Antigen-specific Activity of Carcinoma-reactive BR64-Doxorubicin Conjugates Evaluated in Vitro and in Human Tumor Xenograft Models


ABSTRACT

The anticarcinoma antibody BR64 was conjugated to a doxorubicin derivative, doxorubicin 13-[3-(2-pyridyldithio)propionyl]hydrazone, and the resulting conjugates (BR64-DOX) were evaluated for activity and immunological specificity in vitro and in human tumor xenograft models. The BR64-DOX immunoconjugates retained immunoreactivity and cytotoxicity and demonstrated antigen-specific cytotoxicity in vitro. The potency of BR64-DOX immunoconjugates in vitro was related to the drug:monoclonal antibody mole ratio of the conjugates. The antitumor activity of BR64-DOX conjugates was consistently superior to the maximal activity obtained with the parent drug, doxorubicin (DOX), in established human lung and human breast carcinoma xenograft models. The superior antitumor activity of BR64-DOX conjugates was reflected both in tumor growth inhibition and in regressions and cures of established tumors following the administration of tolerated doses of BR64-DOX. The antitumor activity of BR64-DOX conjugates was not the result of synergism between monoclonal antibody BR64 and DOX, because mixtures consisting of monoclonal antibody and optimized DOX were not more active than an equivalent dose of DOX administered alone. The antitumor activity of BR64-DOX conjugates was antigen specific; equivalent doses of nonbinding isotype-matched conjugates were not active against established tumor xenografts.

INTRODUCTION

Successful treatment of solid tumors is a difficult therapeutic challenge. Although a variety of chemotherapeutic drugs have been shown to be active, the therapeutic index of these agents is, in general, low due to nonspecific toxic effects on normal tissues. One promising means of increasing the efficacy of chemotherapy is to utilize MAbs2 directed against tumor-associated antigens to selectively deliver cytotoxic agents to tumor sites. Site-directed delivery may increase the intratumor concentration of the cytotoxic agent while decreasing systemic toxicity. Recently, the concept of MAb-directed delivery has received significant attention and MAbs have been conjugated to a variety of toxic moieties, including chemotherapeutic agents (1–9), radionuclides (10–12), and plant (13–15) and bacterial (16–18) toxins.

The use of MAb-directed delivery may significantly reduce the toxic side effects of therapy. However, because the majority of MAbs available react to some degree with normal cells, it is critical to develop immunoconjugates in which normal tissue reactivity will not produce unacceptable levels of toxicity. For this reason immunoconjugates produced with clinically known chemotherapeutic drugs offer a significant safety advantage over more potent, but less well defined, agents such as the plant and bacterial toxins. The conventional chemotherapeutic drugs have established clinical profiles of antitumor activity and the dose-limiting toxicities of the drugs have been well characterized. In particular, anthracyclines such as DOX have been shown to have a broad spectrum of activity against solid tumors, and the toxic effects of the drug are dose related and predictable (19, 20). A variety of immunoconjugates have been produced in which anthracyclines are linked to MAbs using several different conjugation strategies (1, 3, 4, 9, 21–28).

In the present study, DOXHZZN (26, 27), which contains an acid-labile acyl hydrazone bond, was conjugated to an anticarcinoma MAb termed BR64. The BR64 MAb is a murine IgGl antibody which identifies a Lea-related tumor-associated antigen expressed on the surface of carcinoma cells of the breast, colon, lung, and ovary. The antibody is internalized following antigen-specific binding (29). This report describes the antigen-specific activity of BR64-DOX immunoconjugates in vitro and the antitumor activity of BR64-DOX compared to that of the optimized parent compound, DOX, in human carcinoma models.

MATERIALS AND METHODS

Monoclonal Antibodies. MAb BR64 (IgGl) identifies a Lea-related tumor-associated antigen expressed on carcinomas of the lung, colon, breast, and ovary (29). The SN7 hybridoma line was received from B. Seon (Roswell Park Memorial Institute, Buffalo, NY). The SN7 MAb identifies an antigen expressed on human B cells and was used in these studies as a nonbinding, isotype-matched, control antibody. Both the BR64 and SN7 MAbs were produced as tissue culture supernatants (Brumswick BioTecnetics, San Diego, CA).

