]. The precise position of rubidazone as an antitumor agent among the anthracycline antibiotics has not been established.

We undertook the present study to determine the position of rubidazone in the family of clinically available anthracycline antibiotics and to explain its biological effects on a pharmacological basis, particularly in comparison with DNR and ADM.

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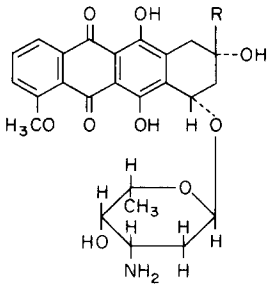
The abbreviations used are: DNR = daunorubicin, daunomycin hydrochloride; ADM = adriamycin, doxorubicin hydrochloride; PBS = phosphate buffer saline.

MATERIALS AND METHODS

Rubidazone, RP 22050 (Lot HB 5140,



R:Rubidazone $C(=N-NHOC-C_6H_5)(CH_3)$

DNR $COCH_3$

ADM $COCH_2OH$

Fig. 1. Molecular structures of rubidazone, DNR and ADM.

50 mg of base per vial), formulated by Société Parisienne D'expansion Chimique, 21 Rue Jean-Goujon, Paris (8e), France, was a gift from Professor C. Jacquillat, Department of Hematology, Institut de Récherches sur les Leucémies, Hôpital Saint-Louis, Université de Paris, France.

DNR (Batch No. 162, 20 mg of base per vial) and ADM (Batch No. 77, 10 mg of base per vial) were obtained from Farmitalia Research Laboratories, Milan, Italy (the distributor of ADM is Adria Laboratories, Inc., Wilmington, DE) through the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

The drugs were dissolved in 0.15 M NaCl and compared on an equimolar basis. The molecular weights of rubidazone, DNR and ADM were calculated to be 681.45, 563.45 and 579.45, respectively.

Determination of anthracycline fluorescence in biological samples

The method for extracting and quantifying total anthracycline fluorescence has been described previously [6].

The fluorescence associated with the anthracycline moiety of the drug and metabolites was determined with a Perkin–Elmer MPF 3 fluorescence spectrometer with an excitation wavelength of 485 nm and an emission wavelength of 580 nm. The fluorescent level was compared with that of a known concentration of a corresponding anthracycline compound in a similar solution. Since this method does not differentiate between the parent drug and fluorescent metabolites in biological tissue [7], the results were expressed as equivalents.

The recovery of anthracycline compounds from animal tissues *in vitro* was studied as follows: 50% homogenates of combined whole tissues of liver, kidney, heart, lung, spleen and muscle from DBA/2 Ha-D mice (Roswell Park Memorial Institute, Buffalo, NY) were made in phosphate buffer saline (PBS), pH 7 (Gibco, Grand Island, NY). One half of a milliliter of the tissue homogenate was mixed with 50 μl of a solution containing 10 or 20 nmole of the compound. After 1 and 24 hr exposures (in order to cover an ample time period for the *in vivo* study) at room temperature, the compound was extracted from the homogenate with 0.3 N HCl/50% ethanol. The fluorescence of the 20,000 *g* supernatant of the homogenate was determined and compared with the similarly treated buffer control.

For the determination of plasma drug level, ICR/Swiss mice (Roswell Park Memorial Institute) were given 50 μmole/kg of each compound by intravenous administration into the tail vein, ICR/Swiss mice were chosen for the ease with which their tail vein could be injected. At intervals blood was collected from a set of 3–6 mice by cardiac puncture with a heparinized syringe and then pooled. Plasma was diluted 3 times with the extraction solution. The samples were centrifuged at 20,000 *g* for 20 min and the fluorescence of the supernatant solution was determined. Non-specific plasma fluorescence from control mice that received 0.15 M NaCl alone was subtracted for the calculation of the equivalents of each anthracycline compound.

For the determination of tissue distribution, tissue specimens from DBA/2 Ha-D mice were obtained 30 min and 2 hr after i.v. administration of 50 μmole/kg of each compound, rinsed in 0.15 M NaCl, blotted and homogenized in 20 vol/g of a 0.3 N HCl/50% ethanol solution with a Sorval Omnimixer homogenizer (Ivan Sorval Inc., Newton, CO) for 3 min. The homogenates were centrifuged at 20,000 *g* and fluorescence in the supernatant was determined as above. Non-specific fluorescence from control mice that received 0.15 N NaCl was subtracted for the calculation of the equivalents.

