Title: COMBINATION CHEMOTHERAPY FOR TREATING CANCER

Abstract: A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer.
COMBINATION CHEMOTHERAPY FOR TREATING CANCER

This application claims the benefit of U.S. Provisional Application No. 61/451,925, filed March 11, 2011, which is incorporated herein by reference in its entirety.

BACKGROUND

Significant research into new cancer treatments is ongoing. One area of research focuses on inducing apoptosis. However, other cell death mechanisms have been less explored in the context of cancer treatment. In addition, there remains a need for treatments with beneficial therapeutic properties while avoiding side effects.

SUMMARY

Disclosed herein are combination therapies for treating cancer.

In one embodiment, there is provided a method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer. In another embodiment, there is disclosed a method for treating cancer in a subject, comprising administering to a subject having a cancer susceptible to treatment with an autophagy mediating agent a therapeutically effective amount of a composition comprising an autophagy inducing agent and an autophagy inhibiting agent, thereby treating the cancer.

In a further embodiment, there is described a method of inhibiting growth of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.

Additionally disclosed is a method for inducing cytotoxicity of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of
a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.

Also disclosed herein is a composition comprising (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell.

A further embodiment disclosed herein involves the use of an autophagy inducing agent and an autophagy inhibiting agent in the manufacture of a medicament(s) for treating cancer in a subject.

Also disclosed herein is a composition comprising an emulsion comprising:
(i) a vinca alkaloid and a pharmaceutically acceptable amphiphilic agent associated together in the form of a hydrophobic ion pairing; and
(ii) a ceramide.

The foregoing will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1A and IB are graphs showing the results of a C₆-ceramide/vinblastine cytotoxicity assay in human hepatocarcinoma cells (Hep G2) cells. Hep G2 cells were treated with vinblastine diluted to 0.008-1 µM in cell culture media, alone or with 0, 12, 23, 47 µM liposomal C₆-ceramide (NCL 170-3) co-treatment, 24h (FIG. 1A), and 48h (FIG. IB). Cytotoxicity was determined at each time point by the MTT assays, as described in the Hep G2 Hepatocarcinoma Assay (GTA-2) (Potter TM, Stern ST, Methods Mol Biol 2011; 697:157-65). Data represents the % media control mean + SE, N=3.

FIG. 2A and 2B are graphs showing the results of a ghost nanoliposome (not containing C₆-ceramide/vinblastine cytotoxicity assay in Hep G2 cells. Human hepatocarcinoma cells were treated with vinblastine diluted to 0.008-1 µM in cell culture media, alone or with 0, 12, 23, 47 uM C₆-ceramide equivalent volume of...
ghost nanoliposome (NCL 169) co-treatment, for 24h (FIG. 2A), and 48h (FIG. 2B). Cytotoxicity was determined at each time point by the MTT assays, as described in the Hep G2 Hepatocarcinoma Assay (GTA-2). Data represents the % media control mean ± SE, N=3.

FIG. 3A and 3B are graphs showing the ghost nanoliposome (not containing ceramide)-vinblastine cytotoxicity assay in LS 174T (human colon cancer cell lines) cells. LS 174T cells were treated with vinblastine diluted to 0.008-1 µM in cell culture media, alone or with 0, 12, 23, 47 uM C₆-ceramide equivalent ghost nanoliposome (NCL 169) co-treatment, 24h (3A), and 48h (3B). Cytotoxicity was determined at each time point by the MTT assay, as described in the methods. Data represents the % media control mean ± SE, N=3.

FIG. 4A and 4B are graphs showing the C₆-ceramide liposomes/vinblastine cytotoxicity assay in LS 174T cells. LS 174T cells were treated with vinblastine diluted to 0.008-1 µM in cell culture media, alone or with 0, 12, 23, 47 uM liposomal C₆-ceramide (NCL 170-3) co-treatment, 24h (4A), and 48h (4B). Cytotoxicity was determined at each time point by the MTT assay, as described in the methods. Data represents the % media control mean ± SE, N=3.

