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Probing the mechanism of drug/lipid membrane interactions using Biacore

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Abstract

Assay conditions were established to screen a panel of drugs for binding to liposome surfaces using a surface plasmon resonance (SPR) biosensor. Drugs were found to bind negligibly or reversibly or were retained on the liposome surface. Cationic amphiphilic drugs fell into the last class and correlated with drugs that induce phospholipidosis in vivo. To a first approximation, a single-site model yielded apparent binding affinities that adequately described a drug's dose-dependent binding to liposome surfaces. Affinities ranged at least 1000-fold within the drug panel. A liposome's drug-binding capacity and affinity depended on both the lipid headgroup and the drug's structure. Although a drug's charge state generally dominated whether or not it remained bound to the liposome, subtle structural differences between members of certain drug families led to them having widely differing binding affinities. A comparison between the dissociation of drugs from liposome surfaces by Biacore and the lipid retention measurements determined by a parallel artificial membrane permeability assay was drawn. The results from this study demonstrate the potential of using SPR-based assays to characterize drug/liposome-binding interactions.

Keywords: Plasmon; Permeability; Affinity; Binding; Phospholipidosis

The interaction of a drug candidate with biological membranes is an important aspect of preclinical drug discovery. Since the bioavailability of drugs depends largely on their solubility in cell membranes, the ability to predict or calculate this property would facilitate rational drug design and screening methods. Various methods can be used to characterize drug/lipid interactions in vitro, including microscopy, calorimetry, analytical electrophoresis, analytical ultracentrifugation, circular dichroism, and mass spectrometry. Most of these analytical tools provide only low-resolution information and have a low throughput. In contrast, surface plasmon resonance (SPR)[1] can provide information-rich data on drug/lipid-binding interactions in an automated mode.

The goal of the current SPR-based study was to extend the scope of our previous work that had focused on the interactions of a small drug panel to immobilized phospholipids [1]. Here we explore a much larger panel of diverse drugs varying in molecular weight (from 138.1 to 664.8 Da) and chemical functionality, rank compounds according to their apparent liposome-binding affinity, and compare dissociation phase data with lipid retention measurements obtained from a parallel artificial membrane permeability assay (PAMPA). The results of this study provide examples on how to develop a ranking assay for the passive diffusion of drugs through lipid membranes.

Materials and methods

General

Interaction analysis was performed at 20 °C using a Biacore 2000 biosensor equipped with an L1 sensor chip.

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[1] *Abbreviations used:* CAD, cationic amphiphilic drug; SPR, surface plasmon resonance; PAMPA, parallel artificial membrane permeability assay; DMSO, dimethyl sulfoxide.

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Two consecutive 30-s pulses of 20 mM zwittergent 3–14 detergent followed by 50 mM HCl in 50% v/v isopropanol were applied at a flow rate of 100 μL/min to precondition new sensor chips.

Liposome preparation

Fig. 1 displays the molecular structures of the five different lipids used in this study, which were purchased from Avanti Polar Lipids (Alabaster, AL), in sealed glass ampoules as either chloroform solutions or lyophilized powders. Lipids were chosen for different experiments. For example, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was used to optimize the assay conditions with respect to liposome size, surface capacity, and DMSO concentration because it is considered a standard lipid in liposome research. A large drug panel was analyzed against 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes, since this lipid was used to coat the filter used in PAMPA (see later). To assess the influence of the headgroup on drug/liposome interactions, DOPC and three other lipids were selected that differed only in their headgroup moiety, namely 1, 2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(glutaryl) (glutaryl DOPE).

Lipids were prepared in the same way, regardless of their structure. Typically, a 0.1-mL aliquot of lipid reconstituted in chloroform to a concentration of 30 mg/mL was dried by evaporation under nitrogen and then lyophilized. Lipid films were hydrated at room temperature in a suitable buffer (final 4 mM), which was usually the running buffer used in the interaction analysis. Lipid suspensions were dispensed into glass vials and subjected to four cycles of freezing (−80 °C), thawing (20 °C), and vortexing (5 s) to ensure that they were thoroughly agitated prior to extrusion through a polycarbonate filter of defined pore diameter (typically 100 nm, unless stated otherwise) using an Avanti Mini-Extruder kit. This involved sandwiching a membrane between two syringes, loading the crude lipid suspension into one syringe, and passing it 19 times through the membrane. The extruded product, containing uniformly sized liposomes, was unloaded from the opposing syringe to ensure that it was free of contaminants found in the loading syringe.

Liposome captures

Typically, liposomes were diluted in running buffer to 2 mM and captured to saturation (5 min) across isolated flow cells at 2 μL/min. Lipid titrations were performed by injecting a liposome concentration series spanning a wide range (typically 4000-fold) prepared by twofold dilutions into running buffer. The flow rate was switched to 100 μL/min and fresh lipid surfaces were washed by applying three 30-s pulses of buffer. Unmodified (lipid-free) flow cells served as reference and control surfaces.

