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Comparative Responsiveness and Pharmacokinetics of Doxorubicin in Human Tumor Xenografts*

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Abstract—*The antineoplastic activity of the anthracycline antibiotic doxorubicin (Adriamycin®) differs in its cytotoxic effectiveness against different types of human tumors. In the present study the effect of doxorubicin on the growth of two human lung carcinomas and one human mammary carcinoma transplanted into athymic mice was correlated with the pharmacokinetics of doxorubicin in the same tumors after intraperitoneal administration. Doxorubicin produced a greater inhibition of tumor growth in the lung carcinomas than in the mammary carcinoma. Furthermore, the pharmacokinetic characteristics of doxorubicin differed widely within the three human solid tumors. No apparent correlation was found to exist between the different tumor growth sensitivities to doxorubicin and the pharmacokinetic parameters of doxorubicin within the tumor tissue. It is suggested that the differences in the demonstrated antitumor effectiveness of doxorubicin may be due to differences in the "intrinsic sensitivity" of the three human solid tumors.*

INTRODUCTION

INDIVIDUAL tumors, even if derived from the same tissue origin, differ from each other in many respects. Most important for cancer chemotherapy, they differ in their responsiveness to various drug treatments. In a number of experimental tumor model systems, the anthracycline antibiotic doxorubicin (DOX) has been found to exert a greater antitumor activity in some types of human cancers than in others. The mechanisms responsible for this are, however, still a matter of discussion [1–3].

The mechanism of the cytotoxic action of anthracyclines is attributed mainly to an interaction with DNA resulting in a premitotic block [4]. As the therapeutic target is localized intracellularly, cellular uptake is a decisive factor in the therapeutic effect of these drugs. Several studies have confirmed that acquired resistance to anthracyclines *in vitro*, as well as *in vivo*, is accompanied by a reduced cellular drug uptake [5–7]. However, the mechanism of decreased drug uptake in tumor cells with acquired resistance has been shown to be multifactorial, including such factors as decreased cellular permeability, increased efflux, and a lower affinity for intracellular binding sites [7–10].

It has also been reported that the cytotoxic activity of DOX on different tumor cells *in vitro* or tumor systems *in vivo* cannot always be correlated to the total cellular or tissue drug concentration [5, 11–13]. Therefore, it seemed appropriate to investigate whether the differential effectiveness of antineoplastic activity was due to differences in the entry and/or intracellular retention of DOX.

To evaluate this possibility, we compared the effect of DOX on the growth of two human lung carcinomas and one human mammary carcinoma heterotransplanted into athymic mice with the tumor pharmacokinetics of DOX after intraperitoneal administration.

MATERIALS AND METHODS

Animals

Specific pathogen-free 4–6-week-old, male, nude mice (nu/nu-Balb/c/ABom) weighing 18–22 g, were supplied by Gl.Bomholtgaard, 8680 Ry, Denmark. The animals were kept under sterile conditions in laminar sterile air-flow clean benches. Room temperature: 25 ± 2° C, relative

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humidity: 60 ± 5%. Sterile food pellets and water were given *ad libitum*.

Tumors and transplantation

T54B: This tumor was established from a cloned human small cell carcinoma of the lung as previously described [14]. The amount of diploid stromal cells was calculated (4–6%) by flow cytometric DNA analysis [15, 16]. Tumors from passage 17–22 were used.

T60: The tumor was established in nude mice from a human estrogen receptor-negative breast carcinoma as previously described [17, 18]. The fraction of diploid stromal cells was calculated to 20–25% by flow cytometric DNA analysis [15, 16]. Tumors from passage 34 and 37 were used.

T84: The tumors used in the experiments were established from a human small cell carcinoma of the lung cell line. The cell line was produced from a lymph node metastasis from a previously untreated patient. The histological subclassification was a small cell carcinoma of the lung of the intermedial type (WHO: 22). After cloning, subpopulations with different DNA content were isolated [19]. The DNA index (= 2.5) of the tumor cells used in this study was calculated by flow cytometric DNA analysis [16]. The heterotransplanted tumor was established by injection of $5 \cdot 10^6$ cells into the flank of a nude mouse. Hereafter the tumor was serial transplanted as described elsewhere [20]. Histology of the heterotransplanted tumors was identical to that of the original tumor. Electron microscopy demonstrated typical dense core bodies. The fraction of diploid stromal cells (5%) was calculated by flow cytometric DNA analysis [15, 16] (Engelholm SA, University Institute of Pathological Anatomy, Copenhagen Denmark, personal communication). Tumors from passage 7 and 12 were used.

