Eur J Cancer Clin Oncol, Vol. 25, No. 4, pp. 679–685, 1989.
Printed in Great Britain

0277-5379/89$3.00 + 0.00

Increasing Therapeutic Effect and Reducing Toxicity of Doxorubicin by *N*-Acyl Dehydroalanines

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Abstract—*Doxorubicin toxicity is generally accepted to be free radical-mediated. N-Substituted dehydroalanines (indexed as AD compounds) are capto-dative olefins which react and scavenge free radicals, especially the superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$). AD-20, an ortho-methoxyphenylacetyl dehydroalanine derivative, decreases the mortality of mice when administered before an acute single dose or multiple non-toxic doses of doxorubicin. Doxorubicin administered to mice induces elevated serum transaminase levels, and the pretreatment of mice with AD-20 decreases significantly these serum enzymatic activities. Preliminary histological examinations suggest that these serum transaminase elevations reflect most likely liver injury. In addition to its cardiotoxicity, doxorubicin induces a severe bone marrow depletion. Although this initial decrease in the peripheral leukocytes induced by doxorubicin is not prevented by the administration of AD-20, it produces a fast recuperation in the white blood cells levels after 1 week, supporting a protective effect at this level. Moreover, the antitumor effect of doxorubicin in L1210 tumor-bearing mice was enhanced when AD-20 was injected before doxorubicin. We postulate that these effects may be related to the free radical scavenging ability of AD-20.*

INTRODUCTION

DOXORUBICIN is an anticancer drug widely used for different human malignancies [1, 2]. However, the clinical therapeutic application of doxorubicin is limited by the appearance of selective organ toxicities: myelosuppression and gastrointestinal mucositis after a single i.v. dose (up to 60–75 mg/m^2 body surface area) and the risk of cardiomyopathy and congestive heart failure when a total cumulative dose up to 550 mg/m^2 is exceeded [3].

Although the mechanism of doxorubicin cardiotoxicity still remains unclear, evidence from several experimental studies suggests that it may be the consequence of the production of reactive oxygen species during its cardiac metabolism [4–7]. Indeed, doxorubicin belongs to the redox cycling drugs, generating an electron flow from reduced pyridine nucleotides to molecular oxygen, which leads to a significant increase in $O_2^{\cdot-}$ and H_2O_2 formation in mitochondria and sarcoplasmic reticulum [8–11]. Furthermore, cardiac tissue produces small amounts of catalase and superoxide dismutase and doxorubicin inhibits glutathione peroxidase [8].

In order to protect cells against the deleterious effects resulting from oxidative damage induced by doxorubicin metabolism, some experimental approaches have been developed to prevent that toxicity by the use of antioxidant molecules such as α-tocopherol [12] or ascorbate [13], SH donor compounds like *N*-acetyl cysteine [14] or glutathione [15], and a radical dimer rescue agent [16]. Recently we have developed a new class of molecules with free radical scavenging properties: the capto-dative olefins [17]. Among them, of particular interest are the so-called *N*-acylaryl dehydroalanines (Fig. 1), indexed as AD compounds, which react and scavenge *in vitro* both $O_2^{\cdot-}$ and $HO^{\cdot}$ and inhibit their deleterious effects, in particular lipid peroxidation [18, 19]. These capto-dative olefins stop the free radical chain reaction by decreasing the reactivity of free radicals. They probably achieve this reaction by forming stabilized radical adducts which often disappear by dimerization or by reaction with another free radical [20].

Accepted 18 November 1988.
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List of abbreviations: AD-20 = *N*-(*o*-methoxyphenylacetyl) dehydroalanine; MST = median survival time; mst = mean survival time; SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvic transaminase; ILS = increase in life span.

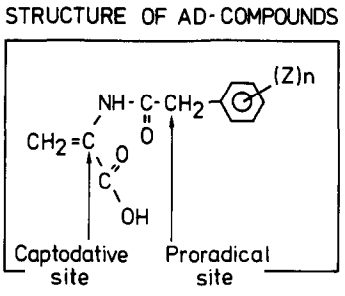

Fig. 1. Structure of AD compounds.

Since the accumulation of drug-induced reactive oxygen species may explain the toxicity of doxorubicin, and based on the premise that its antitumor effects operate in a separate way from its toxic effects, we have tested whether AD-20 [an *N*-(*ortho*-methoxyphenylacetyl)-dehydroalanine derivative], by reducing the toxicity, may decrease the lethality induced by doxorubicin. Previous data [19, 21] have indicated that, *in vitro*, AD-20 protects heart mitochondria from some of the effects of doxorubicin which could be related to its toxicity.

