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(54) **METHODE DE DETECTION DE LA TOXICITE CELLULAIRE
POTENTIELLE DE COMPOSES**

(54) **METHOD FOR DETECTING POTENTIAL CELLULAR
TOXICITY OF COMPOUNDS**

(57) L'invention concerne une procédure d'essai pour le criblage de la toxicité cellulaire potentielle d'un composé d'essai, comprenant les étapes suivantes: (a) fabrication de liposomes à partir d'un mélange de lipides, pour la simulation d'une composition de membrane cellulaire à analyser, les liposomes étant fabriqués dans un milieu aqueux ayant un pH correspondant ainsi au pH intérieur des liposomes; (b) incorporation des liposomes à un milieu d'essai aqueux ayant un pH d'au moins environ 0,5 pH unités supérieur au pH intérieur des liposomes, un gradient de pH existant entre le milieu d'essai et l'intérieur des liposomes; (c) incorporation d'un pigment fluorescent au milieu d'essai, le pigment ayant tendance à pénétrer dans les liposomes en raison du gradient de pH et étant sensible au pH de sorte que la fluorescence du pigment soit éliminée à l'intérieur des liposomes; (d) incorporation du composé d'essai au milieu d'essai; si le composé d'essai interrompt la structure des liposomes ou induit la dissipation du gradient de pH entre le milieu d'essai et l'intérieur des liposomes, la fluorescence du pigment n'est pas éliminée; (e) détermination de la fluorescence du milieu d'essai et comparaison avec celle des témoins.

(57) The subject invention involves a test procedure for screening for potential cellular toxicity of a test compound comprising the following steps: a) making liposomes from one or a mixture of lipids to simulate a cell membrane composition of interest, the liposomes being made in an aqueous medium having a pH which is thereby the inside pH of the liposomes; b) incorporating the liposomes in an aqueous test medium having a pH at least about 0.5 pH units above the inside pH of the liposomes, whereby a pH gradient exists between the test medium and the insides of the liposomes; c) incorporating a fluorescent dye in the test medium, the dye having a tendency to penetrate into the liposomes due to the pH gradient, the dye being pH sensitive such that the fluorescence of the dye is quenched inside the liposomes; d) incorporating the test compound in the test medium; whereby, if the test compound disrupts the structure of the liposomes or otherwise causes dissipation of the pH gradient between the test medium and the insides of the liposomes, the fluorescence of the dye is not quenched; e) determining the fluorescence of the test medium and comparing the determination with those of controls.

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(21) International Application Number: PCT/IB99/00998 (22) International Filing Date: 1 June 1999 (01.06.99) (30) Priority Data: 60/087,977 4 June 1998 (04.06.98) US (71) Applicant (for all designated States except US): THE PROC- TER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): GRINIUS, Leonas, Leono [LT/US]; 5810 Squire Hill Court, Cincinnati, OH 45241 (US). MORRIS, Charles, McClellan [US/US]; 12066 Free- stone Court, Cincinnati, OH 47240 (US). (74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217-1087 (US).	(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: METHOD FOR DETECTING POTENTIAL CELLULAR TOXICITY OF COMPOUNDS		
(57) Abstract <p>The subject invention involves a test procedure for screening for potential cellular toxicity of a test compound comprising the following steps: a) making liposomes from one or a mixture of lipids to simulate a cell membrane composition of interest, the liposomes being made in an aqueous medium having a pH which is thereby the inside pH of the liposomes; b) incorporating the liposomes in an aqueous test medium having a pH at least about 0.5 pH units above the inside pH of the liposomes, whereby a pH gradient exists between the test medium and the insides of the liposomes; c) incorporating a fluorescent dye in the test medium, the dye having a tendency to penetrate into the liposomes due to the pH gradient, the dye being pH sensitive such that the fluorescence of the dye is quenched inside the liposomes; d) incorporating the test compound in the test medium; whereby, if the test compound disrupts the structure of the liposomes or otherwise causes dissipation of the pH gradient between the test medium and the insides of the liposomes, the fluorescence of the dye is not quenched; e) determining the fluorescence of the test medium and comparing the determination with those of controls.</p>		

METHOD FOR DETECTING POTENTIAL CELLULAR TOXICITY OF COMPOUNDS

TECHNICAL FIELD

The subject invention relates to methods for screening compounds to detect those which are potentially toxic to cells due to their effects on cell membranes.

