Enhanced Pharmacodynamic and Antitumor Properties of a Histone Deacetylase Inhibitor Encapsulated in Liposomes or ErbB2-Targeted Immunoliposomes

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Abstract ErbB2-overexpressing human cancers represent potentially sensitive targets for therapy by candidate histone deacetylase (HDAC) inhibitors as we have shown that HDAC inhibitors can selectively reduce ErbB2 expression by repressing the ErbB2 promoter and accelerating the decay of cytoplasmic ErbB2 transcripts. To extend these *in vitro* findings and enhance the *in vivo* pharmacodynamic properties of HDAC inhibitors, we stably encapsulated a potent hydroxamate-based HDAC inhibitor (LAQ824) within long-circulating liposomes (Ls-LAQ824) and immunoliposomes (ILs-LAQ824) bearing >10,000 LAQ824 molecules per nanovesicle. Liposomal LAQ824 exhibits prolonged *in vivo* stability and, unlike free LAQ824, circulates with a half-life of 10.8 hours following a single i.v. injection. Three weekly i.v. injections of 20 to 25 mg/kg Ls-LAQ824 in nude mice with ErbB2 overexpressing BT-474 breast tumor xenografts significantly impairs tumor growth, and administration of ErbB2-targeted ILs-LAQ824 may further improve this antitumor activity. Studies of tumor-bearing mice 24 hours after single treatment indicate that: (*a*) >10% of injected liposomal LAQ824 is still circulating (whereas free LAQ824 is undetectable in the blood after 15 minutes); and (*b*) tumor uptake of Ls-LAQ824 and ILs-LAQ824 is >3% injected drug per gram of tumor, producing levels of acetylated tumor histones that are 5- to 10-fold increased over those following free LAQ824 or saline treatments and resulting in concordantly reduced levels of tumor ErbB2 mRNA. These preclinical results support the clinical evaluation of HDAC inhibitors against ErbB2-overexpressing malignancies, and further indicate that encapsulation into targeted and nontargeted liposomes substantially improves the *in vivo* pharmacokinetics, tumor uptake, and antitumor properties of hydroxamate-based HDAC inhibitors.

ErbB2 (HER2)-overexpressing cancers are potentially excellent candidates for treatment with histone deacetylase (HDAC) inhibitors (1, 2). The ErbB2 receptor is vital for tumor progression, and treatment with HDAC inhibitors has been shown to down-regulate the receptor, decrease synthesis of new ErbB2 transcripts, increase decay of mature ErbB2 mRNA, increase proteasomal degradation of the overexpressed receptor protein, and sensitize ErbB2-overexpressing cells to a variety of antitumor agents including trastuzumab (1, 2). HDACs, capable of regulating gene expression through diverse mechanisms including modulation of the histone code (3, 4) and acetylation of many non–histone proteins (5–7), are inhibited by a structurally diverse group of drugs showing preclinical and clinical promise as cancer therapeutics (7, 8). There are six distinct classes of HDAC inhibitors that bind to various segments of the catalytic domain of HDACs (9–11). Of these, the hydroxamate class of HDAC inhibitors has probably received the most attention; all hydroxamate inhibitors are thought to act by binding to critical zinc and water molecules present in the active site of both class I and II HDACs (12, 13).

LAQ824 (Novartis Pharmaceuticals) is a structurally novel cinnamyl acid in the hydroxamate class of HDAC inhibitors (14). LAQ824 has been shown to induce acetylation of heat shock protein 90, resulting in proteasomal degradation of heat shock protein 90 client oncoproteins like Bcr-Abl and ErbB2 (15); in addition, LAQ824 can induce caspase-dependent apoptosis (14, 16, 17) and inhibit tumor angiogenesis (18). When given i.v. daily for 5 consecutive days and 15 total doses, LAQ824 shows significant antitumor activity against colon and lung tumor xenografts with relatively little host toxicity (19). Numerous studies have also shown that LAQ824 works synergistically with various tyrosine kinase inhibitors including the vascular endothelial growth factor receptor inhibitor PTK787 (18), trastuzumab (2), the Bcr-Abl inhibitor imatinib

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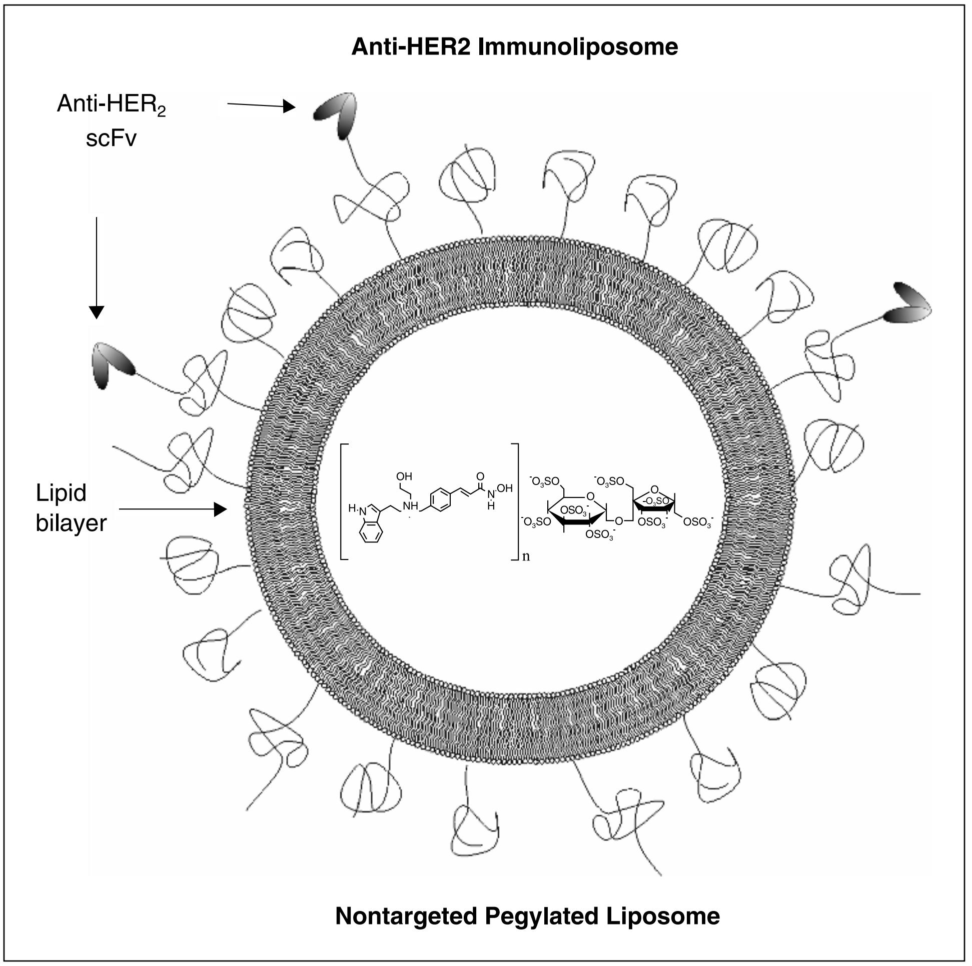

