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Effect of Linker Variation on the Stability, Potency, and Efficacy of Carcinoma-reactive BR64-Doxorubicin Immunoconjugates

Pamela A. Trail,[1] David Willner, Jay Knipe, Arris J. Henderson, Shirley J. Lasch, Mary E. Zoeckler, Mark D. TrailSmith,[2] Terrence W. Doyle,[3] H. Dalton King, Anna Maria Casazza, Gary R. Braslawsky,[4] Joseph Brown, Sandra J. Hofstead, Robert S. Greenfield,[5] Raymond A. Firestone, Kathleen Mosure, Kathleen F. Kadow, Michael B. Yang, Karl Erik Hellström, and Ingegerd Hellström

Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543 [P. A. T., A. J. H., S. J. L., A. M. C., M. B. Y.]; Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492 [D. W., J. K., M. E. Z., H. D. K., S. J. H., R. A. F., K. M., K. F. K.]; and Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121 [K. E. H., I. H.]

ABSTRACT

The internalizing anti-Le^y monoclonal antibody (MAb) BR64 was conjugated to the anticancer drug doxorubicin (DOX) using an acid-labile hydrazone bond to the DOX and either a disulfide or thioether bond to the MAb. The resulting disulfide (BR64-SS-DOX) and thioether (BR64-S-DOX) conjugates were evaluated for stability, potency, and antigen-specific activity in both *in vitro* and *in vivo* model systems. The BR64-SS-DOX conjugates demonstrated antigen-specific activity both *in vitro* and when evaluated against antigen-expressing, DOX-sensitive human carcinoma xenografts. However, the stability and potency of disulfide conjugates were poor, and *in vivo* activity superior to unconjugated DOX was seen only at doses approaching the maximum tolerated dose. Furthermore, BR64-SS-DOX conjugates were not active against antigen-expressing, DOX-insensitive colon tumor xenografts. In contrast, the BR64-S-DOX conjugates demonstrated good stability both *in vitro* and *in vivo*. The increased stability of the BR64-S-DOX conjugates resulted in the delivery of more biologically active DOX to tumors with a concomitant increase in potency and efficacy over that which could be achieved with either unconjugated DOX or BR64-SS-DOX conjugates. Delivery of DOX by BR64-S-DOX conjugates resulted in complete regressions and cures of both DOX-sensitive lung xenografts and DOX-insensitive colon tumor xenografts. These results demonstrate the importance of linker stability when delivering drugs such as DOX to carcinomas via internalizing antibodies and are likely to have direct relevance to the clinical utility of MAb-directed delivery.

INTRODUCTION

MAbs[6] to tumor-associated antigens have been used with variable success to prepare immunoconjugates for the delivery of toxic moieties to malignant cells. The immunoconjugates include both chemical conjugates in which MAbs are covalently coupled to cytotoxic drugs (1–4), radionuclides (5, 6), enzymes (7, 8) and plant or bacterial toxins (9–11) as well as single-chain fusion proteins, expressed in bacteria, in which the genes encoding the MAb variable regions are fused to genes encoding protein toxins (11, 12). The use of MAbs to deliver conventional cytotoxic agents offers a potential method to increase antitumor efficacy by increasing the intratumoral drug concentration and increasing the therapeutic index of the targeted drug. Several strategies involving different MAbs, drugs, and linkers have been evaluated. These studies have included: MAbs that internalize rapidly as well as with MAbs that internalize slowly, if at all (4, 11, 13–15); drugs with varying levels of potency (4, 11, 16, 17); and linkers with different mechanisms of drug release (1, 14, 16, 18–21) and differential stability *in vitro* and *in vivo* (4, 10, 22–24).

Clearly, the selection of an appropriate combination of MAb, linker, and drug is critical to the design of immunoconjugates, which can offer a significant advantage over the unconjugated parent drug. However, few studies have systematically evaluated the relative importance of these parameters. Rather, the efficacy of immunoconjugates prepared using different MAbs, drugs, and linkers has been evaluated under a variety of different *in vitro* and *in vivo* experimental conditions.

The MAb BR64 identifies a Le^y-related tumor-associated antigen expressed at high level (100,000 molecules/cell) on the surface of cells of the majority of human carcinomas (25). Following antigen-specific binding, BR64 is rapidly internalized into the acidic compartment of lysosomes/endosomes (26). In the studies described here, BR64 DOX immunoconjugates were produced using either disulfide (BR64-SS-DOX) or thioether (BR64-S-DOX) bonds to the MAb, and the effect of varying the linker was evaluated with respect to the *in vitro* and *in vivo* stability, potency, and efficacy of the conjugates. The disulfide and thioether conjugates had similar DOX:MAb molar ratios, used the same mechanism of intracellular drug release, an acid-labile hydrazone bond to DOX, and were compared in the same *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Monoclonal Antibodies. MAb BR64 (murine IgG1) identifies a Le^y-related tumor-associated antigen that is expressed on carcinomas of the lung, colon, breast, and ovary and is rapidly internalized following antigen-specific binding (25, 26). The BR64 MAb is used here as a model because of its favorable conjugation characteristics; however, it is not suitable for clinical development because it demonstrates binding to cardiac tissue from some human patients whereas the related anti-Le^y MAb BR96 does not (3, 26). The SN7 hybridoma, which was received from B. Seon (Roswell Park Memorial Institute, New York, NY), identifies an antigen expressed on human B cells and was used as a non-binding, isotype-matched control antibody. The BR64 and SN7 MAbs were produced as tissue culture supernatants (Brunswick BioTechnetics, San Diego, CA).

