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(54) Title: AUTOPHAGY AND PHOSPHOLIPIDOSIS PATHWAY ASSAYS

(57) Abstract: Provided are assays useful for detecting and monitoring autophagy and phospholipidosis, including the progression of lysosomal storage diseases. Drugs and treatments for lysosomal storage diseases can be monitored for effectiveness in lysosomal storage disease conditions. Drug candidates and suspected toxic agents can also be screened for toxicity to cells, tissues and organs. Also provided are methods for distinguishing between phospholipidosis activators and autophagy pathway perturbation agents.



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AUTOPHAGY AND PHOSPHOLIPIDOSIS PATHWAY ASSAYS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is an international application of prior United States Patent Application No. 12/584,958, filed September 14, 2009.

FIELD OF THE INVENTION

This invention relates to lysosomal storage diseases, and more particularly to methods for detecting and monitoring the progression of lysosomal storage diseases as well as methods for monitoring the effectiveness of drugs or remedies used to manage, treat or cure such diseases. This invention also relates to toxicity screening methods, and more particularly, methods and assays for screening drug candidates and suspected toxic agents to determine their toxicity to cells, tissues or organs. This invention also relates to phospholipidosis and autophagy, and more particularly, to methods for distinguishing phospholipidosis activators and autophagy pathway perturbation agents.

BACKGROUND OF THE INVENTION

Cell-based assays are increasingly gaining in popularity in the pharmaceutical industry due to their high physiological relevance. Additional advantages include their ability to predict compound usefulness, evaluate molecular interactions, identify toxicity, distinguish cell type-specific drug effects, and determine drug penetration. Cell-based assays are relevant throughout the drug discovery pipeline, because they are capable of providing data from target characterization and validation to lead identification (primary and secondary screening) to terminal stages of toxicology. Current industry trends of performing drug screening with cell context demand easily monitored, non-invasive reporters. This fundamental approach increases efficiency, reduces costs, and results in shorter time to market for new drugs. In order to fail compounds early, information-rich data for accurate early-stage decision making is required. Such data may be derived by screening compounds in context, that is, by screening in relevant living systems, rather than with classical biochemical assays. These screening systems

often incorporate sophisticated imaging platforms, such as high-content screening (HCS) workstations. The industrialization of fluorescent microscopy has led to the development of these high-throughput imaging platforms capable of HCS. When coupled with fluorescent reporter technology, HCS has provided information-rich drug screens, as well as access to novel types of drug targets.

While suitable for analysis of cell surfaces and permeabilized cells, fluorescently-labeled antibodies have few practical applications for intracellular imaging in living cells, due to their inherent inability to penetrate to their targets, which has given rise to development of cell-permeable small molecule organic fluorochromes. Certain ones of these fluorochromes naturally sequester inside-specific organelles, based upon biophysical or biochemical properties favoring that distribution. Acceptable small molecule organic probes for cell imaging and analysis need to be minimally perturbing, versatile, stable, easy-to-use, and easy to detect using non-invasive imaging equipment. A problem with the classical organic probes from a histological standpoint is that many of them require cofactors or, by requiring fixation or staining, report only on the static condition of a dead cell. The required additional steps may be time consuming and expensive and, in the case of fixing and staining, may lack biological relevance. In the context of the analyses described above, an organic probe must be able to report upon events in living cells and in real time. Simplicity is of key importance, especially in the context of drug screening.

While various organic fluorochromes have been developed in the past for live cell analysis, typically they were devised using unperturbed cells. Thus, such organic fluorochromes statically report upon organelle spatial position within cells without the ability to monitor dynamic changes. For instance, US Patent Nos. 5,338,854, 5,459,268, 5,686,261, 5,869,689, 6,004,536, 6,140,500 and 6,291,203, as well as US Patent Application Publications 2005/0054006 and 2007/0111251 A1 disclose organic fluorochromes which are described as useful for visualizing membranes, mitochondria, nuclei and/or acidic organelles. Additional examples of various fluorochromes and their application in biological imaging may be found in the published literature (see, for example, Pagano et al, 1989; Pagano et al, 1991; Deng et al, 1995; Poot et al, 1996;

Diwu et al, 1999; Rutledge et al, 2000; Lee et al, 2003; Bassøe et al, 2003; Rosania et al, 2003, Li et al 2007; Boldyrev et al, 2007; Nadrigny et al, 2007).

These dyes have been created using a number of fluorophores, most commonly dipyrrometheneboron difluoride (BODIPY), cyanine, carbocyanine, styryl and diaminoxanthene core structures. Typical emission maxima for these organic fluorophores span from 430 to 620 nm. Many of these dyes display certain suboptimal properties, such as a propensity to photo-bleach, metachromasy and even a tendency to photo-convert to different emission maxima upon brief exposure to broad-band illumination.

JC-1 dye is notable in its ability to monitor the dynamics of mitochondrial membrane potential changes and consequently the energetic state of cells (Smiley et al, 1991). Unfortunately, with respect to lysosomes and lysosome-like vesicles (autophagosomes, autophagolysosomes), there has been a notable lack of probes that have the ability to track organelle dynamics arising from drug treatment or intracellular membrane trafficking anomalies.

Spectral problems associated with previously devised organic fluorochromes for analysis of lysosomes

Fluorescence co-localization imaging is a powerful method for exploring the targeting of molecules to intracellular compartments and for screening of their associations and interactions. In these kinds of experiments, distinct fluorochromes and/or fluorescent proteins of interest are imaged as spectrally separated detection channels. The fluorescence intensity in each channel is ideally dominated by spatial and concentration information derived from one fluorophore only. Many commercially available organic fluorophores for subcellular analysis are disadvantaged in displaying suboptimal properties relating to these types of applications.

Lysotracker Red DND-99 (Invitrogen, Carlsbad, CA) contains a BODIPY fluorophore in the form of a conjugated multi-pyrrole ring structure and also contains a weakly basic amine that causes the fluorochrome to accumulate selectively in acidic compartments, exhibiting red fluorescence upon appropriate illumination (excitation: 577 nm, emission: 590 nm) (Freundt et al, 2007). Lysotracker Red is structurally related to

Lysotracker Green but the former has an additional pyrrole ring in conjugation with the primary structure, which produces a longer wavelength emission. Lysotracker Red has commonly been used in multi-color imaging studies as a lysosomal marker to determine intracellular localization of green fluorescent protein (GFP)-tagged proteins by fluorescence or confocal microscopy. Excitation of the red-emitting molecule with broad-band illumination induces molecular changes, however, thus rendering its photochemical properties similar to those of Lysotracker Green. The similarities between the spectra of Lysotracker Green and converted Lysotracker Red suggest that the third pyrrole ring is taken out of conjugation during the photo-conversion process, leading to a shorter wavelength dye emission. Thus, Lysotracker Red staining for epifluorescence or confocal microscopy, in conjunction with visualization of GFP or FITC, leads to spurious results due to photo-conversion of the fluorophore (Freundt et al, 2007).

Acridine orange (Sigma-Aldrich, Saint Louis, MO and other sources) has also been used extensively as a fluorescent probe of lysosomes and other acidic subcellular compartments. Acridine orange's metachromasy results, however, in the concomitant emission of green and red fluorescence from stained cells and tissue (Nadrigny et al, 2007). Evanescent-field imaging with spectral fluorescence detection, as well as fluorescence lifetime imaging microscopy demonstrate that green fluorescent acridine orange monomers inevitably coexist with red fluorescing acridine orange dimers in labeled cells. The green monomer emission spectrally overlaps with that of GFP and produces a false apparent co-localization on dual-color images. Due to its complicated photochemistry and interaction with cellular constituents, acridine orange is a particularly problematic label for multi-color fluorescence imaging, both for dual-band and spectral detection. Extreme caution is required, therefore, when deriving quantitative co-localization information from images of GFP-tagged proteins in cells co-labeled with acridine orange.

Cationic amphiphilic drug tracers have been disclosed. See, for example, acridine-propranolol disclosed in Dickens et al., *J Mol Cell Cardiol* 34:129-137 (2002); Bondok et al., *J. Anat.* 160:161-174 (1988); and Cornett et al., *J. Histochem. Cytochem.*

28:462-464 (1980); and mitoxantrone disclosed in Vibet et al., *Drug Metabolism and Disposition* 35:822-828 (2007); each incorporated by reference.

Problems associated with previously described fluorochromes targeted to lysosomes and lysosome-like bodies

The accumulation of cationic amphiphilic drugs, toxic agents and other basic compounds inside acidic subcellular organelles is referred to as lysosomotropism. While many drugs require the presence of a cationic moiety for intrinsic bioactivity, their accumulation into subcellular organelles can also lead to undesirable tissue distribution, alkalization of lysosomes, phospholipidosis and aberrant pharmacokinetic disposition (Ikeda et al, 2008). Over fifty cationic amphiphilic drugs, including antibiotics, antidepressants, antipsychotics, and antimalarial and antiarrhythmic agents are known to trigger phospholipidosis, which is typified by the excessive intracellular accumulation of phospholipids within lysosomes as lamellar bodies (Anderson and Borlak, 2006). Some commonly prescribed drugs known to perturb lysosomes by inducing phospholipidosis include propranolol, triparanol, chlorpromazine, chloroquine, fluoxetine, clindamycin and ketoconazole.

The origins of drug-induced lamellar bodies remain unresolved, though they appear to be generated by autophagic or heterophagic processes (Anderson and Borlak, 2006). Several mechanisms have been proposed to explain drug-induced phospholipidosis including the formation of drug-phospholipid complexes that are resistant to degradation by lysosomal phospholipases, direct inhibition of phospholipases themselves, and inhibition of intracellular pathways regulating phospholipid metabolism.

Only one instance can be found wherein a cationic amphiphilic fluorophore has been employed for monitoring drug-induced phospholipidosis (Lemieux et al, 2004). LysoTracker Red DND-99 (Invitrogen) was used to stain lysosomes and inhibition or displacement of the accumulated dye by test agents was subsequently monitored by fluorescence microscopy. Thus, the drug-induced vacuoles were not explicitly stained using the fluorescent probe, but rather an absence of signal was used as an indirect indication that the vacuoles were formed.

Numerous other *in vitro* methods for detecting phospholipidosis using different cell types including primary hepatocytes, peripheral blood monocytes and various cell lines, in combination with electron microscopy, flow cytometry, fluorescent microscopy or spectrofluorometry have previously been described (Ulrich et al, 1991; Cramer and Ulrich, 1994; Kasahara et al, 2006; Ikeda et al 2008; Gum et al, 2001; Lemieux et al 2004; Fujimura et al 2007; Tomizawa et al, 2006; Natalie et al, 2009; Nioi et al, 2008; Nioi et al, 2007; Bhandari et al, 2008; Morelli et al, 2006; Casartelli et al, 2003). Due to their inherent low throughput, however, many of these *in vitro* methods are not ideal for routine compound screening during early stage drug development. For example, electron microscopy, fluorescence microscopy and flow cytometry are generally considered low throughput platforms for routine drug screening purposes, especially when contrasted with fluorescence-based multi-well microplate assays. Additionally, some of the above cited assays depend upon difficult to procure cells. For instance, the preparation of primary hepatocytes or peripheral blood monocytes is too laborious for routine drug screening assay programs.

Many of the previously described cell-based methods require a relatively long period of treatment with the test agent before phospholipidosis can be detected (24-72 h). Additionally, the vast majority of the assays are based upon fluorescently labeled phospholipid analogs, such as NBD-PE, NBD-PC and Lipid-Tox reagent (Invitrogen, Carlsbad, CA), which require co-incubation of the analog with the drug during the extended incubation period. Distinct types of phospholipids appear to accumulate differently during phospholipidosis, however, and no explicit comparison of the performance of the different fluorescent lipid analogs has been performed to date (Diez-Blanco et al., 1987). The methods also depend upon permeabilization and fixation of the cells prior to analysis, which adds considerably to the overhead associated with the workflow. What is needed is a fluorescent probe that faithfully highlights increases in vacuolar number and volume associated with exposure of cells and/or tissues to xenobiotic compounds.

SUMMARY OF THE INVENTION

This invention provides a method of detecting the presence of a lysosomal storage disease in a subject in which the following steps are carried out: (a) obtaining a sample containing cells from the subject; (b) contacting the sample with a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; and (c) detecting the cationic amphiphilic tracer compound, thereby determining whether there exists an excess above normal accumulation of vacuoles within the cells of the sample, such an excess above normal accumulation of vacuoles being indicative of the lysosomal storage disease sought to be detected.

This invention also provides a method of monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of ($T_2 - T_1$) time interval(s). In this monitoring method, the following steps are carried out: (a) obtaining from the subject at time T_1 a first sample containing cells; (b) contacting the first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (c) detecting the first cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within the cells of the first sample at time T_1 ; (d) obtaining from the subject at a later time T_2 a second sample containing cells; (e) contacting the second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (f) detecting the second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and (g) comparing the accumulation of vacuoles between the first sample at time T_1 and the second sample at time T_2 , thereby monitoring the progression of lysosomal storage disease in the subject.

Also provided by this invention is a method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease in a subject. To monitor the effectiveness of such a drug or remedy, the following steps are carried out: (a) obtaining from the subject at time T_1 a first sample containing cells; (b) contacting the first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (c) detecting the first cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within the cells of the first

sample at time T_1 ; (d) obtaining from the subject at a later time T_2 a second sample containing cells; (e) contacting the second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (f) detecting the second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and (g) comparing the accumulation of vacuoles between the first sample at time T_1 and the second sample at time T_2 , thereby monitoring the effectiveness of the drug used to manage, treat or cure lysosomal storage disease.

Further provided by the invention herein is a method of screening drug candidates to determine their toxicity to cells, tissues or organs. To screen drug candidates for toxicity, the following steps are carried out: (a) providing: (i) at least one first sample and one second sample of mammalian cells; (ii) a drug candidate for screening; (iii) a drug diluting vehicle or carrier; and (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (b) administering: (i) the drug candidate (ii) to the first sample for a time sufficient to allow the drug candidate to partition into subcellular compartments within the cells to form treated cells; and (ii) the vehicle or carrier (iii) to the second sample to form untreated reference cells; (c) contacting the treated cells and the untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and (d) detecting any increase in vacuole accumulation of the cationic amphiphilic tracer compound in the treated cells relative to said untreated reference cells, thereby determining whether said drug candidate has accumulated in the vacuoles and is toxic to cells, tissues or organs.

The present invention also provides a method of screening suspected toxic agents to determine their toxicity to cells, tissues or organs. In this method for screening suspected toxic agents for their toxicity, the following steps are carried out: (a) providing: (i) at least one first sample and one second sample of mammalian cells; (ii) a suspected toxic agent for screening; (iii) a diluting vehicle or carrier; and (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (b) administering (i) the suspected toxic agent (ii) to the first sample for a time sufficient to allow the suspected toxic agent to partition into subcellular compartments within the

cells to form treated cells; and (ii) the diluting vehicle or carrier (iii) to the second sample to form untreated reference cells; (c) contacting the treated cells and the untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and (d) detecting any increase in vacuole accumulation of the cationic amphiphilic tracer compound in the treated cells relative to the untreated reference cells, thereby determining whether the suspected toxic agent has accumulated in the vacuoles and is toxic to cells, tissues or organs.

The present invention further provides a method of distinguishing between phospholipidosis activators and autophagy pathway perturbation agents. To distinguish such activators and agents, the following steps are carried out: (a) providing: (i) a fluorescent phospholipid analog compound that can incorporate into multilamellar vesicles; (ii) a cationic amphiphilic fluorophore tracer compound that localizes in a vacuole in a cell; (iii) a test agent; and (iv) a sample of mammalian cells; (b) administering to the cells in the sample (iv) the fluorescent phospholipid analog compound (i) and the test agent (iii) to form a testing mixture; (c) subsequently contacting the testing mixture with the cationic amphiphilic fluorophore tracer (ii); and (d) detecting any signals generated from the fluorescent phospholipid analog compound (i) and the cationic amphiphilic fluorophore tracer compound (ii), thereby differentiating between phospholipidosis activators and autophagy pathway perturbation agents based upon differential accumulation patterns of the fluorescent phospholipid analog and the cationic amphiphilic fluorophore tracer.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: Fluorescent microscopy images of cells treated with increasing doses of chloroquine.

FIG. 2: Graphs of relative fluorescent intensity of U2OS cells treated with known cationic amphiphilic drugs.

FIG. 3: Graphs of relative fluorescent intensity of U2OS cells treated with chloroquine (Panel A) or bafilomycin A1 (Panel B), using a fluorescent phospholipid analog versus a fluorescent cationic amphiphilic tracer for detection of vacuolation.

FIG. 4: Graphs of positive leads from a compound library screen obtained using the fluorescent cationic amphiphilic tracer method.

FIG. 5: Schematic illustration for multiplexed detection of phospholipidosis inducers and autophagy pathway inhibitors.

FIG. 6: Fluorescent microscopy image (Panel A) and bright field image (Panel B) of cells stained with 5 μ M Dye 2.

FIG. 7: Graph of relative fluorescent intensities of HeLa cells treated with 200 μ M chloroquine for 5 hours or untreated cells.

FIG. 8: Fluorescent microscopy image (Panel A) and bright field image (Panel B) of cells stained with 10 μ M Dye 5.

FIG. 9: Fluorescent microscopy images of HeLa cells under control and starvation conditions.

FIG. 10: Fluorescent microscopy images of cells stained (a) with Dye 8 and (b) for RFP-LC3.

FIG. 11: Fluorescent microscopy images of cells stained with Dye 8, and the effect of that staining when the cells are treated with Rapamycin or Rapamycin + an inhibitor of the early stages of autophagy (3MA).

FIG. 12: Graph of starvation kinetics with dye 8 by flow cytometry.

FIG. 13: Graph profiling starvation and recovery with Dye 8 by flow cytometry, with associated data.

FIG. 14: Graph profiling autophagic flux with dye 8 by flow cytometry, with associated data.

DETAILED DESCRIPTION OF THE INVENTION

The invention generally relates to an assay suitable for applications involving wide-field fluorescence microscopy, flow cytometry, confocal microscopy, fluorimetry, microplate-based cytometry, high-content cell analysis, cell microarray analysis, high-content cell screening, laser-scanning cytometry and other imaging and detection modalities. The invention relates to fluorescent probes that are selectively sequestered into vacuoles derived from the lysosome catabolic pathway. Without wishing to be

bound by theory, it appears that through careful selection of titratable groups on the probe, labeling can be expanded into lamellar inclusion bodies of cells pre-treated with weakly basic, cell-permeant compounds, such as the anti-malarial drug chloroquine. The probes can be employed for highlighting lysosome-like organelles, e.g., under conditions wherein cells produce vacuoles that contain most of the degradative enzymes of the lysosome, but are not as acidic as the parent organelle. The assay measures generalized dysfunction of the lysosomal-dependent catabolic pathway, which leads to the accumulation of vacuoles such as autophagosomes, and thus is relevant to macroautophagy in general (herein referred to as autophagy), as well as to phospholipidosis in particular. One potential application of the new assay is in preclinical drug safety assessment (ADME-Tox) using *in vitro* cell culture models to aid in the drug development process.

Cationic drugs often exhibit large apparent volumes of distribution, which is consistent with various forms of sequestration within cells and tissues. This form of drug uptake can occur in intact cell types that are not necessarily specialized for the handling of xenobiotics. The drugs are sequestered into cells by a variety of metabolically-driven, but receptor-independent means including mitochondrial membrane potential-driven concentration, nuclear concentration via DNA affinity and vacuolar-ATPase-driven trapping into lysosomes that subsequently swell by an osmotic mechanism. Drug concentration within cells may arise from the innate membrane permeability of the uncharged forms of these drugs, as well as by means of specific transporters (organic cation transporters, choline transporters, etc.). Such drug sequestration could contribute to overall toxicity, as well as prolong the duration of drug action. Fluorescence-based assays are disclosed that assess the impact of xenobiotics on overall cell function, with particular emphasis on the lysosomal and related compartments. In one embodiment, a semi-automated multi-well cell-based assay workflow is presented that provides a rapid and quantitative high-throughput approach for determining drug- or toxic agent-induced live cell response, offering throughput advantages relative to methods based upon electron microscopy, fluorescence microscopy or flow cytometry. Early secondary screening of candidate drugs for

potentially adverse cell activity in the drug discovery phase could predict later risks in drug development arising from drug safety issues. Such a screening approach could aid in selecting the best candidate compounds for further drug development efforts, as well as provide preliminary benchmarking of dosing limits in preclinical toxicity studies.

Definitions:

By fluorescence is meant the emission of light as a result of absorption of light-emission, occurring at a longer wavelength than the incident light.

By fluorophore is meant a component of a molecule which causes a molecule to be fluorescent.

By fluorochrome is meant any of a group of fluorescent dyes used to stain biological specimens.

By anthraquinone is meant the quinone derivative of anthracene, a tricyclic aromatic hydrocarbon containing two opposite carbonyl groups (C=O) at the 9, 10 positions of anthracene. These compounds may also be referred to as anthracenediones or as 9,10-dioxoanthracenes.

By autophagy is meant an evolutionarily conserved subcellular degradation process decomposing folded proteins, protein complexes and entire organelles, such as aggregates of misfolded proteins or damaged mitochondria, involving the import of cytoplasmic components into the lysosome.

By xenobiotic is meant a compound or material foreign to the body of a living organism, including a chemical compound, such as a drug, toxin or pesticide, or a material, such as a particle, nanoparticle, nanocrystal or fiber.

By cationic amphiphilic tracer compound is meant a member of a class of compounds sharing common physiochemical properties: hydrophobic ring structure on the molecule and a hydrophilic side chain with a charged cationic amine group. The cationic amphiphilic tracer compounds of the present invention generally localize to a vacuole of a cell within about one hour.

By phospholipidosis is meant an excessive accumulation of intracellular phospholipids in cells and tissues triggered by a cationic amphiphilic drug or an inborn error of lipid metabolism (lysosomal storage disease).

By lysosomal storage disease is meant any one of a group of approximately 40 or so rare inherited metabolic disorders that result from defects in lysosomal function. Lysosomal storage diseases are characterized by a congenital or acquired deficiency of an enzyme so that one or more specific metabolic processes are not completed. As a result there is an accumulation of metabolic products in the cellular lysosomes. The following is a list of known lysosomal storage diseases: Activator deficiency/GM2 gangliosidosis, Alpha-mannosidosis, Aspartylglucosaminuria, Cholesteryl ester storage disease, Chronic hexosaminidase A deficiency, Cystinosis, Danon disease, Fabry disease, Farber disease, Fucosidosis, Galactosialidosis, Gaucher Disease, GM1 gangliosidosis, I-Cell disease/Mucopolysaccharidosis II, Infantile Free Sialic Acid Storage Disease/ISSD, Juvenile Hexosaminidase A Deficiency, Krabbe disease, Metachromatic Leukodystrophy, Mucopolysaccharidoses disorders, Pseudo-Hurler polydystrophy/Mucopolysaccharidosis IIIA, MPS I Hurler Syndrome, MPS I Scheie Syndrome, MPS I Hurler-Scheie Syndrome, MPS II Hunter syndrome, Sanfilippo syndrome Type A/MPS III A, Sanfilippo syndrome Type B/MPS III B, Sanfilippo syndrome Type C/MPS III C, Sanfilippo syndrome Type D/MPS III D, Morquio Type A/MPS IVA, Morquio Type B/MPS IVB, MPS IX Hyaluronidase Deficiency, MPS VI Maroteaux-Lamy, MPS VII Sly Syndrome, Mucopolysaccharidosis I/Sialidosis, Mucopolysaccharidosis IIIC, Mucopolysaccharidosis type IV, Multiple sulfatase deficiency, Niemann-Pick Disease, Neuronal Ceroid Lipofuscinoses, CLN6 disease, Batten-Spielmeyer-Vogt/Juvenile NCL/CLN3 disease, Finnish Variant Late Infantile CLN5, Jansky-Bielschowsky disease/Late infantile CLN2/TPP1 Disease, Kufs/Adult-onset NCL/CLN4 disease, Northern Epilepsy/variant late infantile CLN8, Santavuori-Haltia/Infantile CLN1/PPT disease, Beta-mannosidosis, Pompe disease/Glycogen storage disease type II, Pycnodysostosis, Sandhoff disease/Adult Onset/GM2 Gangliosidosis, Sandhoff disease/GM2 gangliosidosis – Infantile, Sandhoff disease/GM2 gangliosidosis – Juvenile, Schindler disease, Salla disease/Sialic Acid Storage Disease, Tay-Sachs/GM2 gangliosidosis and Wolman disease.

By metachromasy is meant the hypsochromic (shift in absorption to shorter wavelength) and hypochromic (decrease in intensity of emitted fluorescence) change in color exhibited by certain dyes in aqueous-based media under conditions such as: (1),

increase in dye concentration; (2), temperature decrease; (3), salting out; and (4), interaction with substrates that favor water intercalation and/or proximity or stacking of dye monomers.

Basic fluorophore core structures

The present invention pertains to the preparation and use of fluorescent cationic amphiphilic tracers comprising polycyclic fused ring systems, such as anthraquinone, anthrapyrazole, and benzophenoxazine fluorophores in cell imaging and detection. Also included herein are styryl cyanine dyes and acridine derivatives that are conjugated to amphiphilic moieties such as propranolol. Generally, these types of dyes are electrically neutral and lipophilic, properties which permit them to be better solubilized in non-polar environments, such as cell membranes thereby rendering them cell permeable. More particularly, the invention relates to modifications of these dyes with functional groups that target the dyes to lysosomes and related vacuolar organelles. In one embodiment of the present invention, the functional groups attached to the dyes do not have a propensity for vacuolar organelles in and of themselves, but their addition to a dye endows the modified dye with such properties. In another embodiment of the present invention, functional groups are added that intrinsically have their own affinity for this particular class of organelles and the addition of such groups to a dye conveys this property to the dye. Such tracer compounds, dyes, derivatives, modifications and functional groups are described in related U.S. Patent Application Publications US2010/0062460 and US2010/0062429.

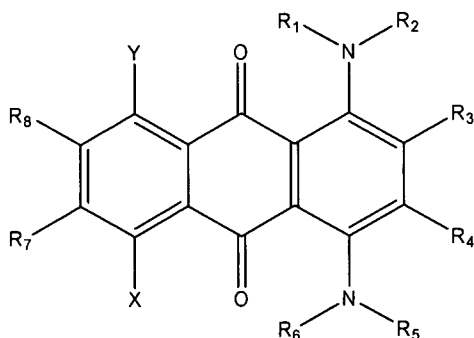
This invention in part provides compositions and methods for identifying catabolic pathway-derived organelles and related cellular structures and elements. Specifically, the present invention provides compounds and methods for measuring generalized dysfunction of the lysosomal-dependent catabolic pathway, which leads to the intracellular accumulation of vacuoles, such as autophagosomes, phagophores or autophagolysosomes. The compounds and methods further allow identification of xenobiotics that interfere with membrane trafficking during autophagy as well as those that induce the phenomenon of phospholipidosis.

The invention discloses compositions comprising cationic amphiphilic fluorophore tracers that are capable of partitioning into vacuoles arising from generalized dysfunction of the lysosomal-dependent catabolic pathway.

The invention further discloses methods for identifying the intracellular accumulation of vacuoles within cells of interest comprising the steps of (A) providing (i) the cells of interest; (ii) any xenobiotic agent (iii) any of the compositions described herein below; (B) incubating the cells of interest (i) with a xenobiotic agent (ii); subsequently incubating cells of interest (i) with any composition (iii) and (C) monitoring the accumulation of the composition (iii) within the cells.

Other aspects and embodiments are described in further detail below.

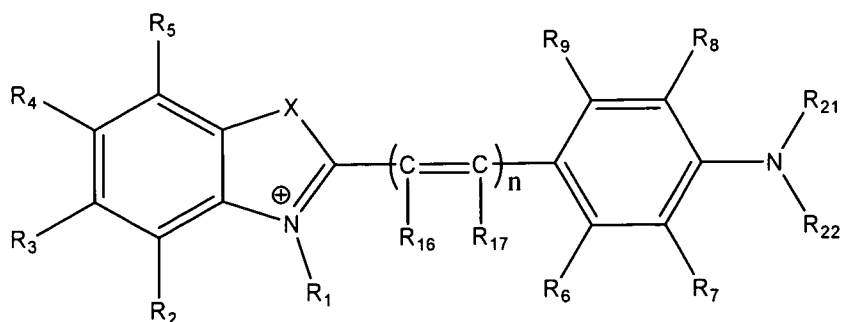
The present invention provides a dye having the formula:



wherein each of X, Y, R₃, R₄, R₇, and R₈ are independently H, OH, F, Cl, Br, I, CN, sulfonate or its salt, sulfoxide (SOR₁₁), a sulfone (SO₂CR₁₁R₁₂R₁₃), a sulfonamide (SO₂NR₁₁R₁₂), a phosphate monoester (PO₃⁻ER₁₁), a phosphate diester (PO₂ER₁₁ER₁₂), a phosphonate monoester (PO₂⁻ER₁₁), a phosphonate diester (POER₁₁ER₁₂), a thiophosphate (PSO₃⁻), a thiophosphate monoester (PSO₂⁻ER₁₁), a thiophosphate diester (PSOER₁₁ER₁₂), a thiophosphonate (PSO₂⁻), a thiophosphonate monoester (PSO⁻ER₁₁), a thiophosphonate diester (PSER₁₁ER₁₂), a phosphoramidate (PONR₁₁R₁₂NR₁₄R₁₅), its thioanalogue (PSNR₁₁R₁₂NR₁₄R₁₅), a phosphoramidite (PO₂R₁₄NR₁₁R₁₂), its thioanalogue (POSR₁₄NR₁₁R₁₂), ketone (COR₁₁), thioketone (CSR₁₁), amide (CONR₁₁R₁₂), thioamide (CSNR₁₁R₁₂), an amino, a nitro, or an alkyl group, wherein any of E can independently comprise O or S. The alkyl group is

saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein the alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted. R_1 , R_2 , R_5 and R_6 are independently H or $-L-Q$ wherein L is C_{0-18} linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof, wherein the linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted, or any combinations thereof. Q comprises a sulfoxide (SOR_{11}), a sulfone ($SO_2CR_{11}R_{12}R_{13}$), a sulfonamide ($SO_2NR_{11}R_{12}$), a phosphate monoester ($PO_3^-ER_{11}$), a phosphate diester ($PO_2ER_{11}ER_{12}$), a phosphonate monoester ($PO_2^-ER_{11}$), a phosphonate diester ($POER_{11}ER_{12}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($PSO_2^-ER_{11}$), a thiophosphate diester ($PSOER_{11}ER_{12}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester (PSO^-ER_{11}), a thiophosphonate diester ($PSER_{11}ER_{12}$), a phosphoramidate ($PONR_{11}R_{12}NR_{14}R_{15}$), its thioanalogue ($PSNR_{11}R_{12}NR_{14}R_{15}$), a phosphoramidate ($PONR_{11}R_{12}NR_{13}NR_{14}R_{15}$), its thioanalogue ($PSNR_{11}R_{12}NR_{13}NR_{14}R_{15}$), a phosphoramidite ($PO_2R_{14}NR_{11}R_{12}$), its thioanalogue ($POSR_{14}NR_{11}R_{12}$), ketone (COR_{11}), thioketone (CSR_{11}), amide ($CONR_{11}R_{12}$), or thioamide ($CSNR_{11}R_{12}$), wherein any of E can independently comprise O or S. Q can also comprise ZR_9R_{10} wherein Z can be N, O, S, Se or any combinations thereof and wherein R_9 and R_{10} can independently be hydrogen or an alkyl group wherein the alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group that is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R_9 and R_{10} may form a heterocyclic ring. Q can also comprise a substituted anthraquinone moiety such that the whole molecule is either a homo or hetero dimer.

In another aspect of the present invention, novel dyes that are based upon styryl dyes are disclosed. In one embodiment the dyes have the general structure:



wherein X comprises CR₁₁R₁₂, NR₁₁, O, S or Se where R₁₁ and R₁₂ independently comprise hydrogen, a halogen, an amino group, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkyl group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R₁₁ and R₁₂ form a 5 or 6 membered ring;

wherein n can be 1, 2 or 3;

wherein at least one of R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈, R₉, R₁₁, R₁₂, R₂₁ or R₂₂ comprises Q, wherein Q comprises a sulfoxide (SOR₁₃), a sulfone (SO₂CR₁₃R₁₄R₁₅), a sulfonamide (SO₂NR₁₃R₁₄), a phosphonate (PO₃⁻), a phosphonate monoester (PO₂⁻ER₁₃), a phosphonate diester (POER₁₃ER₁₄), a thiophosphate (PSO₃⁻), a thiophosphate monoester (PSO₂⁻ER₁₃), a thiophosphate diester (PSOER₁₃ER₁₄), a thiophosphonate (PSO₂⁻), a thiophosphonate monoester (PSO⁻ER₁₃), a thiophosphonate diester (PSER₁₃ER₁₄), a phosphoramidate (PONR₁₃R₁₄NR₁₉R₂₀), its thioanalogue (PSNR₁₃R₁₄NR₁₉R₂₀), a phosphoramidite (PO₂R₁₉NR₁₃R₁₄), or its thioanalogue (POSR₁₉NR₁₃R₁₄), wherein any of E can independently comprise O or S;

wherein Q is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof and wherein when Q(2) is a sulfonamide, it does not comprise a terminal reactive group or a linker joining the dye to a target molecule;

wherein R_{13} , R_{14} , R_{15} , R_{19} and R_{20} can be hydrogen, a halogen, an amino group, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together R_{13} and R_{14} form a five or six membered ring;

wherein R_{16} , R_{17} and the remaining R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{11} , R_{12} , R_{16} , R_{17} , R_{21} and R_{22} can independently be hydrogen, Z, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R_2 and R_3 , R_3 and R_4 , R_4 and R_5 , R_6 and R_7 , R_7 and R_8 , R_1 and R_{16} , R_{11} and R_{16} , R_{16} and R_{17} , R_{17} and R_{18} , and R_{18} and R_9 may form a 5 or 6 membered ring;

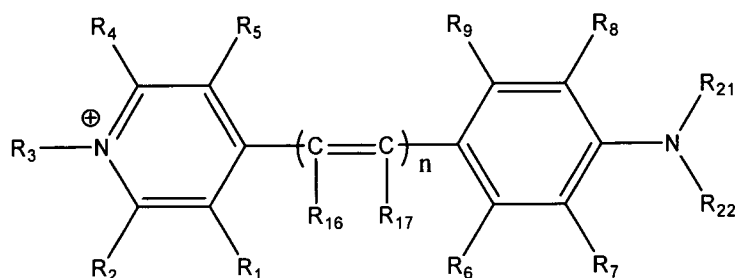
wherein Z comprises a carboxyl group (CO_2^-), an alkyl amino, a carbonate ester (COER_{13}), a sulfonate (SO_3^-), a sulfonate ester ($\text{SO}_2\text{ER}_{13}$), a sulfoxide (SOR_{13}), a sulfone ($\text{SO}_2\text{CR}_{13}\text{R}_{14}\text{R}_{15}$), a sulfonamide ($\text{SO}_2\text{NR}_{13}\text{R}_{14}$), a phosphate (PO_4^-), a phosphate monoester ($\text{PO}_3^-\text{ER}_{13}$), a phosphate diester ($\text{PO}_2\text{ER}_{13}\text{ER}_{14}$), a phosphonate (PO_3^-), a phosphonate monoester ($\text{PO}_2^-\text{ER}_{13}$), a phosphonate diester ($\text{POER}_{13}\text{ER}_{14}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($\text{PSO}_2^-\text{ER}_{13}$), a thiophosphate diester ($\text{PSOER}_{13}\text{ER}_{14}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester ($\text{PSO}^-\text{ER}_{13}$), a thiophosphonate diester ($\text{PSER}_{13}\text{ER}_{14}$), a phosphoramidate ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PO}_2\text{R}_{19}\text{NR}_{13}\text{R}_{14}$), or its thioanalogue ($\text{POSR}_{19}\text{NR}_{13}\text{R}_{14}$), where E can be independently O or S;

wherein Z is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, and any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted, or any combination thereof;

and wherein any of R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{11} , R_{12} , R_{21} or R_{22} may further comprise a heteroatom containing side chain wherein said side chain is joined to

the R group by a linkage which comprises an ether linkage ($-\text{OR}_{25}$), a thioether linkage ($-\text{SR}_{25}$), or an amine linkage ($-\text{NR}_{25}\text{R}_{26}$ or $-\text{N}^+\text{R}_{25}\text{R}_{26}\text{R}_{27}$), and wherein R_{25} , R_{26} and R_{27} independently comprise hydrogen, Z, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group that is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R_{25} and R_{26} , and R_{26} and R_{27} independently comprise a five or six membered ring, and wherein any of R_{25} , R_{26} or R_{27} may further comprise said heteroatom containing side chain.

In another embodiment of the present invention, the styryl dye comprises a picoline or quinoline moiety instead of a benzazolium group. As such, these dyes have the structure:



wherein n can be 1, 2 or 3;

wherein at least one of R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} or R_{22} comprises Q wherein Q comprises a sulfonate (SO_3^-), a sulfonate ester ($\text{SO}_2\text{ER}_{13}$), a sulfoxide (SOR_{13}), a sulfone ($\text{SO}_2\text{CR}_{13}\text{R}_{14}\text{R}_{15}$), a sulfonamide ($\text{SO}_2\text{NR}_{13}\text{R}_{14}$), a phosphate ($\text{PO}_4^{=}$), a phosphate monoester ($\text{PO}_3^-\text{ER}_{13}$), a phosphate diester ($\text{PO}_2\text{ER}_{13}\text{ER}_{14}$), a phosphonate (PO_3^-), a phosphonate monoester ($\text{PO}_2^-\text{ER}_{13}$), a phosphonate diester ($\text{POER}_{13}\text{ER}_{14}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($\text{PSO}_2^-\text{ER}_{13}$), a thiophosphate diester ($\text{PSOER}_{13}\text{ER}_{14}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester ($\text{PSO}^-\text{ER}_{13}$), a thiophosphonate diester ($\text{PSER}_{13}\text{ER}_{14}$), a phosphoramidate ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PO}_2\text{R}_{19}\text{NR}_{13}\text{R}_{14}$), or its thioanalogue ($\text{POSR}_{19}\text{NR}_{13}\text{R}_{14}$), where E can be independently O or S;

wherein Q is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted, or any combinations thereof and wherein when Q is a sulfonamide, it does not comprise a terminal reactive group or a linker joining the dye to a target molecule;

wherein R₁₃, R₁₄, R₁₅, R₁₉ and R₂₀ can be hydrogen, a halogen, an amino group, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together R₁₃ and R₁₄ form a five or six membered ring;

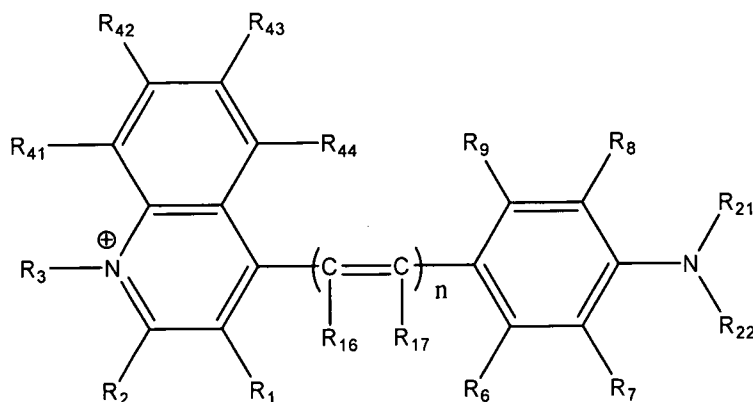
wherein R₁₆, R₁₇ and the remaining R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈, R₉, R₂₁ or R₂₂ can independently be hydrogen, Z, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R₁ and R₂, R₂ and R₃, R₃ and R₄, R₄ and R₅, R₅ and R₁₆, R₁₆ and R₁₇, R₁₇ and R₉, R₉ and R₈, R₈ and R₂₁, R₂₁ and R₂₂, R₂₂ and R₇, and R₇ and R₆ may form a 5 or 6 membered ring;

wherein Z comprises a carboxyl group (CO₂⁻), a carbonate ester (COER₁₃), a sulfonate (SO₃⁻), a sulfonate ester (SO₂ER₁₃), a sulfoxide (SOR₁₃), a sulfone (SO₂CR₁₃R₁₄R₁₅), a sulfonamide (SO₂NR₁₃R₁₄), a phosphate (PO₄⁼), a phosphate monoester (PO₃⁻ER₁₃), a phosphate diester (PO₂ER₁₃ER₁₄), a phosphonate (PO₃⁼), a phosphonate monoester (PO₂⁻ER₁₃), a phosphonate diester (POER₁₃ER₁₄), a thiophosphate (PSO₃⁼), a thiophosphate monoester (PSO₂⁻ER₁₃), a thiophosphate diester (PSOER₁₃ER₁₄), a thiophosphonate (PSO₂⁼), a thiophosphonate monoester (PSO⁻ER₁₃), a thiophosphonate diester (PSER₁₃ER₁₄), a phosphoramidate (PONR₁₃R₁₄NR₁₉R₂₀), its thioanalogue (PSNR₁₃R₁₄NR₁₉R₂₀), a phosphoramidite (PONR₁₃R₁₄NR₁₅NR₁₉R₂₀), its thioanalogue (PSNR₁₃R₁₄NR₁₅NR₁₉R₂₀), a phosphoramidite (PO₂R₁₉NR₁₃R₁₄) or its thioanalogue (POSR₁₉NR₁₃R₁₄), where E can be independently O or S;

wherein Z is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, and any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted, or any combination thereof;

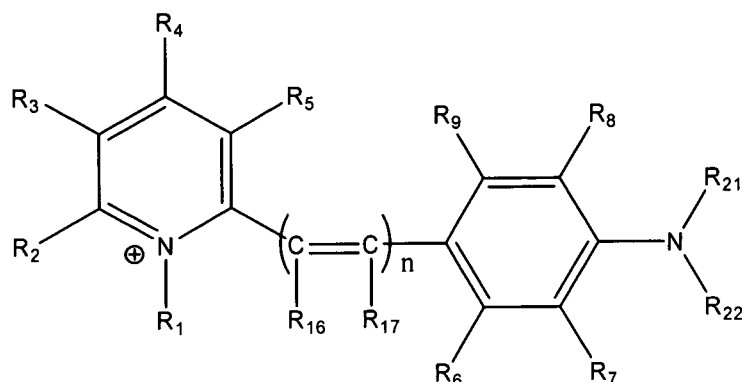
and wherein any of R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} or R_{22} may further comprise a heteroatom containing side chain wherein said side chain is joined to the R group by a linkage which comprises an ether linkage ($-OR_{25}$), a thioether linkage ($-SR_{25}$), or an amine linkage ($-NR_{25}R_{26}$ or $-N^+R_{25}R_{26}R_{27}$), and wherein R_{25} , R_{26} and R_{27} independently comprise hydrogen, Z, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group that is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R_{25} and R_{26} , and R_{26} and R_{27} independently comprise a five or six membered ring, and wherein any of R_{25} , R_{26} or R_{27} may further comprise said heteroatom containing side chain.

When R_4 and R_5 comprise alkyl chains that are joined together, a quinoline moiety can be formed, the dye thereby having the general structure:



where R_{41} , R_{42} , R_{43} and R_{44} are as described previously for R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} and R_{22} .

In yet another embodiment of the present invention, the styryl dye comprises a 2-picoline or quinaldine moiety instead of a benzazolum group. As such, these dyes have the structure:



wherein n can be 1, 2 or 3;

wherein at least one of R₁, R₂, R₃, R₄, R₅, R₆, R₇, R₈, R₉, R₂₁ or R₂₂ comprises Q wherein Q comprises a sulfonate (SO₃⁻), a sulfonate ester (SO₂ER₁₃), a sulfoxide (SOR₁₃), a sulfone (SO₂CR₁₃R₁₄R₁₅), a sulfonamide (SO₂NR₁₃R₁₄), a phosphate (PO₄⁼), a phosphate monoester (PO₃⁻ER₁₃), a phosphate diester (PO₂ER₁₃ER₁₄), a phosphonate (PO₃⁼), a phosphonate monoester (PO₂⁻ER₁₃), a phosphonate diester (POER₁₃ER₁₄), a thiophosphate (PSO₃⁼), a thiophosphate monoester (PSO₂⁻ER₁₃), a thiophosphate diester (PSOER₁₃ER₁₄), a thiophosphonate (PSO₂⁼), a thiophosphonate monoester (PSO⁻ER₁₃), a thiophosphonate diester (PSER₁₃ER₁₄), a phosphoramidate (PONR₁₃R₁₄NR₁₉R₂₀), its thioanalogue (PSNR₁₃R₁₄NR₁₉R₂₀), a phosphoramidite (PO₂R₁₉NR₁₃R₁₄), or its thioanalogue (POSR₁₉NR₁₃R₁₄), where E can be independently O or S;

wherein Q is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof and wherein when Q is a sulfonamide, it does not comprise a terminal reactive group or a linker joining the dye to a target molecule;

wherein R₁₃, R₁₄, R₁₅, R₁₉ and R₂₀ can be hydrogen, a halogen, an amino group, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or

unsaturated, branched or linear, substituted or unsubstituted, or when taken together R_{13} and R_{14} form a five or six membered ring;

wherein R_{16} , R_{17} and the remaining R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} or R_{22} can independently be hydrogen, Z, an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group wherein said alkoxy group is saturated or unsaturated, branched or linear, substituted or unsubstituted, or when taken together, R_1 and R_2 , R_2 and R_3 , R_3 and R_4 , R_4 and R_5 , R_5 and R_{16} , R_{16} and R_{17} , R_{17} and R_9 , R_9 and R_8 , R_8 and R_{21} , R_{21} and R_{22} , R_{22} and R_7 , and R_7 and R_6 may form a 5 or 6 membered ring;

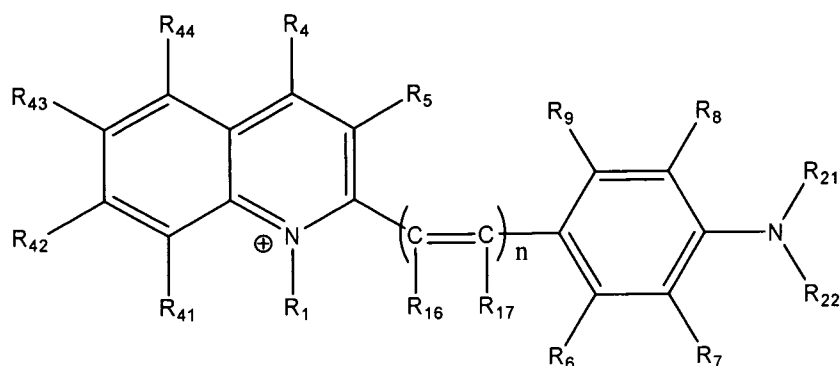
wherein Z comprises a carboxyl group (CO_2^-), a carbonate ester (COER_{13}), a sulfonate (SO_3^-), a sulfonate ester ($\text{SO}_2\text{ER}_{13}$), a sulfoxide (SOR_{13}), a sulfone ($\text{SO}_2\text{CR}_{13}\text{R}_{14}\text{R}_{15}$), a sulfonamide ($\text{SO}_2\text{NR}_{13}\text{R}_{14}$), a phosphate (PO_4^-), a phosphate monoester ($\text{PO}_3^-\text{ER}_{13}$), a phosphate diester ($\text{PO}_2\text{ER}_{13}\text{ER}_{14}$), a phosphonate (PO_3^-), a phosphonate monoester ($\text{PO}_2^-\text{ER}_{13}$), a phosphonate diester ($\text{POER}_{13}\text{ER}_{14}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($\text{PSO}_2^-\text{ER}_{13}$), a thiophosphate diester ($\text{PSOER}_{13}\text{ER}_{14}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester ($\text{PSO}^-\text{ER}_{13}$), a thiophosphonate diester ($\text{PSER}_{13}\text{ER}_{14}$), a phosphoramidate ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{15}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{15}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PO}_2\text{R}_{19}\text{NR}_{13}\text{R}_{14}$), or its thioanalogue ($\text{POSR}_{19}\text{NR}_{13}\text{R}_{14}$), where E can be independently O or S;

wherein Z is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, and any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted, or any combination thereof;

and wherein any of R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} or R_{22} may further comprise a heteroatom containing side chain wherein said side chain is joined to the R group by a linkage which comprises an ether linkage ($-\text{OR}_{25}$), a thioether linkage ($-\text{SR}_{25}$), or an amine linkage ($-\text{NR}_{25}\text{R}_{26}$ or $-\text{N}^+\text{R}_{25}\text{R}_{26}\text{R}_{27}$), and wherein R_{25} , R_{26} and R_{27} independently comprise hydrogen, Z, an alkyl group wherein said alkyl group is

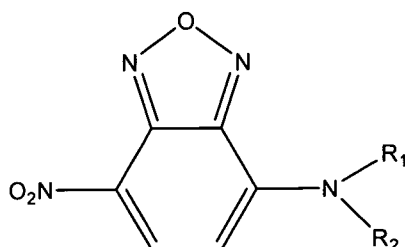
saturated or unsaturated, linear or branched, substituted or unsubstituted, an alkoxy group that is saturated or unsaturated, branched or linear; substituted or unsubstituted, or when taken together, R_{25} and R_{26} , and R_{26} and R_{27} independently comprise a five or six membered ring, and wherein any of R_{25} , R_{26} or R_{27} may further comprise said heteroatom containing side chain.

When R_2 and R_3 comprise alkyl chains that are joined together, a quinaldine moiety can be formed, the dye thereby having the general structure:



where R_{41} , R_{42} , R_{43} and R_{44} are as described previously for R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{21} and R_{22} .

In still another embodiment of the invention, dyes are provided that have the structure:



wherein R_1 and R_2 are independently H, OH, or comprise

an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted,

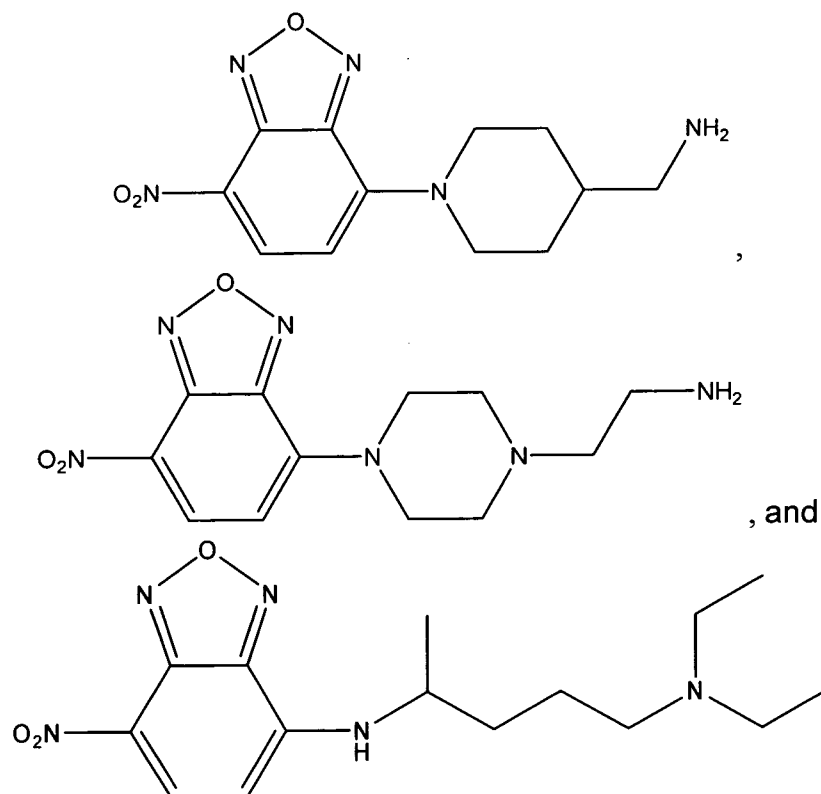
a phenyl group wherein said phenyl group is substituted or unsubstituted, or when taken together, R_1 and R_2 may form a 5 or 6 membered ring comprising carbon, sulfur, oxygen or nitrogen, or any combinations thereof; wherein the 5 or 6

membered ring is saturated or unsaturated, wherein the ring may be further substituted with a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof.

In some of these compounds, at least one of R_1 or R_2 comprises Q wherein Q comprises a carboxyl group (CO_2^-), a carbonate ester (COER_{13}), a sulfonate (SO_3^-), a sulfonate ester ($\text{SO}_2\text{ER}_{13}$), a sulfoxide (SOR_{13}), a sulfone ($\text{SO}_2\text{CR}_{13}\text{R}_{14}\text{R}_{15}$), a sulfonamide ($\text{SO}_2\text{NR}_{13}\text{R}_{14}$), a phosphate (PO_4^-), a phosphate monoester ($\text{PO}_3^-\text{ER}_{13}$), a phosphate diester ($\text{PO}_2\text{ER}_{13}\text{ER}_{14}$), a phosphonate (PO_3^-), a phosphonate monoester ($\text{PO}_2^-\text{ER}_{13}$), a phosphonate diester ($\text{POER}_{13}\text{ER}_{14}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($\text{PSO}_2^-\text{ER}_{13}$), a thiophosphate diester ($\text{PSOER}_{13}\text{ER}_{14}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester ($\text{PSO}^-\text{ER}_{13}$), a thiophosphonate diester ($\text{PSER}_{13}\text{ER}_{14}$), a phosphoramidate ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{15}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{15}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PO}_2\text{R}_{19}\text{NR}_{13}\text{R}_{14}$) or its thioanalogue ($\text{POSR}_{19}\text{NR}_{13}\text{R}_{14}$) where E can be independently O or S, wherein Q can further comprise a nucleophilic reactive group, an electrophilic reactive group, a terminal alkene, a terminal alkyne, a coordinate group or an alkylating agent. Q is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof.

