Anti-HER2 Immunoliposomes: Enhanced Efficacy Attributable to Targeted Delivery[1]

John W. Park,[2] Keelung Hong, Dmitri B. Kirpotin, Gail Colbern,[3] Refaat Shalaby, Jose Baselga,[4] Yvonne Shao, Ulrik B. Nielsen, James D. Marks, Dan Moore, Demetrios Papahadjopoulos, and Christopher C. Benz

Division of Hematology/Oncology [J. W. P., C. C. B.] and Department of Anesthesia [U. B. N., J. D. M.], University of California, San Francisco, San Francisco, California 94143; California Pacific Medical Center Research Institute, San Francisco, California 94115 [K. H., D. B. K., G, C., R, S., Y. S., D. M., D. P.]; Memorial Sloan-Kettering Cancer Center, New York, New York [J. B.]

ABSTRACT

Purpose: **Anti-HER2 immunoliposomes combine the tumor-targeting of certain anti-HER2 monoclonal antibodies (MAbs) with the pharmacokinetic and drug delivery capabilities of sterically stabilized liposomes. We previously showed that anti-HER2 immunoliposomes bind efficiently to and internalize in HER2-overexpressing cells *in vitro,* resulting in intracellular drug delivery.**

Experimental Design: **Here we describe the pharmacokinetics and therapeutic efficacy of anti-HER2 immunoliposomes containing doxorubicin (dox) in a series of animal models.**

Results: **Immunoliposomes displayed long circulation that was identical to that of sterically stabilized liposomes in single- and multiple-dose studies in normal rats. Anti-HER2 immunoliposome-dox produced marked therapeutic results in four different HER2-overexpressing tumor xenograft models, including growth inhibition, regression, and cures. These results demonstrated that encapsulation of dox in anti-HER2 immunoliposomes greatly increased its therapeutic index, both by increasing antitumor efficacy and by reducing systemic toxicity. Immunoliposome-dox was significantly superior to all other treatment conditions tested, including free dox, liposomal dox, and anti-HER2 MAb (trastuzumab). When compared with liposomal dox in eight separate therapy studies in HER2-overexpressing models, immunoliposome delivery produced significantly superior antitumor efficacy in each study ($P < 0.0001$ to 0.04). Anti-HER2 immunoliposome-dox containing either recombinant human MAb HER2-Fab′ or scFv C6.5 yielded comparable therapeutic efficacy. Cure rates for immunoliposome-dox reached 50% (11 of 21) with optimized immunoliposomes and Matrigel-free tumors and overall was 16% (18 of 115) *versus* no cures (0 of 124) with free dox or liposomal dox. Finally, anti-HER2 immunoliposome-dox was also superior to combinations consisting of free MAb plus free dox or free MAb plus liposomal dox.**

Conclusions: **Anti-HER2 immunoliposomes produced enhanced antitumor efficacy via targeted delivery.**

INTRODUCTION

Immunoliposomes represent a novel strategy for tumor-targeted drug delivery (1). Immunoliposomes have become feasible as a result of parallel advances in the areas of liposome research and MAb[5] technology, which in principle can now be combined for tumor-targeted drug delivery.

The development of stable, long-circulating liposomes has led to a new era in liposome drug delivery (2). For example, sterically stabilized liposomes, consisting of small, neutrally charged unilamellar liposomes with a polymeric coating of PEG, display retarded clearance by the RES, resulting in substantially prolonged drug circulation (3). In addition, the long circulation times and small sizes of these liposomes allow preferential extravasation in many solid tumors because of vascular abnormalities associated with tumor angiogenesis (4). However, sterically stabilized liposomes do not interact directly with tumor cells *in vitro* or *in vivo*, and instead release drug for eventual diffusion into tumor cells (5).

Similarly, progress in MAb-based therapy of cancer has finally led to clinical validation after two decades of research (reviewed in Ref. 6). A leading example has been the development of MAbs directed against the $p185^{HER2}$ (HER2; ErbB2) receptor tyrosine kinase, the product of the *HER2* (c-*erb*B2) proto-oncogene. HER2 plays an important role in the pathogenesis of breast and other cancers (reviewed in Ref. (7). HER2 is highly overexpressed in a significant proportion of these can-

Received 6/4/01; revised 1/23/02; accepted 1/30/02.
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[1] This work was supported by grants from the National Cancer Institute Specialized Programs of Research Excellence (SPORE) in Breast Cancer (Grant P50-CA 58207-01), the Department of Defense Breast Cancer Research Program (Grant DAMD17-94-J-4195), and the American Society of Clinical Oncology Young Investigator Award (to J. W. P.) sponsored by the Don Shula Foundation.
[2] To whom requests for reprints should be addressed, at UCSF Comprehensive Cancer Center, 1600 Sutter Street, 2nd Floor, San Francisco, CA 94115-1710. Phone: (415) 502-3844; Fax: (415) 353-7021; E-mail: jpark@cc.ucsf.edu.
[3] Present address: Alza Corporation, Mountain View, CA 94043.
[4] Present address: Hospital General Universitari Vall D'Hebron, 08035 Barcelona, Spain.

[5] The abbreviations used are: MAb, monoclonal antibody; PEG, polyethylene glycol; RES, reticuloendothelial system; PK, pharmacokinetic; rhuMAb, recombinant human MAb; dox, doxorubicin; Ls, liposome; DTPA, diethylenetriaminepentaacetic acid; IHC, immunohistochemical; MTD, maximum tolerated dose.

cers, and HER2 overexpression is clearly associated with poor prognosis in breast cancer (8, 9). Hence, HER2 is an attractive target for MAb-based therapeutic strategies. rhuMAb HER2 (trastuzumab; Herceptin), a humanized MAb directed against HER2, represents one such therapy (10) and has demonstrated clinical benefit in the treatment of advanced breast cancer. Trastuzumab can induce antitumor responses as a single agent (11, 12), but it is most efficacious when combined with chemotherapy (13).

We have developed anti-HER2 immunoliposomes to combine the tumor-targeting properties of MAbs such as trastuzumab with the drug delivery properties of sterically stabilized liposomes. Anti-HER2 immunoliposomes efficiently bind to and internalize in HER2-overexpressing cells, resulting in intracellular drug delivery (14, 15). Here we show the PK and therapeutic properties of dox-loaded anti-HER2 immunoliposomes in a series of animal models.

MATERIALS AND METHODS

Preparation of MAb Fragments. Immunoliposomes were constructed with several different MAb fragments. Most studies included rhuMAb HER2-Fab′ (Genentech, Inc.), a recombinant Fab′ derived from trastuzumab. Cloned trastuzumab heavy- and light-chain genes were coexpressed in *Escherichia coli*, and Fab′ was recovered as described (16). Later studies included scFv C6.5, an alternative anti-HER2 MAb fragment generated by phage display screening of a human antibody gene library (17). scFv was subsequently modified for immunoliposome conjugation by addition of a cysteine residue near the COOH terminus of the recombinant sequence. The modified scFv C6.5 was expressed in *E. coli* and purified as described (17). Immunoliposomes were also constructed with rhuMAb H52-Fab′, an irrelevant humanized Fab′ that differs from rhuMAb HER2-Fab′ only in the antigen-binding loops and shows no detectable binding to any known antigen (14). Another control consisted of inactivated scFv C6.5, in which the scFv was exposed to additional reduction, resulting in elimination of an internal disulfide bond in the antigen-binding domain essential for binding activity.