Synthesis of MAb-DOX Immunoconjugates. MAbs were thiolated with SPDP. Conjugates were prepared by linking 3-(2-pyridinyldithio)propanoyl DOX hydrazone or 6-maleimidocaproyl DOX hydrazone to SPDP-thiolated MAbs to produce disulfide (BR64-SS-DOX) or thioether (BR64-S-DOX) conjugates, respectively (27). Briefly, the MAbs were treated with 8 molar equivalents of SPDP at 30°C, followed by reduction with excess DTT on ice. Excess reagents were removed by dialysis or diafiltration in Amicon Cells, and the thiol and MAb concentrations were determined. A molar equivalent per thiol of 3-(2-pyridinyldithio) propanoyl DOX hydrazone or 6-maleimidocap-

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[1] To whom requests for reprints should be addressed, at Bristol-Myers Squibb Pharmaceutical Research Institute, P. O. Box 4000, K22-01, Princeton, NJ 08543.

[2] Present address: Wyeth Ayerst Research, Radnor, PA 19087.

[3] Present address: VION Pharmaceuticals, Inc., 4 Science Park, New Haven, CT 06511.

[4] Present address: IDEC Corporation, 11011 Torreyana Dr., San Diego, CA 92121.

[5] Present address: Sym Biotech, Inc., 8 Fairfield Boulevard, Wallingford, CT 06492.

[6] The abbreviations used are: MAb, monoclonal antibody; Le^y, Lewis ^{y}; DOX, doxorubicin; BR64-SS-DOX, BR64 disulfide conjugate; SN7-SS-DOX, SN7 disulfide conjugate; BR64-S-DOX, BR64 thioether conjugate; SN7-S-DOX, SN7 thioether conjugate; SPDP, *N*-succinimidyl 3-(2-pyridinyldithio)propionate; HPLC, high-performance liquid chromatography; TVDD, tumor volume doubling delays; PR, partial tumor regression; CR, complete tumor regression; MTD maximum tolerated dose; AUC, area under the curve.

royl DOX hydrazone was added, and the formed conjugates were purified by dialysis or chromatography on Bio-Beads, SM-2 (Bio-Rad). The molar concentrations of DOX and MAb were determined from their UV absorption measured at 495 and 280 nm, respectively, including a correction for the absorption of DOX at 280 nm, as described previously (27).

The immunoconjugates were evaluated by HPLC to assess free drug and by FACS to assess retention of MAb binding activity, as described previously (14). The conjugates used in these studies contained <5% free DOX or DOX linker, retained >90% of the original MAb binding activity, and were of comparable DOX:MAb molar ratios.

Human Carcinoma Lines. L2987 is a lung adenocarcinoma line. RCA is a colorectal carcinoma line obtained from M. Brattain (Medical College of Ohio, Toledo, OH). Both L2987 and RCA express the BR64-defined antigen and were established as tumor xenografts in athymic mice as described previously (14). Tumors were measured in two perpendicular directions at weekly or biweekly intervals using calipers. Tumor volume was calculated according to the equation: $V = l \times w^2/2$, where V = volume (mm^3), l = measurement of longest axis (mm), and w = measurement of axis perpendicular to l (mm). There were 8–10 mice per control or treatment group. Data are presented as median tumor size. Antitumor activity is expressed in terms of median tumor volume doubling delays (*TVDD*), where $TVDD = T - C/TVDT$. $T - C$ is defined as the median time (days) for treated tumors to reach 500 mm^3 in size minus the median time for control tumors to reach 500 mm^3 in size, and *TVDT* is the time (days) for control tumors to double in volume (250–500 mm^3). A tumor growth delay equivalent to ≥3.3 *TVDD* was considered evidence of biological activity. PR reflects a decrease in tumor volume to ≤50% of the initial tumor volume; CR refers to a tumor that has regressed completely and is not palpable for a period of time equal to the TVDT; and cure is defined as an established tumor that has regressed completely and that, after regression, is not palpable for a period of time ≥10 TVDTs.

***In Vitro* Cytotoxicity Assays.** Antigen-specific cytotoxicity was evaluated according to a modification of a method described previously (14). Briefly, monolayer cultures of L2987 lung carcinoma cells were harvested using trypsin-EDTA (Life Technologies, Inc., Grand Island, NY), and the cells resuspended to 1×10^5/ml in RPMI 1640 containing 10% heat-inactivated FCS. The cells were added to flat-bottomed, 96-well microtiter plates (0.1 ml/well) and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. Media were removed from the plates, and serial dilutions of DOX or conjugates were added to each of the wells. Samples were assayed as quadruplicates. The cells were exposed to the drug or the individual conjugates for different exposure times (1–48 h) at 37°C in a humidified atmosphere of 5% CO_2 in air. The drug or conjugate was then removed, and the cells were washed three times with RPMI and cultured in RPMI containing 10% heat-inactivated FCS. Approximately 48 h after the addition of conjugate or DOX, the cells were pulsed for 2 h with 1.0 μCi/well of [^{3}H]thymidine (DuPont NEN, Boston, MA). The media were removed, and trypsin (2.5×) was added to the wells. The cells were harvested (Skatron Instruments, Sterling, VA) onto glass fiber filter mats and dried, and filter-bound [^{3}H]thymidine radioactivity was determined (β-plate scintillation counter; Pharmacia Biotech, Inc., Piscataway, NJ). Inhibition of [^{3}H]thymidine uptake was determined by comparing the mean cpm for treated samples with the mean cpm of the untreated control.