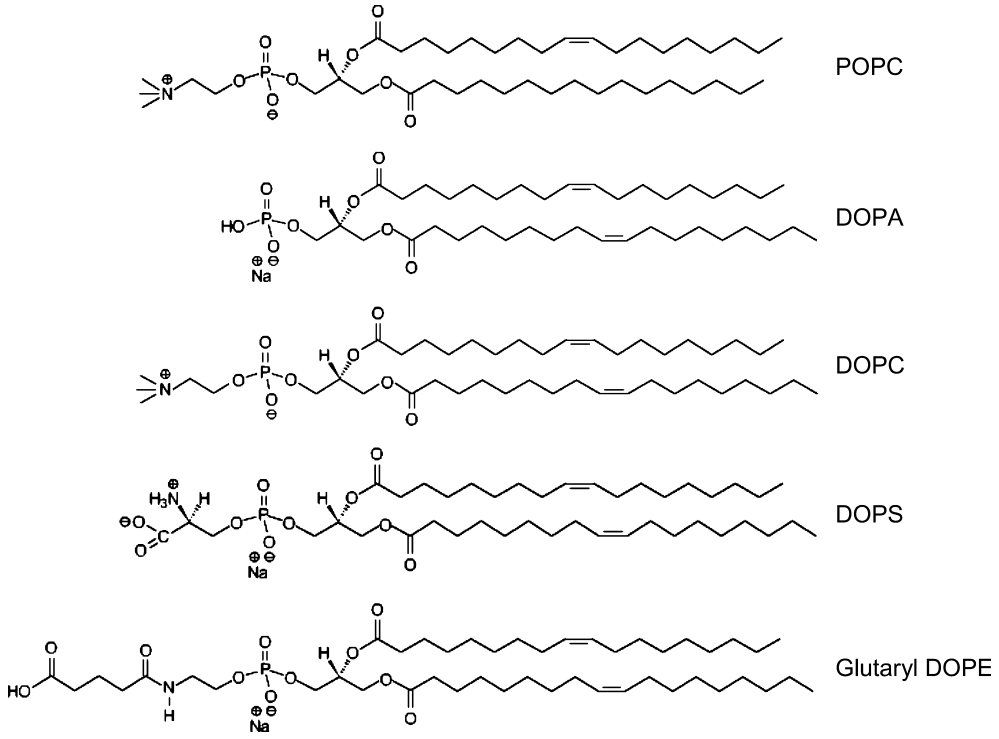

Fig. 1. Molecular structures of the five lipids used in this study.

Drug-binding analyses

All drugs were purchased from Sigma–Aldrich (St. Louis, MO, USA), except formoterol, which was purchased from Tocris (Ellisville, MO, USA). Unless stated otherwise, all data were collected in a proprietary *p*ION system solution (*p*ION, Woburn, MA) supplemented with 3% v/v DMSO and adjusted to pH 5.5, to mimic the experimental conditions used in PAMPA. The acidic pH intentionally simulated the environment of the gastrointestinal tract, which is often used to model absorption in natural biological systems. Depending on their solubility, drugs were dissolved either directly into the running buffer to a concentration of 1 mM or in 100% DMSO to > 10 mM. Drugs prepared by the latter method were then diluted into DMSO-free running buffer to give a final drug concentration of 1 mM and a DMSO concentration that matched the running buffer, e.g., 3:100 v/v to obtain a final concentration of 3% v/v DMSO. Either way, drug solutions at 1 mM were diluted twofold and serially into running buffer to give a concentration series spanning a 30-fold range, typically 15.6–500 μM. Drug samples were dispensed into single-use snap-capped vials, randomized in the rack base, and injected across lipid and lipid-free surfaces in a single step. Highly resolved analyses were conducted using duplicate measurements of a wider drug concentration series. Association and dissociation phases were monitored at 100 μL/min. After each binding cycle, the sensor surface was regenerated to the original matrix by injecting either 1:1 v/v isopropanol/100 mM HCl (to remove phosphatidylcholine-based lipids, POPC or DOPC) or 4:6 v/v isopropanol/50 mM NaOH (to remove any of the five lipids tested). The sensor surface was recoated with a fresh liposome solution for the next binding cycle.

Data analysis

Response data collected across the reference surface was used to correct for instrumental artifacts that are common to all flow cells such as bulk refractive index changes, matrix effects, and noise. Subtracting an average buffer response from all sample responses within each flow cell eliminated artifacts that were flow cell

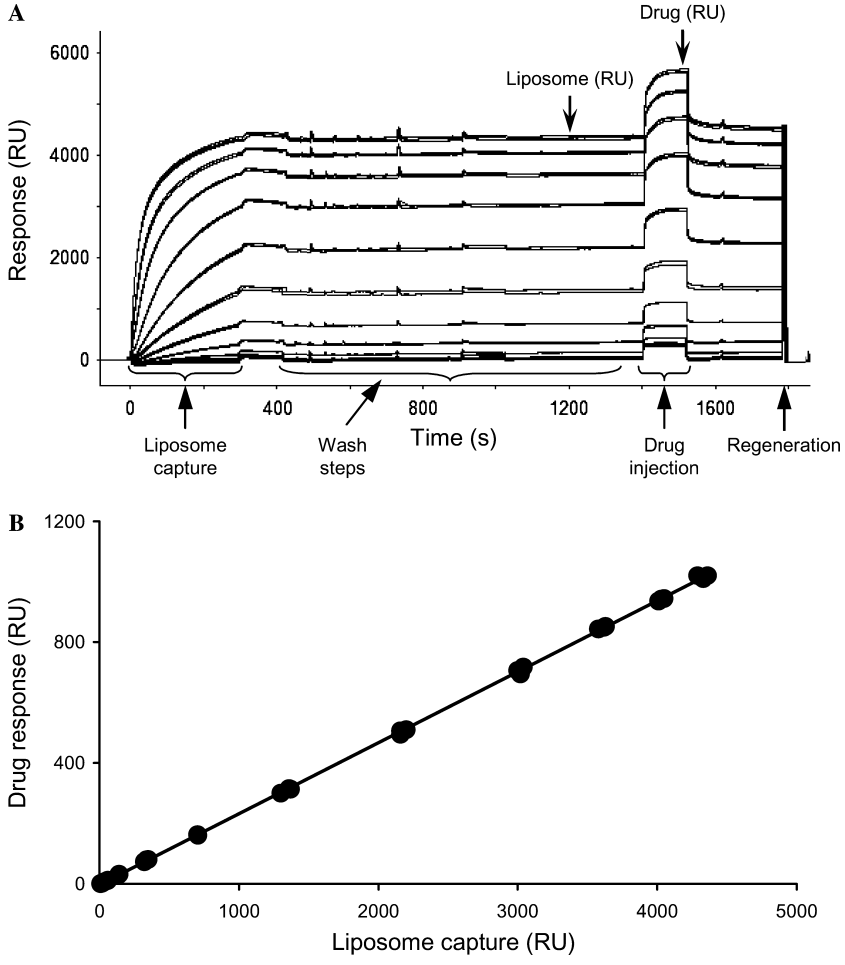

Fig. 2. Effect of surface capacity on drug/liposome-binding interactions. (A) Triplicate analysis of 500 μM homochlorcyclizine across POPC liposomes at varying capture levels (from 9 to 4330 RU). (B) Drug-binding capacity of liposome surfaces as a function of their lipid capture level.

dependent. Since the drug-binding responses (RU_{drug}) were linearly related to the lipid capture level (RU_{lipid}, Fig. 2B), an appropriate correction for cycle-to-cycle variability in lipid capture levels was achieved by dividing RU_{drug} by RU_{lipid}. When comparing different drugs, response data were also divided by the molecular weight of the drug under investigation (MW_{drug}). Normalized response data were then multiplied by an arbitrary scaling factor of 10^6 to return the value to one that resembled an "RU." "Scaled responses" were therefore given in units of $10^6\,RU_{drug}/(MW_{drug}\,RU_{lipid})$.