Preparation of Liposomes and Immunoliposomes. Liposomes were prepared by a lipid film hydration-extrusion method as small unilamellar vesicles (100 ± 10 nm) containing hydrogenated soy phosphatidylcholine and cholesterol as described (14, 15). Liposomes of this composition have increased rigidity and decreased permeability because of the high transition temperature of hydrogenated soy phosphatidylcholine (T_m = 54°C) at 37°C. PEG (M_r 1900)-derivatized distearoyl-phosphatidylethanolamine was included at 0–7 mol % of total lipid for steric stabilization. dox was encapsulated into liposomes with high efficiency by the ammonium sulfate remote loading method (18). For immunoliposomes, Fab′ or scFv was covalently conjugated to liposomes after drug loading. Conjugation was via a thioether linkages between the free thiol near the COOH terminus of the MAb fragment and terminal maleimide groups at the liposome surface (Ls-MAb linkage) as described (14). Alternatively, the MAb thiol was conjugated to maleimide groups at the termini of PEG chains (PEG-MAb linkage) as described (15).

Animal Studies. PK studies were performed in healthy rats, and therapeutic efficacy studies were performed in tumor xenograft nude mouse models, as described below. Institutional approvals for animal use were obtained at each site at which animal studies were performed (Memorial Sloan-Kettering Cancer Center, University of California, San Francisco, and California Pacific Medical Center Research Institute), and the requirements were followed in each of these studies.

PK Studies. Healthy adult Sprague Dawley rats received single or multiple i.v. injections of immunoliposomes, liposomes, or free drug via indwelling jugular venous catheters. After the injection, blood was serially sampled via catheter up to 48 h postinjection, and plasma was assayed for dox, Fab′, or liposome. Dox concentration was determined by spectrofluorimetry after extraction of plasma with acidified alcohol. For two-component PK studies, plasma samples were divided and analyzed for both dox and rhuMAb HER2-Fab′ concentrations. The Fab′ concentration was determined by ELISA, using wells coated with recombinant HER2 extracellular domain for capture and horseradish peroxidase-linked goat antihuman IgG for detection. For multiple-dose PK studies, rats were given i.v. injections of immunoliposomes every week for 3 weeks as in the therapy studies (see below). The initial two doses consisted of "empty" (no encapsulated agent) immunoliposomes, whereas the final dose consisted of immunoliposomes stably loaded with ^{67}Ga-DTPA chelates. Plasma levels were determined by radioactivity counting.

Tumor Cell Lines. Cell lines BT-474, MDA-MB-453, and MCF-7 were obtained from American Type Culture Collection. These cell lines have been extensively characterized for HER2 expression by flow cytometry, ELISA, and immunohistochemistry (19). MCF-7/HER2 was derived by stable transfection of *HER2* cDNA in MCF-7 cells as described (20). IHC scoring of HER2 expression on a semiquantitative scale (0–3+) was first developed for trastuzumab clinical trials (21) and is now routinely used clinically (22). Recent studies have suggested that the presence of *HER2* gene amplification as detected by fluorescence *in situ* hybridization may be more predictive of response to trastuzumab than IHC assays of HER2 (23, 24).

Tumor Xenograft Models. Highly tumorigenic clones were generated from BT-474, MDA-MB-453, MCF-7, and MCF-7/HER2 lines by *in vivo* selection in nude (athymic) mice. In separate studies, tumorigenic clones of BT-474 were independently derived at Memorial Sloan-Kettering Cancer Center (by J. B.) and California Pacific Medical Center (by G. C.), and the resulting xenograft models were designated BT-474/MSKCC and BT-474/SF, respectively. HER2 expression in tumor xenografts was evaluated by IHC analysis and confirmed to be still overexpressed.[6] Estradiol pellets were also implanted to increase tumorigenicity as described (25). In initial experiments, the BT-474/SF, MDA-MB-453, and MCF-7/HER2 models involved s.c. injection of tumor cells mixed with Matrigel (Collaborative Biomedical Associates, Oxford, MA), a complex

[6] M. R. Shalaby, D. Kirpotin, K. Hong, B-M. Ljung, Z. Meng, and J. W. Park. The use of Matrigel in xenograft models: new implications, submitted for publication.

extracellular matrix substitute. Matrigel was omitted in subsequent studies (see text).

Therapy Studies. For each model, tumor cells were implanted s.c. with or without Matrigel in the dorsum of nude mice. When tumor xenografts had become fully established and tumors were $\geq$200 mm^3, mice were randomly assigned to different treatment groups (5–15 mice/group). In the nude mice, all i.v. treatments were performed via retro-orbital injection. Anti-HER2 immunoliposomes were administered i.v. at 5.0–7.5 mg dox/kg/dose every week for 3 weeks, for a total dox dose of 15.0–22.5 mg/kg. The corresponding lipid dose was typically 0.6–1.0 μmol of phospholipid per dose and 1.8–3.0 μmol total. Additional treatment groups were included as indicated in the text. Saline (PBS) was administered i.v. at the same injection volume and schedule as immunoliposomes. Free dox (Adriamycin) was administered i.v. at its MTD of 7.5 mg/kg on the same schedule as immunoliposomes. Sterically stabilized liposomal dox was either prepared identically as immunoliposome-dox except for omission of the MAb conjugation step or consisted of commercial PEGylated liposomal dox (Doxil; Alza Pharmaceuticals). Both versions of liposomal dox were administered i.v. at the same dose and schedule as immunoliposomes. Empty immunoliposomes were administered i.v. at the same lipid dose as anti-HER2 immunoliposome-dox and by the same weekly schedule or three times per week. Free trastuzumab (Genentech, Inc.) was administered i.p. at 0.3 mg/kg twice a week for 3 weeks as described (25). Although all other treatments were administered i.v. (retro-orbital injection) weekly, trastuzumab was administered i.p. twice per week based on extensive preclinical optimization with this regimen (21, 25).

Tumors were measured twice a week by caliper, and tumor volumes were calculated using the equation: length $\times$ width $\times$ depth. Mice with complete tumor regressions by measurement were necropsied at the end of the study and classified as "cured" if no residual tumor cells were detected on histopathological examination of the tumor injection site. Although these xenograft models do not typically support metastatic tumor growth, necropsy also included gross examination of viscera to confirm absence of metastases. Studies were terminated at ~2 months after treatment initiation in these models because of their requirement for estrogen supplementation; estrogen pellets implanted at the time of tumor injection potentially lose potency over extended periods. Mice were also weighed and examined for toxicity twice a week. In most of the studies, the experimenter measuring tumor volumes was blinded to treatment condition.

