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Antitumor Activity of Carcinoma-reactive BR96-Doxorubicin Conjugate against Human Carcinomas in Athymic Mice and Rats and Syngeneic Rat Carcinomas in Immunocompetent Rats[1]

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ABSTRACT

The internalizing monoclonal antibody BR96 was conjugated to the anticancer drug doxorubicin (DOX) using an acid-labile hydrazone bond to DOX and a thioether bond to the monoclonal antibody. The resulting conjugate, termed BR96-DOX, binds to a tumor-associated Lewis ^{y} antigen that is abundantly expressed on the surface of human carcinoma cells. BR96-DOX binds to RCA, a human colon carcinoma cell line, and BN7005, a transplantable colon carcinoma induced in a Brown Norway (BN) rat by 1,2-dimethyl-hydrazine. BR96-DOX produces cures of established s.c. RCA human colon carcinomas in athymic mice and rats. BR96-DOX also cured both s.c. and intrahepatic BN7005 tumors in immunocompetent BN rats. Unconjugated DOX, given at its maximum tolerated dose, and matching doses of nonbinding IgG-DOX conjugate were not active against RCA or BN7005 carcinomas. An anticonjugate antibody response was produced in BN rats treated with BR96-DOX. However, this could be largely prevented by administering the immunosuppressive drug deoxyspergualin. These results confirm the concept of antibody-directed therapy in models in which the targeted antigen is expressed both in normal tissues and tumors. The findings in BN7005 further demonstrate efficacy of BR96-DOX therapy in a model in which the tumor is syngeneic and the host is immunocompetent.

INTRODUCTION

MAbs[3] to tumor-associated antigens have been used with variable success in the treatment of cancer. With the exception of treatment of minimal residual disease colon carcinoma (1), unmodified MAbs have shown little antitumor activity. MAbs have also been used to deliver a variety of toxic moieties to malignant cells (2–4). Antigen-specific activity has been demonstrated *in vitro* and in nude mouse models with several such conjugates. However, to date, the clinical efficacy of MAb-mediated delivery has been demonstrated definitively only for radiolabeled MAb conjugates used in the therapy of B cell lymphoma (5, 6).

Several factors contribute to the lack of clinical predictability of the models commonly used to evaluate MAb conjugates. Typically, treatment starts shortly after xenografting of human tumors to congenitally athymic (nude) mice when the tumor burden is very small, and conclusions about efficacy are commonly based on a delay in tumor outgrowth rather than on regression or cure of established tumors. The tumors used are usually sensitive to the free drug administered by an optimized route and schedule, although activity of the drug may not be observed at "conjugate equivalent" doses. The majority of MAbs identified to date and, therefore, conjugates of these MAbs are tumor selective rather than tumor specific in patients because binding to cells of some normal tissues is typically observed. However, the preclinical models typically used do not address the issue of tumor-selective targeting because rodents do not, for the most part, express the target antigen in normal tissues. Furthermore, patients in Phase I/II clinical trials usually have bulky tumors, into which immunoconjugates penetrate poorly (7), and the tumors are frequently resistant to chemotherapeutic drugs as a consequence of extensive prior therapy. In addition, whereas an antibody response to the conjugate is common in patients, it cannot occur in athymic rodents.

MAb BR96 recognizes a Le^{y}-related epitope that is expressed (>100,000 molecules/cell) at the surface of cells from the majority of human cancers of breast, colon, and lung (8, 9). The conjugate, termed BR96-DOX, was prepared using a chimeric (mouse-human) form of murine BR96 (10) and the DOX derivative 6-maleimidocaproyl DOX hydrazone so that it contained both an acid labile bond and a thioether linker (11, 12). Upon binding to the tumor cell surface, BR96-DOX internalizes into the acidic environment of lysosomes/endosomes and liberates DOX. The BR96-DOX conjugate was shown to produce cures of antigen-expressing tumors, to be at least 8-fold as potent as the unconjugated parent drug DOX, and to be active at a dose equivalent to 5% of its MTD in athymic mice (11).