Fig. 1. Depiction of a liposome encapsulating the HDAC inhibitor, LAQ824, in a stable complex with the polyanionic polyol, SOS. The intraliposomal complex of LAQ824-SOS allows for stable entrapment of the drug while in the general circulation and selective delivery to solid tumors. The liposome is also shown both with a PEG-modified surface for improved circulation lifetimes (PEG-DSPE is also present in the inner monolayer of the liposome membrane, but has been removed for clarity to better show the entrapped drug). Human anti-ErbB2/HER2 scFv antibody fragments can be covalently coupled to the termini of the PEG conjugate, effectively transforming the nontargeted liposome into a molecularly targeted liposomal delivery system for intracellular delivery of LAQ824.

mesylate (15), and the FLT-3 inhibitor PKC412 (20). However, these antitumor effects all require prolonged and continuous tumor cell exposure to LAQ824, typically steady-state concentrations lasting 24 to 72 hours (14, 15). Thus, like other hydroxamate-based HDAC inhibitors, LAQ824 exhibits promising anticancer properties only when given daily to sustain its *in vivo* drug levels, suggesting that this pharmacokinetic limitation might be overcome by introducing a new formulation strategy such as liposome encapsulation.

Unlike most other hydroxamate HDAC inhibitors, LAQ824 contains a titratable amine functionality that makes it amenable to selective and stable drug loading into long-circulating liposomes (Fig. 1; ref. 14). Liposomes have been widely used to improve the pharmacokinetic properties, alter toxicity profiles, modify the metabolic profiles, provide sustained release, and generally improve the therapeutic efficacy of various chemotherapeutic agents (21, 22). Remote-loading technologies have been developed that rely on the selective permeation of the neutral form of an amphipathic amine across liposomal membranes, followed in some instances by complexation of the charged form of the drug with anions in the liposomal lumen (23, 24). Two important results of this loading process are (*a*) quantitative encapsulation of drug within the liposome carrier and (*b*) stable retention of the encapsulated drug for the duration of its *in vivo* circulation. The first of these, quantitative drug loading, can occur in the absence of stable encapsulation if the drug loads but then fails to precipitate in the liposome interior, or if the precipitate is unstable at physiologic temperatures and high dilutions. We have recently described a loading process using triethylammonium sucrose octasulfate (TEA-SOS) as the trapping agent that stably encapsulates a number of drugs that were previously difficult to formulate as liposomes, including irinotecan and vinorelbine.[5,6]

Molecularly targeted liposomes offer even greater specificity in the drug delivery process and further improvement in the therapeutic index. When properly optimized, ligand-targeted liposomes work to deliver therapeutic agents intracellularly and specifically to various sites of disease, most commonly cancers (25, 26). ErbB2-targeted immunoliposomes employing human scFv or Fab′ fragments have been shown to specifically enhance drug delivery to ErbB2-overexpressing tumors (27–29). An ErbB2-targeted liposomal doxorubicin construct proved to be considerably more efficacious than nontargeted liposomal doxorubicin, free doxorubicin, or nontargeted liposomal doxorubicin plus trastuzumab in the treatment of

[5] D.C. Drummond et al., Pharmacokinetics and efficacy of novel liposomal vinorelbine formulations with tunable drug release rates, submitted for publication.
[6] D.C. Drummond et al., Antitumor efficacy and reduced toxicity of a novel liposomal CPT-11 in colon and breast carcinoma xenografts, submitted for publication.

ErbB2-overexpressing breast tumor xenografts (27). Other molecularly targeted liposomes with promising properties include folate- (30, 31), CD19- (32), epidermal growth factor receptor- (33), GD2- (34), and hyaluronate- (35) targeted liposomes.

Here we describe the development and characterization of highly stable liposomal and immunoliposomal formulations of LAQ824. Liposomal LAQ824 (Ls-LAQ824) was prepared using a novel gradient loading strategy, employing TEA salts of either poly(phosphate) or sucrose octasulfate, to load LAQ824 quantitatively into the aqueous interior of liposomes. Ls-LAQ824 was shown to be both long-circulating and highly stable *in vivo*. ErbB2-targeted liposomes were shown to have targeted cytotoxic activity in cell culture using ErbB2-overexpressing breast cancer (SKBR3) cells, even surpassing that observed for unencapsulated LAQ824. *In vivo*, Ls-LAQ824 was found to be active in the treatment of ErbB2-overexpressing breast tumor (BT474) xenografts, even when given in a once weekly schedule, and treatment with anti-ErbB2 immunoliposomal LAQ824 (F5-ILs-LAQ824) seemed to further enhance this antitumor activity. A single injection of liposomal or immunoliposomal LAQ824 resulted in a sustained increase in tumor histone (H4) acetylation and reduction in tumor ErbB2 transcript levels over that seen with free LAQ824 and saline injections. These improvements in the pharmacokinetic and pharmacodynamic profiles afforded by liposomal encapsulation and tumor targeting of LAQ824 seem to have significantly improved the *in vivo* therapeutic index of this promising HDAC inhibitor.

Materials and Methods

Materials. LAQ824 was a kind gift from Novartis Pharmaceuticals, Inc. (East Hanover, NJ). Distearoylphosphatidylcholine (DSPC) and poly(ethylene)glycol (PEG)-derivatized distearoyl-phosphatidylethanolamine (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol was obtained from Calbiochem (La Jolla, CA), mPEG-distearoylglycerol (PEG-DSG) from NOF Corporation (White Plains, NY), and [3H]cholesteryl hexadecyl ether from Perkin-Elmer (Boston, MA). Sucrose octasulfate (sodium salt) was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). Sepharose CL-4B and Sephadex G-75 size exclusion resins, Dowex 50W-8X-200 cation exchange resin, and triethylamine were all obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of triethylammonium salts of poly(PO_4) or sucrose octasulfate. To prepare TEA_8SOS, 6 g of sucrose octasulfate (sodium salt) was dissolved in 16.57 mL of water to give a final concentration of 0.3 mol/L. A Dowex 50W-8X-200 cation exchange resin was employed to prepare the acidic form of sucrose octasulfate. Defined resin was washed twice with two volumes of 1 N NaOH, followed by double-distilled water to neutrality, washed twice with two volumes of 1 N HCl, and finally washed again to neutrality with double-distilled water and then repeated. After the column is poured, it is washed with two volumes of 1 N HCl, one volume of 3 N HCl, and finally two volumes of double-distilled water or until the conductivity reaches 0. The sucrose octasulfate (sodium salt) solution (10% of column capacity) was loaded on the column (120 mL) and eluted with double-distilled water. The column eluent was monitored using a conductivity detector to detect the elution of the sulfated sucrose from the column. The acidic sulfated sucrose was then titrated with triethylamine and the pH and osmolarity were determined. The solution was finally diluted to a concentration corresponding to 0.65 mol/L TEA. The pH was typically in the range of 5.5 to 6.0 and an osmolarity of 480 to 530 mOsmol. Residual sodium was determined using a sodium electrode and any solution with residual sodium >1% was not used further.

TEA poly(phosphate) was prepared similarly using linear sodium poly(phosphate) having 13 to 18 phosphate units per molecule (phosphate glass, Calgon, Sigma-Aldrich, St. Louis, MO), dissolved in deionized distilled water to a concentration of about 1.3 mol/L phosphate. The solution having residual sodium content of <1% was diluted to a final phosphate concentration of 0.55 mol/L. The solution typically has a TEA concentration of 0.52 to 0.55 mol/L (pH 5.5-6.0) and osmolality of 430 to 480 mmol/kg.