Apparent binding affinities (K_D) for drug interactions with liposome surfaces were estimated by plotting equilibrium drug-binding responses (RU) as a function of injected drug concentration (C) and fitting a single-site binding isotherm, $RU = R_{max}/((K_D/C)+1)$, where R_{max} = maximum surface-binding capacity.

An estimate of a drug's "lipid retention" was provided by focusing on only the dissociation phase of the binding cycle. The amount (RU) of drug that remained bound after it was allowed to dissociate from the liposome surface for 30 s was compared to lipid retention measurements made by PAMPA.

PAMPA measurements

The retention of 85 drugs in phospholipid membranes was determined by *p*ION using a parallel artificial membrane permeability method [2] at pH 5.5 in a proprietary *p*ION system solution supplemented with 3% v/v DMSO. The assay was conducted at room temperature and all compounds were filtered after dilution into the buffer solution. DOPC in dodecane was immobilized onto a filter and sandwiched between donor and acceptor buffer compartments. Drugs were introduced into the donor compartment and allowed to diffuse passively through the membrane into the acceptor compartment. Following the permeation period, retention by the membrane was estimated by mass balance by measuring the concentration of the compound in the aqueous acceptor and donor compartments and comparing these measurements with a separate reference solution. Triplicate measurements were made. Drug concentrations were determined by detecting UV–VIS absorbance, scanning between wavelengths of 190 and 500 nm.

Results

Capacity of lipid surfaces

To test how the drug-binding capacity of a lipid surface was related to the amount of liposome captured, drugs were monitored at a single concentration across varying-capacity liposome surfaces. The binding of 500 μM homochlorcyclizine to fresh surfaces of 9–4,330 RU POPC serves as an example (Fig. 2). Liposomes were hydrated in 50 mM Hepes, 150 mM NaCl, pH 7.4, and prepared as a twofold concentration series spanning 750–0.18 μM. Liposome captures were highly reproducible at each concentration tested, as shown by the excellent overlay of triplicate binding cycles in Fig. 2A. Drug binding was analyzed in 10 mM Hepes, 150 mM NaCl, 0.1% v/v DMSO, pH 5.5, and found to be linearly related to the amount of liposome captured on the surface (Fig. 2B). From a technical standpoint, this

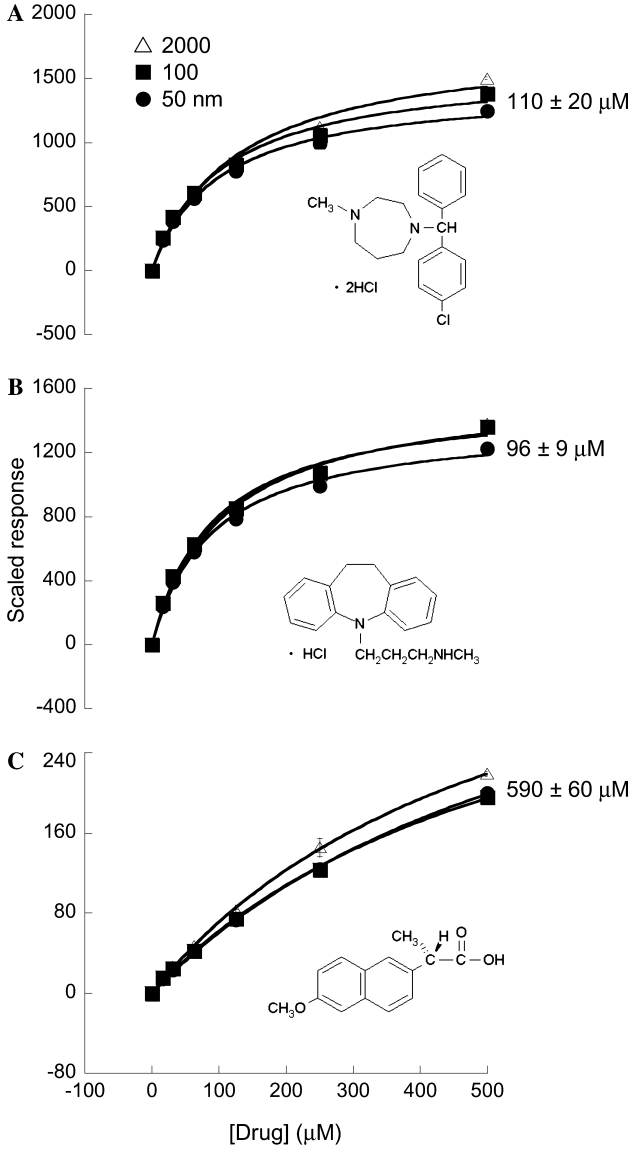

Fig. 3. Effect of liposome diameter (50, 100, or 2000 nm) on the binding of three drugs: (A) homochlorcyclizine, (B) desipramine, and (C) naproxen. Symbols and error bars represent the mean equilibrium-binding drug response and standard deviation for duplicate determinations of drugs at 0, 15.6, 31.3, 62.5, 125, 250, and 500 μM across differently sized POPC liposomes. Trend lines represent the fitted equilibrium-binding isotherms for each drug/liposome pair. Mean apparent binding affinities are provided for each drug, where the standard deviation is for the three different liposome preparations.

had two useful consequences. First, drugs could be analyzed across any capacity surface resulting from liposome captures exceeding approximately 200 RU (below which the correlation broke down). Second, cycle-to-cycle variability in the amount of lipid captured could be corrected for by dividing the observed drug-binding response by the lipid capture level.

Effect of liposome size on drug/liposome-binding interactions

To test whether a liposome's size influenced drug binding, POPC lipids were hydrated in 50 mM NaH_2PO_4, 150 mM NaCl, pH 7.0, and extruded through filters containing pores of differing defined diameters of 0.05, 0.1, and 2 μM. Liposomes prepared by extrusion are slightly larger (20–50%) than the average pore size of the filter. All three liposome preparations were captured to similar levels (~9000 RU) onto individual flow cells. Three drugs, namely homochlorcyclizine, desipramine, and naproxen, were selected for analysis based on their differing kinetic profiles and liposome-binding affinities. Each drug was monitored at concentrations of 0, 15.6, 31.3, 62.5, 125, 250, and 500 μM across all surfaces simultaneously in *p*ION system solution, 0.1% DMSO, pH 5.5. Drugs rapidly attained equilibrium binding and showed dose-dependent responses that were modeled by a simple binding isotherm (Fig. 3).