As the reduction of doxorubicin toxicity could modify its therapeutic effect, it was important to test whether AD-20 would also modify its cytostatic effect against cancer cells. Therefore, the antitumor effect of doxorubicin was evaluated in the absence and in the presence of AD-20 by using as a model L1210 leukemia-bearing mice.

The results obtained and reported in this paper indicate that the treatment of animals with AD-20, before doxorubicin administration, provides a survival benefit to mice against its toxicity, and also an enhancement of its antitumor effect in tumor-bearing mice.

MATERIALS AND METHODS

Drugs and chemicals

Doxorubicin hydrochloride was obtained from Farmitalia-Carlo Erba (Milan), and AD-20 was synthesized by Professor H.G. Viehe and his coworkers at the Organic Chemistry Laboratory (Université Catholique de Louvain). Diagnostic reagents for the determination of both glutamate–oxaloacetate and glutamate–pyruvate transaminase serum levels were obtained from Boehringer (Mannheim).

Experimental animals

Mice were housed in groups of 10 per cage in a constant (22°C) temperature environment with alternating 12 h wake–sleep cycles, and they received standard food and water *ad libitum*.

Drug administration and treatments

Doxorubicin was dissolved in sterile deionized water and administered intraperitoneally (i.p.). AD-20 was also i.p. administered 15 min before doxorubicin (excepting for the kinetic studies) as a water suspension of gum arabic (2%). The solutions were freshly made prior to each treatment.

Doxorubicin lethality. Male mice of the NMRI strain obtained from 'Animalerie Centrale-UCL' were used in these experiments. They were 3 months old and had a mean body weight of 25 g. To evaluate the effect of AD-20 on survival after an acute single dose of doxorubicin (15 mg/kg i.p.), randomized groups of mice received either sterile deionized water or a single i.p. injection of AD-20 at different doses varying from 15 to 90 mg/kg, administered at different times either before or after doxorubicin administration. Mortality was recorded daily and the effect of AD-20 was evaluated by comparing the median survival time (MST) and the number of survivors in doxorubicin and water treated groups at the end of the experiment. The same parameters were used to evaluate the effects of AD-20 on survival of mice after a multiple dose treatment with doxorubicin (3 × 5 mg/kg). In that protocol AD-20 was administered at a dose of 10 mg/kg by i.p. injection 15 min before each doxorubicin administration.

Doxorubicin toxicity. (a) Transaminase (GPT and GOT) serum levels: Male mice of the NMRI strain were divided in three groups. The mice in the first group were treated with two sterile 0.9% NaCl i.p. injections within 15 min. In group 2, the animals received an equal volume of saline and 15 min later they were injected with a single acute i.p. dose of doxorubicin (15 mg/kg). The third group of mice was treated with AD-20 (30 mg/kg i.p.) 15 min before doxorubicin injection as in group 2. Twenty-four hours after the treatment, the animals were killed by exsanguination, and the serum was separated by centrifugation. SGOT and SGPT levels were determined spectrophotometrically at 25°C following the method described by Reitman and Frankel [22]. The transaminase levels were expressed as International Units per liter (IU/l).

(b) Myelotoxicity study: Male mice of NMRI strain were divided in three groups. The mice of group 1 received 0.9% NaCl by i.p. administration. The second group of mice was treated with doxorubicin alone (6 mg/kg i.p.), while in group 3 mice received AD-20 (50 mg/kg i.p.) 15 min before doxorubicin. Animals were anesthesized with Nembutal on the days indicated, and blood was collected by axillary puncture with a syringe containing EDTA as anticoagulating agent. White blood cells were counted in a Neubauer cell by using Turk solution and a micropipette for leukocyte count.

Doxorubicin antitumor effect. Male 3-month-old BDF/1 mice weighing 25–27 g were obtained from

Iffa-Credo (Les Oncins). The L1210 murine lymphocytic leukemia strain was obtained from Institute Jules Bordet (Brussels) and it was maintained *in vivo* by weekly serial passage of 10^6 cells obtained from ascites and injected i.p. into DBA/2 mice. 10^5 L1210 tumor cells were injected i.p. to mice on day 0. On day 1, the mice were randomized into groups receiving either deionized sterile water or a single i.p. injection of AD-20 (25 mg/kg). After 15 min all animals (except those of the non-treated control group) were i.p. injected with a single dose of doxorubicin (7 mg/kg). Each experimental group consisted of 10 mice. The efficacy of each chemotherapeutic treatment was determined by calculating the mean survival time (mst), the increase in life span (ILS), and by counting the long-term survivors. In addition, the animals were weighed daily in order to evaluate the toxicity of the treatments. The weight loss at day 5 expressed as a percentage of weight on day 0 was used to compare the toxicity of the treatments.