Preparation of liposomes. Liposomes were prepared by extrusion of hydrated lipid suspensions through polycarbonate membranes (Nucleopore, Corning-Costar, Acton, MA) having pore sizes that averaged 0.08 or 0.1 μm, depending on the experiment. The primary lipid composition employed in these studies was composed of DSPC, cholesterol, and PEG-DSPE (3:2:0.015, mol/mol/mol) or DSPC/cholesterol/PEG-DSG (3:2:0.3 mol/mol/mol). For *in vivo* pharmacokinetic and formulation stability studies, [3H]cholesterylhexadecyl ether was included in the composition at a concentration of 1 μCi/μmol phospholipid. The dried lipids were dissolved in ethanol at 60°C and subsequently mixed with a TEA salt solution (0.65 mol/L) also heated to 60°C in a 1:9 (vol/vol) ratio. Details as to the salt solutions employed are given in the figure legends and described in Results. The lipid suspension was then extruded 15 times through 0.1 or 0.08 μm polycarbonate filters. Liposomes were characterized by photon correlation spectroscopy using a Nicomp C370 Particle Size Analyzer. Liposomes extruded through 0.08 μm filters averaged from 88 to 95 nm, and after extrusion through 0.1 μm filters averaged from 100 to 115 nm.

Liposome encapsulation of LAQ824. Liposomes were initially purified by gel filtration chromatography using a Sepharose CL-4B gel filtration resin and eluting with HEPES-buffered dextrose [5 mmol/L HEPES, 5% dextrose (pH 6.5)] to remove unencapsulated TEA_8SOS and generate the gradient. LAQ824 in the form of stock solution of 10 mg/mL LAQ824 free base was added to the liposomes in aqueous 5 mmol/L HEPES-Na, 5% dextrose (pH 6.5), at a drug-to-phospholipid ratio of 200 μg/μmol, the pH was adjusted to 6.5 using 1 N NaOH, and the mixture was incubated at 60°C for 30 minutes. For characterizing the effect of PEG-DSPE or PEG-DSG on drug loading, the amount of PEG-DSPE was varied from 0.5 to 10 mol% of the total phospholipid. The mixture was then chilled on ice for 15 minutes, and unencapsulated LAQ824 was removed using a Sephadex G-75 gel filtration chromatography, eluting with HEPES-buffered saline [5 mmol/L HEPES-Na, 145 mmol/L NaCl (pH 6.5)]. Aliquots of purified liposomes were then solubilized in acid isopropanol or acidic methanol and analyzed for LAQ824 using spectrophotometry at 277 nm. Liposomal phospholipid was quantified using a standard phosphate assay (36) of the purified liposome samples directly, or after methanol-chloroform extraction for samples containing poly(phosphate) as the intraliposomal trapping agent. Drug loading was typically quantitative under these conditions.

Preparation of anti-ErbB2 immunoliposomes. F5-Maleimide-PEG-DSPE was prepared by conjugation of reduced F5 scFv (through a COOH-terminal cysteine) to Maleimide-PEG-DSPE at pH 6.5, as described previously (37, 38). A micellar insertion strategy was employed to incorporate the lipid-anchored targeting ligand into the liposomes (37, 38). Micellar solution of F5-PEG-DSPE was incubated at 60°C for 30 minutes with drug-loaded liposomes, quenched on ice for 15 minutes, and subsequently chromatographed using Sepharose CL-4B gel filtration chromatography eluted with HEPES-buffered saline (pH 6.5) to remove uninserted conjugates and any released free drug. This insertion process has been shown previously to yield ~90% incorporation of the F5-PEG-DSPE conjugate into the liposomal formulation (37, 38). Both LAQ824 and phospholipid were reanalyzed in the resulting F5-immunoliposomal LAQ824 (F5-ILs-LAQ824) and the resulting drug-to-phospholipid ratio compared with that obtained prior to insertion. The ratios were always >95% of that obtained prior to insertion, demonstrating no significant drug leakage resulted during the insertion process.

In vitro *cytotoxicity against ErbB2-overexpressing cancer cells.* The cytotoxic activity of free LAQ824, Ls-LAQ824, and F5-ILs-LAQ824 were evaluated against cultured ErbB2-overexpressing SKBR3 breast carcinoma cells. Cells were cultured in McCoy's 5A medium containing 10% FCS and penicillin/streptomycin as antibiotics. SKBR3 cells were plated at a density of 5,000 cells per well in 96-well cell culture plates and allowed to grow overnight. Free, Ls-, or F5-ILs-LAQ824 were then added to the cells for 4 hours, before washing once with HBSS, and finally re-addition of growth medium. The drug was added in triplicate wells for each dilution and a series of 10 1:3 dilutions were prepared for each sample. The liposomes used in this study were composed of DSPC/cholesterol/PEG-DSPE (3:2:0.015, mol/mol/mol) and loaded with LAQ824 at a drug-to-phospholipid ratio of 200 g/mol using the $TEA_n poly(PO_4)$ loading method described previously. After washing, the cells were allowed to grow for an additional 72 hours before being analyzed for cell viability using a tetrazolium-based assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ref. 39]. The IC_{50}'s were estimated by linear interpolation between the two nearest data points.

Pharmacokinetic properties of liposomal LAQ824. The *in vivo* pharmacokinetic properties of both the liposomal lipid and drug components and the characteristics of drug release from the liposomes were studied in female Sprague-Dawley rats (190-210 g) bearing indwelling central venous catheters. The rats were injected with a 0.2- to 0.3-mL bolus of [3H]CE-labeled Ls-LAQ824 (10 mg LAQ824 per kilogram of body weight). The liposome formulation employed was composed of DSPC/cholesterol/PEG-DSG (3:2:0.3 mol/mol/mol) and was loaded using the TEA_8SOS gradient loading method described above. Blood samples (0.2-0.3 mL) were drawn at various times post-injection using heparin-treated syringes, and the withdrawn blood volume was replenished by physiologic PBS. The blood samples were diluted with 0.3 mL of ice-cold PBS containing 0.04% EDTA, weighed, and the RBC were removed by centrifugation. The plasma was collected and assayed for LAQ824 by HPLC analysis as follows. The plasma/PBS (50 μL) was extracted with 450 μL of methanol by vortexing for 10 seconds followed by precipitation of the proteins by freezing at −80°C for 2 hours. The insoluble portion was pelleted by centrifugation at 13,000 rpm for 15 minutes and the supernatant transferred to an autosampler vial kept at 4°C until analysis by HPLC. LAQ824 spiked plasma recovery was >95%. The samples (50 μL) were injected by autosampler on a C_{18} reversed-phase silica column (Supelco C-18 column, 250 mm × 4 mm i.d., particle size of 5 μm) preceded by a C-18 guard column. A gradient elution was used with a mobile phase consisting of 25 mmol/L phosphate at pH 3.0/acetonitrile. The acetonitrile content was increased from 20% to 62% in 7.5 minutes with a flow rate of 1.0 mL/minute. LAQ824 was detected using an absorbance detector at 350 nm; its typical retention time was 6.2 minutes.