Experimental Animals. Congenitally athymic female mice of BALB/c background (BALB/c *nu/nu*; Harlan Sprague Dawley, Indianapolis, IN) were used. Mice were housed in Thoren caging units on sterile bedding with controlled temperature and humidity and received sterile food and water *ad libitum*.

Therapy. DOX was diluted in normal saline, and conjugates were diluted in PBS. Therapy was administered every 4 days for a total of three injections. Control animals were not treated. Doses are presented as mg/kg/injection with immunoconjugate doses reported as mg/kg/injection of equivalent DOX. The MTD for a treatment regimen was defined as the highest dose on a given schedule that resulted in ≤20% lethality.

Pharmacokinetic Analysis. Plasma samples were collected at various times after administration of conjugate to mice bearing L2987 tumors. The concentrations of DOX that had been released *in vivo* from conjugates (released DOX) and that remaining bound to the BR64 MAb (bound DOX) in plasma and tumors were determined. In each sample, the concentrations of both released DOX and total DOX (the total DOX detected after treatment of samples by chemical hydrolysis) were determined; the difference between these values represented conjugate-bound DOX. Tumors were homogenized (Brinkmann Polytron, Westbury, NY) in seven volumes of water, and the resulting homogenate divided in half; one-half was analyzed for free DOX, and the other for total DOX. Plasma samples were also split 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 and 0.01 ml of a 10 μg/ml aqueous solution of an internal standard consisting of daunomycin was added. The mixture was centrifuged, and 0.2 ml of the supernatant fluid was applied to a preconditioned C8 Bond-Elut cartridge (Analytichem, Harbor City, CA). For plasma samples, an aliquot (0.2 ml) was mixed with 0.01 ml of the daunomycin solution, and the mixture was applied to C8 cartridges. After sample loading, each cartridge was washed with water followed by 30% methanol/water and the retained material then eluted with 0.30 ml of 75% acetonitrile/triethylammonium formate buffer (0.05 M, pH 7); 0.05 ml of this eluant was injected onto the HPLC. For analysis of total DOX in samples, separate portions of each plasma or tumor homogenate were mixed with 0.01 ml of a 1 mg/ml aqueous solution of dithioerythritol (Sigma Chemical Co.). After 30 min at room temperature, the pH was lowered to approximately 2.5 with 1 N HCl, and the mixture was incubated at 37°C for 2 h to liberate the total DOX content. After incubation, plasma samples were mixed with daunomycin and subjected to solid-phase extraction, while tissue samples were mixed with daunomycin followed by silver nitrate prior to extraction. HPLC analyses were performed on a Waters (Milford, MA) system comprised of two 510 pumps, a 680 gradient controller, and a 712 autosampler. DOX was detected after chromatography on a Waters μBondapak C_{18} column by fluorescent detection (495 nm excitation, 550 nm emission) using a Waters 470 detector. The mobile phase was 68% triethylammonium formate buffer (0.05 M, pH 2.8)/32% ACN at a flow of 1 ml/min. Standard curves (peak area ratio of DOX:daunomycin *versus* DOX concentration) for DOX quantitation were generated by fortifying control plasma samples or tumor homogenates with known amounts of DOX and processing the samples as described. The recovery of DOX using the above extraction procedures ranged from 82% (tumor samples) to 97% (plasma samples). The total recovery of DOX liberated from the immunoconjugates by hydrolysis followed by solid phase extraction ranged from 75% (tumor samples) to 90% (plasma samples). These extraction efficiencies were used to correct the raw data. The concentrations of DOX in plasma samples were calculated on a μg/ml basis, whereas those in tissue samples were calculated on μg/g of tissue weight basis. The areas under the plasma and tumor concentration *versus* time curves were calculated by the trapezoidal rule using RSTRIP (Micromath Scientific Software, Salt Lake City, UT).

RESULTS

Antigen-specific Cytotoxicity of BR64-DOX Conjugates *in Vitro*. The *in vitro* potency and specificity of disulfide and thioether conjugates, evaluated following various exposure times, is shown in Table 1. The IC_{50}s of both BR64-S-DOX and BR64-SS-DOX conjugates decreased with longer exposure times. However, the thioether-linked conjugates maintained antigen-specific cytotoxicity (specificity ratio of IC_{50} SN7:IC50 BR64, ≥5) for at least 24 h of exposure, whereas the antigen-specific cytotoxicity of the disulfide conjugates was lost within the first 4 h of exposure. The loss of antigen specificity in both cases likely reflects cytotoxicity of DOX released nonspecifically from the BR64 and SN7 MAbs; the rate of nonspecific DOX hydrolysis of disulfide conjugates was significantly faster than that of the thioether conjugates.