A tissue block of ~2 mm was inoculated subcutaneously in each flank of the recipient mice [20]. The procedure was performed under general anesthesia with propanidid (Epontol®). In the chemotherapeutic studies there were 6–8 mice per group. None of the tumors had previously been treated with doxorubicin. Tumors were excluded if the animals died before the end of the experimental period, which was at least 24 days following the treatment.

Drug administration

Doxorubicin hydrochloride (DOX), doxorubicinol, and doxorubicinone were kindly supplied by FarmItalia, Milan, Italy. DOX was freshly dissolved in saline and administered by a single intraperitoneal injection in a volume of 10 ml/kg body wt. The mice were treated with the maximal tolerated dose (10 mg/kg) for the chemotherapeutic studies and with 12.5 mg/kg for the pharmacokinetic studies. The tumors investigated comprised 25 treated and 18 untreated control tumors of T54B, 29 treated and 11 untreated controls of T60, and 20 treated and 18 untreated controls of T84. The treatment was given at day 39 after transplantation in mice bearing T54B, at day 52 in mice bearing T60, and at day 25 in mice bearing T84.

Evaluation of antitumor activity

The body wt of all mice as well as the growth of the heterotransplanted T54B and T84 tumors were measured three times a week. The T60 tumors were measured only twice a week, since they grew at a much lower rate. Length and width of tumors were determined by means of vernier calipers on coded animals. The tumor growth was described mathematically on the basis of a transformed Gompertz function [21] and tumor weight was calculated according to the following formula:

$$TW\ (\text{mg}) = [L\ (\text{mm}) \times (W\ (\text{mm}))^{2}1/2$$

where L is the length and W the width of the tumor [22].

Treatment was delayed until five consecutive tumor growth measurements could be depicted rectilinearly on a pretherapeutic line in the transformed Gompertz growth curve. Tumor growth was estimated for individual tumors and expressed as the relative tumor weight according to the following formula:

$$\text{Relative tumor weight} = \frac{\text{Tumor weight at the indicated days}}{\text{Tumor weight on the day of treatment}}.$$

The data reported in Fig. 1 represent the average tumor weight. Individual growth of heterotransplanted tumors showed considerable variation; converting growth data from absolute to relative values allows for easier visualization of the antineoplastic activity of the drug and standardizes the tumor size at the start of treatment. The antitumor activity was established by the tumor weight doubling time calculated from the individual tumor growth. Furthermore, specific growth delay (SGD) values were calculated [23]. SGD was calculated according to the following formula:

$$\text{Specific growth delay} = \frac{\text{Tumor doubling time (treated)} - \text{Tumor doubling time (control)}}{\text{Tumor doubling time (control)}}$$

The SGD parameter can be regarded as an estimate of the number of tumor doubling times by which growth is delayed; it, therefore, provides a basis for comparison of therapeutic response between tumors of different growth rates. Groups of control mice were treated in parallel with saline. Statistical analysis was performed by a Mann–Whitney test.

Pharmacokinetics

Nude mice with heterotransplanted T54B, T60, and T84 tumors were treated with DOX at a time when the tumor weights were approx 300 mg. The mice were killed by cervical dislocation and the tumors were excised at 5 min, 30 min, 2 hr, 8 hr, 24 hr and 48 hr following drug administration. Tissue samples were frozen at −20° C and analyzed within 2 weeks.

After dissection of necrotic tissue, tumor samples weighing 100–200 mg were analyzed in triplicate according to Johansen [24] with minor modifications. In brief, tissue samples were homogenized in 0.1 M phosphate buffer (pH 7.4) at 0° C. An equal volume of 0.1 M borate buffer (pH 9.8) was added to the homogenate. Samples were extracted with chloroform : methanol (9 : 1). The organic phase was removed and evaporated under nitrogen in siliconized test tubes. The residue was redissolved in 100 μl of methanol followed by addition of 100 μl mobil phase. Samples were stored at −20° C overnight.