BACKGROUND OF THE INVENTION

Testing of chemical agents for toxicity is important for the drug discovery process. It is essential to weed out compounds which cause general toxic effects by altering permeability of biological membranes. Use of *in vitro* toxicity screening systems is desirable prior to using more expensive and time-consuming toxicology experiments with animals. An example is testing for lysis of red blood cells, an accepted standard for hemolytic activity. Such lytic activity can also be assayed using liposomes loaded with fluorescent dyes or other molecules suitable for convenient analytical determination. However, determination of the lytic activity of compounds covers only a fraction of the potentially toxic effects which can be caused at the cell membrane level. A serious limitation of the lytic tests is that they do not detect agents which alter functions of biological membrane by increasing their permeability to ions.

A successful application of liposomes to elucidate a toxic mechanism of thallium has been reported. (Diaz & Monreal, "Thallium mediates a rapid chloride/hydroxyl ion exchange through myelin lipid bilayers", Molecular Pharmacology, Vol. 46, (1994), pp. 1210-1216.) These authors have studied the effects of thallium ions on the H⁺/OH⁻ and Cl⁻ permeabilities of liposomes prepared from brain myelin lipids. Their studies indicate that thallium ions catalyze an electroneutral Cl⁻/ OH⁻ exchange, e.g., antiport of different ion species.

SUMMARY OF THE INVENTION

The subject invention involves test procedures, for screening for potential cellular toxicity of test compounds, comprising the following steps:

- (a) making liposomes from one or a mixture of lipids to simulate a cell membrane composition of interest, the liposomes being made in an aqueous medium having a pH which is thereby the inside pH of the liposomes;
- (b) incorporating the liposomes in an aqueous test medium having a pH at least about 0.5 pH units above the inside pH of the liposomes, whereby a pH gradient exists between the test medium and the insides of the liposomes;
- (c) incorporating a fluorescent dye in the test medium, the dye having a tendency to penetrate into the liposomes due to the pH gradient, the dye being pH sensitive such that the fluorescence of the dye is quenched inside the liposomes;
- (d) incorporating the test compound in the test medium; whereby, if the test compound disrupts the structure of the liposomes or otherwise causes dissipation of the pH gradient between the test medium and the insides of the liposomes, the fluorescence of the dye is not quenched;
- (e) determining the fluorescence of the test medium and comparing the fluorescence with those of controls.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention methods use a fluorescent assay for identification of membrane-active agents which may cause toxic effects by dissipating ion gradients of cellular membranes.

The subject methods involve the use of liposomes to identify a spectrum of potential toxic effects on cells, including, but not limited to, cell membrane lysis and transport of ions across the cell membrane. The methods can be made suitable for high throughput screening. A multiple-well system, such as a 96-well apparatus, allows the use of lipids from different sources in conjunction with different methods of liposome

preparation, as well as testing of multiple compounds at the same time. The liposomes are preferably made from purified phospholipids in a buffer. A pH gradient across the liposomal membrane is generated by immersing liposomes in a medium more alkaline than that in which the liposomes were made. A fluorescent indicator, such as acridine orange, is used to detect the pH gradient. Introduction of the liposomes into the more alkaline medium containing acridine orange results in quenching of the fluorescence due to diffusion of the acridine orange into the liposomes in which the internal environment is more acidic. The quenched state continues as long as the pH gradient is present across the liposome membranes. When the pH gradient across the membranes is dissipated, the fluorescence of the acridine orange returns to its initial level observed in the medium before the addition of the liposomes, provided that the outside medium is buffered such that it has sufficient alkaline capacity to counter any acidic effect from the liposomes.