For MTD studies, nude mice with established tumor xenografts were treated with various doses of free dox, liposomal dox, or anti-HER2 immunoliposome-dox (5–15 mice/group). As in the therapy studies, treatments were administered via retro-orbital i.v. injection over three weekly doses. Total dox administered ranged from 2.5 to 22.5 mg/kg. Mice were monitored for weight and gross toxicity as described over 2 months, the typical duration of the therapy studies. The MTD was defined as the highest tested dose that gave <20% weight loss and no treatment-related deaths.

Statistical Analysis. Tumor growth delay and tumor growth ratio were determined as described in Table 2. Confidence intervals for tumor growth ratios were based on the assumption that the log of the ratios follows a normal distribution, which was consistent with observed data. A separate analysis was performed by fitting a modified Norton-Simon model of tumor growth (26) to the observed data using the method of maximum likelihood to estimate model parameters. In two cases, application of this model to the data did not provide an adequate fit. In these cases, data were analyzed by two-way ANOVA using two factors, treatment group and day. Scheffé *post hoc* comparisons were then used to compare pairs of treatments (Table 2). Cure rates were compared using Fisher's exact test for comparing two proportions.

RESULTS

Preparation of Anti-HER2 Immunoliposomes. Anti-HER2 immunoliposomes were constructed by conjugation of HER2-specific MAb fragments to sterically stabilized liposomes to create a tumor-targeted drug carriers for the treatment of HER2-overexpressing breast cancers. Immunoliposomes were designed to optimize intracellular delivery of encapsulated drug into tumor cells (1). Immunoliposome components included (*a*) a MAb fragment (Fab′ or scFv) to avoid accelerated clearance and immunogenicity associated with intact IgG; (*b*) sterically stabilized liposomes for long circulation and preferential tumor extravasation; (*c*) MAb fragments conjugated to the termini of derivatized PEG chains ("PEG-MAb linkage") to facilitate immunoliposome binding and internalization in target cells; and (*d*) encapsulated anticancer agents (*e.g.,* dox) for which tumor-targeted delivery might enhance the therapeutic index.

dox-loaded anti-HER2 immunoliposomes were initially prepared as described (14), using liposomes containing 0–7 mol % PEG and with rhuMAb HER2-Fab′ conjugated to derivatized head groups of phosphatidylethanolamine on the liposome surface (Ls-MAb linkage). However, immunoliposomes combining the Ls-MAb linkage with a high-density PEG coating showed reduced binding and endocytosis because of steric inhibition (14, 15). This problem was circumvented by use of immunoliposomes with MAb fragments conjugated to the termini of PEG chains (PEG-MAb linkage; Ref. 15). Our optimized construct design therefore consisted of anti-HER2 MAb fragments conjugated via a PEG-MAb linkage to dox-loaded sterically stabilized liposomes containing 6–7 mol % PEG. MAb fragments were either rhuMAb HER2-Fab′ as described (14, 15), or alternatively, scFv C6.5, a fully human anti-HER2 single-chain Fv cloned from a phage antibody library (17). Conjugations were highly efficient, resulting in 30–50 MAb fragments/liposome (70–90% of added MAb fragment).

Pharmacokinetics of Anti-HER2 Immunoliposomes. Although prolonged circulation does not ensure improved efficacy, we sought to construct immunoliposomes with long circulating properties to maximize the effects of tumor targeting. PK studies of anti-HER2 immunoliposome-dox constructs were performed in healthy adult rats. dox-loaded immunoliposomes were prepared with 0–7 mol % PEG and with MAb fragments linked either to the liposome surface (Ls-MAb linkage) or to the termini of PEG chains (PEG-MAb linkage). After a single i.v. dose, all immunoliposome-dox constructs showed a biphasic plasma PK profile for dox (Fig. 1*A*), with terminal $t_{1/2}$ =

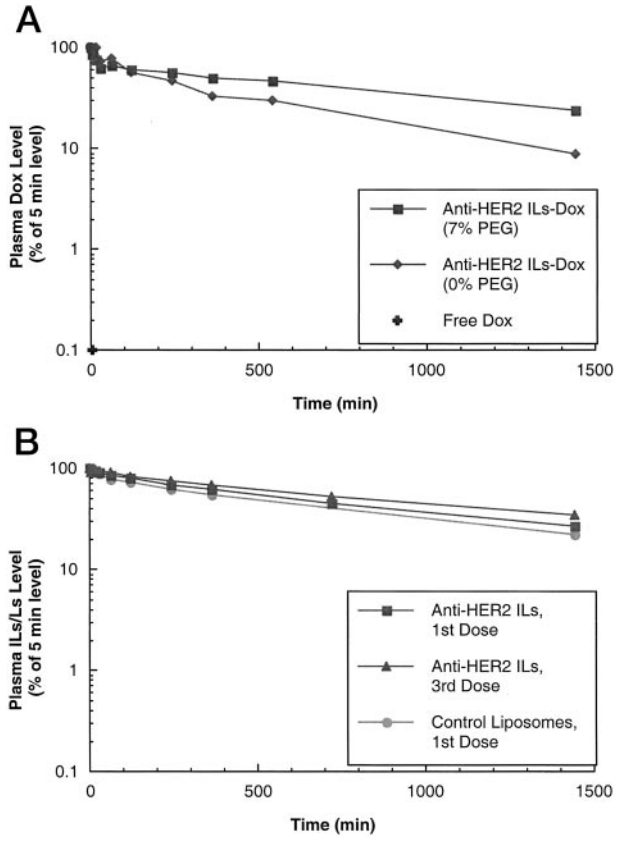

Fig. 1 Plasma pharmacokinetics of anti-HER2 immunoliposomes in rats. *A*, Plasma pharmacokinetics of anti-HER2 immunoliposome-dox *versus* free dox after single i.v. injections in rats. Immunoliposomes containing 0 mol % PEG and Ls-MAb linkage (▨), 7 mol % PEG, PEG-MAb linkage (◈), or free dox (✚) were administered i.v. at time 0. Immunoliposome dose was 5 μmol of total lipid, 0.9 mg of dox; free dox dose was 0.9 mg. Dox levels were determined at the indicated times and expressed as a percentage of the dox concentration at 5 min; dox was undetectable in plasma beyond 5 min after injection of free dox. *B*, Plasma pharmacokinetics of radiolabeled anti-HER2 immunoliposomes after multiple *versus* single i.v. injection in rats. Immunoliposomes (6 mol % PEG, PEG-MAb linkage) were administered i.v. in rats every week for 3 weeks as in the therapy studies. For the third dose, immunoliposomes were loaded with ^{67}Ga-DTPA chelates, and immunoliposome levels were determined at the indicated times by radioactivity counting (△). For comparison, ^{67}Ga-DTPA-loaded immunoliposomes (▨) and sterically stabilized liposomes (○) were also administered to naive rats. Plasma levels of immunoliposomes (*ILs*) or liposomes (*Ls*) are expressed as a percentage of the radioactivity present at 5 min.