The liposome lipid label ([^{3}H]CE) was determined by scintillation counting using conventional methods. Liposome preparations with known drug and [^{3}H]CE-lipid concentrations were used as standards. Radioactivity standards contained an equal amount of diluted rat plasma to account for quenching. The amount of LAQ824 and liposomal lipid in the blood was calculated assuming the blood volume was 6.5% of the body weight, and a hematocrit of 40%. The total amount of the lipid and the drug in the blood was expressed as the percentage of injected dose (%ID) and plotted against post-injection time. The percentage of drug remaining in the liposomes was calculated by dividing the drug/lipid ratio in the blood samples by the drug/lipid ratio of the injected liposomes (taken as 100%). Because the plots generally showed good agreement with monoexponential kinetics (linearity in semilogarithmic scale), blood half-lives of the drug, the lipid, and of the drug release from the liposomes, were calculated from the best fit of the data to monoexponential decay equation using the TREND option of the Microsoft EXCEL computer program (Microsoft Corp.,). Pharmacokinetic variables including the volume of distribution (V_d), clearance (CL), the mean residence time in the circulation (MRT), and the area under the concentration versus time curve (AUC_∞) were all determined by noncompartmental pharmacokinetics data analysis using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

BT474 human breast tumor xenograft model and antitumor properties of liposomal LAQ824. BT474 human breast carcinoma cells were obtained from American Type Culture Collection (Rockville, MD) and used to generate xenograft tumors in nude mice as previously described (27). The cells were propagated *in vitro* in RPMI 1640 with 10% FCS, 0.1 mg/mL streptomycin sulfate, and 100 units/mL penicillin G. NCR *nu/nu* female mice (4-6 weeks old, Taconic Farms, Germantown, NY or Charles River, Wilmington, MA) were s.c. implanted (at the base of the tail) with 60-day sustained release 0.72 mg 17-estradiol pellets (Innovative Research of America, Inc., Sarasota, FL), and after 1 day were inoculated s.c. in the upper back area with a 0.1 mL suspension containing 2×10^7 BT474 cells. Tumor progression was monitored by twice weekly palpation and caliper measurements along the longest (length) and shortest (width) axes, with tumor volumes determined by the formula: $[(\text{length}) \times (\text{width})^2] / 2$ (40). Two independent efficacy experiments were conducted using nude mice from the different vendor sources; surprisingly, this factor resulted in different tumor take rates (40% versus 70%) and variable tumor growth rates under control treatment conditions. In the first experiment, at day 28 post-tumor cell inoculation and when the tumors reached ~500 mm^3 in size (range 203-944 mm^3), mice were randomized into two groups of seven animals per group, and treated i.v. with saline or Ls-LAQ824 [prepared with $poly(PO_4)$ as the counter-ion], at a drug dose of 25 mg/kg LAQ824 once weekly for a total of three injections. Liposomes for this experiment were composed of DSPC/cholesterol/PEG-DSPE (3:2:0.015, mol/mol/mol). In the second experiment, at day 24 post-tumor cell inoculation and when the tumors reached ~175 mm^3 in size (range 106-316 mm^3), mice were randomized into three groups with four to five animals per group, and i.v.-treated with saline, Ls-LAQ824, or F5-ILs-LAQ824 (prepared with sucrose octasulfate as the counter-ion), at a drug dose of 20 mg/kg LAQ824 once weekly for a total of three injections. This dose was reduced slightly from the first experimental dose because the 25 mg/kg Ls-LAQ824-treated animals experienced mild weight loss (averaging 10.4%) a week after the final injection. Liposomes for the second study were composed of DSPC/cholesterol/PEG-DSG (3:2:0.3, mol/mol/mol). In both experiments, all animals were weighed twice weekly and assessed for changes in activity level and general appearance. Both efficacy experiments are shown as plots of time-dependent mean values (±SE) of the tumor volumes for each treatment condition. Differences in tumor growth according to treatment condition were assessed and tested for statistical significance by previously described methods; these include determining tumor growth rates and modeling tumor growth curves, calculating tumor growth delay, and tumor growth ratios (27).

Tumor histone and RNA responses to liposomal LAQ824. Mice implanted with BT474 tumor cells as described above, but whose tumors were allowed to grow for ~60 days after implantation, were given a single i.v. 20 mg/kg dose of free, Ls-, or F5-ILs-LAQ824, versus an equal volume of saline, and sacrificed 24 hours after injection for immediate tumor harvesting and cryopreservation (−80°C). Tumor lysates were prepared for histone protein and total RNA extraction as described below. Tumor samples were ground to a fine powder under liquid nitrogen. For histone protein extraction, tumor powders (0.5 g per sample) were homogenized by sonication (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA) thrice for 15 seconds each in 200 μL of ice-cold DNaseI digestion buffer [125 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, 2 mmol/L DTT and a protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics)]. Extracts were incubated with 100 units of DNaseI on ice for 5 minutes. Following DNaseI digestion, extracts were solubilized by addition of SDS to 1% of the total reaction volume. Extracts were then centrifuged for 10 minutes at 4°C and the supernatant was stored at −20°C prior to Western analyses. Protein content of the tumor extracts was determined by Bradford assay (Bio-Rad, Hercules, CA). For Western analyses, tumor sample extracts were heated to 95°C in 2× sample buffer [125 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol] and electrophoresed into 4% to 12% Nu-Page Bis-Tris gels

(Invitrogen, Carlsbad, CA) with MOPS running buffer (Invitrogen). Separated proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), blocked with 5% nonfat milk in PBS containing 0.05% Tween 20 and probed with an anti-acetyl-histone H4 rabbit polyclonal antiserum (Upstate Biotechnology, Lake Placid, NY) and an anti-histone H3 rabbit polyclonal IgG (Abcam, Cambridge, MA). Membranes were then incubated with horseradish peroxidase-linked secondary antibody (Bio-Rad), and the histone signals were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ) followed by autoradiography. For Northern analyses, total RNA samples were prepared from the frozen tumor powders using an RNEasy Mini Kit (Qiagen, Valencia, CA). Following formaldehyde-agarose gel electrophoresis, the separated RNA was transferred to nylon membranes (Nytran SuperCharge), and the membranes hybridized with 32P-labeled pTRI-eErbB2 antisense RNA probe (Ambion, Austin, TX), or control pTRI-GAPDH and pTRI-Actin (Ambion) probes. After overnight hybridization (5× SSC, 50% formamide, 1% SDS) at 60°C, blots were washed four times at room temperature (2× SSC, 0.1% SDS) followed by two 30-minute washes at 60°C (0.1× SSC, 0.1% SDS) before visualizing the bands by autoradiography and quantitating transcript levels by densitometry.