The antigen to which BR96 binds is not expressed in mice. It is, however, detectable in the gastrointestinal epithelium of humans, dogs, and rats. It was encouraging, therefore, that cures were obtained also in nude rats xenotransplanted with DOX-sensitive human lung tumors (11). Although athymic rats provide an appropriate model for conjugate efficacy and toxicity, they lack the ability to produce an anticonjugate response. Athymic animals may also mount a limited host response to xenotransplanted tumors, which may facilitate regression after an initial damage inflicted by the immunoconjugate (13). Therefore, a model of a syngeneic tumor in immunocompetent animals that expresses the target antigen in normal tissues would reflect the clinical situation more closely.

This study was designed to address several of these issues. The efficacy of BR96-DOX was investigated against several types of colon tumor models. These were s.c. implanted RCA human colon tumor xenografts studied in athymic mice and rats and a rat colon carcinoma, BN7005, which was evaluated as a s.c. metastasis model and an experimental liver metastasis model in syngeneic, immunocompetent rats. In the rat models, the BR96 MAb was tumor selective rather than tumor specific. The effect of an anticonjugate response on the efficacy of BR96-DOX against BN7005 carcinomas was also evaluated.

MATERIALS AND METHODS

MAbs and Immunoconjugates

MAb BR96 has been described previously (8, 9). Human IgG (Rockland Inc., Gilbertsville, PA) was used to produce nonbinding control immunoconjugates. Immunoconjugates consisting of BR96 (BR96-DOX) or human IgG (IgG-DOX) conjugated to DOX were prepared as described previously (12).

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[3] The abbreviations used are: MAb, monoclonal antibody; DOX, doxorubicin; Le^{y}, Lewis ^{y}; MTD, maximum tolerated dose; TVDD, tumor volume doubling delay; PR, partial tumor regression; CR, complete tumor regression; DSG, 15-deoxyspergualin; BN, Brown Norway.

Assay for Rat Antibodies against BR96-DOX

To evaluate rat anticonjugate antibody responses, sera of blood samples drawn from treated rats were assayed in an ELISA measuring specific binding of rat serum antibodies to chimeric BR96-DOX conjugate expressed as $A_{450/630\ nm}$ at a dilution of 1:100.

Carcinoma Lines

RCA is a human adenocarcinoma of the colon obtained from M. Brattain (Medical College of Ohio, Toledo, OH). BN7005 is a rat adenocarcinoma of the colon, induced by 1,2-dimethylhydrazine in a BN rat and cloned by limiting dilution in the absence of selection pressure.

In Vitro Cytotoxicity Assays

Antigen-specific cytotoxicity was evaluated as described previously (14). Briefly, monolayer cultures of colon carcinoma cells were harvested using trypsin-EDTA (Life Technologies, Inc., Grand Island, NY), and the cells were 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 BR96-DOX, IgG-DOX, or DOX were added to each of the wells. Quadruplicate samples were assayed. The cells were exposed to the drug or conjugates for 2 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 later, the cells were pulsed for 2 h with 1.0 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston MA). The media were removed, and trypsin 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 LKB Biotechnology, 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

Immunocompetent inbred male rats, 3–5 months old, of the BN strain were obtained from a closed colony maintained at the Wallenberg Laboratory. Athymic female mice of BALB/c background and athymic (Rowett) female rats were obtained from Harlan Sprague Dawley. Animals received food and water *ad libitum*.

Tumor Models

s.c. tumors were measured in two perpendicular directions at weekly or biweekly intervals using calipers. Tumor size was defined as follows: $l \times w^2/2$, where l = measurement of longest axis (mm) and w = measurement of axis perpendicular to l (mm). Data are presented as median tumor size. Antitumor activity is expressed in terms of TVDDs as described previously (15). 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 tumor volume doubling time; 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.

Human Carcinoma Line RCA

Studies in athymic mice used s.c. tumors maintained by *in vivo* passage as described previously (15). For athymic rat studies, RCA cells were harvested in logarithmic growth using trypsin-EDTA and washed in serum-free medium, and 5×10^6 cells were injected s.c. in the left axillary region.