11.6–13.6 h, area under the curve of 57,539–93,133 min %, and mean residence time of 15.8–24.3 h (Table 1). In contrast, dox levels were undetectable beyond 5 min after administration of the equivalent dose of free dox. The long circulation times of anti-HER2 immunoliposomes are fully equivalent to previous reports of sterically stabilized liposomal dox and greatly superior to those of "conventional" liposomes (27).

Direct comparison of anti-HER2 immunoliposome-dox *versus* sterically stabilized liposomal dox prepared identically except for omission of MAb fragments showed indistinguishable pharmacokinetics (Table 1). The presence of MAb fragments on immunoliposomes therefore did not measurably alter clearance in normal rats.

Table 1 Plasma pharmacokinetics of anti-HER2 immunoliposome-dox in rats

Anti-HER2 immunoliposomes (0–7 mol % PEG; Ls-MAb or PEG-MAb linkage) were loaded with dox and administered i.v. at a dose of 5.0 μmol of total lipid (0.8–1.0 mg of dox).

Immunoliposome type	Terminal $t_{1/2}$ (h)	AUC[a] (min %)	MRT ± SE (h)
0% PEG, Ls-MAb	11.6	70,954	15.8 ± 1.1
1% PEG, Ls-MAb	13.4	85,695	19.3 ± 3.2
2% PEG, Ls-MAb	12.7	57,539	17.7 ± 3.0
7% PEG, PEG-MAb	16.3	93,133	24.3 ± 0.9
Liposome: 1% PEG, no MAb	13.6	69,882	20.7 ± 1.2

[a] AUC, area under the curve; MRT, mean residual time.

Notably, all anti-HER2 immunoliposome-dox constructs were long circulating. Thus, immunoliposomes of this type (*i.e.*, small unilamellar vesicles, solid bilayer, neutral charge) did not require a polymeric coating such as PEG to achieve long circulation. Nevertheless, PEGylation of immunoliposomes, although not necessary for long circulation, was associated with incremental prolongation of mean residence time (Fig. 1*A* and Table 1).

Stability of Anti-HER2 Immunoliposome-dox *in Vivo*. The stability of dox-loaded anti-HER2 immunoliposomes in circulation was evaluated in two-component PK studies, in which dox and MAb levels were simultaneously assayed to evaluate possible drug leakage or dissociation of MAb fragments from liposomes. For these studies, plasma pharmacokinetics of dox (assayed by spectrofluorimetry) and of rhuMAb HER2-Fab′ fragments (assayed by ELISA) were codetermined from the same plasma samples after single i.v. injection of anti-HER2 immunoliposome-dox. Both the dox and Fab′ components showed concordant plasma PK values after immunoliposome administration, with terminal $t_{1/2}$ = 10 h for both. In contrast, free dox was again undetectable beyond 5 min, whereas free rhuMAb HER2-Fab′ has a terminal $t_{1/2}$ in rats of 1–2 h.[7] These results indicated that immunoliposomes greatly prolonged the circulation of both Fab′ and dox components and suggested that anti-HER2 immunoliposome-dox remained intact in circulation, with negligible drug leakage or MAb dissociation.

Multiple-Dose Pharmacokinetics of Anti-HER2 Immunoliposomes. A major limitation of many immunoconjugate strategies has been the immunogenicity of the construct because the MAb component, the effector/cytotoxic component, or the linker can elicit a host immune response that precludes repeated administrations. To circumvent this problem, anti-HER2 immunoliposomes were designed to have components of minimal immunogenicity: sterically stabilized liposomes and humanized/human MAb fragments. Trastuzumab, a humanized IgG1, induced minimal levels of circulating anti-antibodies in clinical trials (11). MAb fragments rather than intact IgG were used to

[7] D. Maneval, Canji, Inc., San Diego, CA, personal communication.

further reduce the potential for immune recognition associated with Fc sequences. To confirm that repeated administrations of anti-HER2 immunoliposomes do not lead to accelerated clearance, multiple-dose PK studies were performed in normal rats. Radiolabeled anti-HER2 immunoliposomes were administered once a week for three doses, using the same lipid dose and schedule as in the therapy studies (see below). Anti-HER2 immunoliposomes showed equivalent plasma pharmacokinetics in rats after the third and final dose as in naive rats without prior immunoliposome treatment (Fig. 1*B*). Furthermore, anti-HER2 immunoliposomes after one or three doses demonstrated plasma pharmacokinetics that were indistinguishable from those of sterically stabilized liposomes (prepared identically except for omission of Fab′ fragments). Thus, the presence of MAb fragments on immunoliposomes did not result in accelerated clearance over a multiple dose schedule, even when humanized Fab′s were introduced into an immunocompetent rodent host. In humans, immunoliposomes containing humanized Fab′ or fully human scFv are designed to further minimize the potential for immunogenicity.

Antitumor Efficacy of anti-HER2 Immunoliposome-dox against HER2-overexpressing Human Breast Cancer Xenografts. The antitumor efficacy of anti-HER2 immunoliposome-dox constructs was evaluated extensively in four different HER2-overexpressing breast cancer xenograft models: BT474/MSKCC and BT474/SF, independently derived xenograft models from the BT-474 cell line ($\sim 10^6$ HER2 receptors/cell; IHC score, 3+); MCF-7/HER2, MCF-7 cells stably transfected with HER2 ($\sim 10^6$ HER2/cell; IHC score, 3+; Ref. 20); and MDA-MB-453 ($\sim 10^5$ HER2/cell; IHC score, 2+). For each model, tumor cells were implanted in nude mice with or without Matrigel, and treatment was initiated subsequently after tumors had become fully established. Tumor volumes at the start of treatment were 200-1000 mm^3 (0.2–1.0 g) in 20-g mice, thus representing a spectrum from well-established moderate tumors to very large (5% of body weight) tumors.

Anti-HER2 immunoliposomes, prepared with 0–7 mol % PEG and either the Ls-MAb or PEG-MAb linkage, were loaded with dox and administered i.v. at a total dox dose of 15.0–22.5 mg/kg, divided over three weekly doses. Immunoliposomes were directly compared with multiple other treatment conditions, including saline, free dox at its MTD of 7.5 mg/kg, and sterically stabilized liposomal dox at the same dose and schedule as immunoliposome-dox. Additional controls included empty anti-HER2 immunoliposomes (no dox), at the same lipid dose as immunoliposome-dox and free anti-HER2 MAb (trastuzumab) administered at 0.3 mg/kg i.p. twice a week as described (25). Other controls included dox-loaded immunoliposomes containing an irrelevant MAb fragment, rhuMAb H52-Fab′ (14), as well as dox-loaded immunoliposomes containing inactivated anti-HER2 scFv C6.5, in which binding affinity was abrogated by reduction of internal disulfides in the antigen-binding domain before immunoliposome conjugation. Both irrelevant and inactivated immunoliposomes yielded results comparable to those of liposomal dox (data not shown). For this reason, liposomal dox was used as the nontargeted control liposomes for most studies, either by preparing sterically stabilized liposomes identical to anti-HER2 immunoliposome-dox except for omission of MAb fragments or by use of commercial PEGylated liposomal dox (Doxil). The treatment groups are summarized in Table 2.