For the determination of drug uptake *in vitro* into L1210 murine leukemia cells (obtained from the Department of Experimental Therapeutics, Roswell Park Memorial Institute and maintained in our laboratory by serial transplantation into DBA/2 Ha-D mice), cells were collected from tumor bearing animals, contaminated red blood cells were lysed by hypotonic shock and then washed once in 0.15 M NaCl. Test tubes (17

× 100 mm, Falcon Plastics, Oxnard, CA) containing 2 ml of minimal essential medium (Gibco), 10^7 L1210 cells and 25 μg/ml of an anthracycline compound were incubated in a Dubnoff shaking incubator at 37°C or in an ice bath. At appropriate intervals the incubated tubes were placed in ice and the medium was removed by washing twice with cold 0.15 M NaCl. Two ml of 0.3 n HCl/50% ethanol were added to the cell pellet, mixed thoroughly, centrifuged at 10,000 ***g*** for 20 min and the supernatant assayed for fluorescence.

Drug uptake into and retention in L1210 cells *in vivo* was determined by the method described by Kessel *et al.* [8] with a minor modification. DBA/2 Ha-D mice bearing 6 day old L1210 ascites (initial inoculum 10^5 cells per mouse) received 0.05 μmole i.p. per mouse of either rubidazone, DNR or ADM. Ascites samples containing 40–400 mg (wet weight) of L1210 cells were removed with needle and syringe at intervals into preweighed tubes. Contaminated red blood cells were lysed by hypotonic treatment and then washed twice with cold 0.15 M NaCl. The resulting cell pellets were weighed in the tube and fluorescence was extracted with 2 ml per tube of the acid ethanol solution. Similarly treated L1210 cells from mice which received 0.15 M NaCl were used to correct endogenous fluorescence.

Therapeutic activity of rubidazone, DNR and ADM

These experiments were carried out as described previously [6]. DBA/2 Ha-D female mice 3–4 months old weighing 18–22 g received 10^3 viable L1210 cells in 0.2 ml of PBS on day 0. The mice were randomized on day 1 and the compounds were given i.p. for 5 days (0.5 ml/day/mouse) from days 2 to 6. Therapeutic activity was determined by the survival observed daily for 45 days.

For the study of the effects of the anthracycline compounds on immunosuppressed mice, DBA/2 Ha-D female mice 3–4 months old received i.p. administration of cyclophosphamide 150 mg/kg on day −1 [9]. On the following day, 10^3 L1210 cells were given i.p. and the anthracycline compounds were administered for 5 days. The treated and control mice were observed for 45 days.

RESULTS

When rubidazone, DNR and ADM standards in 0.3 N HCl/50% ethanol were plotted on an equimolar basis, essentially identical

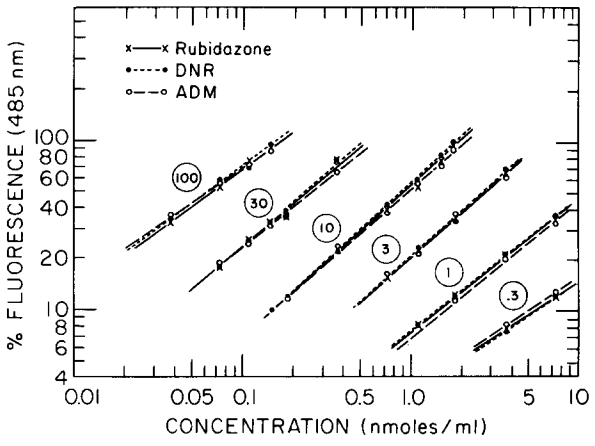


Fig.2. Relationship between fluorescence value vs concentration of rubidazone, DNR or ADM plotted on a molar basis. The spectrofluorimeter was adjusted to 100% *at a sensitivity setting of* "10" *for DNR* 1 μg/ml *in* 0.3 N HCl/50% *ethanol, and the fluorescence was measured using excitation wavelength of* 485 nm *and emission wavelength of* 580 nm. *The number in the circle represents the sensitivity setting.*

fluorescence was obtained (Fig. 2), indicating that comparison of the 3 anthracycline compounds on an equimolar basis is possible with this method. Recovery of the anthracycline compounds from biological tissue incubated up to 2 hr was complete with a range of 90–110% of control. These results indicate the fluorescence was not significantly altered in the presence of biological tissue within the experimental conditions described.