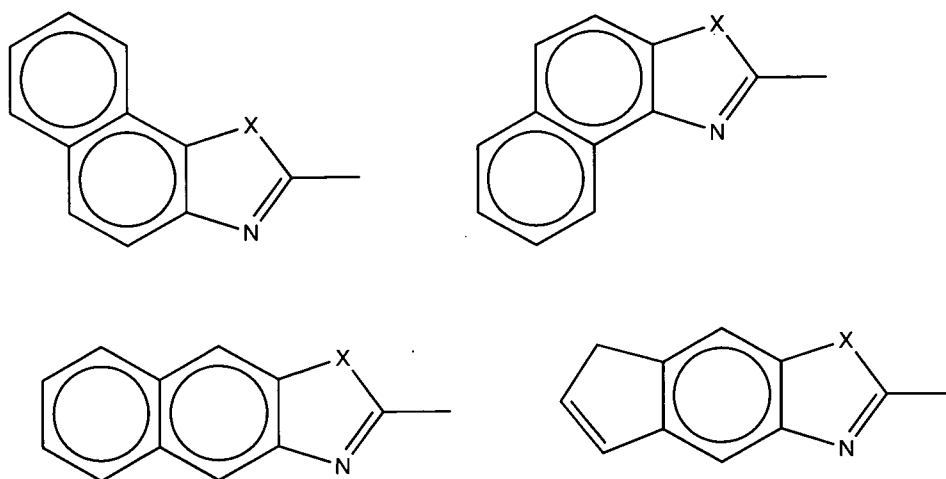
In other embodiments of these compounds, R_1 and R_2 are independently H, or a saturated alkyl group that may be substituted at any one or multiple positions with an N, where R_1 and R_2 may form a 6 membered ring, provided that, when one of R_1 and R_2 is H, the other of R_1 and R_2 is not H.

Specific embodiments of these compounds are



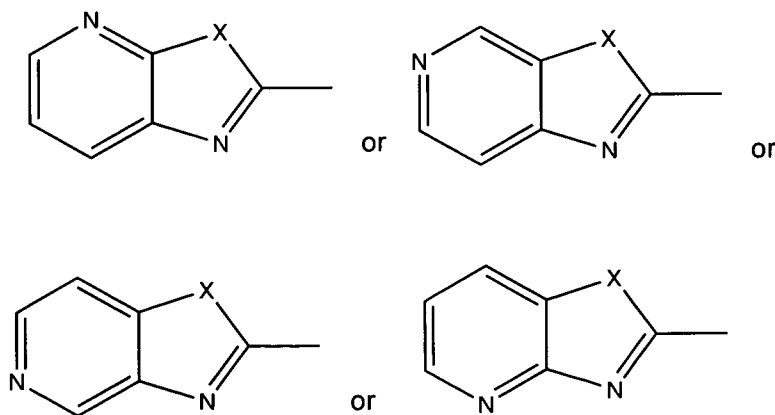
Complex ring structures

As described above some of the R groups may be joined together to form one or more fused 5 or 6 membered ring structures. It is understood that the complex rings that are formed by closure of R groups may be further substituted with any of the R groups described previously. Examples of complex rings that may be formed for the benzazolum portion of cyanine and asymmetric cyanine dyes can comprise but not be limited to:

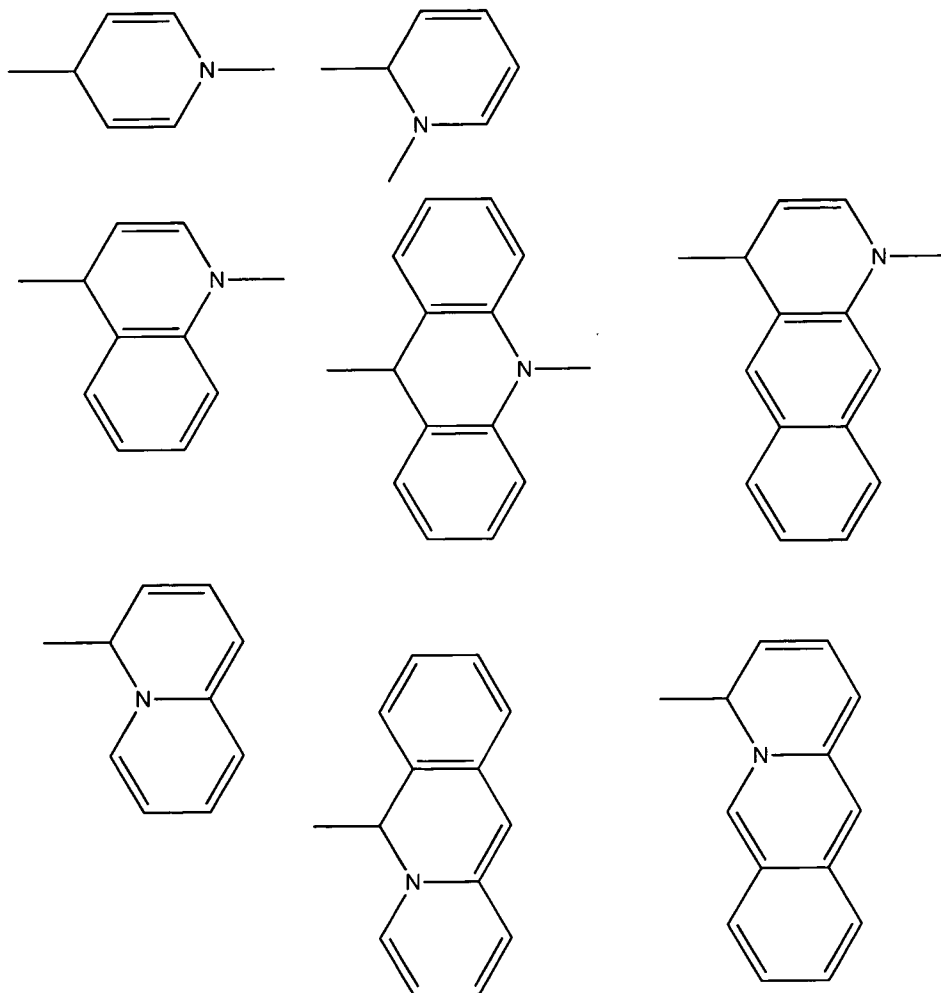


In addition, "rigid" cyanine dyes have been described where a fused ring is formed where the nitrogen of the benzazolium is linked to the nearest carbon of the methine bridge (see, e.g., U.S. Patents 6,133,445 and 6,686,145). Similarly in a cyanine dye with a monomethine bridge (i.e. when $n = 0$), a rigid linkage can be formed by joining the nitrogens of the benzazolium group to each other (see, e.g., U.S. Patents 5,852,191 and 5,981,747).

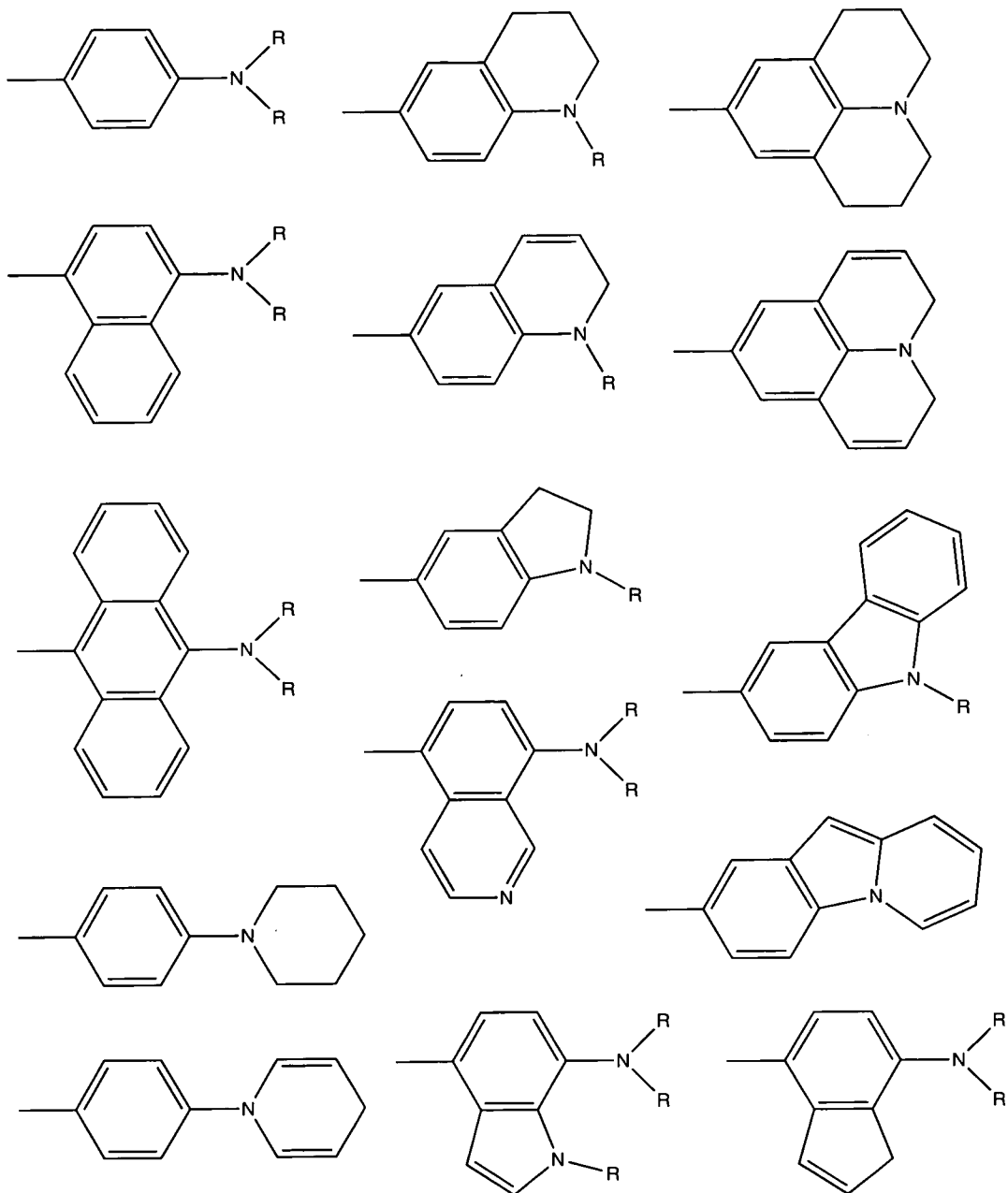
If desired, a variation of the preceding dyes can be the substitution of an azabenzazolium instead of a benzazolium moiety in the cyanine, asymmetric cyanine and styrene dyes; *i.e.* a nitrogen replaces the carbon in the positions where R^2 , R^3 , R^4 , R^5 , R^7 , R^8 , R^9 or R^{10} are connected to the benzazolium moiety of cyanine dyes or to the R^2 , R^3 , R^4 or R^5 positions of the asymmetric cyanine and styrene dyes disclosed previously. Methods for the synthesis and use of an azabenzazolium based dyes are disclosed in U.S. Patent 6,664,047. As such these moieties would have the structures:



Examples of rings and complex rings that may comprise the non-benzazolum portion of an asymmetric cyanine dye can comprise but not be limited to:



Examples of rings and complex rings that may be part of the non-benzazolum portion of a styryl dye can comprise but not be limited to:



Reactive Groups and Targets

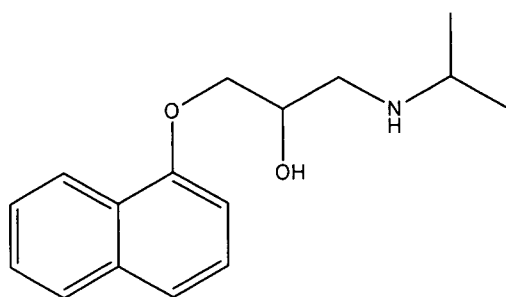
In another aspect of the present invention, one of the R groups is a reactive group thereby allowing the dyes of the present invention to be attached to a useful target molecule. Examples of reactive groups that may find use in the present invention can include but not be limited to a nucleophilic reactive group, an electrophilic reactive group, a terminal alkene, a terminal alkyne, a platinum coordinate group or an alkylating agent.

There are a number of different electrophilic reactive groups that may find use with the present invention; examples can include but not be limited to isocyanate, isothiocyanate, monochlorotriazine, dichlorotriazine, 4,6,-dichloro-1,3,5-triazines, mono- or di-halogen substituted pyridine, mono- or di-halogen substituted diazine, maleimide, haloacetamide, aziridine, sulfonyl halide, acid halide, hydroxysuccinimide ester, hydroxysulfosuccinimide ester, imido ester, hydrazine, azidonitrophenol, azide, 3-(2-pyridyl dithio)-propionamide, glyoxal and aldehyde groups. Nucleophilic reactive groups can include but not be limited to reactive thiol, amine and hydroxyl groups. For purposes of synthesis of dyes, reactive thiol, amine or hydroxyl groups can be protected during various synthetic steps and the reactive groups generated after removal of the protective group. Use of a terminal alkene or alkyne groups for attachment of markers has been previously described in U.S. Patent Application Publication US2003/0225247. The use of platinum coordinate groups for attachment of other dyes has been previously disclosed in U.S. Patent 5,580,990 and the use of alkyl groups has been previously described in U.S. Patent 6,593,465.

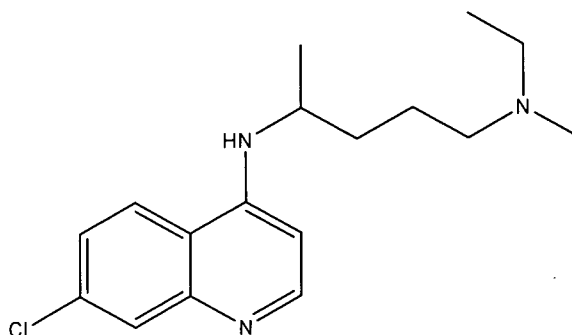
Examples of useful target molecules can include but not be limited to a cationic amphiphilic drugs, nucleoside, nucleotide, oligonucleotide, polynucleotide, peptide nucleic acid, protein, peptide, enzyme, antigen, antibody, hormone, hormone receptor, cellular receptor, lymphokine, cytokine, hapten, lectin, avidin, streptavidin, digoxigenin, carbohydrate, oligosaccharide, polysaccharide, lipid, liposomes, glycolipid, viral particle, viral component, bacterial cell, bacterial component, eukaryotic cell, eukaryotic cell component, natural drug, synthetic drug, glass particle, glass surface, natural polymers,

synthetic polymers, plastic particle, plastic surface, siliceous particle, siliceous surface, organic molecule, dyes and derivatives thereof.

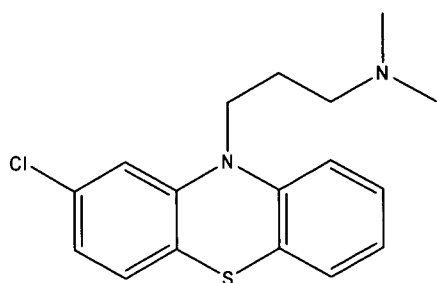
Specific examples of cationic amphiphilic drugs includes but not limited to propranolol, chloroquine, chlorpromazine, gentamicin, chlorphentermine, AY9944, amiodarone, fluoxetine, imipramine, chlorcyclizine and tamoxifen. Structures of some of these drugs are shown below:



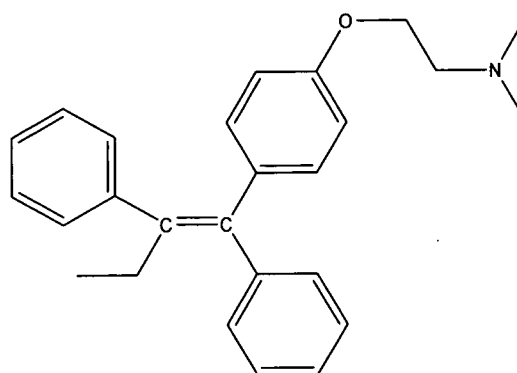
Propranolol



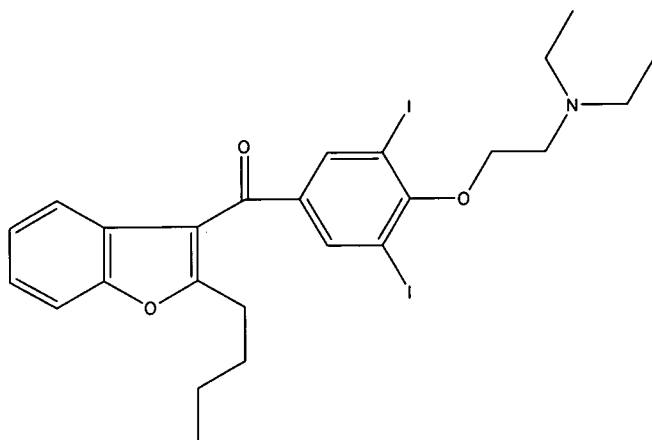
Chloroquine



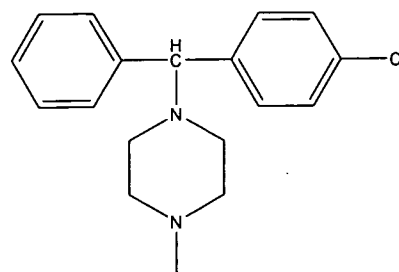
Chlorpromazine



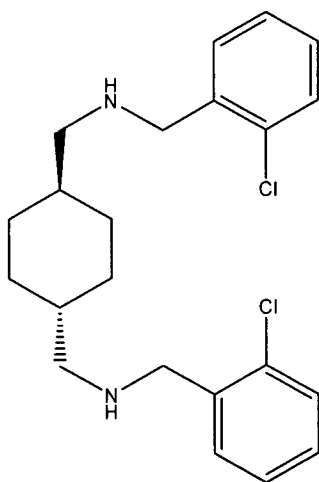
Tamoxifen



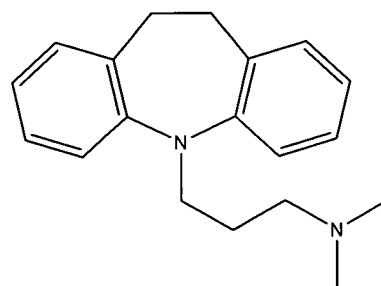
Amiodarone



Chlorcyclizine



AY 9944



Imipramine

The nucleoside, nucleotide, oligonucleotide, or polynucleotide can comprise one or more ribonucleoside moieties, ribonucleotide moieties, deoxyribonucleoside moieties, deoxyribonucleotide moieties, modified ribonucleosides, modified ribonucleotides, modified deoxyribonucleosides, modified deoxyribonucleotides, ribonucleotide analogues, deoxyribonucleotide analogues and any combination thereof.

As described above, the dyes of the present invention may have dyes as targets thereby creating composite dyes. By joining the dyes of the present invention to another dye, unique properties may be enjoyed that are not present in either dye alone. For instance, if one of the dyes of the present invention is joined to another dye such

that it creates an extended conjugation system, the spectral characteristics of the dye may be different than either dye component. Another example of this method is where the conjugation systems do not overlap but the proximity allows an internal energy transfer to take place thereby extending the Stokes shift. For an example of this, see U.S. Patents 5,401,847, 6,008,373, and 5,800,996. Other properties may also be enhanced by this joining, for example, it has been previously described that the joining together of two ethidium bromide molecules generates a dye that has enhanced binding to nucleic acids (U.S. Patent Publication 2003/0225247). Other composite dyes have been described that simultaneously enjoy both properties, i.e. enhanced binding and energy transfer (U.S. Patent 5,646,264). Furthermore, these composite dyes are not limited to binary constructs of only two dyes, but may comprise oligomeric or polymeric dyes. These composite dyes may be comprised of the same dye or different dyes may be joined together depending upon the properties desired.

Utility may also be achieved by attaching a dye of the present invention to a target specific moiety. Thus, binding between the target specific moiety and its corresponding target may be monitored by essentially determining the presence or amount of dye that is bound to the target. Well-known examples of such assays are hybridizations between complementary nucleic acids as well as binding that take place between antibodies and their corresponding antigens. Other binding pairs that may be of interest can include but not be limited to ligand/ receptor, hormone/hormone receptor, carbohydrate/lectin and enzyme/substrate. Assays may be carried out where one component is fixed to a solid support and a corresponding partner is in solution. By binding to the component fixed to the support, the partner now becomes attached to the support as well. A well-known example of this method is the microarray assays where labeled analytes become bound to discrete sites on the microarray. Homogeneous probe dependent assays are also well known in the art and may take advantage of the present invention. Examples of such methods are energy transfer between adjacent probes (U.S. Patent 4,868,103), the Taqman exonuclease assay (U.S. Patents 5,538,848 and 5,210,015), Molecular Beacons (U.S. Patents 5,118,801 and 5,925,517) and various real time assays (U.S. Patent Publication US2005/0137388).

Various applications may enjoy the benefits of binding the dyes of the present invention to appropriate targets. As described above, staining of macromolecules in a gel is a methodology that has a long history of use. More recent applications that also may find use are real time detection of amplification (U.S. Patents 5,994,056 and 6,174,670 and U.S. Patent Publication US2005/0137388), and binding of nucleic acids to microarrays. *In situ* assays may also find use where the binding of dyes of the present invention is used to identify the location or quantity of appropriate targets.

Amphiphilic compounds containing a cationic (aka basic) moiety often accumulate in lysosomes or other acidic subcellular compartments. This lysosomotropism is thought to be due to the protonation of the dye within acidic organelles leading to the formation of a membrane-impermeable form. Highly lipophilic dyes show a greater propensity to accumulate in lysosomes than those with a lower lipophilicity.

Some generalized approaches for identifying dyes that statically accumulate in lysosomes have previously been reported (Rashid, et al, 1991). While these guidelines for creating lysosome-targeting probes are known, the basis of the selectivity of specific fluorochromes for this group of subcellular organelles in live cells is sometimes elusive. To clarify this, interactions of living cells with series of different probe molecules, having systematically varied physicochemical properties, may be analyzed experimentally and numerically using approaches such as quantitative structure activity relationship analysis (QSAR) and Fick-Nernst-Planck analysis. Typically, a single cell line or a panel of cell lines is incubated with different concentrations (typically 1-100 μ M) of the potential vacuole-targeting compounds and subcellular distribution is monitored by wide-field fluorescence microscopy. Combinatorial synthesis of panels of dye derivatives may be subjected to cell-based screening in order to identify lead compounds with desired localization properties. The lysosome probes described in the literature tend to dissipate upon treatment of cells with drugs and agents that impinge upon the lysosomal catabolic pathways, rendering them unsuitable for xenobiotic screening in any dynamic context. Identification of probes that encompass both unperturbed and perturbed states requires screening both control cells and cells treated

with a perturbing agent, such as propranolol, triparanol, chlorpromazine, chloroquine, fluoxetine, clindamycin, ketoconazole or bafilomycin A.

Dyes conjugated to organelle specific moieties

In another embodiment of the present invention, the inventors have found that the combination of nuclear dye with a moiety that has an affinity for the lysosome can result in a conjugate that retains the ability to target the lysosomal organelle and endow it with the spectral properties of the dye. It has been found that a variety of subcellular organelle, region or domain targeting functional groups may be covalently affixed to a fluorophore core. Typically, either one or two such functional groups are affixed to the core structure, though in certain circumstances as many as four such groups can potentially be affixed to the fluorophore core. These are non-limiting examples of targeting groups that may find use with the present invention by being conjugated to an anthraquinone, anthrapyrazole or benzophenoxazine. Another important example is the conjugation of a fluorophore to a cationic amphiphilic drug, such as propranolol, triparanol, chlorpromazine, chloroquine, fluoxetine, clindamycin, or ketoconazole. Halogenated analogs of these drugs are especially suitable when compared to nonhalogenated analogs for profiling the lysosomal catabolic pathway. Without wishing to be bound by theory, this appears to be due to a stronger affinity of halogenated analogs for the lysosome and lysosome-related compartments.

Detecting Lysosomal Storage Diseases

Because the cationic amphiphilic tracer compounds exhibit specificity in localizing to cellular vacuoles, the present invention is particularly well suited for detecting the presence of a lysosomal storage disease in a subject or patient. For a good review of lysosomal disorders and lysosomal storage diseases, see Ballabio and Gieselmann, *Biochimica et Biophysica Acta* 1793:684-696 (2009).

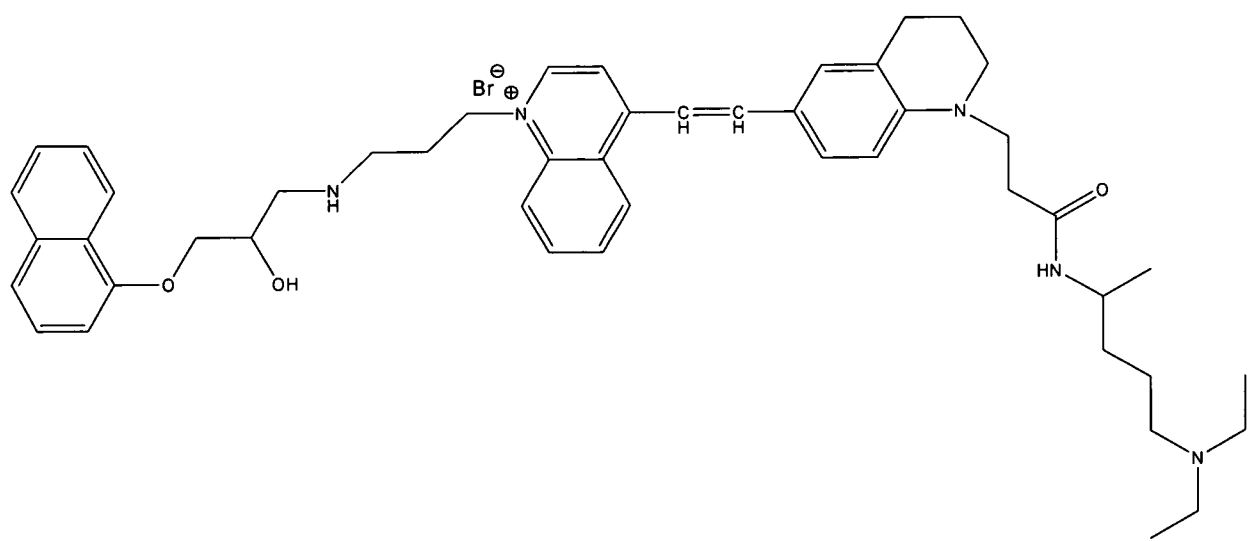
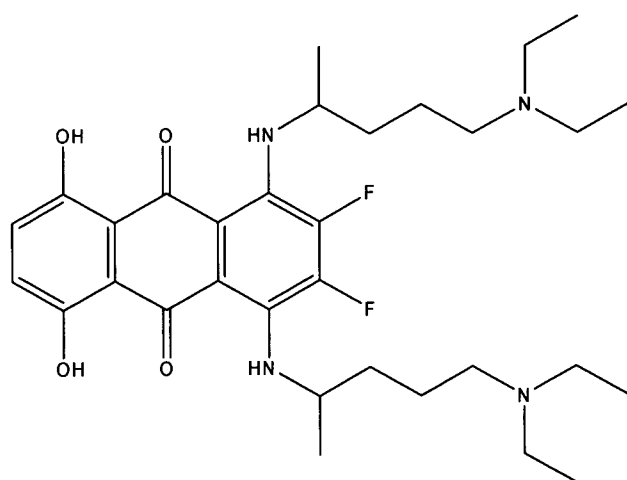
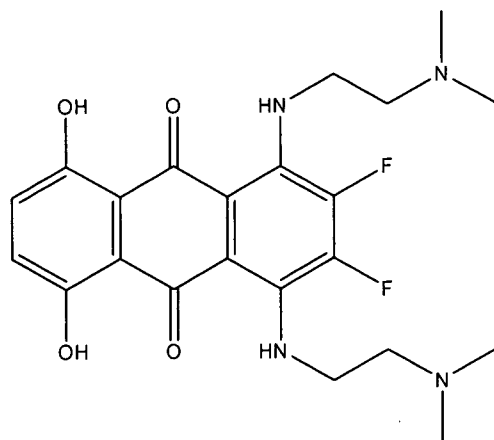
Briefly, the method for detecting lysosomal storage diseases is carried out by (a) obtaining a sample containing cells from the subject or patient followed by (b) contacting the sample with a cationic amphiphilic tracer compound that localizes to a vacuole in a cell. By next (c) detecting the cationic amphiphilic tracer compound, it can be determined whether there exists an excess above normal accumulation of vacuoles

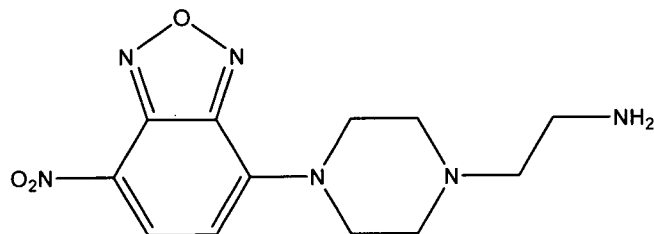
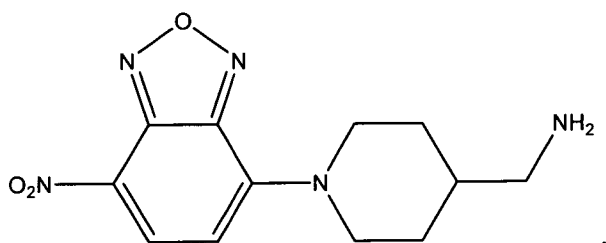
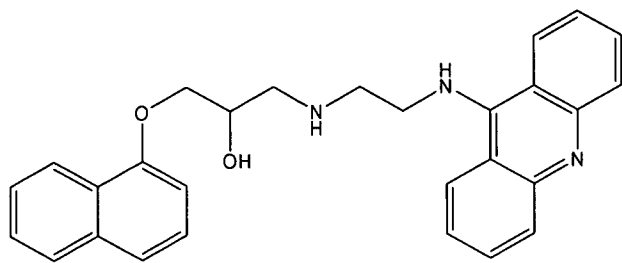
within the cells of the sample. Such an excess above normal accumulation of vacuoles is indicative of the lysosomal storage disease.

The lysosomal storage disease sought to be detected can arise from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome. Such storage diseases can also arise from a defect in one or more lysosomal membrane proteins. Further, these lysosomal storage diseases can arise from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus or an endosomal pathway, as well as combinations of any of the foregoing. In other instances, the lysosomal storage disease may comprise a mucopolysaccharidoses, a lipidoses or a glycogenosis. Moreover, this method is applicable to a subject is known to have a lysosomal storage disease based upon previous genetic or metabolic testing.

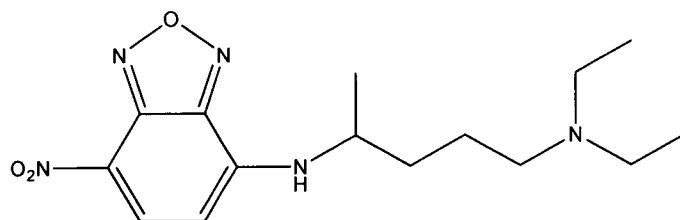
As discussed above, the cationic amphiphilic tracer compound provides for specific localization in vacuoles of cells. Such vacuoles include, for example, lysosomes, phagophores, autophagosomes or autophagolysosomes, and combinations of the foregoing. The cationic amphiphilic tracer compound can comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis. The cationic amphiphilic tracer compound can also comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis. Among useful cationic amphiphilic tracer compound of the present invention are those which comprise nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, or 9-amino-acridin-propanolol, and combinations of any of the foregoing.

Of particular interest in the present invention are cationic amphiphilic tracer compound having any of the following structures:

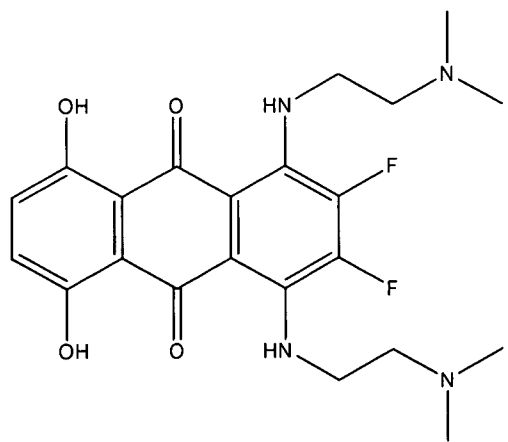


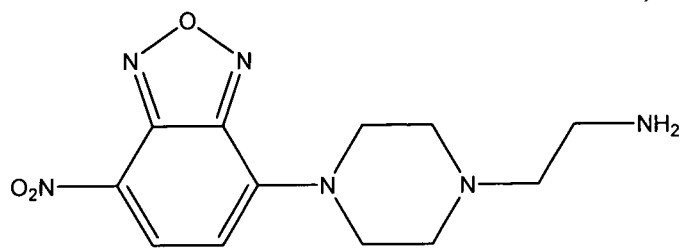
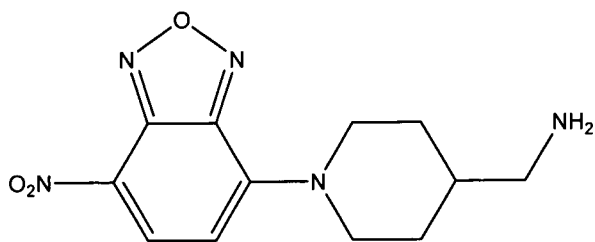
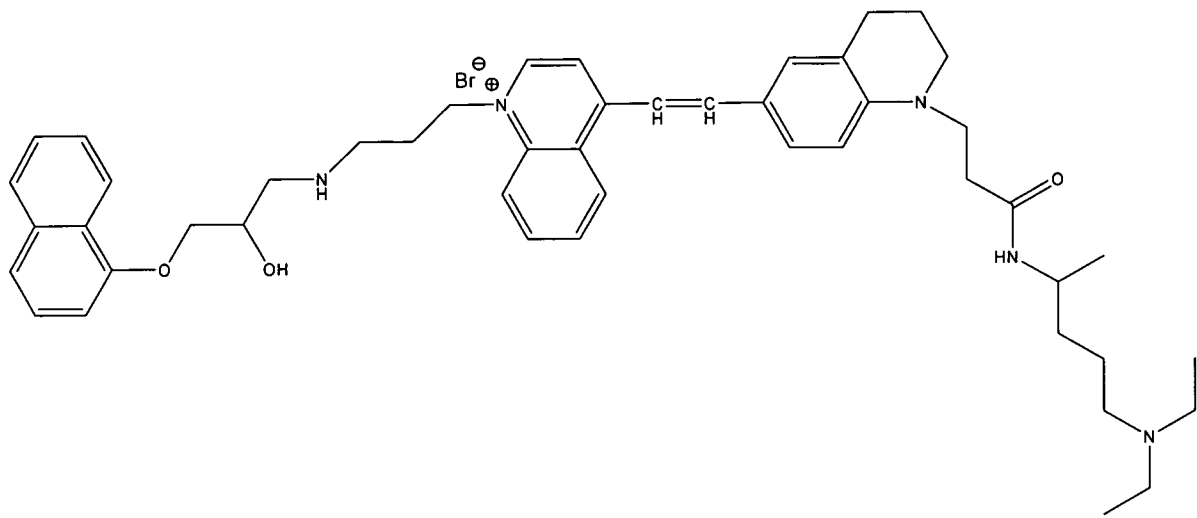


or

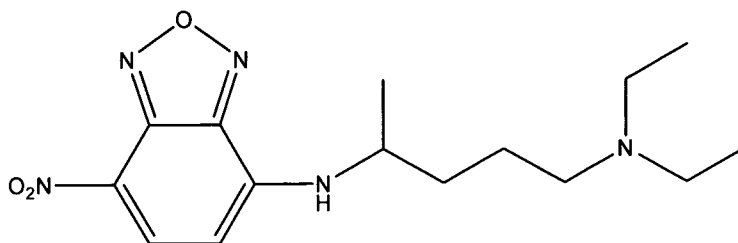


Particularly well suited for detecting lysosomal storage diseases are compound dyes having the structures:





or



The above detection method can also be modified by providing a reference sample of normal cells from a subject or patient who is known not to have a lysosomal storage disease. Thus, the above-described method would further comprise the following steps (a') of providing a reference sample of normal cells from a subject

known not to have lysosomal storage disease; (b') contacting the sample of normal cells with the same cationic amphiphilic tracer compound of step (b); and (c') comparing the accumulation of vacuoles in the sample obtained from the subject known or suspected of having a lysosomal storage disease with the accumulation in the reference sample of normal cells.

The cells which are contained in the sample obtained from a subject or patient can comprise lymphocytes, granulocytes, macrophages or monocytes or a combination of any or all of these. The subject may be a mammal, for example a human.

Formulations and compositions

Compositions according to the invention comprise a compound of the invention and are intended to include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like, compatible with the application. Except insofar as any conventional media or agent is compatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

For example, when a composition of the invention is applied to cells or tissues, it is formulated to be compatible with the intended route of entry into the cells or tissues. For example, isotonic saline solutions, mildly hypertonic saline solutions, phosphate-buffered saline, cell culture media, isotonic sucrose solutions, or mildly hypertonic sucrose solutions may serve as the vehicle for delivery of the compound to the cells. Polyethylene glycols, glycerin, dimethylsulfoxide, dimethylformamide, propylene glycol, or other co-solvents may be included to facilitate solubilization of the compound. Antibacterial agents, such as benzyl alcohol or methyl parabens; antioxidants, such as ascorbic acid or sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid (EDTA) or cyclodextrin; buffers, such as acetates, phosphates or citrates; agents for adjusting tonicity, such as sodium chloride or sucrose; and agents that adjust the pH value of the delivery vehicle, such as sodium hydroxide and hydrochloric acid may be incorporated in the formulation of the compound.

Compositions of the said invention may include certain anions and cations (e.g. alkyl metal chlorides) to facilitate penetration of the compound thru cell membranes.

Non-limiting examples of anions include barbitol, bicarbonate, borate, chloride, oxylate or EDTA. Not all anions have been found suitable for promoting penetration of cell membranes. Non-limiting examples of cations include sodium (as in sodium chloride), potassium (as in potassium chloride), tris-hydroxymethylaminomethane (TRIS), tris-hydroxymethylaminomethane-hydrochloric acid (TRIS-HCl), or triethanolamine (TEA).

Reagent kits

Commercial kits are valuable because they eliminate the need for individual laboratories to optimize procedures, saving both time and resources. They also allow better cross-comparison of results generated from different laboratories. The present invention additionally provides reagent kits, i.e., reagent combinations or means, comprising all of the essential elements required to conduct a desired assay method. The reagent system is presented in a commercially packaged form, as a composition or admixture where the compatibility of the reagents will allow, in a test kit, i.e., a packaged combination of one or more containers, devices or the like holding the necessary reagents, and usually written instructions for the performance of the assays. Reagent systems of the present invention include all configurations and compositions for performing the various labeling and staining formats described herein.

The reagent kits may generally comprise (1) one or more fluorochrome designed to target the lysosome and/or related organelles; and (2) instructions for usage of the included reagents. Generic instruction, as well as specific instructions for the use of the reagents on particular instruments, such as a wide-field microscope, confocal microscope, flow cytometer or microplate-based detection platform may be provided. Recommendations regarding filter sets and/or illumination sources for optimal performance of the reagents for a particular application may also be provided.

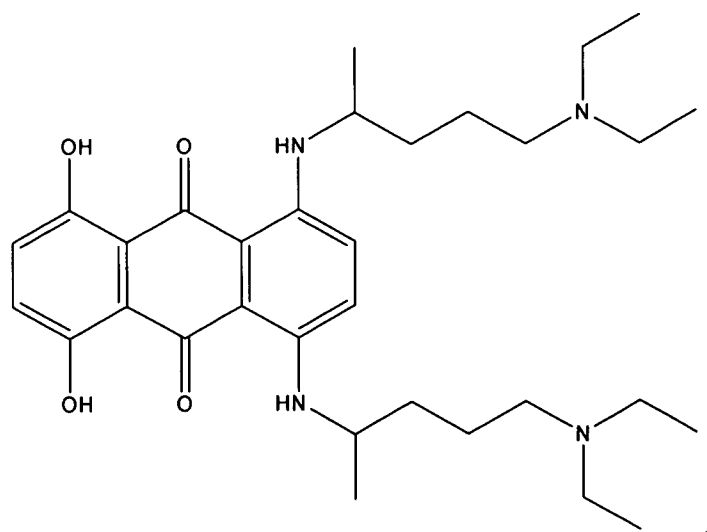
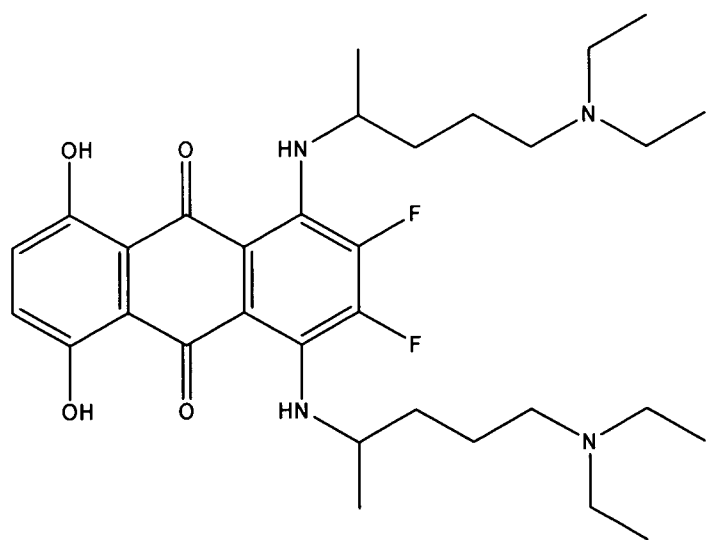
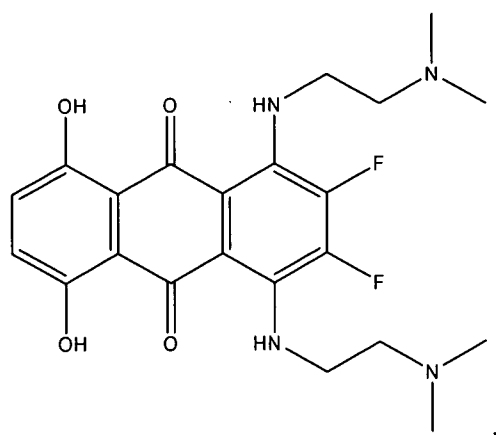
A test kit form of the system for detecting xenobiotics that modify the lysosomal-dependent catabolic pathway, for example, can contain one or more substituted anthraquinone, phenoxazines, anthrapyrazole or benzophenoxazine fluorochromes that localize to the lysosome, and additional ancillary chemicals, such as dilution buffer, live-cell DNA stain, live-cell mitochondrial stain and/or an antibody, a lectin, a Ca^{2+} -dependent, phospholipid binding protein (such as Annexin V), or other reporter labeled

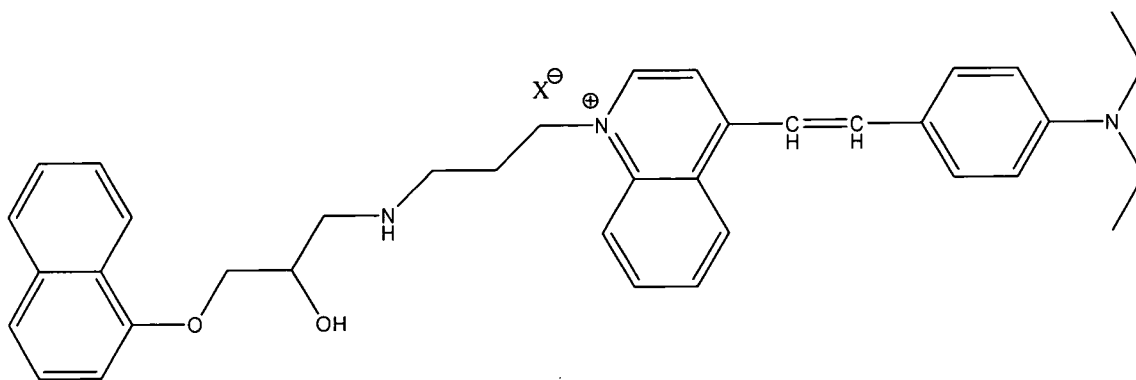
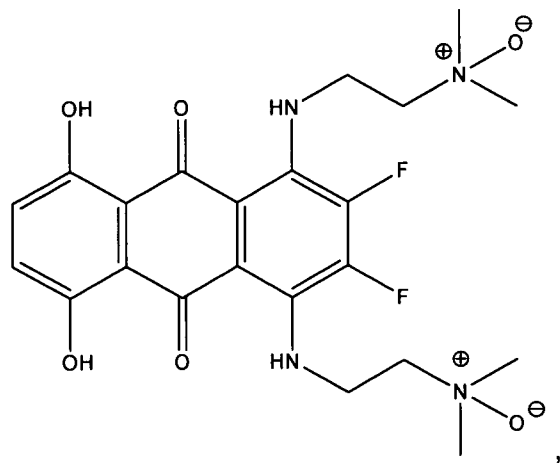
with a fluorophore. In some instances one or more fluorochrome may be combined within a single container for easier use. In some instances, calibrants are included, such as microsphere or bead standards of known fluorescent output.

Phenotypic monitoring of the progression of lysosomal storage diseases

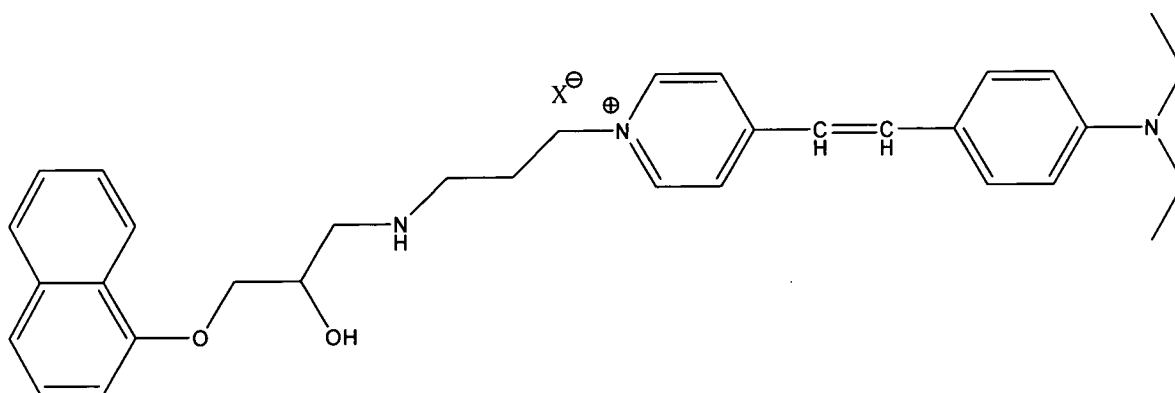
Often with inherited lysosomal storage diseases, there is no strict correlation between disease-causing genetic mutations and the severity of clinical phenotype. For example, in Nieman-Pick disease, some patients develop severe neurological symptoms during the first two years of life. These patients demonstrate delayed motor development and hypotonia. They typically die within their first five years of life. More commonly, patients exhibit first symptoms between the ages of 3 and 15 years, often first noted as a loss of speech and increasing clumsiness. Neurological symptoms may progress to include cerebellar ataxia, dysarthria, dysphagia, cataplexy, seizures, dystonia, vertical gaze palsy and dementia. These patients usually die by 8 to 15 years of age. Adult onset cases of the disease have also been documented, usually occurring in the patient's twenties or thirties, though occasionally as late as in their mid-sixties. These patients develop similar symptoms to the classical; form of juvenile Nieman-Pick disease and often expire in their 30's and 40's. Though there are currently no effective treatments for Nieman-Pick and many of the other lysosomal storage diseases, it is recognized that the described assays hold promise in the routine phenotypic assessment of lysosomal dysfunction in patients, which could eventually lead to appropriate therapeutic intervention prior to neurological compromise.