***In Vivo* Stability of BR64 Disulfide and Thioether Conjugates.** The *in vivo* stability of conjugates was assessed by measuring the quantity of intact conjugate (quantified as protein-bound DOX) in plasma at various times after the administration of a single dose of BR64-SS-DOX or BR64-S-DOX to tumor-bearing mice. Conjugates were administered at a dose equivalent to 5 mg/kg DOX. Free DOX, released from the conjugates, could not be detected in these plasma samples, presumably due to the expected rapid uptake of unconjugated DOX into tissues (28). As shown in Fig. 1, higher levels of bound DOX were observed for BR64-S-DOX than BR64-SS-DOX at

Table 1 *In vitro cytotoxicity and specificity of disulfide- and thioether-linked conjugates*

	IC_{50} (μM equivalent DOX)					
	Disulfide conjugates		Thioether conjugates		Specificity ratio[a]	
Exposure time (h)	BR64	SN7	BR64	SN7	Disulfide	Thioether
1	2.0	10.0	2.0	50.0	5.0	25.0
2	0.90	5.50	2.0	20.0	6.1	10.0
4	0.45	1.50	0.80	10.0	3.3	12.5
8	0.45	1.50	0.35	2.50	3.3	7.1
12	0.40	0.90	0.18	1.50	2.3	8.3
24	0.20	0.55	0.15	0.75	2.8	5.0
48	0.15	0.25	0.18	0.50	1.7	2.8

[a] IC_{50} SN7:IC_{50} BR64.

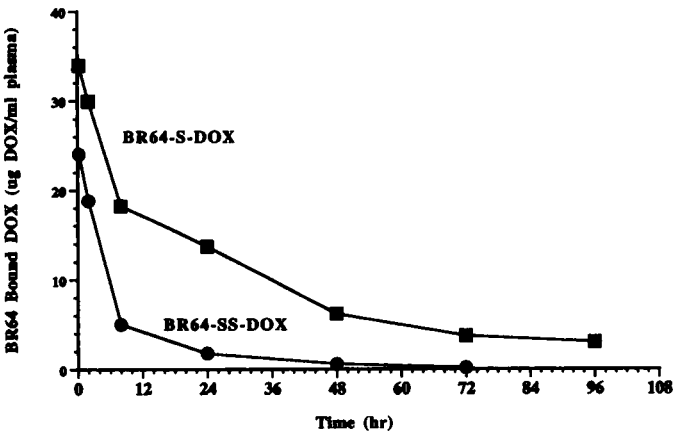

Fig. 1. Plasma levels of conjugate-bound DOX at various times following the administration of BR64-SS-DOX (●) and BR64-S-DOX (■) conjugates (5 mg/kg equivalent DOX).

all times evaluated. The plasma terminal $T½$ of BR64-S-DOX was 30.1 h, whereas that of BR64-SS-DOX was 17.4 h. The total systemic exposure to bound DOX, as assessed by plasma AUC values, was 4.2-fold higher for BR64-S-DOX than for BR64-SS-DOX (Table 2), indicating that the thioether conjugate was more stable *in vivo* than the disulfide.

***In Vivo* Pharmacokinetics of BR64 Disulfide and Thioether Conjugates.** The ability of BR64-S-DOX and BR64-SS-DOX conjugates to deliver DOX to L2987 tumors was evaluated in parallel with measurements of plasma stability. The greater plasma stability of BR64-S-DOX *in vivo* resulted in a higher peak concentration (C_{max}) of bound DOX in tumors (Fig. 2*A*). In the case of BR64-S-DOX, the C_{max} obtained was 3.5-fold higher than that obtained with the BR64-SS-DOX conjugate (5.71 μg DOX/g tumor as compared to 1.63 μg/g tumor, respectively). The levels of intratumoral bound DOX reached peak concentrations 2–8 h after the administration of either BR64-S-DOX or BR64-SS-DOX. As shown in Fig. 2*B*, maximum levels of biologically active, conjugate-liberated DOX were observed in tumors 48 h after the administration of BR64-S-DOX. The peak intratumoral concentration for the BR64-S-DOX conjugate was 5.1 ± 1.2 μg DOX/g tumor. In contrast, with BR64-SS-DOX, the maximum intratumoral concentration of biologically active DOX (Fig. 2*B*) was 0.45 ± 0.35 μg DOX/g tumor with levels of 0.3–0.45 μg DOX/g tumor observed during the first 48 h after administration. Based on AUC values, BR64-S-DOX delivered 12.7-fold more biologically active DOX to tumors than an equivalent dose of BR64-SS-DOX. The levels of bound and free DOX observed in tumors at various times after administration of BR64-S-DOX are presented in Fig. 2*C*. Initially, the majority of intratumoral DOX was present in conjugated form; however, within the first 24 h of administration, the levels of free DOX increased with a compensatory decline in conjugate-bound DOX. The increase in intratumoral levels of free DOX likely reflects the specific release of DOX from the BR64-S-DOX conjugate following internalization by antigen-expressing tumor cells and subsequent exposure to the acidic environment of lysosomes.

