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ORIGINAL ARTICLE

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Disposition of conjugate-bound and free doxorubicin in tumor-bearing mice following administration of a BR96-doxorubicin immunoconjugate (BMS 182248)

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Abstract *Purpose*: The chimeric BR96–doxorubicin (DOX) immunoconjugate, BMS 182248, has induced remissions and cures of human lung adenocarcinoma (L2987) implanted in athymic mice. The purpose of this study was to evaluate the biodistribution of DOX after BMS 182248 administration to tumor-bearing mice and to evaluate the ability of BMS 182248 to target DOX to tumors. *Methods*: For this evaluation, L2987-implanted mice were given BMS 182248 (5 mg DOX/kg; three doses 4 days apart) and the levels of both conjugate-bound and free DOX in plasma, tumor, liver and heart were determined. *Results*: Conjugate-bound DOX comprised the majority of plasma DOX, with relatively low levels of free DOX present. From plasma, conjugate-bound DOX distributed to the tissues examined with the order of concentration (per gram of tissue) being tumor > liver > heart. Free DOX was also detected in liver and heart, but at concentrations lower than those present after an equivalent DOX dose (5 mg/kg; three doses 4 days apart). The total exposure of heart to free DOX after BMS 182248 administration was about one-quarter of that found after the administration of DOX alone. The elimination kinetics of both conjugate-bound and free DOX from heart and liver after BMS 182248 administration paralleled those observed from plasma, indicating that equilibrium had been attained between these nontumor tissues and plasma. The elimination kinetics of both entities from tumors, however, were different from those from plasma, liver and heart. BMS 182248 produced sustained levels of both conjugate-bound and free DOX which were present throughout the experiment. This suggested that, in contrast to normal tissues, tumor tissue retention of BMS 182248 by antigen-promoted binding had occurred and that the kinetics of free DOX in the tumors were controlled by the rate of release of DOX from tumor-associated BMS 182248. As a result of this retention, the tumor concentrations of free DOX after BMS 182248 administration exceeded those produced by i.v. administration of DOX at the same dose, a finding consistent with the greater antitumor activity of BMS 182248 relative to DOX. BMS 182248 also liberated DOX upon incubation with rat liver lysosomes and was accumulated by L2987 cells in culture, with the subsequent intracellular release of DOX. *Conclusions*: BMS 182248 effectively delivered DOX to L2987 xenografts implanted in athymic mice and produced higher and more prolonged tumor concentrations of free DOX than the administration of DOX alone. Following BMS 182248 administration, normal tissues (liver and heart) were exposed to lower overall concentrations of free DOX than were produced by administration of an equivalent DOX dose.

Key words Doxorubicin · Immunoconjugate · Biodistribution · Tumor-bearing mice

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Introduction

The selective delivery to tumor cells of cytotoxic agents by virtue of their conjugation with tumor-specific monoclonal antibodies is an area of ongoing intense interest [11, 19, 20]. One aspect of such research has centered on the formation of an acid-sensitive linkage between the cytotoxic anthracycline, doxorubicin (DOX), and tumor-specific monoclonal antibodies [26]. Ideally, such conjugates, after internalization into tumor cells, would be selectively cleaved in acidic cellular compartments (lysosomes, endosomes) to liberate DOX, which would exert its cytotoxic effects [2, 25]. Such antibody-directed targeting followed by selective release within tumor cells should spare normal cells some or all of the toxicity typically associated with DOX administration.

Recently, a novel chimeric monoclonal antibody–DOX conjugate (BMS 182248) has been prepared and

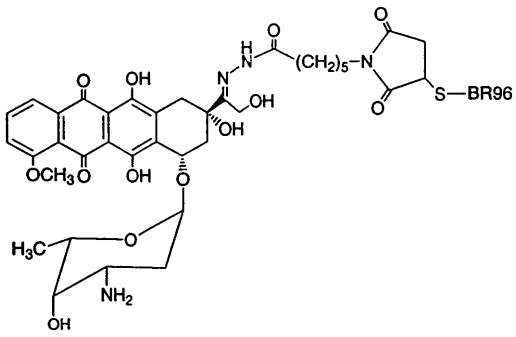

Fig. 1 Structure of BMS 182248

evaluated in animal models. This conjugate (Fig. 1) is a C-13 hydrazone of DOX, prepared by linking the DOX derivative, maleimidocaproyl DOX hydrazone [27], to cysteine residues of a chimeric (mouse–human) monoclonal antibody, BR96. This antibody binds to a tumor-associated cell surface antigen closely associated with Lewis Y(Le^y) and, following binding, is rapidly internalized into such cells [10, 12, 21]. This conjugate induces complete regressions and cures of xenografted human lung, breast and colon carcinomas growing in athymic mice [24].