Selected embodiments of the compounds of this invention include but are not limited to following:

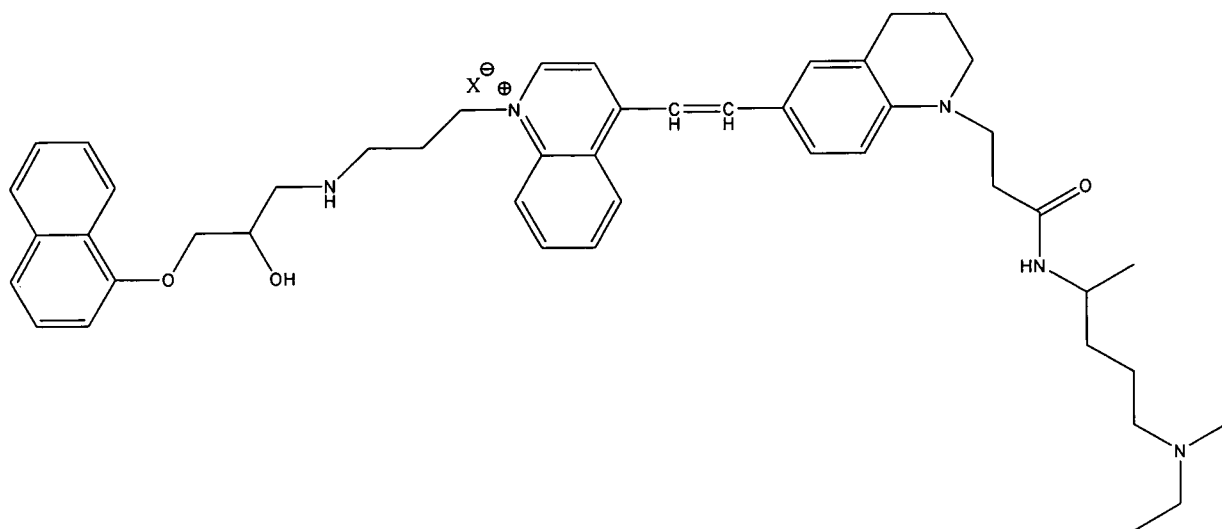




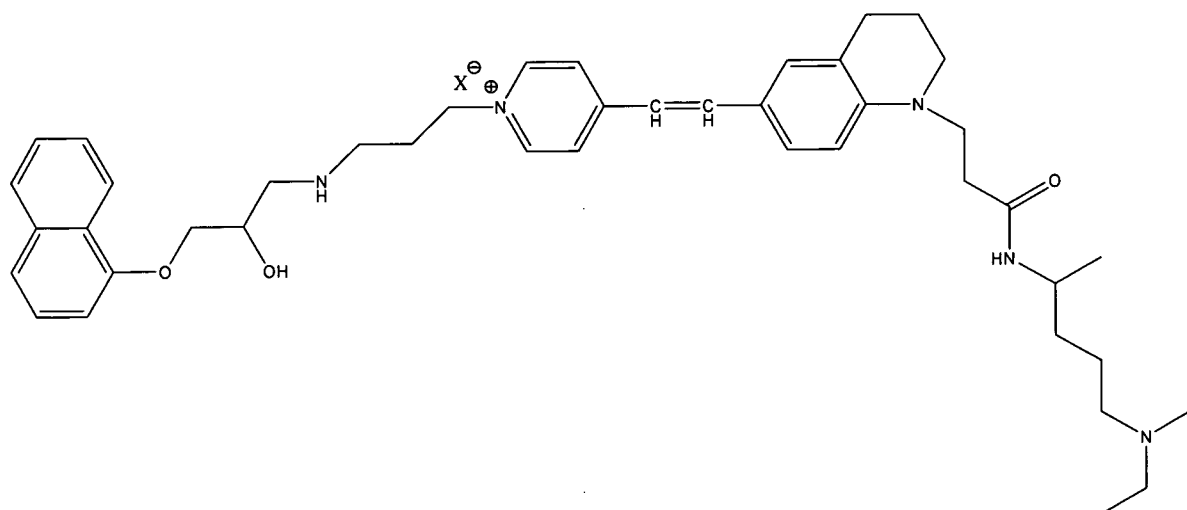
where X comprises an anion,



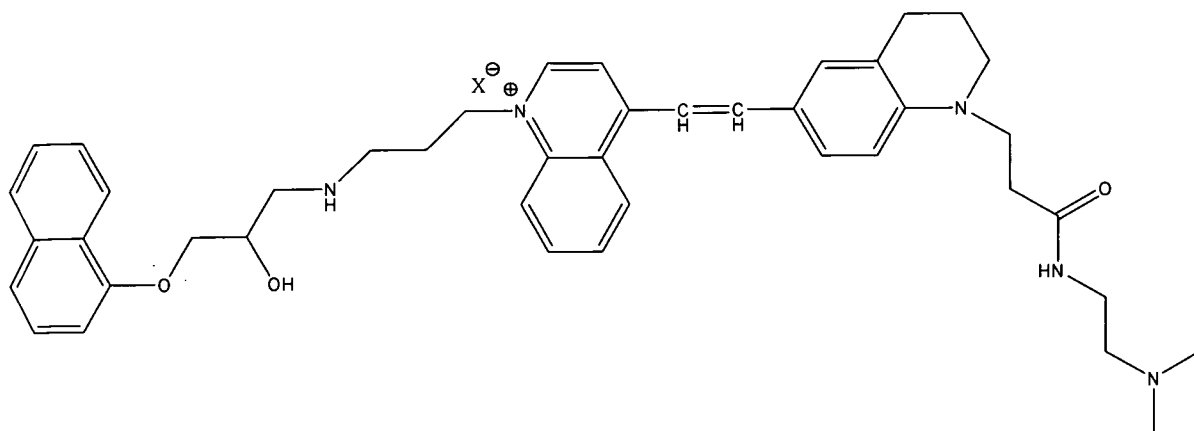
where X comprises an anion,



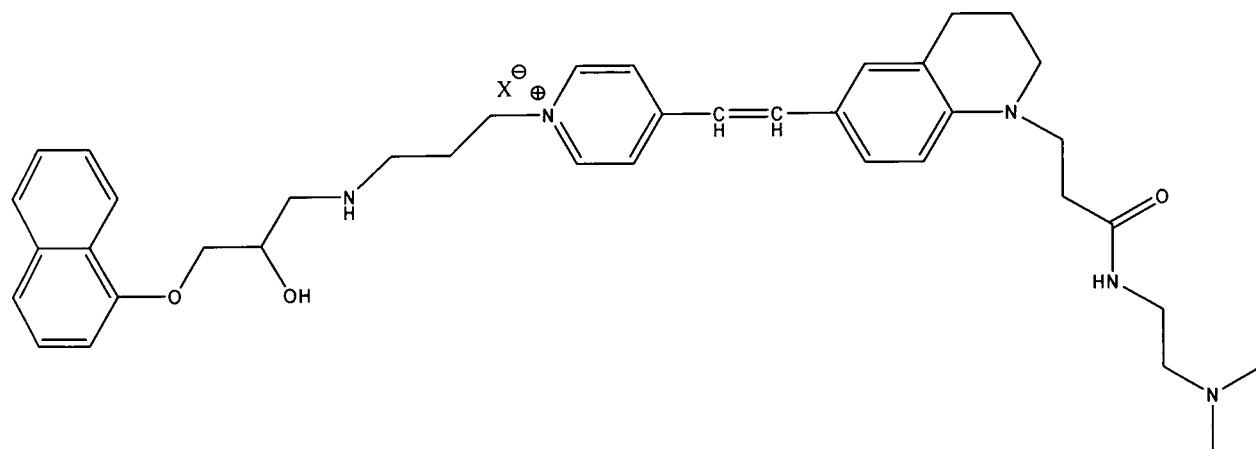
where X comprises an anion,



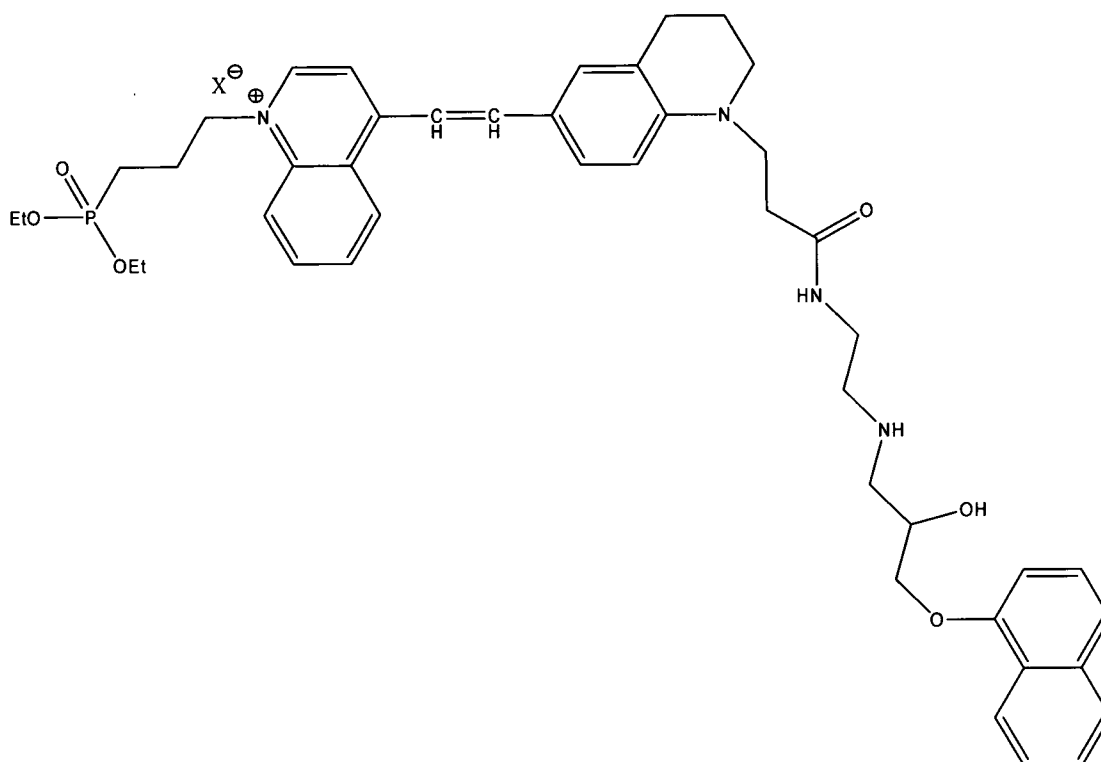
where X comprises an anion,



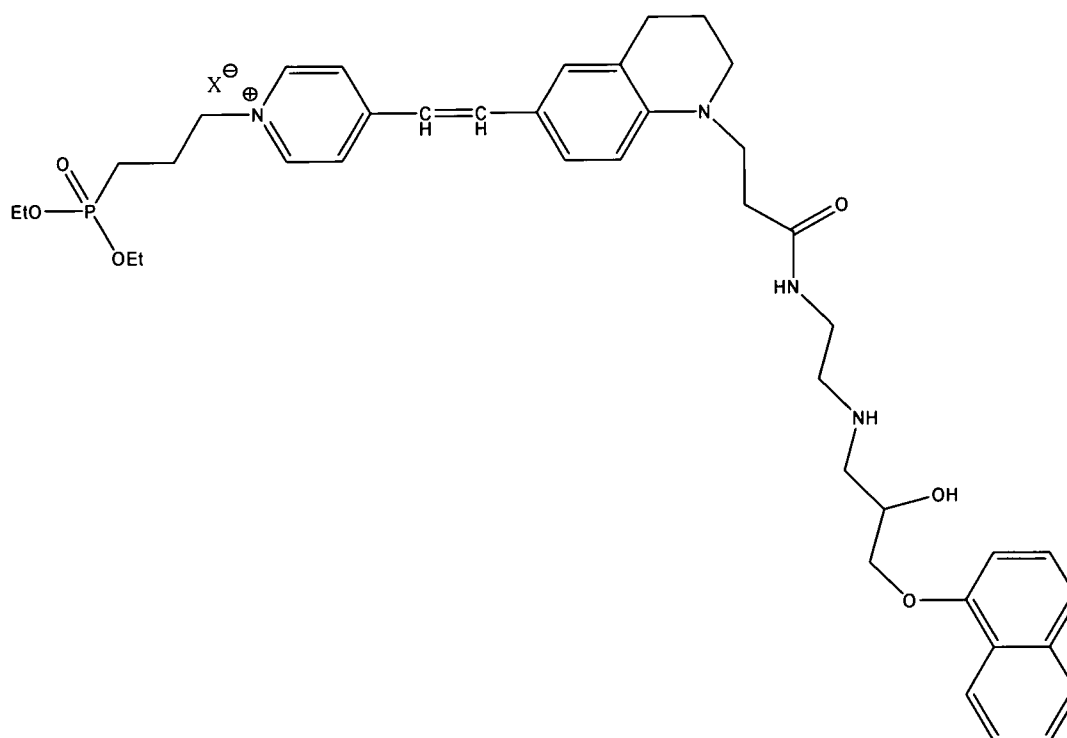
where X comprises an anion,



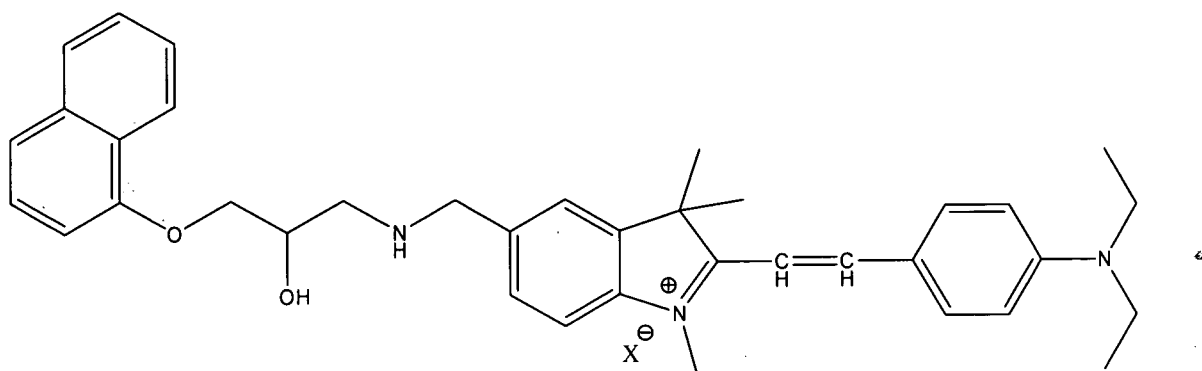
where X comprises an anion,



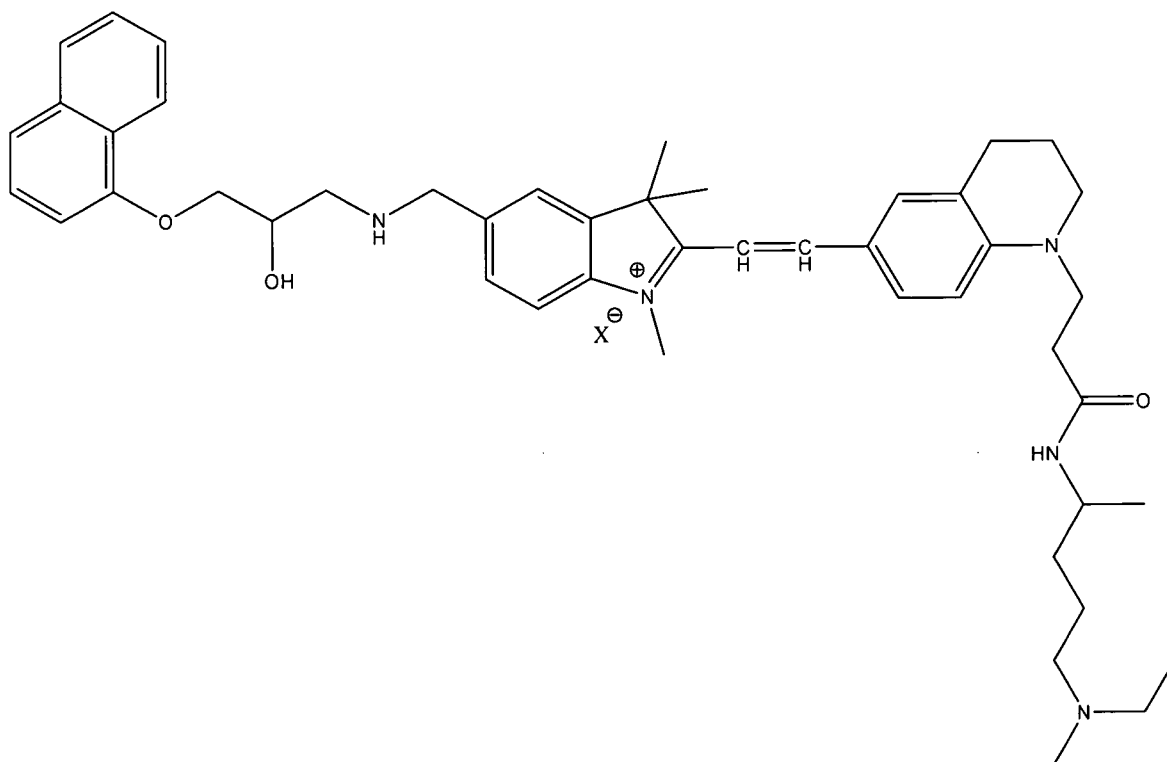
where X comprises an anion,



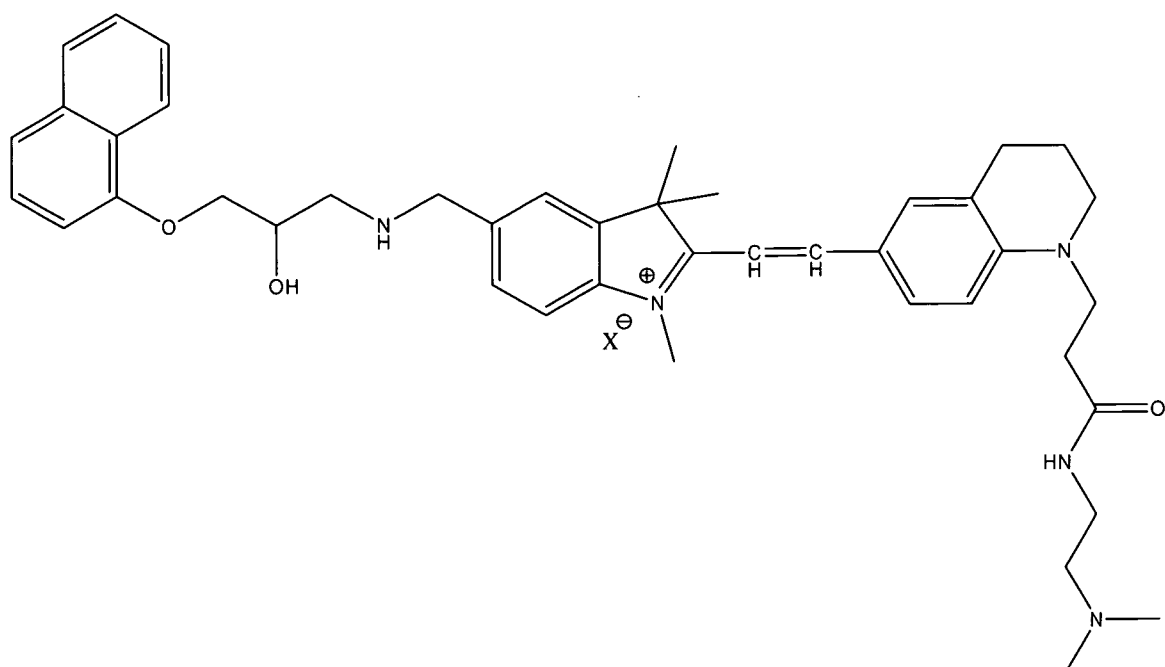
where X comprises an anion,



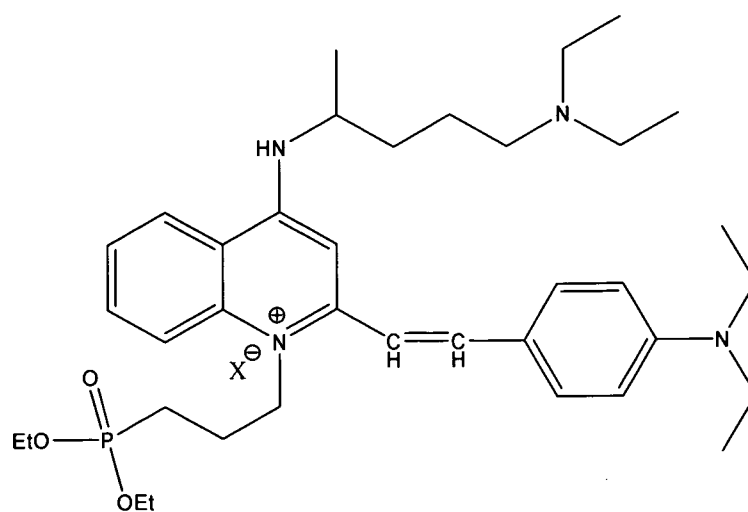
where X comprises an anion,



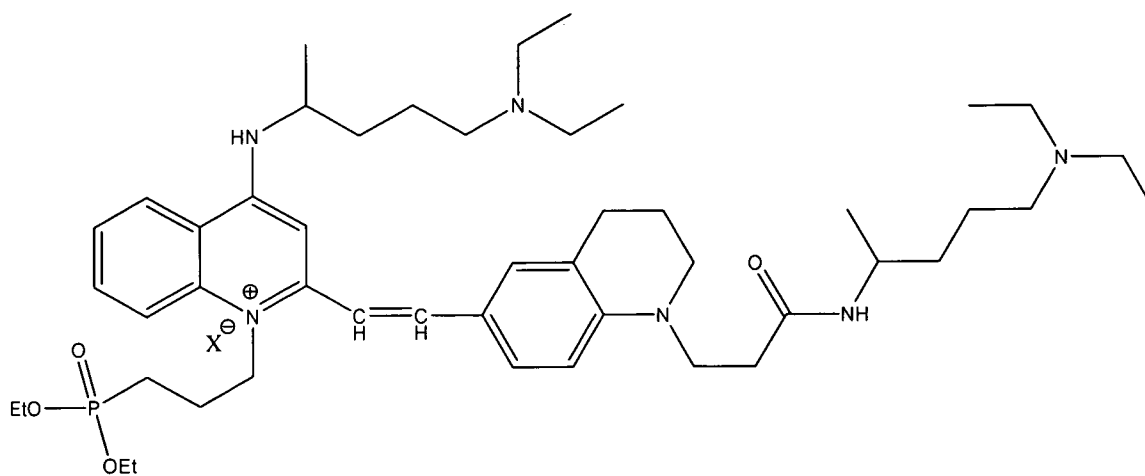
where X comprises an anion,



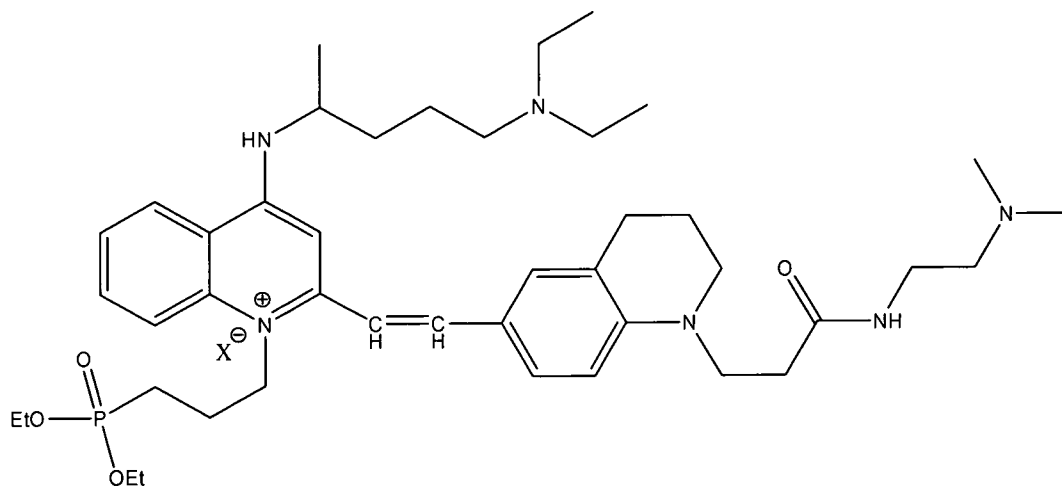
where X comprises an anion,



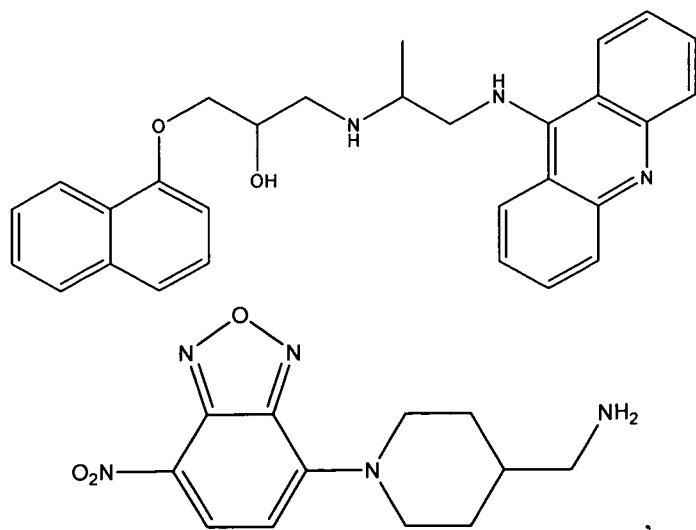
where X comprises an anion,

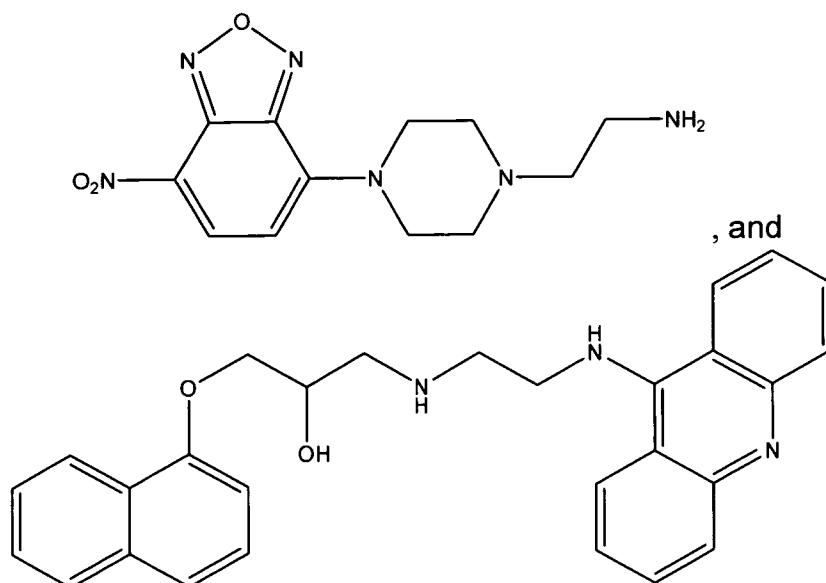


where X comprises an anion,



wherein X comprises an anion,





Monitoring Progression of Lysosomal Storage Disease

This invention further provides a method of monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of ($T_2 - T_1$) time interval(s). Briefly, this method comprises carrying out the steps of: (a) obtaining from the subject at time T_1 a first sample containing cells; (b) contacting the first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (c) detecting the first cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within the cells of the first sample at time T_1 ; (d) obtaining from the subject at a later time T_2 a second sample containing cells; (e) contacting the second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (f) detecting the second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and (g) comparing the accumulation of vacuoles between the first sample at time T_1 and the second sample at time T_2 , thereby monitoring the progression of lysosome storage disease in the subject.

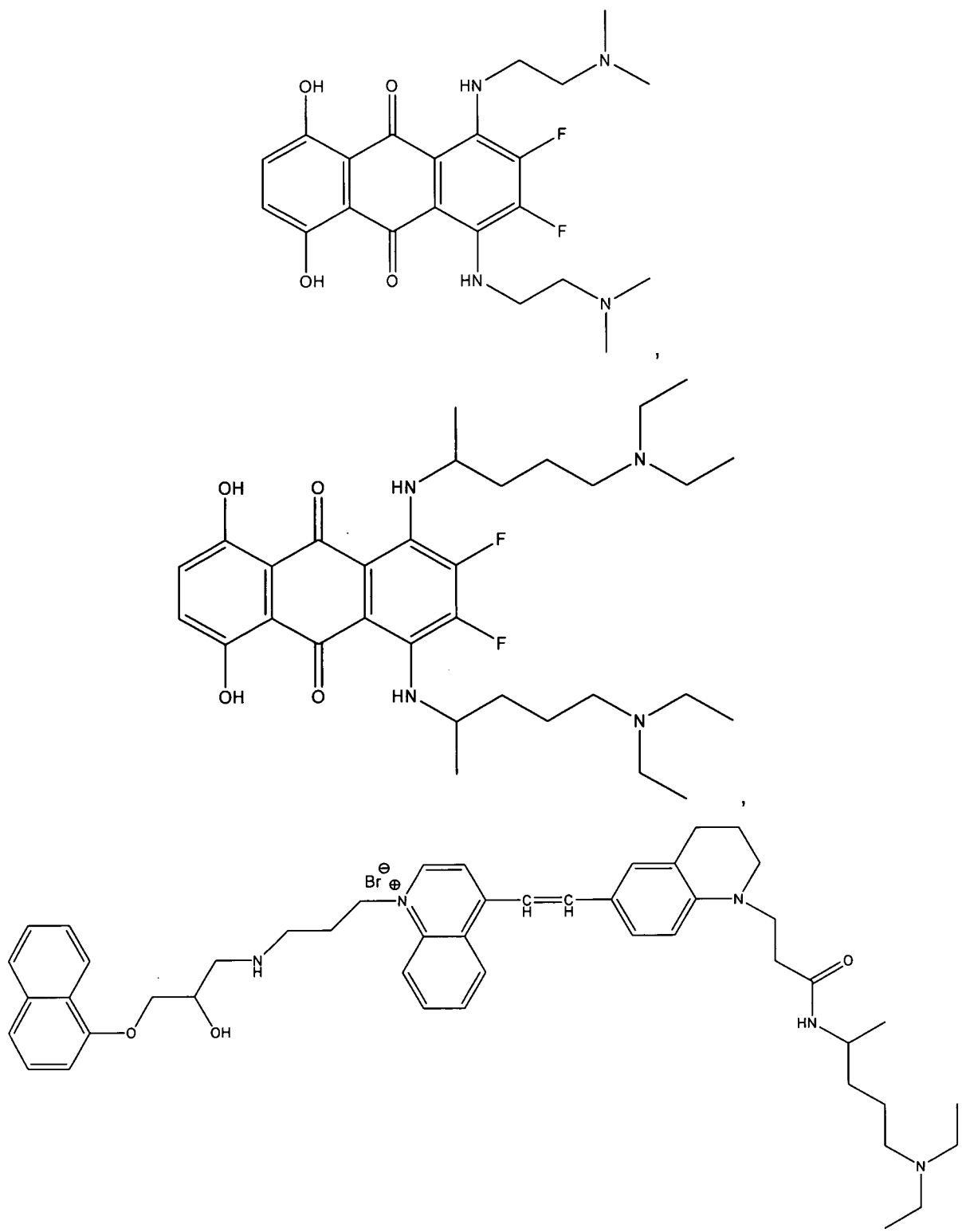
As described earlier, the lysosomal storage disease sought to be detected can arise from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome. Alternatively, the lysosomal storage disease can arise from a defect in one or more lysosomal membrane proteins. In other embodiments, the lysosomal

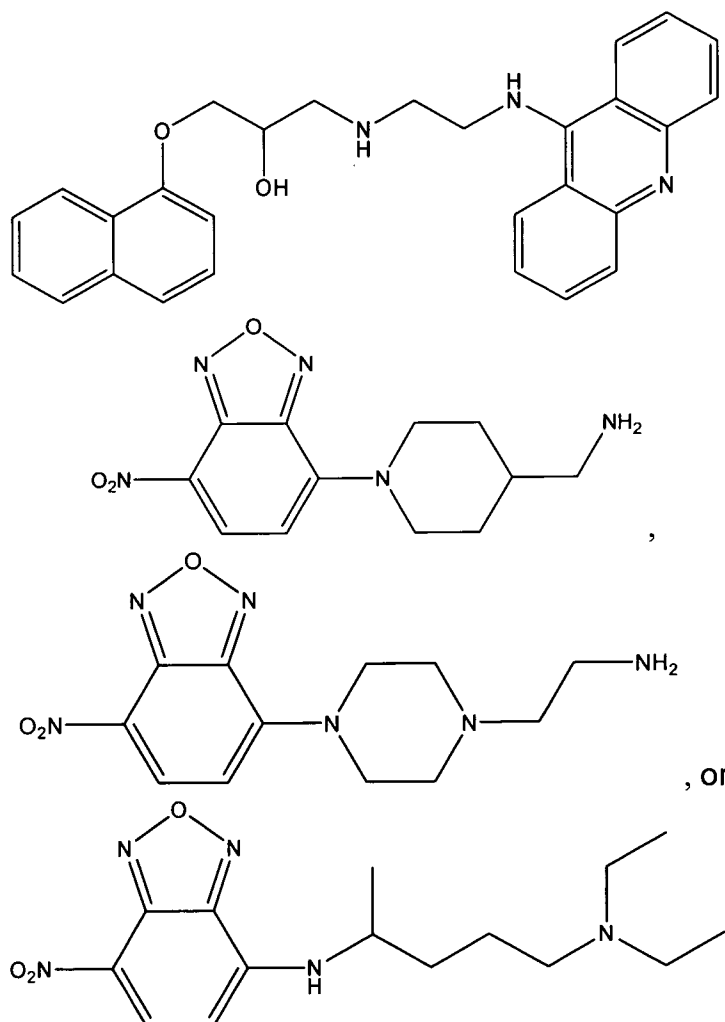
storage disease can arise from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus or an endosomal pathway, as well as combinations of any of the foregoing. In yet other embodiments, the lysosomal storage disease can comprise a mucopolysaccharidoses, a lipidoses or a glycogenosis. Moreover, in carrying out the present detection method, the subject can be one known to have a lysosomal storage disease based upon previous genetic or metabolic testing. The list of lysosomal storage diseases (some 40 in number) are provided above in the definition of *lysosomal storage disease* and need not be repeated here.

The vacuole in which the cationic amphiphilic tracer compound localizes can comprise a lysosome, a phagophore, an autophagosome or an autophagolysosome, or any combination thereof. The first cationic amphiphilic tracer compound and the second cationic amphiphilic tracer compound can comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis. As a further aspect, the first cationic amphiphilic tracer compound and the second cationic amphiphilic tracer compound can comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis. Indeed, the first cationic amphiphilic tracer compound and the second cationic amphiphilic tracer compound can be the same compound, or they can be different compounds.

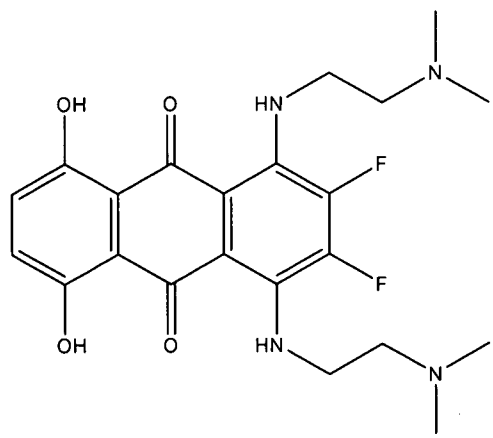
Among useful cationic amphiphilic tracer compounds for the present invention and method are those comprising nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, or 9-amino-acridin-propranolol, and combinations of any of the foregoing.

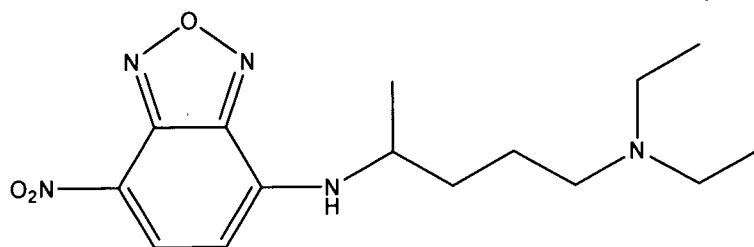
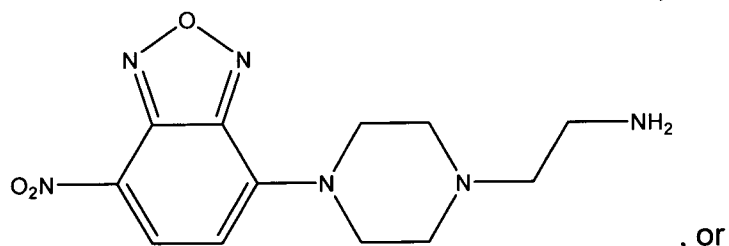
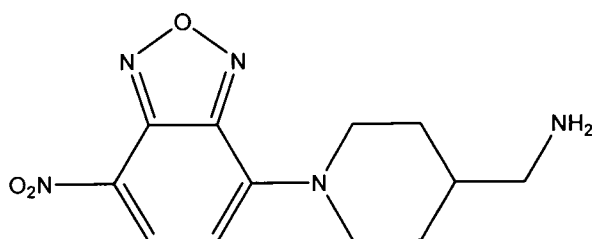
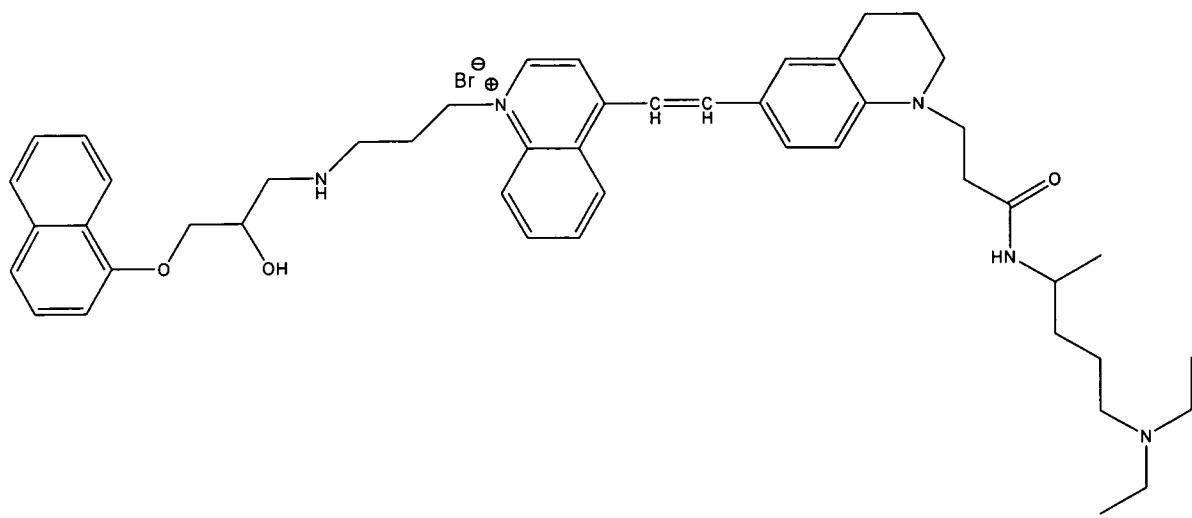
In other embodiments, the cationic amphiphilic tracer compounds have any of the the structures:





Preferred cationic amphiphilic tracer compounds for carrying out the method for monitoring lysosomal storage disease progression are those having the structures:





In carrying out this method to monitor the progression of lysosomal storage disease, the difference ($T_2 - T_1$) comprises time units can be measured in hours, days, weeks, months or years.

The cells contained in the sample obtained from the subject being monitored can comprise lymphocytes, granulocytes, macrophages or monocytes, or any combination thereof. Further, the subject or patient can be a mammal which can be human.

The cations described above serve as counterions for the compounds and dyes of the present invention. Examples of cations that may serve as counterions include but are not be limited to hydrogen, sodium, potassium, lithium, calcium, cesium, ammonium, alkyl ammonium, alkoxy ammonium and pyridinium. When a dye comprises a cationic group, an anionic counterion will also be present. Any anion may serve this purpose as long as it doesn't interfere with the use of the dye. Examples of anions that may serve as counterions include but are not limited to halides such as a bromide, chloride, fluoride and iodide. Other examples of anions that can serve as counterions include but are not limited to perchlorate (ClO_4^-), sulfate (SO_4^{2-}), sulfonate, alkane sulfonate, aryl sulfonate, phosphate, tosylate, mesylate and tetrafluoroborate moieties.

As also described above, in some cases the counterion or counterions are provided by the dye being presented as a salt where it exists as separate ionic species. In other cases, the counterion or counterions may be present as part of the compound (sometimes called inner salts). It is understood that a combination of ions may be provided by the compound and salts. With regard to acid moieties that are shown in forms such as COOH , it should be understood and appreciated that these compounds may be found in ionized forms such as COO^- . It should also be appreciated by those skilled in the art that the stoichiometric number of counterion or counterions which balance the charge or charges on the compounds of the present invention can be the same or they can be different, provided that the counterions balance the charge(s) on the compound. The combination of counterions can be selected from any of the anions described above. Similarly, the combination of counterions can also be selected from any of the cations described above.

Monitoring Drug/Remedy Effectiveness for Lysosomal Storage Disease

The present invention further provides a method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease in a subject, comprising the steps of: (a) obtaining from the subject at time T_1 a first sample containing cells; (b) contacting the first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (c) detecting the first cationic amphiphilic

tracer compound, thereby determining the accumulation of vacuoles within the cells of the first sample at time T_1 ; (d) obtaining from the subject at a later time T_2 a second sample containing cells; (e) contacting the second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (f) detecting the second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and (g) comparing the accumulation of vacuoles between the first sample at time T_1 and the second sample at time T_2 , thereby monitoring the effectiveness of the drug used to manage, treat or cure lysosome storage.

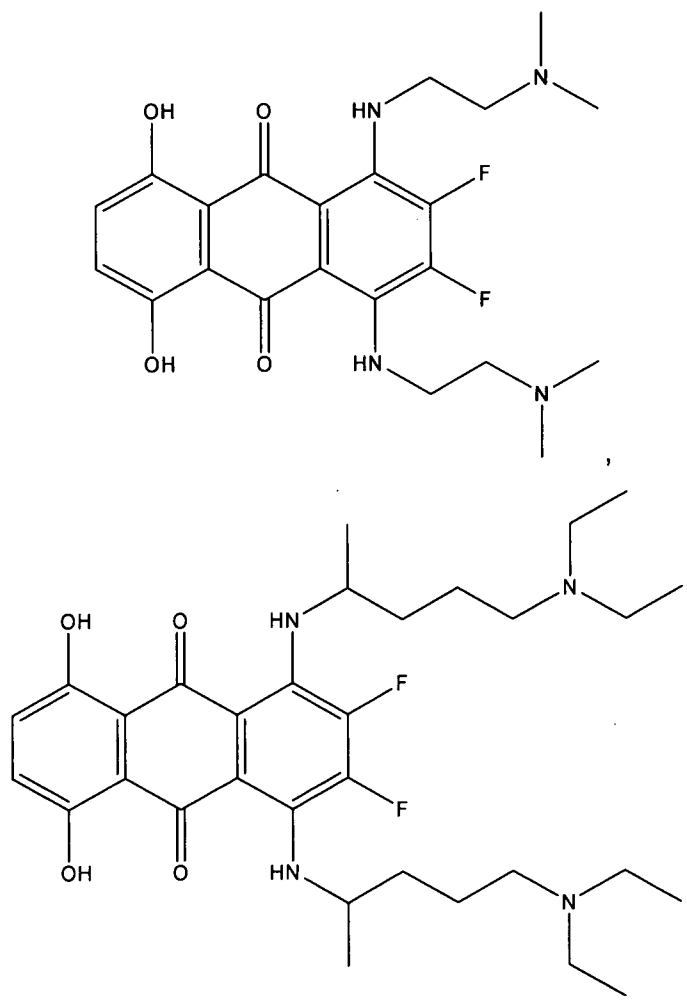
As explained above, lysosomal storage disease can arise from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome. Alternatively, the lysosomal storage disease can arise from a defect in one or more lysosomal membrane proteins. In other aspects, the lysosomal storage disease can arise from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus or an endosomal pathway, and combinations of any of the foregoing. In further aspects, the lysosomal storage disease comprises a mucopolysaccharidoses, a lipidoses or a glycogenosis. Moreover, the method can be usefully carried out in a subject is known to have a lysosomal storage disease based upon previous genetic or metabolic testing.

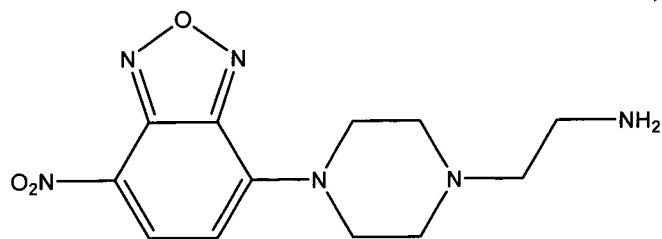
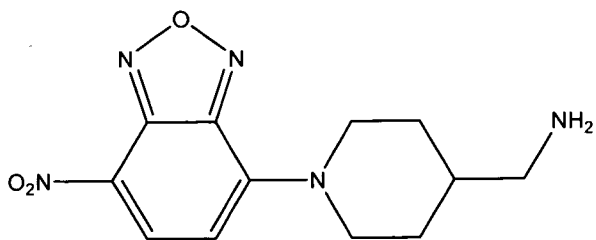
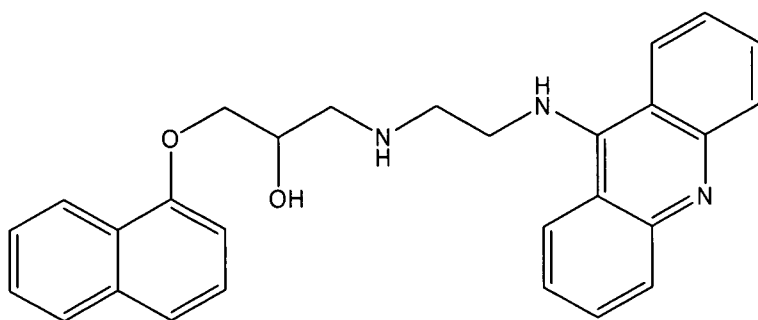
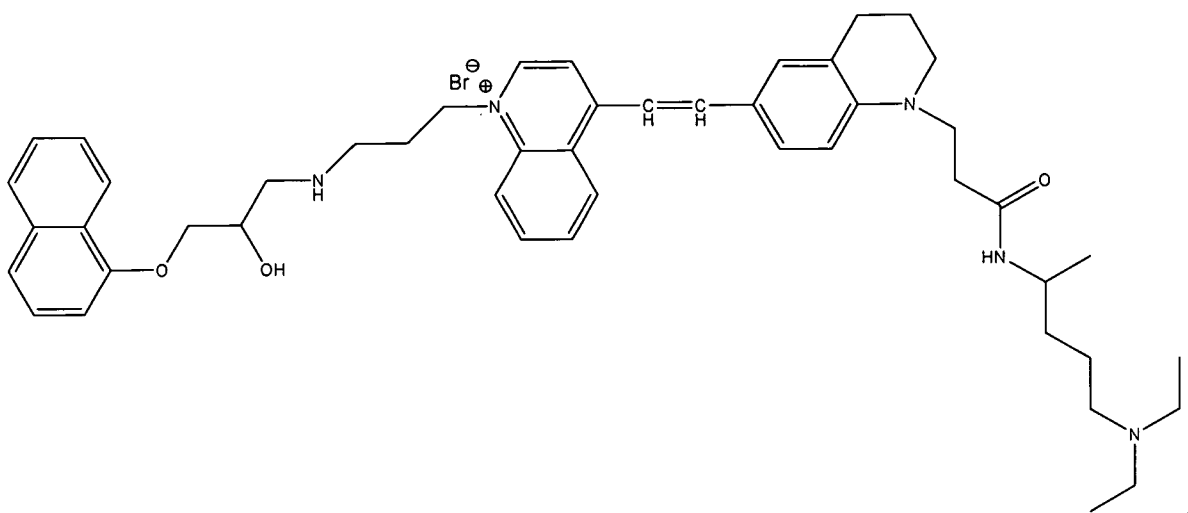
The first and second cationic amphiphilic tracer compounds can localize in vacuoles, the latter comprising lysosomes, phagophores, autophagosomes or an autophagolysosomes, and combination of the foregoing. Useful in the present invention are first cationic amphiphilic tracer compounds and the second cationic amphiphilic tracer compounds comprising fluorescent drugs that induces phospholipidosis or a fluorescently labeled analogs of a drug that induces phospholipidosis. In other aspects, the first cationic amphiphilic tracer compounds and the second cationic amphiphilic tracer compound comprise halogenated and fluorescent drugs that induce phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.

It should be appreciated by those skilled in the art that the first cationic amphiphilic tracer compound and the second cationic amphiphilic tracer compound can be the same compounds, or they can be different compounds.

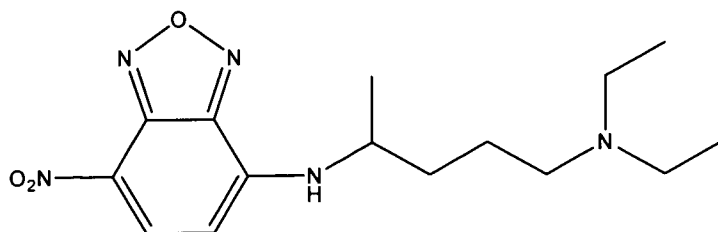
Among useful cationic amphiphilic tracer compounds in the present invention and method are those comprising nitrobenzoxadiazole (NBD)-spiperone, NBD-propranolol, or 9-amino-acridin-propanolol, and combinations of any of the foregoing.

In other embodiments, the cationic amphiphilic tracer compounds have any of the following the structures:

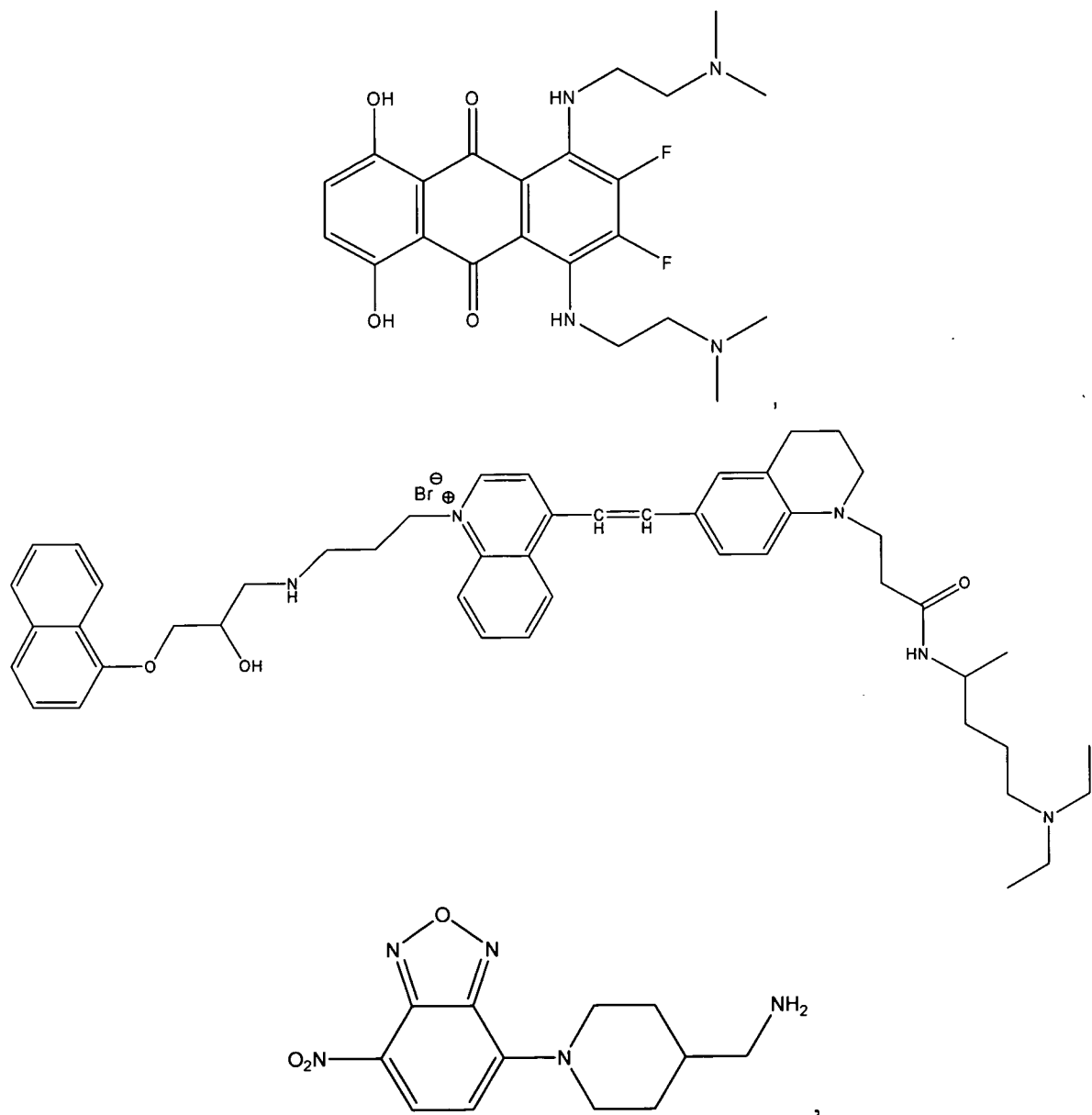


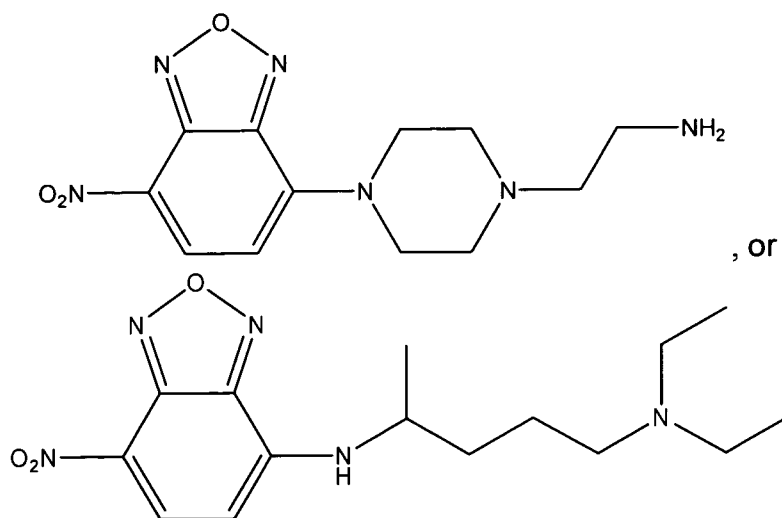


, or



Particularly preferred in carrying out the present invention and method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease in a subject are those cationic amphiphilic tracer compounds having the structures:





In monitoring the effectiveness of any drug or remedy used to manage, treat or cure lysosomal storage disease, the difference ($T_1 - T_2$) can comprise time units measured in various units, such as hours, days, weeks, months or years.

The cells which are contained in the samples obtained from the subject or patient can comprise lymphocytes, granulocytes, macrophages or monocytes, or any combination thereof. The subject can be a mammal (e.g., a human).

Screening Drug Candidates For Toxicity

This invention also provides for screening drug candidates to determine their toxicity to cells, tissues or organs. This method comprises the steps of: (a) providing: (i) at least one first sample and one second sample of mammalian cells; (ii) a drug candidate for screening; (iii) a drug diluting vehicle or carrier; and (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (b) administering: (i) the drug candidate (ii) to the first sample for a time sufficient to allow the drug candidate to partition into subcellular compartments within the cells to form treated cells; and (ii) the vehicle or carrier (iii) to the second sample to form untreated reference cells; (c) contacting the treated cells and the untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and (d) detecting any increase in vacuole accumulation of the cationic amphiphilic tracer compound in the treated cells relative to the untreated reference cells, thereby

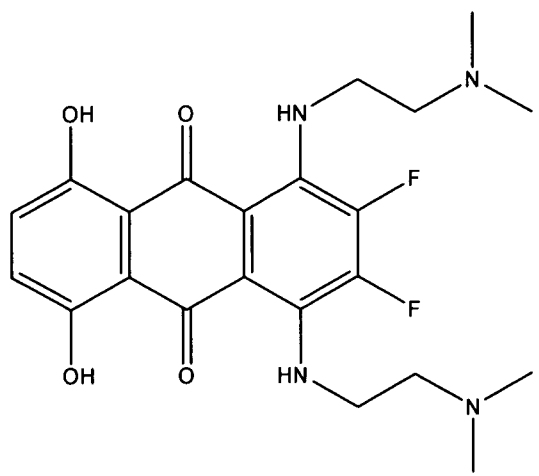
determining whether the drug candidate has accumulated in the vacuoles and is toxic to cells, tissues or organs.

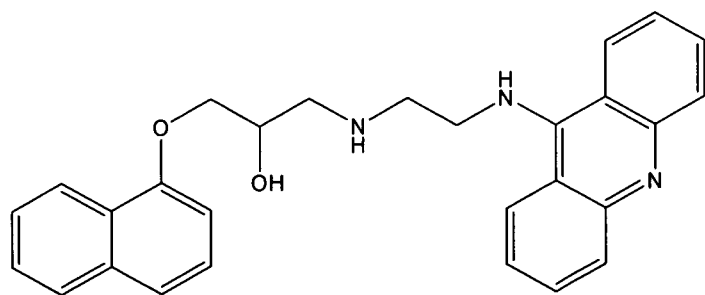
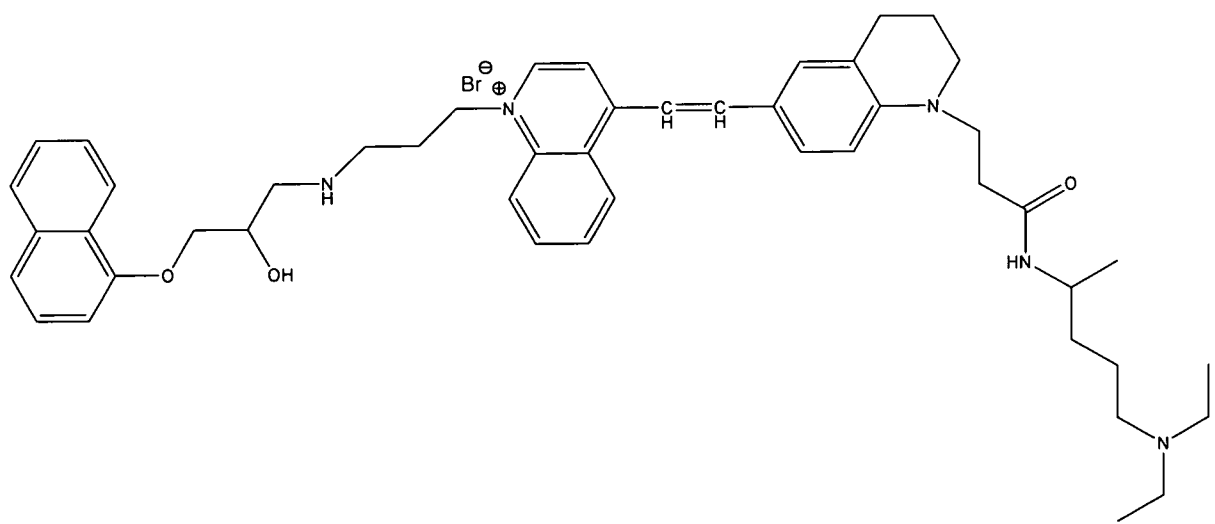
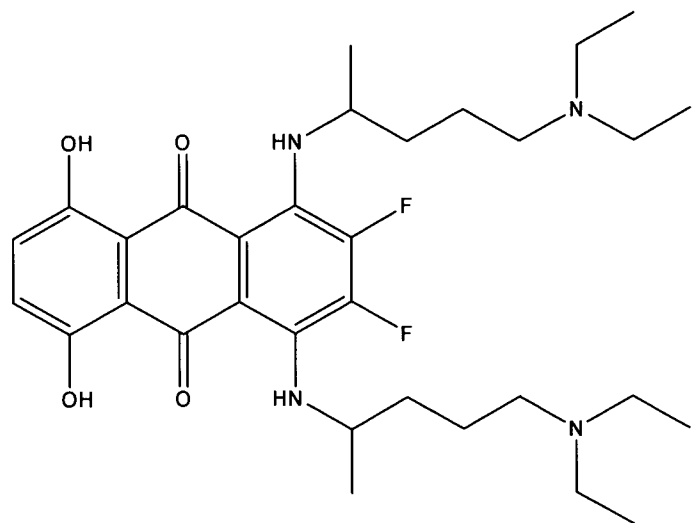
In other aspects of this method, the vehicle or carrier comprises cell culture medium, buffer, organic solvent or water, and combinations of the foregoing. The organic solvent can comprise dimethylsulfoxide (DMSO).