Homochlorcyclizine and desipramine both bound with high affinity (K_D ~100 μM) and dissociated slowly from liposomes, while naproxen bound weakly (K_D ~600 μM) and dissociated rapidly. Despite a 40-fold difference in liposome diameter (equivalent to a 64,000-fold difference in volume), the binding affinity of each drug was relatively unaffected and was identical for the three lipid preparations within the errors associated with their fits (see Fig. 3). However, the binding capacity of liposomes appeared to increase slightly with liposome size. Filters containing a 100-nm pore diameter were chosen for the preparation of liposomes (120–140 nm) in all further experiments since extrusion through them was physically easy, they are commonly used in lipid research, and pharmacokinetic studies suggest that liposomes of this size are optimal for drug delivery [3].

Effect of DMSO on drug/liposome-binding interactions

The running buffer was supplemented with an organic solvent to carry multifarious drugs that would otherwise be insoluble at concentrations relevant to the drug-binding assay (up to 500 μM drug). Although DMSO is the usual solvent for biosensor assays, its effect on drug/liposome-binding interactions was unknown. To test how much DMSO the assay could tolerate, desipramine was injected across POPC liposomes in four replicate experiments, differing only in the concentration of DMSO (0.1, 1, 5, or 10% v/v) used in the running buffer (*p*ION system solution; pH 5.5). Lipids were hydrated in running buffer to match the vesicle interior with the bulk buffer. Duplicate injections of desipramine, spanning concentrations of 1–500 μM in twofold increments, were analyzed across freshly deposited liposomes.

Increasing the concentration of DMSO in the running buffer 100-fold, from 0.1 to 10% v/v, had two effects on drug/liposome binding. The amount of lipid captured and hence the surface's drug-binding capacity decreased 30% (Fig. 4A), which was substantial but irrelevant for the comparative analysis of drug-binding constants, since the affinity of the desipramine/POPC-binding interaction weakened only twofold from 66 ± 7 to 102 ± 6 μM (Fig. 4B). Further drug-binding analyses were conducted in 3% v/v DMSO as a compromise between surface capacity and solubilizing power.

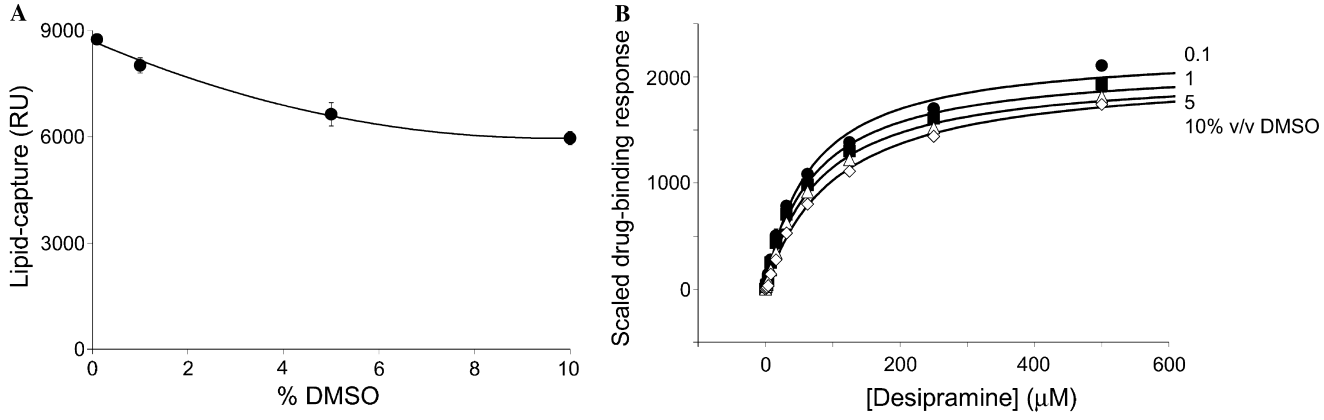

Fig. 4. Effect of DMSO concentration on desipramine/POPC liposome-binding interactions. (A) DMSO-dependent liposome captures, where the error bars represent the variability within 22 capture cycles per experiment. (B) Duplicate equilibrium-binding analyses of 1–500 μM desipramine in running buffer containing varying concentrations of DMSO (0.1, 1, 5, or 10% v/v). Symbols and error bars represent the mean values and standard errors for duplicate determinations. Trend lines indicate the fitted equilibrium-binding isotherms.

Analysis of a large panel of drugs binding to liposomes

The scope of the drug/lipid-binding assay was broadened in an effort to generate a drug database from which comparisons could be drawn between SPR and permeability measurements using PAMPA (discussed later). The two assays were conducted using the same lipid (DOPC) and proprietary buffer (*p*ION system solution, 3% v/v DMSO, pH 5.5). Compounds amenable to both techniques were chosen in assembling a large drug panel. The PAMPA method demanded that compounds absorbed UV–VIS light within the range 190–500 nm and were soluble as 10 mM stock solutions in 100% DMSO. A total of 85 compounds spanning molecular weights of 138.1 Da (salicylic acid) to 664.8 Da (metoprolol), representing various chemical functionalities, and belonging to different therapeutic groups, were selected based on these two criteria.

Using Biacore, a concentration series of each drug (typically spanning 16–500 μM in twofold increments) was analyzed across DOPC liposome surfaces (typically ~7000 RU). Three distinct liposome-binding behaviors were discerned: (1) nonbinders, (2) reversible binders, and (3) drugs that formed stable liposome complexes. Fig. 5 depicts five drugs that were binned to each class. No liposome binding was detected for some drugs (Fig. 5A) even when compounds were injected at concentrations as high as 500 μM. Drugs that bound to liposome surfaces differed markedly in their dissociation phase, which was found to be either rapid or extremely slow. Fig. 5B depicts the former behavior that gave rise to transient complexes. In contrast, Fig. 5C depicts the latter behavior, namely the formation of a very stable drug/liposome complex: these drugs were likely retained in the lipid membrane by intercalation or liposome trapping. The transition from reversible to stable binders was a little floating (compare dapsone with norclomipramine, Figs. 5B and C, respectively). Drugs were considered to be stable binders if they did not dissociate fully from the lipid surface after 3 min.