Dissipation of the pH gradient indicates that the liposome membrane is no longer a barrier to both protons and their counterions. Due to the requirement of maintaining macroscopic electroneutrality, the pH gradient is not dissipated if the membrane is permeable only to one ionic species, e.g. protons. Diffusion only of protons (from inside the liposome) causes a negative electric potential inside the liposomes; and this prevents further proton escape from the liposomes. Therefore, dissipation of the pH gradient (indicated by a lack of quenching of the fluorescence of the acridine orange) is an indication that either the liposome is disrupted (is no longer intact); or although still intact, it has become permeable to ion flow both out of (protons) and into (counterions) the liposome.

Lipids from either mammals (e.g., pork brain) or bacteria (e.g., *E. coli*) can be used to prepare liposomes for the subject invention methods. The pH gradient is adequately stable in both types of liposomes to allow measurements of effects within a 30 minute period. This feature permits the use of the subject liposome fluorescence assays in 96 well format suitable for high throughput screening. Membrane-disrupting agents (e.g., mellitin and Triton X-100) demonstrate similar activity against both types of liposomes, but the *E. coli* liposomes are intrinsically more permeable to ionic species compared to the pork brain liposomes. The pork brain liposomes can be used to determine the mechanism of

membrane activity of chemical compounds, because of easier handling and greater stability of the pH gradient. Bacterial liposomes offer an opportunity to investigate specific effects on bacterial membranes.

The origin of the phospholipids from which the liposomes are made generally determines the class of organisms for which the toxicity screen is designed. If the phospholipid is of mammalian origin, this screen is most likely useful for predicting toxicity to mammalian cells. If the phospholipid is from bacterial origin, the screen is most likely useful for predicting toxicity to bacterial cells. Examples of phospholipids useful for making liposomes for the subject test procedures include *E. coli* total phospholipids, pork brain polar lipids, and egg phosphatidylcholine, all available from Avanti Polar Lipids, Inc., Alabaster, Alabama.

The liposomes are made in a buffered medium at a pH which is more acidic than the pH of the medium containing the test compound to which the liposomes will be added. The pH of the medium in which the liposomes are made, and thereby the inside pH of such liposomes, is preferably at least about 4, more preferably at least about 5, more preferably still at least about 6, and preferably at most about 10, more preferably at most about 9, more preferably still at most about 8.

Methods for making liposomes useful in the subject invention test procedures are disclosed in: Grinius and Goldberg, "Bacterial Multidrug Resistance Is Due to a Single Membrane Protein Which Functions as a Drug Pump", J. Biological Chemistry, vol. 269, no. 47 (1994), pp. 29998-30004, incorporated herein by reference. The liposomes are preferably made from a mixture of a lipid and a phospholipid. The lipids and phospholipids are selected to simulate a cell membrane composition of interest.

The test procedure takes place in an aqueous test medium having a pH at least about 0.5 pH units above the inside pH of the liposomes (the pH of the medium in which the liposomes were made). Preferably the pH of the test medium is at least about 1 pH unit above, more preferably at least about 1.5 pH units above, more preferably still at least about 2 pH units above, and preferably at most about 5 pH units above, more preferably at most about 4 pH units above, more preferably still at most about 3 pH units above, the inside pH of the liposomes. The pH of the aqueous test medium is preferably at most about

12, more preferably at most about 11, more preferably still at most about 10, and preferably at least about 6, more preferably at least about 7, more preferably still at least about 8, still more preferably at least about 9. The aqueous test medium is preferably buffered at the selected pH, such that when the liposomes are added to it, the pH does not change substantially.

For general toxicity testing, the aqueous test medium preferably comprises sodium chloride and/or potassium chloride, preferably at a total concentration of both together of at least about 10 mM, more preferably at least about 100 mM, and preferably at most about 400 mM, more preferably at most about 200 μ M. While either salt can be used alone, a mixture of the two is more preferred. The test medium preferably comprises sodium chloride preferably at a concentration of at least about 50 mM, more preferably at least about 120 mM, and preferably at most about 250 mM, more preferably at most about 180 mM; and preferably comprises potassium chloride at a concentration of preferably at least about 5 mM, more preferably at least about 10 mM, and preferably at most about 50 mM, more preferably at most about 20 mM.