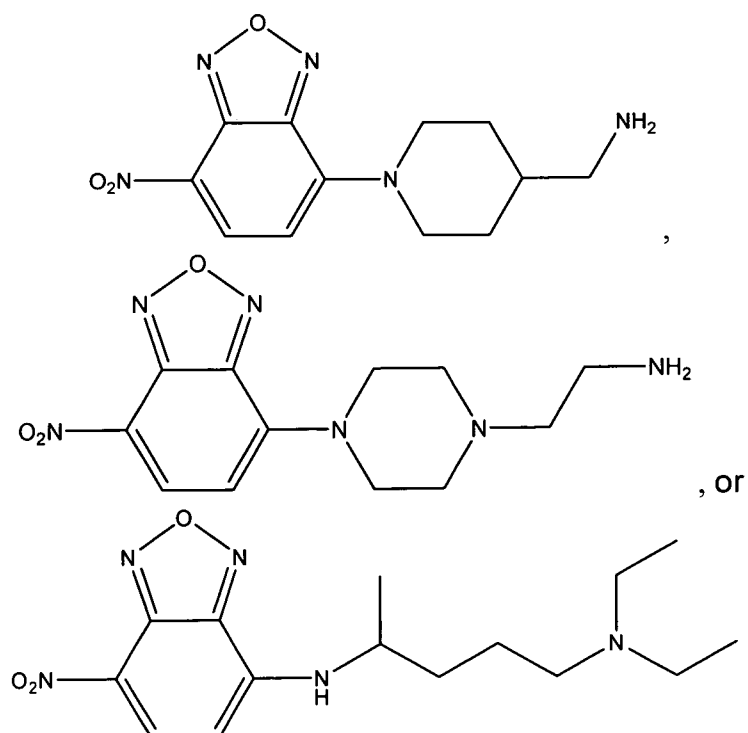
The vacuoles in the mammalian cells can comprise lysosomes, phagophores, autophagosomes or autophagolysosomes, or any combination thereof.

In other embodiments, the first cationic amphiphilic tracer compound and the second cationic amphiphilic tracer compound can comprise fluorescent drugs that induce phospholipidosis or fluorescently labeled analogs of a drug that induce phospholipidosis. In yet further embodiments, the first cationic amphiphilic tracer compounds and the second cationic amphiphilic tracer compounds can comprise halogenated and fluorescent drugs that induce phospholipidosis or halogenated and fluorescently labeled analogs of a drug that induce phospholipidosis.

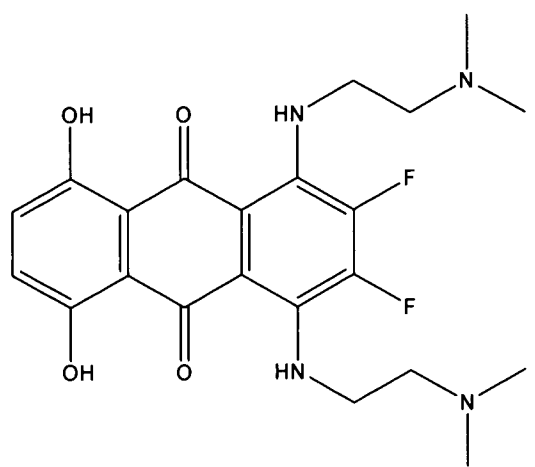
In certain aspects of this invention and method, the cationic amphiphilic tracer compounds comprise nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, or 9-amino-acridin-propranolol and combinations of any of the foregoing. In other aspects, the cationic amphiphilic tracer compounds have any of the following structures:

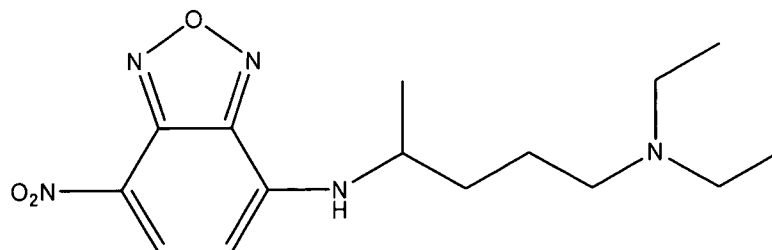
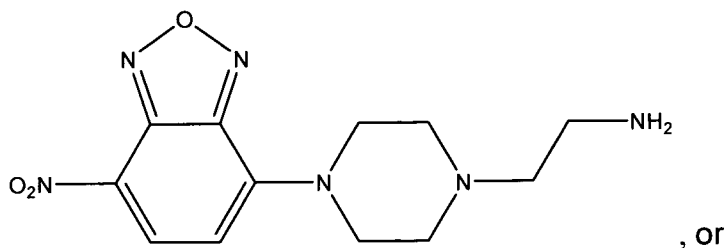
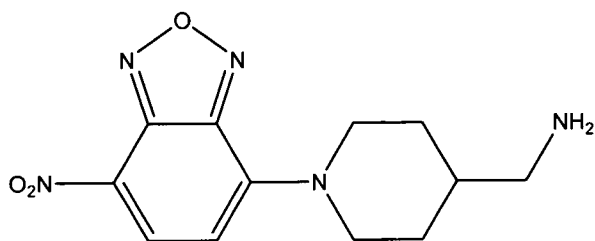
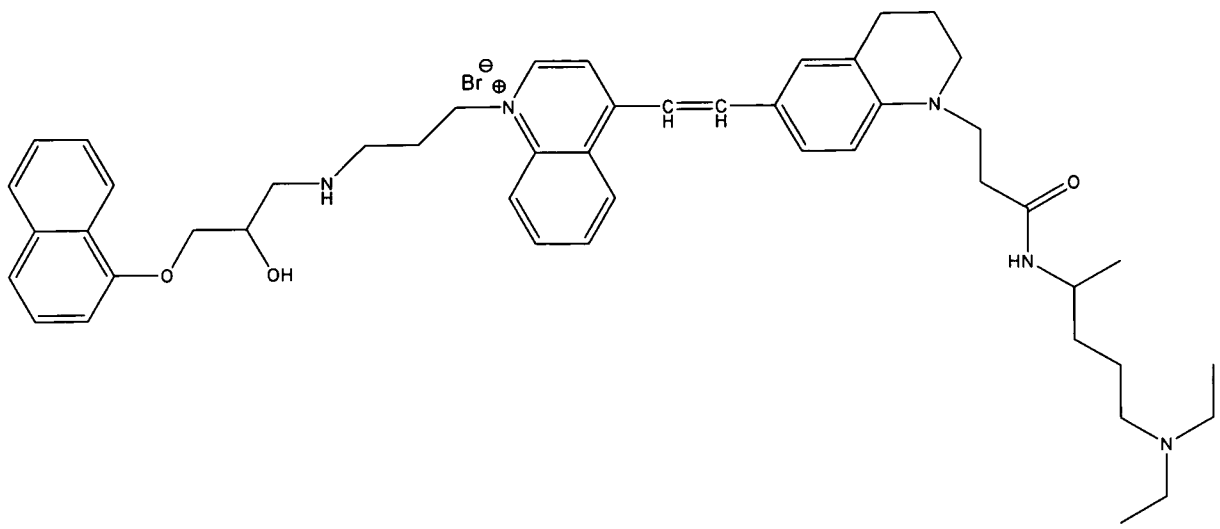






Preferred in the methods of the present invention for screening drug candidates to determine their toxicity to cells, tissues or organs are the cationic amphiphilic tracer compounds having the structures:





The mammalian cells used in the method of the present invention can be from a cell line which can be a human cell line. Furthermore, the mammalian cells can be from a subject who can be a human. The cells used in this method can comprise lymphocytes, granulocytes, macrophages or monocytes, or any combination thereof.

Screening Suspected Toxic Agents for Toxicity

This invention also provides a method of screening suspected toxic agents to determine their toxicity to cells, tissues or organs. In carrying this method, the following steps are carried out: (a) providing: (i) at least one first sample and one second sample of mammalian cells; (ii) a suspected toxic agent for screening; (iii) a diluting vehicle or carrier; and (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; (b) administering: (i) the suspected toxic agent (ii) to the first sample for a time sufficient to allow the suspected toxic agent to partition into subcellular compartments within the cells to form treated cells; and (ii) the diluting vehicle or carrier (iii) to the second sample to form untreated reference cells; (c) contacting the treated cells and the untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and (d) detecting any increase in vacuole accumulation of the cationic amphiphilic tracer compound in the treated cells relative to the untreated reference cells, thereby determining whether the suspected toxic agent has accumulated in the vacuoles and is toxic to cells, tissues or organs.

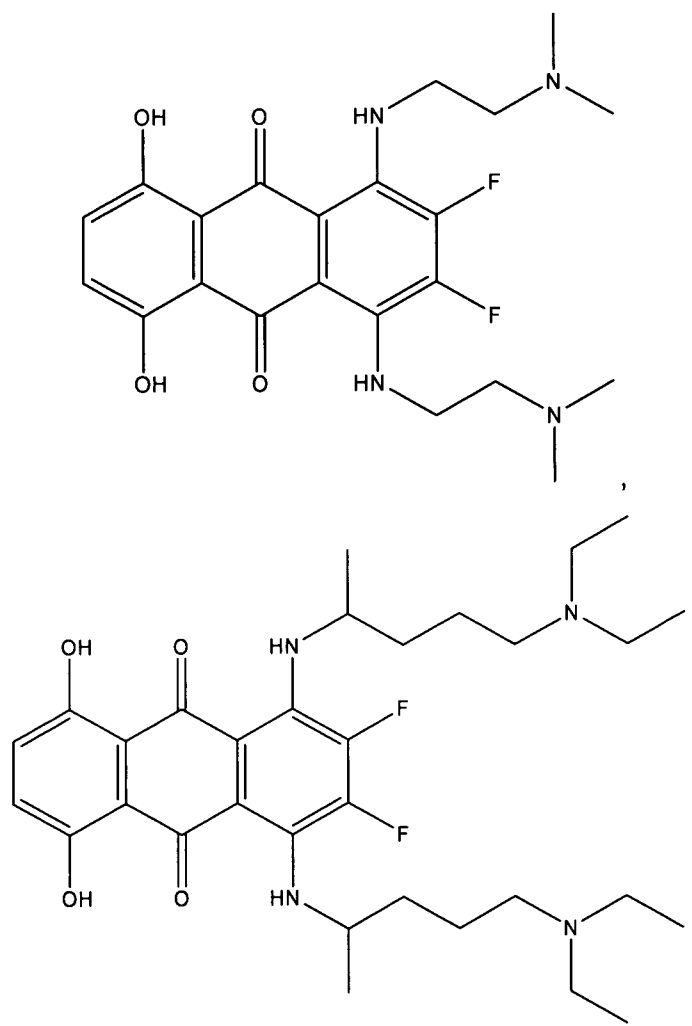
In other embodiments of this invention, the vehicle or carrier comprises cell culture medium, buffer, organic solvent or water, and combinations of the foregoing. In one embodiment, the organic solvent can comprise dimethylsulfoxide (DMSO).

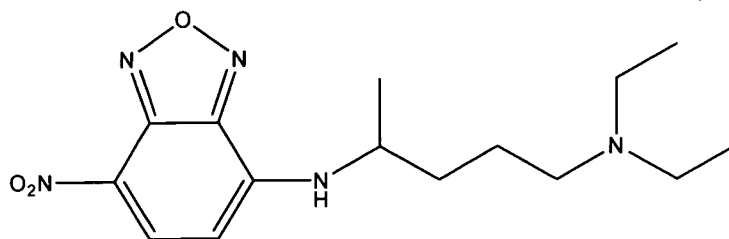
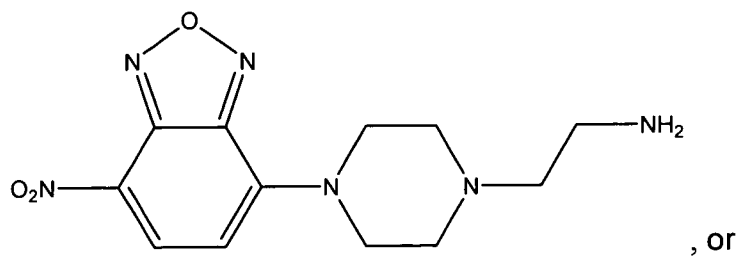
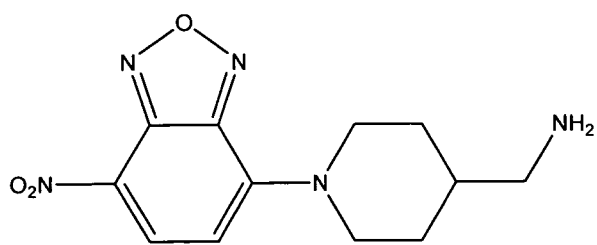
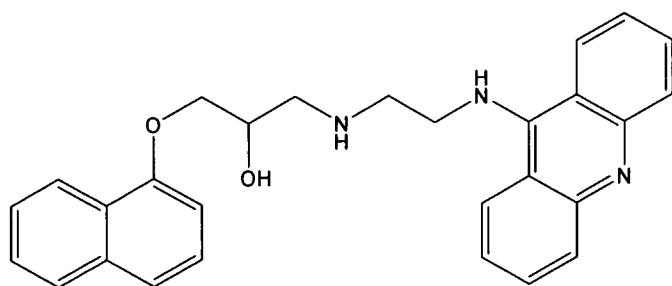
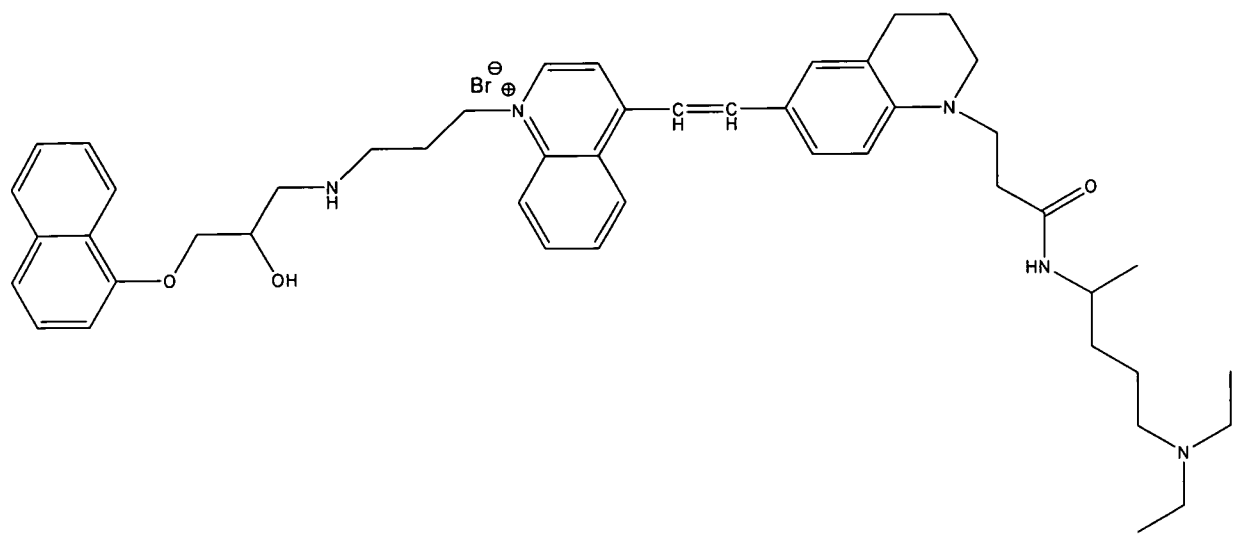
The vacuoles contained in the mammalian cells can comprise lysosomes, phagophores, autophagosomes or autophagolysosomes, or any combination thereof.

In certain aspect of this invention and method, the first cationic amphiphilic tracer compounds and the second cationic amphiphilic tracer compounds can comprise fluorescent drugs that induce phospholipidosis or fluorescently labeled analogs of a drug that induce phospholipidosis. In other aspects of this invention, the first cationic amphiphilic tracer compounds and the second cationic amphiphilic tracer compounds comprise halogenated and fluorescent drugs that induce phospholipidosis or halogenated and fluorescently labeled analogs of drug that induce phospholipidosis.

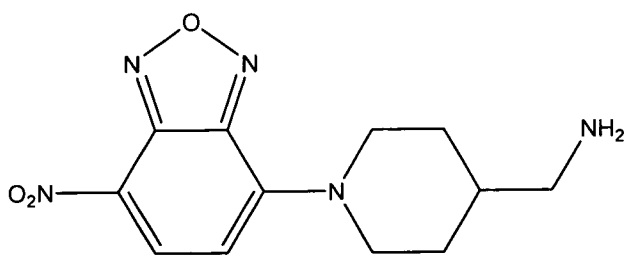
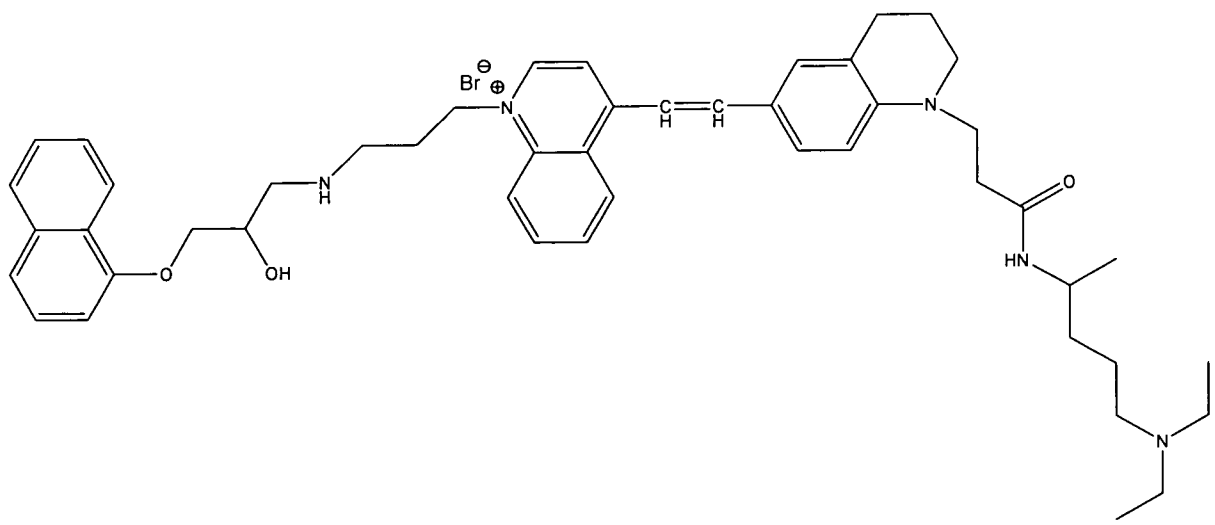
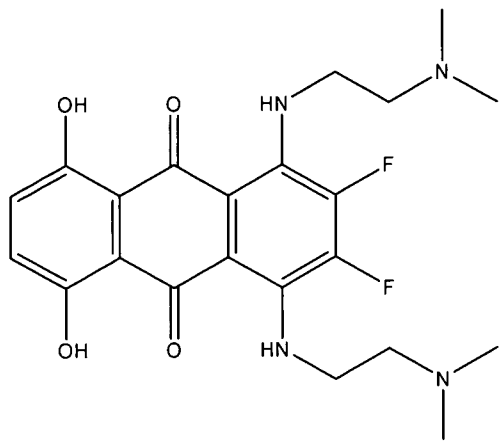
Among the preferred cationic amphiphilic tracer compounds are those comprising nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, or 9-amino-acridin-propranolol and combinations of any of the foregoing.

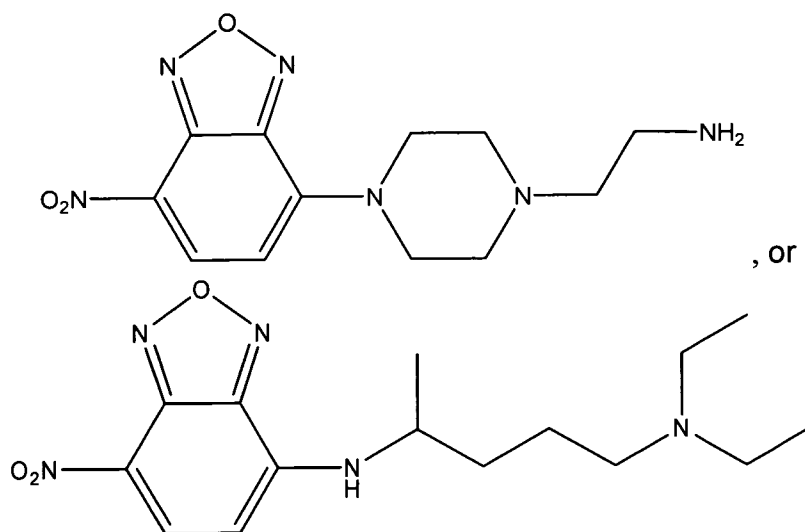
Also useful in the present method are cationic amphiphilic tracer compounds having any of the following structures:





Preferred for carrying out the method of screening suspected toxic agents to determine their toxicity to cells, tissues or organs are the cationic amphiphilic tracer dye compounds having the structures:





In carrying out the present method, the mammalian cells can be from a cell line which can be a human cell line. Further, the mammalian cells can be from a subject who can be human.

In another embodiment, the mammalian cells can comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing.

Distinguishing Phospholipidosis Activators from Autophagy Pathway Perturbation

Agents

This invention additionally provides a method of distinguishing between phospholipidosis activators and autophagy pathway perturbation agents. To distinguish these activators from such agents, the following steps are carried out: (a) providing: (i) a fluorescent phospholipid analog compound that forms multilamellar vesicles; (ii) a cationic amphiphilic fluorophore tracer compound that localizes in a vacuole in a cell; (iii) a test agent; and (iv) a sample of mammalian cells; (b) administering to the cells in the sample (iv) the fluorescent phospholipid analog compound (i) and the test agent (iii) to form a testing mixture; (c) contacting the testing mixture with the cationic amphiphilic fluorophore tracer (ii); and (d) detecting any signals generated from the fluorescent phospholipid analog compound (i) and the cationic amphiphilic fluorophore tracer compound (ii), thereby differentiating between phospholipidosis activators and

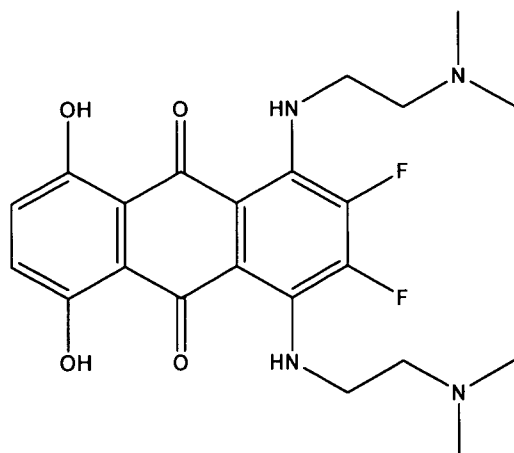
autophagy pathway perturbation agents based upon differential accumulation patterns of the fluorescent phospholipid analog and the cationic amphiphilic fluorophore tracer.

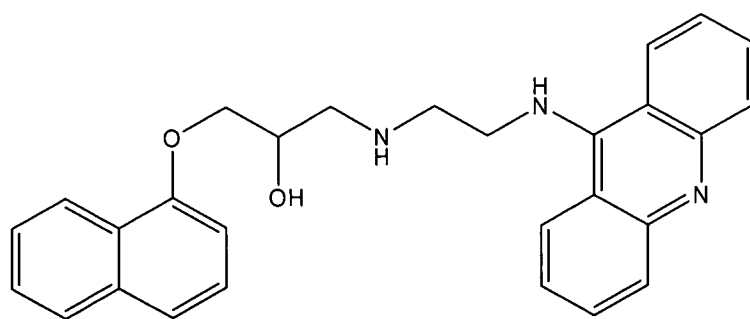
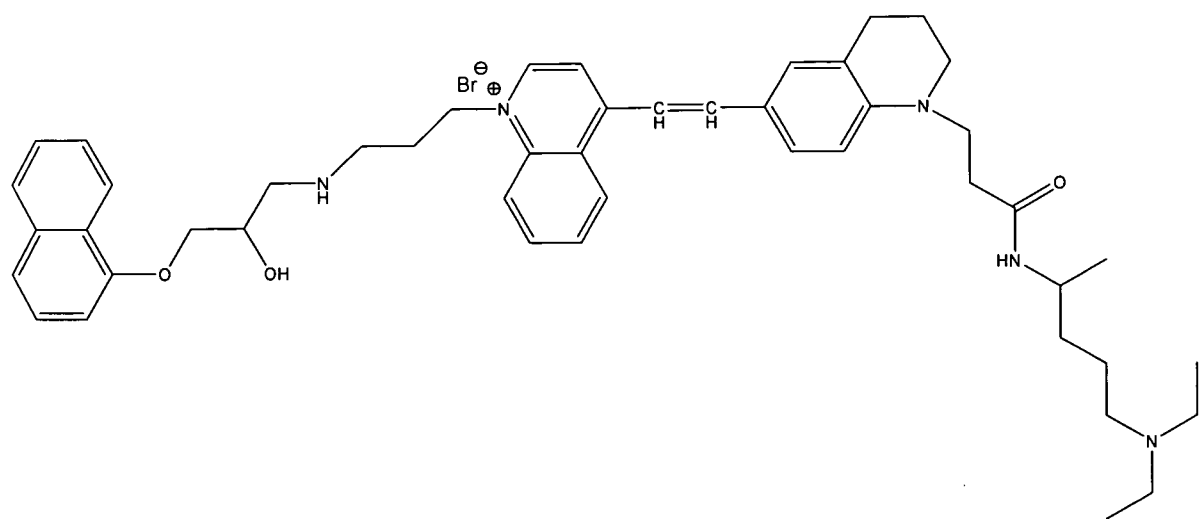
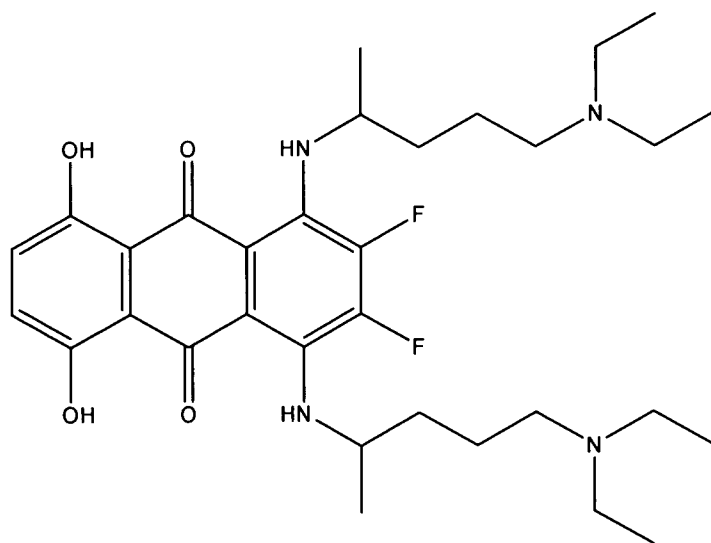
In certain aspects of this invention and method, the fluorescent phospholipid analog compound comprises NBD-PE (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine), NBD-PC (1-acyl-2-(12[(7-nitro-2-1-3-benzoxadiazol-4-yl)amino] dodecanoyl) phosphatidylcholine), CF-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) or Lipid-Tox reagent, and a combination of any of the foregoing.

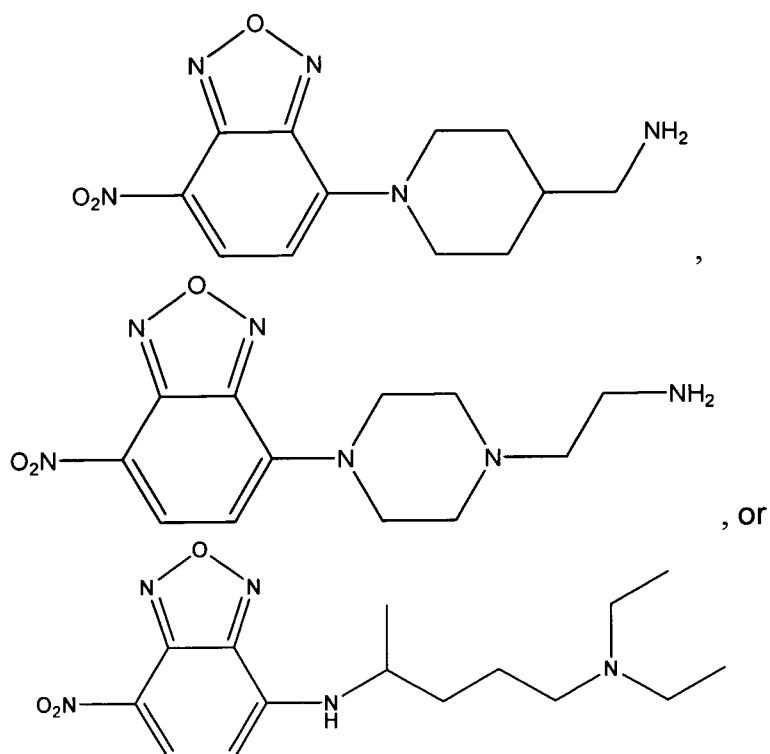
The vacuoles can comprise lysosomes, phagophores, autophagosomes or autophagolysosomes, and a combination of the foregoing.

The cationic amphiphilic tracer compounds can comprise fluorescent drugs that induce phospholipidosis or fluorescently labeled analogs of drugs that induce phospholipidosis. In some aspects, the cationic amphiphilic tracer compound can comprise halogenated and fluorescent drugs that induce phospholipidosis or halogenated and fluorescently labeled analogs of drugs that induce phospholipidosis.

In certain other aspects, the cationic amphiphilic tracer compounds have any of the following structures:

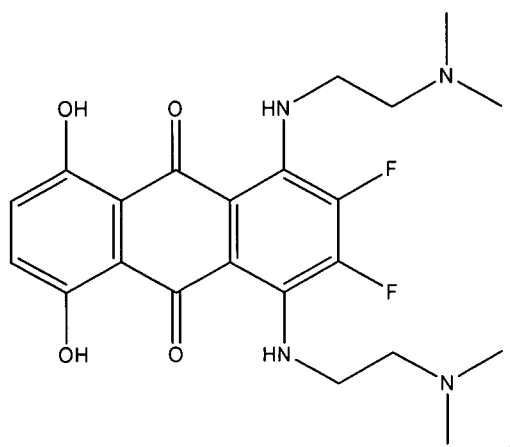


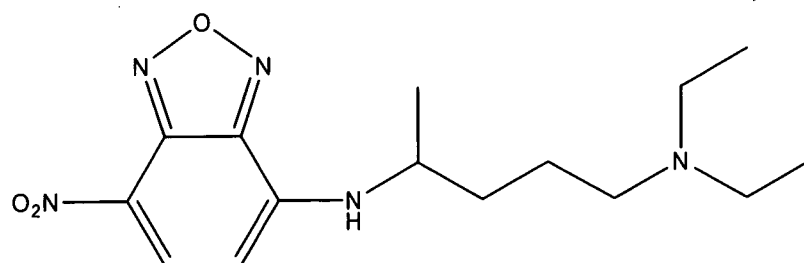
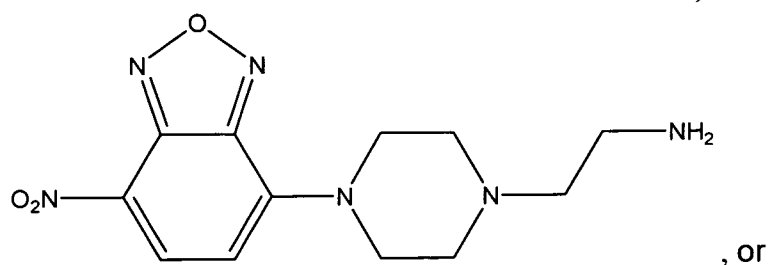
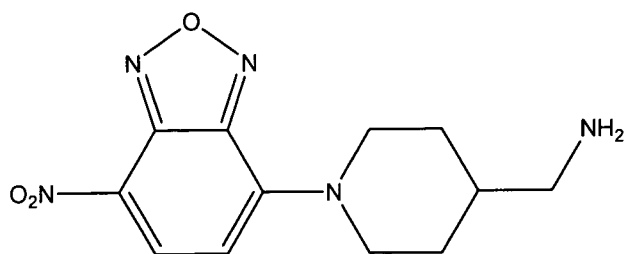
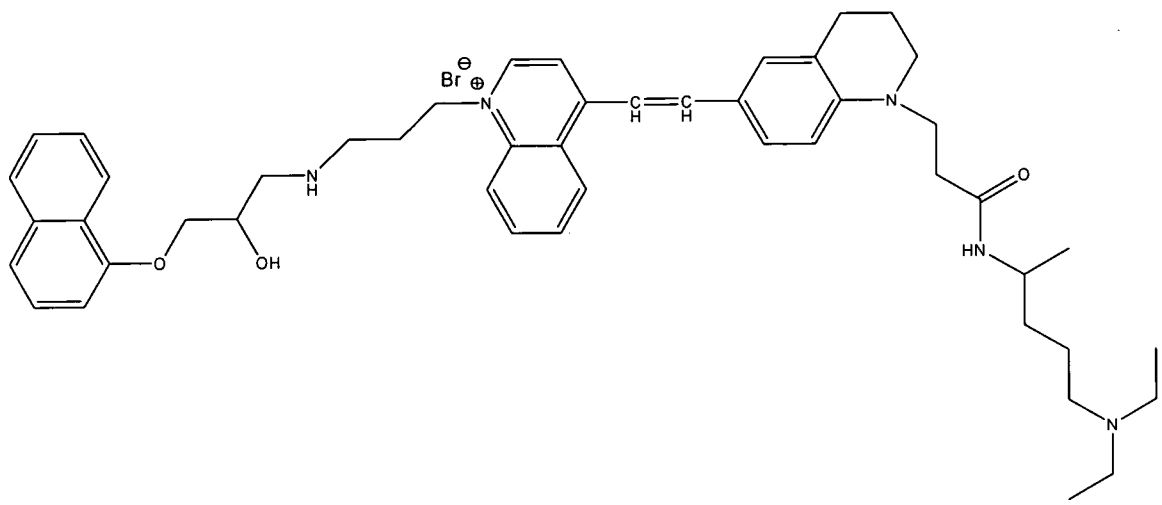




Preferred in the present invention and method for distinguishing phospholipidosis activators from autophagy pathway perturbation agents are the cationic amphiphilic tracer dye compounds having the structures:

compounds having the structures:





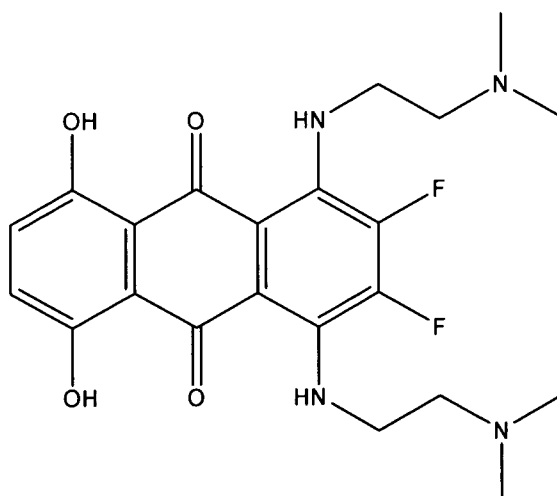
In carrying out this method, the test agent can be a drug candidate or an environmental contaminant. The mammalian cells contained in the sample can comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing. The mammalian cells can be from a cell line, including a human cell line. The mammalian cells can be from a subject who can be a human.

In distinguishing between activators and agents using the method of the present invention, phospholipidosis is identified as increased accumulation of both the fluorescent phospholipid analog compound and the cationic amphiphilic fluorophore tracer compound. Additionally, the autophagy pathway perturbation agent is identified by no increased accumulation of the fluorescent phospholipid analog compound, but increased accumulation of the cationic amphiphilic fluorophore tracer compound.

The examples which follow are set forth to illustrate various aspects of the present invention but are not intended in any way to limit its scope as more particularly set forth and defined in the claims that follow thereafter.

Example 1 Synthesis of 1,4-bis(2-(dimethylamino)ethylamino)-2,3-difluoro-5,8-dihydroxyanthracene-9,10-dione (Dye 1)

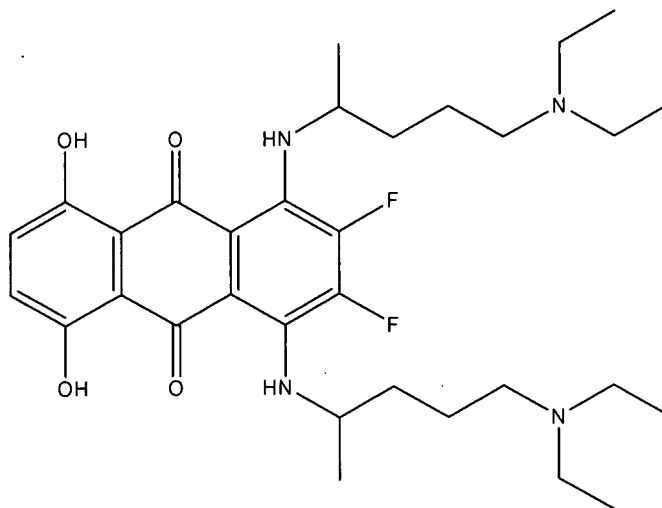
A mixture of 1,2,3,4-tetrafluoro-5,8-dihydroxyanthraquinone (1.0 g, 3.2 mmol) and *N,N*-dimethylethylenediamine (3 mL) in CH_2Cl_2 (30 mL) was stirred at room temperature for 12 hours. After evaporation of the solvents, the residue was purified by silica gel chromatography using isocratic solvent system of EtOAc/MeOH/ Et_3N (10:10:1) yielding 830 mgs of Dye 1 as dark blue product. Abs (max, PBS pH 7.4) = 568 nm; Em = 675 nm. The structure of Dye 1 is given below:



Dye 1

Example 2 Synthesis of 1,4-bis(5-(diethylamino)pentan-2-ylamino)-2,3-difluoro-5,8-dihydroxyanthracene-9,10-dione (Dye 2)

A mixture of 1,2,3,4-tetrafluoro-5,8-dihydroxyanthraquinone (0.47 g, 1.5 mmol) and 2-Amino-5-diethylaminopentane (2.3 mL) in CH_2Cl_2 (20 mL) was stirred at room temperature for 12 hours. After evaporation of the solvents, the residue was purified by silica gel chromatography using isocratic solvent system of $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{Et}_3\text{N}$ (100:10:1) yielding 364 mgs of Dye 2 as dark blue product. Abs (max, PBS pH 7.4) = 667 nm; Em = 700 nm. The structure of Dye 2 is given below:

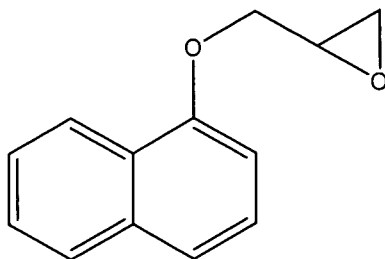


Dye 2

Example 3 Synthesis of Dye 3**(a) Preparation of 2-((naphthalene-1-yloxy)methyl)oxirane (Compound 1)**

To a solution of 1-naphthol (3.0 g, 0.02 mol) in DMSO (10 mL), potassium hydroxy (flakes, 2.0 g, 0.06 mol) was added. The combined mixture was stirred at room temperature for 30 min and then epichlorohydrin (5.6 g, 4.7 mL, 0.06 mol) was added slowly over a period of 45 min and stirring was continued for 16 hours. The reaction was quenched with water (30 mL) and extracted with chloroform (2 x 50 mL). The combined organic layers were washed with 1N aqueous NaOH (2 x 50 mL), water (2 x 100 mL) and brine (2 x 100 mL) and dried over MgSO_4 . The solvent was removed

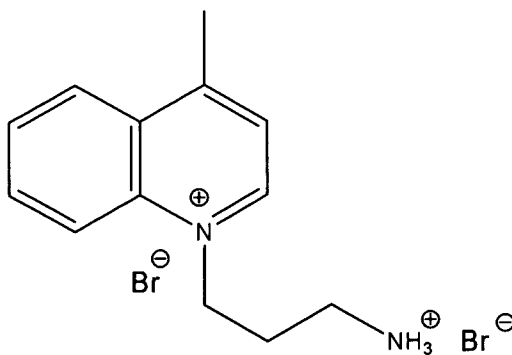
under reduced pressure to provide Compound 1 as a yellow liquid (3.26 g, 78%). This product was used without further purification. The structure of Compound 1 is given below:



Compound 1

(b) Preparation of 1-(3-ammoniopropyl)-4-methylquinolinium bromide (Compound 2)

A mixture of lepidine (3.0 g, 21 mmol) and 3-bromopropylamine hydrobromide (4.6 g, 21 mmol) in 1,2-dichlorobenzene (50 mL) was heated at 135 °C for 16 hours. The mixture was cooled and 1,2-dichlorobenzene was decanted. The residue thus obtained was suspended in methanol (25 mL) and refluxed for 10 min. It was cooled and separated solid was collected by centrifugation, washed with methanol (2 x 30 mL) and dried under high vacuum to yield Compound 2 as an light gray solid (1.4 g, 19%). This product was used without further purification. The structure of Compound 1 is given below:

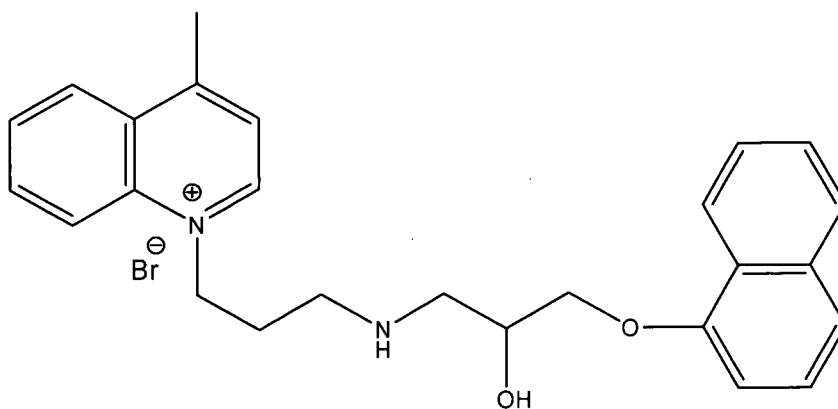


Compound 2

(c) Preparation of 1-(3-(2-hydroxy-3-(naphthalen-1-yloxy)propylamino)propyl)-4-methylquinolinium bromide (Compound 3)

To a suspension of Compound 1 (0.17 g, 0.83 mmol) and Compound 2 (0.30 g, 0.83 mmol) in water (1 mL), triethylamine (0.10 g, 0.99 mmol) was added. The

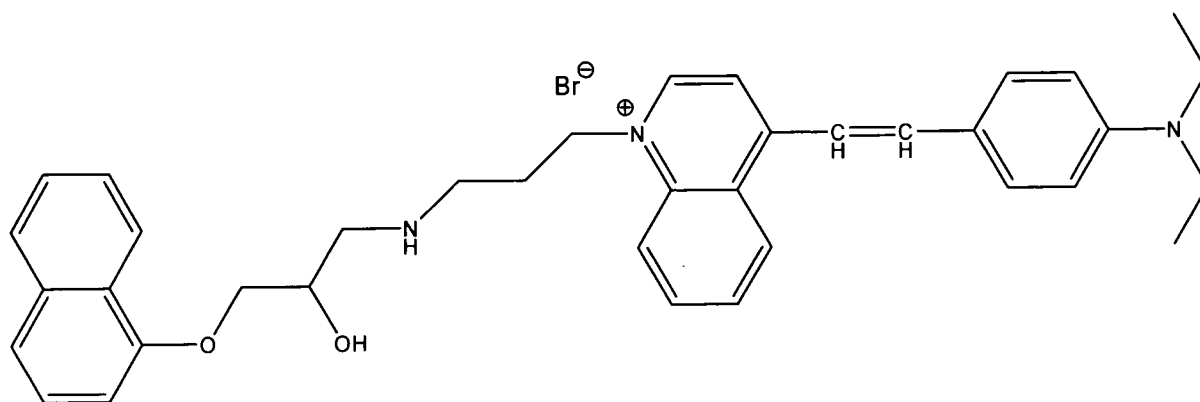
combined mixture was stirred at room temperature for 24 hours and then evaporated in the rotary evaporator to provide Compound 3 as a solid. This product was used without any workup and further purification. The structure of Compound 3 is given below:



Compound 3

(d) Preparation of Dye 3

To a mixture of Compound 3 (0.20 g, 0.5 mmol) and 4-dimethylamino benzaldehyde (0.09 g, 0.5 mmol) in glacial acetic acid (5 mL), piperidine (0.043 g, 0.5 mmol) was added. The combined mixture was heated in an oil bath at 110 °C for 5 hours. Upon cooling the reaction mixture was added to 40% aqueous sodium iodide solution (15 mL) and kept at room temperature for 18 hours. Precipitated dye was collected by centrifugation, washed with water, dried under vacuum and purified by Biotage (SNAP 25 g column) using a gradient of methanol in methylene chloride (1% to 10% over 10 column volume). Appropriate fractions pooled together and dried in a rotary evaporator to provide Dye 3 as a purple solid (128 mg). Abs (max, methanol) = 565 and 295 nm. The structure of Dye 3 is given below:

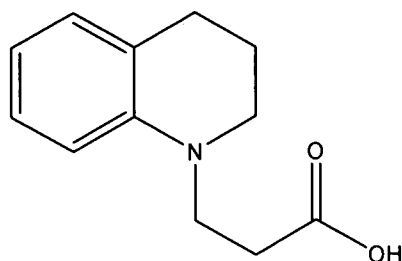


Dye 3

Example 4 Synthesis of Dye 4

(a) Preparation of 3-(3,4-dihydroquinolin-1(2*H*)-yl)propanoic acid (Compound 4)

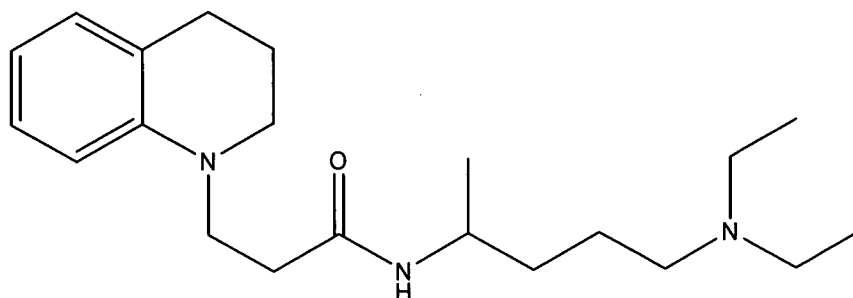
A mixture of 1,2,3,4-tetrahydroquinoline (3.0 g, 22.5 mmol) and methylacrylate (3.88 g, 45.1 mmol) in glacial acetic acid (15 mL) was refluxed for 16 hours. Volatiles were then removed in the rotary evaporator and the residue thus obtained was co-evaporated with methanol (3 x 20 mL) and then dissolved in 15 mL methanol. To this mixture, an aqueous solution of LiOH (1.9 g, 45 mmol, 15 mL) was added and combined mixture was stirred at room temperature for 2 hours. Reaction mixture was made acidic with conc. HCl (pH ~ 2) and extracted with ethyl acetate (2 x 50 mL). The combined organic layers were washed with water (2 x 100 mL) and brine (2 x 100 mL) and dried over MgSO₄. The solvent was removed under reduced pressure to provide Compound 4 as a dark brown viscous liquid (2.91 g). This product was used without further purification. The structure of Compound 4 is given below:



Compound 4

(b) Preparation of *N*-(5-(diethylamino)pentan-2-yl)-3-(3,4-dihydroquinolin-1(2*H*)-yl)propanamide (Compound 5)

To a solution of Compound 4 (1.9 g, 9.3 mmol) in DMF (35 mL), 2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate (3.4 g, 11.2 mmol) and *N,N*-diisopropyl ethylamine (3.4 mL, 19.5 mmol) were added. Combined mixture was stirred at room temperature for 30 mins and then a solution of 2-amino-5-diethylaminopentane (2.2 g, 13.9 mmol) in DMF (5 mL) was added. After stirring the mixture for additional 1 hour, it was poured in brine (100 mL) and extracted with ethyl acetate (2 x 75 mL). The combined organic layers were washed with brine (3 x 100 mL) and dried over MgSO₄. The solvent was removed under reduced pressure to provide Compound 5 as a dark yellow viscous liquid (2.64 g, 82.5%). This product was used without further purification. The structure of Compound 5 is given below:

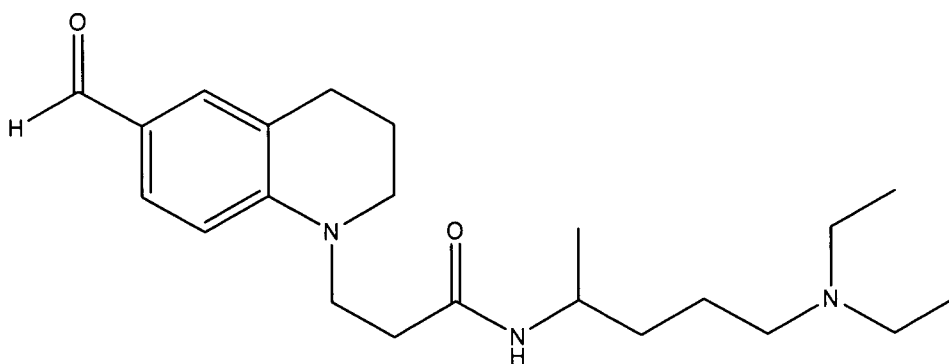


Compound 5

(c) Preparation of *N*-(5-(diethylamino)pentan-2-yl)-3-(6-formyl-3,4-dihydroquinolin-1(2*H*)-yl)propanamide (Compound 6)

POCl₃ (2.37 g, 15.3 mmol) was added dropwise to DMF (1.8 mL) which was cooled in an ice bath. The combined mixture was stirred for 30 minutes and to this a solution of Compound 5 (2.64 g, 7.6 mmol) in DMF (25 mL) was added dropwise over a period of 20 min. After the addition was complete, the ice bath was removed and the reaction mixture was stirred at room temperature for 17 hour. An aqueous solution of sodium acetate (25% w/w, 25 mL) was then added to the reaction mixture and it was heated in an oil bath (T= 110 °C) for 15 min. The reaction mixture was cooled and pH was adjusted with 10 N NaOH to ~11. It was diluted with water (100 mL) and extracted with ethyl acetate. The organic layer was washed twice with water (75 mL) followed by

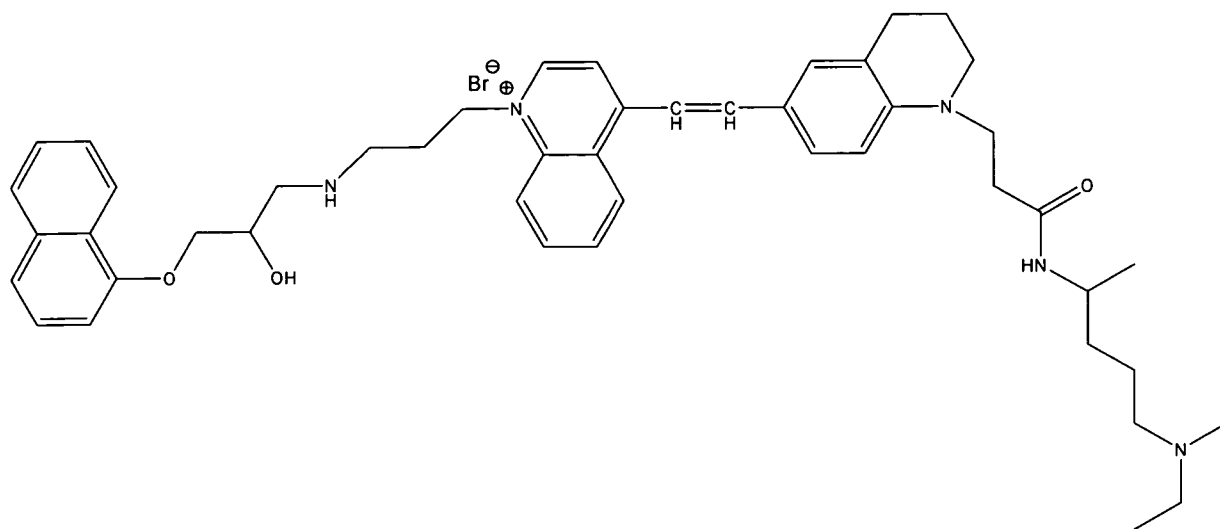
brine (3 x 50 mL), dried over magnesium sulfate and then evaporated to dryness to yield 1.44 g of an orange viscous liquid which was used without any further purification. The structure of Compound 6 is given below:



Compound 6

(d) Preparation of Dye 4

To a mixture of Compound 3 (0.15 g, 0.3 mmol) and Compound 6 (0.13 g, 0.34 mmol) in glacial acetic acid (4 mL), piperidine (0.029 g, 0.34 mmol) was added. The combined mixture was heated in an oil bath at 110 °C for 5 hours. Upon cooling the reaction mixture was added to 40% aqueous sodium iodide solution (12 mL) and kept at room temperature for 18 hours. Precipitated dye was collected by centrifugation, washed with water, dried under vacuum and purified by Biotage (SNAP 25 g column) using a gradient of methanol in methylene chloride (1% to 10% over 10 column volume). Appropriate fractions pooled together and dried in a rotary evaporator to provide Dye 4 as a purple solid (75 mg). Abs (max, methanol) = 577 and 297 nm. The structure of Dye 4 is given below:

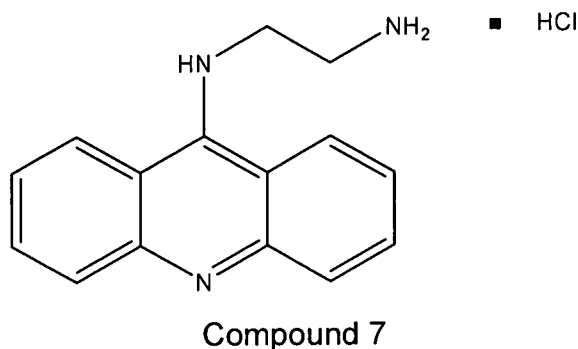


Dye 4

Example 5 Synthesis of Dye 5

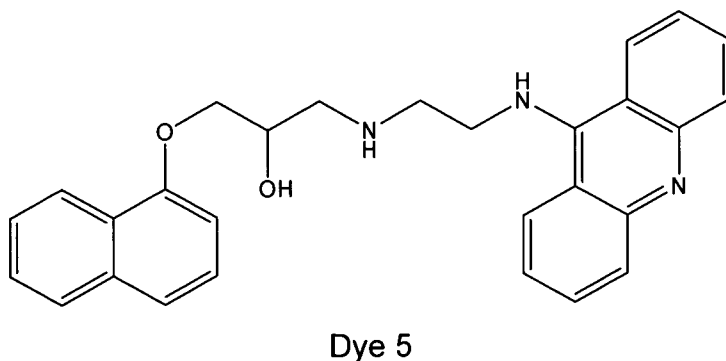
(a) Preparation of *N*¹-(acridin-9-yl)ethane-1,2-diamine hydrochloride (Compound 7)

A mixture of 9-chloroacridine (1.21 g, 5.7 mmol) and phenol (10.8 g, 57 mmol) was mixed and heated at 110 °C for 45 min under argon. Distilled ethylene diamine (3.4 g, 57 mmol) was added via syringe. After the reaction mixture was heated at 110 °C for additional 2 hrs, it was poured into 2N NaOH solution (60 mL). The product was extracted with ethyl ether (3 × 100 mL). The combined organic layer was washed with 2N NaOH solution (40 mL) and water (2 × 100 mL) and dried with anhydrous potassium carbonate. The solution was concentrated to 15 mL. Ethanol (15 mL) was added, followed by the addition of concentrated hydrochloric acid (10 mL). The mixture was cooled in freezer (-20 °C) for 4 hrs. The yellow solid formed was collected by centrifugation, washed with ethyl ether (10 mL) and dried under vacuum to provide Compound 7 as yellow powder 0.82 g (53 %) which was used without any further purification. The structure of Compound 7 is given below:



(b) Preparation of Dye 5

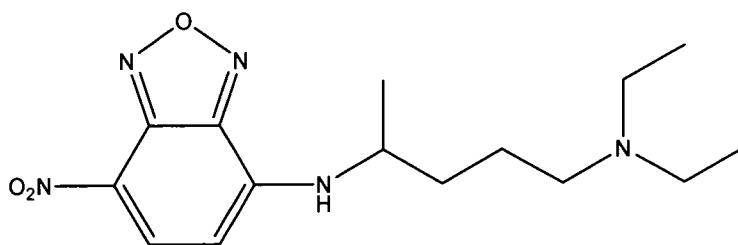
To a solution of Compound 1 (200 mg, 1mmol) in diisopropylethylamine (0.6 mL) and dioxane (4 mL), was added Compound 7 (274 mg, 1 mmol). The reaction mixture was heated at 50 °C for 20 hrs. The yellow precipitate was collected by centrifugation and then further purified by flash chromatography (gradient of mobile phase: dichloromethane/methanol = 50/1 to 5/1). The solvent was removed under vacuum to provide Dye 5 as a yellow solid (34.5 mg, 8%). Abs (max, methanol) = 412 and 440 nm; Em (max, methanol) = 426, 452 and 480 nm. The structure of Dye 5 is given below:



Example 6 Preparation of Dye 6

A mixture of 4-chloro-7-nitro-2,1,3-benzoxadiazole (0.5 g, 2.5 mmol), 2-amino-5-diethylaminopentane (0.58 mL, 3 mmol) and cesium carbonate (0.98 g, 3 mmol) in acetonitrile (5 mL) was refluxed for 1 hour. Reaction mixture cooled and volatiles were removed in the rotary evaporator. The residue thus obtained was dissolved in a mixture of ethyl acetate (40 mL) and water (40 mL). The organic layer was separated, washed

with water (2X), brine (2X), dried (MgSO_4) and evaporated. The crude dye thus obtained was purified on Biotage (Si, SNAP 25 g) using a gradient of methanol (2% to 40% over 18 column volume) in methylene chloride to provide Dye 6 as a yellow residue (61 mg). Abs (max, methanol) = 467 nm; $\epsilon = 30,200 \text{ M}^{-1}\text{cm}^{-1}$. The structure of Dye 6 is given below:



Dye 6

Example 6 Fluorescence Microscopy-Based Assay of Lysosomal Perturbation

Portions of Examples 6-10 are published as Coleman et al., 2010, J.