Results

Stable liposome encapsulation of LAQ824. A new method for stabilizing amphipathic weak amines in the liposomal interior was used to encapsulate the HDAC inhibitor, LAQ824. Although a wide variety of structurally different HDAC inhibitors are under evaluation, including more than 10 members from the hydroxamate class of inhibitors, only one of these structurally reported inhibitors contains a weakly basic amine group facilitating remote-loading into liposomes. Using TEA salts of both poly(phosphate) and sucrose octasulfate, quantitative loading of liposomes with LAQ824 was accomplished at a drug-to-phospholipid ratio of 200 g/mol of phospholipids (Fig. 1). Although there may be problems with loading and/or stability with certain drug classes using liposomes containing negatively charged PEG-DSPE, use of the neutral PEG-DSG or PEG-ceramide seems to obviate these problems. Here we observe that LAQ824 can be quantitatively loaded into DSPC/cholesterol liposomes containing 10 mol% (of the total phospholipid) PEG-DSG. These LAQ824 loaded liposomes can be stably stored for months at physiologic ionic strength (pH 6.5) and 4°C.

***Cytotoxic comparison of Ls-LAQ824, anti-ErbB2 ILs-LAQ824, and free LAQ824* in vitro.** Liposome and immunoliposome formulations of LAQ824 were compared in an *in vitro* cytotoxicity assay against ErbB2-overexpressing SKBR3 breast carcinoma cells. The liposomes employed in this experiment were minimally PEGylated (0.5 mol% PEG-DSPE) to reduce aggregation and loaded using the $TEA_n poly(PO_4)$ loading method described in Materials and Methods. Growth-inhibiting activity after 4-hour exposure to Ls-LAQ824, F5-ILs-LAQ824, or free LAQ824 was compared over the drug concentration range shown in Fig. 2, and IC_{50} values determined. F5-ILs LAQ824 (IC_{50} = 0.065 μg/mL) is shown to have greater *in vitro* activity than either the free drug (IC_{50} = 0.29 μg/mL) or the nontargeted Ls-LAQ824 (IC_{50} = 1.33 μg/mL).

Stability and prolonged* in vivo *circulation of liposomal LAQ824. The pharmacokinetics of both the liposomal carrier (Fig. 3A) and free or liposomal LAQ824 (Fig. 3B) were determined in normal healthy rats. The time-dependent plasma concentrations of free drug compared with liposome encapsulated LAQ824 are shown in Fig. 3B, and the resulting pharmacokinetic variables calculated for both are listed in Table 1. As seen in Fig. 3B, the clearance of LAQ824 is greatly reduced when the drug is stably encapsulated in the liposome formulation employed. Because free LAQ824 is cleared considerably faster than the liposomal carrier ($t_{1/2}$ of 0.2 versus 18.1 hours), the ratio of LAQ824-to-phospholipid is a good determinant of the degree of stability of the liposomal LAQ824 formulation. If the drug were to leak rapidly from the carrier, then this ratio would decrease quickly (Fig. 3C); however, the half-life for LAQ824 release from the liposomes is 26.1 hours, comparable to that previously shown for a variety of other liposome-encapsulated antineoplastic drugs, including CPT-11[5] and vinorelbine.[6]

Antitumor activity with infrequent dosing of Ls-LAQ824. The antitumor efficacy of Ls-LAQ824 [loaded using $TEA_n poly(PO_4)$ into 0.5 mol% PEG-DSPE liposomes] was studied against the ErbB2-overexpressing BT474 human breast cancer xenograft model (Fig. 4A). Unlike the daily i.v. dosing with up to 100 mg/kg in order to see *in vivo* antitumor activity with free LAQ824 (18), once weekly dosing of 25 mg/kg encapsulated LAQ824 produces significant tumor growth inhibition despite delay of treatment initiation until tumors had obtained an average volume of 500 mm^3 (Fig. 4A). At this dose and treatment schedule (once weekly ×3), an average loss in tumor-corrected animal body weight of 10.4 ± 7.4% was noted following the final injection, and ultimately, one of the treated mice died, prompting a dose reduction in the following efficacy treatment.

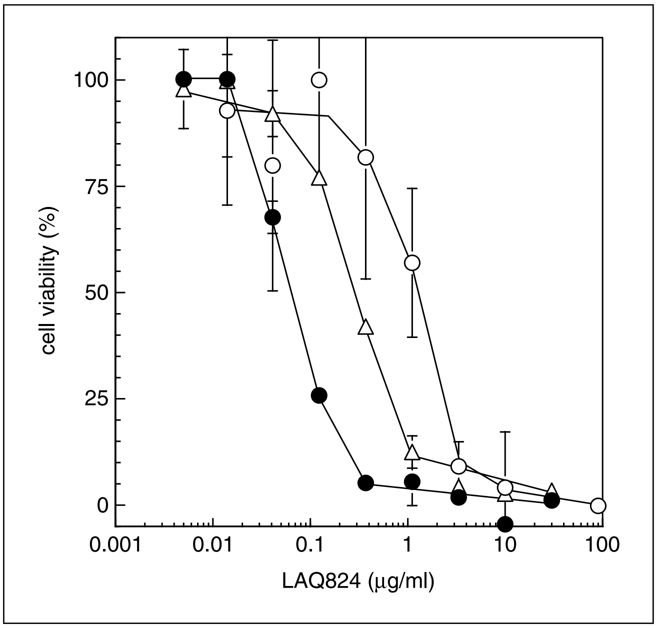

Fig. 2. *In vitro* sensitivity of ErbB2-overexpressing human breast carcinoma cells (SKBR3) to free (△), liposomal (○), and anti-ErbB2 immunoliposomal (●) LAQ824. SKBR3 cells were plated at a density of 5,000 cells per well in 96-well plates and incubated with a series of 1:3 dilutions of the three different formulations of LAQ824 for 4 hours at 37°C. The cells were subsequently washed and allowed to grow for an additional 72 hours, at which time cell viability was determined using a standard tetrazolium-based assay. The percentage of cell viability is plotted as a function of LAQ824 concentration. All samples were examined in triplicate. Best fit trend lines are provided to follow the trends of the data, but do not represent actual model-dependent fits.