The aqueous test medium comprises a fluorescent dye. The dye is selected to have the ability to penetrate into the liposomes when there is a pH gradient between the medium and the inside of liposomes. The dye is selected such that the dye fluoresces at the pH of the test medium, but the fluorescence is measurably quenched at the lower pH inside the liposomes. A preferred dye useful in the subject invention test methods is acridine orange, available from Sigma Chemical Company, St. Louis, Missouri.

The fluorescent dye is incorporated in the aqueous test medium at a concentration of preferably at least about 0.5 μ M, more preferably at least about 1 μ M, and preferably at most about 4 μ M, more preferably at most about 2 μ M.

A test sample is incorporated in the aqueous test medium, except for control samples used to determine the quenching of the fluorescent dye by the liposomes without a test sample present. Such control samples are always needed in order to determine the effect of the compound being tested. The test compound is generally incorporated in the aqueous test medium at different concentrations in separate test samples, in order to determine the minimum concentration of the test compound at which a measurable effect

on the liposomes can be detected. Therefore, the concentration of the test compound in the aqueous test medium will vary greatly, preferably being at least about 0.01 $\mu\text{g/ml}$, more preferably at least about 0.1 $\mu\text{g/ml}$, more preferably still at least about 1 $\mu\text{g/ml}$, and preferably at most about 500 $\mu\text{g/ml}$, more preferably at most about 100 $\mu\text{g/ml}$.

The fluorescence of the dye in the test medium is determined after the test compound is incorporated in the medium, but before the liposomes are incorporated in the test medium, in order to obtain a baseline of the fluorescence without any quenching. Fluorescence is preferably measured by using a luminescence spectrometer.

The liposomes are then incorporated in the test medium. The amount of liposomes incorporated in the test medium is preferably at least about 1 $\mu\text{g/ml}$, more preferably at least about 5 $\mu\text{g/ml}$, more preferably still at least about 8 $\mu\text{g/ml}$, and preferably at most about 350 $\mu\text{g/ml}$, more preferably at most about 50 $\mu\text{g/ml}$, more preferably still at most about 24 $\mu\text{g/ml}$, still more preferably at most about 16 $\mu\text{g/ml}$. Mixing of the test medium to suspend the liposomes in the medium is continued preferably for at least about 10 sec after adding the liposomes to the test medium.

The fluorescence of the dye in the test medium after incorporation of liposomes therein is determined in the same manner as prior to the addition of the liposomes. The determination must be made quickly, since readings will slowly drift with increased time as the pH gradient across the liposome membrane slowly dissipates. Spectrometer readings are preferably completed within at most about 30 min, more preferably within at most about 15 min, of addition of the liposomes to the test medium. The temperature of the test medium during addition of the liposomes and taking of the spectrometer readings is preferably at least about 5°C, more preferably at least about 15°C, more preferably still at least about 20°C, and preferably at most about 42°C, more preferably at most about 30°C, more preferably still at most about 24°C.

The fluorescence determinations for the various samples are compared. A spectrometer reading for a test medium with dye and test compound but without liposomes provides the maximum reading (no quenching), while the reading for a test medium with dye and liposomes but no test compound provides the minimum reading (maximum quenching). These are compared to the readings for test mediums with dye, liposomes, and

various amounts of a test compound to determine whether the test compound reduces the quenching (dissipates the pH gradient across the liposome membrane), and if so, to what degree at different concentrations.

Example

Materials. The following lipids are used (all from Avanti Polar Lipids, Inc.): *E. coli* total phospholipids, pork brain polar lipids and egg phosphatidylcholine. The lipids are dissolved in chloroform at 20 mg/ml, and stored at -70°C. Acridine orange, ammonium chloride, carbonyl cyanide m-chlorophenylhydrazone (CCCP), dimethylsulfoxide, dithiotreitol, gramicidin, mellitin, 3-(N-morpholino)-propanesulfonic acid (MOPS), nigericin, Tris, Triton X-100, and valinomycin are obtained from Sigma Chemical Co., St. Louis, Missouri. Octylgalactoside (n-octyl- β -D-galactopyranoside) is obtained from Antrace Inc., Maumee, Ohio.