Plasma levels of rubidazone, DNR and ADM

The plasma levels and clearance of rubidazone, DNR and ADM equivalents after i.v. injection of 50 μmole/kg of the anthracycline compounds are shown in Fig. 3. The initial plasma levels appear to be on the order of rubidazone > ADM > DNR but in 30 min the

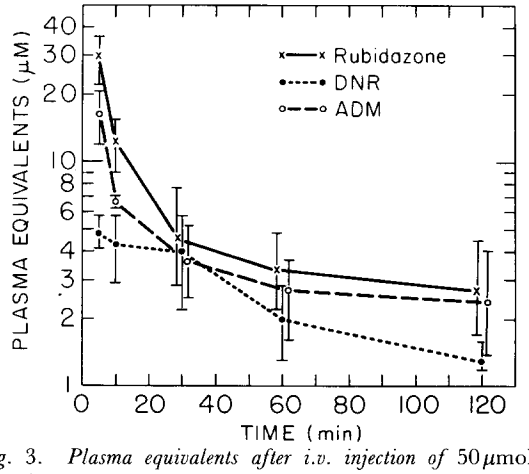


Fig. 3. Plasma equivalents after i.v. injection of 50 μmole/kg *of rubidazone, DNR and ADM. Results are plotted as the mean and range of* 3 *separate experiments.*

equivalents of all these compounds seemed to overlap and were slowly cleared from plasma.

The plasma levels 10 min after injection of the 3 compounds ranged from only 4 to 10% of an estimated total body water distribution (600 ml/kg), indicating that all 3 compounds bound rapidly to the tissue. ADM plasma levels at early time points were higher than those of DNR and are consistent with an earlier report [10]. Rubidazone-related plasma fluorescence levels were even higher. thus, observed therapeutic differences (see below) could not be explained by plasma fluorescence levels alone.

Tissue distribution of rubidazone, DNR and ADM

Rubidazone, DNR and ADM equivalents in various organs of 2 mice at 30 min and at 2 hr after i.v. injection of each compound are shown in Table 1. Fluorescence of the 2 hr heart homogenate after DNR injection appeared to be about half that after ADM and the fluorescence of the rubidazone group was midway between. Uptake of the equivalents into spleen and kidney was appreciably higher in mice receiving rubidazone and DNR compared to those receiving ADM.

Somewhat higher DNR equivalents than ADM equivalents in the kidney are consistent with increased urinary excretion of the former compound [10].

Uptake of rubidazone, DNR or ADM equivalents into L 1210 leukemia cells in vitro

The uptakes of rubidazone, DNR and ADM equivalents into L1210 leukemic cells *in vitro* are shown in Fig. 4. All were temperature dependent. DNR uptake was rapid at 37°C, reaching a high plateau by 30 min. In contrast, there was a gradual and progressive increase in ADM uptake which did not reach a plateau in 24 hr. These findings are consistent with work reported by Meriwether *et al.* [11].

Rubidazone uptake appeared to be a mixture of these 2 different patterns. Thus, there was rapid and incomplete uptake occurring in 30 min, followed by a slow progressive uptake lasting for at least 24 hr. The temperature dependence of rubidazone uptake was similar to that of other anthracycline antibiotics.

At later hours a definite decrease in the DNR fluorescence was noted. This suggested an efflux from the cells of DNR but not of ADM and rubidazone.