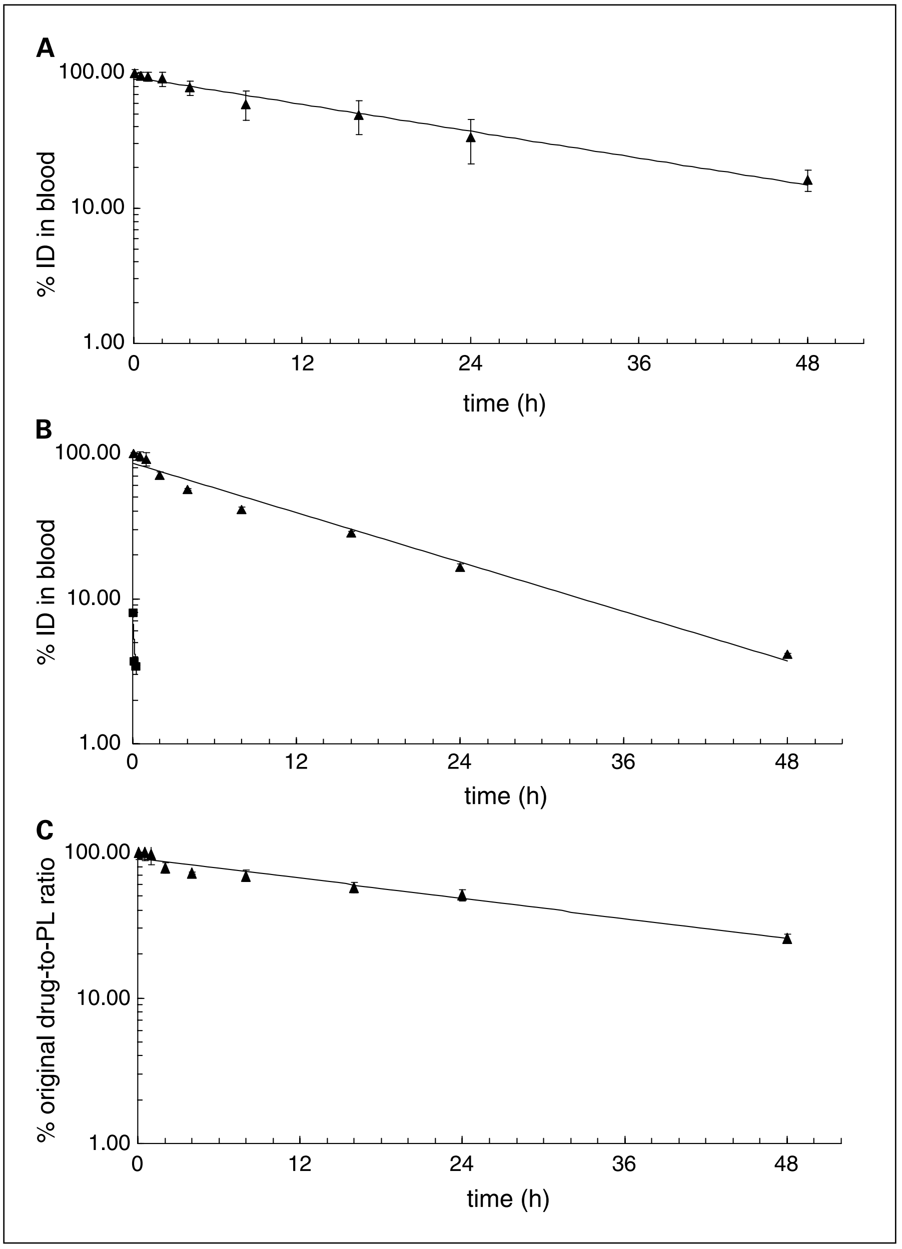

Fig. 3. Pharmacokinetics of free and liposomal LAQ824 after i.v. bolus administration in rats. The liposomes were loaded using pre-entrapped TEA-SOS at a drug-to-phospholipid ratio of 200 μg LAQ824/μmol phospholipid as described in Materials and Methods. Free LAQ824 was dissolved in 5% dextrose and injected. The liposomes used in this study were composed of DSPC/cholesterol/PEG-DSG (3:2:0.3, mol/mol/mol). The pharmacokinetics of liposomal lipid (*A*) and free or liposomal LAQ824 (*B*) were followed as a function of time using scintillation counting of [^{3}H]cholesteryl hexadecylether for determination of the lipid label (*A*) and HPLC analysis for liposomal or free LAQ824 (*B*) quantitation in the plasma as described in Materials and Methods. Free (■) and liposomal (▲) LAQ824 were injected at a dose of 10 mg LAQ824/kg. *C,* the dynamics of the drug-to-liposomal lipid ratio in the blood of a rat *in vivo* following i.v. bolus administration of liposomal LAQ824 is also shown. Two rats were used for determination of the pharmacokinetics and the average and cumulative errors of those measurements are plotted.

In vivo ***efficacy of anti-ErbB2 ILs-LAQ824 relative to Ls-LAQ824.*** The second *in vivo* efficacy experiment was designed to evaluate targeted therapy of ErbB2-overexpressing BT474 xenografts with F5-ILs-LAQ824 relative to nontargeted Ls-LAQ824, at the reduced LAQ824 dose of 20 mg/kg (Fig. 4B). The long-circulating liposomes used in this second experiment contained 10 mol% PEG-DSG (rather than the previously used 0.5 mol% PEG-DSPE liposomes) and were loaded using the TEA_8SOS [instead of the poly(PO_4)] counter-ion gradient loading procedure. Results from this experiment show that both Ls-LAQ824 and F5-ILs-LAQ824 are effective in retarding the growth of BT474 tumors, with a trend suggesting that tumor-targeted F5-ILs-LAQ824 is more effective than non-targeted Ls-LAQ824 (Fig. 4B). However, the limited number of tumor-bearing animals in each arm and the greater than anticipated variability in control tumor growth prevented this apparent increase in ILs-LAQ824 efficacy from reaching statistical significance. Given by the same once weekly ×3 injection schedule, the reduced 20 mg/kg doses of liposomal LAQ824 seemed no more toxic than saline in the control treatment group of animals.

Enhanced tumor histone acetylation and reduced ErbB2 transcript levels following liposomal LAQ824 injection. Treatment effects on fully grown BT474 xenografts (~60 days post-implantation) were determined by measuring histone acetylation and ErbB2 mRNA levels 24 hours after a single 20 mg/kg injection of free LAQ824, Ls-LAQ824, or F5-ILs-LAQ824 and compared with saline-treated controls (Fig. 5). After 24 hours, only a small increase in acetylation can be observed for the tumors treated with unencapsulated LAQ824. The minimal increase in tumor histone H4 acetylation content 24 hours after injection of free LAQ824 (relative to saline treatment and gel loading of unacetylated H3 protein) contrasts with the substantially increased level of H4 acetylation observed

Table 1. Pharmacokinetic variables for free and liposomal LAQ824 in rats

Formulation	$t_{1/2}$ (h)	AUC_{∞} (μg × h/mL)	CL (mL/h)	V_d (mL)	MRT (h)
Free LAQ824	0.2	13.6	737.2	808	1.1
Ls-LAQ824 (TEA_8SOS)*	10.8	2198.5	4.5	70.7	15.5

NOTE: The data used to calculate the pharmacokinetic variables for LAQ824 when formulated either in the free form or liposomal form refers to the actual drug concentrations measured in the blood that were then used to calculate the %ID values found in the corresponding curves for Fig. 3B.
Abbreviations: AUC_{∞}, area under the concentration versus time curve in plasma based on the sum of exponential terms; MRT, mean residence time calculated from exponential terms; CL, clearance calculated from exponential terms; V_d, volume of distribution.
*The liposomal LAQ824 formulation examined was prepared from DSPC/cholesterol/PEG-DSG (3:2:0.3, mol/mol/mol) and loaded with LAQ824 at a ratio of 200 g LAQ824/mol phospholipid using the TEA_8SOS gradient loading method as described in Materials and Methods.

following treatment with LAQ824 encapsulated in either nontargeted or tumor-targeted liposomes (Fig. 5A). At this single 24-hour time point, we were unable to detect any drug in the plasma of tumor-bearing mice treated with free LAQ824 as compared with the 11.18 ± 3.07% of injected dose detected following Ls-LAQ824 and 8.27 ± 1.24% injected dose following F5-ILs-LAQ824 injection. Quantitation of the tumor liposome content at this same time point showed a level of 3.01 ± 1.37% injected dose per gram of tumor following Ls-LAQ824 and 4.79 ± 1.63% injected dose per gram of tumor following the single dose of F5-ILs-LAQ824. Concordant with these tumor measurements, RNA purified from the same treated samples analyzed by Northern blotting showed that ErbB2 mRNA content was also significantly reduced at 24 hours, but only after liposomal LAQ824 treatment and more so by the tumor-targeted F5-ILs-LAQ824 than the nontargeted Ls-LAQ824 injections (Fig. 5B). There was no significant difference in tumor ErbB2/GAPDH mRNA ratios detectable at 24 hours after treatment with free LAQ824 as compared with saline-treated control tumors.