We report here the plasma and tissue (tumor and nontumor) pharmacokinetics of DOX following its administration as BMS 182248 to athymic mice bearing implanted human lung tumor xenografts. These data are compared with those obtained following the administration of DOX itself to tumor-bearing mice using the same dosing schedule.

Materials and methods

Chemicals

The BR-96 DOX immunoconjugate, BMS 182248 (molecular weight ca. 160 000 Da), was synthesized by the Central Chemistry Department of Bristol-Myers Squibb and supplied as a solution in physiologically buffered saline (pH 7.4), with a protein concentration of 9.01 mg/ml and a DOX concentration of 256 μg/ml. The Central Chemistry Department similarly supplied DOX, while daunorubicin (DNM), the internal standard for the HPLC assay was from Sigma (St. Louis, Mo.). All solvents were HPLC grade (Fisher Scientific, Fair Lawn, NJ) and other chemicals were ACS reagent grade or better.

Animals, dosing and sample collections

All animal experimentation was conducted in accordance with USDA guidelines under the Animal Welfare Act. Female, athymic mice (20–25 g; Balb c Nude; Harlan Sprague Dawley, Indianapolis, Ind.) were subcutaneously implanted with the human lung adenocarcinoma xenograft, L2987. Following implantation, tumors were allowed to grow 14 days until well established, at which time dosing with either DOX or BMS 182248 was performed. Multiple intravenous (i.v.) doses of either entity were administered to groups of mice via a tail vein on a schedule (three doses 4 days apart) previously shown to have antitumor efficacy [24]. Each DOX dose was 5 mg/kg, while the dose of antibody protein administered as BMS 182248 was 176 mg/kg. At selected times after dosing, groups of mice (three per time point) were anesthetized with diethyl ether and a terminal blood sample was drawn from the inferior vena cava of each mouse into a heparinized syringe. Each blood sample was transferred to a 1.5-ml microcentrifuge tube and stored on ice until the plasma was separated by centrifugation (12 500 *g*). Plasma samples were then stored frozen (−20 °C) until analysis. At the time of blood collection, livers, hearts and tumors were excised, washed, blotted dry, weighed and frozen (−20 °C) until analysis.

Sample analysis

After DOX dosing, its concentrations in tissue and plasma samples were determined as described below for free DOX. After BMS 182248 dosing, the concentrations of both free DOX (that released in vivo from BMS 182248) and conjugate-bound DOX (that remaining bound to BR96) in plasma and tissue samples were determined. In each sample, the concentrations of both free and total DOX (the amount of DOX liberated by chemical hydrolysis of BMS 182248) were determined; the difference between these values represented conjugate-bound DOX. A similar strategy has been applied to the analysis of other anthracycline conjugates [4, 22]. Tissue samples were homogenized (Brinkmann Polytron, Westbury, N.Y.) in seven volumes of water and the resulting homogenate divided in half; one-half was analyzed for free DOX, the other for total DOX. Plasma samples were also divided in half prior to analysis. For analysis of free DOX in tissue homogenates, 0.20 ml of each homogenate was mixed on ice with 0.04 ml of a cold 33% aqueous (w/v) solution of silver nitrate [6], 0.01 ml of a 10 μg/ml aqueous solution of DNM added and the mixture centrifuged. A portion (0.2 ml) of the supernatant fluid was applied to a preconditioned C8 Bond-Elut cartridge (Analytichem, Harbor City, Calif.). For plasma samples, an aliquot (0.2 ml) was mixed with 0.01 ml of the DNM solution and the mixture applied to C8 cartridges. After sample loading, each cartridge was washed (water followed by 30% methanol in water) and the retained material then eluted with 0.30 ml of 75% acetonitrile/triethylammonium formate (TEAF) buffer (0.05 *M*, pH 7), and 0.05 ml of this eluant was injected onto the HPLC column.