Liposome Preparation. An aliquot (1200 μ l) of the selected lipid (32 mg) is mixed with 320 μ l of egg phosphatidylcholine (3.2 mg) in a 16 mm glass tube and dried under nitrogen gas stream. The residue is kept under a vacuum (attached to a water aspirator) for three hours (or overnight). An aliquot (1200 μ l) of 0.1 M MOPS buffer, pH 7, supplemented with 1.2% octylgalactoside and 1 mM dithiotreitol ("Buffer") is added to the dried residue. The suspension is sonicated in a bath-type sonicator (Branson 1200) for 10-15 min until clear. The clear suspension of phospholipids is divided into four aliquots (0.3 ml each), and the aliquots are kept at -70°C before use. An aliquot of the lipid suspension is thawed on ice before use and sonicated briefly in a Tekmar TM250B Sonic Disruptor (Heat Systems Ultrasonics) equipped with a microtip. A 300 μ l aliquot of the phospholipid suspension is mixed with 400 μ l Buffer. The surface of the suspension is flushed with nitrogen, and the suspension is sonicated for 1 min in the sonicator. An aliquot of the resulting suspension (0.7 ml) is diluted under vigorous stirring into 23.1 ml of NH₄Cl buffer supplemented with 15 mM MOPS, pH 7, 0.15 M NH₄Cl, and 1 mM dithiotreitol; the stirring is continued for 20 min at room temperature. Aliquots of the diluted mixture (about 10 ml each) are loaded into ultracentrifuge tubes. The liposomes are pelleted at 50,000 rpm in a Beckman 70Ti rotor for 1 h at 4°C. The supernatant is discarded. The pellets are combined and resuspended in an aliquot of the NH₄Cl buffer by

pipeting at the following ratio: the pellets obtained from 35.2 mg of lipid resuspended in 0.8 ml of the buffer. The liposomes are frozen in dry ice/ethanol mixture and stored at -70°C.

Measurement of pH Gradient in Liposomes. Changes in intraliposomal pH are monitored by using the pH gradient-sensitive probe acridine orange. As a weak base, acridine orange is in its neutral form at physiological pH. This unprotonated form of the dye is able to move across the lipid membranes into the liposomes. When the unprotonated molecule of acridine orange enters an acidic environment, it binds protons and forms a protonated species. The protonated form of the dye accumulates inside the liposomes where the pH is lower. The increase in the concentration of the dye in the liposomes leads to its dimerization and quenching of its fluorescence. To measure fluorescence of acridine orange, a spectrofluorimeter (Perkin-Elmer Model LS 50B) is set at 495 nm for excitation and 530 nm for emission, with both slits at 10. The time scale is set for 60 seconds. An aliquot (2 ml) of the reaction buffer (15 mM Tris-Cl, pH10, 150 mM KCl, 2 μ M acridine orange) is added to a cuvette equipped with an automatic stirrer. A base line of the acridine orange fluorescence is recorded for 10 sec, and then an aliquot (8 μ l) of liposomes is injected. The measurement continues until the end of the chosen time interval (60 seconds). Injection of liposomes into a media with pH 10 results in fluorescence quenching due to diffusion of acridine orange into the liposomes where the environment is more acidic (pH 7). Dissipation of pH gradient across the liposome membrane results in a decrease in the amplitude of the quenching in response to liposome addition.

Measurements in a 96-well format. The spectrofluorimeter is equipped with a 96-well reader supplied by the vendor. Aliquots (195 μ l) of a reaction buffer are loaded to certain wells of a 96-well plate using an 8-lane multi-channel pipettor. These wells are marked as "no liposome" wells. Other wells of the same plate are loaded with 175 μ l aliquots of the same buffer, and they are marked as "plus liposome" wells. Some of each set of wells are marked as "solvent" wells, and they received 5 μ l aliquots of the appropriate solvent (H₂O, ethanol, or dimethylsulfoxide). Other wells from both sets are marked as "test compound" wells; they received 5 μ l aliquots of test compounds. Stock solutions of test compounds are made at 40-fold higher concentration than the desired final