Tumor Cell Lines. L2987 is a human lung adnocarcinoma line which expresses the BR64 antigen, and it was obtained from I. Hellstrom (Bristol-Myers Squibb, Seattle, WA). MCF7 is an estrogen-dependent human breast carcinoma line which expresses the BR64 antigen, and it was obtained from the American Type Culture Collection. HCT116 is a human colon carcinoma line, obtained from M. Brattain (Baylor College of Medicine, Houston, TX), which does not express the BR64 antigen.

Synthesis of MAb-DOX Immunoconjugates. DOXHZZN and DOX conjugates were prepared as described previously (26, 27). Briefly, immunoconjugates were prepared by linking DOXHZZN to MAbs which had been thiolyzed with n-succinimidyl 3-(pyridyldithio)propionate. The thiopyridyl protecting groups were removed from the MAb by reduction with excess dithiothreitol, and the free thiol-containing MAbs were condensed with DOXHZZN. Unconjugated drug was removed by dialysis and passage over SM-2 Biobeads (Bio-Rad Laboratories, Richmond, CA). Immunoconjugates were evaluated for free drug contamination by high performance liquid chromatography and for retention of MAb binding activity as described previously (30). Immunoconjugates used in these studies contained <5% free DOX and retained >90% of the original antibody binding activity.
conjugates are denoted by the conjugate mole ratio (moles drug/mole immunoglobulin).

**In Vitro Cytotoxicity Assays.** Monolayer cultures of the various human carcinoma cells were harvested using trypsin-EDTA (GIBCO, Grand Island, NY), and the cells were counted and resuspended to $1 \times 10^5$/ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum (RPMI-10% FCS). Cells (0.1 ml/well) were added to each well of 96-well microtiter plates and incubated overnight at 37°C in a humidified atmosphere of 5% CO$_2$. Medium was removed from the plates and serial dilutions of DOX or MAb-DOX conjugates were added to the wells. All dilutions were performed in quadruplicate. Cells were exposed to DOX or MAb-DOX conjugates for 2 h at 37°C in a humidified atmosphere of 5% CO$_2$. Plates were then centrifuged (200 x g, 5 min), the drug or conjugate was removed, and the cells were washed 3 times with RPMI-10% FCS. The cells were cultured in RPMI-10% FCS (37°C, 5% CO$_2$) for an additional 48 h. At this time the cells were pulsed for 2 h with 1.0 µCi/well of $[^3]$H]thymidine (New England Nuclear, Boston, MA). The cells were harvested onto glass fiber mats (Skatron Instruments, Inc., Sterling, VA) and dried, and filter-bound $[^3]$H]thymidine radioactivity was determined ($\beta$-Plate scintillation counter; Pharmacia LKB Biotechnology, Piscataway, NJ). Inhibition of $[^3]$H]thymidine uptake was determined by comparing the mean cpm for treated samples with the mean cpm for the untreated control.

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

**Human Tumor Xenograft Models.** The L2987 and MCF7 human tumor lines were established as tumor xenografts in athymic mice. All tumor lines were maintained by serial passage as described previously (31). The MCF7 tumor is an estrogen-dependent human breast tumor line. Athymic mice were implanted with 0.65-mg (65-day release rate) estradiol pellets (Innovative Research of America, Toledo, OH) 1 day prior to the implantation of MCF7 tumors. L2987 and MCF7 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 $l$.

In general, there were 8–10 mice in each control or treatment group. Data are presented as median tumor size for control or treated groups. Antitumor activity is expressed in terms of median TVDD values, 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$^3$). Partial tumor regression reflects a decrease in tumor volume to ≤50% of the initial tumor volume, complete tumor regression refers to a tumor which for a period of time is not palpable, and cure is defined as an absence of either 5 mm$^3$ in size. The MTD of DOX was dependent on the schedule and route of administration. With i.p. administration the MTD of DOX was 4 mg/kg given q4dx3 or 8 mg/kg given q7dx2. DOX administered i.p. at tolerated doses was not active against established L2987 tumors. The MTD was significantly higher when DOX was administered by the i.v. route. Maximal antitumor activity was observed when DOX was administered by the i.v. route either at a dose of 8 mg/kg on a q4dx3 schedule or at a dose of 10 mg/kg on a q7dx2 schedule. At these doses the antitumor activity was equivalent to 4.5 ± 0.7 and 4.6 ± 0.7 TVDDs, respectively. Regressions and cures of established tumors occurred in ≤5% of animals treated with tolerated doses of DOX.