The determination of doxorubicin and metabolites was performed with high pressure liquid chromatography equipment consisting of Waters U6K universal injector, a Waters pump 6000A and a Schoeffel fluorescence detector FS-970. The reversed-phase RP-8 column was eluted isocratically at ambient temperature with an acetonitrile : 0.01 M phosphoric acid (50 : 50 v/v) mixture (pH 2.0). The flow rate was 1.5 ml min^{-1} and the column effluent was monitored spectrofluorimetrically with excitation wavelength at 470 nm and emission wavelength at 550 nm. The identity of the products in the sample injected (100 μl) into the column were determined by comparing the peak heights with those of external standards containing doxorubicin, doxorubicinol and doxorubicinone. Tumor tissue concentrations of DOX were calculated and expressed as ng/mg wet weight. Recovery of DOX and doxorubicinol added to tumor homogenate was 80–85%, and recovery of doxorubicinone was 90–95%.

The tumor tissue concentration-time data of DOX were submitted to a one-compartment open model analysis with first order influx and efflux according to the equation:

$$\text{conc(DOX)} = B \exp(-k_2 t) - A \exp(-k_1 t).$$

The half lives were calculated as:

$$t_{\frac{1}{2}} = \ln 2/k_2.$$

The area under the tissue concentration–time curves (AUC_0^∞) as:

$$AUC_0^\infty = [B/k_2] - [A/k_1].$$

The time for maximal tissue concentration (t_{max}) as:

$$t_{max} = [1/(k_1 - k_2)] \ln (k_1/k_2)$$

and the maximal tissue concentration (c_{max}) as:

$$c_{max} = [(A+B)/2][1-(k_1/k_2)] \exp(-k_2 t_{max}).$$

The curve fittings were performed by a non-linear least square fitting program. Statistical analysis was performed by Student's *t*-test.

RESULTS

Effect of doxorubicin chemotherapy

As seen in Fig. 1, all the heterotransplanted tumors (T54B, T60, T84) exhibited an exponential growth phase in the nude mice during these studies (rectilinearity in a semilogarithmic plot). The tumor doubling times for T54B and T84, however, were lower than that of T60 indicating a difference in growth rate.

The chemosensitivity of T54B, T60, and T84 to DOX is indicated in Table 1. The tumor growth of all heterotransplanted tumors were all significantly delayed as a result of DOX treatment as judged by the tumor weight doubling time (Table 1). However, the SGD values of T54B, T60, and T84 indicated difference in sensitivity to DOX upon treatment, with T84 being the most sensitive and T60 the least.

Toxicity of DOX treatment in the nude mice was monitored as a change in relative body wt. As indicated in the upper panel of Fig. 1 there was a significant weight loss in T54B, T60, and T84 bearing mice treated with DOX compared with controls.

Tumor levels of doxorubicin

The tumor tissue DOX concentration after intraperitoneal injection of 12.5 mg/kg DOX in T54B, T60, and T84 bearing nude mice is presented in Fig. 2 together with the fitted curves. The results of the pharmacokinetic analysis are presented in Table 2.

The maximal DOX concentration in the tumors was calculated from the primary parameters and varied from 0.74 ng/mg in T54B to 1.84 ng/mg in T84. Furthermore, the time for maximal DOX concentration in the tumor varied from approx. 4 hr in T54B to 23 hr in T84. After injection of DOX, drug entry into T54B as evaluated by the