Statistical significance was estimated using Student's *t*-test for paired or impaired observations. A *P* value of less than 0.05 was considered significant. The statistical analysis of the treatment of mice for both toxicological and therapeutic studies were evaluated by the use of the parameters median survival time (MST), mean survival time (mst), and increase in life span (ILS) as described by Geran *et al.* [23].

RESULTS

Effect of AD-20 on survival of mice following the administration of a single dose of doxorubicin

(a) *Influence of the administered doses of AD-20.* Increasing doses of AD-20, administered i.p. 15 min before doxorubicin, results in a non-linear increase in the survival time (Table 1). Among the four doses of AD-20 tested, only 30 mg/kg gives real protection as measured by the MST (>56 days) and the number of survivors (11/20), when compared to doxorubicin alone. A dose of 90 mg/kg of AD-20 increases the toxicity of doxorubicin.

Table 1. *Effect of increasing doses of AD-20 on survival of mice after receiving a single dose of doxorubicin*

	Dose (mg/kg)	MST (days)	S*
Doxorubicin	15	7.5	4/20
AD-20	15	10.5	4/20
AD-20	30	>56	11/20
AD-20	60	10.5	2/20
AD-20	90	7.0	2/20

AD-20 was i.p. administered at the indicated doses 15 min before doxorubicin.
*Number of mice surviving/total number of mice.

(b) *Influence of the time of AD-20 administration.* Table 2 shows that AD-20 protects against doxorubicin lethality when it is administered a short time before doxorubicin. After doxorubicin administration there is no protection. Even though the simultaneous administration of doxorubicin and AD-20 gives some protection (MST = 11.5 days against MST = 5.5 days of doxorubicin alone), it has a less protective effect as compared to the administration 5 and 15 min before doxorubicin. A higher effect is obtained with AD-20 administered 15 min before doxorubicin.

Table 2. *Effect of AD-20 administered at different times on survival of mice after receiving a single dose of doxorubicin*

	Time (min)	MST (days)	S*
Doxorubicin	—	5.5	4/30
AD-20 + doxorubicin	−30	11.5	2/10
AD-20 + doxorubicin	−15	42.0	4/10
AD-20 + doxorubicin	− 5	21.5	4/10
AD-20/doxorubicin†	0	11.5	2/10
Doxorubicin + AD-20‡	+15	7.5	1/10

Both treatments were administered i.p., the dose of doxorubicin was 15 mg/kg and that of AD-20 was 30 mg/kg.
*Number of mice surviving/number of total mice.
†Simultaneous administration.
‡AD-20 administered after doxorubicin.

(c) *Prevention of doxorubicin lethality by AD-20.* When doxorubicin is administered as a single i.p. injection at a dose of 15 mg/kg it produces a significant lethality with 80% of mice dead within the first 10 days (Fig. 2, dashed line). Pretreatment of animals with AD-20 given 15 min before the injection of doxorubicin significantly enhances animal survival (Fig. 2, continuous line). In fact, after 8 weeks from the onset of doxorubicin treatment, AD-20 administration provided a survival advantage: 50% of the mice survived in the group treated with AD-20 and doxorubicin, as compared to only 10% in the doxorubicin-treated group. In addition, the MST at the end of the experiment (day 56) was 5.5 days for animals pretreated with doxorubicin alone and more than 56 days for mice receiving AD-20 and doxorubicin. During the 8 weeks of experimental observation AD-20 alone produced no mortality when given i.p. at doses up to 1 g/kg (data not shown).

Effect of AD-20 on survival of mice following the administration of multiple doses of doxorubicin

Since pretreatment with AD-20 before a single acute dose of doxorubicin significantly increases the survival time of the animals, we examined its protective action in mice treated with multiple non-toxic drug doses. This protocol seemed closer to that of doxorubicin administration in man.