**In Vivo Antitumor Activity of DOX against Established Tumor Xenografts.** The optimal route, dose, and schedule of DOX administration were determined for established L2987 and MCF7 tumor xenografts. The antitumor activity of DOX evaluated against established L2987 tumor xenografts is summarized in Table 1. Therapy was initiated approximately 14 days after tumor implantation, when the tumors were 50–100 mm$^3$ in size. The MTD of DOX was dependent on the schedule and route of administration. With i.p. administration the MTD of DOX was 4 mg/kg given q4dx3 or 8 mg/kg given q7dx2. DOX administered i.p. at tolerated doses was not active against established L2987 tumors. The MTD was significantly higher when DOX was administered by the i.v. route. Maximal antitumor activity was observed when DOX was administered by the i.v. route either at a dose of 8 mg/kg on a q4dx3 schedule or at a dose of 10 mg/kg on a q7dx2 schedule. At these doses the antitumor activity was equivalent to 4.5 ± 0.7 and 4.6 ± 0.7 TVDDs, respectively. Regressions and cures of established tumors occurred in ≤5% of animals treated with tolerated doses of DOX.

**RESULTS**

Antigen-specific Cytotoxicity of BR64-DOX Conjugates *in Vitro*. Several human carcinoma lines, representing various histological types, express the BR64 antigen (29). Among these, the L2987 lung and MCF7 breast carcinoma lines were selected for use in these studies. As shown in Fig. 1, A and B, BR64-DOX conjugates demonstrated antigen-specific cytotoxicity *in vitro* when evaluated against the L2987 and MCF7 human carcinoma lines, respectively. The BR64-DOX conjugate was approximately 15-fold more potent than the SN7-DOX conjugate (IC$_{50}$ values of 2.0 and 30 µM DOX, respectively) against the L2987 line. Antigen-specific cytotoxicity was also demonstrated by blocking of the activity of the BR64-DOX conjugate with the addition of unconjugated BR64 antibody. As shown in Fig. 1A, the potency of the BR64-DOX conjugate (IC$_{50}$ of 2 µM) was significantly reduced in the presence of MAb BR64 (IC$_{50}$ of 20 µM) but was not affected (IC$_{50}$ of 2 µM) by the addition of an isotype-matched nonbinding antibody.

The BR64-DOX conjugate was approximately 25 times more potent than the SN7-DOX conjugate (IC$_{50}$ values of 0.2 and 5 µM DOX, respectively) against the MCF7 breast carcinoma line (Fig. 1B). The BR64-DOX conjugate was not active (IC$_{50}$ of >30 µM) against the HCT116 carcinoma cell line (Fig. 1C). This cell line is sensitive to unconjugated DOX (IC$_{50}$ of 0.04 µM) but does not express the BR64 antigen.

To investigate the relationship between the drug to MAb and *in vitro* potency of BR64-DOX conjugates, a variety of conjugates with conjugate ratios ranging from 1 to 8 were prepared. These immunoconjugates were evaluated *in vitro* against the L2987 lung carcinoma line. The potency of the immunoconjugates varied over a 33-fold range (IC$_{50}$ values of 1–33 µM DOX). As shown in Fig. 2, conjugates of higher mole ratio were significantly ($P < 0.05$) more potent *in vitro* than those conjugates prepared at lower mole ratios.