For analysis of total DOX in samples, separate portions of each plasma or tissue homogenate sample were mixed with 0.01 ml of a 1 mg/ml aqueous solution of dithioerythritol (Sigma). After 30 min at room temperature, the pH was lowered to about 2.5 with 1 *N* HCl and the mixture incubated at 37 °C for 2 h to liberate all the DOX. After incubation, plasma samples were mixed with DNM and subjected to solid-phase extraction, while tissue samples were mixed with DNM followed by silver nitrate prior to extraction. HPLC analyses were performed on a Waters (Milford, Mass.) system comprising two 510 pumps, 680 gradient controller and 712 autosampler. DOX was detected after chromatography on a Waters μBondapak C18 column by fluorescent detection (495 nm excitation, 550 nm emission) with a Waters 470 detector. The mobile phase was 68% TEAF buffer (0.05 *M*, pH 2.8) and 32% acetonitrile at a flow rate of 1 ml/min. Standard curves (peak area ratio of DOX/DNM vs DOX concentration) for DOX quantitation were generated by fortifying control plasma samples or tissue homogenates with known amounts of DOX or BMS 182248 and processing the samples as described. The recovery of DOX using the above extraction procedures ranged from 82 ± 4% (tissue samples) to 97 ± 1% (plasma samples). The total recovery of DOX liberated from BMS 182248 by hydrolysis followed by solid phase extraction ranged from 75 ± 4% (tissue samples) to 90 ± 2% (plasma samples). These extraction efficiencies were used to correct the raw data. The concentrations of DOX in plasma samples were calculated as micrograms per milliliter and those in tissue samples as micrograms per gram of tissue.

Uptake of BMS 182248 into cultured cells

Human lung tumor cells (L2987) were obtained from the ATCC (Rockville, Md.) and grown to an 80–90% confluent monolayer in culture flasks (175 cm^2; Becton-Dickenson) containing RPMI-1640 medium supplemented with 10% fetal bovine serum, 1%

L-glutamine and 1% of a mixture of penicillin, streptomycin and neomycin (Gibco-BRL, Grand Island, N.Y.). The cells were prepared for uptake studies by first removing the culture medium and then washing with phosphate-buffered saline (0.05 m*M*, pH 7.4). To the washed cells was added a solution of BMS 182248 (1 μ*M* DOX equivalent) in phenol red-free RPMI medium, and incubation was carried out on a rocker at 37 °C under an atmosphere containing 5% CO_2. After various incubation times up to 4 h, the medium was removed, the cells were washed twice with cold buffered saline and trypsinized (trypsin-EDTA, Gibco) for 2–3 min at 37 °C. Trypsinized cells were resuspended in ice-cold phenol red-free RPMI and an aliquot was removed for counting in a hemocytometer. The remainder of the cells were pelleted by centrifugation (10 min at 1500 rpm, 4 °C), the supernatant removed, and the cells resuspended in water and lysed by three freeze/thaw cycles. Analysis of both free and bound DOX in these resuspended cells was carried out as described above for tissue homogenates.

Generation of DOX from BMS 182248 in rat liver lysosomes

Rat liver lysosomes were isolated by differential centrifugation according to a previously published method [14]. The protein concentrations in lysosomal pellets were determined by the Bradford assay (Bio-Rad Protein Assay Kit II, Bio-Rad, Hercules, Calif.). For a typical incubation, a known concentration of BMS 182248 (about 0.1 μ*M* DOX equivalent) was added to a pH 5.5 buffer mixture containing 0.1% Triton-X and 6.0 mg lysosomal protein in a volume of 2.0 ml. At specific time-points an aliquot (0.1 ml) of the incubation mixture was removed and the reaction stopped by the addition of 0.01 ml of a silver nitrate solution (33%, w/v), followed by 0.01 ml of a DMN solution (10 μg/ml). The samples were kept on ice for 5 min and then centrifuged (Eppendorf microfuge). The supernatant solution was then extracted and analyzed for free and conjugate-bound DOX as described above for tissue samples. At the end of the 4-h incubation, remaining BMS 182248 was cleaved to liberate its total DOX content by acid hydrolysis as described above. This value for total DOX liberated was used for the endpoint value in determining the half-life of DOX formation. For comparative purposes, the stability of DOX itself was evaluated in the above procedure and the stability of BMS 182248 was determined in the buffer mixture lacking lysosomal protein.

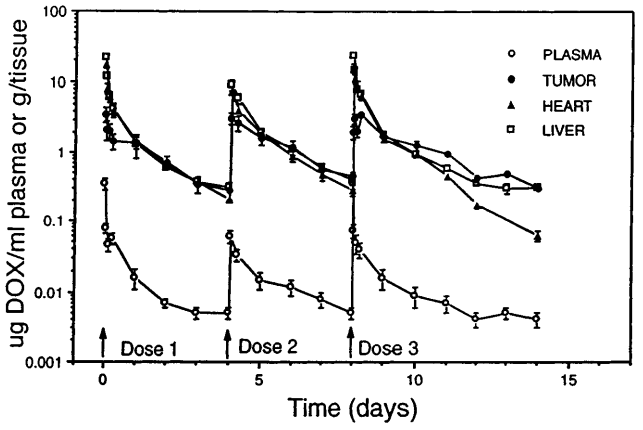

Fig. 2 Plasma and tissue concentrations of DOX following repeated i.v. administration (three doses 4 days apart; 5 mg/kg/dose) to L2987-bearing mice