Uptake and retention of rubidazone, DNR and ADM equivalents into L 1210 leukemia cells in vivo

The results of these experiments are illustrated in Fig. 5. The initial high uptake of

Table 1. *Tissue distribution of rubidazone, DNR and ADM in DBA/2 Ha-D mice expressed as total equivalents* (μmole/g *wet tissue*)

	Rubidazone		DNR		ADM	
	30 min	2 hr	30 min	2hr	30 min	2 hr
Lung	129	65	96	54	99	75
	131	73	115	61	109	86
Heart	66	40	61	27	63	54
	73	43	69	33	66	61
Liver	123	54	124	42	127	42
	154	71	131	57	131	50
Spleen	93	74	77	68	47	42
	98	76	102	88	58	59
Kidnet	140	116	140	78	126	62
	154	118	152	94	131	64
Muscle	18	15	33	20	24	21
	19	21	37	23	24	24

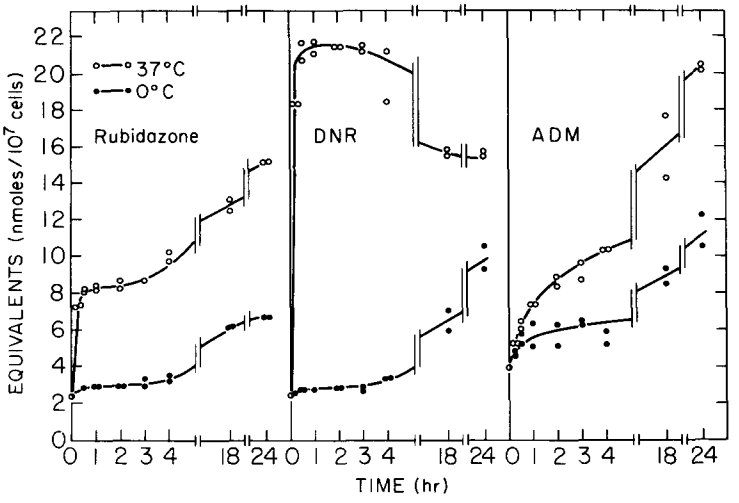


Fig. 4. Uptake of rubidazone, DNR and ADM equivalents into L 1210 leukemia cells in vitro. *An experiment run in duplicate is shown.*

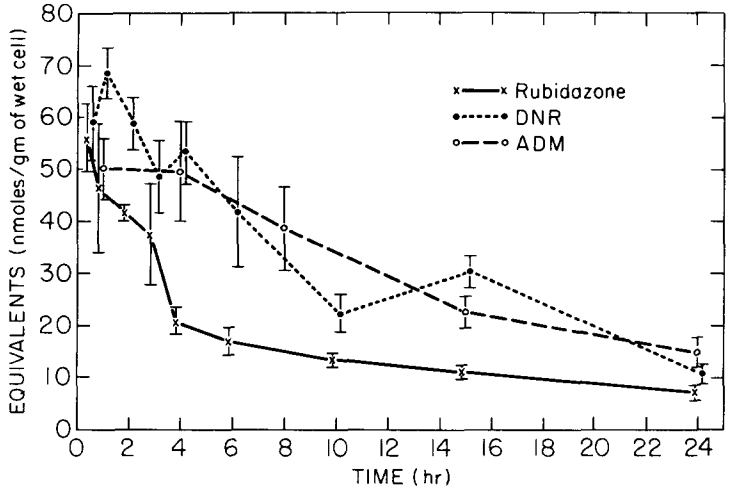


Fig. 5. Retention of rubidazone, DNR and ADM equivalemts in L 1210 leukemia cells in vivo. *Mean* ± S.E.M. *of at least 2 triplicate experiments.*

DNR equivalents at 30 min and 1 hr compared to that of the rubidazone and ADM equivalents is in accord with the *in vitro* observations described.

We noted that the decay slopes of DNR and rubidazone retention were steep in the early hours after administration of the compounds, while ADM retention was stable. Thus, the DNR retention decreased rather rapidly and overlapped with that of ADM in 4 hr and then gradually decreased. Rubidazone retention also decreased rapidly in the initial hours and it reached a level in 6 hr which ADM and DNR did not attain until 24 hr. The better ADM retention in the initial hours appeared to be due to an increased affinity of the tumor cells for this compound and this probably correlates to the superior therapeutic efficacy of ADM.