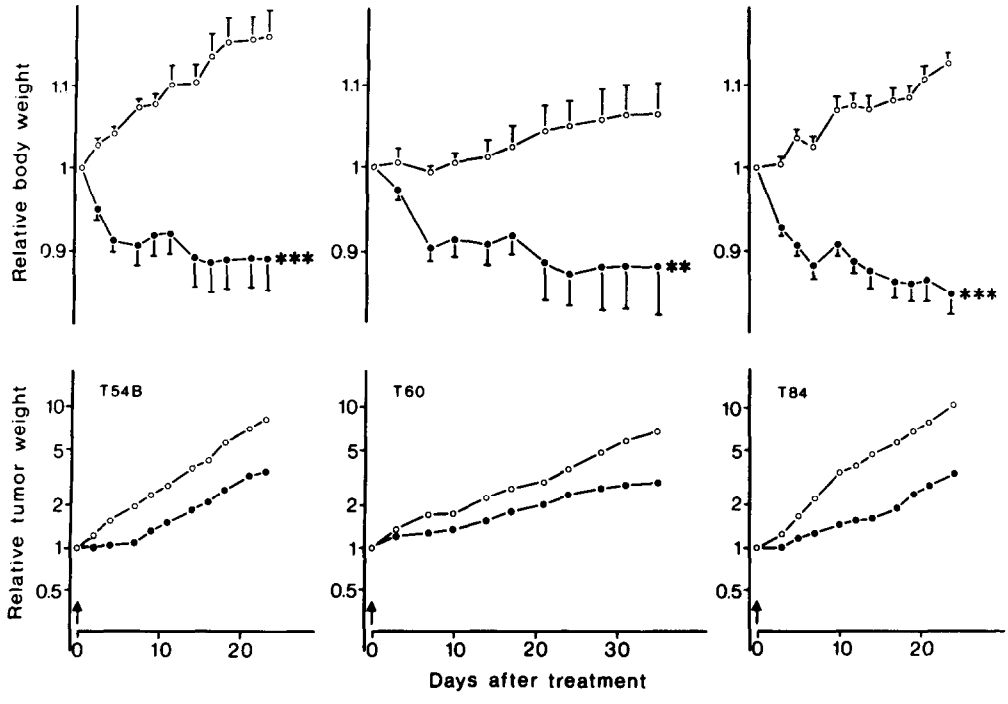

Fig. 1. Response of T54B, T60 and T84 human tumor xenografts in athymic mice to doxorubicin (lower panel) and the relative body wt of the respective heterotransplanted athymic mice after treatment with doxorubicin (upper panel). ○—○ = *controls;* ●—● = *treated; arrows = day of treatment with* 10 mg/kg *i.p. Abscissa: days after initial treatment. Lower panel: Each point represents the median of relative tumor weight* (n = 11–29 *tumors). Ordinate: ratio between tumor weight at the indicated days and tumor weight at the beginning of treatment. Upper panel: Each point represents the mean ± S.E.M. of relative body wt* (n = 6–15 *animals). Ordinate: ratio between body wt at the indicated days and body wt at the beginning of treatment. Significant difference from the control curve* $^{**}P < 0.01$, *and* $^{***}P < 0.001$ (*Student's t-test*).

Table 1. Effect of doxorubicin against human solid tumors heterotransplanted into athymic mice

Tumor	Day of treatment*	Dose (mg/kg)	Tumor weight doubling time† (days)	Specific growth delay‡	P‖
Lung, T54B	39	Saline	7.2 (18)§		
	39	10.0	15.2 (25)**	1.11	
					$P < 0.05$
Breast, T60	52	Saline	12.4 (11)		
	52	10.0	20.6 (29)*	0.66	$P < 0.05$
					$P < 0.01$
Lung, T84	25	Saline	6.4 (18)		
	25	10.0	17.6 (20)**	1.75	

*Doxorubicin was administered i.p. on the indicated days after tumor transplantation.
†The time taken for tumors to double in weight.
‡The ratio between the difference in the time taken for control tumors and treated tumors to double in weight and control tumors to double in weight.
§Data are presented as the median of 11 to 29 tumors (number of tumors is given in parentheses). Statistical significance based on Mann–Whitney test; * = $P < 0.05$ of drug treated compared to respective control, ** = $P < 0.01$.
‖Statistical significance based on Mann–Whitney test.

entry rate constant (k_1) was significantly faster than into T60 and T84 (Table 2); the entry rate constant for T60 was significantly greater than for T84.

Differences in the time course subsequent to the entry phase were also observed. The exit rate constant (k_2) of DOX in T54B was significantly lower than that of T60 and T84 (Table 2), while no significant difference was observed between T60 and T84. The apparent half lives of DOX calculated from k_2 values were approx. 174 hr in T54B, 27 and 43 hr in T60 and T84, respectively.