Drugs bound DOPC liposomes not only with differing kinetic profiles but also with varying capacities and affinities. Dose-dependent drug responses were modeled using a single-site equilibrium-binding isotherm. To a first approximation, the calculated affinities served as a simple way by which to rank the drugs within the panel. Affinities ranged at least 1000-fold within the drug panel. Fig. 6 shows that approximately half the drugs tested had measurable binding affinities (from 10 μM to 2 mM) for DOPC liposomes, whereas the remainder showed weaker interactions that could not be determined accurately. Drugs with the highest affinities formed very stable complexes with liposomes and dissociated extremely slowly from them: their profiles resembled those shown in Fig. 5C. Structurally, they were cationic amphiphilic drugs (CADs) that fell into structural families, such as phenothiazines (e.g., chlorpromazine, perphenazine, and thioridazine) and isoflavone derivatives (e.g., genistein, nafoxidine, raloxifene, and tamoxifen). Chlorinated drugs (e.g., norclomipramine and clomipramine) were found to bind with twice the affinity of their nonchlorinated analogs (respectively, desipramine and imipramine).

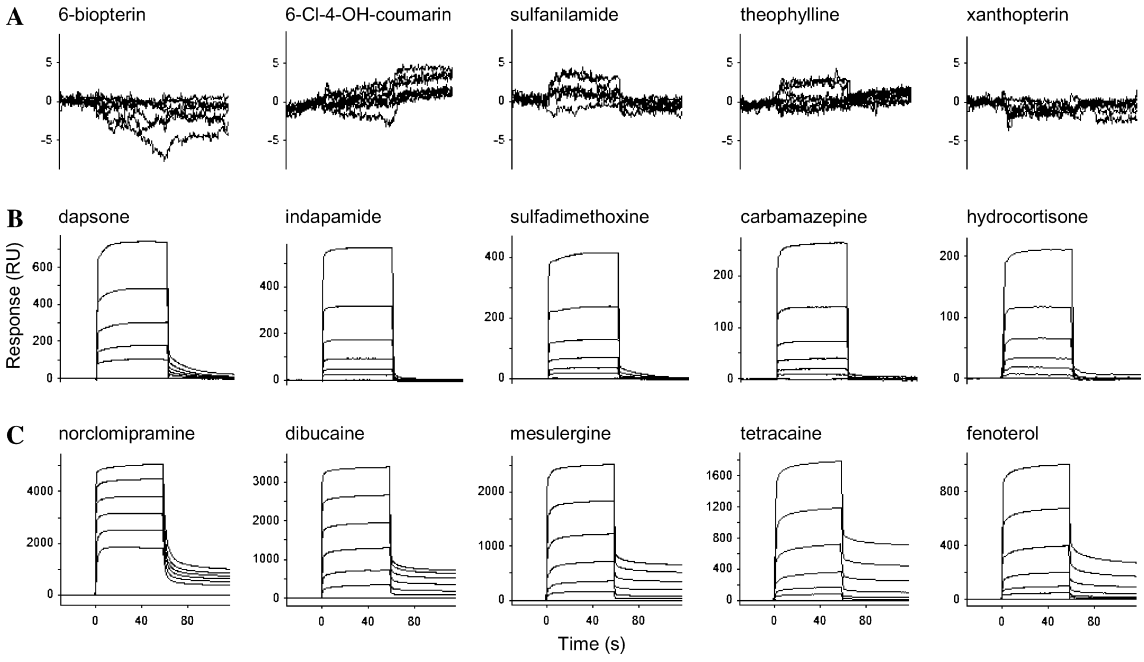

Fig. 5. Representative examples of drug-binding responses collected across DOPC liposome surfaces. Three different behaviors were discerned, namely (A) no binding (B) reversible binding, and (C) drugs that formed stable lipid complexes. Drugs were analyzed across a twofold concentration series typically spanning 15.6–500 μM.

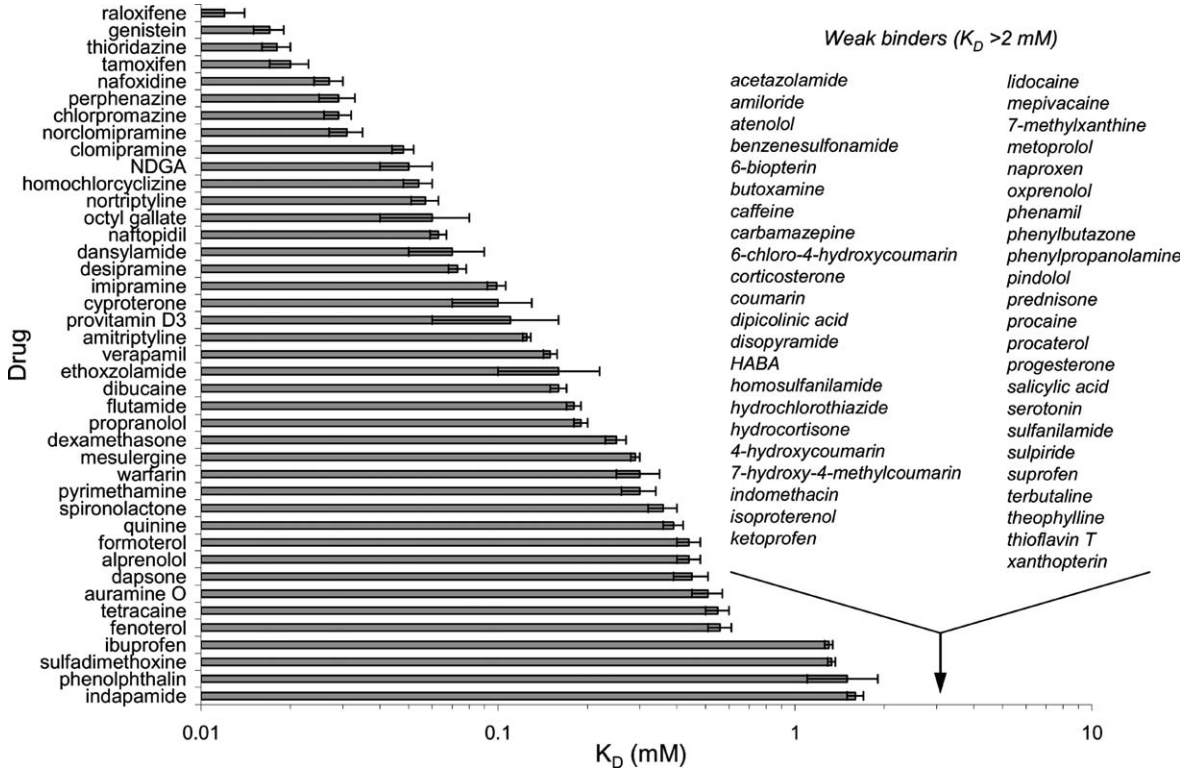

Fig. 6. Drugs ranked by their apparent liposome-binding affinities. Error bars indicate the standard error for each fit.