FIG. 5A and 5B are graphs showing the caspase 3/7 activity in Hep G2 cells. Hep G2 cells were treated with 11, 23, and 45 uM of C₆-ceramide liposomes (NCL170), cell culture media (negative control) or 25 mM acetaminophen (positive control) for 24 and 48 h (5A). In a second experiment, Hep G2 cells were treated with media control (negative control), or vinblastine (0.004 - 1 uM) alone or with 12 uM of C₆-ceramide liposomes (NCL170), for 24 and 48 h (5B). Data are presented as % control caspase 3/7 activity. Error bars correspond to the mean ± SE of three individual samples.

FIG. 6 is an LC3 immunoblot analysis of HepG2 cells co-treated with ceramide and vinblastine showing autophagy induction. Hep G2 cells were treated for 24 h with media control (negative control), 47 uM of C₆-ceramide liposomes (NCL170), 0.2 uM of vinblastine, or co-treatment with C₆ ceramide liposomes (47 uM) and vinblastine (0.2 uM), in duplicate. Protein lysates were separated by
denaturing SDS-PAGE electrophoresis, transferred to PVDF membrane, and probed with antibodies specific to LC3, p62, and β-actin.

FIG. 7 is a TEM photomicrograph of HepG2 cells co-treated with ceramide and vinblastine showing autophagy induction. Hep G2 cells were treated for 24 h with media control (negative control), 47 μM of C₆-ceramide liposomes, 0.2 μM of vinblastine, or co-treatment with 47 μM of C₆-ceramide liposomes and 0.2 μM of vinblastine. A representative low (top panel) and high (bottom panel) magnification photomicrographs for each treatment are shown. Arrows indicate autophagic vacuoles.

FIG. 8A and 8B are Kaplan-Meier survival plots. Kaplan-Meier survival plots are presented for low (10 mg/kg) (A) and high (20 mg/kg) (B) dose vinblastine groups from a study evaluating efficacy of a C₆-ceramide/vinblastine co-treatment of a human colon cancer (LS174T) xenograft in nude mice. Time to endpoint was plotted against the fraction of surviving animals, by treatment group, based on the parameters displayed in Table 2. * C₆ ceramide/vinblastine treatment groups showed statistically significant increase in survival in comparison to vinblastine alone (p ≤ 0.05). (MANOVA, with Duncan’s multiple range test).

FIG. 9A and 9B show tumor growth response curves. Tumor growth responses to C₆ ceramide/vinblastine combination therapy or single agent therapy, in subcutaneously implanted LS 174T human colon cancer model, are displayed. A. Tumor growth responses to all the treatment groups B. Tumor growth responses to high dose groups. Data represents mean ± SD of tumor volumes for the treatment groups for each tumor measurement day.

* indicates significantly different from vinblastine group (20 mg/kg), p<0.05 (MANOVA, with Duncan’s multiple range test).

FIG. 10A and 10B are graphs showing treatment effect on animal body weight. The graphs display mean body weight over the study period for all the treatment groups (A) and high dose vinblastine groups (B). Data represents mean ± SD body weight for the treatment groups at each tumor measurement day.

FIG. 11 is a graph showing the results of a C₆-ceramide/vinblastine combination index (CI) in Hep G2 cells. Based on the 48 h data generated for
Fig. 1B, a combination index graph was produced as described in the methods. As evident from the calculated CI values <1, the combination of vinblastine and ceramide treatment resulted in a synergistic cytotoxicity in the Hep G2 cell line.

Fig. 1A and 1B are graphs showing the results of a C6-ceramide/paclitaxel cytotoxicity assay in Hep G2 cells. Human hepatocarcinoma cells were treated with paclitaxel diluted to 3.9-500 μM in cell culture media, alone or with 0, 12, 23, 47 μM liposomal C6-ceramide (NCL 170) co-treatment, at 24h (Fig. 12A), and 48h (Fig. 12B). Cytotoxicity was determined at each time point by the MTT assays, as described in the methods. Data represents the % media control mean ± SE, N=3.

