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Distribution and Antitumoral Activity of Adriamycin Combined with Warfarin in Mice

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Summary. *The distribution of adriamycin (AM) in C57Bl/6 mice bearing intramuscular Lewis lung carcinoma under the influence of combined treatment with warfarin (W) was investigated by a fluorimetric procedure. AM was injected IV at the dose of 7.5 mg/kg 14 days after tumor transplantation and W was given in the drinking water for 96 h, starting 24 h before AM. No substantial modifications in the serum and tissue distribution of AM fluorescence were observed under combined short-term treatment with W.*

Introduction

Anticoagulants are often associated with cancer chemotherapy with the aim of preventing the formation of fibrin and of microthrombi and thus inhibiting tumor cell adherence to capillary endothelium. Metastasis formation could therefore be hampered and tumor cells, devoid of their usual protective fibrin coating, should be more susceptible to chemotherapy [16, 18, 19].

In experimental tumor systems warfarin (W), among other anticoagulants, has been reported to have an additive or synergistic effect when combined with antineoplastic agents such as cyclophosphamide, bleomycin [9], 5-FU [12], adriamycin [10], or immunostimulants, whose effect on macrophages was enhanced [4, 15].

In addition, W was shown to inhibit DNA and RNA synthesis in vitro [2], to have a direct cytotoxic effect [12, 20], to inhibit cell motility and mitotic activity on different cell populations in culture [19, 20], and to potentiate the effect of nitrogen mustard [3], However, in vivo the maximal antimetastatic effect of W seems to be related to full, prolonged anticoagulation [4, 11], and this is supported by the lack of antitumoral effect observed in a short-term test (7 days) [8].

Whether alterations in the plasma and tissue distribution of antineoplastic agents also occur under the influence of W, accounting for the differences observed in drug activity, as suggested by Kirsch for 5-FU in the Lewis lung carcinoma tumor system and in cancer patients [12], is an interesting matter for investigation. The rationale for this is related to the fact that W binds 99% to plasma albumins [1] and could thus affect the distribution of other compounds by competing for protein-binding sites.

This report describes studies on serum and tissue distribution of the widely used antitumoral agent adriamycin (AM), in mice bearing intramuscular Lewis lung carcinoma (3LL) which also received W. The antitumoral and antimetastatic activity of AM in the same experimental conditions was also assessed in the presence or absence of W.

Materials and Methods

Animals and Tumors

C57Bl/6 male mice (22 ± 2 g body weight) obtained from Charles River, Italy, received an intramuscular (IM) transplant of $2 \cdot 10^5$ viable cells of the syngeneic Lewis lung carcinoma (3LL) maintained by IM passages in the same strain every 2 weeks and known to give rise to macroscopic metastases to the lung. A group of animals bearing tumors of about 2 g (14 days after implantation) was treated as described below and used for pharmacokinetic studies, and another group received the same treatment for evaluation of drug antitumoral activity. On day 25 after tumor implantation this second group was killed, and the animals' weight, primary tumor and lung weight, and the number and weight of metastases were recorded. A previously described procedure was followed for establishing the weight of lung metastases [7]. A third group of animals was used to record survival time; any deaths among treated mice occurring before the death of the first control

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mouse were considered toxic deaths and were not included in the evaluation. The animals were housed in Makrolon cages (20 × 30 × 13 cm) at room temperature (22° C) and relative humidity about 60%, with free access to food and water during the experiments.

Experimental Design

For pharmacokinetic studies and evaluation of antitumoral activity W was given orally (5 mg/l for 24 h and 1 mg/l for another 96 h), starting on day 13 after tumor transplantation. AM was injected IV at the dose of 7.5 mg/kg on day 14.

Serum and tissue sampling for drug assay was performed at different intervals after AM injection: 1, 5, 15, 30, 60 min, 3, 6, 24, 48 h. At each time five animals per group were used.

Drug Assay

AM fluorescence in serum and different tissues was assayed according to biochemical procedures already described elsewhere. For serum determination the Finkel method was used [6]; recovery was 90% and sensitivity about 0.05 μg/ml serum. To measure the AM concentration in tissues the fluorimetric procedure described by Schwartz [17] was modified to improve sensitivity: the samples were centrifuged after adding silver nitrate and the supernatant extracted with n-butanol. In these conditions recovery was 75% and sensitivity about 0.1 μg/g tissue. Although the reported concentrations are AM equivalents, measurement of total fluorescence may well represent the actual concentrations of unchanged AM, at least in tissues, 90% of fluorescence found after AM treatment being accounted for by the compound as such a few minutes after administration.

Data Analysis

Pharmacokinetic analysis for serum was done by the peeling method according to a two-compartment open model. The experimental areas under the AM concentration-versus-time curves (AUC) for serum and tissues were calculated by the trapezoid method and the statistical significance of the differences between AUC values for AM- and (AM + W)-treated groups was established by Student's *t*-test. In the evaluation of antitumoral activity differences between the various parameters were analysed by Duncan's test. The Quantile test was employed for the median survival time.