PAMPA analysis

The Biacore assay had revealed marked differences in the way that different drug types dissociated from liposomes. The entire drug panel was therefore analyzed under similar conditions (with respect to the lipid and buffer) by PAMPA, a complementary biophysical tool that measures lipid retention. The goal was to understand why Biacore showed that some drugs dissociated extremely slowly for lipids, while others dissociated rapidly, in addition to why a lipid (DOPC) varied widely in its capacity for various drug types. By focusing on only the dissociation phase of a drug/liposome-binding interaction measured by Biacore, a comparison was made with PAMPA-based measurements (Fig. 7).

There is reasonable agreement between the two types of measurements for the majority of compounds, despite the inherent differences in the assays. We do not suggest that the relation between SPR- and PAMPA-derived data can be described mathematically, but taken together, the two techniques shed light on the way in which drugs bind lipid membranes. For example, 14 drugs that showed no binding in the Biacore assay (in neither their association nor dissociation phases) were also not retained by PAMPA (0–4%): acetazolamide, atenolol, benzenesulfonamide, 6-biopterin, caffeine, 6-chloro-4-hydroxycoumarin, dipicolinic acid, 4-hydroxycoumarin, 7-methylxanthine, salicylic acid, sulfanilamide, sulpiride, theophylline, and xanthopterin. No drugs appeared to bind by Biacore and not by PAMPA, since the lower right quadrant of the plot (Fig. 7) is vacant. However, a few compounds appeared to bind by PAMPA but not by Biacore (Fig. 7, upper left quadrant, circled) because, although they showed a measurable binding response when injected across liposome surfaces, they dissociated rapidly. The two most highly bound drugs analyzed by Biacore, tamoxifen and

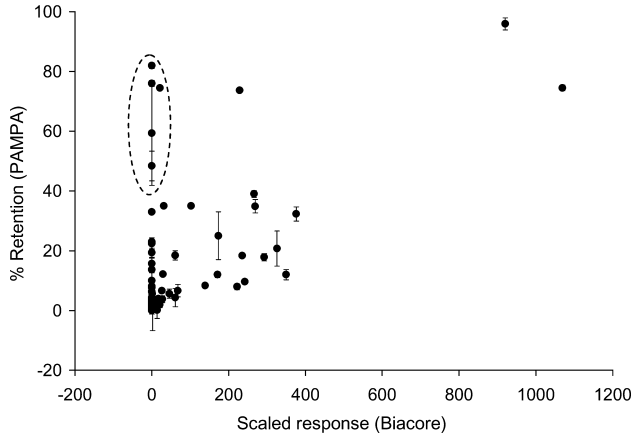

Fig. 7. SPR and PAMPA comparison. Biacore data were obtained from drugs screened at 31 μM. The amount (RU) that remained bound to the liposome surface after 30 s dissociation provided a measure of its "retention" in the liposome preparation. Responses were scaled by the lipid's capture level and the molecular weight of the relevant drug. *y* axis error bars represent the standard deviation for triplicate PAMPA measurements.

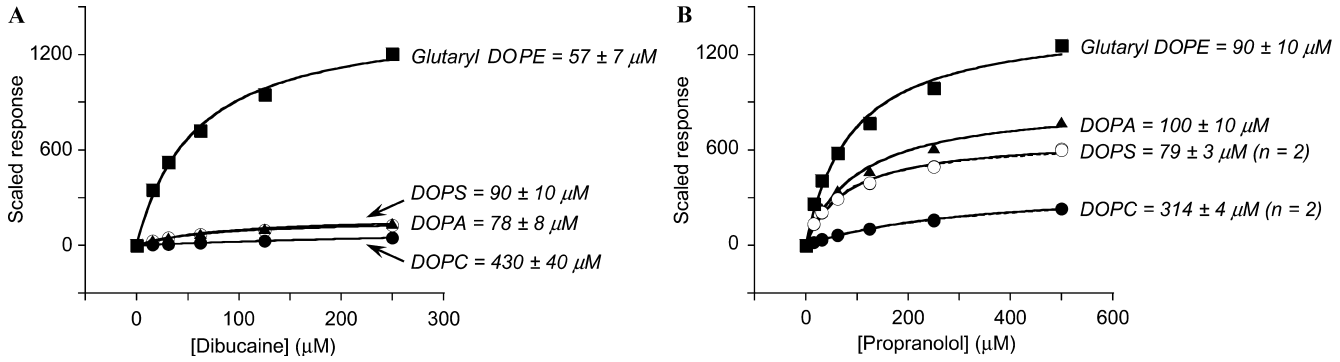

Fig. 8. Comparison of various lipid headgroups. The two test drugs were (A) dibucaine and (B) propranolol. Trend lines indicate the fitted equilibrium-binding isotherms from which binding affinities were estimated (as indicated). Values for propranolol binding to DOPC and DOPS surfaces were each determined from two independent experiments ($n = 2$), where the standard deviation is for the duplicates. The standard deviations for all other experiments are for the fits themselves.

nafoxidine, which had high liposome-binding affinities of $K_D = 20 \pm 3\,\mu M$ and $K_D = 27 \pm 3\,\mu M$, respectively (Fig. 6), were highly retained by PAMPA (95.9 and 74.5%, respectively). Both drugs are positively charged under the assay conditions (pH 5.5) due to their tertiary amine groups ($pK_a \sim 9$). Comparing our Biacore results with PAMPA measurements enabled us to interpret the former. Drugs that formed a stable complex with surface-tethered liposomes on Biacore and remained tightly bound during the dissociation phase (see Fig. 5C) were likely intercalated in the lipid membrane, as suggested by their high retention according to PAMPA.