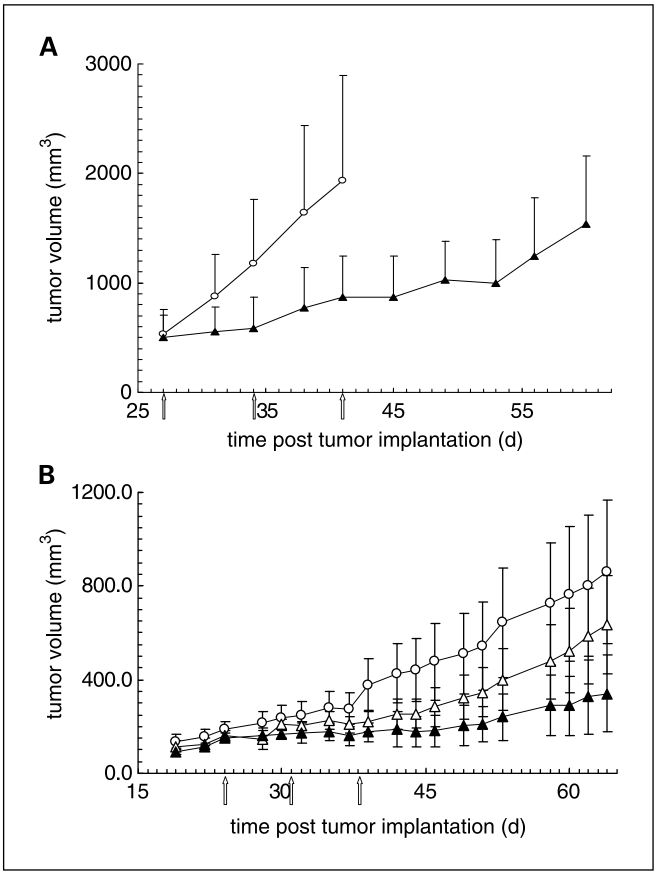

Fig. 4. Antitumor efficacy of minimally PEGylated Ls-LAQ824 (*A*) and anti-HER2-ILs-LAQ824 (*B*) in a human breast (BT-474) tumor xenograft model. Female NCR *nu/nu* mice inoculated s.c. with human breast tumors (BT-474) were treated i.v. with saline (○) or Ls-LAQ824 with poly(PO_4) as a counter-ion (▲), at a dose of 25 mg LAQ824/kg weekly for a total of three injections, beginning on day 27. The liposomes used in this study were minimally pegylated and prepared using the TEA_npoly(PO_4) loading method. *B,* in a second study, the mice were randomized into three groups of four to five animals per group, and treated i.v. with saline (○), Ls-LAQ824 (△), or F5-ILs-LAQ824 (▲), at a dose of 20 mg LAQ824/kg weekly for a total of three injections beginning at day 19 post-tumor cell inoculation. The liposomes used in this study were composed of DSPC/cholesterol/PEG-DSG (3:2:0.3, mol/mol/mol) and were prepared using the TEA_8SOS gradient method as described in Materials and Methods. The control groups for both studies received an equal volume of normal saline. The limited number of tumor-bearing animals in each treatment arm and the greater than anticipated variability in control tumor growth prevented the apparent increase in ILs-LAQ824 efficacy from reaching statistical significance ($P > 0.05$).

Discussion

HDAC inhibitors have been shown to induce differentiation, apoptosis, and growth arrest in cancer cells (7, 8, 10, 41–43). Several hydroxamic acid–based HDAC inhibitors, including LAQ824, have shown potent *in vivo* antitumor activity in animal models with seemingly little host toxicity (18, 19, 42, 44). Against breast cancer cells, HDAC inhibitors have been shown to modulate estrogen receptor and ErbB2 receptor levels as well as the expression of key cell cycle regulatory proteins (1, 2, 42, 45–47). Recently, we have shown that HDAC inhibitors can inhibit ErbB2 promoter activity, repress the synthesis of new ErbB2 mRNA, and accelerate the decay of mature ErbB2 transcripts (1). Among hydroxamates, LAQ824 exhibits exceptional potency for inhibiting ErbB2 promoter and transcript levels (7). In addition to inhibiting ErbB2 mRNA levels, these same HDAC inhibitors are reported to directly alter the chaperone activity of heat shock protein 90, targeting the ErbB2 receptor and other heat shock protein 90 client oncoproteins for proteasomal decay (2, 15). Because HDAC-dependent mechanisms seem to regulate ErbB2 expression at multiple levels, ErbB2-dependent malignancies may be particularly sensitive to HDAC inhibitors like LAQ824. Unfortunately, like other hydroxamates that have entered clinical trials, LAQ824 requires daily dosing due to its rapid (<1 hour) blood clearance. Thus, these HDAC-inhibiting hydroxamates would seem to be excellent candidates for reformulation into long-circulating liposomes, which have been shown to improve the pharmacokinetic properties and enhance the therapeutic index