**In Vivo Antitumor Activity of DOX against Established Tumor Xenografts.** The optimal route, dose, and schedule of DOX administration were determined for established L2987 and MCF7 tumor xenografts. The antitumor activity of DOX evaluated against established L2987 tumor xenografts is summarized in Table 1. Therapy was initiated approximately 14 days after tumor implantation, when the tumors were 50–100 mm$^3$ in size. The MTD of DOX was dependent on the schedule and route of administration. With i.p. administration the MTD of DOX was 4 mg/kg given q4dx3 or 8 mg/kg given q7dx2. DOX administered i.p. at tolerated doses was not active against established L2987 tumors. The MTD was significantly higher when DOX was administered by the i.v. route. Maximal antitumor activity was observed when DOX was administered by the i.v. route either at a dose of 8 mg/kg on a q4dx3 schedule or at a dose of 10 mg/kg on a q7dx2 schedule. At these doses the antitumor activity was equivalent to 4.5 ± 0.7 and 4.6 ± 0.7 TVDDs, respectively. Regressions and cures of established tumors occurred in ≤5% of animals treated with tolerated doses of DOX. The antitumor activity of DOX tested against established MCF7 tumors is presented in Table 2. Maximal antitumor activity was observed when DOX was administered i.v. at a dose of either 6 mg/kg on a q4dx3 schedule or 8 mg/kg on a q7dx2 schedule. At these doses the antitumor activity was equivalent to 3.0 ± 0.4 and 2.9 ± 0.6 TVDDs, respectively. Regressions and cures of established tumors were not observed. The MTD
**Fig. 1.** Cytotoxicity of BR64-DOX conjugates against L2987 lung (A), MCF7 breast (B), and HCT116 colon (C) carcinoma lines. Cells were exposed to DOX or DOX conjugates for 2 h at 37°C, washed, and cultured for an additional 48 h prior to the addition of [3H]thymidine. Data are presented as percentage of inhibition (mean ± SD) of [3H]thymidine uptake, relative to control cells. A, L2987 cells treated with DOX (△), BR64-DOX (mole ratio = 6.74) (●), or nonbinding SN7-DOX (mole ratio = 5.52) (○). The effect of adding unconjugated MAb BR64 (●) or a nonbinding isotype-matched MAb (○) to BR64-DOX conjugates is also shown. B, MCF7 cells treated with DOX (△), BR64-DOX (mole ratio = 4.88) (●), or nonbinding SN7-DOX (mole ratio = 3.89) (○). C, HCT116 cells treated with DOX (△) or BR64-DOX (mole ratio = 6.74) (●).

**Fig. 2.** Relationship between conjugate mole ratio and in vitro potency of BR64-DOX conjugates. Conjugates were prepared with mole ratios of 1–8 and were tested against L2987 lung carcinoma cells. Cells were exposed to BR64-DOX conjugates for 2 h at 37°C, as described in “Materials and Methods.” Data are presented as the IC50 value (mean ± SD) of BR64-DOX conjugates.

Antitumor Activity of BR64-DOX Immunoconjugates Evaluated against Established Human Tumor Xenografts. The antitumor activity of BR64-DOX conjugates was evaluated against established (50–100-mm3) L2987 human lung tumor xenografts and compared to that of optimized DOX and equivalent doses of nonbinding immunoconjugates. Mice were treated i.v. with optimized DOX or i.p. with BR64-DOX. Data presented in Fig. 3A compare the antitumor activity of DOX administered at the MTD on a q4dx3 schedule with that of a BR64-DOX conjugate (mole ratio = 5.37) administered i.p. on the same schedule. DOX (8 mg/kg) produced a significant delay in the growth of established L2987 tumors. The antitumor activity of DOX in this experiment was equivalent to 5.0 TVDDs, and tumor regressions were not observed. The BR64-DOX conjugate tested at the MTD (35 mg/kg equivalent DOX) was significantly (P < 0.01) more active than optimized DOX and produced activity equivalent to 11.9 TVDDs. At this dose 63% partial regressions and 13% cures were observed. Administration of the BR64-DOX conjugate at a lower dose (25 mg/kg equivalent DOX) resulted in antitumor activity equivalent to 10.6 TVDDs. At this dose 33% partial regressions and 17% cures of established tumors were observed. These animals are long term cures and the mice have remained tumor free for >1 year.

The antitumor activity of BR64-DOX conjugates was also compared to that of nonbinding (SN7-DOX) conjugates administered at equivalent doses and with the same schedule and...