Antigen-specific Activity of BR64-SS-DOX and BR64-S-DOX Conjugates Evaluated against DOX-sensitive Human Lung and DOX-insensitive Human Colon Tumor Xenografts. Antigen-specific antitumor activity of thioether and disulfide conjugates was evaluated against established (50–100 mm^3) xenografts of L2987 human lung carcinomas. Representative data are presented in Fig. 3. The L2987 xenografts were sensitive (≥3.3 TVDD) to DOX administered at tolerated doses (Fig. 3*A*); however, tumor regressions were not achieved, even at the MTD. The antitumor activity of BR64-SS-DOX and nonbinding SN7-SS-DOX conjugates and DOX is presented in Fig. 3*A*. At a dose of 25 mg/kg/injection equivalent DOX, BR64-SS-DOX produced antitumor activity equivalent to 10.6 TVDD with 33% PR. The activity of BR64-SS-DOX was superior to that obtained with the MTD (8 mg/kg) of unconjugated DOX (5.0 TVDD, with no tumor regressions). An equivalent dose of nonbinding SN7-SS-DOX conjugate was not active (2.4 TVDD), indicating that the efficacy of BR64-SS-DOX was antigen specific.

The BR64-S-DOX conjugate (Fig. 3*B*), administered at a dose of 10 mg/kg equivalent DOX, resulted in regression of 100% of established tumors (78% cures, 11% CR, and 11% PR) and a tumor growth delay of >16 TVDD. The antitumor activity of BR64-S-DOX was antigen specific because an equivalent dose of nonbinding SN7-S-DOX produced only 5.6 TVDD and no regressions. The BR64-S-DOX conjugate, administered at a dose of 5 mg/kg equivalent, produced 100% regressions of established tumors (55% cures and 45% CR) and a growth delay of >16 TVDD. In contrast, DOX, administered at its MTD (8 mg/kg), produced a tumor growth delay equivalent to 5.5 TVDD and did not cause any tumor regressions. Therefore, the BR64-S-DOX conjugate was both more active and more potent than optimized, unconjugated DOX.

The BR64-S-DOX conjugate was both significantly more efficacious and more potent than a BR64-SS-DOX conjugate of similar DOX:MAb molar ratio evaluated in parallel (Fig. 3*C*). Administration of BR64-S-DOX at a dose of 10 mg/kg equivalent DOX resulted in regression of 100% of tumors (50% cures, 25% CR, and 25% PR) and

Table 2 *Pharmacokinetic analysis of plasma and tumor exposure following administration of BR64-SS-DOX or BR64-S-DOX*

	AUC (μg/ml·h)		
	BR64-SS-DOX	BR64-S-DOX	AUC ratio
Plasma			
Bound DOX	214.74	908.47	4.23
Released DOX	<LLQ[a]	<LLQ[a]	
Tumor			
Bound DOX	54.89	282.39	5.15
Released DOX	28.35	280.99	9.91

[a] Below the lower level of quantitation.