concentration. An aliquot (20 μ l) of the stock suspension of the liposomes is diluted into 1980 μ l of 15 mM MOPS, pH 7.0, adjusted with 2 M Tris, pH 10. Aliquots (20 μ l) of diluted liposomes are loaded into the "plus liposome" wells. Before the measurement, the plate is placed inside the spectrofluorimeter and incubated 15 minutes at room temperature. The plate is read at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. The fluorescence quenching is recorded in arbitrary units. Each measurement is run in triplicate to calculate an average level of fluorescence intensity in a given set of wells. The fluorescence quenching by liposomes, (the "liposome effect") is calculated by subtracting the level of fluorescence observed in the presence of liposomes from the level of fluorescence observed in the absence of liposomes. Accordingly, the liposome effect in the presence of solvent alone is the difference between the fluorescence of the solvent/no liposome wells and the fluorescence of the solvent/plus liposome wells. Similarly, the liposome effect in the presence of a test compound is the difference between the fluorescence of the test compound/no liposome wells and the fluorescence of the test compound/plus liposome wells. To calculate the effect of a test compound on the fluorescence quenching by liposomes, the following equation is used: ((the liposome effect in the presence of solvent alone - the liposome effect in the presence of test compound)/(the liposome effect in the presence of solvent-alone))x 100%.

As indicated above, the subject invention test methods can be used as a general toxicity screen which detects effects of test compounds on liposome membranes which dissipate a pH gradient across the membrane, either due to general disruption of the integrity of the membrane or by allowing ions to flow across the membrane. Another aspect of the subject invention test procedures is the determination of which of these mechanisms is the cause of a dissipation of pH gradient across the liposome membrane.

In the test method described above, the test medium into which the liposomes are introduced contains a variety of different ions, so that whether the test compound generally disrupts the liposome membrane or facilitates transport of a particular ion across the membrane, dissipation of the pH gradient is observed since whatever ion is needed is in the test medium and available for transport across the liposome membrane.

Once a test compound is found to cause dissipation of the pH gradient across the liposome membrane in the general test method described above, the test method can be rerun altering the ion content of the test medium in order to determine which mechanism is operative. The test method is first rerun without any ions in the test medium to determine whether dissipation of the pH gradient is due to general disruption of the liposome membrane. If this does not result in dissipation of the pH gradient (the fluorescence of the acridine orange is quenched when liposomes are added to the test medium both in the presence and absence of the test compound), then the test procedure can be rerun with single ion pairs present in the test medium, until the ones being transported across the liposome membrane is determined.

Using the subject invention methods, the following classes of toxic compounds can be identified: (a) detergents and other membrane disrupting agents, (b) agents which form low specificity ion channels in the membrane which are permeable to monovalent cations, e.g., gramicidin, (c) ion antiporters which exchange H^+ for other ions, e.g., for K^+ as for nigericin, and (d) agents which induce specific ion permeability, e.g., valinomycin for K^+ , and protonophoric uncouplers for H^+ .

Based on the forgoing, the subject invention procedures include a two tier procedure of detecting membrane-active agents and determining mechanism of their action. In the first tier, compounds are tested on liposomes suspended in a salt-free media. Dissipation of the proton gradient under these conditions indicates that a test compound has detergent-like properties. Most likely that involves disruption of the membrane structure.

It must also be taken into consideration that some compounds can dissipate the proton gradient of the liposomes by virtue of direct interaction with protons, e.g., $NH_3 + H^+ = NH_4^+$. Such an effect is generally observed within tens and hundreds millimolar concentration ranges. This is generally well above any meaningful therapeutic range for a compound, and is therefore not of real concern.

Compounds which do not dissipate the proton gradient of the liposomes in a salt-free media can be tested in the second tier of the liposome assay to determine whether they cause more specific alteration(s) of the membrane which may be toxic. If dissipation of the proton gradient by a test compound is observed in the presence of specific ion

species, that indicates either an ion antiport or the formation of ion channels with properties similar to gramicidin-formed channels. Both types of compounds likely interfere with gradients of monovalent cations which are of vital importance.