Each of the anti-HER2 immunoliposome-dox constructs produced marked antitumor effects, including tumor growth inhibition, tumor regression, and cures of mice. These effects were observed in all sizes of tumors. Anti-HER2 immunoliposome-dox consistently showed activity superior to that of all other treatment conditions. For example, initial studies of anti-HER2 immunoliposome-dox containing 1 mol % PEG and Ls-MAb linkage demonstrated suppression of tumor growth that persisted after treatment. Tumor inhibition was clearly superior to that associated with free dox or liposomal dox (Fig. 2 and Table 2).

Comparison of this initial anti-HER2 immunoliposome-dox (1 mol % PEG, Ls-MAb linkage) with the optimized anti-HER2 immunoliposome construct design (6 mol % PEG, PEG-MAb linkage) indicated superior antitumor activity for the latter (data not shown). This result was presumably attributable to the incremental PK advantage associated with the higher PEG concentration (Table 1) and/or the unimpeded internalization associated with the PEG-MAb linkage (15). Subsequent studies therefore evaluated the optimized anti-HER2 immunoliposomes containing 6 mol % PEG and a PEG-Fab′ linkage. In addition, subsequent studies used a Matrigel-free version of the BT-474/SF tumor xenograft model. Matrigel was omitted once tumor localization studies revealed that liposomes and immunoliposomes were nonspecifically trapped in Matrigel deposits.[6] Establishment of tumors without Matrigel coimplantation removed this artifact and produced improved tumor penetration of liposomes and immunoliposomes.

Therapy studies using the optimized anti-HER2 immunoliposome-dox construct in the Matrigel-free BT-474/SF model further demonstrated that targeted delivery of dox was associated with enhanced efficacy (Fig. 3). Anti-HER2 immunoliposome-dox produced substantial tumor regressions in this model, including high rates of cured mice (complete regression of tumor confirmed histopathologically at sacrifice). Indeed, anti-HER2 immunoliposomes containing either rhuMAb HER2-Fab′ or an alternative anti-HER2 antibody fragment, scFv C6.5, produced significantly superior growth inhibition than liposomal dox ($P < 0.0001$ for either anti-HER2 immunoliposome-dox construct *versus* liposomal dox). Furthermore, cure rates in this model reached 50% (5 of 10 mice) for rhuMAb HER2-Fab′ immunoliposomes and 55% (6 of 11 mice) for scFv C6.5 immunoliposomes *versus* 0% (0 of 11 mice) for liposomal dox. These differences were also highly significant, with $P < 0.0001$ for either anti-HER2 immunoliposome-dox construct *versus* liposomal dox.

Treatment with anti-HER2 immunoliposome-dox containing rhuMAb HER2-Fab′ also produced superior efficacy compared with either anti-HER2 MAb (trastuzumab) or empty anti-HER2 immunoliposomes lacking dox (Table 2). In fact, empty anti-HER2 immunoliposomes, administered at the same lipid dose and schedule (once a week) as anti-HER2 immunoliposome-dox, produced no effects on tumor growth (data not shown), indicating that the therapeutic activity of anti-HER2 immunoliposome-dox was attributable to targeted delivery of dox and not to the antiproliferative activity of the rhuMAb HER2-Fab′ fragments on the immunoliposome itself. When

Table 2 Therapeutic efficacy of anti-HER2 immunoliposome-dox

Tumor xenograft-nude mouse model[a]	Cures[b] (*n*)	Tumor growth delay[c] (days)	Tumor growth ratio[d]	*P*, anti-HER2 immunoliposomes-dox *vs.* Lipo-dox[e,f]
BT474/MSKCC				
Study 1				
dox	0/11	3	4.51 ± 2.00	
Lipo-dox	0/9	5	2.62 ± 0.81	
Anti-HER2 ILs-dox	1/7	>40	0.62 ± 0.25	<0.0001
Study 2				
dox	0/8	1	14.95 ± 3.25	
Lipo-dox	0/8	1	4.85 ± 0.78	
Anti-HER2 ILs-dox	0/8	>40	2.63 ± 0.55	<0.0001
BT474/SF				
Study 1 (+Matrigel)				
dox	0/15	1	19.13 ± 1.14	
Lipo-dox	0/15	>40	2.59 ± 0.28	
Anti-HER2 ILs-dox	3/30	>40	0.63 ± 0.12	0.001
Study 2 (+Matrigel)				
Lipo-dox	0/8	>40	1.28 ± 0.25	
Anti-HER2 ILs-dox	0/8	>40	0.60 ± 0.08	0.04
Study 3				
rhuMAb HER2, BIW i.p.	2/8	8	3.61 ± 1.18	
Empty ILs, TIW	0/11	6	8.80 ± 2.02	
Lipo-dox	0/3	9	2.58 ± 0.43	
Anti-HER2 ILs-dox	1/11	>40	1.00 ± 0.24	0.04
Study 4				
Lipo-dox	0/11		2.30 ± 0.70	
Anti-HER2 ILs-dox (Fab′)	6/11	>40	0.74 ± 0.28	<0.0001
Anti-HER2 ILs-dox (scFv)	5/10	>40	0.21 ± 0.06	<0.0001
MCF-7/HER2 (+Matrigel)				
Lipo-dox	0/12	>40	2.14 ± 0.12	
Anti-HER2 ILs-dox	1/12	>40	1.48 ± 0.15	<0.0001
MDA-MB-453 (+Matrigel)				
dox	0/12	17	3.54 ± 0.53	
Lipo-dox	0/12	>40	2.15 ± 0.29	
AntiHER2 ILs-dox	1/18	>40	1.17 ± 0.16	0.004

[a] Tumor xenograft models as described in text, with/without Matrigel as indicated.
[b] Complete regression of established tumor during the study and no evidence of tumor at sacrifice.
[c] Difference in time (days) for treated *vs.* control tumors to increase 3-fold in mean volume. A value >40 indicates mean tumor volume never attained a 3-fold increase during the study.
[d] Ratio of tumor volume at end of study over volume at initiation of treatment, per animal (±SE).
[e] Statistical analysis of the antitumor therapeutic effect of anti-HER2 immunoliposome-dox *vs.* liposomal dox using a modified Norton-Simon model of tumor growth. In BT474/SF studies 3 and 4, statistical analysis was performed by a two-way ANOVA with two factors, treatment and day.
[f] Lipo, liposomal; ILs, immunoliposome; BIW, twice a week; TIW, three times a week.

given three times per week instead of once a week, empty anti-HER2 immunoliposomes containing rhuMAb HER2-Fab′ showed only modest inhibition of tumor growth (Table 2), which again was much less than that of anti-HER2 immunoliposome-dox. These results are consistent with previous studies of trastuzumab, which requires steady-state pharmacokinetics and serum trough concentrations ≥10 μg/ml for tumor inhibition (11, 28). The three weekly doses of anti-HER2 immunoliposome-dox did not lead to steady-state levels.