Biomolecular Screening 15:398-405.

The Lysosomal Storage Disorder known as Fabry disease arises from a deficiency of the enzyme alpha-galactosidase A, which leads to an accumulation of the glycolipid known as globotriaosylceramide (aka ceramide trihexoside) within endothelium, striated muscle (skeletal, cardiac), smooth muscle, and renal epithelium. This accumulation leads to an impairment of their proper function. The condition affects hemizygous males, as well as both heterozygous and homozygous females; males tend to experience the most severe clinical symptoms, while females vary from virtually no symptoms to those as serious as males. This variability is thought to be due to X-inactivation patterns during embryonic development. Diagnosis of Fabry disease can be difficult even in a homozygous male. Some patients, renal variants, may lack classic manifestation of Fabry disease such as acroparesthesia, angiokeratoma and corneal lesion. This difficulty is compounded in a heterozygous female who may be variably affected from lyonization (from Dr. Mary Lyon's theory of random X-inactivation). It should also be recognized that serum-galactosidase A is frequently normal in female patients. It has recently been noted that chloroquine, hydroxychloroquine and

amiodarone therapy can result in abnormal storage of biochemically and ultrastructurally similar vacuolar inclusions in many of the same cells as Fabry disease and consequently results in similar clinical manifestations (see Müller-Höcker et al, *Human Pathology* 34:285-289 (2003); Albay et al, *Modern Pathology* 18:733-738 (2005); and Bracamonte et al, *Am J Kidney Disease* 48:844-850 (2006), incorporated herein by reference).

Stable cell lines derived from patients with Fabry disease can not be found in cell line repositories, such as American Type Tissue Collection (Manassas, VA), and are not readily available to the research community. Consequently, an in vitro cell culture model mimicking Fabry disease was generated by incubation of cells with chloroquine.

U2OS human osteosarcoma cells were maintained in McCoy's 5a modified medium supplemented with 10% FBS and 100 units/ml penicillin/ streptomycin (Invitrogen, Carlsbad, CA) in a humidified 37°C, 5% CO₂ environment. Cells were allowed to proliferate on glass slides or were seeded into 96-well plates at a density of 2×10^4 cells/well and allowed to attach overnight. The cells were plated such that at the end of the experiment, they reached about 90% confluency.

The fluorescence-based lysosomal perturbation assay was established by first incubating cells cultivated on glass slides for 4-24 hours with chloroquine (8-64 μ M final concentration), a well known phospholipidosis-inducing agent. Control cells were incubated with 0.1% (v/v) dimethyl sulfoxide (DMSO), which served as the vehicle control. Cells were then briefly (15 min) incubated with a combination of Dye 1 and Hoechst 33342 dye. Following incubation, cells grown on glass microscope slides were covered with glass coverslips, sealed with nail polish and observed using an inverted Axiovert 200M microscope (Carl Zeiss, Inc., Oberkochen, Germany). Images were acquired with a 63X objective lens (Zeiss). Fluorescence emission intensity and wavelength maximum of Dye 1 is not significantly altered in the pH range of 4.0 to 9.0, allowing interpretation of any increase in fluorescence intensity as an indication of the accumulation of the probe within the cells, rather than as a variation in dye response to lysosomal pH values.

Examination of the distribution of the Dye 1 in cells treated with chloroquine, revealed a punctate pattern of cytoplasmic staining that increased in a dose-dependent manner (FIG. 1). Cells were subsequently stained with Dye 1 for 15 minutes. Nuclei were counter-stained with Hoechst 33342 dye. In FIG. 1, control U2OS cells are shown in (A), cells pre-treated for 18 hours with 8 μ M chloroquine in (B), cells pre-treated for 18 hours with 16 μ M chloroquine in (C) and cells pre-treated for 18 hours with 64 μ M chloroquine in (D). This staining pattern was also consistently observed for other drugs that are known to induce phospholipidosis, such as chlorpromazine, fluoxetine, pimozide and verapamil (further described in Example 4). For all the cited drugs the levels of Dye 1 in the cells increased in a dose-dependent manner until cytotoxic doses of the test agent were attained, which led to a loss of cells. Typically at high doses of test agent the cells remaining on the slide displayed diffuse cytoplasmic staining with Dye 1. Negative control compounds, the vehicle control and untreated medium showed minimal accumulation of Dye 1, which was strictly confined to the lysosomes. The ability of Dye 1 to detect vacuole accumulation in the described in vitro cell culture model mimicking Fabry disease strongly supports the present invention and its suitability for the detection of genetically derived lysosomal storage diseases.

Example 7 Fluorescence Microscopy with Dye 6

HeLa cells were maintained in Dulbecco's modified Eagles medium supplemented with 10% FBS, 100 units/ml penicillin/ streptomycin (Invitrogen, Carlsbad, CA) and MEM non essential amino acids in a humidified 37°C, 5% CO₂ environment. Cells were allowed to proliferate on glass slides at a density of 2×10^4 cells/well and allowed to attach overnight. The cells were plated such that at the end of the experiment, they reached about 90% confluency.

The fluorescence-based lysosomal perturbation assay was established by first incubating cells cultivated on glass slides for 6 hours with chloroquine (120 μ M final concentration), a well known phospholipidosis-inducing agent. Control cells were incubated with 0.1% (v/v) dimethyl sulfoxide (DMSO), which served as the vehicle

control. Cells were then briefly (15 min) incubated with Dye 6 at room temperature. Following incubation, cells grown on glass microscope slides were covered with glass coverslips, sealed with nail polish and observed using an Olympus BX51 microscope. Images were acquired with a 40X objective lens. Examination of the distribution of the Dye 6 in cells treated with chloroquine, revealed an increase in fluorescence intensity of lysosomal staining. The untreated cells gave a low level of fluorescent staining.

Example 8 Fluorescence Microplate Based Assay of Lysosomal Perturbation

In order to increase throughput and reduce handling time, the fluorescence microscopy-based lysosomal perturbation assay was scaled up to a 96-well microplate plate assay format and analyzed using a fluorescence microplate reader.

Monitoring cell number per well by determining overall blue fluorescence emission intensity, as indicated by the nuclear Hoechst 33342 dye, was performed to identify generalized compound toxicity. Fluorescence intensity of the cells cultured on the 96-well plates was measured using a FLUOstar OPTIMA Multifunction Microplate Reader (BMG LabTech, Offenburg, Germany). Dye 1 and Hoechst 33342 nuclear counter-stain can be read with Texas Red filter set and DAPI filter set, respectively (Hoechst excitation ~300 nm and emission ~480 nm, Dye 1 excitation ~570 nm and emission ~670 nm). Fluorescence intensity was expressed as percentage of the vehicle control value. Values for Dye 1 fluorescence can be normalized to those of Hoechst 33342 dye fluorescence to control for any loss in cell number. Results are provided in FIG. 2. Cells were treated with verapamil in (A), fluoxetine in (B), chlorpromazine in (C) and pimozide in (D) for 18 hours, and then stained with Dye 1 (solid lines with solid diamonds) and Hoechst 33342 (dashed line with squares). Concentrations of compounds that resulted in a greater than 30% decrease in overall cell number using the described assay were flagged as being toxic doses, as especially evident in FIGS. 2B and 2D. This benchmark has previously been established in a phospholipidosis assay using a fluorescent lipid analog as well as in a high content screening assay of autophagy (Nioi et al 2008; Zhang et al, 2007). Microscopic examination of wells highlighted as containing generally toxic doses of a test compound demonstrated

diffuse cytoplasmic staining of Dye 1 instead of the anticipated localized punctuate staining associated with lysosomes and lamellar bodies. Scaling-up to the higher throughput microplate format did not affect the sensitivity of detection nor the reproducibility of the assay, as demonstrated by re-testing the reference compounds and comparing the results with those obtained in the fluorescence microscopy assay. However, it was found to be critical to check for any generalized toxicity-related artifacts thru monitoring Hoechst 33342 dye signal.

In high-throughput screening activities, experimenters often compare a large number (hundreds of thousands to tens of millions) of single measurements of unknown samples to positive and negative control samples. The particular choice of experimental conditions and measurements is called an assay. Large screens are expensive in time and resources. Therefore, prior to starting a large screen, smaller test (or pilot) screens are used to assess the quality of an assay, in an attempt to predict if it would be useful in a high-throughput setting. The Z-factor is an attempt to quantify the suitability of a particular assay for use in a full-scale, high-throughput screen. Extreme conservatism is used in high throughput screening, due to the large number of tests being performed.

The Z-factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls (μ_p , σ_p , and μ_n , σ_n). Given these values, the Z-factor is defined as:

$$\text{Z-factor} = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

In practice, the Z-factor is estimated from the sample means and sample standard deviations:

$$\text{Estimated Z-factor} = 1 - \frac{3 \times (\hat{\sigma}_p + \hat{\sigma}_n)}{|\hat{\mu}_p - \hat{\mu}_n|}.$$

When tested in the 96-well assay format using the cited cationic amphiphilic drugs, the Dye 1-based assay demonstrated a Z-factor value of at least 0.60. This falls within the range of 0.5 and 1, which is defined as a high quality assay by the applied

statistical parameter (Zhang et al, 1999). EC₅₀ values for the test drugs ranged from 6 μ M for chlorpromazine to 30 μ M for verapamil, with chloroquine itself having an EC₅₀ value of 15 μ M.

Example 9 Comparison with a Prototypical Fluorescent Phospholipid Analog-Based Assay

Many assays of phospholipidosis, including the Lipid-Tox assay (Invitrogen, Carlsbad, CA), are based upon co-incubating cells with a fluorescent phospholipid analog for an extended period of time, along with the drug or test agent being evaluated, and then monitoring signal generation in fixed and permeabilized cells. Dye 1 is not a fluorescent phospholipid analog, but instead is a cationic amphiphilic fluorophore tracer. Cells are incubated with the drug or test agent in isolation and then briefly treated with the tracer dye before evaluation in live cells. This prompted us to speculate that since the two assays are based upon fundamentally different mechanisms, they might also differ in terms of the types of test agents that elicited a positive response. Chloroquine, a prototypical phospholipidosis-inducing agent, and bafilomycin A, an agent well known to induce the accumulation of autophagosomes, were evaluated using both Dye 1 and Lipid-Tox dye-based assays. FIG. 3 shows relative fluorescent intensity of U2OS cells treated with chloroquine (A) or bafilomycin A1 (B) at different concentrations for 24 hours. Cells stained with Lipid-Tox dye (solid triangles dash-dot line) were incubated in the presence of the fluorescent lipid for 24 hours during treatment with the drugs. Cells stained with Dye 1 (solid diamond solid line) or Hoechst 33342 (solid square dashed line) were stained for 15 minutes post drug incubation. Both assays reliably predicted that chloroquine induced an expansion in the lysosomal compartment (FIG. 3A). However, only Dye 1 identified the increased vacuolation arising from bafilomycin A treatment (FIG. 3B). The calculated EC₅₀ value for this particular compound was determined to be 3 nM in Dye 1 based assay, easily making it the most potent agent screened in our study. This suggests that the Lipid-Tox assay is strictly speaking a phospholipidosis assay while Dye 1 assay is capable of

detecting vacuolation arising from both phospholipidosis and disruption of trafficking in the autophagy pathway.

Thus, in addition to authentic compounds that cause phospholipidosis, agents that cause the accumulation of autophagosomes by blocking the downstream lysosomal pathway and/or intracellular trafficking of autophagosomes may also lead to increases in the accumulation of intracellular Dye 1 signal in the described assay. For example, Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces griseus*, is a particularly potent agent in our assay. Initial screening of this compound using the ICCB library revealed it to be cytotoxic, but rescreening at much lower doses demonstrated its ability to increase vacuole number and volume. Bafilomycin A1 is a rather specific vacuolar type H⁺-ATPase inhibitor which is known to increase the numbers of intracellular autophagosomes, purportedly by blocking the ability of the lysosome to degrade them [Yamamoto et al, 1998]. The bafilomycin A1 results suggest that the described assay should be considered a measure of generalized dysfunction of the lysosomal-dependent catabolic pathway, which leads to the accumulation of autophagosomes, and thus relevant to autophagy in general, as well as phospholipidosis in particular.

Example 10 Compound Library Screening

Small molecule activators and inhibitors are commonly used to provide insight into both the mode of regulation and physiological roles of various subcellular targets. However, more extensive screening of these compounds generally reveals that most have overlapping specificities, complicating interpretation of their overall effects on biological systems. As proof-of-principle, U2OS cells were incubated with the 480 compounds in the ICCB known bioactives compound library (Enzo Life Sciences) overnight at concentrations of 0.1–53 μ M (1000-fold dilution of the stock), and then evaluated using the described lysosomal perturbation assay. In the preliminary screen, test agents were employed at a single dose in an effort to identify any potential lysosome-modifying agents. Our objective was to benchmark the assay workflow, not to exhaustively classify all of the compounds in the library. It would be advisable to use

a range of concentrations for each test agent in order to reduce the number of false negative results obtained with the described assay. The compounds listed in Table 1 increased staining by greater than three standard deviations compared with untreated control cells.

Table 1: Positive compound hits from a screen of the ICCB Known Bioactive Compounds Library. U2OS cells were treated with the highlighted compounds at the indicated concentrations for 18 hours. Compounds that displayed increased fluorescence from incubation with Dye 1 by greater than 3 standard deviations relative to the untreated cells are summarized in the table. Relative Lyso stain represents the intensity of Dye 1 fluorescence of treated cells compared to the untreated control. Relative Hoechst stain represents the intensity of the Hoechst 33342 fluorescence of treated cells compared to the untreated control, which indicates the number of cells remaining after the assay. Compounds listed in boldface do not decrease the signal from Hoechst by more than 30%.

NAME	Classification	Action	μM used	Relative Lyso Stain	Relative Hoechst Stain
Ro 20-1724	Inhibitors	phosphodiesterase (PDE4) inhibitor	18 μM	190%	53%
Bafilomycin A1	Inhibitors	vacuolar ATPase inhibitor	1 μM	176%	58%
E6 Berbamine	Inhibitors	calmodulin inhibitor	7 μM	230%	100%
Ro 31-8220	Kinase inhibitors	PKC inhibitor	9 μM	146%	89%
Propranolol (S-)	CNS receptor ligands	adrenoceptor antagonist (beta)	17 μM	142%	88%
SB-431542	Kinase inhibitors	ALK4, ALK5, ALK7 inhibitor	13 μM	154%	101%
Rottlerin	Kinase inhibitors	PKC delta inhibitor	10 μM	187%	34%
GF-109203X	Kinase inhibitors	PKC inhibitor	12 μM	197%	87%
Tyrphostin 9	Kinase inhibitors	PDGF-R tyrosine kinase inhibitor	18 μM	200%	31%

FIG. 4 highlights representative results obtained from four of the test compounds determined to generate a positive response in the assay. FIG. 4 shows relative fluorescent intensity of U2OS cells treated with propranolol in FIG. 4A, E6-berbamine in FIG. 4B, GF-109203X in FIG. 4C or rottlerin in 4D for 18 hours, after staining with Dye 1 (solid lines with solid diamonds) and Hoechst 33342 dye (dashed line with squares). Many of the agents used in the screen caused a loss of cells, an indication of generalized cellular toxicity at the compound concentration evaluated. Rottlerin (FIG. 4D) is an example of a generally cytotoxic compound, which was apparent from a substantial decrease in the Hoechst 33342 dye signal. Observation of rottlerin-treated

cells by fluorescence microscopy confirmed the microplate-based results, with those cells remaining attached to the tissue culture surface displaying diffuse cytoplasmic red staining. The cursory screening activity led to the identification of five compounds that induced increases in vacuolation without causing obvious cellular toxicity (boldface entries, Table 1). These agents all elicited a dose-dependent response, with estimated EC₅₀ values ranging from 1 to 7 μ M.

Two of the five agents, propranolol and GF-109203X, have previously been reported to induce phospholipidosis in cells. Propranolol is a well-known and extensively investigated cationic amphiphilic drug that induces phospholipidosis (Cramb, 1986; Lemieux et al, 2004). High micromolar concentrations of the drug are rapidly taken up and accumulated by a wide range of cultured cells, with a steady state being reached after 40-60 min administration and half maximum uptake within 4-10 min. The rate of propranolol dissociation is much slower than the rate of uptake, with 10% of the propranolol remaining associated with the cells after a 90 minute incubation period in the absence of the drug (Cramb, 1986).

In a recent cell-based HTS screen for anthrax toxin inhibitors, GF-109203X was identified as an agent displaying both protein kinase C-inhibitory and lysosomotropic activities (Sanchez et al, 2007). Screening of bisindolylmaleimide compounds related to GF-109203X which lack protein kinase C inhibitory activity but retain tertiary amines and thus the potential to neutralize endosomal pH, was subsequently undertaken by the investigators. This established that the observed toxin inhibitory activity correlated strongly with the presence of the tertiary amines, but not with protein kinase C inhibition (Sanchez et al, 2007). In our own screen, Ro 31-8220, a bisindolylmaleimide compound closely related to GF-109203X, but not to our knowledge previously described as a lysosome-perturbation agent, was also found to be a positive hit. A structurally unrelated protein kinase C δ -inhibitor, rottlerin, increased subcellular accumulation of Dye 1 but displayed significant toxicity, as demonstrated by loss of Hoechst 33342 dye signal in the assay. Re-examination of this compound over a broad range of concentrations revealed it to be generally cytotoxic (data not shown). Intracellular accumulation of Dye 1 in treated cells was always diffuse and cytoplasmic,

demonstrating it to be a false positive hit. Our results, taken together with that of Sanchez et al, 2007, suggests that the observed accumulation of Dye 1 in vacuoles correlates strongly with the cationic amphiphilic nature of the bisindolylmaleimide compounds, rather than any protein kinase C inhibitory activity.

Two other positive hits in our screen were SB-431542 and berbamine. SB-431542 is reported to be a transforming growth factor- β -receptor antagonist that serves as a potent competitive ATP binding site kinase inhibitor for the downstream activin receptor-like kinases, ALK4, ALK5 and ALK7 (Hjelmeland et al, 2004). Physiologically, SB-431542 has previously been shown to inhibit cell proliferation and block cell motility. Berbamine is reported to selectively induce caspase-3- dependent apoptosis of leukemia NB4 cells via a survivin-mediated pathway (Xu et al, 2006). We are not aware of any previous reports that these two agents have a propensity to induce vacuolation in cells, and indeed their chemical structures are not consistent with them belonging to the cationic amphiphilic class of compounds.

Example 11 Distinguishing between induction of phospholipidosis and inhibition of the autophagy pathway

Example 8 demonstrates that the fluorescent cationic amphiphilic tracer dyes while sharing with the fluorescent phospholipid analogs an ability to identify phospholipidosis activators also distinctly reveal agents that inhibit the autophagy pathway, leading to accumulation of vacuoles such as autophagosomes and autophagolysosomes. This relationship is highlighted schematically in FIG. 5. Agents such as NBD-PE (N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine), NBD-PC (1-acyl-2-(12[(7-nitro-2-1-3-benzoxadiazol-4yl)amino] dodecanoyl) phosphatidylcholine), CF-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) and Lipid-Tox reagent (Invitrogen) can be used in a consolidated workflow along with cationic amphiphilic fluorophore tracers, like Dye 1 in order to differentiate between phospholipidosis activators and autophagy

pathway inhibitors, providing unprecedented insight into the overall dysfunction of the lysosomal-dependent catabolic pathway.

For example, CF-PE was suspended in ethanol and diluted in the cell culture medium (McCoy's 5a modified medium, as described in Example 3) to give a solution (CF-PE medium) with final concentration of 50 μ M (0.46% ethanol). NBD-PE medium was then sonicated and filtered through a 0.22 μ m filter. The prepared was then applied to U2OS cells growing in a 96-well microplate. After an equilibration period of 24 h, the cell culture medium was replaced with CF-PE medium with or without each test compound, and the cells were incubated at 37°C for an additional 24 h. After incubation, the cells were rinsed with medium and further incubated with the same medium containing Dye 1 and Hoechst 33342 dye for an additional 15 min at 37°C in 5% CO₂ atmosphere.

Fluorescence signal was determined using a standard microplate reader, as described in example 4, except that CF-PE was additionally read at an excitation wavelength of 492 nm and an emission wavelength of 517 nm. Autophagy pathway inhibitors such as bafilomycin A generate an increased red signal but low green signal relative to vehicle-treated controls in the assay. Phospholipidosis inducers like chloroquine lead to both an increased green and red signal. Generalized cytotoxicity is evident as a substantial decrease in blue signal ($\geq 30\%$) relative to vehicle-treated control cells.

Autophagy is known to be substantially up-regulated in colon cancer tissues when compared with the surrounding non-cancerous counterparts. The multiplexed assay of lysosomal catabolic dysfunction should find application in identifying drug candidates that form the basis of new therapeutic strategies based upon the regulation of autophagy.

Example 12 Fluorescence Microscopy-Based and Microplate Assay-Based Staining using Dye 2

HeLa cervical cancer cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 1% MEM non-essential amino acids and 100

units/ml penicillin/ streptomycin (Invitrogen, Carlsbad, CA) in a humidified 37°C, 5% CO₂ environment. Cells were allowed to proliferate on glass slides or were seeded into 96-well plates at a density of 2×10^4 cells/well and allowed to attach overnight.

Cells grown on glass microscope slides were stained with 5 µM Dye 2, washed with PBS containing 2% FBS, covered with glass coverslips, sealed with nail polish and observed using an inverted Axiovert 200M microscope (Carl Zeiss, Inc., Oberkochen, Germany). Images were acquired with a 63X objective lens (Zeiss). Fluorescent and brightfield images are shown in FIG. 6.

The fluorescence-based lysosomal perturbation assay was established by first incubating cells cultivated on a microtiter plate for 5 hours with chloroquine (200 µM final concentration), a well known phospholipidosis-inducing agent. Control cells were incubated with 0.1% (v/v) dimethyl sulfoxide (DMSO), which served as the vehicle control. Cells were then briefly (30 min) incubated with 40 µM Dye 2 in PBS containing 2% FBS. Following incubation, cells were washed with PBS containing 2% FBS. Finally 60 µl of PBS containing 2% FBS was added to each well, and the fluorescence of each well was determined using a Synergy Mx plate scanner (BioTek, Winooski, VT) set to excite at 660 nM and emit at 705 nM. The results are shown in FIG. 7.

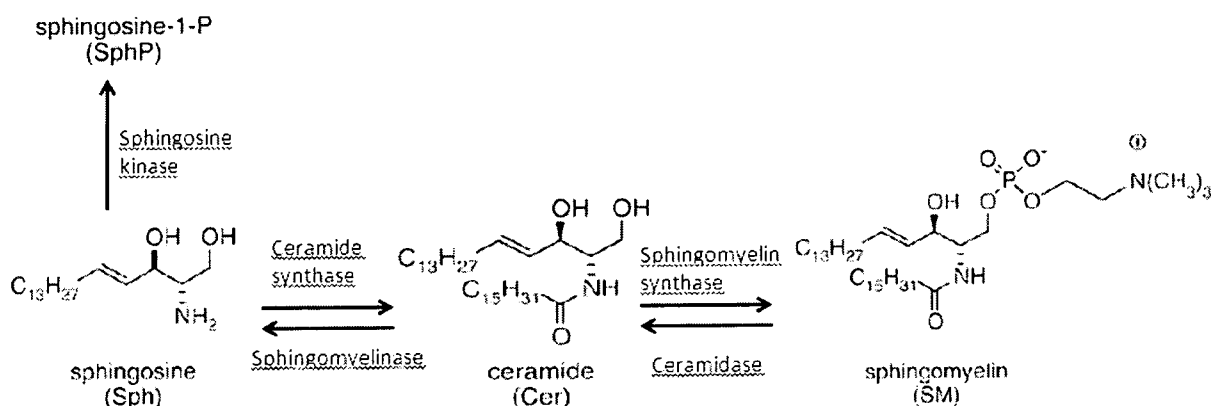
Example 13 Fluorescence Microscopy-Based Staining using Dye 5

HeLa cervical cancer cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% FBS, 1% MEM non-essential amino acids and 100 units/ml penicillin/ streptomycin (Invitrogen, Carlsbad, CA) in a humidified 37°C, 5% CO₂ environment. Cells were allowed to proliferate on glass slides and allowed to attach overnight.

Cells grown on glass microscope slides were stained with 10 µM Dye 5, washed with PBS containing 2% FBS, covered with glass coverslips, sealed with nail polish and observed using an inverted Axiovert 200M microscope (Carl Zeiss, Inc., Oberkochen, Germany). Images were acquired with a 63X objective lens (Zeiss). Fluorescent and brightfield images are shown in FIG. 8.

Example 14: Model system that mimics a lysosomal storage disease (Niemann-Pick Disease)

Sphingomyelin is the primary sphingophospholipid in mammalian cells. A simplified reaction pathway scheme for its biosynthesis is presented below. Sphingomyelinases catalyze the lysosomal degradation of sphingomyelin. Inherited deficiencies of acid sphingomyelinase activity result in various clinical forms of the Niemann–Pick disease, which are characterized by massive lysosomal accumulation of sphingomyelin.

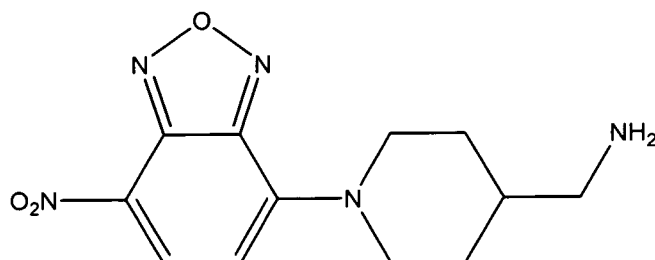


Stable cell lines derived from patients with Niemann-Pick disease cannot be found in cell line repositories, such as American Type Tissue Collection (Manassas, VA), and are not readily available to the research community. Consequently, an *in vitro* tissue culture model of sphingolipidosis was devised using 2R,3S,4E)-N-methyl-5-(4'pentylphenyl)-2-aminopent-4-ene-1,3-diol, designated SK1-I (BML-258), a potent, water soluble, isozyme-specific inhibitor of sphingosine kinase-1. In contrast to pan-sphingosine kinase inhibitors, SK1-I does not inhibit sphingosine kinase-2, protein kinase C, or numerous other protein kinases. U-2-OS human osteosarcoma cells were seeded at 22,500 cells/well, grown overnight, and then treated with a final concentration of 0, 2.5, 5, 10 or 20 μ M of SK1-1 for 18.5 hours. Next, the cells were briefly treated with compound 1 and the nuclear Hoechst 33342 dye, as described in Example 7. Fluorescence signal was read on a BioTek Synergy Mx microplate reader at excitation

values of 340 and 540 nm and emission values of 480 and 680 nm. Dose-dependent increases in emission values at 680 nm were observed upon treatment with SK1-1 ($Z' = 0.55$), signifying excessive accumulation of sphingolipids within the vacuolar compartment. Cell viability was maintained under all treatment conditions, as signified by relatively constant 480 nm emission intensity values from the Hoechst dye. Fluorescence microscope-based examination of the wells confirmed that compound 1 staining of the cells was confined to the vacuolar structures. This *in vitro* model confirms the feasibility of using the assay for detecting aberrant accumulation of sphingolipids in cells, and by extension the feasibility of using the assay in monitoring inherited lysosomal storage diseases, such as Niemann-Pick disease.

Example 15 Preparation of Dye 7

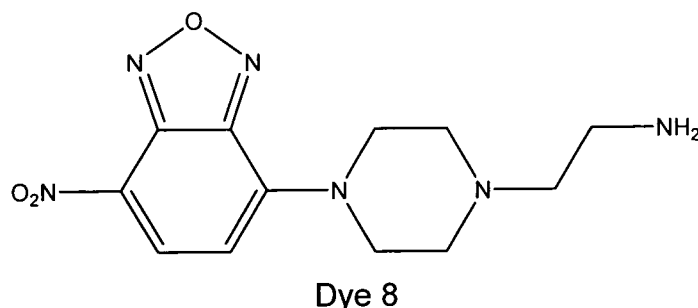
To an aqueous solution (7 mL) of sodium carbonate (105 mg, 1 mmol), acetonitrile (15 mL) was added dropwise. To this mixture 4-(aminomethyl)piperidine (228 mg, 240 μ L, 2 mmol) was added followed by a solution of 4-chloro-7-nitro-2,1,3-benzoxadiazole (200 mg, 1 mmol) in acetonitrile (10 mL). The combined mixture was stirred at room temperature for 15 minutes. Volatiles were then removed by rotary evaporation. The residue thus obtained was adsorbed on silica gel and purified on Biotage (Si, SNAP 25 g) using a gradient of methanol (7% to 60% over 10 column volume) in methylene chloride (+ 1% triethylamine) to provide Dye 7 as a reddish residue (105 mg). Abs (max, methanol) = 343 and 467 nm; Em (max, methanol) = 540 nm; $\epsilon = 11,100 \text{ M}^{-1}\text{cm}^{-1}$. The structure of Dye 7 is given below:



Dye 7

Example 16 Preparation of Dye 8

To an aqueous solution (5 mL) of sodium carbonate (105 mg, 1 mmol), acetonitrile (15 mL) was added dropwise. To this mixture 1-(2-Aminoethyl)piperazine (258 mg, 263 μ L, 2 mmol) was added followed by a solution of 4-chloro-7-nitro-2,1,3-benzoxadiazole (200 mg, 1 mmol) in acetonitrile (10 mL). The combined mixture was stirred at room temperature for 15 minutes. Volatiles were then removed by rotary evaporation. The residue thus obtained was dissolved in 7% methanol in dichloromethane and purified on Biotage (Si, SNAP 25 g) using a gradient of methanol (7% to 60% over 10 column volume) in methylene chloride (+ 1% triethylamine) to provide Dye 8 as a red powder (105 mg). Abs (max, methanol) = 335 and 467 nm; Em (max, methanol) = 534 nm; ϵ = 16,867 $M^{-1}cm^{-1}$. The structure of Dye 8 is given below:

**Example 17 Green autophagy dye (Dye 8)-accumulation increases during starvation**

The most well-known inducer of autophagy is nutrient starvation, both in cultured cells and in intact organisms, ranging from yeast to mammals. We evaluated the incorporation of Dye 8 green autophagy dye in cells where autophagy was stimulated by amino acid deprivation, a physiological stimulus of this process. Human cervical adenocarcinoma epithelial cell line HeLa, HepG2 and Human leukemic Jurkat cells were used. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). HeLa & HepG2 cells were routinely cultured in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) with low glucose, supplemented with 10% FBS (ATCC, Manassas, VA) and 100 U /ml penicillin, 100 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Jurkat cells were grown in suspension in RPMI medium

supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U /ml), streptomycin (100 µg/ml), and glutamine (200 mM). Cells were maintained in a saturated humidified atmosphere at 37°C, 5% CO₂ and 95% air.

For induction of starvation, HeLa, Jurkat or HepG2 cells were washed three times with Earle's balanced salts solution (EBSS) (Sigma-Aldrich, St. Louis, MO). at 37°C incubated with EBSS medium at 37°C for one hour. Control cells were incubated in d-MEM medium. Following this incubation period, both starved and control cells were incubated with 20 µM of Dye 8 green autophagy dye for 10 minutes at 37°C and then washed once with 1x PBS. Cells were observed using a fluorescence microscope (Carl Zeiss, Inc) using an FITC filter set. Images were acquired with a 63X objective lens (Zeiss). As shown in Fig. 9, starvation conditions with HeLa cell induces an increase in Dye 8 green autophagy fluorescence intensity in punctate cytoplasmic structures. Similar results were obtained with HepG2 and Jurkat cell line (data not shown).

Example 18 A population of the Dye 8-labeled vesicles co-localizes with LC3, a specific autophagosome marker

Under physiological conditions, autophagy is a constitutive self-degradative process involved both basal turnover of cellular components and an induced response to nutrient starvation in eukaryotes. During autophagy, portions of the cytoplasm are sequestered by elongation of double-membrane structures called phagophores, which form vesicles called autophagosomes. These vesicles then fuse with lysosomes to form autolysosomes, where their contents are degraded by acidic lysosomal hydrolases for subsequent recycling. LC3, a mammalian homologue of the ubiquitin-like (UBL) protein Atg8, has been used as a specific marker of autophagosomes in mammals.

In this example, HeLa cells at 70% confluence were transfected with RFP-LC3 using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. After 24 hours transfection, cells were treated with either vehicle or 10 µM tamoxifen overnight. The cells were then stained with Dye 8 green autophagy dye and observed using fluorescence microscope (Carl Zeiss, Inc) using an FITC filter set as indicated in Example 16. Images were acquired with a 63X objective lens (Zeiss).

Tamoxifen is a drug that is widely used in the treatment of breast cancer. It competes with estrogen for receptor binding and prevents the transcription of estrogen responsive genes, which results in reduced cell growth. There is some evidence that tamoxifen causes increased synthesis of Beclin 1 (BECN1; also known as Atg6), which may stimulate autophagy. We have demonstrated that Tamoxifen induces an increase in Dye 8 green autophagy fluorescence intensity in punctuate structures (FIG. 10a) that co-localize with RFP-LC3 (FIG. 10b). Note RFP-LC3 is not uniformly expressed in all cells due to the limited efficiency of transfection.

Example 19 Validating small molecules that affect the autophagy pathway

Besides Tamoxifen and starvation treatment, autophagy also can be induced by the various compounds indicated in Table 2. The compounds in Table 2 (Chloroquine, verapamil, Lithium, Trehalose, Bafilomycin A1, Hydroxychloroquine, Loperamide, Clonidine, MG-132, Norclomipramine), were obtained from Sigma-Aldrich Chemical Company (Saint Louis, MO). Following treatment, the cells were incubated with Dye 8 green autophagy detection reagent and analyzed using fluorescence microscope (Carl Zeiss, Inc) by the method indicated in Example 17.

Table 2.

Treatment	Target	Effect	μM used	Induction Time (hrs)	Cell Line
Starvation	Inhibits mTOR	Activates autophagy	N/A	1~4	HeLa, HepG2, Jurkat
Rapamycin	Inhibits mTOR	Activates autophagy	0.2~3	6~18	HeLa & Jurkat
PP242	ATP-competitive inhibitor of mTOR	Activates autophagy	1	18	HeLa
Lithium	mTOR-independent	Activates autophagy	10,000	18	HeLa & Jurkat
Trehalose	Unknown, mTOR-independent	Activates autophagy	50,000	6	HeLa & Jurkat
Bafilomycin A1	Inhibits Vacuolar-ATPase	Inhibits lysosome function	$3\sim9*10^{-3}$	18	HeLa
Chloroquine	Alkalinizes Lysosomal pH	Inhibits lysosome function	10~50	18	HeLa
Tamoxifen	abolishes the inhibitory effect of PI3K	Activates autophagy & inhibits lysosome function	4~10	6~18	HeLa, HepG2, Jurkat
Verapamil	mTOR-independent	Activates autophagy	40~100	18	HeLa & Jurkat
Hydroxy-chloroquine	Alkalinizes Lysosomal pH	Inhibits lysosome function	10	18	HeLa
Loperamide	mTOR-independent	Activates autophagy	5	18	HeLa
Clonidine	mTOR-independent	Activates autophagy	100	18	HeLa
MG-132	Selective proteasome inhibitor	Activates autophagy	2~5	4~12	HeLa, Jurkat
Norclomipramine	Alkalinizes Lysosomal pH	Inhibits lysosome function	5~20	18	HeLa

A popular autophagy target is mTOR, which is a potent suppressor of autophagy. Rapamycin, an inhibitor of mTOR, increases Dye 8 signal (FIG. 11B). It is known that 3-methyladenine (3-MA) is a specific inhibitor of early stages of the autophagic process

(Seglen and Gordon, 1982). To test if the incorporation of Dye 8 was indeed via autophagy, HeLa cells were pre-incubated for 3 hours with 10 mM 3-MA (Sigma) and autophagy was induced by 3 μ M Rapamycin (Sigma) overnight. FIG. 11C shows that uptake of Dye 8 is almost completely inhibited in this situation. Taken together, the results indicate that the accumulation of Dye 8 in vesicles correlates with the induction of autophagy by rapamycin, and is abrogated by proven inhibitors of the autophagic pathway such as 3-MA. Therefore, autophagocytosis can be followed both biochemically and morphologically in living cells using this system based upon the incorporation of Dye 8.

Several mTOR-independent autophagy activators have also been validated using the Dye 8 (Table 2). Lithium induces autophagy through inhibition of inositol monophosphatase (an mTOR-independent pathway). Trehalose and small molecule enhancers of rapamycin (SMERs) also induce autophagy by mechanisms that are not well understood. Two FDA-approved compounds that induce autophagy in an mTOR-independent manner, loperamide hydrochloride and clonidine, also substantially increase green fluorescent signal in the assay. Bafilomycin A1 is a selective inhibitor of vacuolar (V)-type ATPases, which results in elevated lysosomal pH. Chloroquine, verapamil, norclomipramine and hydroxychloroquine are small molecule modulators that passively diffuse into the lysosome and become trapped upon protonation. All these agents also cause an increase in lysosomal pH, which inhibits lysosome function and blocks fusion of the autophagosome with the lysosome. The agents generate a positive signal with the Dye 8 green autophagy dye. Furthermore, MG-132, a potent cell-permeable and selective proteasome inhibitor, has been shown to induce autophagy, as demonstrated with the described assay. The ubiquitin-proteasome system (UPS) and autophagy serve as two complementary, reciprocally regulated protein degradation systems. Blockade of UPS by MG-132 is well known to activate autophagy.

Example 20 Quantifying autophagic activity by flow cytometry using Dye 8 in living cells

Autophagy is an important cellular process and has recently been recognized to be involved in various developmental processes and various diseases including cancer and neurodegeneration. However, experimental methods to accurately detect and quantify this process in mammalian cells are limited due to lack of autophagic markers. Recently, LC3, a mammalian homologue of the ubiquitin-like (UBL) protein Atg8, has been used as a specific marker of autophagosomes in mammals. However, current methods to quantify autophagic activity using LC3 by (1) microscopy, (2) biochemical methods, and (3) detection of protein modifications through SDS-PAGE and Western blotting are time-consuming, labor-intensive and require much experience for accurate interpretation. Thus, there is need to have a simple, reproducible and sensitive method to determine autophagic activity in living cells. We have developed a novel flow cytometry cell-based autophagy assay using Dye 8 to measure autophagic activity in living cells.

In this example, autophagy was induced by amino acid starvation using the method described in Example 17. Following this incubation period, both starved and control cells were incubated with 2.5 μ M of Dye 8 for 10 minutes at 37°C. Experiments were performed using a FACS Calibur benchtop flow cytometer (BD Biosciences, San Jose, CA) equipped with a blue (488 nm) laser, and Dye 8 fluorescence was measured either in the FL1 or FL2 channel. No washing was required prior to the flow cytometry analysis.

All of the experiments were performed at least three times. Data analysis was carried out with the FlowJo software package. Specifically, data were analyzed by comparison of median fluorescence, by calculating Autophagic Activity Factor (AAF) values using the following formula: $AAF = 100 * ((MFI_{inducer} - MFI_0) / MFI_{inducer})$, wherein $MFI_{inducer}$ and MFI_0 are the mean fluorescence intensity values measured from control and treated samples. This is using a similar formula used in evaluating multiple drug resistance (MAF values) (Hollo et al., 1998).

We show that Dye 8 fluorescent signal is increased during starvation in a time-dependent manner (FIG. 12). To analyze the kinetics of Dye 8 signal change, Jurkat

cells were incubated in EBSS medium for different periods of time and subsequently stained with Dye 8 green autophagy dye. Thirty minutes of amino acid deprivation was enough to increase by more than 50% the amount of Dye 8 signal that accumulated in the starved cells. This is consistent with previous results indicating that autophagy is rapidly induced following amino acid deprivation (Dunn, 1990). After 2 hours of starvation, the Dye 8 signal was maximal.

FIG. 13 shows the histogram overlays. Control Jurkat cells were stained as well but displayed low fluorescence. Dye 8 signal increases about 2.5-fold after 2 hour starvation, indicating that starvation causes an increase in autophagic vesicles in Jurkat cells. The AAF value is about 59 as compared with a typical cut-off value of about 20-25 in most MDR flow cytometry experiments (Hollo et al., 1998). This indicated that the peak from starved cells is well separated from the control group. Starved cells switched back to growth medium for 1 hour demonstrate complete recovery.

Example 21 Utilizing Flow Cytometry to measure autophagic flux with Dye 8 green autophagy dye

Induction of autophagy results in the accumulation of insoluble, autophagosome-associated LC3-II in the cytoplasm of cells. However, the same observation can be made when autophagic degradation is inhibited. Therefore, it is important to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors. Currently autophagic flux is measured either by western blot (Klionsky et al., 2008) or by EGFP-LC3 reporter-transfected cells using saponin extraction (Eng et al., 2010). However, both methods are labor intensive and time consuming.

We present a rapid quantitative assay using Dye 8 to measure autophagic flux. Staining protocol, data acquisition and analysis are the same as described in Example 20. FIG. 14 shows that 300 μ M of chloroquine treatment for 2 hours lead to a modest increase in fluorescence intensity of Dye 8. A 2 hour starvation in Jurkat cells induces an increase in fluorescence intensity of Dye 8 about 3 fold. However, starvation in the presence of chloroquine increases Dye 8 fluorescence intensity about 4 fold. The

increase in signal represents the accumulation of the signal due to the ongoing constitutive generation of new autophagosomes under conditions where their lysosomal degradation is inhibited. These results demonstrate that the assay can easily study autophagic flux (separate from inhibition of degradation) by simply using Dye 8, in the presence of chloroquine, with flow cytometry.

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U.S. Pat. No. 5401847
U.S. Pat. No. 5538848
U.S. Pat. No. 5580990
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U.S. Pat. No. 5800996
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U.S. Pat. Pub. No. 2010/0062460

In view of the above, it will be seen that several objectives of the invention are achieved and other advantages attained.

Many obvious variations will no doubt be suggested to those of ordinary skill in the art, in light of the above detailed description and examples of the present invention. It will be appreciated by those skilled in the art that any arrangement which is calculated to achieve the same purpose may be substituted for the specific embodiments shown. This application and invention are intended to cover any adaptations or variations of the present invention. All such variations are fully embraced by the scope and spirit of the invention as more particularly defined in the claims that now follow.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

WHAT IS CLAIMED IS:

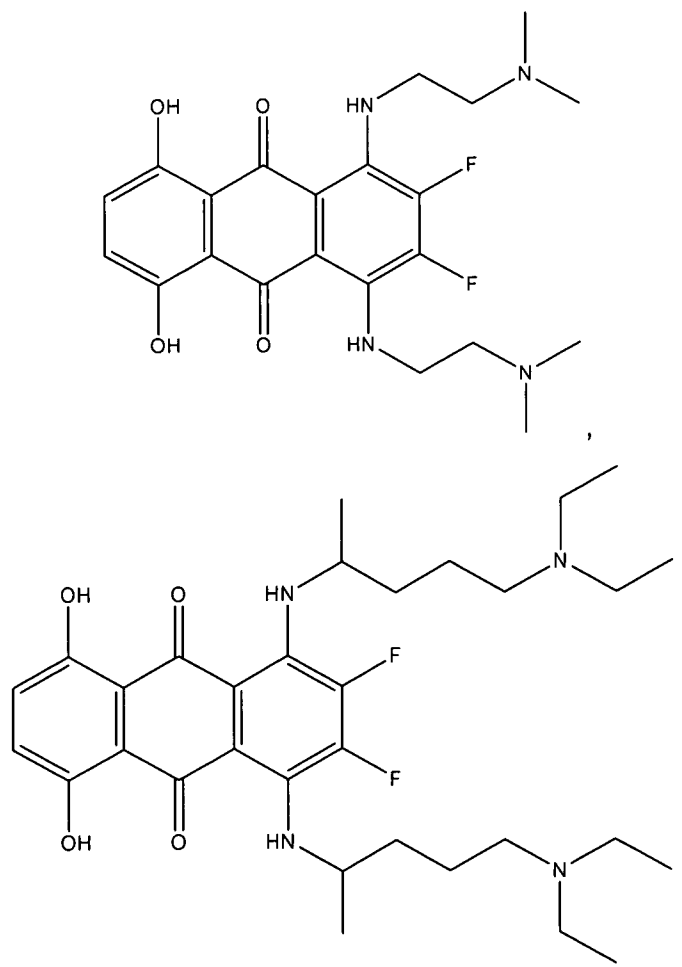
1. A method of detecting the presence of a lysosomal storage disease in a subject comprising the steps of:
 - (a) obtaining a sample containing cells from said subject;
 - (b) contacting said sample with a cationic amphiphilic tracer compound that localizes to a vacuole in a cell; and
 - (c) detecting said cationic amphiphilic tracer compound, thereby determining whether there exists an excess above normal accumulation of vacuoles within said cells of the sample, said excess above normal accumulation of vacuoles being indicative of the lysosomal storage disease.
2. The method of claim 1, wherein said lysosomal storage disease arises from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome.
3. The method of claim 1, wherein said lysosomal storage disease arises from a defect in one or more lysosomal membrane proteins.
4. The method of claim 1, wherein said lysosomal storage disease arises from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus, an endosomal pathway, or any combination thereof.
5. The method of claim 1, wherein said lysosomal storage disease comprises a mucopolysaccharidoses, a lipidoses or a glycogenosis.
6. The method of claim 1, wherein a subject is known to have a lysosomal storage disease based upon previous genetic or metabolic testing.

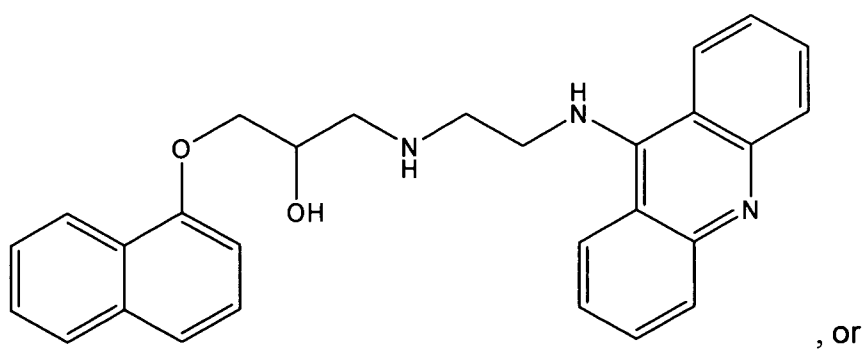
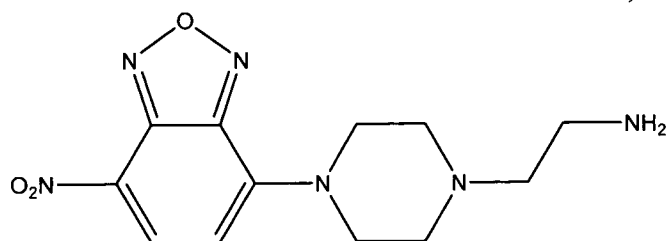
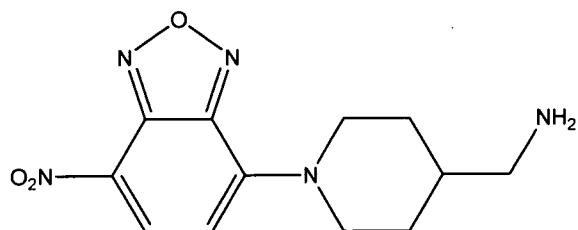
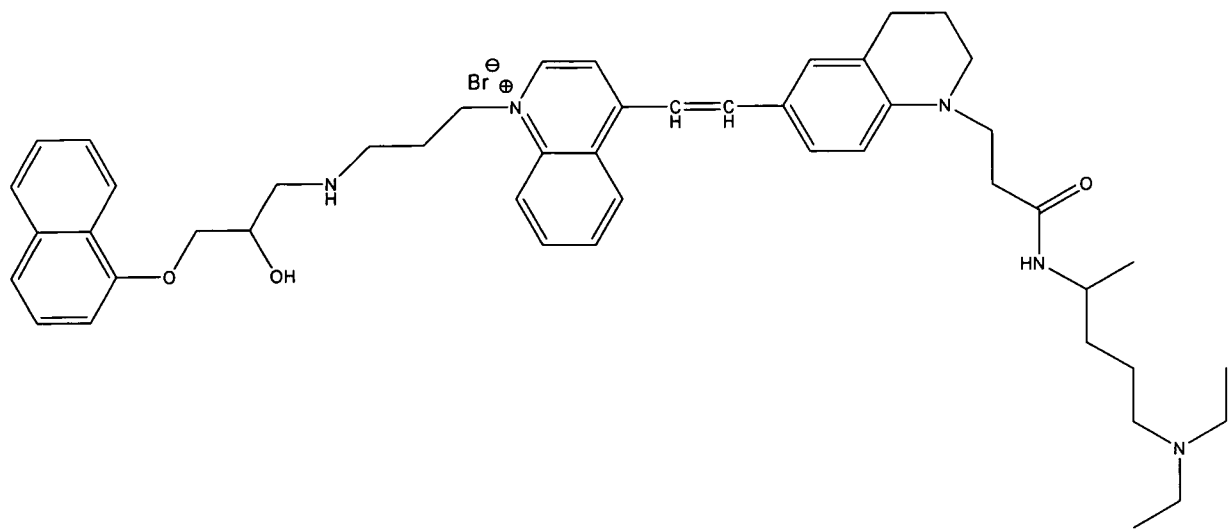
7. The method of claim 1 wherein said lysosomal storage disease comprises Activator Deficiency/GM2 Gangliosidosis, Alpha-mannosidosis, Aspartylglucosaminuria, Cholesteryl Ester Storage Disease, Chronic Hexosaminidase A Deficiency, Cystinosis, Danon Disease, Fabry Disease, Farber Disease, Fucosidosis, Galactosialidosis, Gaucher Disease, GM1 Gangliosidosis, I-Cell Disease/Mucopolidosis II, Infantile Free Sialic Acid Storage Disease/ISSD, Juvenile Hexosaminidase A Deficiency, Krabbe Disease, Metachromatic Leukodystrophy, Mucopolysaccharidoses Disorders, Pseudo-Hurler Polydystrophy/Mucopolidosis IIIA, MPSI Hurler Syndrome, MPSI Scheie Syndrome, MPS I Hurler-Scheie Syndrome, MPS II Hunter Syndrome, Sanfilippo syndrome Type A/MPS III A, Sanfilippo syndrome Type B/MPS III B, Sanfilippo Syndrome Type C/MPS III C, Sanfilippo Syndrome Type D/MPS III D, Morquio Type A/MPS IVA, Morquio Type B/MPS IVB, MPS IX Hyaluronidase Deficiency, MPS VI Maroteaux-Lamy, MPS VII Sly Syndrome, Mucopolidosis I/Sialidosis, Mucopolidosis IIIC, Mucopolidosis Type IV, Multiple Sulfatase Deficiency, Niemann-Pick Disease, Neuronal Ceroid Lipofuscinoses, CLN6 Disease, Batten-Spielmeyer-Vogt/Juvenile NCL/CLN3 Disease, Finnish Variant Late Infantile CLN5, Jansky-Bielschowsky Disease/Late infantile CLN2/TPP1 Disease, Kufs/Adult-onset NCL/CLN4 Disease, Northern Epilepsy/Variant Late Infantile CLN8, Santavuori-Haltia/Infantile CLN1/PPT Disease, Beta-Mannosidosis, Pompe Disease/Glycogen Storage Disease Type II, Pycnodysostosis, Sandhoff disease/Adult Onset/GM2 Gangliosidosis, Sandhoff Disease/GM2 Gangliosidosis – Infantile, Sandhoff Disease/GM2 Gangliosidosis – Juvenile, Schindler disease, Salla Disease/Sialic Acid Storage Disease, Tay-Sachs/GM2 Gangliosidosis, Wolman Disease, or any combination thereof.