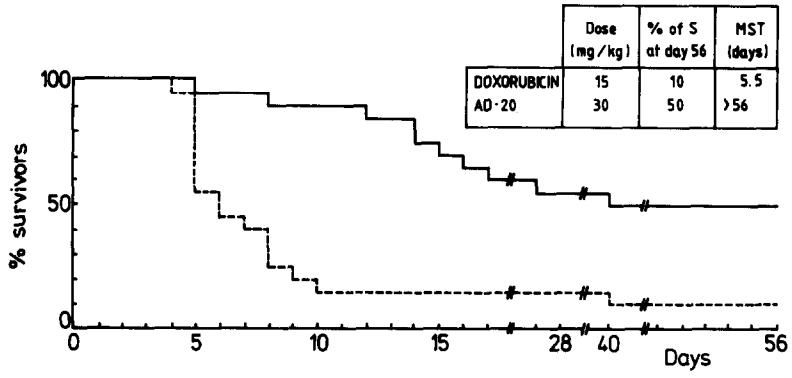

Fig. 2. Effect of AD-20 on lethality of mice after receiving a single dose of doxorubicin. Doxorubicin alone (dashed line) at a dose of 15 mg/kg *was injected i.p. AD-20* (30 mg/kg) *and later on doxorubicin at the same dose (continuous line) were injected i.p. separately with an interval of* 15 min. *AD-20 was administered as a water suspension of gum arabic* (2%). *Each experimental group consisted of* 30 *mice.*

AD-20 treatment (10 mg/kg) given to mice 15 min prior to doxorubicin injection (Fig. 3, continuous line) significantly improves both short- and long-term survival after repeated, small doses of the drug (3 × 5 mg/kg). One week after the last injection of doxorubicin (day 35), the survival rate was 68% and 32% for the groups of mice receiving doxorubicin and AD-20 and doxorubicin alone respectively. At the end of the experiment (12 weeks after the last injection of doxorubicin), survival in the group pretreated with AD-20 was 20% as compared to 6% for animals receiving doxorubicin alone (Fig. 3, dashed line). The median survival time (MST) for animals receiving AD-20 and doxorubicin was 40.0 days, whereas in mice pretreated with doxorubicin alone the parameter has a value of 31.5 days only.

Effect of AD-20 on doxorubicin toxicity

(a) *Transaminase serum levels following the administration of a single acute dose of doxorubicin.* Cell injury induced by doxorubicin leads to hepatic and cardiac tissue damage, producing a pattern of morphological abnormalities [24]. In order to see whether the pretreatment with AD-20 before a single acute i.p. dose of doxorubicin (15 mg/kg) would prevent damage caused in the liver of mice, we measured the serum levels of GPT and GOT. For the animals receiving 0.9% NaCl (group 1) the serum GPT and GOT levels, 24 h after doxorubicin administration, were 34 ± 3 UI/l and 54 ± 5 UI/l respectively (Table 3). In the experimental group receiving doxorubicin alone (group 2), the GPT and GOT values were significantly elevated (260 ± 22 UI/l and 121 ± 5 IU/l), compared to the saline control group ($P < 0.05$). The SGPT and SGOT activities of mice treated with AD-20 (30 mg/kg) 15 min before doxorubicin (group 3) were still significantly elevated when compared to the saline control group (150 ± 36 IU/l and 96 ± 11 IU/l respectively) ($P < 0.05$), but they were significantly lower than those of the group treated with doxorubicin alone ($P < 0.05$). These results show that AD-20 protects, to a certain extent, the hepatic tissue of mice receiving a toxic dose of doxorubicin.

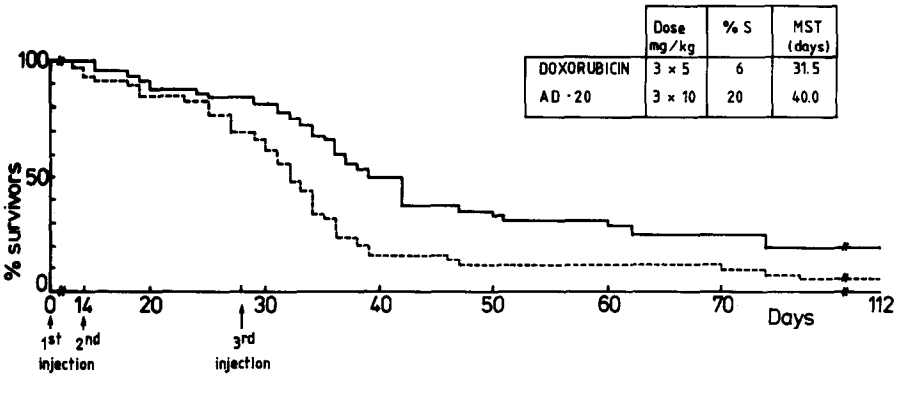

Fig. 3. Effect of AD-20 on lethality of mice after receiving multiple doses of doxorubicin. Doxorubicin alone (dashed line) at a dose of 5 mg/kg *was injected i.p. at days* 1, 14 *and* 28. *AD-20* (10 mg/kg) *and later on doxorubicin (continuous line) were injected i.p. separately with an interval of* 15 *min on the same days. AD-20 was administered as a water suspension of gum arabic* (2%). *Each experimental group consisted of* 50 *mice.*