Results

Figures 1 and 2 report the distribution of AM fluorescence under the influence of W in serum, tumor, and heart of mice bearing intramuscular 3LL.

In serum a transient increase in AM fluorescence was seen when W was given. When the data were elaborated according to a pharmacokinetic model, the extrapolated concentration at time zero (Co) and the area under the concentration-versus-time curve (AUC), either theoretical or experimental, turned out to be somewhat higher, although not at the level of significance, in the (AM + W)-treated group (Table 1): the relative error between mean experi-

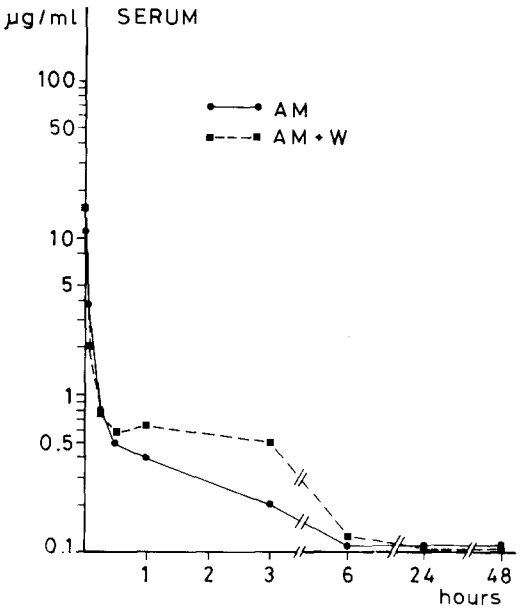

Fig. 1. Serum decay of adriamycin combined with warfarin in C57Bl/6 mice bearing 14-day intramuscular Lewis lung carcinoma. Warfarin was given orally (5 mg/l × 24 h + 1 mg/l × 96 h) starting on day 13 after tumor transplantation. AM was injected IV at the dose of 7.5 mg/kg on day 14. Five mice per point were used. ●——●, AM; ■– –■, AM + W

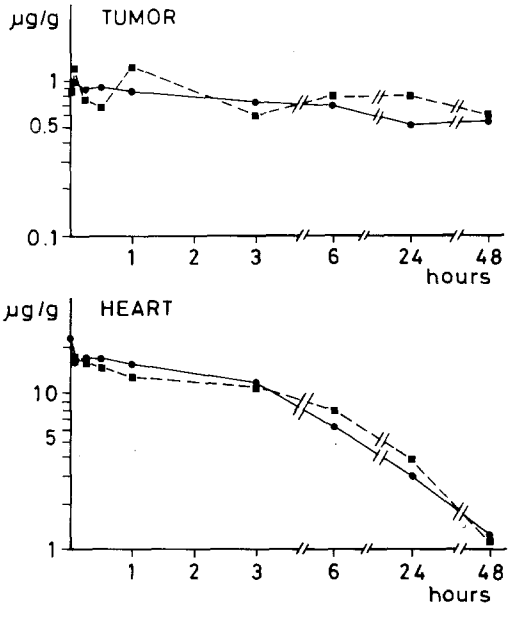

Fig. 2. Heart and tumor distribution of adriamycin combined with warfarin in C57Bl/6 mice bearing 14-day intramuscular Lewis lung carcinoma. See legend to Fig. 1. ●——●, AM; ■– –■, AM + W

mental values and the theoretical curve calculated on the model proposed ranged between 0.1% and 10%, reaching 25% only at 3 h in the (AM + W) group.

However, this apparent elevation of circulating AM does not result in modifications of other pharmacokinetic parameters, such as $\beta T_{1/2}$, Vd, and total body clearance, or in greater drug availability at the target tissues of tumor and heart. The time course of AM accumulation in tumor and heart did not seem to be affected by the combined treatment with W, as AM distribution followed exactly the same pattern in the presence as in absence of W.

Statistical analysis of total AM exposure in tumor and heart values, as reported in Table 1 never revealed significant differences between W-treated and untreated mice, such as were found in serum.

Investigation of the antitumoral and antimetastatic activity of AM combined with W in the same experimental conditions (Table 2) indicated that the therapeutic result was no better than with AM alone. The same inhibition of body, tumor and metastases weight and the same decrease in number of metastases in (AM + W)-treated mice was accompanied by a somewhat shorter median survival time than with AM alone, although the difference did not reach the level of statistical significance. It may be noted that, in our conditions, the antitumoral and antimetastatic activity of W alone was not significantly different from control values.