If a test compound does not dissipate the proton gradient even in the presence of salts, it could be tested for ability to increase membrane permeability specifically to H^+ or K^+ . Discrimination between those two possibilities can be achieved in separate steps. To determine specific membrane permeability to H^+ , a test compound has to be tested in a media which is supplemented with both K^+ and valinomycin. Dissipation of the proton gradient under this specific set of conditions indicates the ability of a test compound to make a membrane permeable to protons. To strengthen a conclusion about specific permeability to protons, a compound can be re-tested in the K^+ medium from which valinomycin is omitted. Indication for a specific protonophore, such as CCCP, would be lack of dissipation of the proton gradient in the absence of valinomycin.

While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the arts that various changes and modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of this invention.

What is claimed is:

1. A method for screening a test compound for potential cellular toxicity comprising the steps:
 - (a) making liposomes from one or a mixture of lipids to simulate a cell membrane composition of interest, the liposomes being made in an aqueous medium having a pH which is thereby the inside pH of the liposomes;
 - (b) incorporating the liposomes in an aqueous test medium having a pH at least 0.5 pH units above the inside pH of the liposomes, whereby a pH gradient exists between the test medium and the insides of the liposomes;
 - (c) incorporating a fluorescent dye in the test medium, the dye having a tendency to penetrate into the liposomes due to the pH gradient, the dye being pH sensitive such that the fluorescence of the dye is quenched inside the liposomes;
 - (d) incorporating the test compound in the test medium; whereby, if the test compound disrupts the structure of the liposomes or otherwise causes dissipation of the pH gradient between the test medium and the insides of the liposomes, the fluorescence of the dye is not quenched;
 - (e) determining the fluorescence of the test medium and comparing the determination with those of controls.
2. The method of Claim 1 wherein:
 - (a) the inside pH of the liposomes is within the range of from 4 to 10, preferably 5 to 9;
 - (b) the pH of the test medium is within the range of from 6 to 12, preferably 6 to 12.
3. The method of Claim 1 or 2 wherein the dye is acridine orange, and the fluorescence is read using a spectrofluorimeter.

4. The method of any of Claims 1-3 wherein the pH of the test medium is buffered at a pH at least 1 pH unit, preferably at least 2 pH units, above the inside pH of the liposomes.
5. The method of any of Claims 1-4 wherein:
 - (a) the test compound is incorporated in the test medium at a concentration of from 0.01 $\mu\text{g/ml}$ to 500 $\mu\text{g/ml}$, preferably from 0.1 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$;
 - (b) the liposomes are incorporated in the test medium at a concentration of from 1 $\mu\text{g/ml}$ to 350 $\mu\text{g/ml}$, preferably from 5 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$;
 - (c) the dye is incorporated in the test medium at a concentration of from 0.5 μM to 4 μM ;
 - (d) the test medium preferably comprises sodium chloride, potassium chloride, or both, at a total concentration within the range of from 10 mM to 400 mM.
6. The method of any of Claims 1-5 wherein:
 - (a) the liposomes are incorporated in the test medium at a concentration of from 8 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$;
 - (b) the temperature of the test medium is maintained within the range of from 20°C to 24°C;
 - (c) the spectrometer readings are taken within 15 min. of the time of incorporation of the liposomes in the test medium;
 - (d) the test medium comprises sodium chloride at a concentration of from 120 mM to 180 mM, and potassium chloride at a concentration of from 5 mM to 20 mM.
7. The method of any of Claims 1-6 wherein the liposomes consist essentially of lipids from mammals.
8. The method of any of Claims 1-6 wherein the liposomes consist essentially of lipids from bacteria.

9. The methods of any of Claims 1-8 wherein a multiple-well system is used for high throughput screening of multiple test samples.
10. A two-tier system of testing for potential cellular toxicity of a test compound comprising:
- (1) using the method of any of Claims 1-9 without additional ions in the test medium to determine whether general disruption of the liposome occurs, and if not;
 - (2) repeating the method with a series of tests in which single ion pairs are present in the test medium to determine which ions are being transported across the liposome membrane.