Table 3. Effect of AD-20 on the serum levels of GPT and GOT in mice receiving a single dose of doxorubicin

Group	Treatment		SGPT (UI/l)	SGOT (UI/l)
1	Control	(13)	34 ± 3	54 ± 5
2	Doxorubicin	(16)	260 ± 22*	121 ± 5*
3	AD-20	(10)	150 ± 36*†	90 ± 11*†

Doxorubicin was injected i.p. at a dose of 15 mg/kg. AD-20 was also injected i.p. 15 min prior to doxorubicin at a dose of 30 mg/kg.
( ): number of mice utilized.
*Statistically significant difference from control values ($P < 0.05$).
†Statistically significant difference from doxorubicin values ($P < 0.05$).

Table 4. Effect of AD-20 on bone marrow depression induced by doxorubicin

Days	WBC ($\times 10^2$/mm^3 ± S.D.) Control Group 1	Doxorubicin Group 2	Doxo + AD-20 Group 3
0	85.5 ± 18.6	76.5 ± 7.5	78.2 ± 10.2
2	82.4 ± 17.4	34.9 ± 6.6*	43.4 ± 17.7*
4	92.7 ± 13.0	18.7 ± 9.2*	18.6 ± 7.8*
7	71.4 ± 15.1	60.7 ± 10.8	82.8 ± 13.7
9	72.6 ± 18.3	51.5 ± 6.1*	83.7 ± 16.9
11	84.3 ± 16.6	55.4 ± 5.6*	83.8 ± 13.8

Doxorubicin was injected i.p. at a dose of 6 mg/kg, while AD-20 was injected i.p. at a dose of 50 mg/kg 15 min before doxorubicin. Results are means ± S.D. from values obtained with five mice per experimental group.
*Values significantly different ($P < 0.05$) in relation to their respective values at day 0.

(b) *Bone marrow toxicity induced by doxorubicin.* Table 4 shows the effects of doxorubicin on peripheral white blood cells, in the absence and presence of AD-20. They were compared to the blood leukocyte count of non-treated mice (group 1). In the blood of doxorubicin-treated animals (group 2), there was a strong decrease in cell count during the first 4 days, reaching 24% of the initial value. One week after, this count increased to 80%, but at day 9, a new and significant decrease appeared which remained constant until day 11. While in group 1 no modifications in the leukocyte count was observed from day 1 to day 11, in group 3 (doxorubicin + AD-20), the leukocytes followed the same pattern as in group 2 (doxorubicin alone) during the first 4 days. Nevertheless, 1 week after the peripheric leukocyte count increased rapidly, reaching more than 100% of the values obtained at day 0.

Improved antitumor effect of doxorubicin

After investigating the protective effect of AD-20 on doxorubicin toxicity we examined its effect on the chemotherapeutic activity of the drug (Table 5). All non-treated mice bearing L1210 tumor cells died approximately at day 10 (mst of 10.4 days). The animals receiving doxorubicin alone (7 mg/kg i.p.) had a mst of 15.3 days, and no survivors after 20 days. When mice are pretreated with AD-20, at doses of 10 and 25 mg/kg, given 15 min before doxorubicin, the mst values are 28.6 and 39.6 days respectively. At day 60, 30 and 50% of the animals survive in the same order of doses administered, compared with 0% in the group receiving doxorubicin alone. Furthermore, the increase in the life span (ILS) of tumor-bearing mice, when compared to mice receiving doxorubicin alone, were 86.9 and 158.8% for the doses 10 and 25 mg/kg of AD-20, showing clearly a significant enhancement of the therapeutic activity of doxorubicin.

Survival of mice bearing L1210 leukemia was not affected by the i.p. administration of AD-20 alone at doses varying from 50 to 400 mg/kg, showing that AD-20 by itself was without cytostatic activity against L1210 cells (data not shown).