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**Notes:**
- DOX is generally administered at 8 mg/kg i.v. or 8 mg/kg i.p. on a q4dx3 schedule.
- TVDDs refer to the total number of days the tumor volume is reduced.
- MTD: Maximum Tolerated Dose.
- Estrogen supplementation required for in vivo growth of the estrogen-dependent MCF7 tumor.
ANTITUMOR ACTIVITY OF BR64-DOX CONJUGATES

Table 1  Antitumor activity of DOX against established L2987 human lung carcinoma xenografts  

<table>
<thead>
<tr>
<th>Schedule, route</th>
<th>Dose (mg/kg)</th>
<th>TVDD</th>
<th>Partial</th>
<th>Complete</th>
<th>Cure</th>
<th>Lethality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>q4dx3, i.v.</td>
<td>10</td>
<td>Toxic</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>47 (19)*</td>
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<tr>
<td></td>
<td>8</td>
<td>4.5 ± 0.7</td>
<td>0.7</td>
<td>0</td>
<td>0</td>
<td>2 (55)*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.6 ± 0.3</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0 (46)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.3 ± 0.7</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0 (20)</td>
</tr>
<tr>
<td>q7dx2, i.v.</td>
<td>12</td>
<td>Toxic</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>33 (63)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.6 ± 0.7</td>
<td></td>
<td>0</td>
<td>1.1</td>
<td>2 (94)*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.0 ± 0.3</td>
<td></td>
<td>0</td>
<td>2.1</td>
<td>0 (48)</td>
</tr>
<tr>
<td>Single injection, i.v.</td>
<td>16</td>
<td>Toxic</td>
<td>100 (10)</td>
<td>0</td>
<td>0</td>
<td>32 (19)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Toxic</td>
<td>33 (9)</td>
<td>0</td>
<td>0</td>
<td>22 (9)</td>
</tr>
<tr>
<td></td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>q4dx3, i.p.</td>
<td>6</td>
<td>Toxic</td>
<td>3.2</td>
<td>0</td>
<td>0</td>
<td>0 (9)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (8)</td>
</tr>
<tr>
<td>q7dx2, i.p.</td>
<td>8</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>0</td>
<td>0</td>
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</tbody>
</table>

* Values in parentheses, numbers of mice.  

Table 2  Antitumor activity of DOX against established MCF7 human breast carcinoma xenografts  

<table>
<thead>
<tr>
<th>Schedule, route</th>
<th>Dose (mg/kg)</th>
<th>TVDD</th>
<th>Partial</th>
<th>Complete</th>
<th>Cure</th>
<th>Lethality (%)</th>
</tr>
</thead>
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<tr>
<td>q4dx3, i.v.</td>
<td>8</td>
<td>Toxic</td>
<td>57.5 (40)*</td>
<td>0</td>
<td>0</td>
<td>7.5 (40)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.0 ± 0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 (10)</td>
</tr>
<tr>
<td>q7dx2, i.v.</td>
<td>12</td>
<td>Toxic</td>
<td>50.0 (26)</td>
<td>0</td>
<td>0</td>
<td>43.5 (44)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Toxic</td>
<td>17.8 (45)</td>
<td>0</td>
<td>0</td>
<td>17.8 (45)</td>
</tr>
</tbody>
</table>

* Values in parentheses, numbers of mice.  

route. Representative data are presented in Fig. 3B. The BR64DOX conjugate (mole ratio = 3.89) administered i.p. at a dose of 15 mg/kg equivalent DOX on a q2dx5 schedule demonstrated antitumor activity which was equivalent to 11.5 TVDDs, and this treatment produced 38% partial tumor regressions. The SN7-DOX conjugate (mole ratio = 5.88), administered at a matching dose and schedule, was not active against L2987 xenografts.

The antitumor activity of BR64-DOX conjugates was compared to that of unconjugated MAb BR64 and mixtures consisting of MAb BR64 and DOX. In these experiments the mixtures were administered either i.p. or i.v. and the DOX dose of the mixtures was equivalent to the MTD for the particular route of administration. As shown in Fig. 4, unconjugated MAb BR64 administered i.p. at a dose of 1000 mg/kg or i.v. at a dose of 500 mg/kg on a q4dx3 schedule did not inhibit the growth of established L2987 tumors. Data presented in Table 1 demonstrate that DOX administered at the MTD by the i.p. route was not active against established L2987 tumors. The antitumor activity of BR64-DOX conjugates was significantly better than that of optimized DOX, equivalent doses of nonbinding conjugates, and mixtures consisting of MAb BR64 and DOX.