Rat Carcinoma Line BN7005

s.c. BN7005 Tumors. Tumors were excised and minced in PBS to obtain a single cell suspension, which was inoculated s.c. in the thigh of the right hind leg.

Intrahepatic BN7005 Tumors. Human colon carcinomas commonly metastasize to the liver, whereas those of rats do not. To evaluate whether a therapeutic response could be achieved against BN7005 growing in the liver, a model was developed in which approximately 10^6 BN7005 cells were implanted under the capsule of two liver lobes. In untreated animals, tumor nodules were visible at laparotomy 7 days after grafting and enlarged rapidly, spreading into the peritoneal cavity and mesenteric lymph nodes yielding ascites and killing recipients within 3–4 weeks. Tumor growth was determined at laparotomy by liver inspections and measurements of perpendicular diameters of intrahepatic tumors, which were detected by a distinct, light color contrasting to the normal liver tissue.

Therapy

Tumors were staged to various sizes prior to therapy. Athymic mice and rats received all therapy i.v., whereas BN rats received DOX i.v. and BR96-DOX and IgG-DOX i.p. Treatments were performed on an individual body weight basis; doses were presented as both mg/m^2 of MAb and equivalent DOX (16). Conjugates typically contained 1 mg of DOX per 35 mg of MAb. To clarify whether the anticipated generation of an anticonjugate response toward BR96-DOX might reduce the efficacy of BR96-DOX therapy, experiments were performed in which BN rats were treated concurrently with DSG, a drug shown previously to suppress the appearance of an antibody response to analogous conjugates in mice (17, 18). DSG was administered i.p. in 11 daily doses of 30 mg/m^2 beginning one day after the first day of BR96-DOX therapy. In one experiment, a second round of BR96-DOX therapy, with or without DSG, was given when tumors had regrown following BR96-DOX-induced PR.

RESULTS

Antigen-specific Cytotoxicity of BR96-DOX Conjugates *in Vitro*. The *in vitro* potency and specificity of BR96-DOX was evaluated against RCA and BN7005 carcinoma cells following a 2-h drug or conjugate exposure. As shown in Table 1, BR96-DOX demonstrated antigen-specific cytotoxicity against both the RCA and BN7005 colon carcinoma lines. The BR96-DOX conjugate was more potent than a nonbinding IgG-DOX conjugate prepared using the same linker chemistry. The BR96-DOX was approximately 10-fold less potent than unconjugated DOX following a 2-h exposure *in vitro*.

Activity of BR96-DOX against s.c. RCA Human Colon Tumors in Athymic Mice and Rats. Previous studies have reported that RCA colon tumors in athymic mice were cured by BR96-DOX, although the xenografts were insensitive to unconjugated DOX (11). Antitumor activity of BR96-DOX was shown to be antigen specific because a nonbinding IgG-DOX conjugate was not active. In the study presented here, the activity of BR96-DOX against established s.c. RCA colon tumors was evaluated in both athymic mice and rats. In each case, unconjugated DOX was administered at its MTD; 24 and 12 mg/m^2 in mice and rats, respectively. BR96-DOX was administered to both mice and rats at a dose of 420 mg/m^2 BR96-DOX (12 mg/m^2 equivalent DOX). In athymic mice (Fig. 1), the BR96-DOX conjugate produced cures of established RCA xenografts, whereas unconjugated

Table 1 *Antigen-specific activity of BR96-DOX against RCA human and BN7005 rat colon carcinoma cells in vitro*

	IC_{50} (μM DOX)			
	BR96-DOX	IgG-DOX	DOX	Specificity ratio[a]
RCA	2.0	20	0.2	10
BN7005	7.0	>30	0.7	>4.3

[a] IC_{50} of IgG-DOX:IC_{50} of BR96-DOX.