Pharmacokinetic analysis

The average maximum plasma or tissue homogenate concentrations of DOX attained after dosing (C_{max}), as well as the times at which these occurred (T_{max}) were obtained by visual inspection of the data. Average terminal elimination half-lives ($T_{1/2}$) as well as the areas under the concentration vs time (AUC) and first-moment vs time (AUMC) curves were calculated using RSTRIP (Micromath Scientific Software, Salt Lake City, Utah). The AUC and AUMC values were calculated from 0 to 96 h after doses 1 and 2 and extrapolated to infinity after dose 3. Both the total body clearance (Cl_{tb}) and the volume of distribution at steady-state (V_{ss}) were calculated from noncompartmental analysis of the plasma level data as follows:

$$Cl_{tb} = \frac{\text{Dose}}{\text{AUC}} \qquad V_{ss} = \text{Dose}\frac{\text{AUMC}}{\text{AUC}^2}$$

Table 1 Plasma pharmacokinetics of DOX following multiple dosing with either DOX or BMS 182248. All values are means ± SD for three animals at each sampling point

Treatment	Parameter	DOX dosing DOX	BMS 182248 dosing	
			Free DOX	Bound DOX
Dose 1	C_{max} (μg/ml)	0.346 ± 0.067	0.301 ± 0.044	60.979 ± 5.450
	T_{max} (h)	0.08	0.08	0.08
	AUC (μg h/ml)[a]	1.6 ± 0.1	2.6 ± 0.3	946.4 ± 84.6
	$T_{1/2}$ (h)	16 ± 3	16 ± 2	22 ± 4
	V_{ss} (ml/kg)	67104 ± 5473	49506 ± 5026	145 ± 13
	Cl (ml/h kg)	3125 ± 289	1923 ± 156	5 ± 1
Dose 2	C_{max} (μg/ml)	0.059 ± 0.014	0.173 ± 0.022	61.147 ± 6.327
	T_{max} (h)[b]	1.0	1.0	1.0
	AUC (μg h/ml)[a]	1.4 ± 0.2	5.9 ± 1.1	1825.2 ± 321.7
	$T_{1/2}$ (h)	44 ± 6	19 ± 3	25 ± 4
	V_{ss} ml/kg	118500 ± 10989	30400 ± 2365	95 ± 12
	Cl (ml/h kg)	3571 ± 439	847 ± 124	3 ± 0.8
Dose 3	C_{max} (μg/ml)	0.353 ± 0.045	0.275 ± 0.032	70.725 ± 8.439
	T_{max} (h)	0.08	0.08	0.08
	AUC (μg h/ml)[a]	1.7 ± 0.3	8.3 ± 1.4	2306.6 ± 165.8
	$T_{1/2}$ (h)	38 ± 7	39 ± 6	38 ± 6
	V_{ss} (ml/kg)	80705 ± 8342	43905 ± 3907	120 ± 18
	Cl (ml/h kg)	2941 ± 276	602 ± 111	2 ± 0.3

[a]AUC values were calculated from 0 to 96 h after doses 1 and 2, and from 0 to infinity after dose 3
[b]The first sample after dose 2 was at 1.0 h, not 5 min, as after doses 1 and 3

Results

Administration of DOX to tumor-bearing mice

Multiple i.v. dosing (three doses 4 days apart; 5 mg/kg per dose) of DOX was performed to generate pharmacokinetic data to compare with those obtained after BMS 182248 administration. The average concentrations of DOX in plasma, liver, heart and tumor are shown as a function of time after dosing in Fig. 2, while the estimated pharmacokinetic parameters are given in Tables 1 (plasma) and 2 (tissues). After each dose, the observed C_{max} in plasma, liver and heart was at the first sampling time (5 min after doses 1 and 3, 1 h after dose 2). Tumor C_{max} was at the first sampling point after doses 1 and 2, but at 6 h after dose 3. As reflected by the relative AUC values, tissues contained higher DOX concentrations than plasma, consistent with a rapid distribution of DOX from plasma. Such high tissue/plasma ratios have previously been observed in rodents [1, 3, 9, 18, 23]. The high V_{ss} calculated for DOX is consistent with such extensive tissue distribution. The elimination of DOX from tissues and plasma occurred at similar rates, as evidenced by the overall similarity of $T_{1/2}$ values for plasma and tissues after each dose. Some tissue accumulation of DOX was noted after multiple dosing (as indicated by the increase in AUC values with successive doses). However, tumor C_{max} and AUC values remained lower than those of either heart or liver, indicating that DOX did not preferentially concentrate in tumor tissue.