The antitumor activity of BR64-DOX conjugates was compared to that of optimized DOX in the MCF7 breast carcinoma model. Therapy was initiated 12–14 days after tumor implantation, when the tumors were 75–125 mm³ in size. Mice were treated i.v. with optimized DOX (6 mg/kg, q4dx3) or i.p. with BR64-DOX immunoconjugates (15 mg/kg equivalent DOX; mole ratio = 3.89). As shown in Fig. 5, the antitumor activity of the BR64-DOX conjugate (5.9 TVDDs) administered at a dose of 10 mg/kg, q2dx4, was superior to that observed for optimized DOX (2.5 TVDDs).

The antitumor activity of several preparations of BR64-DOX and SN7-DOX conjugates is summarized in Table 3. The antitumor activity of BR64-DOX conjugates prepared with mole ratios of 3.89–7.85 was consistently superior to that of optimized DOX (Table 1) or equivalent doses of nonbinding SN7-DOX conjugates. The MTD for the conjugates was 30–35 mg/kg when administered on a q4dx3 schedule and 15–20 mg/kg when administered on a q2dx5 schedule. This is equivalent to a cumulative exposure of approximately 100 mg/kg equivalent DOX. The MTD of the conjugates did not vary as a function of the mole ratio of the conjugate. The antitumor activity observed at optimal doses was not significantly different whether the conjugate was administered on a q2dx5 (7–13 TVDDs) or q4dx3 (9–12 TVDDs) schedule. Although the mole ratio of the conjugates was correlated with in vitro potency (Fig. 2), a similar relationship was not observed in vivo for conjugates prepared with mole ratios in the range of 3–8. For example, the

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Fig. 3. Antitumor activity of DOX and DOX conjugates evaluated against established L2987 human lung tumor xenografts. A, dose-dependent antitumor activity of BR64-DOX conjugates (mole ratio = 5.37), compared to that of optimized DOX. Results are from control animals (A) or animals treated with DOX at 8 mg/kg i.v. (B) or BR64-DOX conjugates at 35 mg/kg i.p. (C) or 25 mg/kg i.p. (D) 14, 18, and 22 days after tumor implantation. B, antigen-specific antitumor activity of BR64-DOX conjugates. The antitumor activity of BR64-DOX conjugates was compared to that of a nonbinding SN7-DOX conjugate. Results are from control animals (A) or animals treated i.p. with 15 mg/kg BR64-DOX (mole ratio = 5.14) (B) or SN7-DOX (mole ratio = 5.88) (C) 22, 24, 26, 28, and 30 days after tumor implantation.

antitumor activity of a BR64-DOX conjugate with a mole ratio of 3.89 (administered q2dx5) was similar to that of a BR64-DOX conjugate with a mole ratio of 7.85 administered on the same schedule.

DISCUSSION

These experiments have described the antigen-specific activity of BR64-DOX conjugates when evaluated in vitro and in human tumor xenograft models. MAb BR64 identifies a Le^ related antigen expressed in high density on carcinomas of several histological types. In addition, the MAb has been shown to be internalized following antigen-specific binding (29). Because of this characteristic of the antibody, immunoconjugates were produced using an acid-labile hydrazone bond (26, 27). With this linker chemistry, internalization of the immunoconjugate into an acidic environment, such as that of lysosomes or endosomes, will result in the release of DOX (26, 33).

The ability to deliver a lethal concentration of drug to cells is dependent upon the density of antigen expressed (i.e., the number of accessible sites for immunoconjugate binding) and the quantity of drug delivered per antibody molecule. Therefore, conjugates prepared with a higher drug:MAb mole ratio should be more potent because more drug is delivered per antibody molecule bound to target cells. An obvious upper limit to increasing potency by increasing the drug:MAb mole ratio is the reduction in immunoreactivity typically observed with high drug loading (5, 34). In the present study immunoconjugates were prepared with drug:MAb mole ratios in the range of 1–8,

Fig. 4. Antitumor activity of MAb BR64, DOX, and mixtures of MAb BR64 and DOX evaluated against established L2987 human tumor xenografts. Therapy was administered i.v. (A) or i.p. (B) on days 15, 19, and 23 after tumor implantation. A, treatment administered i.v. A, control; B, DOX, 8 mg/kg; C, MAb BR64, 500 mg/kg; D, a mixture consisting of 8 mg/kg DOX and 500 mg/kg MAb BR64. B, treatment administered i.p. A, control; B, DOX, 4 mg/kg; C, MAb BR64, 1000 mg/kg; D, a mixture consisting of 4 mg/kg DOX and 1000 mg/kg MAb BR64.