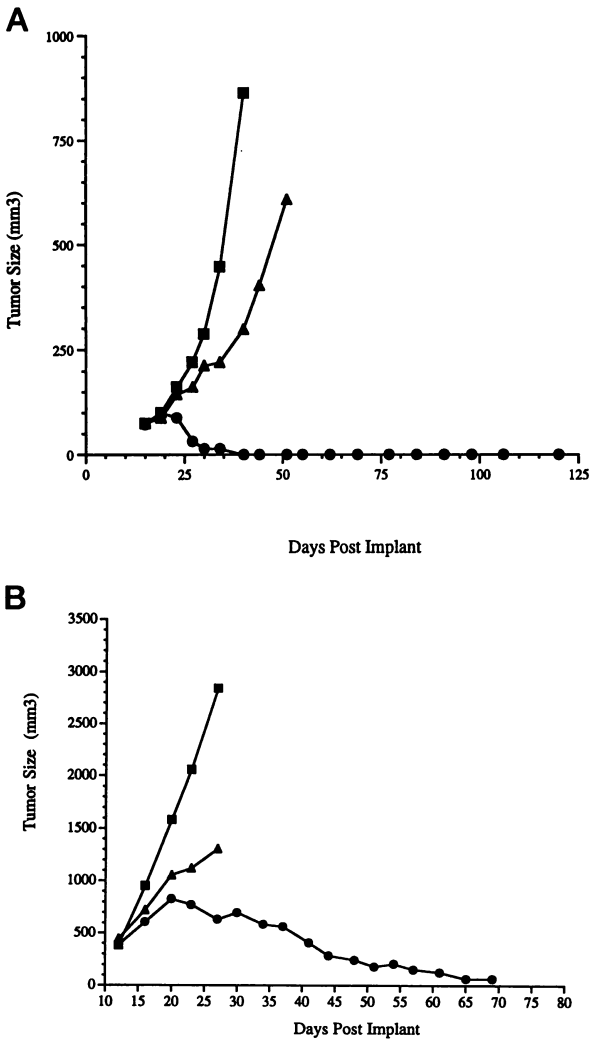

Fig. 1. Antitumor activity of BR96-DOX against s.c. RCA human colon tumor xenografts. *A*, athymic mice. Results are from untreated control mice (■) and mice treated with 420 mg/m^2 (12 mg/m^2 equivalent DOX) BR96-DOX (●) or 24 mg/m^2 of DOX (▲) on days 15, 19, and 23. *B*, athymic rats. Results are from untreated control rats (■) and rats treated with 420 mg/m^2 (mg/m^2 equivalent DOX) BR96-DOX (●) or 12 mg/m^2 of DOX (▲) on days 12, 16, and 20.

DOX administered at twice that dose was not active (<3.3 TVDD). In the RCA colon tumor model in athymic mice, BR96-DOX was more active and more potent than unconjugated DOX. The athymic mouse model, although an appropriate model for demonstrating distal site antigen-specific activity, does not address the issue of normal tissue expression of the targeted antigen. Previous studies demonstrated that BR96-DOX produced cures of established DOX-sensitive human lung tumor xenografts in athymic rats (11). Rats, like humans and in contrast to mice, have been shown by immunohistology to bind BR96; the normal tissue reactivity occurs primarily on cells of the gastrointestinal tract; esophagus, stomach, intestine, and pancreas (acinar cells; Refs. 8 and 11). The athymic rat, therefore, provides a unique model to evaluate the antitumor activity of BR96-DOX against DOX-insensitive human colon tumors in a system in which the targeting MAb is tumor selective rather than tumor specific. As shown in Fig. 1*B*, the BR96-DOX conjugate, administered at a dose equivalent to the MTD of unconjugated DOX, demonstrated a significant tumor growth delay, with 50% CRs and 50% PRs. In contrast, unconjugated DOX administered at the same dose was not active. Therefore, BR96-DOX was active and tolerated in a model of a human DOX insensitive