Taking all of the therapy studies together, in each of four HER2-overexpressing tumor xenograft models (BT474/MSKCC, BT474/SF, MCF-7/HER2, and MDA-MB-453), treatment with anti-HER2 immunoliposome-dox was significantly superior to all other treatment conditions (Table 2). In contrast to free dox, which produced modest tumor growth inhibition in these xenograft models, anti-HER2 immunoliposome-dox showed highly potent antitumor efficacy. In eight separate studies comparing anti-HER2 immunoliposome-dox *versus* liposomal dox, immunoliposome-mediated delivery produced significantly superior therapeutic results ($P < 0.0001$ to 0.04). Furthermore, anti-HER2 immunoliposomes, but not free dox or liposomal dox, produced frequent cures in mice. The total cure rate with immunoliposomes in all studies was 18 of 115 mice (16%), with higher rates of ~50% obtained in later studies using optimized immunoliposomes (PEG-MAb linkage) and non-Matrigel models as described. In contrast, no cures were observed in any of the mice treated with free dox (0 of 46) or liposomal dox (0 of 78). These differences were highly significant ($P < 0.0001$ *versus* free dox or liposomal dox).

Toxicity of Anti-HER2 Immunoliposome-dox. In addition to enhanced efficacy, encapsulation of dox in either liposomes or anti-HER2 immunoliposomes was associated with markedly reduced host toxicity compared with free dox. In studies to determine the MTD in nude mice with tumor xenografts, various doses of free dox, sterically stabilized liposomal dox, or anti-HER2 immunoliposome-dox were administered i.v. once a week for three doses. The MTDs of immunoliposome-dox (≥22.5 mg/kg) and liposomal dox (18.8–

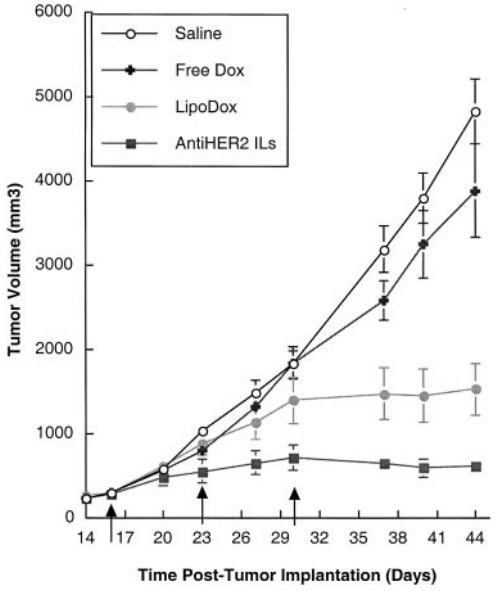

Fig. 2 Efficacy of anti-HER2 immunoliposome-dox in the HER2-overexpressing BT474/MSKCC tumor xenograft model. Anti-HER2 immunoliposome-dox containing 1 mol % PEG/Ls-MAb linkage (▨) was administered by i.v. injection at a total dox dose of 15 mg/kg on the indicated days post-tumor implantation (*arrows*). Other treatment groups included saline (○), free dox (✚) at its MTD of 7.5 mg/kg, and liposomal dox (◉), prepared identically to anti-HER2 immunoliposome-dox but without MAb and given at the same dose and schedule. Anti-HER2 immunoliposome-dox was significantly superior to all other treatment conditions ($P < 0.0001$ *versus* liposomal dox). Data represent mean tumor volumes; *bars*, SE.

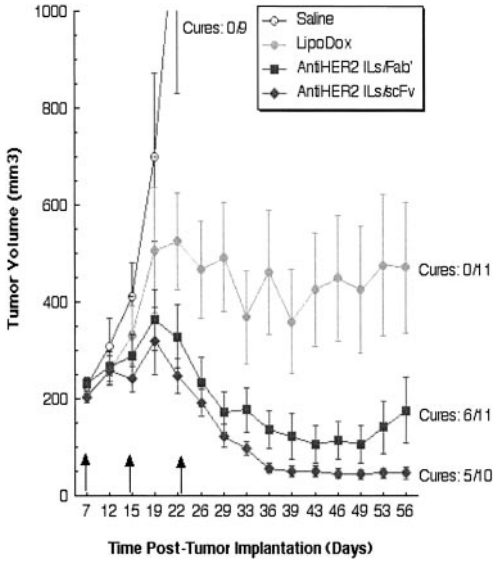

Fig. 3 Efficacy of anti-HER2 immunoliposome-dox in the HER2-overexpressing BT474/SF tumor xenograft model. Anti-HER2 immunoliposome-dox containing 6 mol % PEG/PEG-MAb linkage and either rhuMAb HER2-Fab′ (▨) or C6.5 scFv (◈) were administered i.v. at a total dox dose of 15 mg/kg on the indicated days post-tumor implantation (*arrows*). Other treatment groups included saline (○) and sterically stabilized liposomal dox (◉), prepared identically to anti-HER2 immunoliposome-dox but without MAb and given at the same dose and schedule. Both versions of anti-HER2 immunoliposome-dox were significantly superior to liposomal dox ($P < 0.0001$ for both). Data represent mean tumor volumes; *bars*, SE.

22.5 mg/kg) were 2.5-fold greater than that of free dox (7.5 mg/kg).

Effects of Anti-HER2 Immunoliposome-dox against Non-HER2-overexpressing Tumor Xenografts. As an additional control, anti-HER2 immunoliposome-dox was evaluated in a non-HER2-overexpressing xenograft model. MCF-7 xenografts express low or basal levels of HER2 ($\sim 10^4$ HER2/cell; IHC score, 1+). Previous studies of anti-HER2 immunoliposome binding and internalization in MCF-7 cells *in vitro* showed undetectable uptake by confocal microscopy and quantitative fluorimetry; furthermore, uptake of immunoliposomes was indistinguishable from that of control liposomes lacking MAb fragments (15). To evaluate *in vivo* selectivity, anti-HER2 immunoliposome-dox was administered i.v. in the MCF-7 xenograft model at the same dose and schedule as in the other therapy studies. Equivalent therapeutic effects were obtained for both immunoliposome-dox and nontargeted liposomal dox (Fig. 4). This result confirmed that anti-HER2 immunoliposomes require a threshold HER2 density (or functional activity) to enable appreciable drug delivery *in vivo* as well as *in vitro*.

Antitumor Efficacy of Anti-HER2 Immunoliposome-dox *versus* Combination Therapy. Additional therapy studies in the BT-474/SF model were performed to compare the antitumor efficacy of anti-HER2 immunoliposome-dox *versus* combination therapies consisting of free anti-HER2 MAb (trastuzumab) plus chemotherapy with either free dox or liposomal dox. Trastuzumab has been shown to significantly increase the efficacy of dox-based chemotherapy in both preclinical (25) and clinical studies (13). In a direct comparison of anti-HER2 immunoliposome-dox *versus* the combination of free trastuzumab plus free dox (Fig. 5*A*), anti-HER2 immunoliposome-dox showed efficacy that was significantly superior to that of the combination ($P < 0.0001$). Anti-HER immunoliposome-dox was also directly compared with the combination of free trastuzumab plus liposomal dox, which in this experiment consisted of commercial PEGylated liposomal doxorubicin (Doxil; Fig. 5*B*). Anti-HER2 immunoliposomes again showed significantly superior efficacy than this combination ($P < 0.0001$).