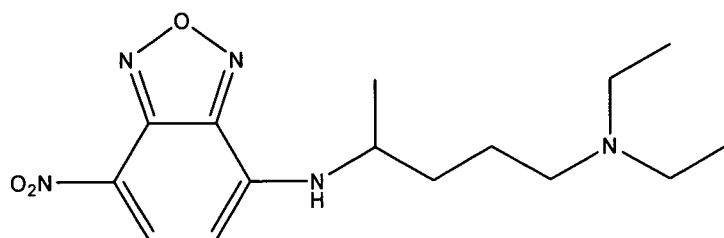
8. The method of claim 1, wherein said vacuole comprises a lysosome, a phagophore, an autophagosomes, an autophagolysosome, or any combination thereof.

9. The method of claim 1, wherein said cationic amphiphilic tracer compound comprises a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.

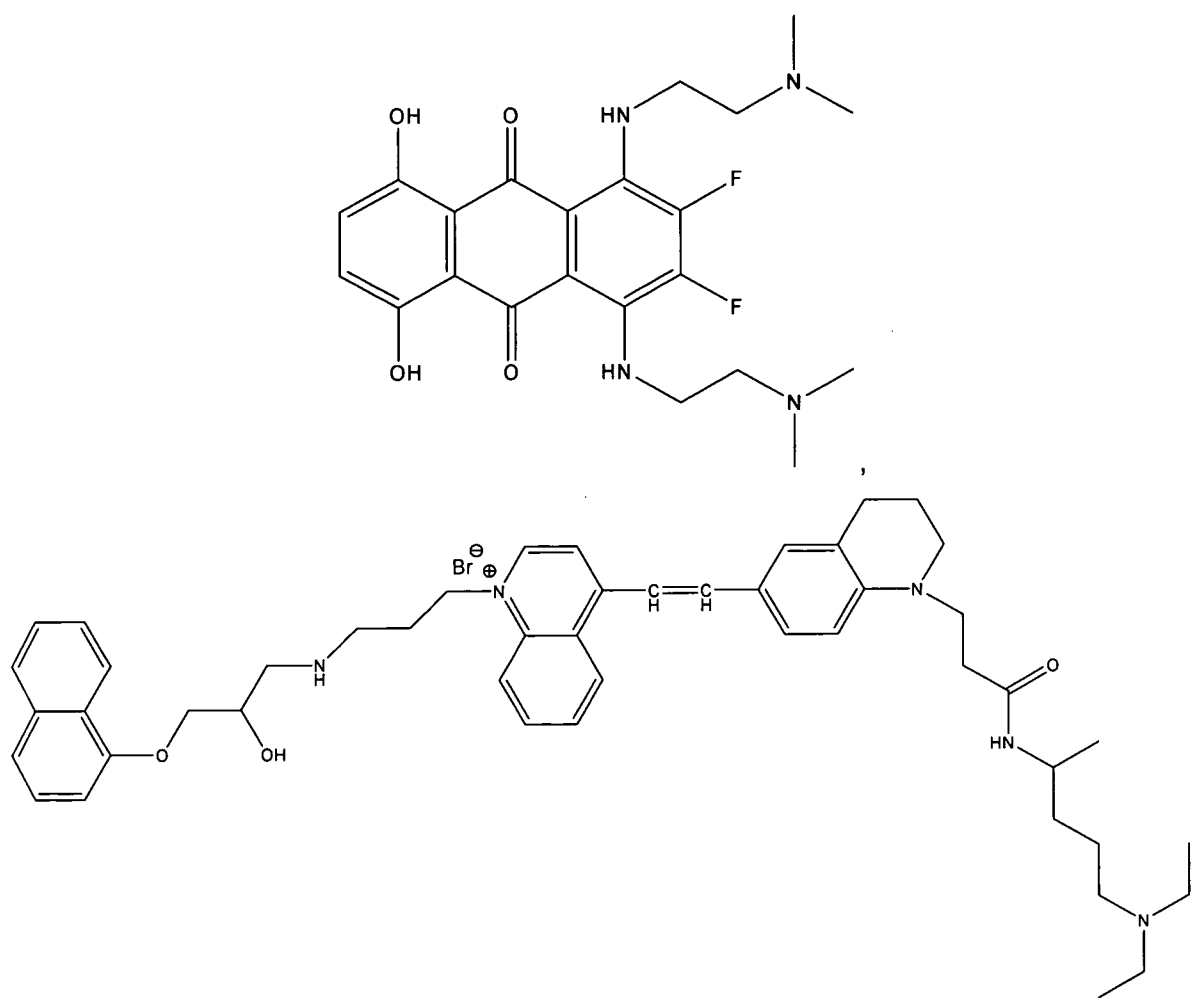
10. The method of claim 1, wherein said cationic amphiphilic tracer compound comprises a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.
11. The method of claim 1, wherein said cationic amphiphilic tracer compound comprises nitrobenzoxadiazole (NBD)-siperone, nitrobenzoxadiazole (NBD)-propranolol, 9-amino-acridin-propanolol, or any combination thereof.
12. The method of claim 1, wherein said cationic amphiphilic tracer compound has the structure:

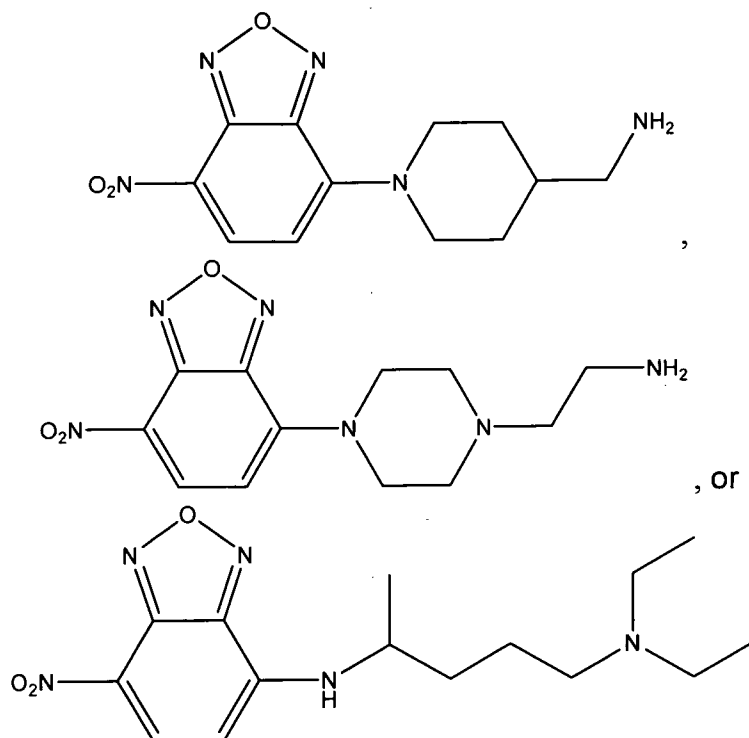






13. The method of claim 12, wherein said cationic amphiphilic tracer compound has the structure





14. The method of claim 1, further comprising the steps (a') of providing a reference sample of normal cells from a subject known not to have lysosomal storage disease; (b') contacting said sample of normal cells with the same cationic amphiphilic tracer compound of step (b); and (c') comparing the accumulation of vacuoles in the sample obtained from the subject known or suspected of having a lysosomal storage disease with the accumulation in said reference sample of normal cells.

15. The method of claim 1, wherein said cells comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing.

16. The method of claim 1, wherein said subject is a mammal.

17. The method of claim 16, wherein said mammal is human.

18. A method of monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of ($T_2 - T_1$) time interval(s), said method comprising the steps of:

- (a) obtaining from said subject at time T_1 a first sample containing cells;
- (b) contacting said first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
- (c) detecting said first cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within said cells of the first sample at time T_1 ;
- (d) obtaining from said subject at a later time T_2 a second sample containing cells;
- (e) contacting said second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
- (f) detecting said second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and
- (g) comparing the accumulation of vacuoles between said first sample at time T_1 and said second sample at time T_2 , thereby monitoring the progression of lysosome storage disease in said subject.

19. The method of claim 18, wherein said lysosomal storage disease arises from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome.

20. The method of claim 18, wherein said lysosomal storage disease arises from a defect in one or more lysosomal membrane proteins.

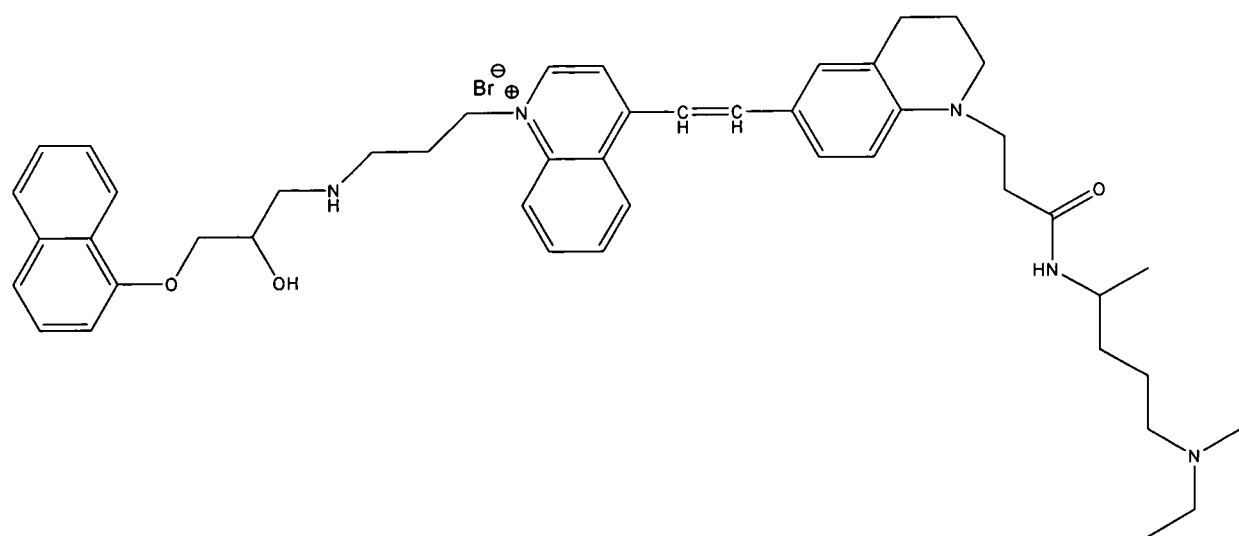
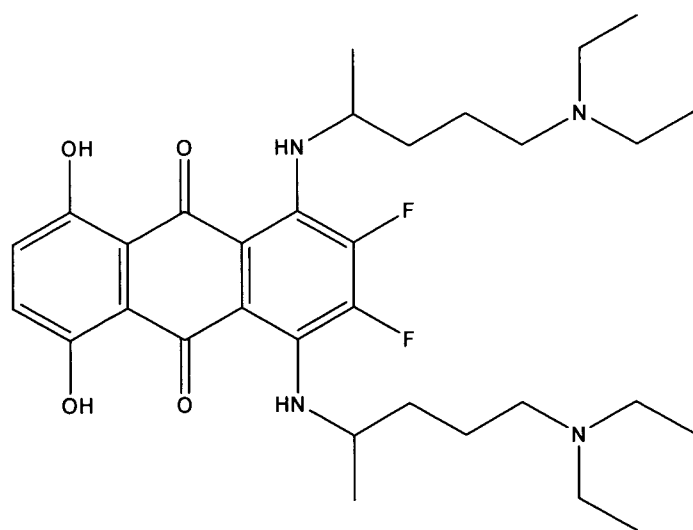
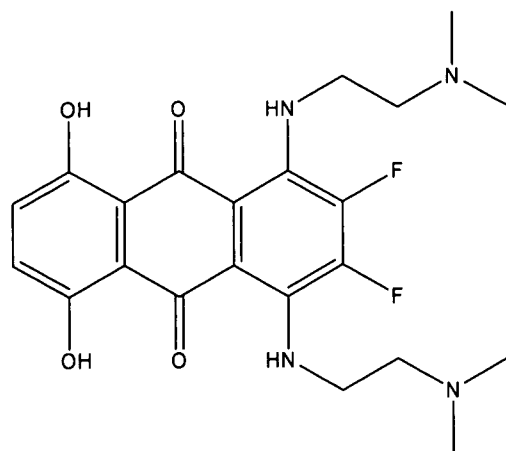
21. The method of claim 18, wherein said lysosomal storage disease arises from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus or an endosomal pathway, and combinations of any of the foregoing.

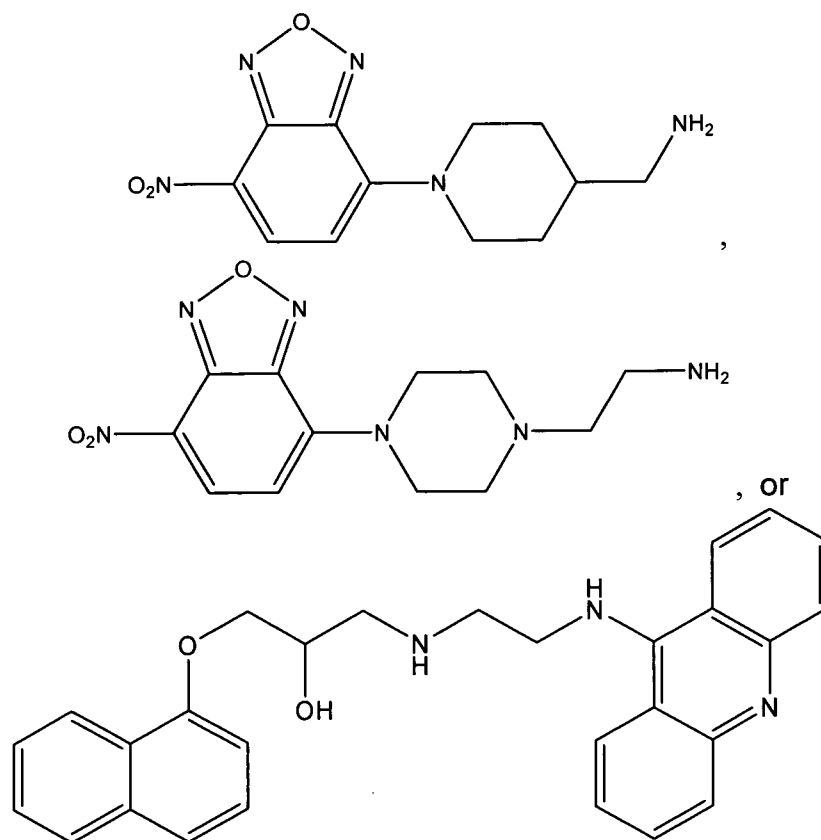
22. The method of claim 18, wherein said lysosomal storage disease comprises a mucopolysaccharidoses, a lipidoses or a glycogenosis.

23. The method of claim 18, wherein a subject is known to have a lysosomal storage disease based upon previous genetic or metabolic testing.

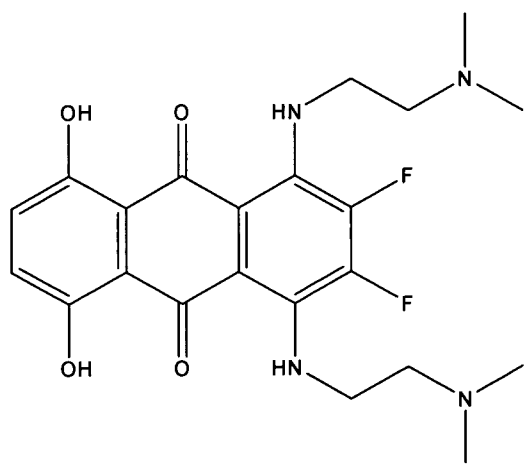
24. The method of claim 18, wherein said lysosomal storage disease comprises Activator Deficiency/GM2 Gangliosidosis, Alpha-mannosidosis, Aspartylglucosaminuria, Cholesteryl Ester Storage Disease, Chronic Hexosaminidase A Deficiency, Cystinosis, Danon Disease, Fabry Disease, Farber Disease, Fucosidosis, Galactosialidosis, Gaucher Disease, GM1 Gangliosidosis, I-Cell Disease/Mucopolipidosis II, Infantile Free Sialic Acid Storage Disease/ISSD, Juvenile Hexosaminidase A Deficiency, Krabbe Disease, Metachromatic Leukodystrophy, Mucopolysaccharidoses Disorders, Pseudo-Hurler Polydystrophy/Mucopolipidosis IIIA, MPSI Hurler Syndrome, MPSI Scheie Syndrome, MPS I Hurler-Scheie Syndrome, MPS II Hunter Syndrome, Sanfilippo syndrome Type A/MPS III A, Sanfilippo syndrome Type B/MPS III B, Sanfilippo Syndrome Type C/MPS III C, Sanfilippo Syndrome Type D/MPS III D, Morquio Type A/MPS IVA, Morquio Type B/MPS IVB, MPS IX Hyaluronidase Deficiency, MPS VI Maroteaux-Lamy, MPS VII Sly Syndrome, Mucopolipidosis I/Sialidosis, Mucopolipidosis IIIC, Mucopolipidosis Type IV, Multiple Sulfatase Deficiency, Niemann-Pick Disease, Neuronal Ceroid Lipofuscinoses, CLN6 Disease, Batten-Spielmeyer-Vogt/Juvenile NCL/CLN3 Disease, Finnish Variant Late Infantile CLN5, Jansky-Bielschowsky Disease/Late infantile CLN2/TPP1 Disease, Kufs/Adult-onset NCL/CLN4 Disease, Northern Epilepsy/Variant Late Infantile CLN8, Santavuori-Haltia/Infantile CLN1/PPT Disease, Beta-Mannosidosis, Pompe Disease/Glycogen Storage Disease Type II, Pycnodysostosis, Sandhoff disease/Adult Onset/GM2 Gangliosidosis, Sandhoff Disease/GM2 Gangliosidosis – Infantile, Sandhoff Disease/GM2 Gangliosidosis – Juvenile, Schindler disease, Salla Disease/Sialic Acid Storage Disease, Tay-Sachs/GM2 Gangliosidosis, Wolman Disease, or any combination thereof.

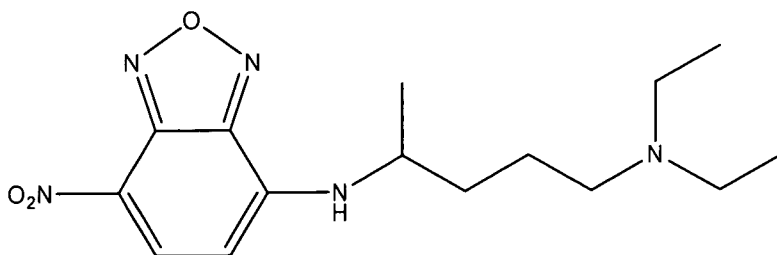
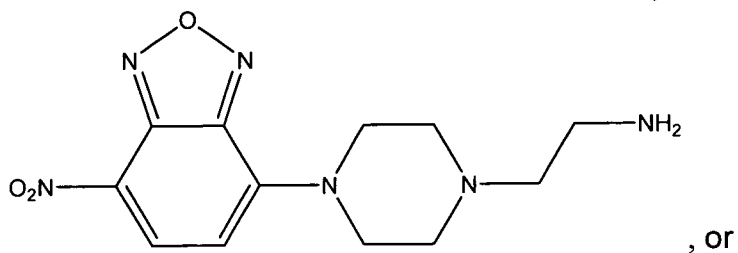
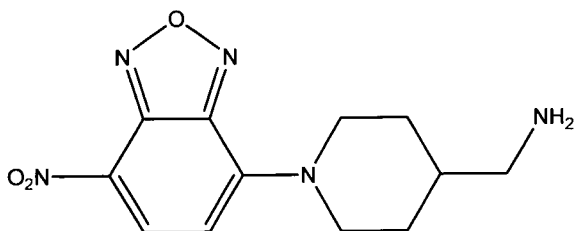
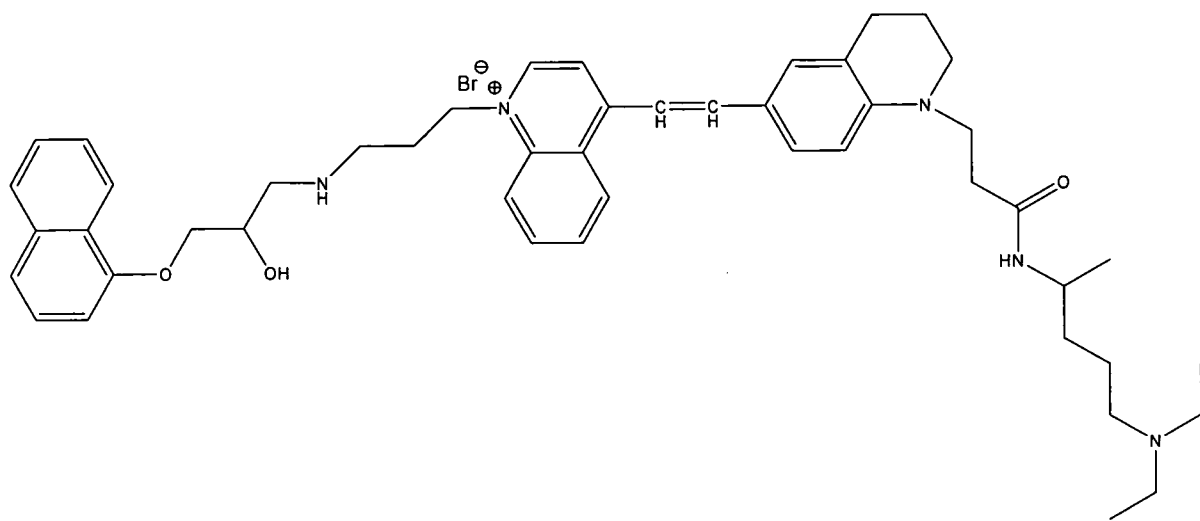
25. The method of claim 18, wherein said vacuole comprises a lysosome, a phagophore, an autophagosome, an autophagolysosome, or any combination thereof.
26. The method of claim 18, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.
27. The method of claim 18, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.
28. The method of claim 18, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound are the same compound.
29. The method of claim 18, wherein said cationic amphiphilic tracer compound comprises nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, 9-amino-acridin-propranolol, or any combination thereof.
30. The method of claim 18, wherein said cationic amphiphilic tracer compound has the structure:





31. The method of claim 18, wherein said cationic amphiphilic tracer compound has the structure





32. The method of claim 18, wherein the difference ($T_2 - T_1$) comprises time units measured in hours, days, weeks, months or years.

33. The method of claim 18, wherein said cells comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing.

34. The method of claim 18, wherein said subject is a mammal.
35. The method of claim 34, wherein said mammal is human.
36. A method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease in a subject, comprising the steps of:
- (a) obtaining from said subject at time T_1 a first sample containing cells;
 - (b) contacting said first sample with a first cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
 - (c) detecting said first cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within said cells of the first sample at time T_1 ;
 - (d) obtaining from said subject at a later time T_2 a second sample containing cells;
 - (e) contacting said second sample with a second cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
 - (f) detecting said second cationic amphiphilic tracer compound, thereby determining the accumulation of vacuoles within cells of the second sample at time T_2 ; and
 - (g) comparing the accumulation of vacuoles between said first sample at time T_1 and said second sample at time T_2 , thereby monitoring the effectiveness of said drug used to manage, treat or cure lysosome storage.
37. The method of claim 36, wherein said lysosomal storage disease arises from deficiencies of one or more soluble lysosomal proteins residing in the lumen of the lysosome.
38. The method of claim 36, wherein said lysosomal storage disease arises from a defect in one or more lysosomal membrane proteins.

39. The method of claim 36, wherein said lysosomal storage disease arises from deficiencies of non-lysosomal proteins residing in a site comprising an endoplasmic reticulum, a Golgi apparatus, an endosomal pathway, or any combination thereof.

40. The method of claim 36, wherein said lysosomal storage disease comprises a mucopolysaccharidoses, a lipidoses or a glycogenosis.

41. The method of claim 36, wherein a subject is known to have a lysosomal storage disease based upon previous genetic or metabolic testing.

42. The method of claim 36, wherein said lysosomal storage disease comprises Activator Deficiency/GM2 Gangliosidosis, Alpha-mannosidosis, Aspartylglucosaminuria, Cholesteryl Ester Storage Disease, Chronic Hexosaminidase A Deficiency, Cystinosis, Danon Disease, Fabry Disease, Farber Disease, Fucosidosis, Galactosialidosis, Gaucher Disease, GM1 Gangliosidosis, I-Cell Disease/Mucopolipidosis II, Infantile Free Sialic Acid Storage Disease/ISSD, Juvenile Hexosaminidase A Deficiency, Krabbe Disease, Metachromatic Leukodystrophy, Mucopolysaccharidoses Disorders, Pseudo-Hurler Polydystrophy/Mucopolipidosis IIIA, MPSI Hurler Syndrome , MPSI Scheie Syndrome, MPS I Hurler-Scheie Syndrome, MPS II Hunter Syndrome , Sanfilippo syndrome Type A/MPS III A, Sanfilippo syndrome Type B/MPS III B, Sanfilippo Syndrome Type C/MPS III C, Sanfilippo Syndrome Type D/MPS III D, Morquio Type A/MPS IVA, Morquio Type B/MPS IVB, MPS IX Hyaluronidase Deficiency, MPS VI Maroteaux-Lamy, MPS VII Sly Syndrome, Mucopolipidosis I/Sialidosis, Mucopolipidosis IIIC, Mucopolipidosis Type IV, Multiple Sulfatase Deficiency, Niemann-Pick Disease, Neuronal Ceroid Lipofuscinoses, CLN6 Disease, Batten-Spielmeyer-Vogt/Juvenile NCL/CLN3 Disease, Finnish Variant Late Infantile CLN5, Jansky-Bielschowsky Disease/Late infantile CLN2/TPP1 Disease, Kufs/Adult-onset NCL/CLN4 Disease, Northern Epilepsy/Variant Late Infantile CLN8, Santavuori-Haltia/Infantile CLN1/PPT Disease, Beta-Mannosidosis, Pompe Disease/Glycogen Storage Disease Type II, Pycnodysostosis, Sandhoff disease/Adult Onset/GM2 Gangliosidosis, Sandhoff

Disease/GM2 Gangliosidosis – Infantile, Sandhoff Disease/GM2 Gangliosidosis – Juvenile, Schindler disease, Salla Disease/Sialic Acid Storage Disease, Tay-Sachs/GM2 Gangliosidosis, Wolman Disease, or any combination thereof.

43. The method of claim 36, wherein said vacuole comprises a lysosome, a phagophore, an autophagosomes, an autophagolysosome, or any combination thereof.

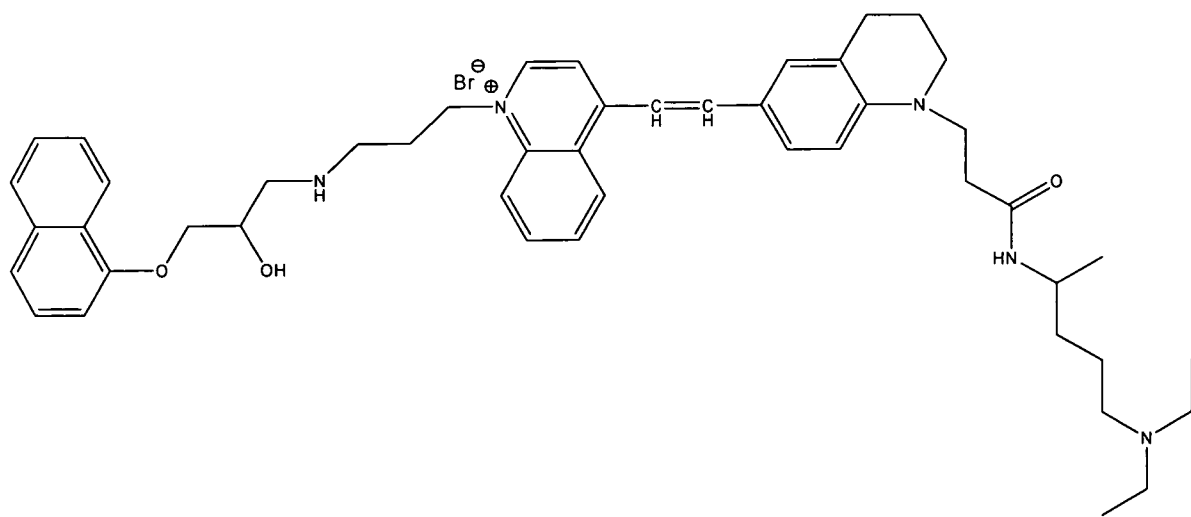
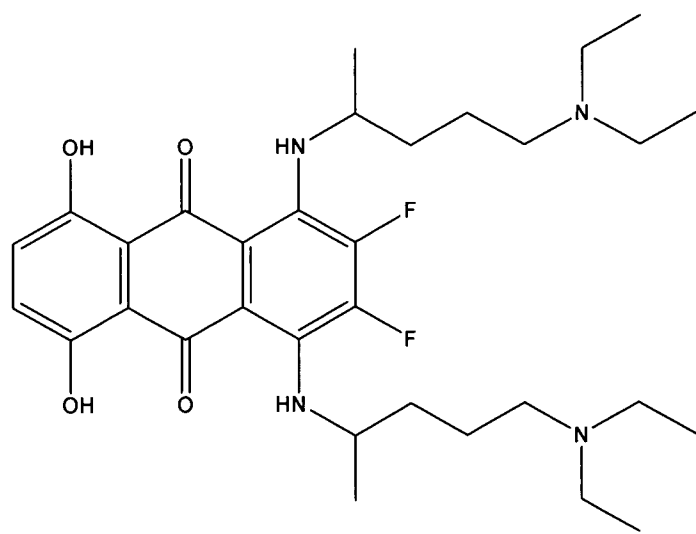
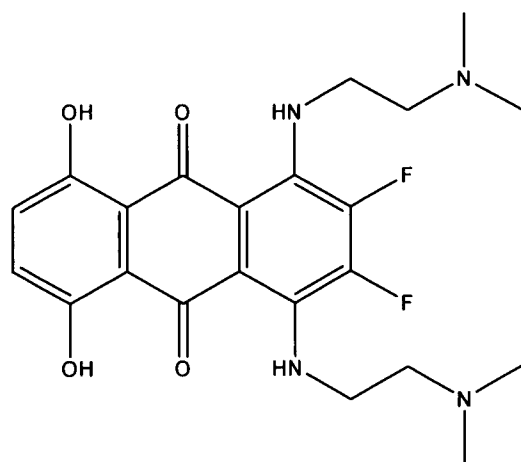
44. The method of claim 36, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.

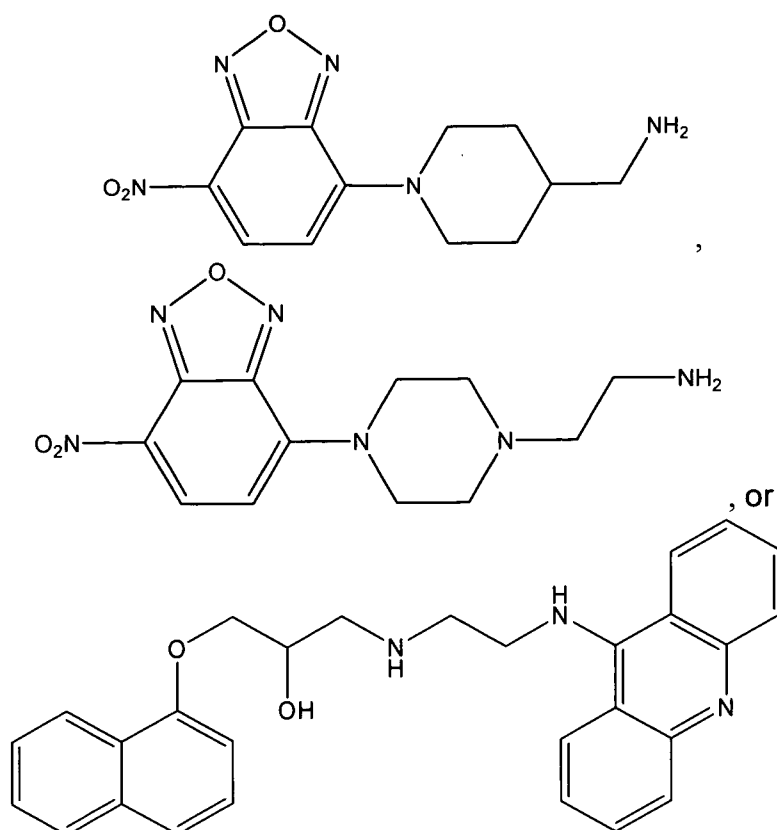
45. The method of claim 36, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.

46. The method of claim 36, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound are the same compound.

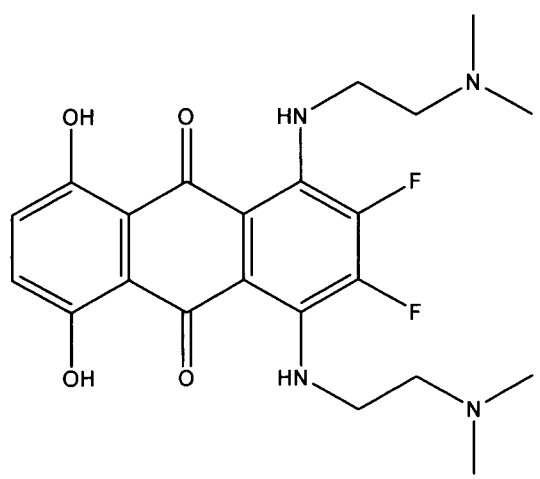
47. The method of claim 36, wherein said cationic amphiphilic tracer compound comprises nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, 9-amino-acridin-propanolol, or any combination thereof.

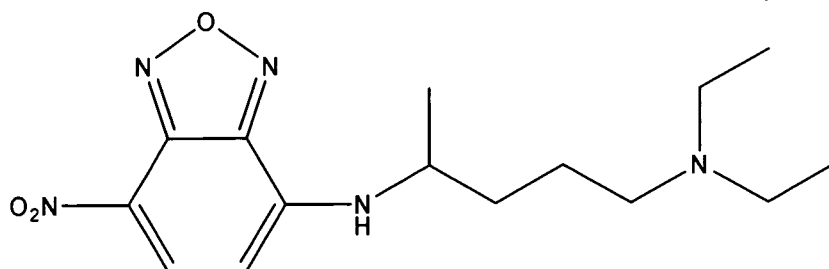
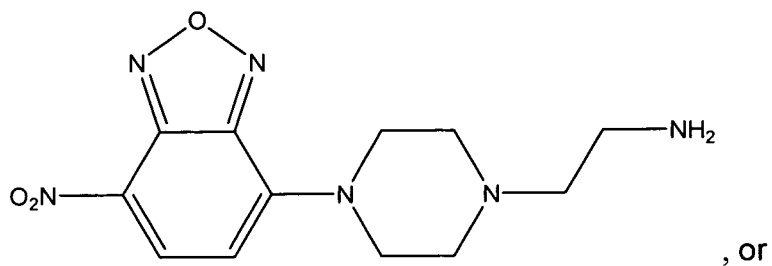
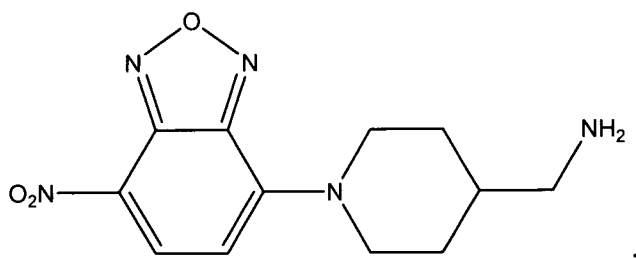
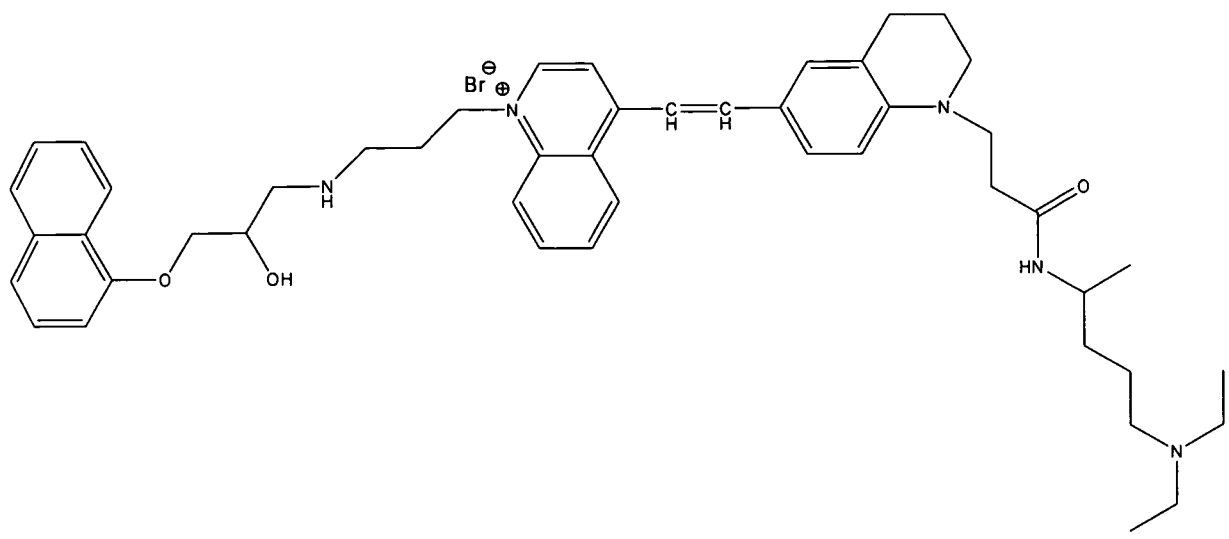
48. The method of claim 36, wherein said cationic amphiphilic tracer compound has the structure:





49. The method of claim 36, wherein said cationic amphiphilic tracer compound has the structure

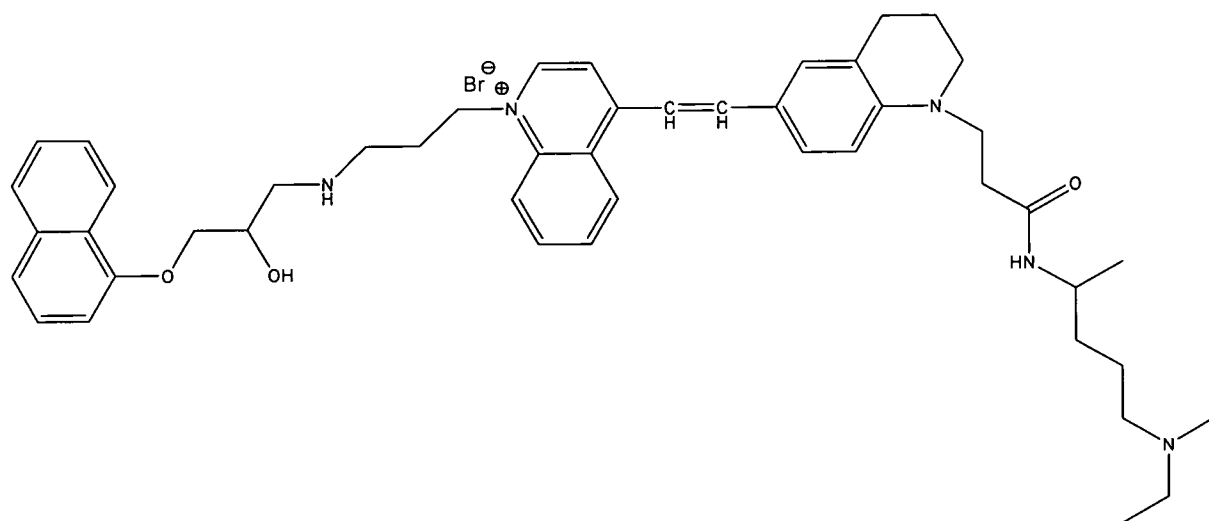
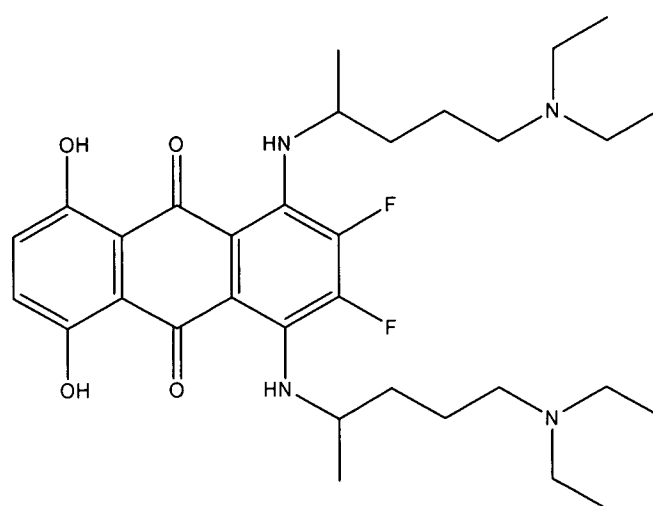
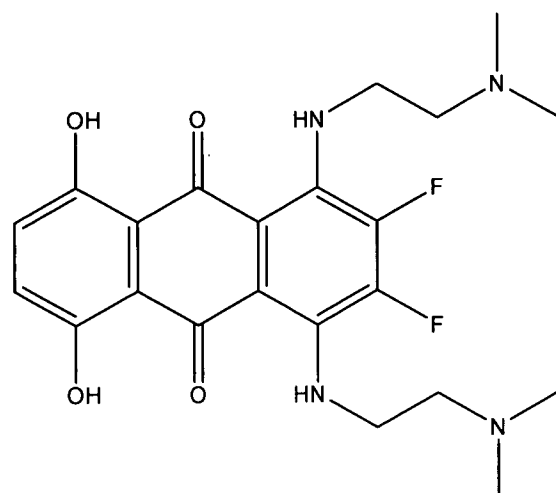


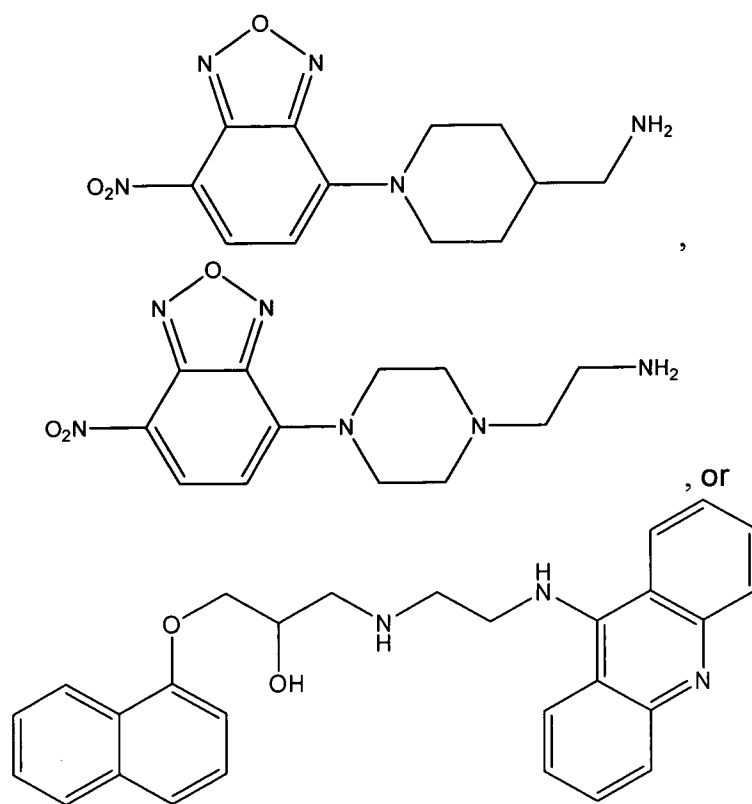


50. The method of claim 36, wherein the difference ($T_1 - T_2$) comprises time units measured in hours, days, weeks, months or years.

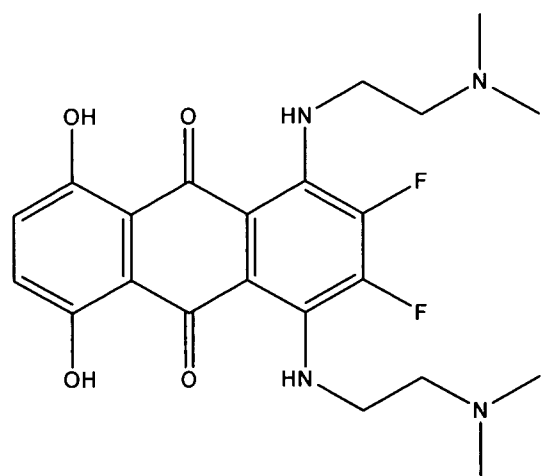
51. The method of claim 36, wherein said cells comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing.
52. The method of claim 36, wherein said subject is a mammal.
53. The method of claim 52, wherein said mammal is human.
54. A method of screening drug candidates to determine their toxicity to cells, tissues or organs, said method comprising the steps of:
- (a) providing:
 - (i) at least one first sample and one second sample of mammalian cells;
 - (ii) a drug candidate for screening;
 - (iii) a drug diluting vehicle or carrier; and
 - (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
 - (b) administering:
 - (i) said drug candidate (ii) to said first sample for a time sufficient to allow the drug candidate to partition into subcellular compartments within said cells to form treated cells; and
 - (ii) said vehicle or carrier (iii) to said second sample to form untreated reference cells;
 - (c) contacting said treated cells and said untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and
 - (d) detecting any increase in vacuole accumulation of said cationic amphiphilic tracer compound in the treated cells relative to said untreated reference cells, thereby determining whether said drug candidate has accumulated in the vacuoles and is toxic to cells, tissues or organs.

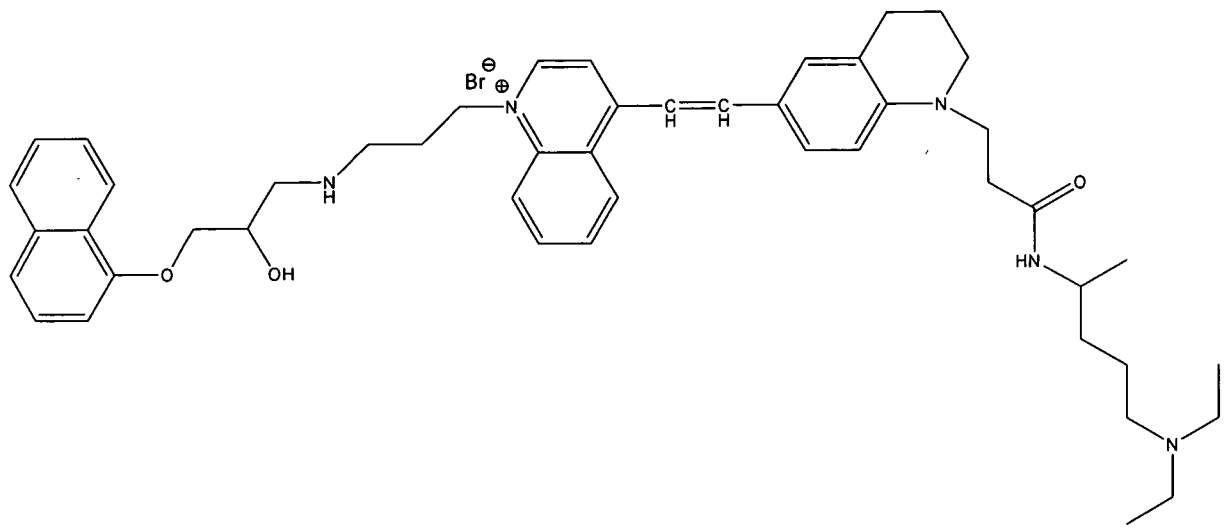
55. The method of claim 54, wherein said vehicle or carrier comprises cell culture medium, buffer, organic solvent or water, and combinations of the foregoing.
56. The method of claim 55, wherein said organic solvent comprises dimethylsulfoxide (DMSO).
57. The method of claim 54, wherein said vacuole comprises a lysosome, a phagophore, an autophagosomes, an autophagolysosome, or any combination thereof.
58. The method of claim 54, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.
59. The method of claim 54, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.
60. The method of claim 54, wherein said cationic amphiphilic tracer compound comprises nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, 9-amino-acridin-propanolol, or any combination thereof.
61. The method of claim 54, wherein said cationic amphiphilic tracer compound has the structure:



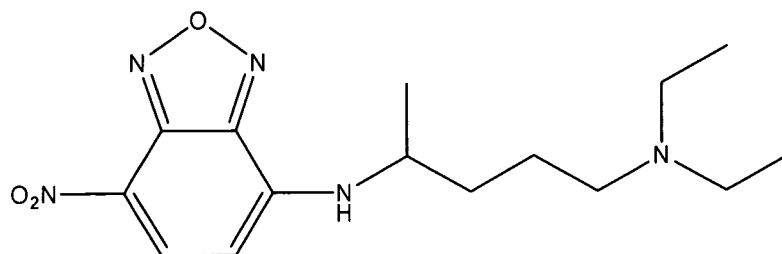


62. The method of claim 54, wherein said cationic amphiphilic tracer compound has the structure





or



63. The method of claim 54, wherein said mammalian cells are from a cell line.
64. The method of claim 63, wherein said cell line is a human cell line.
65. The method of claim 54, wherein said mammalian cells are from a subject.
66. The method of claim 65, wherein said subject is a human.
67. The method of claim 54, wherein said cells comprise lymphocytes, granulocytes, macrophages or monocytes, and a combination of any of the foregoing.
68. A method of screening suspected toxic agents to determine their toxicity to cells, tissues or organs, said method comprising the steps of:

- (a) providing:
 - (i) at least one first sample and one second sample of mammalian cells;
 - (ii) a suspected toxic agent for screening;
 - (iii) a diluting vehicle or carrier; and
 - (iv) a cationic amphiphilic tracer compound that localizes to a vacuole in a cell;
- (b) administering:
 - (i) said suspected toxic agent (ii) to said first sample for a time sufficient to allow said suspected toxic agent to partition into subcellular compartments within said cells to form treated cells; and
 - (ii) said diluting vehicle or carrier (iii) to said second sample to form untreated reference cells;
- (c) contacting said treated cells and said untreated reference cells with the cationic amphiphilic tracer (iv) for a period of time sufficient to accumulate in vacuoles in the cells, and
- (d) detecting any increase in vacuole accumulation of said cationic amphiphilic tracer compound in the treated cells relative to said untreated reference cells, thereby determining whether said suspected toxic agent has accumulated in the vacuoles and is toxic to cells, tissues or organs.

69. The method of claim 68, wherein said vehicle or carrier comprises cell culture medium, buffer, organic solvent or water, and combinations of the foregoing.

70. The method of claim 69, wherein said organic solvent comprises dimethylsulfoxide (DMSO).

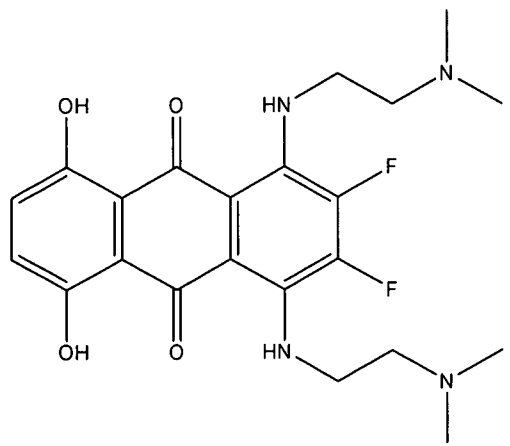
71. The method of claim 68, wherein said vacuole comprises a lysosome, a phagophore, an autophagosomes, an autophagolysosome, or any combination thereof.