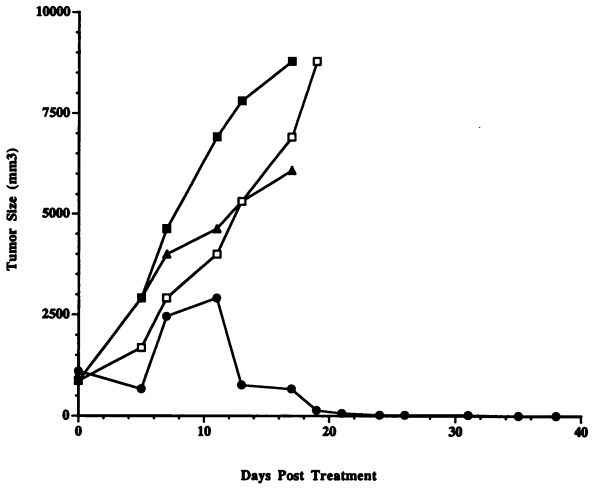

Fig. 2. Antigen-specific antitumor activity of BR96-DOX against s.c. BN7005 colon tumors in BN rats. Tumors were implanted s.c., and therapy was initiated when tumors had reached approximately 12 mm in diameter. Results are from untreated control rats (■) and rats treated with 420 mg/m^2 (12 mg/m^2 equivalent DOX) BR96-DOX (●), IgG-DOX (□), or 12 mg/m^2 of DOX (▲).

Table 2 *Antigen-specific antitumor activity of BR96-DOX against s.c. BN7005 syngeneic rat colon carcinoma*

Treatment	Dose/injection (mg/m^2)[a]		Tumor regressions		Total responding/ no. of Rats
	MAb	DOX	Complete	Partial	
BR96-DOX	90	2.5	0	0	0/5
	175	5.0	1	0	1/5
	350	10.0	3	0	3/5
	420	12.0	3	6	9/13
IgG-DOX	420	12.0	0	0	0/10
DOX		12.0	0	0	0/8
		18.0[b]	0	0	0/20
		24.0[b]	0	0	0/8

[a] Treatments administered every 4 days for 3 injections.
[b] Toxicity observed as weight loss >15% and hind leg paralysis.

Table 3 *Response of established intrahepatic isografts of the BN7005 colon carcinoma to BR96-DOX administered i.p. on four occasions*

Treatment[a]		Wk after tumor isografting			
		1	3	4	6
Untreated	Proportion with tumor	6/6	6/6[b]		
	Tumor diameter (mm)	≥4	23 ± 1.4[c]		
BR96-DOX 420 mg/m^2 (12 mg/m^2 DOX)					
	Proportion with tumor	6/6	6/6	0/6	1/6
	Tumor diameter (mm)	≥4	≤3	ND[d]	Metastases[e]

[a] First day of therapy was considered day 1; treatments were administered on days 1, 4, 7, and 11.
[b] Sacrificed on day 21 because all rats in this group had large tumors in the liver, which spread to the peritoneal wall and mesenteric lymph nodes.
[c] For each rat, the mean diameter of each tumor of two liver tumors was calculated and averaged. Mean ± SE for each group is shown.
[d] ND, not done.
[e] One rat died with multiple lung metastases but no liver tumors. The remaining five rats were sacrificed 5 weeks after the start of therapy and determined to be tumor free on necropsy.