Effect of lipid headgroup on drug/lipid-binding interactions

To test how the lipid headgroup influenced the way in which liposomes bound drugs, compounds were tested against four lipids that shared an identical (symmetric) pair of acyl chains but had variable headgroups (phosphate, choline, serine, and glutaryl ethanolamine moieties). Equivalent levels of each liposome preparation (~4000 RU) were captured onto individual flow cells.

Two drugs (dibucaine and propranolol) were chosen for this work based on their previous analysis with DOPC in the *p*ION system solution (as reported in Figs. 5C and 6). They were readily soluble in 3% v/v DMSO and had formed stable, high-affinity complexes ($K_D = 160 \pm 10\,\mu M$ and $K_D = 190 \pm 10\,\mu M$, respectively) with large, easily measured binding responses. Both drugs were now analyzed at concentrations spanning 15–500 μM in a different buffer (50 mM NaH_2PO_4, 150 mM NaCl, 3% v/v DMSO, pH 5.5) across liposomes generated from DOPA, DOPC, DOPS, and glutaryl DOPE lipids. Their dose-dependent liposome-binding responses were overlaid, as shown in Fig. 8. The most striking observation was that liposomes prepared from lipids containing different headgroups showed varying drug-binding capacities and affinities. The glutaryl DOPE headgroup had the highest binding capacity for both drugs tested. Although different lipid headgroups had varying capacities for a given drug, some had similar affinities (e.g., compare glutaryl DOPE with DOPA), which implied that a Biacore drug/lipid-binding assay can be tailored toward maximum sensitivity by choosing an appropriate lipid.

Another observation from this experiment was that drug/lipid-binding interactions were buffer dependent, since the affinities obtained for dibucaine and propranolol analyzed in a phosphate-based buffer ($K_D = 430 \pm 40\,\mu M$ and $K_D = 314 \pm 4\,\mu M$, respectively, Fig. 8) were twofold weaker than those obtained in the proprietary *p*ION system solution ($K_D = 160 \pm 10\,\mu M$ and $K_D = 190 \pm 10\,\mu M$, respectively, Fig. 6).

Discriminating between members of a drug family using Biacore

The biosensor was able to discriminate between members of a structurally related drug family or therapeutic group. The series of arylethanolamines depicted in Fig. 9 serve as an example. Although all eight compounds existed as cations ($pK_a \sim 6$–7) at pH 5.5, they displayed varying capacities and affinities for liposomes, ranging from stable complexes (e.g., fenoterol and formoterol) to transient ones (e.g., procaterol and salbutamol). This set of compounds illustrated how not only the charge but also the ring substituents and their relative positions affected the overall ability of a compound to bind liposomes. Presumably, this is due to a combination of resonance and inductive effects and the varied potential of a compound to form intra- and intermolecular hydrogen bonds. Another example of differences discerned within a drug family is provided by the study of six β-blocker compounds that existed as cations at neutral pH (Fig. 10), which share one or more aromatic rings and a 3-amino-2-hydroxypropoxy side chain ($pK_a \sim 9.5$). All but one of them formed a stable liposome complex in the Biacore assay: no binding was detected for atenolol.

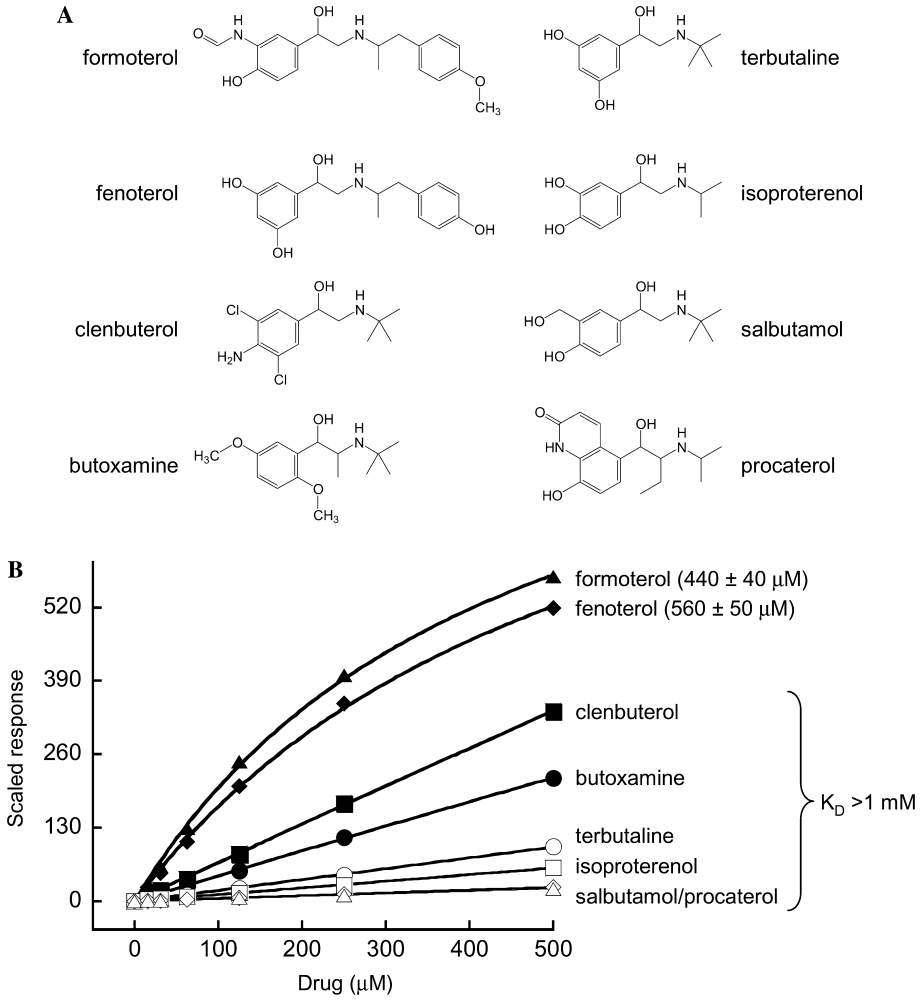

Fig. 9. Discriminating between structurally related compounds. (A) Molecular structures of eight arylethanolamines. Clenbuterol and salbutamol were not included in the drug panel that was tested by PAMPA. (B) Overlay plot of drug/liposome equilibrium-binding isotherms. Only two compounds within this set had measurable liposome-binding affinities (their apparent K_D values are provided along with the standard deviations for the fits).