Table 1. Pharmacokinetic parameters of adriamycin combined with warfarin in mice bearing intramuscular Lewis lung carcinoma[a]

	AM	AM + W
Serum		
Co (μg/ml)	13.3	23.1
T½ (min) α	2.65	1.33
T½ (min) β	143	151
VD (l/kg) β	9.6	7.5
Cl (l/min/kg)	0.04	0.03
AUC (μg/ml × min)[b]		
theoretical	162.1	218.0
experimental	140.5	200.9 n.s.[c]
Tumor		
AUC (μg/ml × min after 48 h)	2,050 ± 295	2,572 ± 219 n.s.[c]
Heart		
AUC (μg/ml × min after 48 h)	11,875 ± 1,274	13,876 ± 1,704 n.s.[c]

[a] Warfarin was given orally (5 mg/l × 24 h + 1 mg/l × 96 h) starting on day 13 after tumor transplantation. Adriamycin was injected IV at the dose of 7.5 mg/kg on day 14
[b] The theoretical AUC was derived according to the peeling method. Whereas the experimental AUC was calculated by the trapezoidal method extrapolated to ∞
[c] n.s. = not significant after statistical analysis (Student's *t*-test) versus AM-treated group (significance level $\alpha \leqq 0.01$)

Discussion

The findings described indicate that a combined treatment with W slightly affects the amount of AM fluorescence in the serum of tumor-bearing mice, as expressed by the pharmacokinetic parameters Co and AUC, and this may possibly be attributable to the fact that our analytical procedure does not discriminate between unchanged AM and metabolites. However, the experimental AUC is not significantly different after statistical analysis in the two groups,

Table 2. Antitumoral and antimetastatic activity of adriamycin combined with warfarin in mice bearing intramuscular Lewis lung carcinoma[a]

Treatment	Body weight[b] (g)	Tumor weight[b] (g)	Lung weight[b] (mg)	Metastases[b]		Survival (days)		Toxic deaths
				No.	Weight (mg)	Mean	Median[c]	
–	26.9 ± 0.7	9.3 ± 0.3	286 ± 46	29.8 ± 5.7	102.5 ± 36.8	22 ± 1.4	21.5	–
W	26.8 ± 0.7	8.1 ± 0.3	226.6 ± 23	16.2 ± 5.1	56.6 ± 17.2	20.4 ± 1.3	19	–
AM	23.4 ± 0.9[e]	7.8 ± 0.3[d]	185 ± 10[d]	10.7 ± 3.4[e]	15.6 ± 5.1[d]	25.5 ± 1.7	26	1/10
AM + W	22.6 ± 0.7[e]	8.3 ± 0.6	159.6 ± 13.1[d]	11.2 ± 2.2[e]	15.1 ± 2.4[d]	21.4 ± 1.2	19	1/10

[a] Warfarin was given orally (5 mg/l × 24 h + 1 mg/l × 96 h) starting on day 13 after tumor transplantation: adriamycin was injected IV at the dose of 7.5 mg/kg on day 14. Groups of ten mice each were used
[b] Animals were sacrificed on day 25 after tumor transplantation
[c] Quantile test for median survival times
[d] $P < 0.05$ relative to untreated mice (Duncan's test)
[e] $P < 0.01$

and moreover no modifications are evident in $\beta T_{1/2}$, Cl, and Vd of AM in serum or in its disposition in tissues, where drug levels are the actual determinants of drug response. This poor pharmacokinetic interaction between W and AM is in good agreement with previous reports indicating that serum and particularly tissue distribution of AM are not easily altered by a series of interacting drugs (anticoagulants or not), such as heparin, bromelain, defibrase, ICRF 159, isoproterenol, alpha-tocopherol and antitumorals [5; E. Piazza et al. work submitted for publication].

A possible explanation for this behaviour in our opinion might be in this antitumoral antibiotic's pharmacokinetic characteristics. As reported in previous studies [13, 14] and as suggested on the basis of this report (Table 1), the drug tends to distribute to the tissue compartment very rapidly (the distributive phase has a $t_{1/2}$ of 1−2 min), and to a very large extent the AUC values, which express drug exposure in time, being one or two orders of magnitude higher in tissues than in the blood.

This maximal AM distribution makes it difficult for another agent to interfere with the time and entity of pharmacokinetics or with the response to this drug. Indeed, in the same experimental situation used for pharmacokinetic studies, the therapeutic efficacy of the antitumoral antibiotic was not improved under the influence of W inhibition of tumor growth, being comparable in AM- and (AM + W)-treated mice, and the somewhat shorter survival time in the group treated with the drug combination not reaching a significant level.

This lack of potentiation of antitumoral effect under the influence of W is in good agreement with the study by Higashi and Heidelberger [8], who observed no increase in the antitumor effect of 5-FU on primary or metastatic L 1210 and adenocarcinoma 755 when W was associated. The fact that other authors found enhanced effectiveness of AM [11], 5-FU [12] or other antitumorals [9] may be related to the different treatment schedules employed for W. These authors gave very early, prolonged W treatment, which as previously mentioned, might have an additive or synergistic effect with antitumoral therapy. It is perhaps worth noting that in studies by other authors [12, 13] on different tumor systems of the mouse, a daily W treatment, which ensures full anticoagulation, displayed a certain antitumoral or antimetastatic effect by itself.

However, short-term therapy was employed by Higashi and Heidelberger [8] and in our study. In fact we were interested in ensuring anticoagulation only for the time the antitumoral agent was present in the body.

These findings suggest that when anticoagulants are combined with antitumoral therapy it must be taken into account that opposite results may be obtained, depending on the treatment schedule employed.

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