Antitumor activity of rubidazone, DNR and ADM on L 1210 murine leukemia

The comparative antitumor activities of the 3 anthracycline compounds given i.p. in mice inoculated with L1210 leukemia are shown in Table 2. The superiority of ADM over DNR in L1210 murine leukemia was as reported by Sandberg *et al.* [12]. The moderate antitumor activity of DNR at a low tumor load was as reported previously [6]. Rubidazone appeared to be an active drug but its activity fell behind that of ADM. It may be concluded that the therapeutic activity of the 3 compounds against L1210 murine leukemia is on the order of ADM > rubidazone ≧ DNR.

Table 2. Comparison of antitumor activity among rubidazone, DNR and ADM on L 1210 bearing DBA/2 Ha-D mice

Dose μmole/kg/day days 1–5	Rubidazone			DNR			ADM		
	MST* (range) days	ILS† %	>45 days	MST* (range) days	ILS† %	>45 days	MST* (range) days	ILS† %	>45 days
Exp. I									
0	11 (10–14)		0/7						
1	15 (11–14)	36	0/7	15 (13–16)	36	2/7	17 (12–>45)	54	2/7
2	16 (12–>45)	45	2/7	15 (13–18)	36	1/7	>45 (11–>45)	>308	4/7
4	16 (13–36)	45	0/7	13 (10–16)	18	0/7	16 (7–>45)	45	2/7
6	9 (8–14)	−18	0/7	9 (7 –13)	−18	0/7	15 (7–>45)	36	0/7
Exp. II									
0	12.5 (10–14)		0/6						
0.5	14 (11–15)	12	0/6	13.5 (12–>45)	8	1/6	14.5 (13–16)	16	0/6
1	19.5 (12–31)	56	0/6	15.5 (13–>45)	24	1/6	>45 (15–>45)	>260	4/6
2	>45 (14–>45)	>260	4/6	18 (12–>45)	44	2/6	>45 all>45)	>260	6/6
3	11.5 (9–19)	−8	0/6	12 9–22)	−4	0/6	>31 (12–>45)	>148	3/6
4	9 (9–22)	−28	0/6	10 (9–11)	−20	0/6	20 (17–>45)	60	1/5

*MST, median survival time.
†The percentage increase in median survival time over control.

Antitumor activity of rubidazone, DNR and ADM on L1210 murine leukemia inoculated into immunosuppressed mice

The comparative antitumor activities of the 3 anthracycline compounds given i.p. in immunosuppressed mice inoculated with L1210 leukemia are shown in Fig. 6. Although the antitumor activities of the compounds were substantially lower in comparison with those in non-immunosuppressed animals, ADM remained relatively superior to DNR and rubidazone.

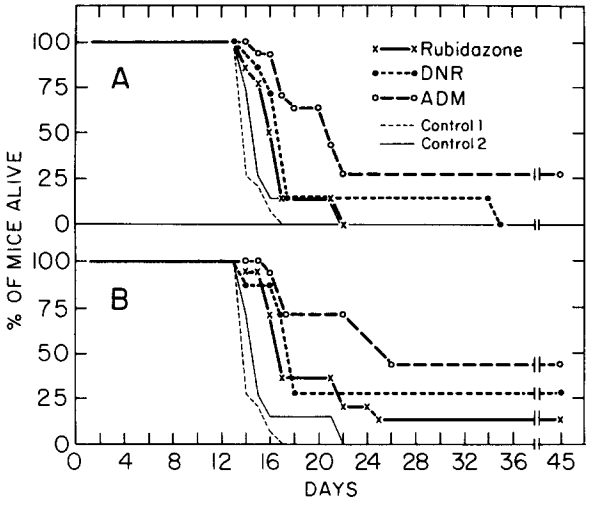


Fig. 6. Therapeutic effects of rubidazone, DNR and ADM in immunosuppressed mice inoculated with L 1210 leukemia. Mice were immunosuppressed with 150 mg/kg *of cyclophosphamide on* day -1. 10^3 *L 1210 cells were given i.p. on* day 0. *Treatment was given from* days 1 *to* 5. *A:* 1 μmole/kg/day ×5; *B:* 2 μmole/kg/day ×5; *control* 1 = *cyclophosphamide and L 1210; and control* 2 = *L 1210 alone. The rubidazone and ADM experiments were done twice using 7 mice in each group and the combined data are presented. The experiments with DNR were carried out once only.*