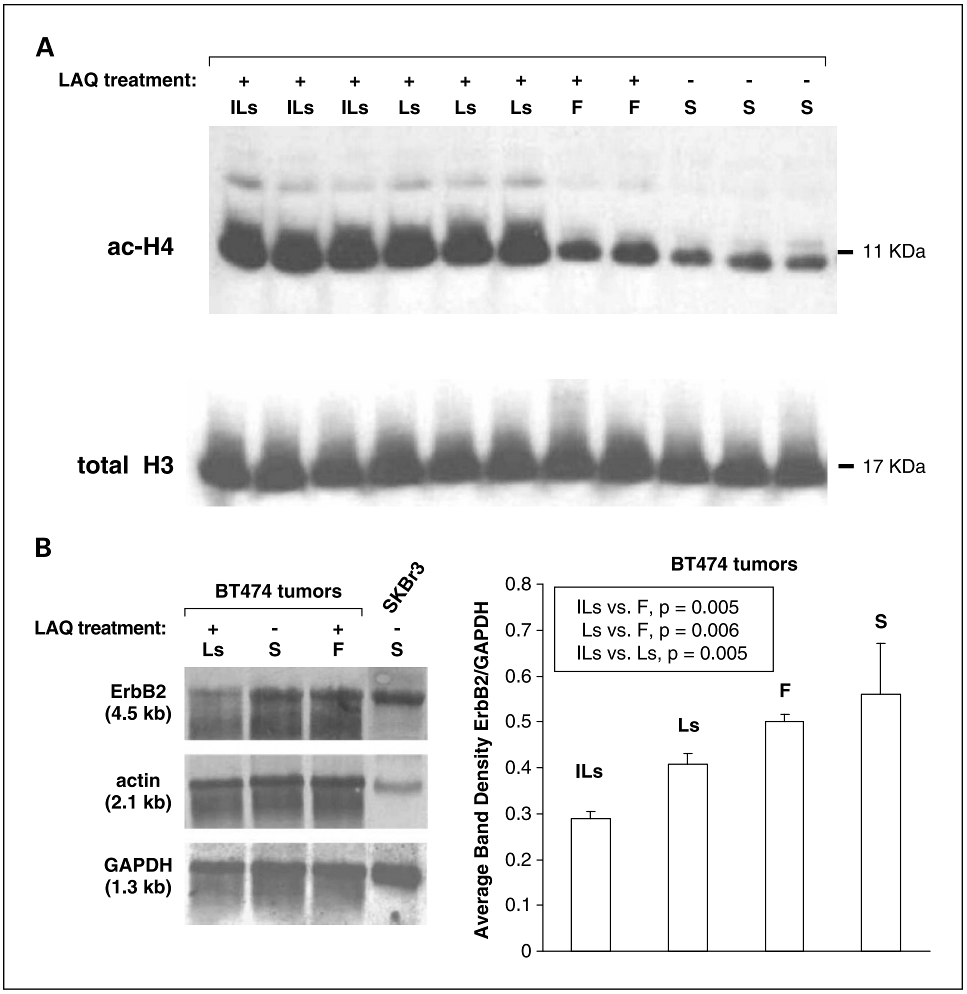

Fig. 5. The effect of treatment with free or liposomal formulations of LAQ824 on acetylated histone levels (A) and ErbB2 RNA transcripts (B). *A,* acetylated histone H4 levels in 11 different human breast (BT474) tumors were determined after treatment with saline (S), free drug (F), Ls-LAQ824 (Ls), or F5-ILs-LAQ824 (ILs) for 24 hours. Western blots of the tumor cell extracts probed with either an anti-acetyl H4 rabbit polyclonal antiserum or an anti-histone H3 rabbit polyclonal IgG. *B,* Northern blot of total RNA from tumor cell extracts of ErbB2-overexpressing BT474 tumors that were treated for 24 hours with saline (S), free LAQ824 (F), or Ls-LAQ824 (Ls). A Northern blot of total RNA purified from saline-treated ErbB2-overexpressing SKBR3 cells is also shown as an internal control for transcript size and integrity. Northern blot mRNA expression levels were determined using a ^{32}P-labeled antisense RNA probe complimentary to human ErbB2 mRNA. Controls used antisense RNA probes complimentary to human Actin or GAPDH mRNA. Columns, means; bars, ±SE of the calculated ErbB2/GAPDG densitometry values for BT474 xenografts treated 24 hours earlier with a single 20 mg/kg i.v. dose of ILs-LAQ824 (ILs; $n = 2$), Ls-LAQ824 (Ls; $n = 4$), free LAQ824 (F; $n = 3$), or saline (S; $n = 2$).

of an increasing number of rapidly cleared cancer therapeutics (21, 22, 48, 49). Because of its structurally unique titratable amine moiety, LAQ824 can be stably loaded into liposomes or immunoliposomes. These liposomal formulations of LAQ824 exhibit a 164-fold reduced *in vivo* clearance rate as compared with free LAQ824. For most long-circulating liposome constructs, maximum tumor accumulation occurs between 24 and 48 hours (22, 49). Thus, as long as the slow rate of drug release from liposomes approximates or is longer than the time required for maximum tumor accumulation of liposomes, then the liposome formulation acts as a sustained drug release system at the tumor site. The half-life of LAQ824 release from liposomes is 26 hours (Fig. 3C), fulfilling this requirement and also accounting for the observed *in vivo* antitumor activity of Ls-LAQ824 with once weekly injections of drug at lower doses than the previously observed higher daily doses of free drug necessary for antitumor activity in tumor xenograft models (19). Furthermore, liposomal LAQ824 seems to be highly active even when treatment is initiated against relatively large tumor masses (~500 mm^3).

In an attempt to assess the relative therapeutic efficacy of liposomal LAQ824 specifically targeted to ErbB2 overexpressing tumors, immunoliposomes were prepared that are capable of specific uptake into the ErbB2 receptor-overexpressing tumor cells that constitute BT474 xenografts (27, 29). These ILs-LAQ824 possess the ErbB2-binding F5 scFv antibody fragment covalently coupled to termini of PEG-derivatized lipids located within the liposomal membrane (28). We have previously shown that F5-ILs are rapidly and efficiently endocytosed into ErbB2-overexpressing tumor cells both *in vitro* and *in vivo*, and that this internalization process accounts for the selectively greater antitumor efficacy of drugs encapsulated within ErbB2-targeted immunoliposomes as compared to drugs encapsulated within nontargeted liposomes (27–29). The efficiency of ErbB2 receptor-mediated internalization by F5-ILs-LAQ824 is shown with the enhanced *in vitro* cytotoxic activity of these immunoliposomes against ErbB2-overexpressing SKBR3 cells, as compared with the activity of the nontargeted Ls-LAQ824 or even the rapidly diffusible free LAQ824 (Fig. 2). This enhanced *in vitro* activity against SKBR3 cells seems consistent with the observed *in vivo* activity of F5-ILs-LAQ824 against the BT474 xenografts (Fig. 4B), where the added pharmacodynamic feature of rapid intracellular drug uptake potentially improves upon the pharmacokinetic advantage of long-circulating liposomal drug, and despite the drug's ultimate intracellular inhibitory effect on tumor ErbB2 receptor expression.

A single *in vivo* injection of either liposomal or immunoliposomal LAQ824 produces a markedly enhanced BT474 tumor level of histone H4 acetylation at 24 hours, as compared with either saline-treated controls or tumors treated with free LAQ824 (Fig. 5A). Thus, the improved pharmacokinetics afforded by liposomal encapsulation of LAQ824 resulted in an enhanced and sustained HDAC-inhibiting molecular target effect within the *in vivo* tumor cells, making it unlikely that other more indirect *in vivo* effects (e.g., antiangiogenic) accounted for the enhanced antitumor efficacy of liposomal LAQ824. Likewise, the dramatic decrease in tumor ErbB2 transcript levels observed within 24 hours of liposomal LAQ824 injection not only supports the evidence for direct intratumor enhancement of bioactivity, but also provides *in vivo* confirmation of the ErbB2 transcript–inhibiting mechanism observed *in vitro* for various HDAC inhibitors including LAQ824 (1, 7).

In summary, stable encapsulation into liposomes or immunoliposomes seems to enhance the pharmacokinetic and pharmacodynamic properties of HDAC inhibitors like LAQ824, reducing the frequency and potentially also the drug dose needed to achieve *in vivo* antitumor effects. Additional *in vivo* studies are needed to compare the maximally tolerated doses of F5-ILs-LAQ824 to Ls-LAQ824 and free LAQ824 for different injection schedules and thus clearly show the magnitude of enhanced therapeutic index achieved by encapsulating and tumor-targeting LAQ824. The ability to deliver antitumor doses of a potent HDAC inhibitor like LAQ824 with only once weekly injections may also be expected to facilitate the testing of sequenced combinations of this HDAC inhibitor with other anticancer agents.

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Enhanced Pharmacodynamic and Antitumor Properties of a Histone Deacetylase Inhibitor Encapsulated in Liposomes or ErbB2-Targeted Immunoliposomes

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