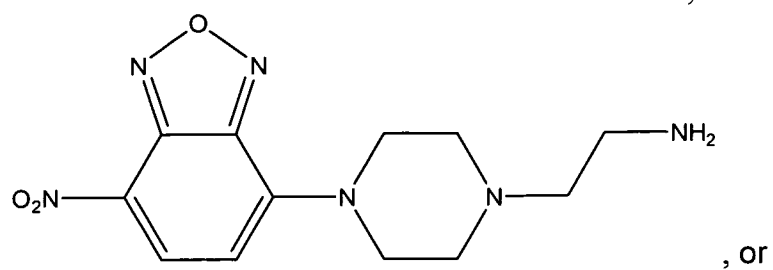
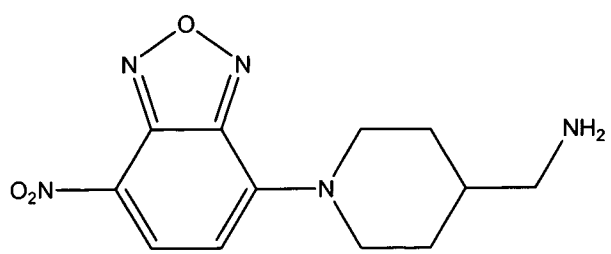
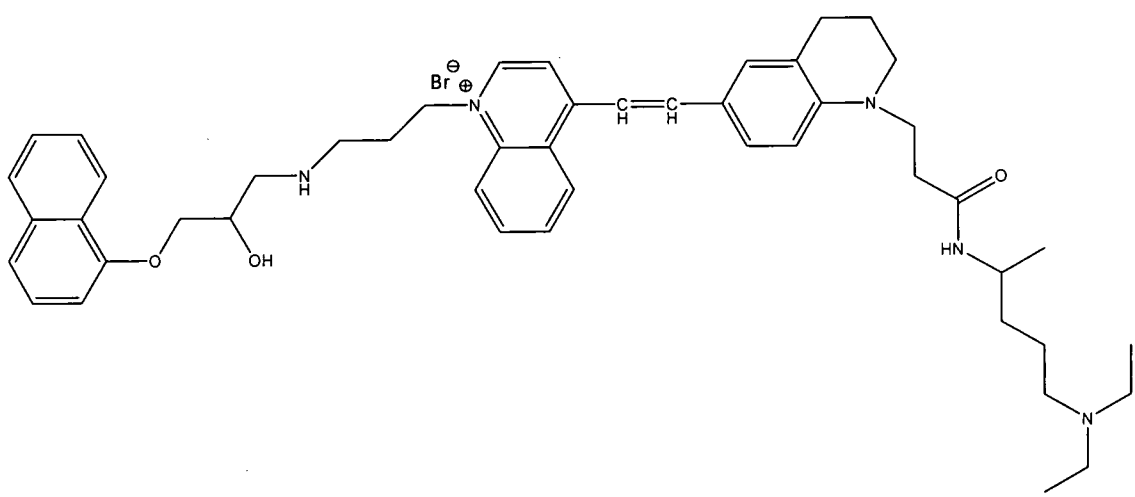
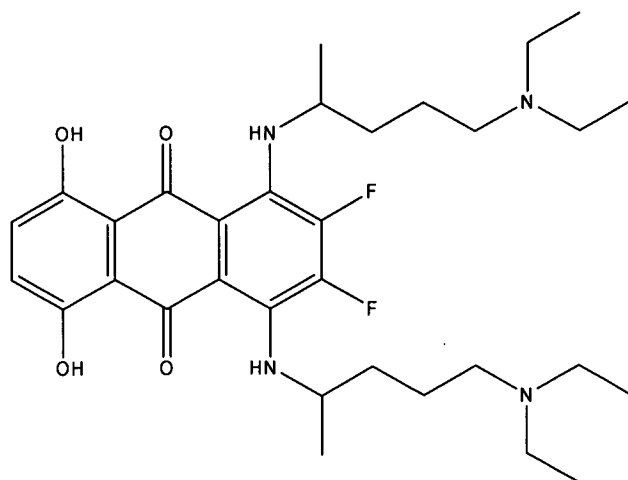
72. The method of claim 68, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.

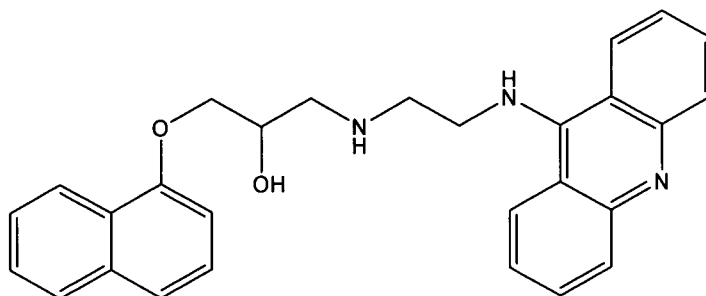
73. The method of claim 68, wherein said first cationic amphiphilic tracer compound and said second cationic amphiphilic tracer compound comprise a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.

74. The method of claim 68, wherein said cationic amphiphilic tracer compound comprises nitrobenzoxadiazole (NBD)-spiperone, nitrobenzoxadiazole (NBD)-propranolol, 9-amino-acridin-propanolol, or any combination thereof.

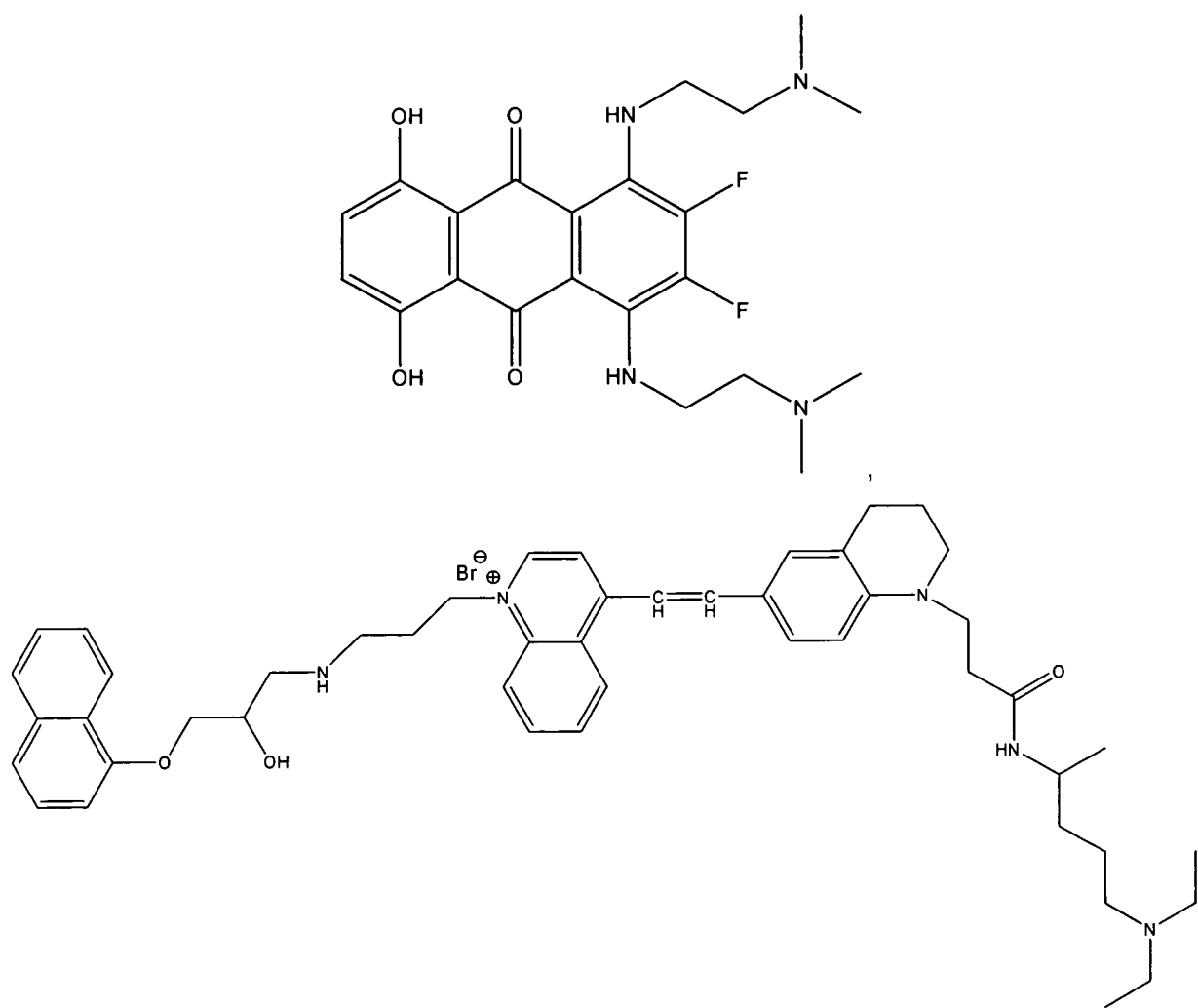
75. The method of claim 68, wherein said cationic amphiphilic tracer compound has the structure:

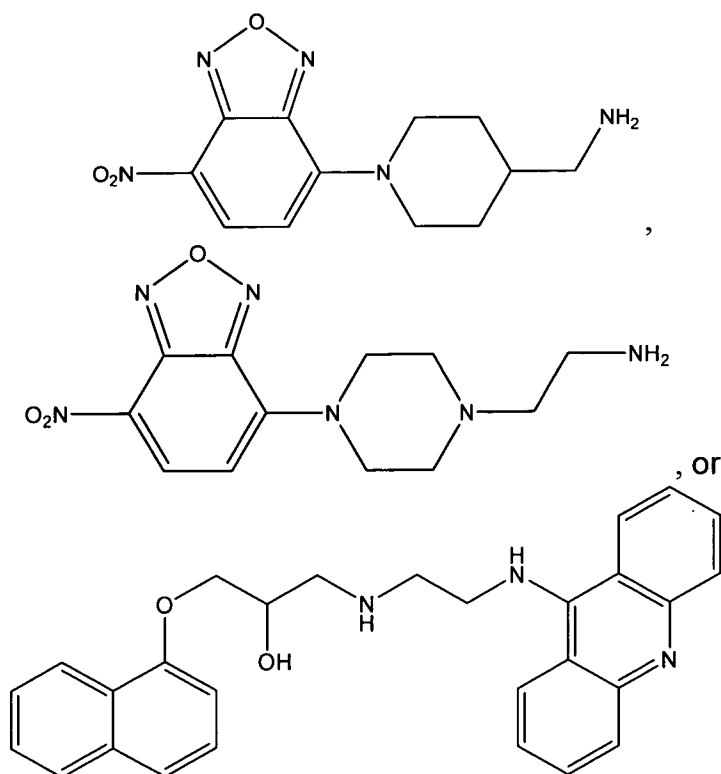






76. The method of claim 68, wherein said cationic amphiphilic tracer compound has the structure





77. The method of claim 68, wherein said mammalian cells are from a cell line.
78. The method of claim 77, wherein said cell line is a human cell line.
79. The method of claim 68, wherein said mammalian cells are from a subject.
80. The method of claim 79, wherein said subject is a human.
81. The method of claim 68, wherein said cells comprise lymphocytes, granulocytes, macrophages, monocytes, or any combination thereof.
82. A method of distinguishing between phospholipidosis activators and autophagy pathway perturbation agents, comprising the steps of:
 - (a) providing:

- (i) a fluorescent phospholipid analog compound that forms multilamellar vesicles;
 - (ii) a cationic amphiphilic fluorophore tracer compound that localizes in a vacuole in a cell;
 - (iii) a test agent; and
 - (iv) a sample of mammalian cells;
- (b) administering to the cells in said sample (iv) the fluorescent phospholipid analog compound (i) and the test agent (iii) to form a testing mixture;
- (c) contacting said testing mixture with the cationic amphiphilic fluorophore tracer (ii); and
- (d) detecting any signals generated from said fluorescent phospholipid analog compound (i) and said cationic amphiphilic fluorophore tracer compound (ii), thereby differentiating between phospholipidosis activators and autophagy pathway perturbation agents based upon differential accumulation patterns of the fluorescent phospholipid analog and the cationic amphiphilic fluorophore tracer.

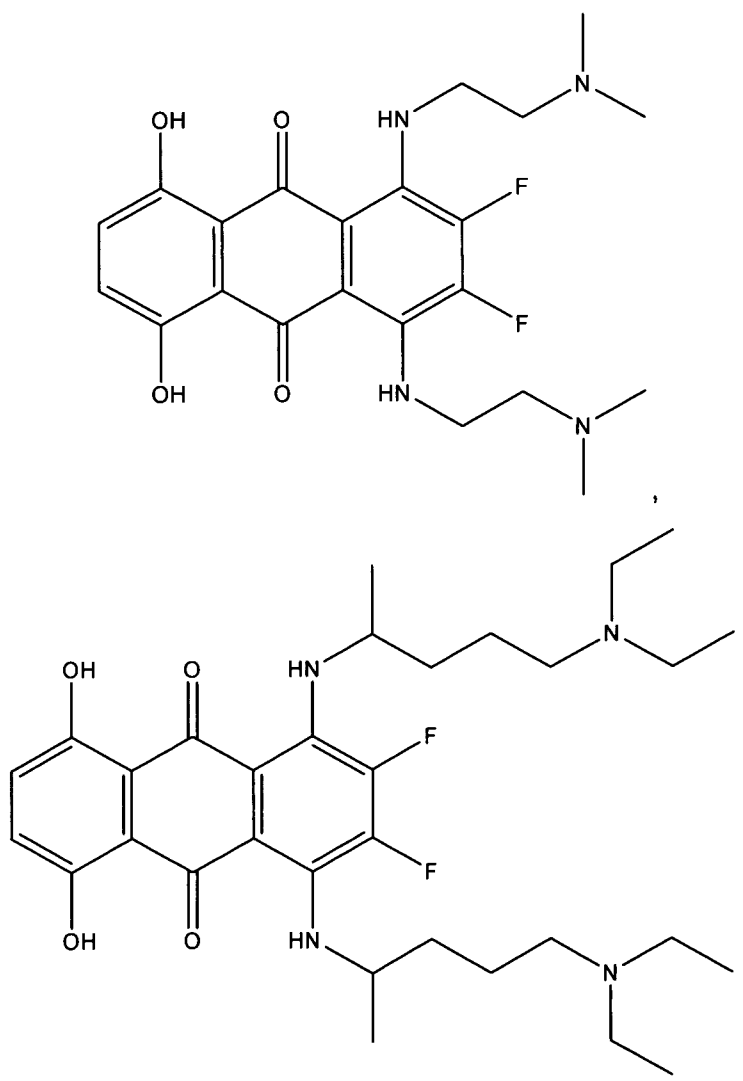
83. The method of claim 82, wherein said fluorescent phospholipid analog compound comprises NBD-PE (N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)-1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine), NBD-PC (1-acyl-2-(12[(7-nitro-2-1-3-benzoxadiazol-4yl)amino] dodecanoyl) phosphatidylcholine), CF-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein)) Lipid-Tox reagent, or any combination thereof.

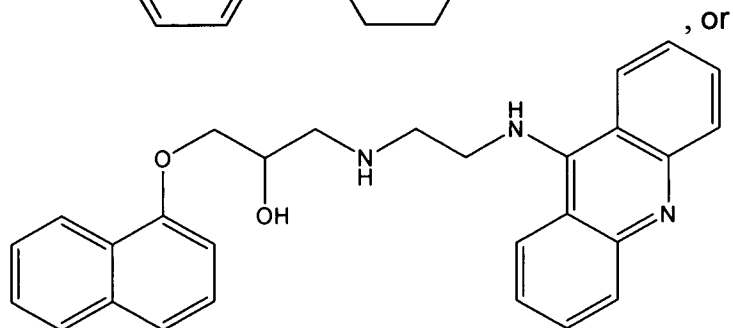
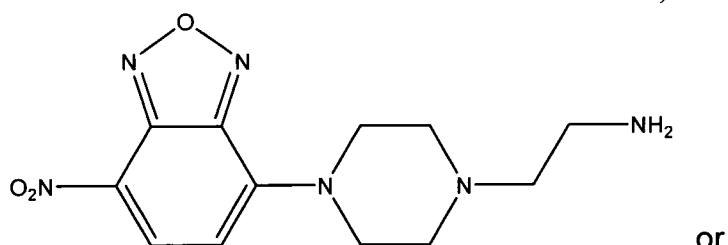
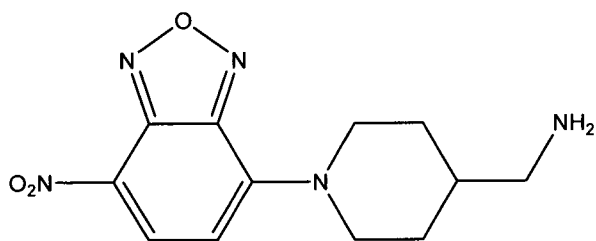
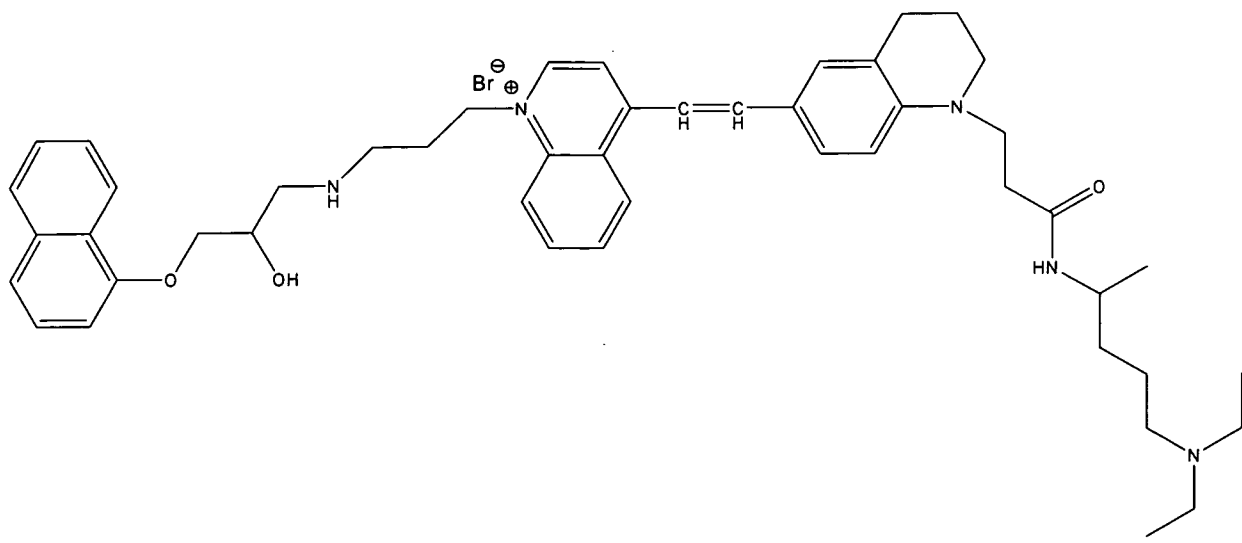
84. The method of claim 82, wherein said vacuole comprises a lysosome, a phagophore, an autophagosomes, an autophagolysosome, or any combination thereof.

85. The method of claim 82, wherein said cationic amphiphilic tracer compound comprises a fluorescent drug that induces phospholipidosis or a fluorescently labeled analog of a drug that induces phospholipidosis.

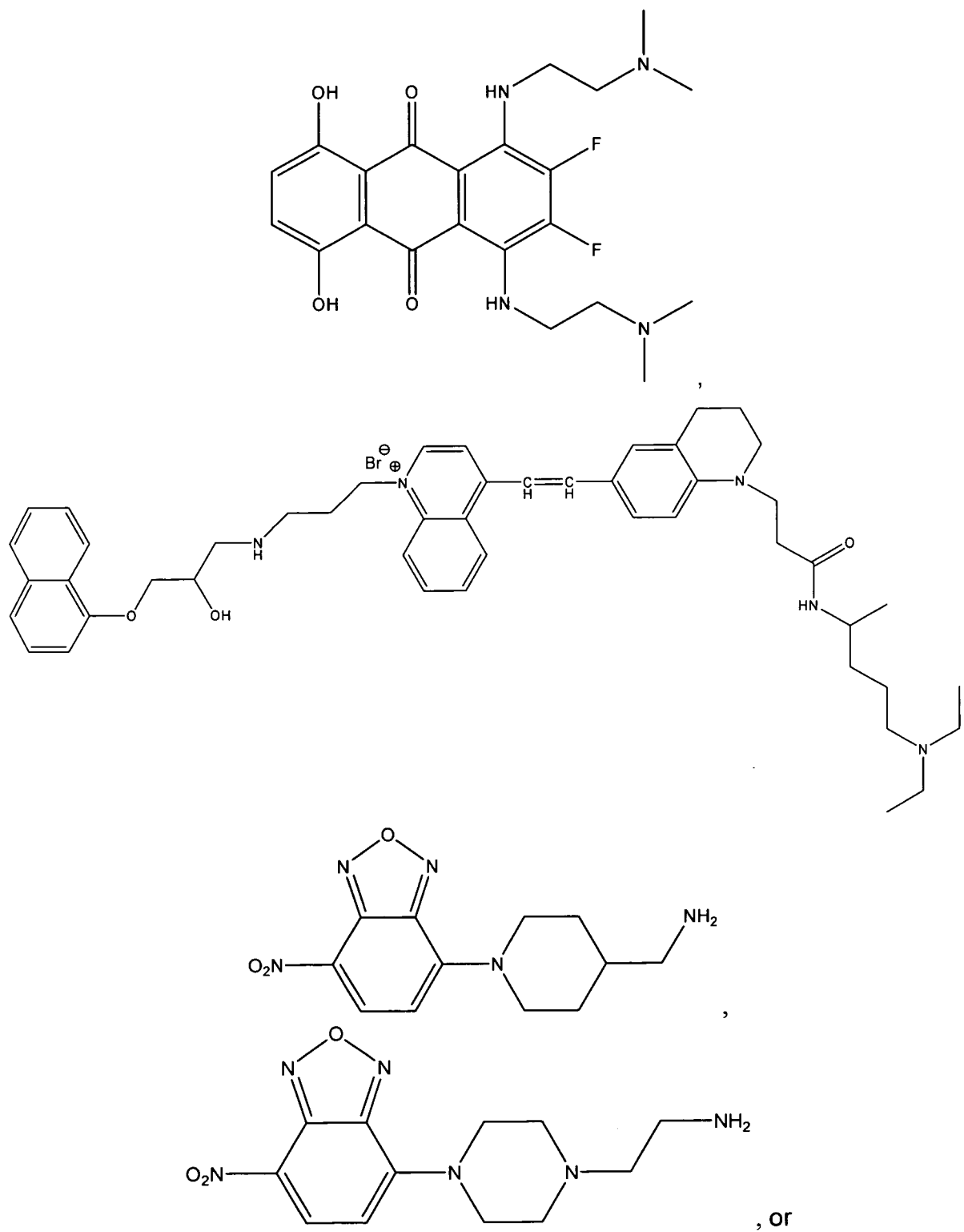
86. The method of claim 82, wherein said cationic amphiphilic tracer compound comprises a halogenated and fluorescent drug that induces phospholipidosis or a halogenated and fluorescently labeled analog of a drug that induces phospholipidosis.

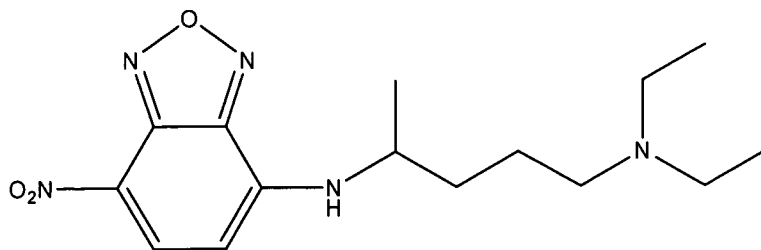
87. The method of claim 82, wherein said cationic amphiphilic tracer compound has the structure:



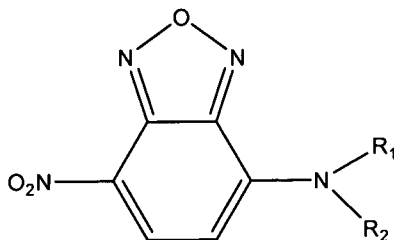


88. The method of claim 82, wherein said cationic amphiphilic tracer compound has the structure





89. The method of claim 82, wherein the test agent is a drug candidate.
90. The method of claim 82, wherein the test agent is an environmental contaminant.
91. The method of claim 82, wherein said cells comprise lymphocytes, granulocytes, macrophages, monocytes, or any combination thereof.
92. The method of claim 82, wherein said mammalian cells are from a cell line.
93. The method of claim 92, wherein said cell line is a human cell line.
94. The method of claim 82, wherein said mammalian cells are from a subject.
95. The method of claim 94, wherein said subject is a human.
96. The method of claim 82, wherein phospholipidosis is identified as increased accumulation of both the fluorescent phospholipid analog compound and the cationic amphiphilic fluorophore tracer compound.
97. The method of claim 82, wherein the autophagy pathway perturbation agent is identified by no increased accumulation of the fluorescent phospholipid analog compound, but increased accumulation of the cationic amphiphilic fluorophore tracer compound.
98. A compound having the structure:



wherein R_1 and R_2 are independently H, OH, or comprise

an alkyl group wherein said alkyl group is saturated or unsaturated, linear or branched, substituted or unsubstituted,

a phenyl group wherein said phenyl group is substituted or unsubstituted, or

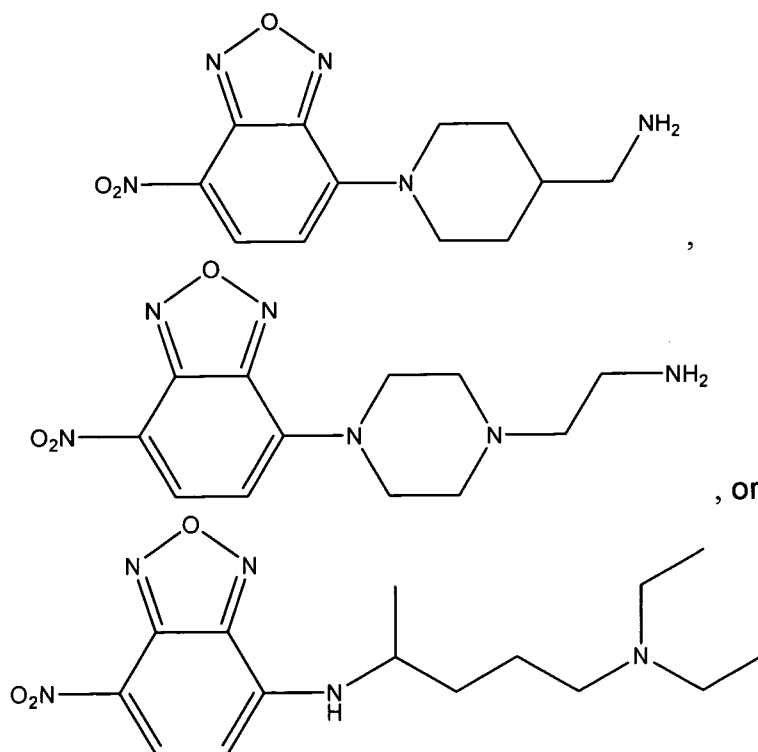
when taken together, R_1 and R_2 may form a 5 or 6 membered ring comprising carbon, sulfur, oxygen or nitrogen, or any combinations thereof; wherein the 5 or 6 membered ring is saturated or unsaturated, wherein the ring may be further substituted with a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof.

99. The compound of claim 98, wherein at least one of R_1 or R_2 comprises Q wherein Q comprises a carboxyl group (CO_2^-), a carbonate ester (COER_{13}), a sulfonate (SO_3^-), a sulfonate ester ($\text{SO}_2\text{ER}_{13}$), a sulfoxide (SOR_{13}), a sulfone ($\text{SO}_2\text{CR}_{13}\text{R}_{14}\text{R}_{15}$), a sulfonamide ($\text{SO}_2\text{NR}_{13}\text{R}_{14}$), a phosphate (PO_4^-), a phosphate monoester ($\text{PO}_3^-\text{ER}_{13}$), a phosphate diester ($\text{PO}_2\text{ER}_{13}\text{ER}_{14}$), a phosphonate (PO_3^-), a phosphonate monoester ($\text{PO}_2^-\text{ER}_{13}$), a phosphonate diester ($\text{POER}_{13}\text{ER}_{14}$), a thiophosphate (PSO_3^-), a thiophosphate monoester ($\text{PSO}_2^-\text{ER}_{13}$), a thiophosphate diester ($\text{PSOER}_{13}\text{ER}_{14}$), a thiophosphonate (PSO_2^-), a thiophosphonate monoester ($\text{PSO}^-\text{ER}_{13}$), a thiophosphonate diester ($\text{PSER}_{13}\text{ER}_{14}$), a phosphoramidate ($\text{PONR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), its thioanalogue ($\text{PSNR}_{13}\text{R}_{14}\text{NR}_{19}\text{R}_{20}$), a phosphoramidite ($\text{PO}_2\text{R}_{19}\text{NR}_{13}\text{R}_{14}$) or its thioanalogue ($\text{POSR}_{19}\text{NR}_{13}\text{R}_{14}$) where E can be independently O or S, wherein Q can further comprise a nucleophilic reactive group, an electrophilic reactive group, a terminal alkene, a terminal alkyne, a coordinate group or an alkylating agent;

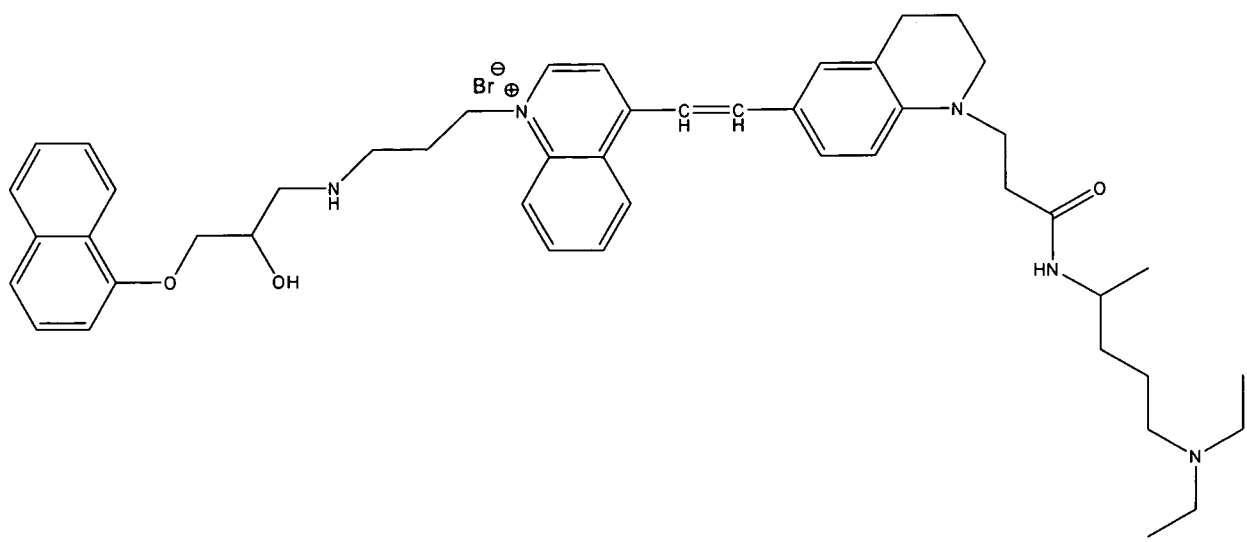
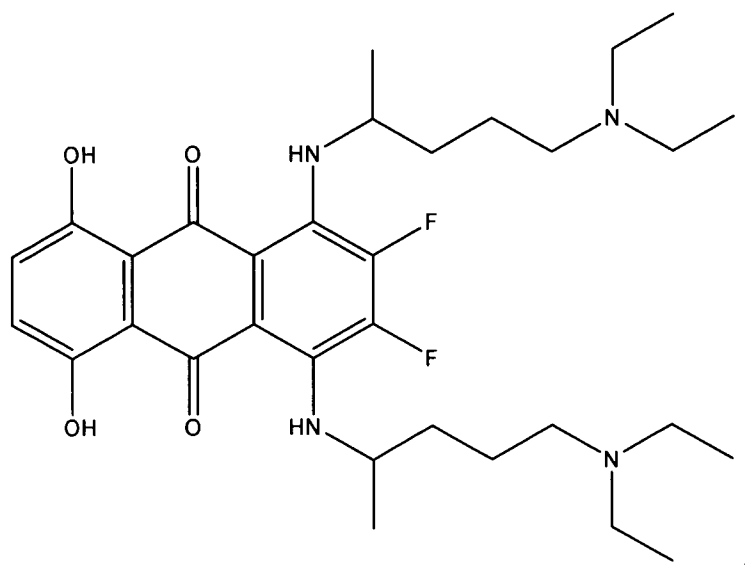
wherein Q is attached directly, or indirectly through a linker arm comprising carbon, sulfur, oxygen, nitrogen, or any combinations thereof and wherein said linker arm may be saturated or unsaturated, linear or branched, substituted or unsubstituted or any combinations thereof.

100. The compound of claim 98, wherein R_1 and R_2 are independently H, or a saturated alkyl group that may be substituted at any one or multiple positions with an N, where R_1 and R_2 may form a 6 membered ring, provided that, when one of R_1 and R_2 is H, the other of R_1 and R_2 is not H.

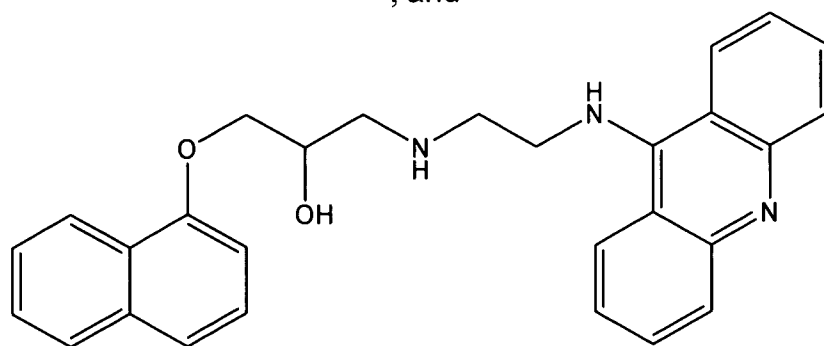
101. The compound of claim 98, consisting of



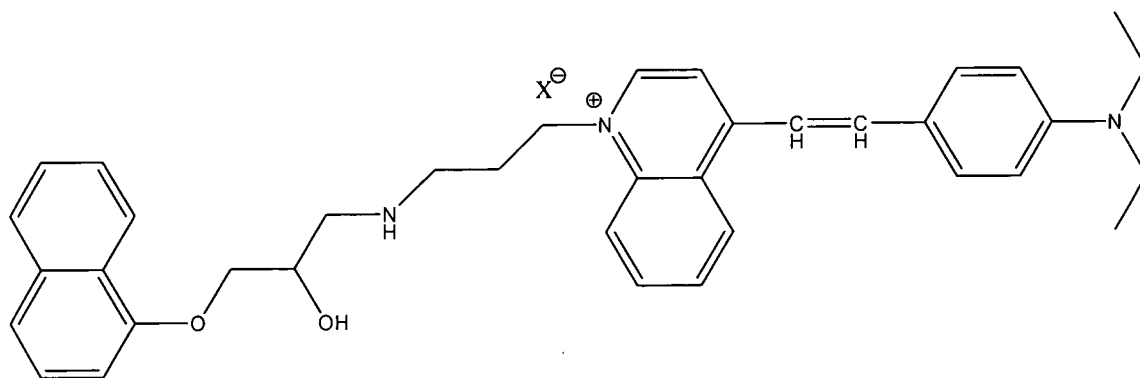
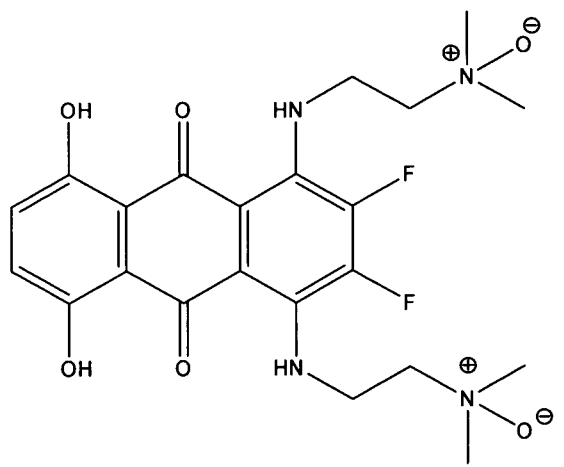
102. A compound selected from the group consisting of



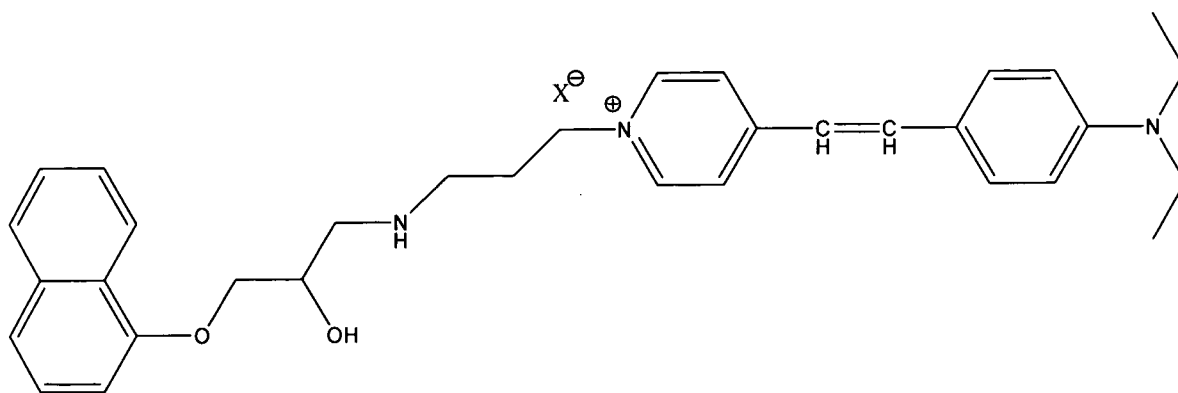
, and



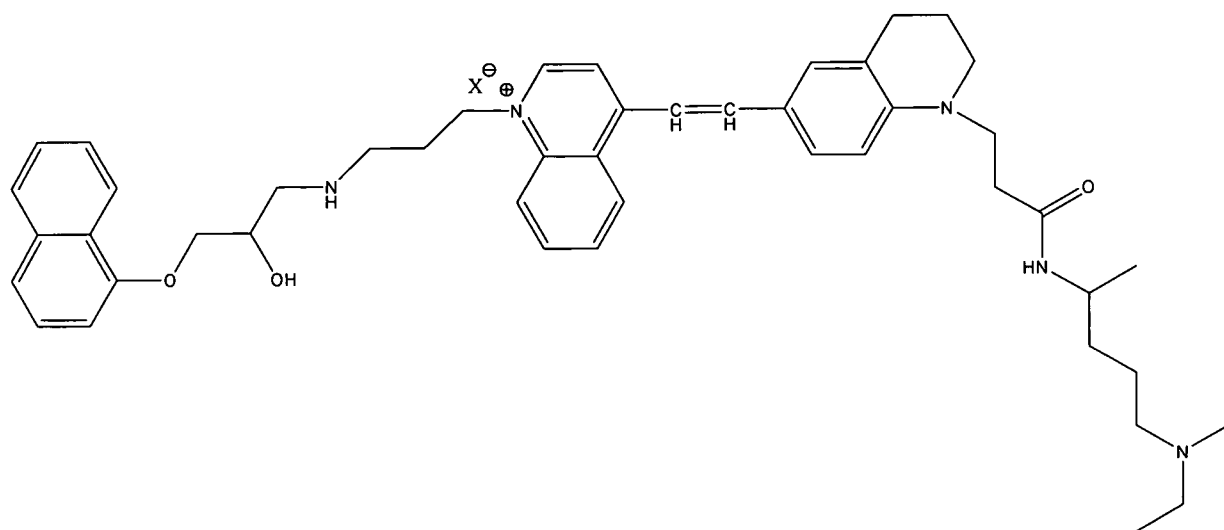
103. A compound selected from the group consisting of