DISCUSSION

The present study revealed that the antineoplastic activity of rubidazone against L1210 murine leukemia was at least equivalent to DNR but was definitely less than that of ADM on an equimolar basis. The therapeutic effects of ADM are greater than those of DNR in a variety of animal tumor systems including L1210 [9, 12–15]. This appears to correlate with the increased activity of ADM in man, especially in patients with carcinomas and sarcomas. The antileukemic effects of rubidazone, essentially in a range with DNR as shown in the present study, are consistent with its reported antileukemic effects in man [4].

The biological superiority of ADM over DNR has not yet been universally explained by pharmacological data alone. Di Fronzo *et al.* showed that uptake and retention of ADM was consistently superior to DNR in Sa 180 after i.v. injection of the compound [16]. Kessel *et al.* were able to relate therapeutic effects of DNR in a variety of animal tumors to the retention of the drug *in vivo* [8]. In contrast, Schwartz *et al.* reported that the uptakes of DNR and ADM into P-288 *in vivo* after a single i.v. dose were similar over a 4 hr period [9]. Their observation that the therapeutic effects of DNR and ADM became identical in cyclophosphamide-treated animals and that DNR fluorescence was higher than that of ADM in the spleen led to the conclusion that ADM's superiority over DNR was related to its lesser immunosuppressive effects. Yet, comparison of the therapeutic effects of the 3 compounds in immunosuppressed animals in the present study showed that there was no preferential decrease in therapeutic activity.

Recently Skovsgaard studied the determinants of net cellular uptake of these 3 compounds using Ehrlich ascites cells *in vitro* [17]. His findings suggested passive transport of the drugs into the cells. He also observed an efflux of DNR and rubidazone but not of ADM. The overall membrane transport of the drugs was termed a "leak-and-pump" system. Our observations on the *in vitro* uptake of the 3 compounds are similar to his and by prolonged observation we were able to differentiate the pattern of uptake between ADM and rubidazone. The decreased fluorescence uptake in the DNR-treated cells at later hours is consistent with the efflux of DNR observed by Skovsgaard.

Our observation of prolonged ADM retention as shown by its gentle decay from L1210 *in vivo* (in contrast with DNR or rubidazone) is consistent with the report of ADM's higher affinity for isolated nuclei and for total cellular homogenate. The markedly low rubidazone retention was again in accord with its reported lowest affinity among the 3 compounds [17]. From these data it appears that the rate of fluorescence decay from tumor cells *in vivo* in the early hours after drug administration correlates with the affinity of drug to the leukemic cells and with therapeutic efficacy. As the slope of fluorescence decay becomes steeper, drug-tumor cell affinity, and, probably, cell kill, decrease as is seen with both DNR and rubidazone.

It is of interest to note that the 2 hr DNR

concentration in the heart was about half that of ADM. In the rabbit model critical cardiotoxic doses of DNR and ADM were reported to be 400 and 250 mg/m^2, respectively (D. Young, presented at the Anthracycline Antibiotics Meeting, National Cancer Institute, 15 July, 1974). We observed a higher uptake of DNR than ADM in the spleen but the peak ADM and DNR concentrations were observed at later hours [10]. Thus our observation on the spleen sample may have little significance.

Attempts have been made to identify various active and inactive metabolites of ADM and DNR in animals and man [15, 18–21]; however, no major metabolites of rubidazone have yet been identified unequivocally. Since the relative antitumor activities and interactions of various metabolites of these compounds have not been delineated fully and the metabolotes are not available for testing of their individual antileukemic effects in the L1210 system *in vivo*, we did not attempt to assay the concentrations of various metabolites from tested compounds in the animal plasma and in tumor cells.

Additional data on the antitumor activities of individual metabolites in defined tumors and the rate of intracellular retention and decay of these metabolotes in neoplastic and normal tissues are necessary before the differential therapeutic efficacies of the anthracycline compounds can be fully elucidated pharmacologically.

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