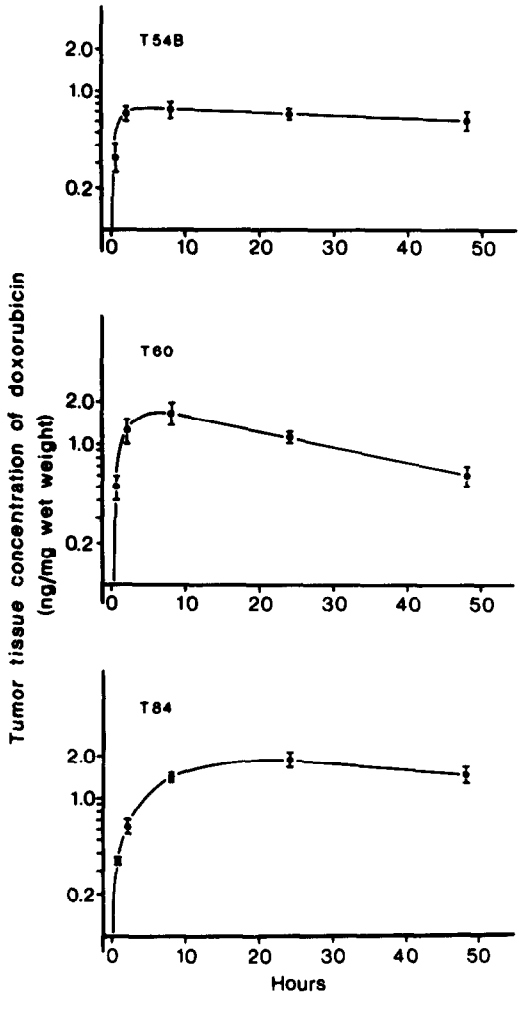

Fig. 2. Tumor tissue concentrations of doxorubicin in T54B, T60, and T84 human tumor xenografts in athymic mice following 12.5 mg/kg *i.p. Each point represents the mean ± S.E.M. of data from five animals.*

The areas under the tumor concentration–time curves (AUC_0^∞) were calculated from the primary parameters. The AUC_0^∞ for T54B and T84 were of the same order of magnitude, while the AUC_0^∞ for T60 was approx. one-half of that of the others (Table 2). Neither doxorubicinol nor doxorubicinone was detected in any of the investigated tumors throughout the 48 hr observation period.

DISCUSSION

Factors determining the responsiveness to DOX of many human and experimental solid tumors and leukemias have not been clarified. The present study compared the antineoplastic activity and pharmacokinetics of DOX in human solid tumors transplanted into athymic mice. The findings demonstrated that no apparent correlation existed amongst tumor response, tumor drug entry rate and the persistence of DOX in tumor tissue when evaluated by SGD values and the apparent half-life of the drug. Furthermore, the ranking of the three tumors according to their SGD values (T84 > T54B > T60) was neither correlated to the amount of drug passing through the tumors within the time (AUC_0^∞) nor to the peak concentration of DOX in the tumor tissue and the fraction of diploid stromal cells. We therefore suggest that the difference in the demonstrated tumor response upon DOX treatment could be due to differences in the intrinsic sensitivity of the three tumors to DOX or other cellular phenomena.

Our findings are comparable with a previous study comparing *in vitro* uptake and cytotoxicity of DOX in primary and metastatic Lewis lung carcinoma [12]. No difference was observed in drug uptake, but DOX caused a greater cytotoxic effect on the secondary tumor cells than on the primary tumor cells. It was suggested that differences in anti-neoplastic response might be due to differences in the intrinsic sensitivity of the two tumor cell populations to DOX. However, their

Table 2. Pharmacokinetic parameters obtained by applying a one-compartment open model to the tumor tissue concentration–time data of doxurubicin