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ANTITUMOR ACTIVITY OF BR64-DOX CONJUGATES

and >90% of immunoreactivity was retained following drug conjugation. As shown above, immunoconjugates produced with higher mole ratios were significantly more potent in vitro than those prepared with lower mole ratios. This relationship was most dramatic for conjugates in which mole ratios were <3; these conjugates were approximately 10-fold less potent than conjugates prepared with mole ratios of ≥3. It is possible that the reduced potency of the low mole ratio immunoconjugates may reflect both the lower drug loading and the presence of unconjugated BR64; the unconjugated BR64 could have competed with BR64-DOX for binding sites on the target cells and thereby reduced the effective potency of the conjugates. Based upon these in vitro data, immunoconjugates with mole ratios of >3 were selected for further studies.

Imunoconjugates have been constructed using chemotherapeutic agents such as methotrexate (5, 34), Vinca derivatives (7, 35–37), chlorambucil (8), and a variety of anthracyclines including DOX (4, 22, 28, 30), daunomycin (1, 3), and morpholinodoxorubicin (9). In general, the immunoconjugates have been active in vitro and in vivo. However, antitumor activity was typically evaluated against newly implanted tumors and the activity was compared to that of equivalent rather than optimal doses of the parent compound. The antitumor activity of BR64-DOX conjugates described in this report is comparable or superior to that which has been demonstrated for other immunoconjugates. In particular, the activity of BR64-DOX conjugates was evaluated under stringent conditions requiring antigen-specific activity against well established, progressively growing, carcinoma xenografts. The antitumor activity of BR64-DOX was compared to the maximal activity which could be achieved with DOX, the clinically relevant parent compound. The antitumor activity of BR64-DOX conjugates was clearly superior to that achieved with optimized DOX in both the L2987 human lung and MCF7 human breast carcinoma models. The superior efficacy of BR64-DOX conjugates was evident in terms of both a prolonged delay in tumor growth and an increase in tumor regressions and cures. Complete tumor regressions and cures were observed following treatment with BR64-DOX but were not observed following therapy with optimized DOX. The antitumor activity of the BR64-DOX conjugates was shown to be dose dependent and may be further improved by evaluating the optimal route and schedule for conjugate administration. Although the conjugate mole ratio was correlated with in vitro potency, a similar relationship was not observed in vivo for conjugates prepared with mole ratios in the range of 3–8.

Experiments were performed to ensure that the superior activity of the BR64-DOX conjugate was not due to a carrier effect in which the conjugate served as a depot for the release of DOX. Nonbinding immunoconjugates were prepared and administered using the same dose, route, and schedule as those for the binding BR64-DOX conjugates. The BR64-DOX conjugates consistently demonstrated excellent antitumor activity, whereas the nonbinding conjugates were not active against established L2987 tumor xenografts. These data indicate that the superior activity of the BR64-DOX conjugates is a result of antigen-specific binding and is not the result of a pharmacokinetic change due to slow release of free DOX from the antibody.

Table 3  Antitumor activity of BR64-DOX and nonbinding SN7-DOX conjugates against established L2987 human lung carcinoma xenografts