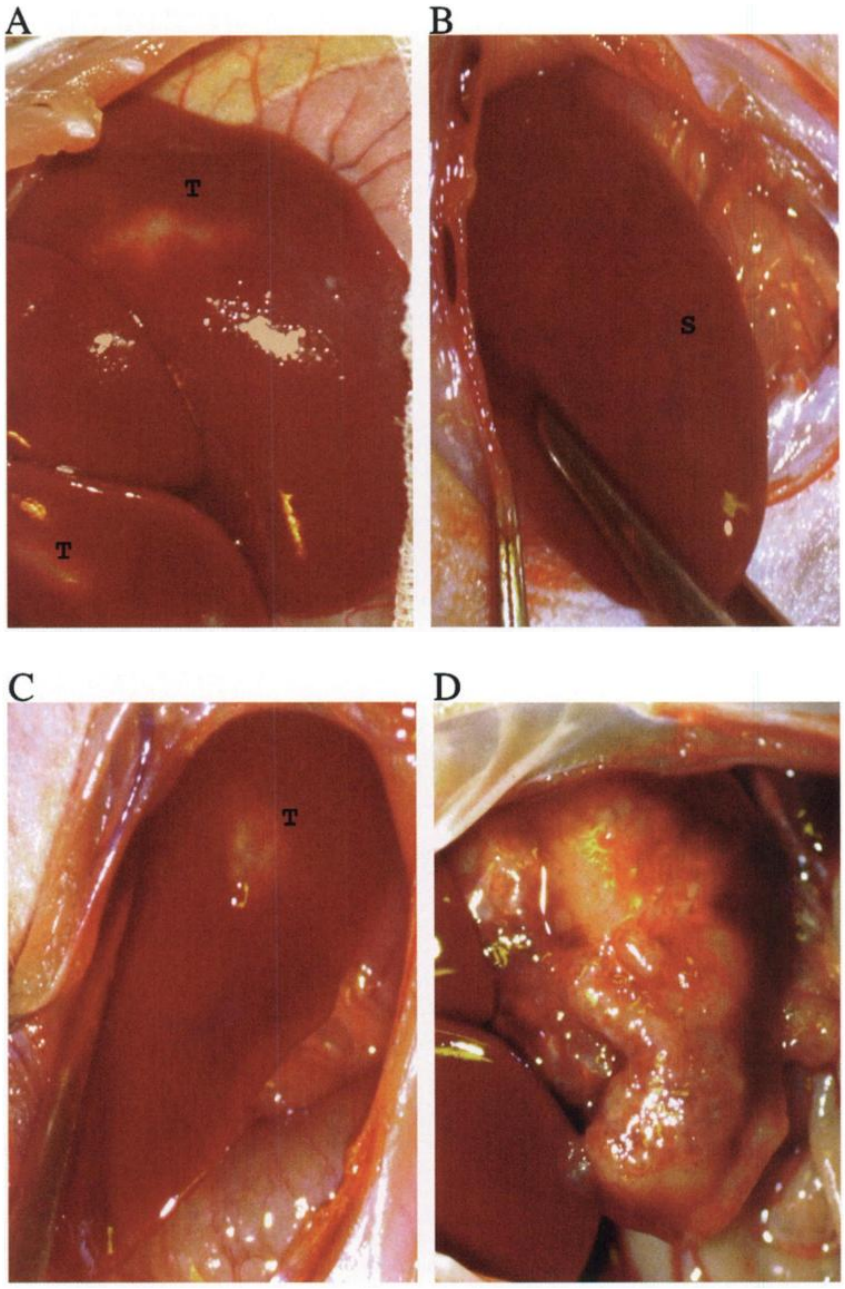

Fig. 3. Macroscopic appearance of rat BN7005 colon carcinoma growing in two liver lobes. *A*, livers prior to therapy; *B*, livers 21 days after treatment with BR96-DOX; *C* and *D*, liver from untreated control rat on days 7 and day 21, respectively. *T*, tumor; *S*, scar tissue.

colon carcinoma although the targeted antigen was expressed in normal tissues.

Antitumor Activity of BR96-DOX against s.c. Rat Colon Carcinoma BN7005. Although athymic rats provide an appropriate model for conjugate efficacy and toxicity, they may also mount a limited host response to xenotransplanted tumors, which may facilitate regression after an initial damage inflicted by the immunoconjugate (13). In addition, athymic rats lack the ability to produce an anticonjugate response and cannot be used to address issues of altered conjugate clearance, pharmacokinetics, and tumor localization when anticonjugate antibodies are present. Therefore, a model of a syngeneic tumor in immunocompetent animals expressing the target antigen in normal tissues would reflect the clinical situation more closely. The BN7005 rat colon carcinoma, derived originally from BN rats, was used to address these issues. Treatment of established BN7005 s.c. tumors with BR96-DOX at a dose of 420 mg/m^2 (12 mg/m^2 equivalent DOX) administered every 4 days for a total of three injections resulted in antitumor activity equivalent to ≥6.7 TVDD; 3 of 5 rats were cured of their tumors (Fig. 2). As shown, BN7005 tumors were not sensitive to unconjugated DOX because treatment with the MTD of 12 mg/m^2 did not result in tumor regressions or even a tumor growth delay. The activity of BR96-DOX against established BN7005 tumors was antigen specific; a matching dose of 420

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