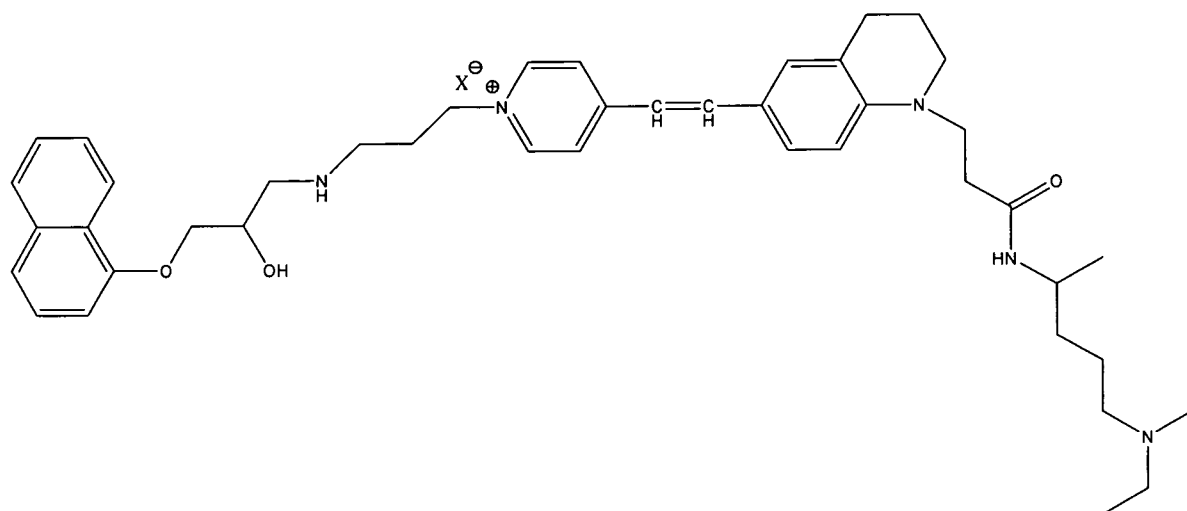
where X comprises an anion,



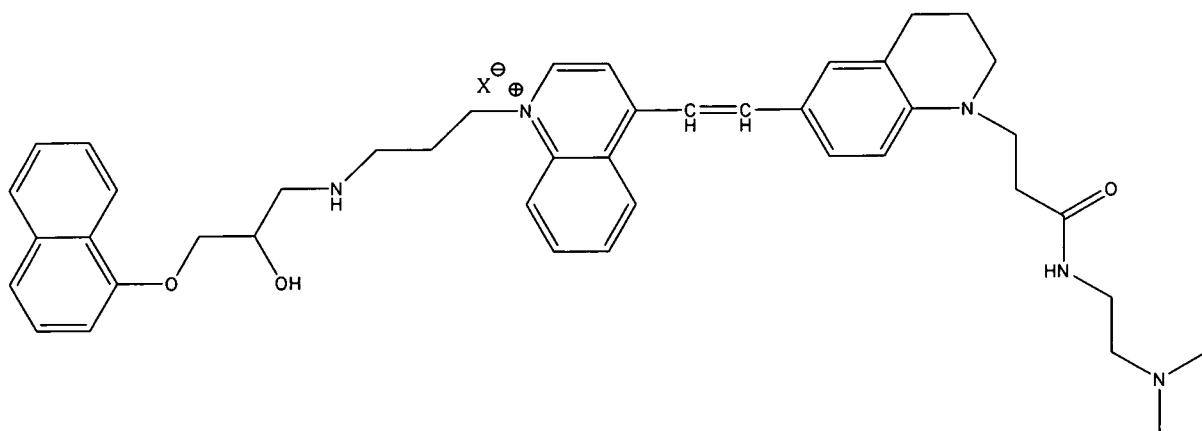
where X comprises an anion,



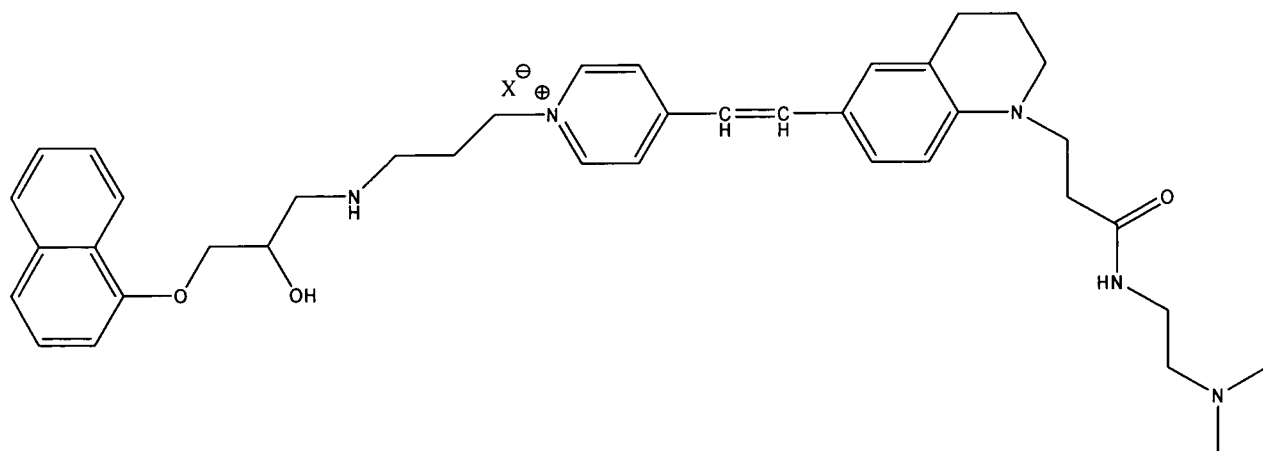
where X comprises an anion,



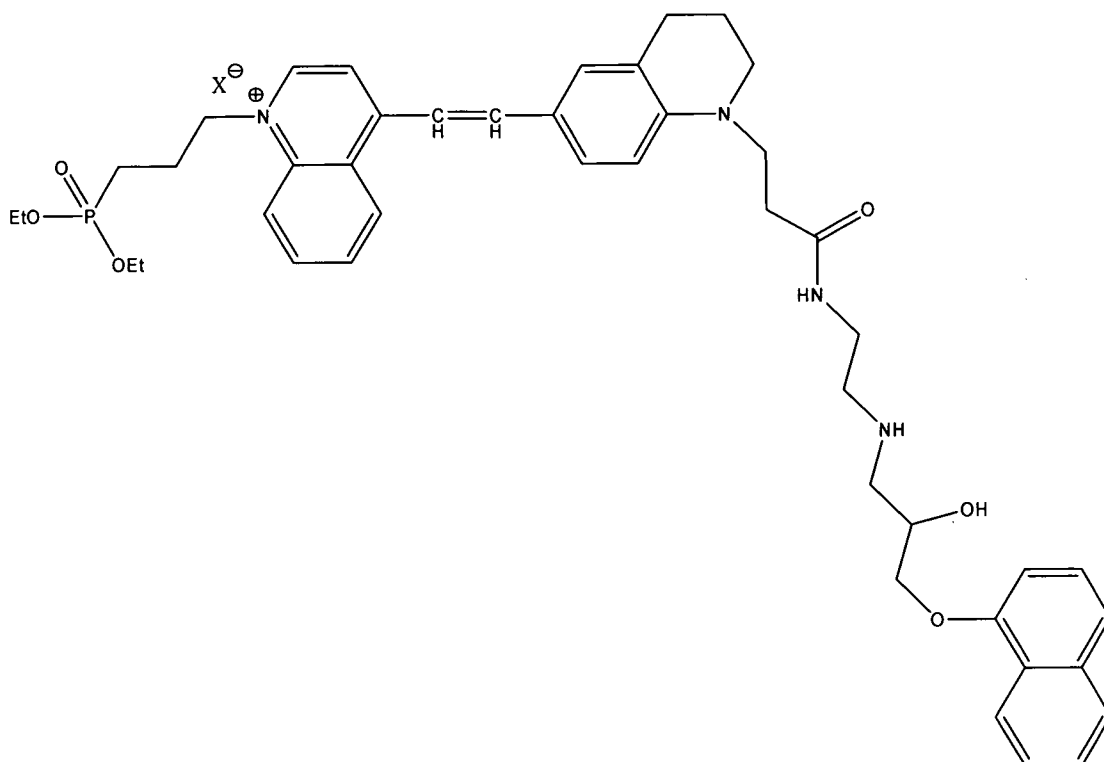
where X comprises an anion,



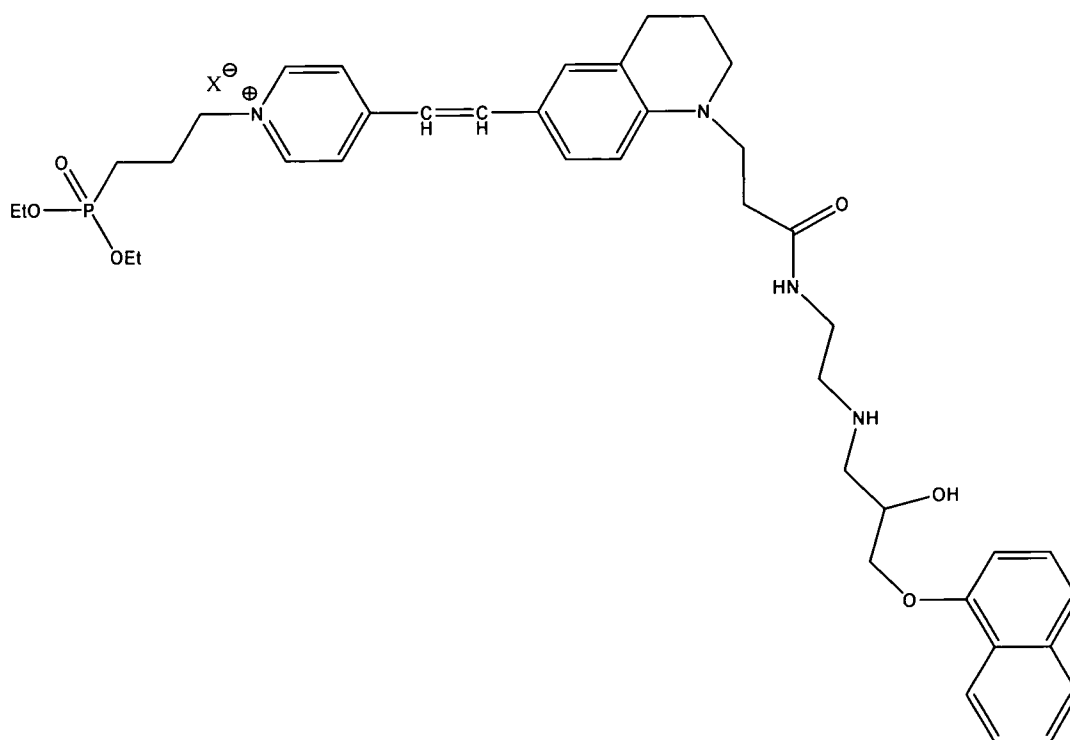
where X comprises an anion,



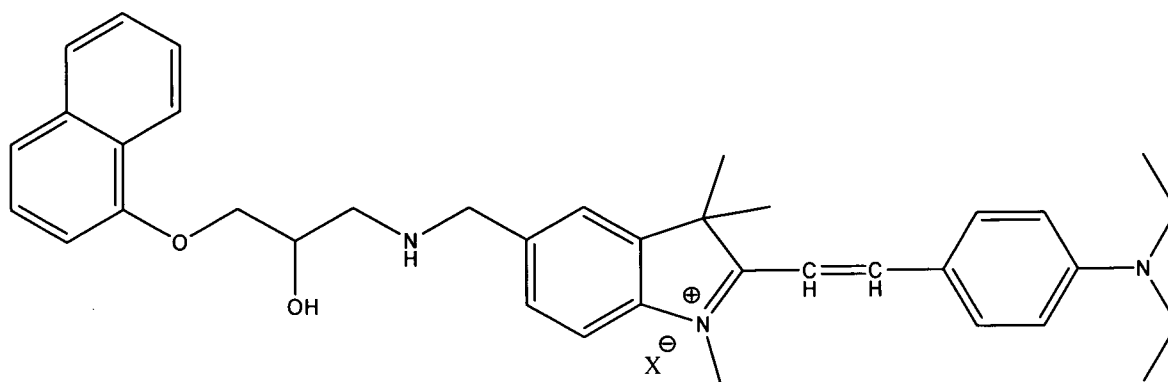
where X comprises an anion,



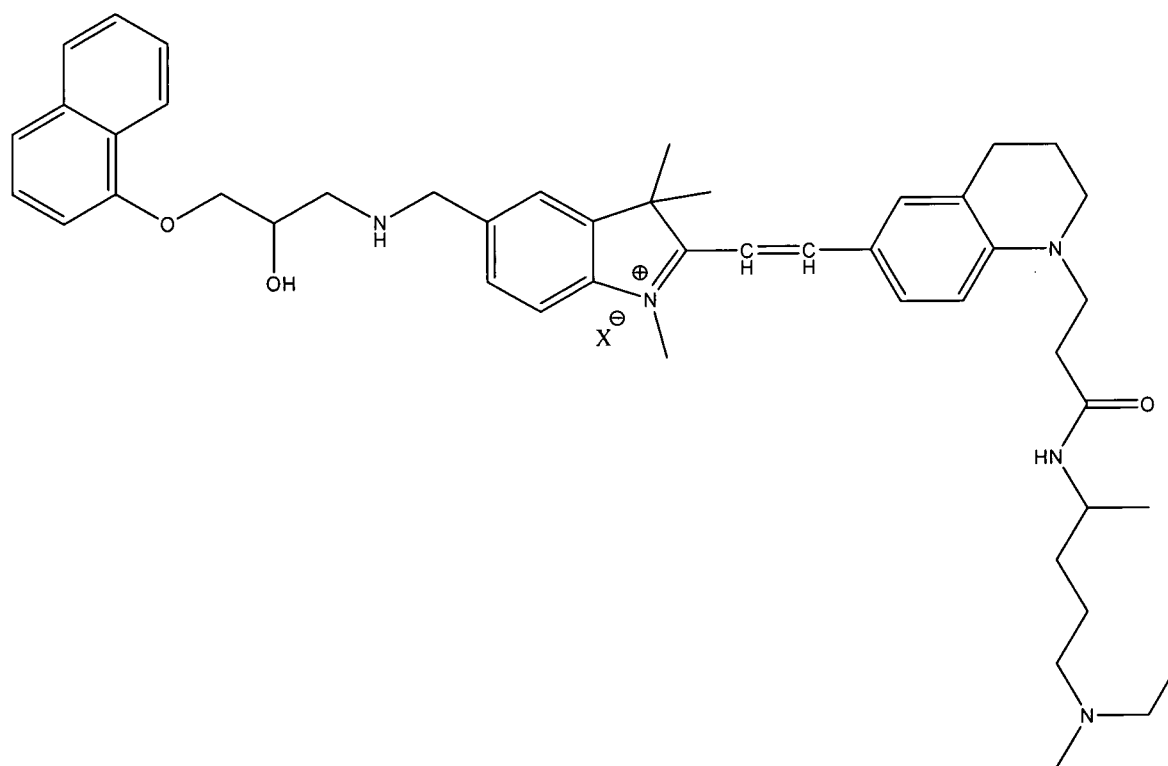
where X comprises an anion,



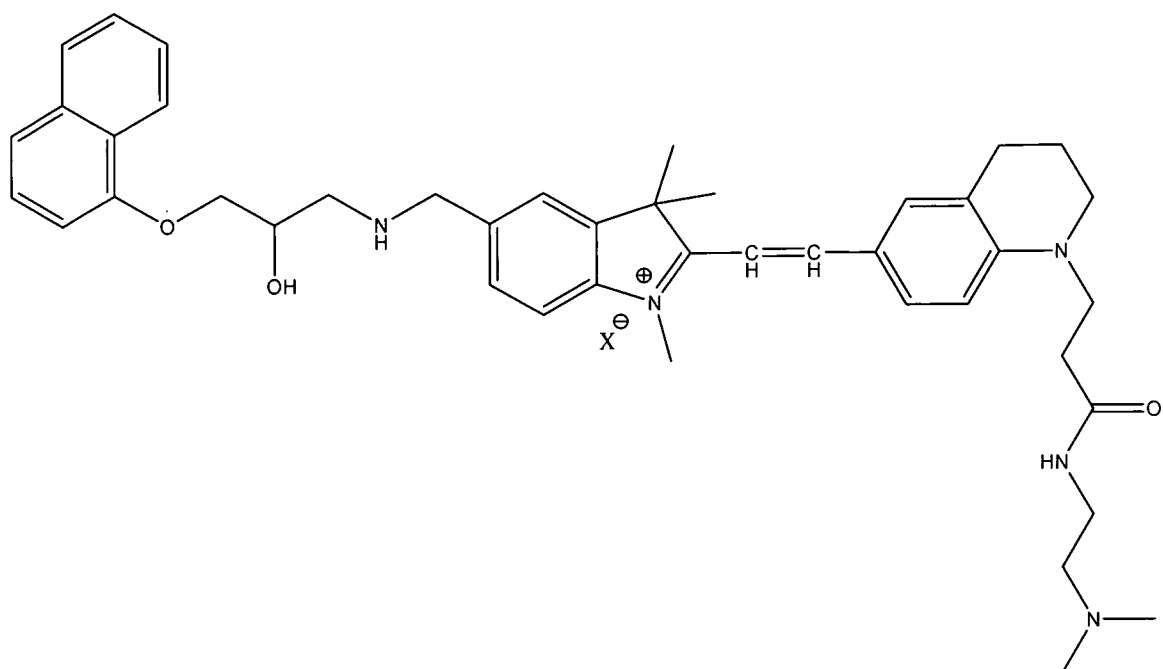
where X comprises an anion,



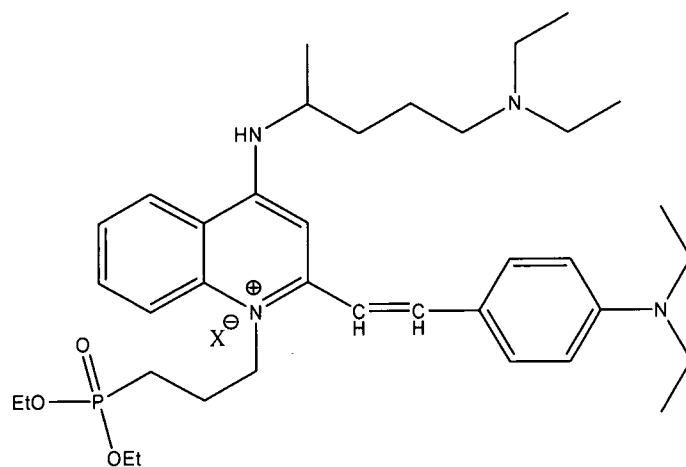
where X comprises an anion,



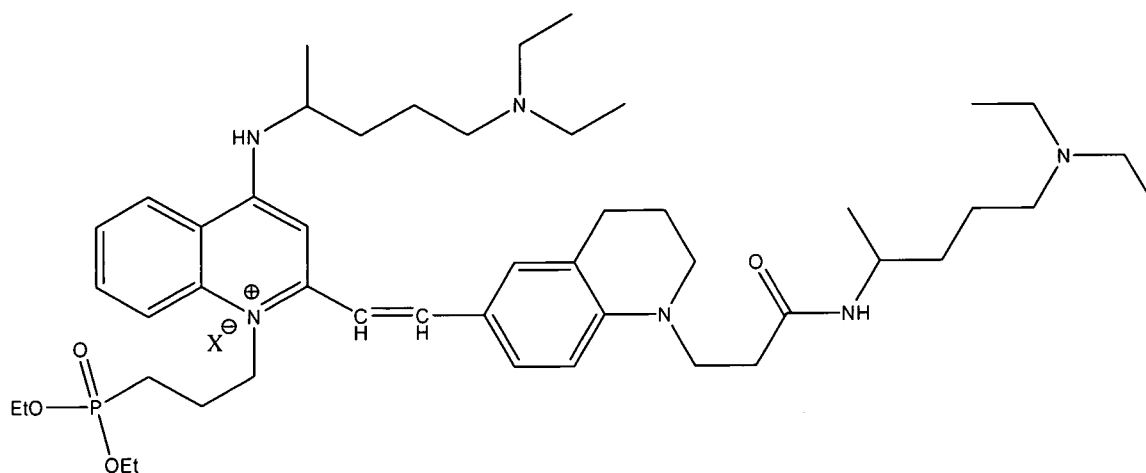
where X comprises an anion,



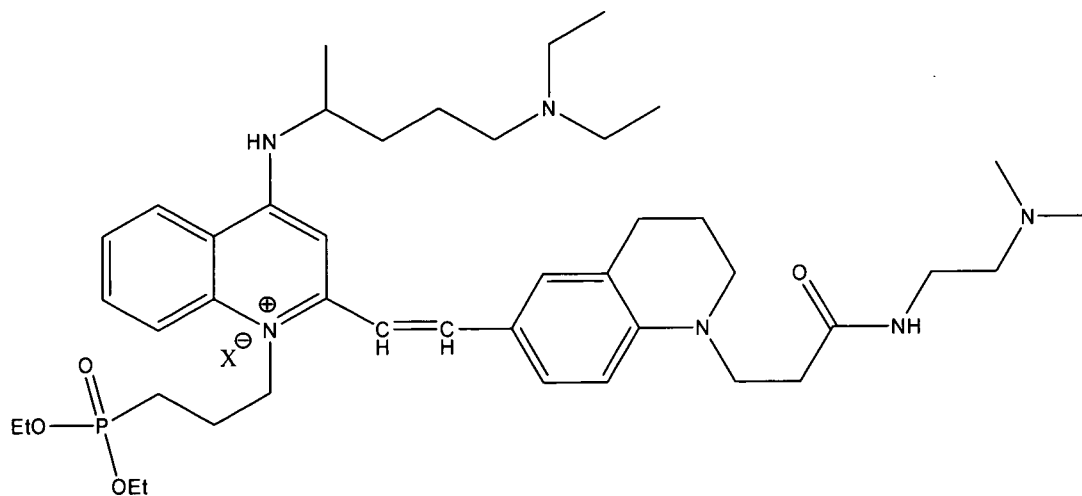
where X comprises an anion,



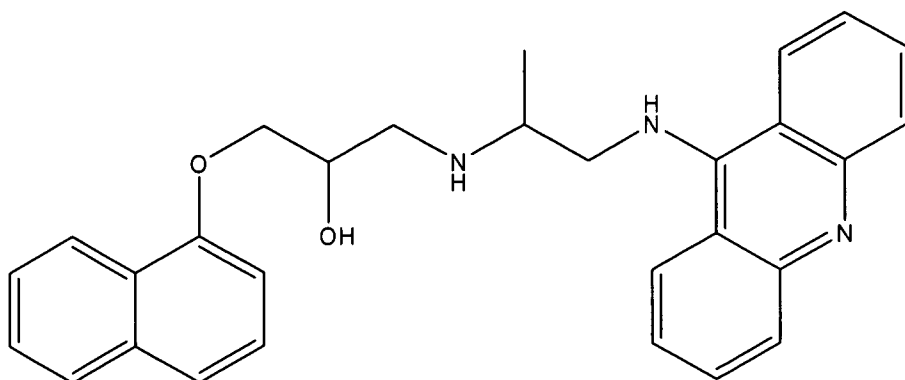
where X comprises an anion,



where X comprises an anion,



wherein X comprises an anion, and



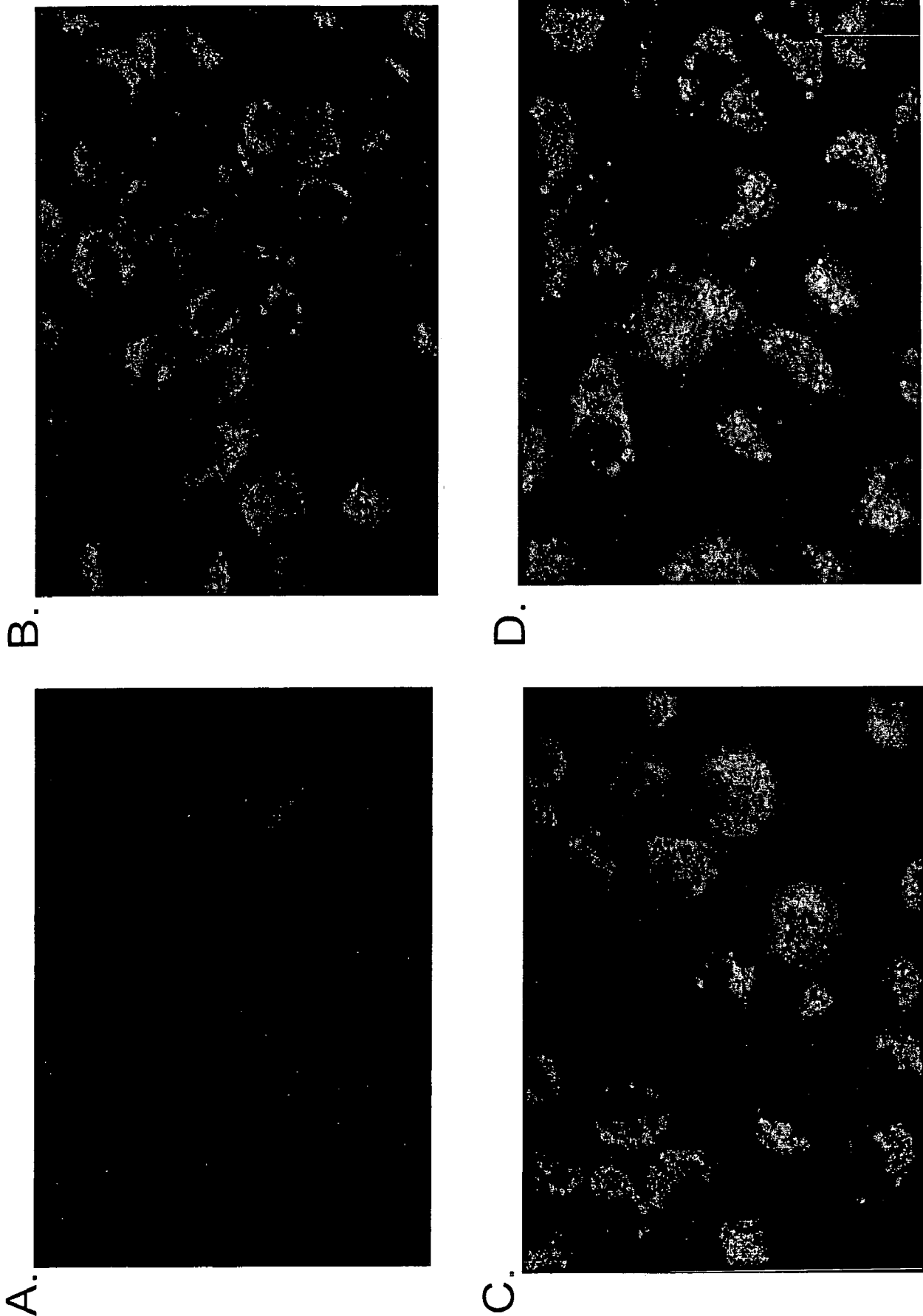


FIG. 1

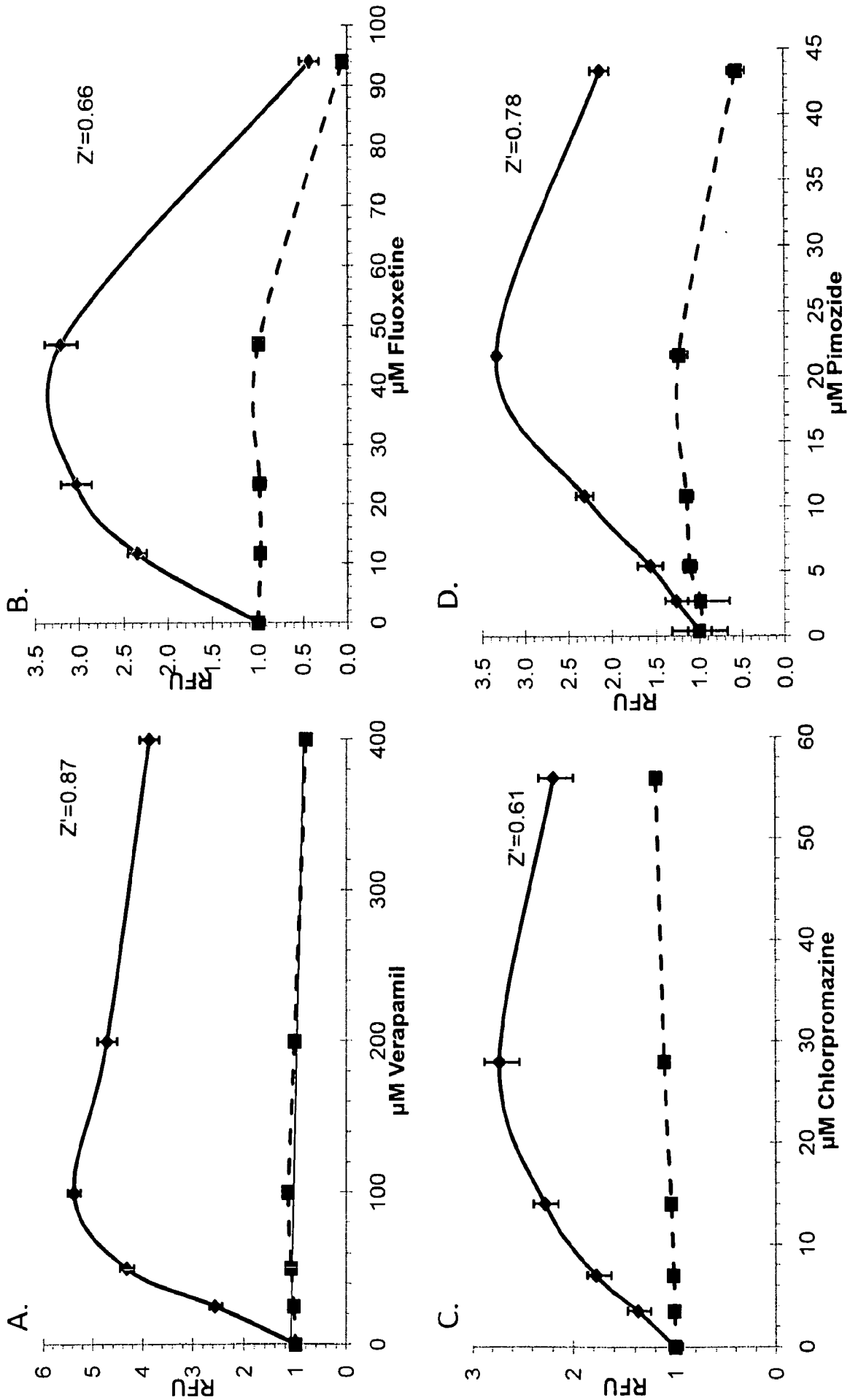
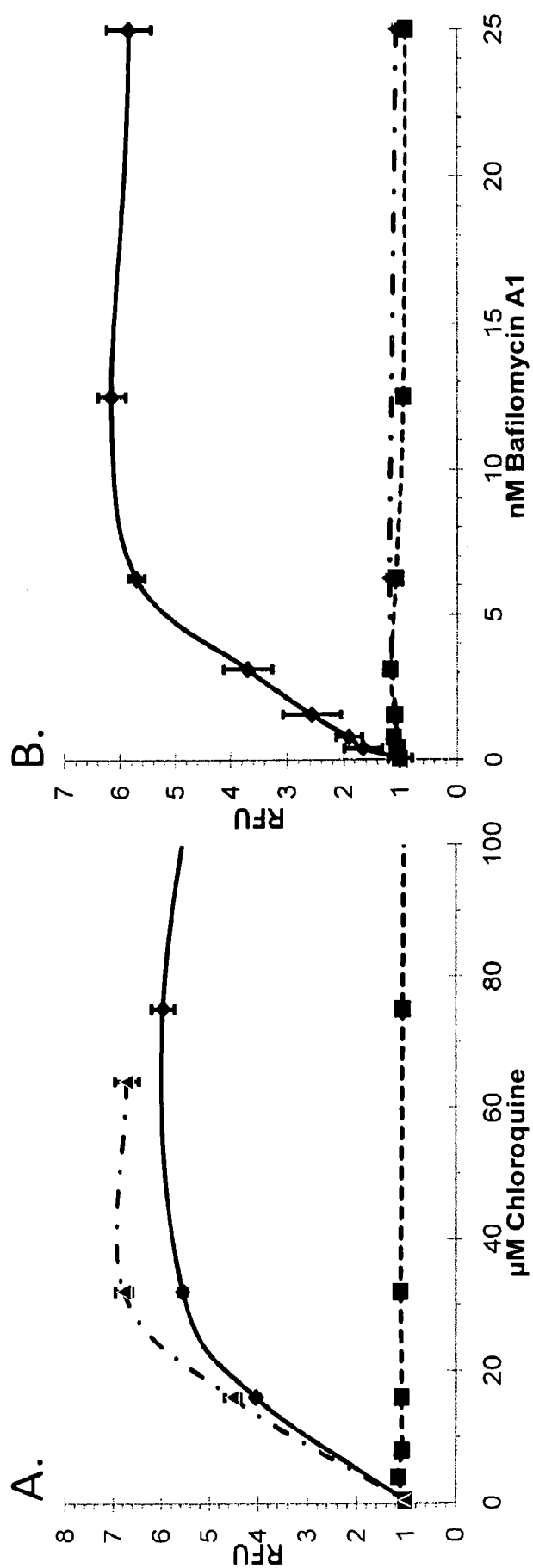


FIG. 2

**FIG. 3**

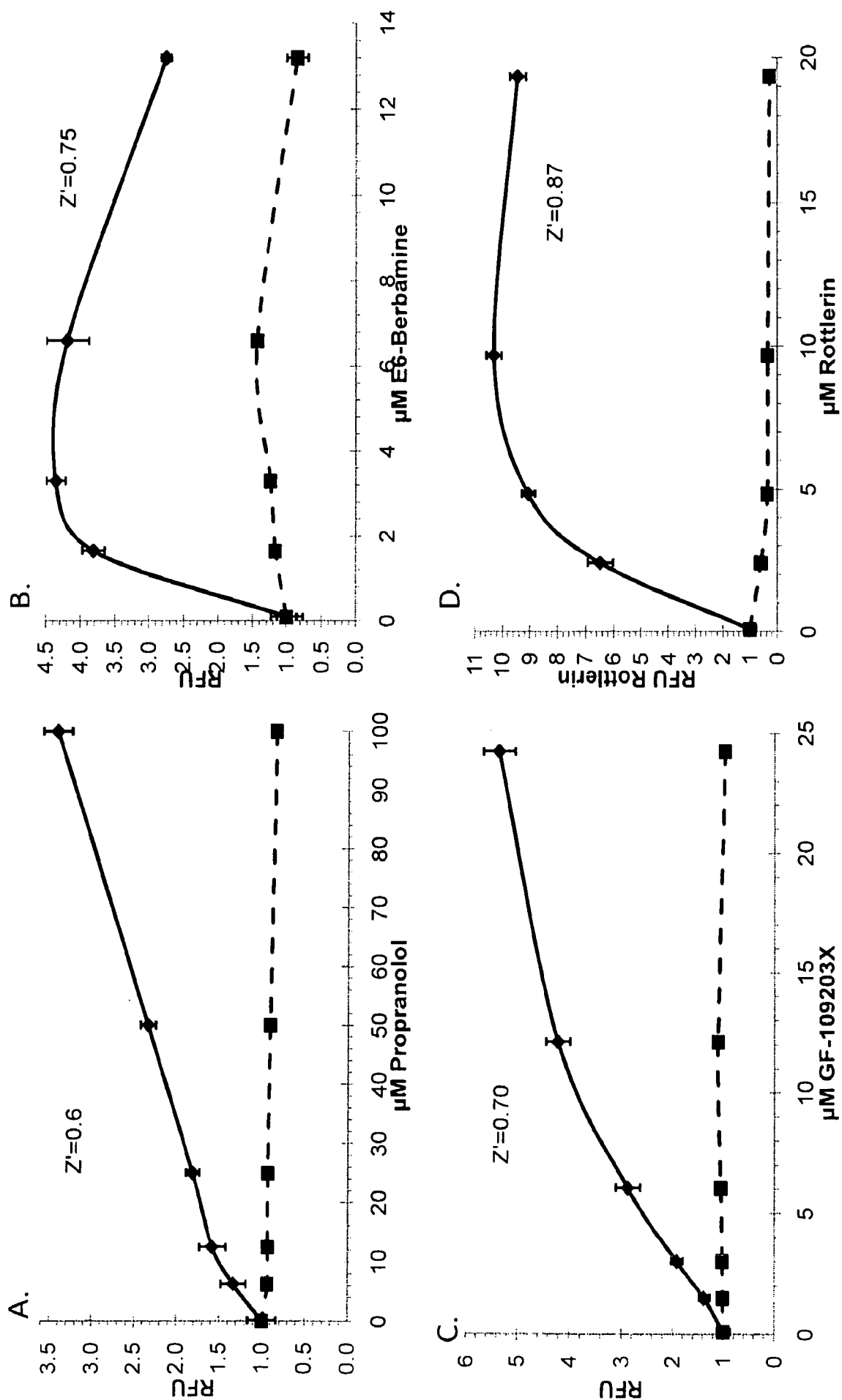


FIG. 4

**Schematic Illustration for Multiplexed Detection of
Phospholipidosis Inducers and Autophagy Pathway Inhibitors**

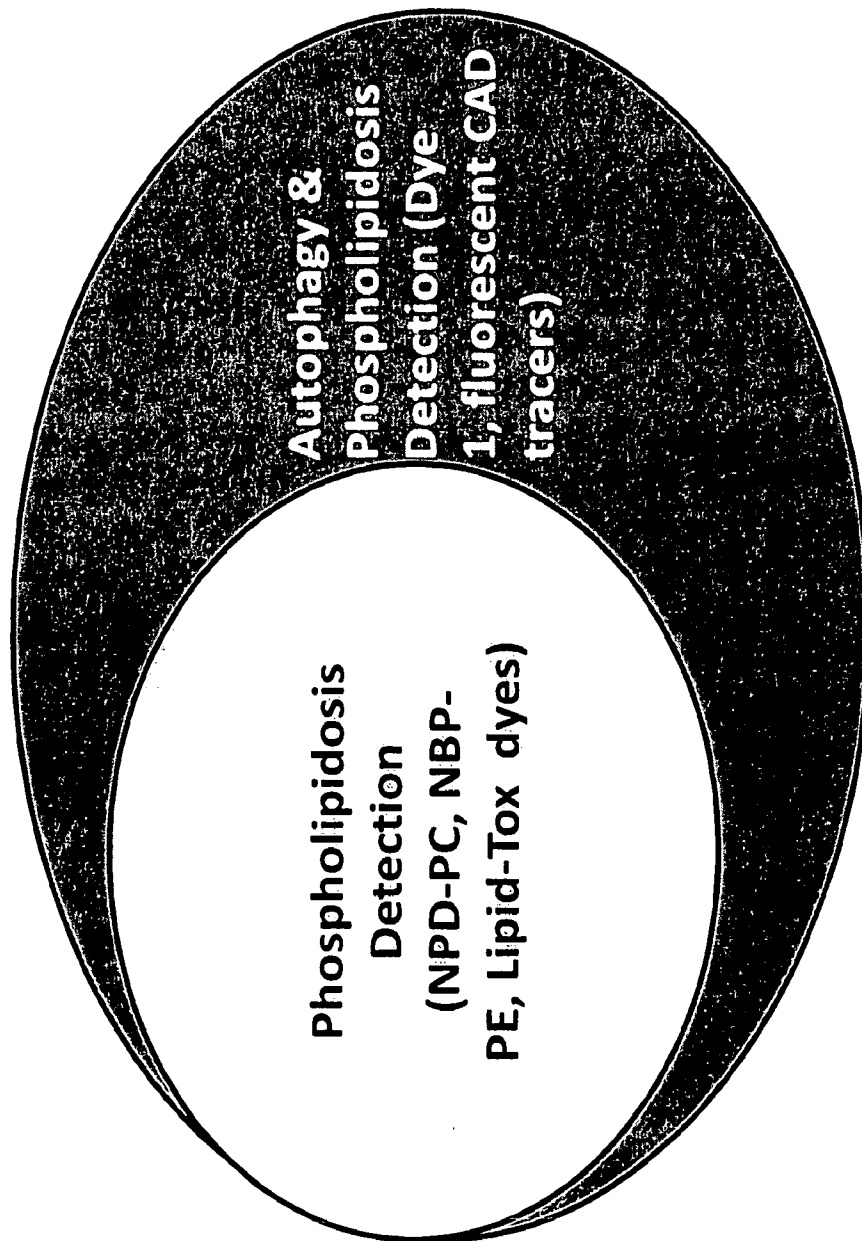


FIG. 5

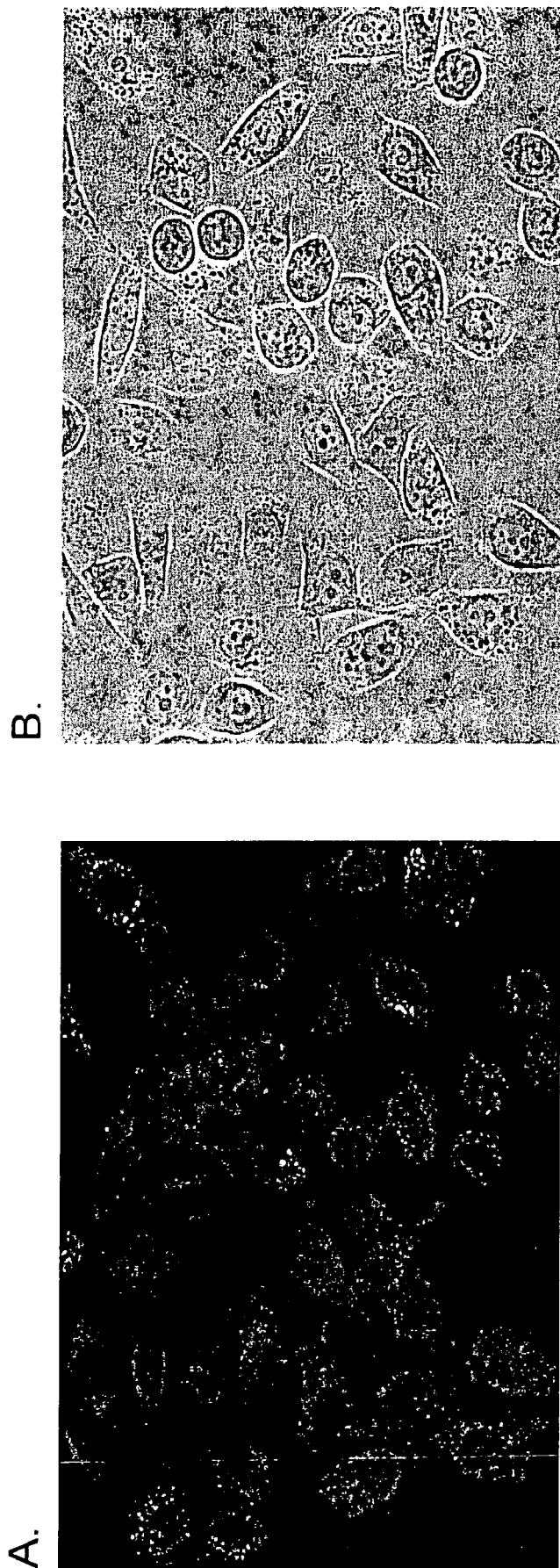


FIG. 6

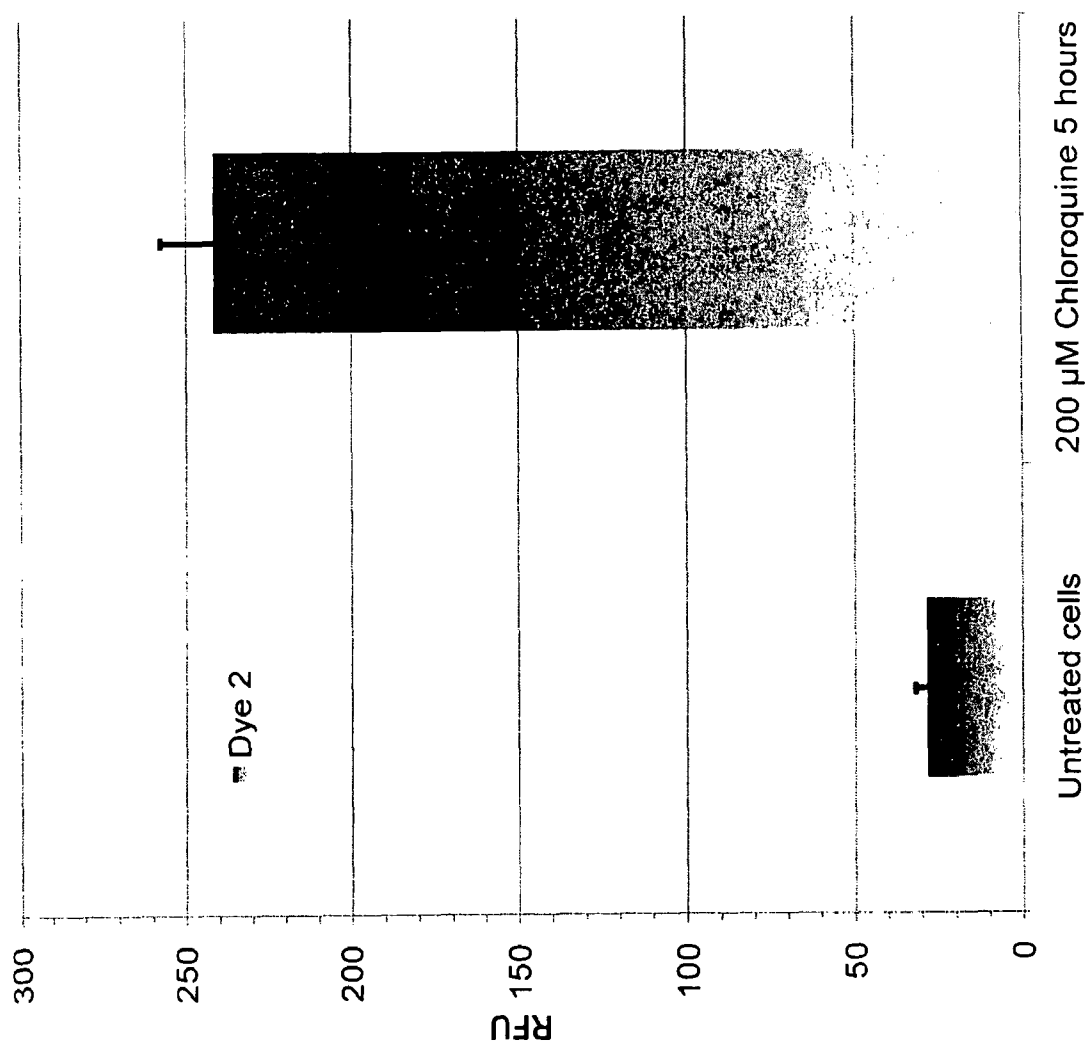


FIG. 7

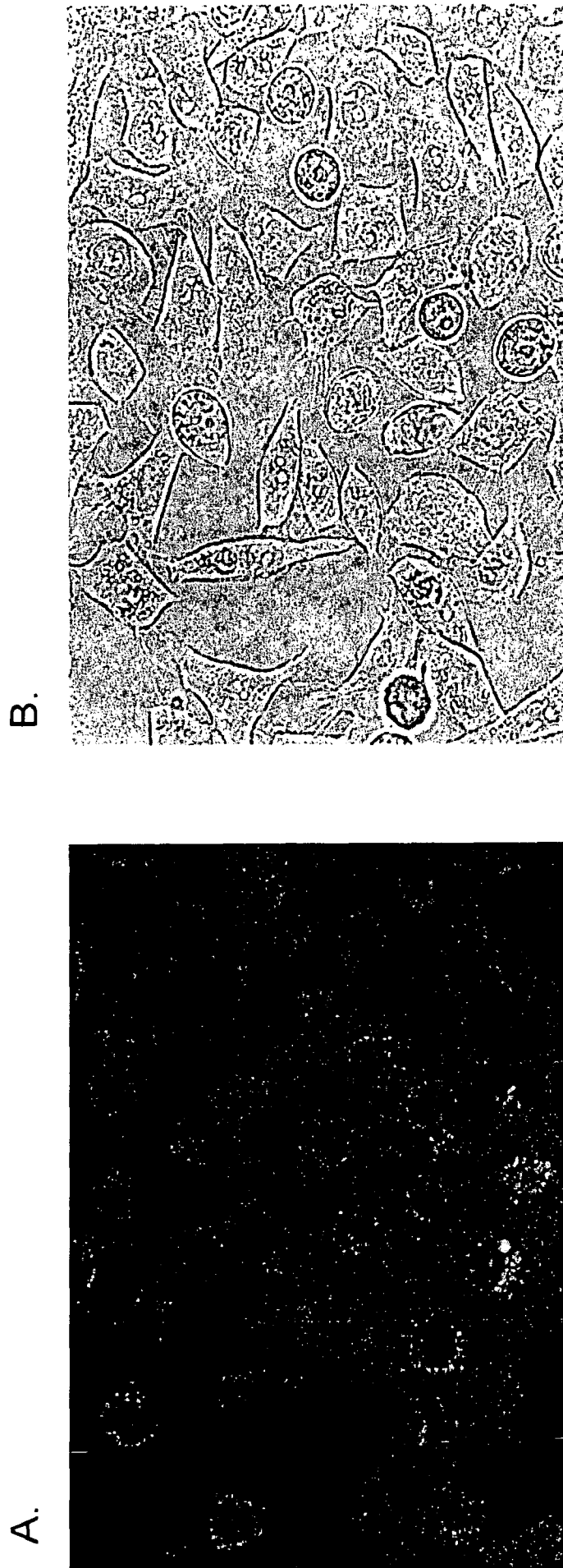


FIG. 8

Dye 8-labeled Vesicles are Induced by Starvation

Control



Starvation

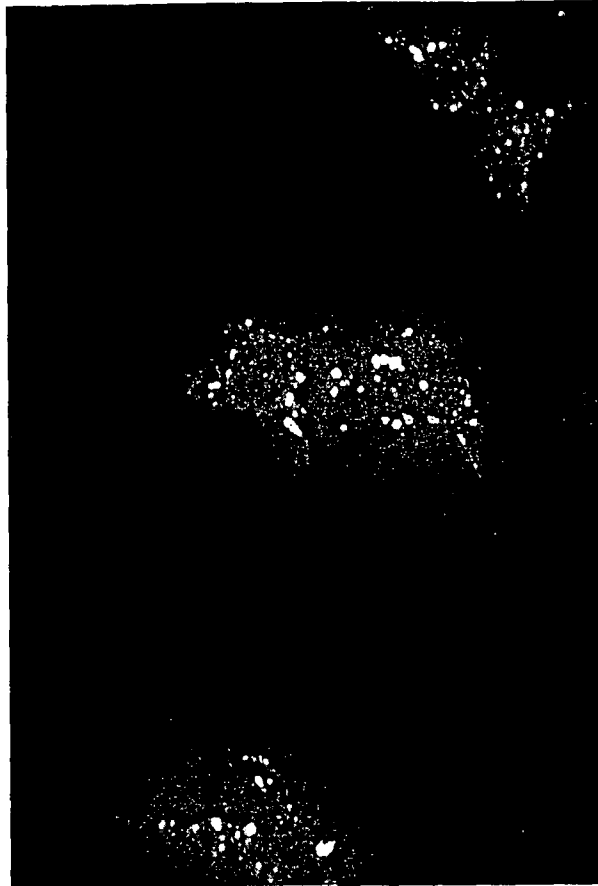


FIG. 9

A Population of Dye 8-Labeled Vesicles Co-Localizes with Specific Autophagosome Marker LC3

(a) Dye 8



(b) RFP-LC3



FIG. 10

**Rapamycin is an mTOR Pathway Inhibitor that Induces Autophagy;
3-MA is an Autophagy Inhibitor**

A Control **B** 3 μ M Rapamycin **C** 3 μ M Rapamycin+10 mM 3MA

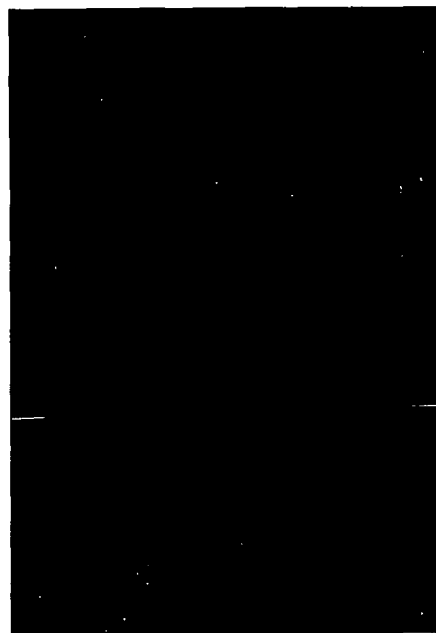


FIG. 11

Profiling Starvation Kinetics with Dye 8 by Flow Cytometry

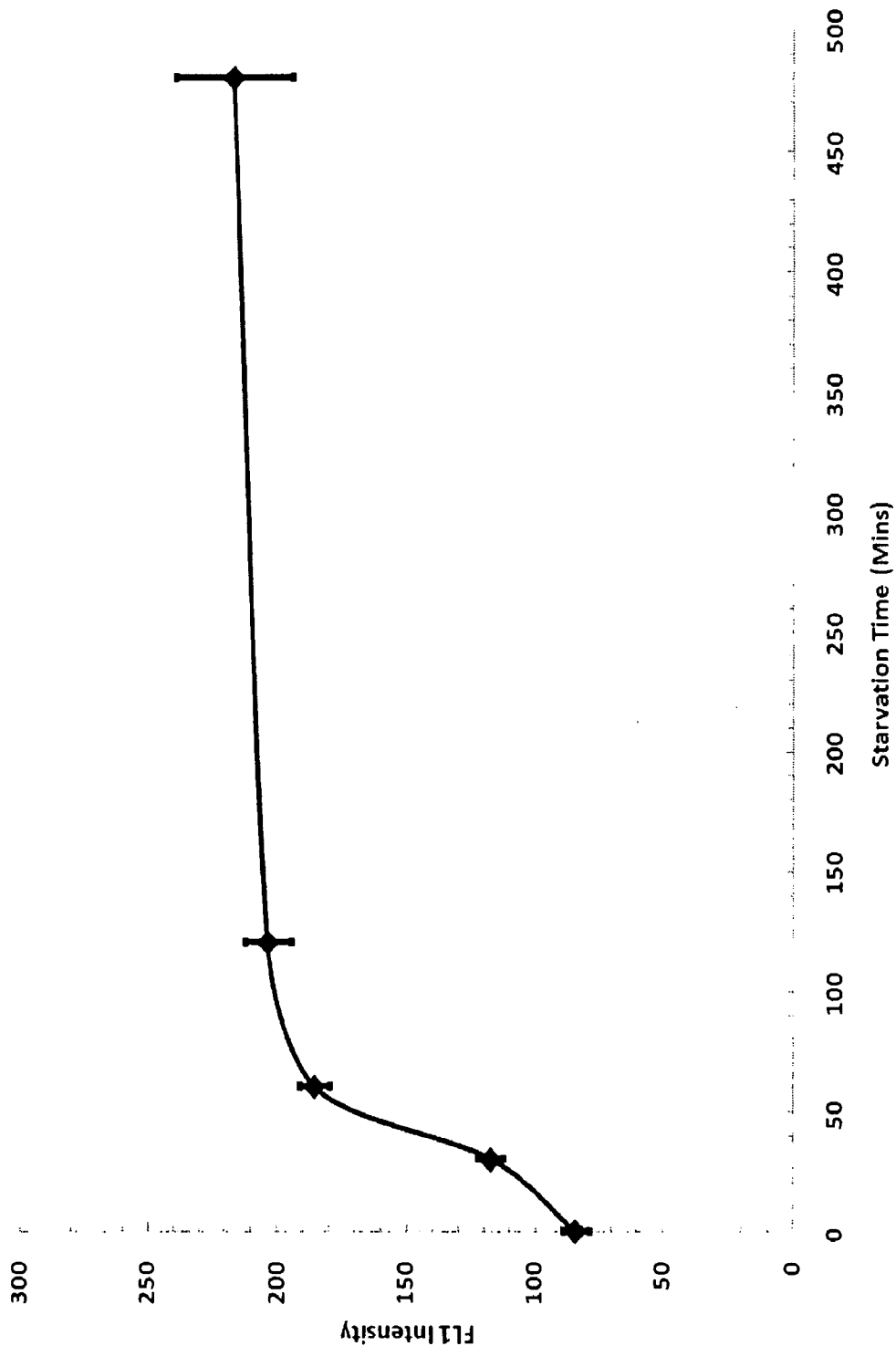
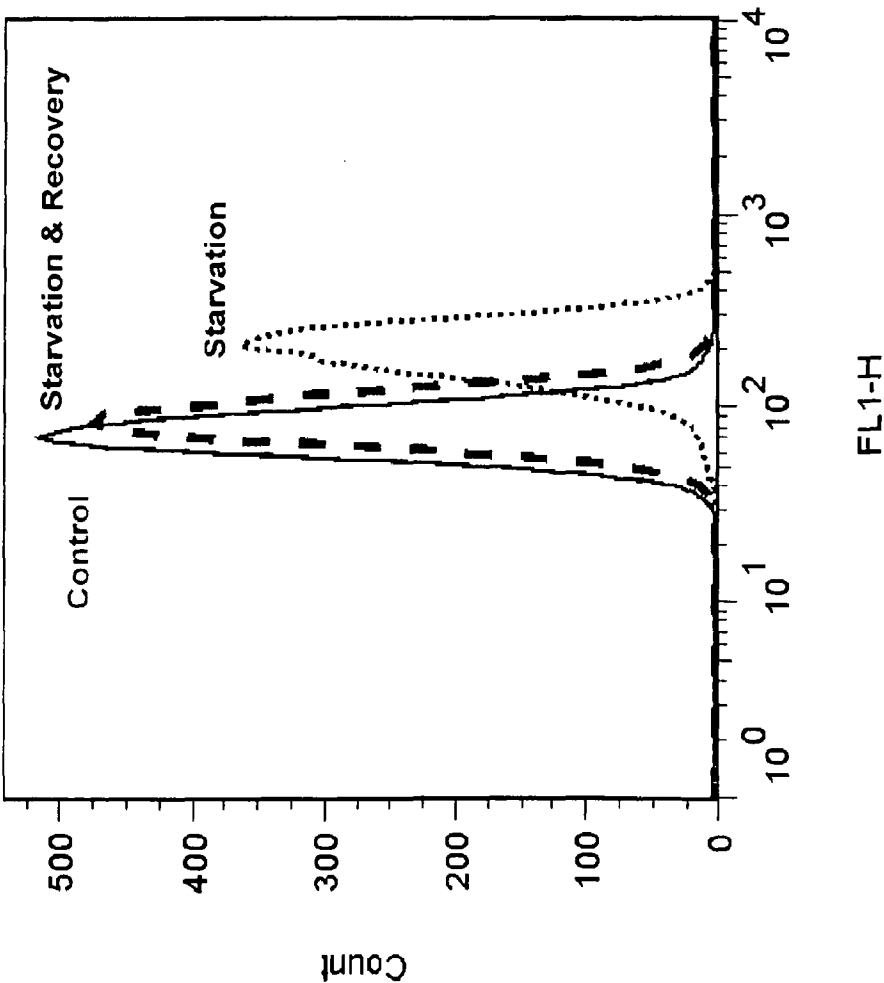


FIG. 12

Profiling Starvation and Recovery with Dye 8 by Flow Cytometry



Treatment	Mean Signal (FL1) N=3
Control	83±5
2 hour starvation	204±8
2hr starvation & 1 hr recovery	91±4

Autophagic activator factor	59
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FIG. 13

Solid line: Control
Dash line: 2hr starvation & recovery
Dotted line: starvation

Profiling Autophagic Flux with Dye 8 by Flow Cytometry

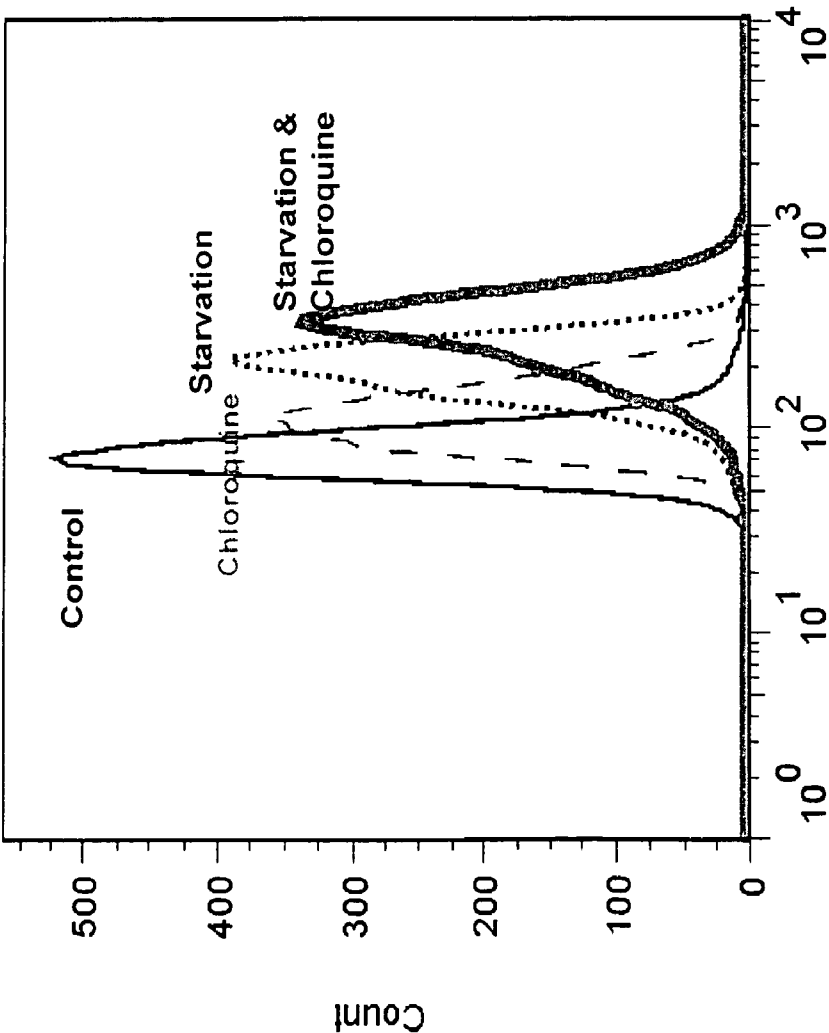


FIG. 14

FL1-H

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data, PAJ, BIOSIS, EMBASE, COMPENDEX, INSPEC, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2002/077366 A1 (BANERJEE RAJKUMAR [IN] ET AL) 20 June 2002 (2002-06-20) Relevant for non unityclaims 1-11 -----	1-17
A	LEMIEUX B ET AL: "Quantitation of the lysosomotropic character of cationic amphiphilic drugs using the fluorescent basic amine Red DND-99" ANALYTICAL BIOCHEMISTRY, vol. 327, no. 2, 15 April 2004 (2004-04-15), pages 247-251, XP004499135 ACADEMIC PRESS INC, NEW YORK ISSN: 0003-2697 DOI: 10.1016/J.AB.2004.01.010 cited in the application abstract Relevant for non unity ----- -/--	1-17

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 November 2010

Date of mailing of the international search report

25/02/2011

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

van der Kooij, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/002494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JESUS ALMELA MARIA ET AL: "Characterization of the phospholipidogenic potential of 4(1H)-pyridone antimalarial derivatives" TOXICOLOGY IN VITRO, vol. 23, no. 8, Sp. Iss. SI, 18 June 2009 (2009-06-18), pages 1528-1534, XP002608592 ISSN: 0887-2333 abstract page 1530, column 1, paragraph 2 - paragraph 3 figure 2 -----	1-17
Y	ISHIGURO KAZUHIRO ET AL: "Novel application of 4-nitro-7-(1-piperazinyl)-2,1,3-benzoxadia zole to visualize lysosomes in live cells" BIOTECHNIQUES, vol. 45, no. 4, 1 January 2008 (2008-01-01), pages 465-468, XP008128839 INFORMA LIFE SCIENCES PUBLISHING, WESTBOROUGH, MA, US ISSN: 0736-6205 abstract figure 1 -----	1-17
A	ZEIGLER, M. ET AL: "Prenatal diagnosis of Krabbe disease using a fluorescent derivative of galactosylceramide" CLINICA CHIMICA ACTA, vol. 142, no. 3, 1984, pages 313-318, XP002608593 ISSN: 0009-8981 abstract figure 1 -----	1-17
A	GATT, S. ET AL: "A fluorometric determination of sphingomyelinase by use of fluorescent derivatives of sphingomyelin, and its application to diagnosis of Niemann - Pick disease" CLINICAL CHEMISTRY, vol. 26, no. 1, 1980, pages 93-96, XP002608594 (WASHINGTON, DC, UNITED STATES) ISSN: 0009-9147 abstract page 95, column 1, paragraph 4 - column 2, paragraph 1 ----- -/--	1-17

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/002494

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RABEN NINA ET AL: "Monitoring autophagy in lysosomal storage disorders." [Online] 15 April 2009 (2009-04-15), pages 1-24, XP002608595 NIH Public Access: Author Manuscript Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2669416/pdf/nihms101000.pdf?tool=pmc entrez> [retrieved on 2010-11-15] the whole document -----	1-17
A	COX T M: "Biomarkers in lysosomal storage diseases: a review." ACTA PAEDIATRICA, vol. 94, no. Suppl. 447, March 2005 (2005-03), pages 39-42, XP002608596 (OSLO, NORWAY : 1992) ISSN: 0803-5326 the whole document -----	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2010/002494

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-17 (partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17(partially)

A method of detecting the presence of a lysosomal storage disease using cationic amphiphilic compounds with a nitrobenzoxadiazole structure.

2. claims: 1-17(partially)

A method of detecting the presence of a lysosomal storage disease using cationic amphiphilic compounds with an acridine structure.

3. claims: 1-17(partially)

A method of detecting the presence of a lysosomal storage disease using cationic amphiphilic compounds with a 5,8-dihydroxyanthracene-9,10-dione structure.

4. claims: 1-17(partially)

A method of detecting the presence of a lysosomal storage disease using quinolinium, 4-[2-[1-[3-[[4-(diethylamino)-1-methylbutyl]amino]-3-oxopropyl]-1,2,3,4-tetrahydro-6-quinoliny]ethenyl]-1-[3-[[2-hydroxy-3-(1-naphthalenyloxy)propyl]amino]propyl]-, bromide as cationic amphiphilic compound.

5. claims: 18-35(partially)

A method for monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of (T2-T1) time interval(s) using cationic amphiphilic compounds with a nitrobenzoxadiazole structure.

6. claims: 18-35(partially)

A method for monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of (T2-T1) time interval(s) using cationic amphiphilic compounds with an acridine structure.

7. claims: 18-35(partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method for monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of (T2-T1) time interval(s) using cationic amphiphilic compounds with a 5,8-dihydroxyanthracene-9,10-dione structure.

8. claims: 18-35(partially)

A method for monitoring the progression of a lysosomal storage disease in a subject by comparing vacuole accumulation over a course of (T2-T1) time interval(s) using quinolinium, 4-[2-[1-[3-[[4-(diethylamino)-1-methylbutyl]amino]-3-oxopropyl]-1,2,3,4-tetrahydro-6-quinoliny]ethenyl]-1-[3-[[2-hydroxy-3-(1-naphthalenyloxy)propyl]amino]propyl]-, bromide as cationic amphiphilic compound.

9. claims: 36-53(partially)

A method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease using cationic amphiphilic compounds with a nitrobenzoxadiazole structure.

10. claims: 36-53(partially)

A method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease using cationic amphiphilic compounds with an acridine structure.

11. claims: 36-53(partially)

A method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease using cationic amphiphilic compounds with a 5,8-dihydroxyanthracene-9,10-dione structure.

12. claims: 36-53(partially)

A method of monitoring the effectiveness of a drug or remedy used to manage, treat or cure a lysosomal storage disease using quinolinium, 4-[2-[1-[3-[[4-(diethylamino)-1-methylbutyl]amino]-3-oxopropyl]-1,2,3,4-tetrahydro-6-quinoliny]ethenyl]-1-[3-[[2-hydroxy-3-(1-naphthalenyloxy)propyl]amino]propyl]-, bromide as cationic amphiphilic compound.

13. claims: 54-67

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

A method of screening drug candidates to determine their toxicity to cells, tissues or organs using a cationic amphiphilic tracer compound.

14. claims: 68-81

A methods of screening suspected toxic agents to determine their toxicity to cells, tissues or organs using a cationic amphiphilic tracer compound.

15. claims: 82-97

a method of distinguishing between phospholipidosis activators and autophagy pathway perturbation agents using a cationic amphiphilic tracer compound.

16. claims: 98-101

compounds having a nitrobenzoxadiazole structure.

17. claim: 102(partially)

compounds having an acridine structure.

18. claims: 102, 103(partially)

compounds having a 5,8-dihydroxyanthracene-9,10-dione structure.

19. claims: 102, 103(partially)

compounds having an ethenyl structure.

20. claims: 1-17(partially)

compounds having an ethenyl structure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2010/002494

Patent document
cited in search report

Publication
date

Patent family
member(s)

Publication
date

US 2002077366 A1 20-06-2002 NONE