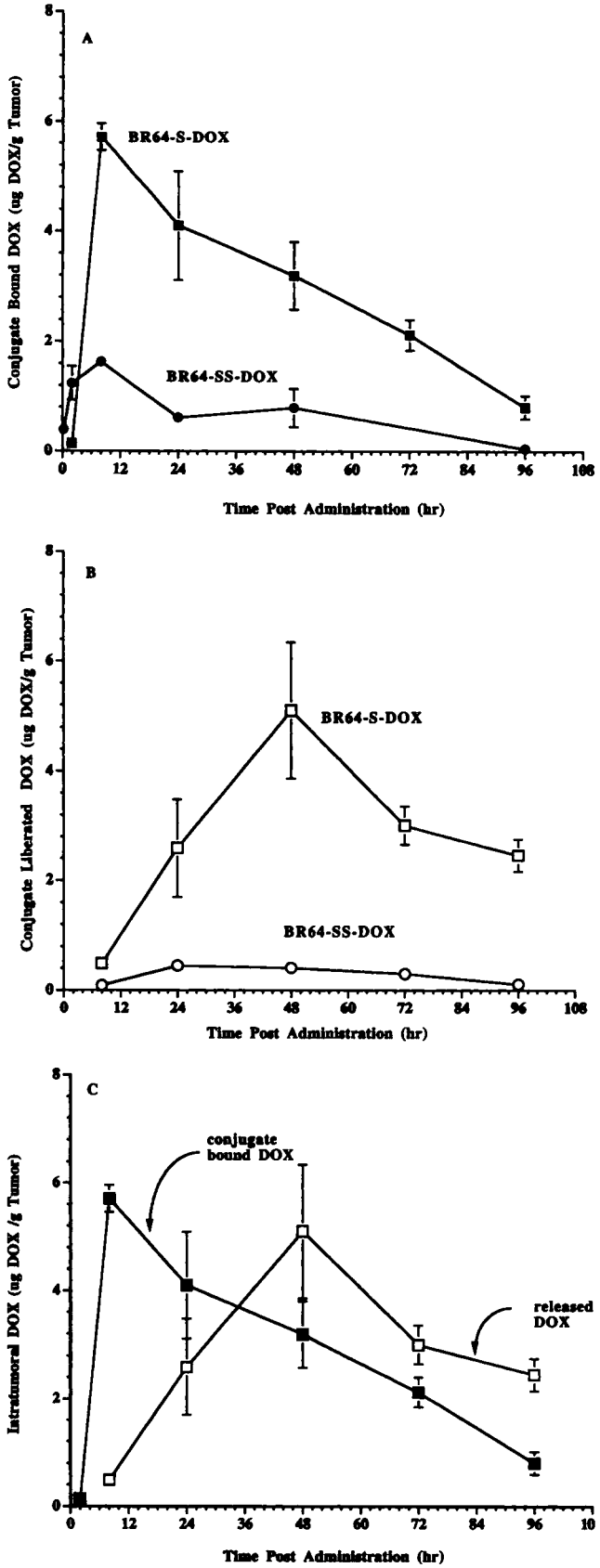

Fig. 2. Intratumoral concentrations of conjugate-bound and conjugate-liberated DOX following the administration of 5 mg/kg BR64-SS-DOX or BR64-S-DOX. *A*, intratumoral concentrations of conjugate-bound DOX: ●, BR64-SS-DOX; ■, BR64-S-DOX. *Bars*, Mean ± SD. *B*, intratumoral concentrations of DOX liberated from BR64-SS-DOX (○) or BR64-S-DOX (□) conjugates. *Bars*, Mean ± SD. *C*, relationship between conjugate-bound DOX (■) and conjugate-liberated DOX (□) following the administration of BR64-S-DOX. *Bars*, Mean ± SD.

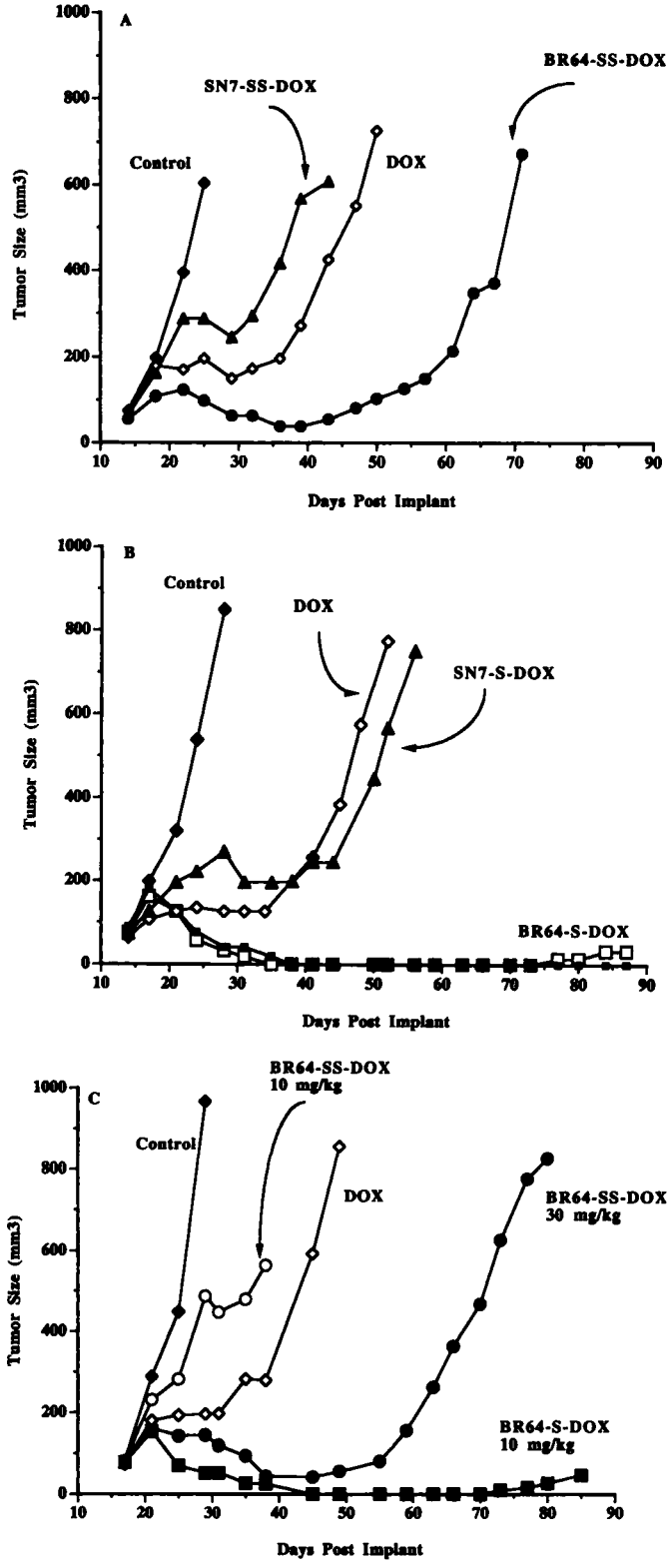

Fig. 3. Antitumor activity of disulfide and thioether conjugates and DOX against established L2987 human lung carcinoma xenografts in athymic mice. *A*, antigen-specific antitumor activity of BR64-SS-DOX conjugates. Results are from control mice (◆) or mice treated with 25 mg/kg BR64-SS-DOX (●), 25 mg/kg nonbinding SN7-SS-DOX (▲), or the MTD (8 mg/kg) of unconjugated DOX (◇) on days 14, 18, and 22 after tumor implant. *B*, antigen-specific antitumor activity of BR64-S-DOX conjugate. Results are from control mice (◆) or mice treated with BR64-S-DOX conjugate at 10 mg/kg (■) or 5 mg/kg (□), nonbinding SN7-S-DOX at 10 mg/kg (▲), or the MTD (8 mg/kg) of unconjugated DOX (◇) on days 14, 18, and 22 after tumor implant. *C*, comparison of efficacy and potency of BR64 thioether and disulfide conjugates. Results are from untreated control mice (◆) or mice treated with BR64-S-DOX at 10 mg/kg (■), BR64-SS-DOX at 30 mg/kg (●), BR64-SS-DOX at 10 mg/kg (○), or the MTD (8 mg/kg) of DOX (◇) on days 17, 21, and 25 after tumor implant.