Administration of BMS 182248 to tumor-bearing mice

The plasma concentrations of free and bound DOX after multiple i.v. doses of BMS 182248 (176 mg/kg per dose; 5 mg DOX equivalents/kg per dose) are shown in Fig. 3. For comparison, DOX levels after multiple DOX dosing are also shown (DOX/DOX). After BMS 182248 administration, plasma DOX existed predominantly as the conjugate-bound form, with relatively little free DOX present. The ratio of AUC values (bound DOX/free DOX; see Table 1) was about 350. As expected for i.v. administration, T_{max} values for bound DOX occurred at the first sampling points after each dose. Plasma half-lives for bound DOX ranged between 22 and 38 h, while those for free DOX ranged between 16 and 39 h (Table 1). The V_{ss} of these entities were markedly different; those for free DOX were similar to those estimated after administration of DOX alone, while those for bound DOX were considerably lower. This difference is consistent with the more limited distribution of conjugate-bound DOX predicted by its relatively high plasma levels. From plasma, bound DOX distributed to tissues, as evidenced by the tissue levels shown in Fig. 4 (tumor), 5 (liver) and 6 (heart). For

Table 2 Tissue pharmacokinetic parameters for DOX following multiple dosing with either DOX or BMS 182248. All values are means ± SD for three animals at each sampling point (*ND* not determined, values could not be calculated from the data obtained)

Treatment	Parameter	Liver			Tumor			Heart		
			BMS 182248			BMS 182248			BMS 182248	
		DOX	Free DOX	Bound DOX	DOX	Free DOX	Bound DOX	DOX	Free DOX	Bound DOX
Dose 1	C_{max} (μg/g)	21.416 ± 2.067	2.933 ± 0.254	8.480 ± 0.921	3.591 ± 0.437	4.716 ± 0.398	12.332 ± 1.549	17.338 ± 2.043	0.794 ± 0.089	7.113 ± 0.675
	T_{max} (h)	0.08	12 ± 10	1.0	0.08	18 ± 10	6.0	0.08	18 ± 10	1.0
	AUC (μg h/g)[a]	141.3 ± 10.6	123.9 ± 14.9	260.1 ± 22.4	83.5 ± 9.5	354.4 ± 43.7	808.1 ± 74.9	135.7 ± 18.9	40.7 ± 5.1	205.6 ± 24.6
	$T_{1/2}$ (h)	35 ± 5	31 ± 6	19 ± 4	26 ± 6	ND	ND	26 ± 5	41 ± 8	46 ± 10
Dose 2	C_{max} (μg/g)	9.076 ± 1.152	2.107 ± 0.357	6.706 ± 0.553	3.137 ± 0.287	9.130 ± 1.003	12.217 ± 2.437	7.356 ± 0.799	0.791 ± 0.103	7.472 ± 0.832
	T_{max} (h)	1.0	18 ± 10	1.0	1.0	32 ± 14	18 ± 10	1.0	4.3 ± 2.9	1.0
	AUC (μg h/g)[a]	178.7 ± 23.7	140.5 ± 15.8	340.9 ± 27.3	121.0 ± 14.6	625.3 ± 42.4	769.3 ± 67.3	140.2 ± 12.4	36.5 ± 4.3	233.9 ± 25.7
	$T_{1/2}$ (h)	30 ± 5	47 ± 6	39 ± 6	44 ± 8	ND	ND	26 ± 4	54 ± 7	26 ± 5
Dose 3	C_{max} (μg/g)	22.898 ± 3.159	2.139 ± 0.275	6.575 ± 0.498	3.552 ± 0.436	7.335 ± 0.712	16.438 ± 2.450	15.450 ± 2.129	0.787 ± 0.102	8.332 ± 0.954
	T_{max} (h)	0.08	6.0	1.0	6.0	18 ± 10	6.0	0.08	1.0	0.08
	AUC (μg h/g)[a]	248.1 ± 30.6	179.5 ± 19.4	408.0 ± 51.8	186.4 ± 15.7	774.9 ± 80.2	625.4 ± 59.6	187.4 ± 22.5	53.6 ± 4.9	402.4 ± 38.8
	$T_{1/2}$ (h)	31 ± 4	46 ± 10	ND	34 ± 6	ND	ND	25 ± 5	77 ± 15	79 ± 15

[a] AUC values were calculated from 0 to 96 h after doses 1 and 2 and from 0 to infinity after dose 3