<table>
<thead>
<tr>
<th>Conjugate, MR</th>
<th>Optimal dose (mg/kg) Conjugate</th>
<th>Schedule</th>
<th>TVDD</th>
<th>Partial</th>
<th>Complete</th>
<th>Cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR64-DOX, 7.85</td>
<td>DOX 20, MAb 718</td>
<td>q2dx5</td>
<td>11.6</td>
<td>60</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>BR64-DOX, 5.37</td>
<td>DOX 15, MAb 787</td>
<td>q2dx5</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BR64-DOX, 5.14</td>
<td>DOX 15, MAb 768</td>
<td>q2dx5</td>
<td>11.5</td>
<td>38</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BR64-DOX, 5.1</td>
<td>DOX 20, MAb 1105</td>
<td>q2dx5</td>
<td>8.6</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>BR64-DOX, 3.89</td>
<td>DOX 20, MAb 1448</td>
<td>q2dx5</td>
<td>12.9</td>
<td>60</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 6.35</td>
<td>DOX 20, MAb 887</td>
<td>q2dx5</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 5.88</td>
<td>DOX 15, MAb 648</td>
<td>q2dx5</td>
<td>2.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 4.82</td>
<td>DOX 15, MAb 877</td>
<td>q2dx5</td>
<td>1.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 2.57</td>
<td>DOX 20, MAb 1645</td>
<td>q2dx5</td>
<td>3.1</td>
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<td>0</td>
</tr>
<tr>
<td>BR64-DOX, 7.85</td>
<td>DOX 30, MAb 1077</td>
<td>q4dx3</td>
<td>8.9</td>
<td>38</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>BR64-DOX, 7.5</td>
<td>DOX 20, MAb 35</td>
<td>q4dx3</td>
<td>10.3</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BR64-DOX, 5.37</td>
<td>DOX 35, MAb 1836</td>
<td>q4dx3</td>
<td>11.9</td>
<td>63</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>BR64-DOX, 4.88</td>
<td>DOX 30, MAb 1732</td>
<td>q4dx3</td>
<td>9.2</td>
<td>50</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 4.82</td>
<td>DOX 25, MAb 1462</td>
<td>q4dx3</td>
<td>3.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 4.82</td>
<td>DOX 20, MAb 1449</td>
<td>q4dx3</td>
<td>2.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SN7-DOX, 3.78</td>
<td>DOX 30, MAb 2237</td>
<td>q4dx3</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* MR, mole ratio.

* All therapy was administered by the i.p. route.
Because unconjugated MAbs have been shown to be active against human tumor xenografts, it was possible that immunoconjugate activity may have been mediated, at least in part, by effector activities of the MAb (22, 31, 38, 39). Also, in several studies un-conjugated mixtures of antibody and drug have been shown to give greater antitumor activity than does a matched dose of drug administered alone (22). In the present study, the murine IgG1 MAb BR64 was not active against established human tumor xenografts. To address the potential synergistic activity of DOX and antibody, mixtures of BR64 antibody and DOX were administered by the i.p. or i.v. route at the drug MTD. The activity of mixtures of BR64 antibody and DOX was not different from that observed for DOX alone. Therefore, the superior activity of BR64-DOX conjugates reflects the efficacy of the conjugate itself and is not a function of synergistic activity of MAB BR64 and DOX.

To achieve antitumor activity superior to that of DOX it was necessary to administer a cumulative dose of approximately 60–90 mg/kg equivalent DOX. It is not clear at this time whether the high doses of BR64-DOX conjugate required reflect low potency of the conjugate or whether a threshold dose of MAB must be administered to achieve sufficient accumulation of the BR64-DOX conjugate in the solid tumor xenografts. The conjugates used in these studies were prepared using the coupling agent N-succinimidyl 3-(pyridydithioli)propionate, which generates a disulfide linkage. Previously, it has been shown that disulfide-linked ricin A chain conjugates are unstable in vivo and that the use of other linkages, such as a hindered disulfide, increases the stability of the conjugates (40, 41). This increase in stability likely reflects the reduced susceptibility of the hindered disulfide to reduction mechanisms, such as that involving glutathione present in liver and plasma. The low potency of the BR64-DOX conjugates may result from the instability of the disulfide linker, and the use of more stable linkers may significantly improve both the potency and the antitumor activity of BR64-DOX conjugates.

In summary, the studies reported here demonstrate that BR64-DOX conjugates mediate antigen-specific cytotoxicity in vitro and are highly effective in inhibiting the growth of established antigen-expressing human tumor xenografts. The antitumor activity of BR64-DOX conjugates is clearly superior to that of matching doses of non-binding conjugate and to optimal doses of the relevant clinical compound, DOX.

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Antigen-specific Activity of Carcinoma-reactive BR64-Doxorubicin Conjugates Evaluated in Vitro and in Human Tumor Xenograft Models

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