DISCUSSION

Anti-HER2 immunoliposomes combine the tumor-targeting properties of certain anti-HER2 MAbs with the PK and drug delivery properties of long-circulating liposomes. Dox-loaded anti-HER2 immunoliposomes displayed prolonged circulation as stable constructs without drug leakage or MAb dissociation. These results contrast with recent reports of other immunoliposomes constructed with intact IgG, which showed significantly accelerated clearance and reduced circulation times compared with nontargeted liposomes (29, 30). Accelerated clearance of immunoliposomes containing intact IgG may be attributable to recognition of Fc sequences by the RES and/or lability of the IgG conjugation *in vivo*. Immunoliposomes containing covalently conjugated MAb fragments (Fab′ or scFv) have circumvented these limitations.

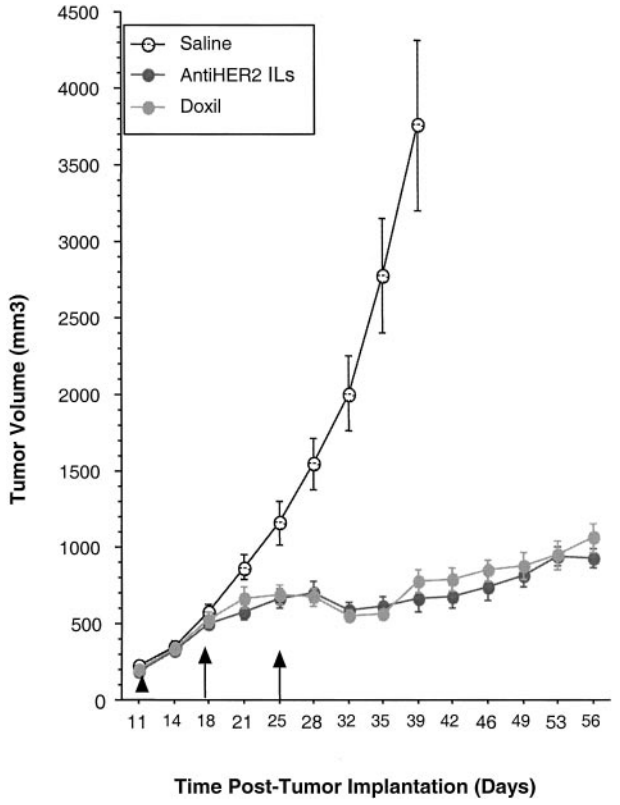

Fig. 4 Lack of enhanced efficacy of anti-HER2 immunoliposome-dox in the non-HER2-overexpressing MCF-7 tumor xenograft model. As a further negative control, therapy studies were performed in nude mice containing xenografted MCF-7 cells, which lack HER2 overexpression (10^4 receptors/cell; IHC score, 1+). Anti-HER2 immunoliposome-dox containing 7 mol % PEG/PEG-MAb linkage and anti-HER2 scFv (●) was administered i.v. at a total dox dose of 15 mg/kg on the indicated days post-tumor implantation (*arrows*). Other treatment groups included saline (○) and sterically stabilized liposomal dox (⊖), prepared identically to anti-HER2 immunoliposome-dox but without MAb fragment and given at the same dose and schedule. Data represent mean tumor volumes; *bars*, SE.

Therapy studies in four different HER2-overexpressing human tumor xenograft models demonstrated that delivery of dox via anti-HER2 immunoliposomes greatly increased the therapeutic index of dox, both by increasing antitumor efficacy and by reducing systemic toxicity. Furthermore, anti-HER2 immunoliposome-dox was significantly superior to all other relevant treatment conditions, including liposomal dox, free trastuzumab, trastuzumab plus free dox, and trastuzumab plus liposomal dox.

These results indicated that anti-HER2 immunoliposome-dox produced enhanced antitumor efficacy via tumor-targeted drug delivery. An alternative explanation is that rhuMAb HER2-Fab′ fragments provided an independent antiproliferative effect on HER2-overexpressing cancer cells, which in turn acted additively or synergistically with dox therapy. Indeed, we have previously shown that empty anti-HER2 immunoliposomes containing rhuMAb HER2-Fab′, when present continuously *in vitro*, do possess antiproliferative activity approaching that of trastuzumab and greatly exceeding that of rhuMAb HER2-Fab′ alone (14). However, two lines of evidence preclude the possibility that this antiproliferative mechanism contributed to the *in vivo* efficacy of anti-HER2 immunoliposome-dox observed in the present studies. The first consideration is that administration of empty anti-HER2 immunoliposomes at the same lipid dose and schedule as dox-loaded immunoliposomes failed to produce significant antitumor efficacy. This result is consistent with preclinical and clinical studies of trastuzumab, which required steady-state pharmacokinetics with a trough serum concentration ≥10 μg/ml for optimal antitumor activity (11, 28). The dose and schedule for anti-HER2 immunoliposomes-dox were far below this, consisting of just three weekly doses. The second consideration is that immunoliposomes containing either rhuMAb HER2-Fab′ or scFv C6.5 produced comparable therapeutic results, although scFv C6.5 does not possess antiproliferative activity as a monomer or when bivalent as a diabody (31).

We previously showed that anti-HER2 immunoliposomes efficiently bind and internalize in HER2-overexpressing cells *in vitro*, leading to intracellular drug delivery (14, 15). Importantly, we have recently evaluated the mechanism by which anti-HER2 immunoliposomes mediate drug delivery *in vivo*.[8] In the same breast cancer xenograft models described here, tumor xenografts were examined histopathologically after i.v. treatment with either gold-loaded anti-HER2 immunoliposomes or gold-loaded sterically stabilized liposomes. Visualization of liposomes and immunoliposomes via a silver enhancement method revealed striking differences in intratumoral localization and mechanism of delivery. Sterically stabilized liposomes were predominantly observed in extracellular areas of tumor stroma and within tissue macrophages and were not observed within tumor cells, consistent with previous reports (32). In contrast, anti-HER2 immunoliposomes were predominantly found internalized within tumor cells in a broad distribution throughout tumor tissue. These results confirm that anti-HER2 immunoliposomes mediate intracellular drug delivery in HER2-overexpressing tumor cells *in vivo* and suggest that the therapeutic advantage obtained with anti-HER2 immunoliposome-dox derives from this novel mechanism of action.