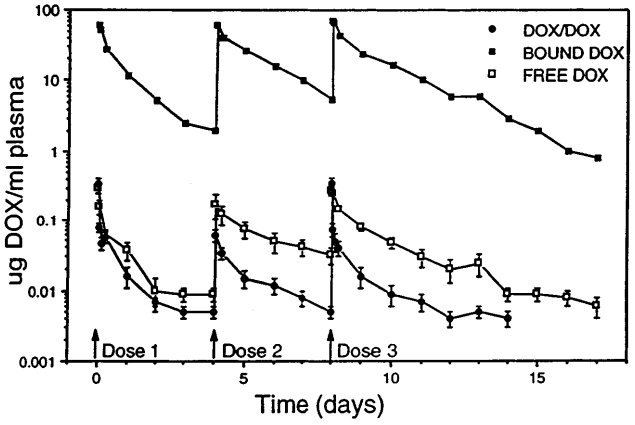

Fig. 3 Plasma concentrations of conjugate-bound and free DOX after i.v. administration of BMS 182248 (three doses 4 days apart; 5 mg DOX/kg/dose) to L2987-bearing mice. Concentrations of DOX after DOX administration (three doses 4 days apart; 5 mg/kg/dose) are shown for comparison

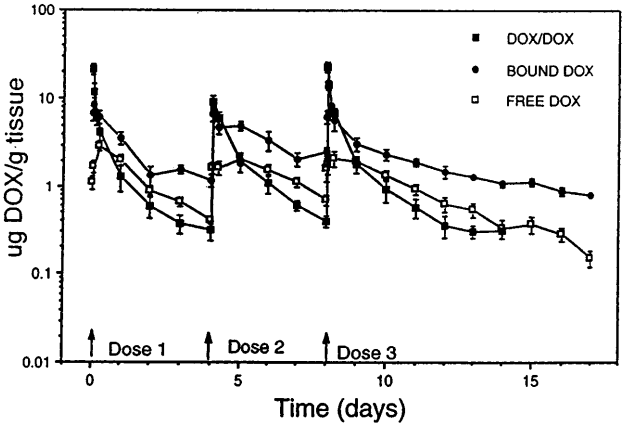

Fig. 5 Liver concentrations of conjugate-bound and free DOX after i.v. administration of BMS 182248 (three doses 4 days apart; 5 mg DOX/kg/dose) to L2987-bearing mice. Concentrations of DOX after DOX administration (three doses 4 days apart; 5 mg/kg/dose) are shown for comparison

comparison in these Figures, the DOX levels after multiple DOX dosing are also shown (DOX/DOX). The pharmacokinetic values estimated from these data are given in Table 2.

After BMS 182248 administration, a substantial concentration of free DOX was present in tumors. This amount was comparable to that which remained conjugate-bound (compare free and bound curves in Fig. 4 and the AUC values in Table 2). Levels of free DOX in tumors after BMS 182248 administration exceeded those after administration of DOX alone. Conjugate-bound DOX also distributed to liver and heart (see Fig. 5 and 6), but attained a lower concentration on a per gram of tissue basis in these normal tissues than in tumors. The AUC values (Table 2) indicated that the order of accumulation (bound DOX/g tissue) was tumor > liver > heart. Similarly, the concentrations of free DOX in liver and heart after BMS 182248 administration were lower than in tumor; only in tumors did the concentrations of free DOX approach those of bound DOX (see relative AUC values of Table 2). Elimination half-lives of both free and bound DOX from liver and heart were slightly longer than those observed for DOX (Table 2). Because of the persistence of both free and bound DOX in tumor (Fig. 4), elimination half-lives could not be determined.

Cellular uptake studies

The monoclonal antibody, BR96, binds to an Le^y-type antigen expressed on the surface of the human lung carcinoma cell line, L2987 [21]. As discussed above, L2987 cells were cultured in monolayers and exposed for 4 h to

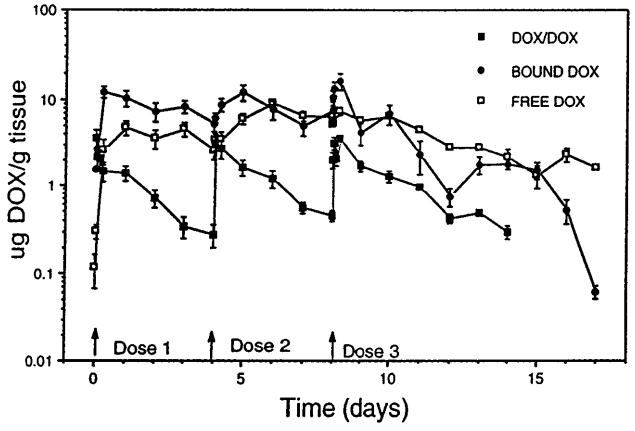

Fig. 4 Tumor concentrations of conjugate-bound and free DOX after i.v. administration of BMS 182248 (three doses 4 days apart; 5 mg DOX/kg/dose) to L2987-bearing mice. Concentrations of DOX after DOX administration (three doses 4 days apart; 5 mg/kg/dose) are shown for comparison