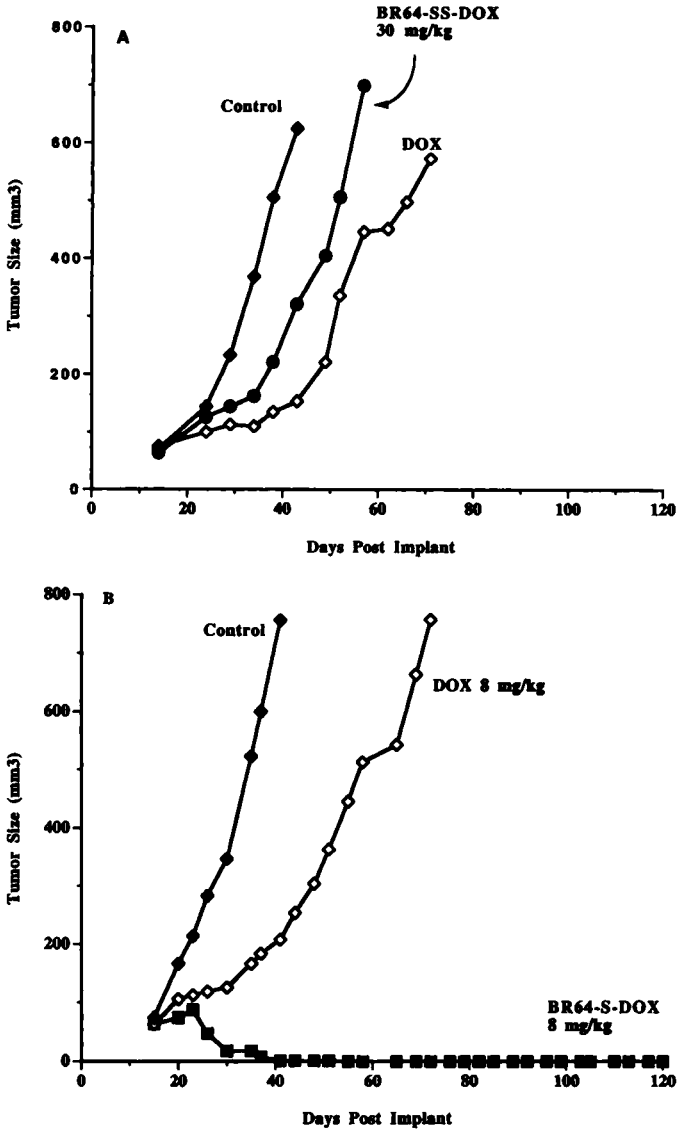

Fig. 4. Antitumor activity of disulfide and thioether conjugates and DOX against established RCA human colon carcinoma xenografts in athymic mice. *A*, antitumor activity of BR64-SS-DOX conjugates. Results are from control mice (♦) or mice treated with 30 mg/kg BR64-SS-DOX (●) or the MTD (8 mg/kg) of unconjugated DOX (◇) on days 14, 18, and 22 after tumor implant. *B*, antitumor activity of BR64-S-DOX conjugate. Results are from control mice (♦) or mice treated with 8 mg/kg of BR64-S-DOX conjugate (■) or unconjugated DOX (◇) on days 15, 19, and 23 after tumor implant.

antitumor activity of >16 TVDD, whereas an equivalent dose of BR64-SS-DOX was not active. In fact, the activity of the BR64-S-DOX conjugate at a conjugate dose of 10 mg/kg was superior to that obtained with BR64-SS-DOX administered at a 3-fold higher dose, 30 mg/kg (9.2 TVDD with 62.5% PR).

RCA human colon tumor xenografts (50–100 mm^3 in size) are not sensitive to unconjugated DOX administered at tolerated doses (3). As shown in Fig. 4*A*, BR64-SS-DOX, evaluated at a dose equivalent to 30 mg/kg DOX, was not active (1.7 TVDD with 0% regressions) against RCA tumors. In contrast, treatment with BR64-S-DOX (Fig. 4*b*) at a dose equivalent to 8 mg/kg DOX resulted in regression of 78% of established tumors (67% cures and 11% PR) and produced >16 TVDD. The thioether-linked BR64 conjugate demonstrated significant antitumor activity against colon tumors that were insensitive to both unconjugated DOX and the BR64 disulfide conjugate.