It is notable that anti-HER2 immunoliposomes constructed using an alternative methodology appear to have properties, including lack of enhanced efficacy, very different from those described here (30). In that report, MAb N-12A5, an intact IgG1 directed against HER2, was conjugated to sterically stabilized liposomal dox. N-12A5 immunoliposomes bound to HER2-overexpressing cells *in vitro*, but were not internalized. Therapy studies showed that these immunoliposomes provided no additional efficacy over sterically stabilized liposomal dox. This result suggests that internalization of anti-HER2 immunoliposome-dox constructs in cancer cells was a critical factor in the enhanced efficacy that we observed.

Targeted delivery of dox by anti-HER2 immunoliposomes may represent a particularly advantageous strategy for the treat-

[8] D. B. Kirpotin, K. Hong, J. W. Park, Y. Shao, R. Shalaby, G. Colbern, C. C. Benz, and D. Papahadjopoulos. Localization of anti-HER2 immunoliposomes in HER2-overexpressing breast cancer xenografts: intracellular delivery via immunotargeting, submitted for publication.

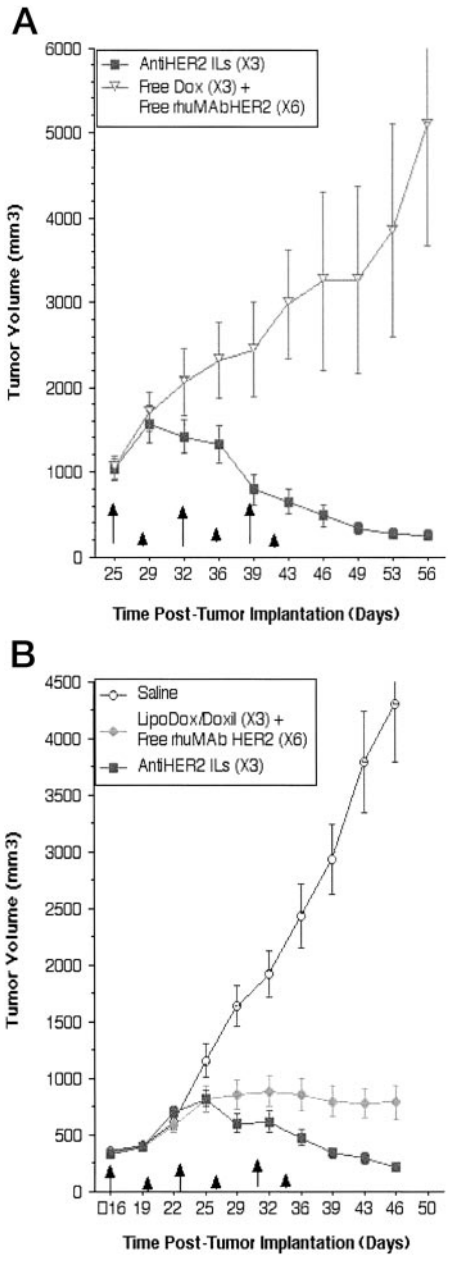

Fig. 5 Efficacy of anti-HER2 immunoliposome-dox *versus* combination therapies in the BT474/SF tumor xenograft model. Data represent mean tumor volumes; *bars*, SE. *A*, dox-loaded anti-HER2 immunoliposomes containing rhuMAb HER2-Fab′ (▨) *versus* combination therapy (▽) of free rhuMAb HER2 (trastuzumab; Herceptin) plus free dox. Anti-HER2 immunoliposomes were administered i.v. at a total dox dose of 15 mg/kg over three doses (*arrows*), free dox was administered i.v. at its MTD of 7.5 mg/kg over three doses (*arrows*), and trastuzumab was administered i.p. at 0.3 mg/kg twice a week over six doses (*arrows* and *arrowheads*). Anti-HER2 immunoliposome-dox was significantly superior to the combination of free MAb plus free drug ($P < 0.0001$). *B*, dox-loaded anti-HER2 immunoliposomes containing rhuMAb HER2-Fab′ (▨) *versus* combination therapy (○) of free anti-HER2 MAb (trastuzumab) + commercial PEGylated liposomal dox (Doxil). Immunoliposomes and liposomal dox were each administered i.v. at a total dox dose of 15 mg/kg over three doses (*arrows*), and trastuzumab was administered i.p. over six doses (*arrows* and *arrowheads*). Anti-HER2 immunoliposome-dox was significantly superior to the combination of free MAb plus liposomal drug ($P < 0.0001$).

ment of HER2-overexpressing cancers. Preclinical and clinical studies have raised the possibility that HER2-overexpressing breast cancers may be relatively resistant to hormone therapy and certain types of chemotherapy (reviewed in Ref. (33), but may be especially sensitive to anthracycline-based chemotherapy (34–36). In principle, anti-HER2 immunoliposome-dox can provide maximal anthracycline delivery to HER2-overexpressing cancers while reducing the toxicity of free dox to the myocardium and hematopoietic cells because HER2 expression is extremely low in these cell types (37) and drug delivery by anti-HER2 immunoliposomes is dependent on receptor density (15). Another relevant issue is that trastuzumab therapy is associated with some cardiotoxicity as a single agent (12) and prohibitive cardiotoxicity when used in combination with dox chemotherapy (13). Although the toxicological mechanism remains to be elucidated, it is presumed that trastuzumab-associated cardiotoxicity is related to its therapeutic antiproliferative and/or immunological effects. As discussed, these effects require high steady-state levels of trastuzumab. Hence, immunoliposomes, which can be administered intermittently, should not produce these effects. Consistent with this, empty immunoliposomes containing rhuMAb HER2-Fab′ did not display antiproliferative activity *in vivo* with a weekly schedule. Furthermore, immunoliposomes can be constructed with scFv C6.5, a ligand devoid of antiproliferative activity, and immunoliposomes containing either Fab′ or scFv lack Fc sequences to mediate immunological functions such as antibody-dependent cellular cytotoxicity. Finally, as noted, this delivery system does not accumulate in low-HER2-expressing cells such as MCF-7 (10^4 HER2/cell; Ref. 15); this threshold suggests that significant delivery or accumulation is unlikely in myocardial cells containing $\sim 10^3$ HER2/cell. Hence, we hypothesize that anti-HER2 immunoliposome-dox may represent a particularly apt strategy to deliver dox to HER2-overexpressing cancer cells, resulting in less cardiotoxicity than dox chemotherapy alone and much less than dox chemotherapy plus trastuzumab. Final determination of the safety profile of anti-HER2 immunoliposome-dox will require formal toxicological evaluation and Phase I clinical studies.

We conclude that tumor-targeted drug delivery using anti-HER2 immunoliposomes can enhance the therapeutic index of dox chemotherapy and therefore may provide a particularly potent and useful therapy for cancers that involve HER2 overexpression. In addition, the strategy of immunoliposome delivery may have broad utility for targeted delivery of other anticancer agents, such as those agents with narrow therapeutic indices, PK limitations, or a requirement for intracellular delivery.

ACKNOWLEDGMENTS

We are grateful to Sharon Baughman and I. Craig Henderson for helpful suggestions.

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Anti-HER2 Immunoliposomes: Enhanced Efficacy Attributable to Targeted Delivery

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Clin Cancer Res 2002;8:1172-1181.