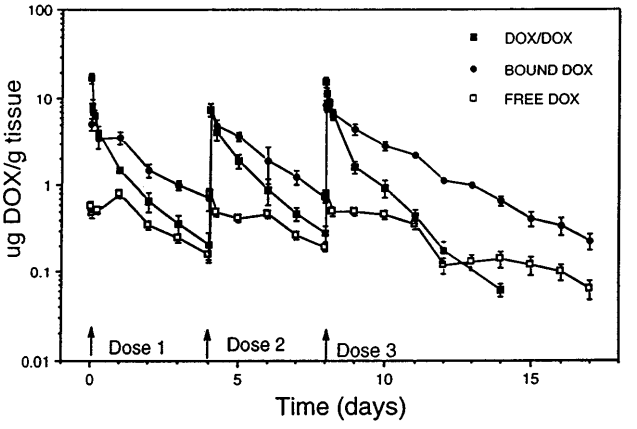

Fig. 6 Heart concentrations of conjugate-bound and free DOX after i.v. administration of BMS 182248 (three doses 4 days apart; 5 mg DOX/kg/dose) to L2987-bearing mice. Concentrations of DOX after DOX administration (three doses 4 days apart; 5 mg/kg/dose) are shown for comparison

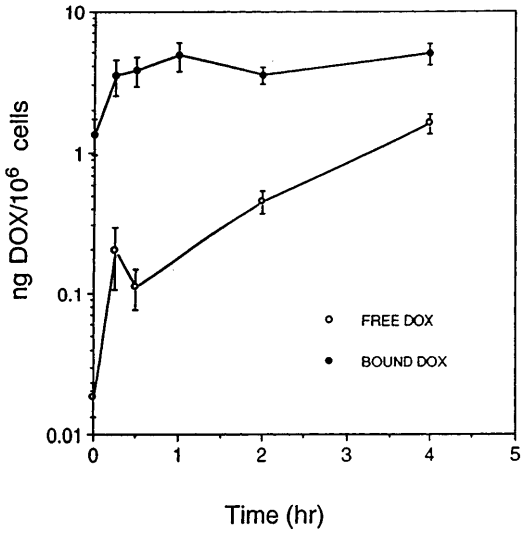

Fig. 7 Accumulation of free and bound DOX within L2987 cells after exposure to BMS 182248

BMS 182248 (final DOX concentration 0.1 μM in the incubation medium). At intervals, cells were harvested, lysed, and their free and bound DOX concentrations determined. Figure 7 shows the cellular free and bound DOX concentrations after BMS 182248 exposure as a function of incubation time. Thus, L2987 cells in culture accumulated bound DOX after exposure to BMS 182248 and liberated free DOX from the immunoconjugate.

Liberation of DOX from BMS 182248 in rat liver lysosomes

Figure 8 shows the time course for appearance of DOX upon incubation of BMS 182248 with either rat liver lysosomes or the buffer (pH 5.5) minus lysosomal protein (average of three determinations). DOX itself was stable in the lysosomal mixture over the time of incubation. From these data a half-life for DOX formation from BMS 182248 of 7.7 h was calculated in buffer, while a value of 1.7 h was calculated in lysosomes.

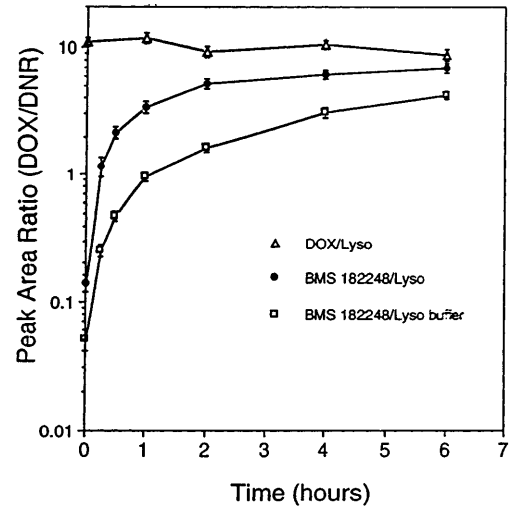

Fig. 8 Generation of DOX from BMS 182248 after incubation with either isolated rat liver lysosomes or lysosomal buffer (pH 5.5). The stability of DOX in lysosomes is shown for comparison