Table 5. Effect of AD-20 on therapeutic effect of doxorubicin in L1210 leukemia bearing mice

Treatment	Dose (mg/kg)	mst (days)	ILS (%)	% S	Body weight (%)*
Control	—	10.4	—	0	+5.7
Doxorubicin	7	15.3	—	0	+0.8
+ AD-20	10	28.6	86.9	30	+0.3
+ AD-20	25	39.6	158.8	50	+0.7

Control non-treated mice, bearing L1210 cells, received an i.p. injection of gum arabic. Each experimental group consisted of 20 mice.
mst: mean survival time calculated at day 60.
ILS: increase in life span calculated at day 60.
% S: percentage of animals surviving at day 60.
*Loss or gain in body weight at day 5 (expressed as %) when compared to day 0.

DISCUSSION

Results of several experiments support the hypothesis that the toxic effects of doxorubicin, at least in the heart, may result from oxidative tissue injury [5, 6]. In the present study we have reported results which demonstrate that a compound belonging to the class of *N*-acyldehydroalanines (the so-called AD-20) is able to reduce the toxic effects induced by doxorubicin *in vivo*. Indeed, these results indicate that the administration of AD-20 before doxorubicin not only decreases both the acute and subchronic toxicity of doxorubicin, but also enhances its antitumor effect. This supports the hypothesis that the toxicity on the one hand and the therapeutic action on the other hand may operate in different and separate ways.

AD-20 pretreatment of mice results in a more elevated number of survivors than doxorubicin alone, whatever the protocol used: a single acute dose (Fig. 2) or multiple non-toxic doses (Fig. 3). These results show that AD-20 may not only offer some specific organ protection, as can be seen by the decrease in the SGPT and SGOT levels (Table 3), but may also offer a systemic protection. However, the mechanism(s) by which AD-20 protects against lethality induced by doxorubicin still remains unclear. On the basis of the results reported elsewhere [19, 21], this protection may be due to the improvement of the cardiac function. In fact, mitochondrial membranes are modified after doxorubicin treatment (as seen by the inhibition of enzymatic activities, the increase of its rigidity and the increase in lipid peroxidation). These parameters were almost restored to normal values when animals received AD-20 before doxorubicin. Nevertheless, the pattern of mortality shown in Figs. 2 and 3, as well as death of mice already at day 5, appears to be more closely related to myelotoxicity than to cardiotoxicity. Furthermore, AD compounds protect mice exposed to 600–800 rads of X-rays [25], a dose which induces bone marrow toxicity leading to death. Based on these results indicating the radioprotective effect of AD-20 (without excluding some protection at the cardiac level) and the results obtained in Table 4 showing a partial protection by AD-20 in white blood cell levels, we postulate that myeloprotection may best explain most of the protective effects obtained with AD-20 in both single- and multiple-dose doxorubicin-induced lethality. On the other hand, since we do not have pharmacokinetic data on the combination doxorubicin and AD-20, we cannot exclude the possibility that AD-20 could modify the distribution of doxorubicin, reducing its concentration in heart and bone marrow tissues, and thus decreasing its toxicity.

It has been proposed that the major cytotoxic effect of doxorubicin on tumor cells is not related to free radical formation [14], but that it probably results from its intercalation in DNA, producing an alteration in the physical structure of the DNA. Inhibition of RNA and DNA synthesis also accompanies DNA intercalation [26, 27]. If AD-20 was without tumoricidal activity against L1210 cells, the mechanism(s) to explain the enhanced therapeutic effect may be connected with those involved in the protection against doxorubicin toxicity: improvement of the cardiac function and bone marrow protection. In fact, in other experimental conditions, AD-20 was also able to potentiate the therapeutic effect of cyclophosphamide in BDF1 mice, bearing L1210 leukemia cells [28]. Since cyclophosphamide induces a bone marrow depression, protection by AD-20 against this myelotoxicity might explain the increase of that chemotherapeutic action.

AD-20 inhibits *in vivo* deleterious processes mediated by doxorubicin and improves its antitumor effect. These results confirm our previous report concerning the enhancement by AD-20 of the antitumor effect of doxorubicin administered intravenously to Lou rats bearing L311 leukemia [19]. Since the toxicity of doxorubicin may be produced separately from its therapeutic action, AD-20 by its free radical scavenging abilities may enhance the anticancer effect of doxorubicin. On the basis of the results obtained previously [18, 19, 21], we postulate that AD-20 protects doxorubicin-treated mice by blocking the radical chain reaction and in this way can limit the adverse effects of a doxorubicin-induced free radical cascade.

Acknowledgements—We thank I. Blave and Ch. Vasseur for their excellent technical assistance.

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