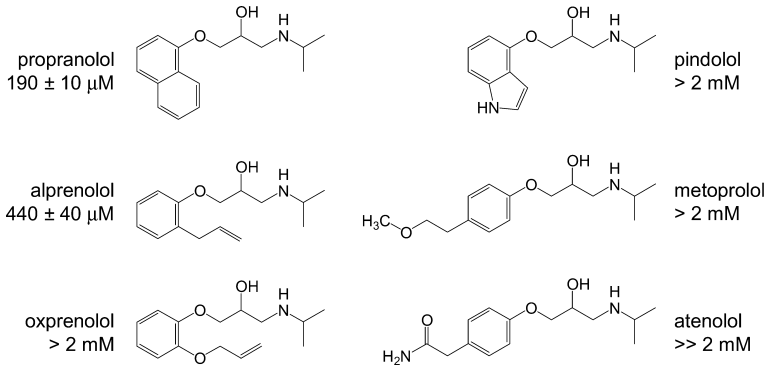

Fig. 10. Molecular structures and liposome-binding affinities of six structurally related β-blocker compounds.

Discussion

The goal of this study was to characterize the binding interactions of drugs with liposome surfaces using SPR biosensors. The biosensor method proved to be a powerful tool in discerning distinct classes of drug-binding behaviors, namely nonbinders, transient binders, and drugs that formed very stable complexes as characterized by a very slow dissociation rate. Electrostatic interactions appeared to dominate drug/lipid binding because

positively charged compounds generally remained bound to liposomes. A comparative analysis by PAMPA revealed that these drugs were highly retained by the lipid bilayer. This suggested that the high-affinity complexes observed by Biacore were due to drugs intercalating into the lipid bilayer.

The Biacore assay allowed drugs to be ranked according to their equilibrium-binding affinities. Drugs with the highest affinities for liposome surfaces dissociated from them extremely slowly, presumably because they had intercalated. These compounds were representative of CADs because they incorporated both hydrophobic and hydrophilic moieties into their structure, commonly an aromatic core and a side chain bearing a protonated amine group ($pK_a \sim 9$–10) under the assay conditions (pH 5.5). Such compounds are known to induce phospholipidosis, a lipid storage disorder that is characterized by the formation of a nonmetabolizable drug/phospholipid complex [4]. We speculate that the lipidosis-inducing potential inherent to a CAD may be diagnosed in the biosensor assay by drugs that bind with high affinity to liposomes and dissociate from them extremely slowly.

Tricyclic antidepressants typify CAD-like compounds, e.g., amitriptyline, nortriptyline, imipramine, desipramine, and their chlorinated analogs, clomipramine, and norclomipramine. All these compounds formed stable liposome complexes with high affinities (ranging from 57–125 μM). In contrast, carbamazepine, which shares a similar aromatic core but lacks an ionizable amine group, formed a transient complex with low affinity (>2 mM) by Biacore. This implied that electrostatic interactions between a drug's ionizable side chain and a phospholipid's polar headgroup were primarily responsible for the stable, high-affinity complexes observed for CAD-like compounds.

Drugs that formed a transient complex with liposome surfaces by Biacore were typically either neutral or anionic due to an ionizable acidic group ($pK_a < 5.5$), e.g., ibuprofen, ketoprofen, naproxen, suprofen, and warfarin. Fourteen drugs that bound negligibly by Biacore were not retained by PAMPA (0–4%).

The ranking of phospholipid-binding affinities determined within several drug families in the current study concurred with those obtained in an independent study of CAD-like compounds binding to phospholipid monolayers by Lullman et al. [5]. For example, in the current study, the affinities of four local anesthetics were determined to be $K_D > 2$ mM (procaine and lidocaine), $K_D = 550 \pm 50$ μM (tetracaine), and $K_D = 163 \pm 8$ μM (dibucaine) (see Fig. 6). Lullman et al. ranked the affinities of these compounds in a similar manner and reported that procaine bound 100-fold weaker than dibucaine. They also tested three β-blocker compounds and noted that atenolol had a 100-fold weaker affinity than propranolol with metoprolol taking an intermediate position. This agreed with the result of our Biacore assay because propranolol bound with high affinity (190 ± 10 μM), metoprolol bound weakly (>2 mM), and atenolol showed negligible binding. Atenolol was also an outlier (for unknown reasons) in another study that investigated the relationship between the structure and the lipophilicity of a panel of β-blockers [6]. Lullman et al. also showed that three psychotropic drugs (chlorpromazine, amitriptyline, and imipramine) had similar binding affinities: we determined them to be 93 ± 6, 99 ± 7, and 125 ± 4 μM, respectively, by Biacore. Lullman et al. suggested that drug/lipid affinity estimates enable tentative predictions about the potency of compounds to induce phospholipidosis in vivo to be made. By analogy, we speculate that the potential for a drug to exert this effect can be predicted from whether it remains bound to the lipid surface with high affinity in the Biacore assay.

A drug's liposome-binding behavior was dictated by an interplay of structural factors reflecting not only the drug's charge state but also its unique blend of functional groups. Mechanistically, drug/lipid interactions are complex since lipid vesicles present multiple binding sites due to their amphiphilic nature and can entrap drugs. The exact configuration of the liposome on the biosensor is controversial and depends on the assay conditions. Some investigators believe that immobilized vesicles remain intact [7] while others argue that they fuse to form a lipid bilayer [8]. Regardless of the exact nature of the lipid surface used in this study, the results demonstrate that drug/liposome-binding interactions were unaffected by the size of the lipid vesicles (differing 64,000-fold in volume) but strongly affected by buffer composition (e.g., *p*ION system solution vs. a simple PBS or HBS system) and the lipid headgroup. Bearing in mind these sources of variability, the use of the SPR assay lies in its ability to discern distinct liposome-binding behaviors of drugs, especially when a therapeutic or structural drug family is investigated. The ability to estimate affinity constants and monitor dissociation rates of drugs from lipid layers could also provide an early predictive tool for phospholipidosis.

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