Discussion

Previous studies have demonstrated that the conjugation of anthracyclines to monoclonal antibodies via acid-sensitive linkers represents a promising approach to selectively directing these cytotoxic agents to tumor tissue [7, 8, 15, 16, 28, 29]. Through a selection process that involved the evaluation of a variety of acid-sensitive hydrazone linkers at the C-13 position of DOX [13, 27] and of suitable tumor-specific monoclonal antibodies [12, 21], the BR96-DOX immunoconjugate, BMS 182248, was chosen for extensive preclinical evaluation. This conjugate demonstrates cytotoxicity against L2987 cells in culture and, in vivo, produces long-term cures when administered at equivalent DOX doses of 5 mg/kg or greater (three doses 4 days apart) to athymic mice bearing subcutaneously implanted L2987 tumors [24]. To understand the biodistribution of DOX following the administration of BMS 182248, athymic mice bearing implanted L2987 carcinomas were administered three doses (5 mg DOX equivalents/kg) of BMS 182248 4 days apart, i.e. the same schedule as that which produced antitumor efficacy. Plasma and tissue samples were then analyzed for both free and bound DOX, as described above. For comparative purposes, DOX was administered at the same dose and schedule.

After administration of BMS 18248, most circulating plasma DOX remained conjugate-bound (see Fig. 3 and the free and bound AUC ratios of Table 1). This bound DOX was eliminated from plasma with half-lives that ranged from 22 h after the first dose to 38 h after the third dose. The plasma concentrations of free DOX were elevated three to five times (based on plasma AUC ratios) over those observed after administration of DOX alone (Table 1). From plasma, BMS 182248 rapidly distributed to the three tissues examined, with the order of bound DOX concentrations attained on a per gram of tissue basis being tumor > liver > heart. Free DOX was also detected in the tissues after BMS 182248 administration, but the concentrations of free DOX in heart and liver were lower than those detected after administration of DOX alone (see Fig. 5 and 6 and the AUC values of Table 2). As assessed by the AUC values, the total exposure of the heart to free DOX was about one-quarter of that observed after the administration of DOX alone. Since cardiotoxicity is a significant clinical problem following DOX administration [17], the reduced exposure of murine heart to DOX following administration of BMS 182248 may signify a potential therapeutic benefit. Indeed, recent evidence has shown that the cardiotoxicity of BMS 182248 in rats is substantially

less severe than that from an equivalent DOX dose [5]. The elimination kinetics of both conjugate-bound DOX and free DOX from liver and heart paralleled those observed from plasma, indicating that BMS 182248 and the free DOX derived therefrom attained equilibrium between those nontumor tissues and plasma.

The results presented above clearly indicate a targeting of DOX to tumor tissue following administration of BMS 182248. This fact is evident by a visual comparison of the data in Fig. 4, which compares the concentrations of free and bound DOX in tumors as a function of time after BMS 182248 administration with those of DOX after administration of an equivalent dose. As is evident from the data of Fig. 4, the elimination kinetics for both conjugate-bound and free DOX from tumors following BMS 182248 administration differed greatly from those for DOX after its administration. Since, in tumors after BMS 182248 administration, substantial prolonged levels of both conjugate-bound DOX and free DOX were present, the elimination kinetics of free DOX appeared to be controlled by the rate of release of DOX from tumor-associated immunoconjugate. Such sustained concentrations within the tumor tissue suggest that retention of BMS 182248 by antigen-promoted binding to tumor cells had occurred. As a result of this retention of BMS 182248 within tumor tissue, the tumor concentrations of free DOX greatly exceeded those produced by the administration of DOX at an equivalent dose, a finding consistent with the greater antitumor activity of BMS 182248 relative to DOX [24].

The AUC ratios of free to bound DOX within tumors increased over the duration of the experiment suggesting that the tumors were capable of converting BMS 182248 to DOX. This supposition was explored by in vitro studies with BMS 182248. The uptake of BMS 182248 and its conversion to DOX was studied in cultured L2987 cells, which, as Le^y expressors, are capable of binding and internalizing BR96 [10, 24]. After incubation of L2987 cells with BMS 182248, both bound and free DOX were detected within the cells (Fig. 7). Upon incubation of BMS 182248 with rat liver lysosomes, DOX was liberated from BMS 182248 with a half-life of 1.7 h, while in buffer (pH 5.5) the half-life was 7.7 h (Fig. 8). Thus, lysosome-mediated cleavage of BMS 182248 to liberate DOX can readily occur.

The data generated in these studies demonstrated that BMS 182248 effectively delivered DOX to human tumor xenografts implanted in athymic mice. Further, BMS 182248 yielded higher and more prolonged tumor concentrations of free DOX than were attained after administration of DOX alone. This increased tumor localization was presumably due to antigen-promoted binding and internalization into tumor cells followed by lysosomal release of DOX. Following BMS 182248 administration, normal tissues, as exemplified by heart and liver, were exposed to lower overall concentrations of free DOX than produced by an equivalent DOX dose.

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