	T54B	*P**	T60	*P*†	T84	*P*‡
Primary parameters						
A (ng/mg)	0.75 ± 0.03	$P < 0.001$	2.08 ± 0.18	NS§	3.30 ± 1.18	$P < 0.05$
k_1 (10^{-3}) (min^{-1})	24 ± 3.7	$P < 0.01$	8.4 ± 1.7	$P < 0.01$	1.5 ± 0.46	$P < 0.001$
B (ng/mg)	0.84 ± 0.04	$P < 0.001$	2.00 ± 0.18	NS	3.05 ± 1.16	$P < 0.05$
k_2 (10^{-5}) (min^{-1})	6.7 ± 2.7	$P < 0.01$	43 ± 6.7	NS	27 ± 17	$P < 0.05$
Derived parameters						
c_{max} (ng/mg)	0.74		1.68		1.84	
t_{max} (hr)	4		6		23	
$t_{\frac{1}{2}}$ (hr)	172		27		43	
AUC_0^∞ (ng/mg) min	12506		4403		9096	

Statistical significance based on Student's t-test, *T54B versus T60, †T60 versus T84,‡ T54B versus T84,‡ §not significant. Data are presented as mean ± S.E.M. where $n = 5$.

data do not exclude the possibility that the different effectiveness of cytotoxicity could be due to a possible difference in the kinetics of DOX in the tumor cells. In the present study, DOX caused a greater growth inhibition of T84 than of T54B. Despite no differences in tumor AUC_0^∞ values, a significantly faster entry rate and a lower exit rate of DOX were calculated for T54B. Furthermore, we found a maximal DOX concentration in T84, which was more than twice of that in T54B. Pharmacokinetically, this can be interpreted as a result of greater volume of distribution within T84 tumor cells than within T54B tumor cells. A comparable finding has also been made by DeGregorio *et al.* [11] in an investigation of uptake and cytotoxicity of DOX in two human sarcoma cell lines *in vitro*. They found that uptake of DOX by Ewing's sarcoma cells was 1.5–3.0 times greater than by rhabdomyosarcoma cells, the latter being more sensitive.

The antitumor activity of DOX after intraperitoneal administration has been reported to be inferior to that observed with intravenous administration [25, 26]. In a study comparing the pharmacokinetics of DOX after intravenous and intraperitoneal administration in the nude mouse (the same strain and the same dose as used in the present study) however, there were similar concentrations of drug in the plasma [24]. Thus, it cannot be assumed that higher antitumor activity of DOX following intravenous administration would necessarily correlate with the pharmacokinetic pattern of the drug in tumor tissues. Furthermore, as shown in this study drug uptake and efflux in tumor tissue may not directly correlate with the drug's antitumor effect in tumors of different size and degree of vascularization.

One possible reason for the lack of correlation between the pharmacokinetic parameters of DOX and the sensitivity may be that the kinetic model considers the tumor as a homogenous compartment. In reality, the tumor is a mass of cells belonging to different cell populations with different cell kinetic characteristics [27–30].

The tumor growth of all three heterotransplanted tumors were reduced by DOX treatment (Table 1). Drug sensitivities deduced from the SGD values, however, indicated that T60 was not sensitive to DOX since the SGD value was below 1.0 [23] whereas, SGD values for T54B and T84 (1.11 and 1.75, respectively) correctly indicated tumor sensitivity to DOX. The fact that the SGD value of T60 was below 1.0 together with the sensitivity ranking of T54B and T84 along with the pharmacokinetic parameters in our experiments indicated no apparent qualitative correlation to the previously described mechanisms of resistance or reduced sensitivity to anthracycline antibiotics [7, 8, 10]. Therefore, other cellular mechanisms may account for the differential effectiveness of cytotoxicity.

The importance of strict monitoring of the weight of the animals during the experiments has been emphasized [31, 32]. In fact, in animals with a body wt loss the growth of transplanted tumors is impaired [33]. As depicted from our experiments, a significant weight loss was observed in the DOX treated groups (Fig. 1), indicating an unspecific toxicity. Thus, a consequent reduction in the transplanted tumors could result in an overestimation of the chemotherapeutic effect of DOX.

In conclusion, with the present investigation, it has not been possible to indicate a pharmacokinetic parameter predictive as to the sensitivity to DOX. Further studies are, however, necessary to confirm or contradict these findings.

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