DISCUSSION

In the present study, the contribution of the linker to the efficacy of MAb immunoconjugates was evaluated. The MAb, drug, drug:MAb molar ratios and the mechanism of intracellular drug release were kept constant, and the linkers changed from disulfide to thioether. The effect of this change in linker on the antigen-specific activity, potency, efficacy, and pharmacokinetics of the conjugates was determined. Earlier studies reported the *in vitro* and *in vivo* activity of BR64-DOX conjugates produced with disulfide linkers (14). Although antigen-specific antitumor activity against established human carcinoma xenografts was observed, activity superior to that of unconjugated DOX was seen only at doses approaching the MTD of the conjugate (~30 mg/kg DOX, 1200 mg/kg MAb administered every 4 days for a total of three injections). The low potency of the BR64-SS-DOX conjugates may have been due to poor MAb localization, inefficient intracellular drug release, and/or the instability of the disulfide linker. Previous studies with ricin A conjugates have shown that the disulfide linkage is unstable *in vivo* and that use of other linkages, such as a thioether or hindered disulfide, can significantly improve stability and efficacy of immunotoxins and immunoconjugates (10, 23, 24, 29, 30). The increased stability of these conjugates likely reflects the reduced susceptibility of thioether and hindered disulfide bonds to reductive mechanisms such as those of glutathione and other thiol-containing molecules present in liver and plasma. In fact, another anti-Le^y MAb, BR96, conjugated to DOX via an acid-labile hydrazone linker to DOX and a thioether linker to the MAb has demonstrated excellent potency and efficacy, and cures have been observed in several established tumor models of various histological types (3). However, because both the MAb and linker were changed when the BR96-DOX conjugate was prepared, it was not clear if the improved potency and efficacy resulted from the change in linker from disulfide to thioether or whether the BR96 MAb was better than BR64 in localizing and delivering DOX to tumors.

To address the issue of the relative importance of linker stability, disulfide and thioether conjugates of BR64-DOX were evaluated in this study. The data clearly demonstrate the superiority of conjugates prepared with a thioether linker. BR64-S-DOX conjugates demonstrated significantly better extracellular stability *in vitro*, as evidenced by the kinetics of antigen-specific cytotoxicity of thioether relative to disulfide conjugates (Table 1). Furthermore, antigen-specific cytotoxicity was maintained for at least 24 h, whereas the antigen-specific cytotoxicity of the disulfide linked was lost within the first 4 h of *in vitro* incubation, because DOX was released extracellularly from both the BR64-SS-DOX and the nonbinding SN7-SS-DOX conjugates. Several types of immunoconjugates have been described that rely on an extracellular mechanism of drug release (13, 20). For these conjugates, extended stability, as demonstrated *in vitro*, is probably not a prerequisite, and it may in fact be a detriment for *in vivo* efficacy. In contrast, conjugates using acid-labile linkers, such as hydrazone (3, 14, 21, 27, 31–33) or *cis*-aconityl linkers (19, 34, 35), must have sufficient extracellular stability so that the majority of drug is released only after internalization of the conjugate into antigen-expressing cells. It is also unlikely that conjugates with poor *in vitro* stability will have sufficient metabolic stability to effect tumor localization and drug delivery prior to release of the drug. The data on *in vivo* stability of BR64-DOX disulfide and thioether conjugates support this concept (Fig. 1). The plasma terminal $T_{1/2}$ of bound DOX was 30 h for BR64-S-DOX and only 17 h for BR64-SS-DOX. The total systemic exposure to bound DOX, as assessed by plasma AUC values, was 4-fold higher for BR64-S-DOX than for BR64-SS-DOX conjugates (Table 2). These data indicate that BR64-S-DOX was more stable than the BR64-SS-DOX both *in vitro* and *in vivo*. The increased stability of the BR64-S-DOX conjugate resulted in a significant increase in the intratumoral

levels of biologically active, conjugate-released DOX relative to that achieved with BR64-SS-DOX conjugates (Table 2 and Fig. 2*B*).

The efficacy and potency of thioether and disulfide-linked conjugates were compared in both DOX-sensitive (L2987 lung) and DOX-insensitive (RCA colon) tumor xenografts. The BR64-S-DOX conjugates were both more active and more potent than optimized DOX against DOX-sensitive tumors (Fig. 3*B*). In contrast, the BR64-SS-DOX conjugates (Fig. 3*A*) demonstrated poor *in vivo* potency, and activity superior to that of optimized DOX was obtained only at doses approaching the MTD. The difference was even more striking against RCA colon tumor xenografts, which are insensitive to unconjugated DOX. Delivery of DOX via BR64 thioether-linked conjugates produced 78% responses with 67% cures at a dose of 8 mg/kg, whereas the disulfide conjugate, administered at a 4-fold higher dose was not active.

The increased efficacy and potency of BR64-S-DOX relative to BR64-SS-DOX reflects the greater plasma stability of BR64-S-DOX and the subsequent ability to successfully deliver higher concentrations of biologically active DOX to tumors. These results compare favorably with that of anticarcinoma BR96-DOX (thioether/hydrazone) conjugates described previously (3) as well as that of antimelanoma conjugates of a similar DOX:MAb molar ratio (36).

In summary, the results presented here clearly demonstrate the importance of using linkers with sufficient stability to deliver an anticancer drug such as DOX to solid tumors via internalizing MAbs. The use of targeted drug delivery was shown to result in improvements in both the efficacy and potency of DOX, and cures of established tumors were obtained in models where the relevant parent drug, DOX, was not active.

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