Fig. 13A and 13B are graphs showing the results of a C6-ceramide/paclitaxel combination index in Hep G2 cells. Based on the 24 and 48 h data generated for Fig. 12, combination index graphs were produced as described in the methods. As evident from the calculated CI values >1, the combination of paclitaxel and ceramide treatment resulted in a less than additive response.

Fig. 14 is graph depicting the results of an analysis of caspase 3/7 activation in LS174T cells. LS174T cells were treated for 24 h with cell culture media or vinblastine (0.004 - 1 μM) alone, or in combination with 24 μM C6-ceramide nanoliposom, Data are presented as % control caspase 3/7 activity. Data presented is the mean ± SE of three individual samples. * indicates statistically significant over vinblastine treated samples p<0.05 (ANOVA with Dunnett’s T test).

Fig. 15. Induction of autophagy markers (LC3 and P62) by combination treatment in LS174T cell lines. LS174T cells were treated for 24 h with cell culture media, or C6-ceramide nanoliposom, (12 μM for LS174T cells), alone or in combination with 0.2 μM of vinblastine. Experiment was performed in duplicate.

Fig. 16. TEM photomicrographs of LS174T cells. LS174T cells were treated with cell culture media, or 12 μM of C6-ceramide nanoliposom, alone or in combination with 0.2 μM of vinblastine. A representative low (top row) and high (bottom row) magnification for each treatment are shown. Arrows indicate autophagic vacuoles.
FIG. 17A and 17B. Graphs showing the averaged intensity distribution plots for A) NE 004-1 (C₆Cer + VB Oleate) and B) NE 005-1 (VB Oleate) nanoemulsions.

FIG. 18A and 18B. Graphs showing the ζ Potential distribution for A) NE 004-1 (C₆Cer + VB Oleate) and B) NE 005-1 (VB Oleate).

FIG. 19A and 19B. Graph showing standard calibration curves for A) vinblastine and B: C₆ ceramide.

DETAILED DESCRIPTION

The following explanations of terms and methods are provided to better describe the present compositions and methods, and to guide those of ordinary skill in the art in the practice of the present disclosure. It is also to be understood that the terminology used in the disclosure is for the purpose of describing particular embodiments and examples only and is not intended to be limiting.

As used herein, the singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Also, as used herein, the term "comprises" means "includes." Hence "comprising A or B" means including A, B, or A and B.

"Administration" as used herein is inclusive of administration by another person to the subject or self-administration by the subject.

An "animal" refers to living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and non-human subjects, including birds and non-human mammals, such as non-human primates, companion animals (such as dogs and cats), livestock (such as pigs, sheep, cows), as well as non-domesticated animals, such as the big cats. The term subject applies regardless of the stage in the organism's life-cycle. Thus, the term subject applies to an organism in utero or in ovo, depending on the organism (that is, whether the organism is a mammal or a bird, such as a domesticated or wild fowl).
The term "co-administration" or "co-administering" refers to administration of an autophagy inducing agent with an autophagy inhibiting agent within the same general time period, and does not require administration at the same exact moment in time (although co-administration is inclusive of administering at the same exact moment in time). Thus, co-administration may be on the same day or on different days, or in the same week or in different weeks. The autophagy inducing agent and the autophagy inhibiting agent may be included in the same composition or they may each individually be included in separate compositions. In certain embodiments, the two agents may be administered during a time frame wherein their respective periods of biological activity overlap. Thus, the term includes sequential as well as coextensive administration of two or more agents.

The term "derivative" refers to a compound or portion of a compound that is derived from or is theoretically derivable from a parent compound.

"Inhibiting" refers to inhibiting the full development of a disease or condition. "Inhibiting" also refers to any quantitative or qualitative reduction in biological activity or binding, relative to a control.

The term "neoplasm" refers to an abnormal cellular proliferation, which includes benign and malignant tumors, as well as other proliferative disorders.

"Optional" or "optionally" means that the subsequently described event or circumstance can but need not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

The term "subject" includes both human and veterinary subjects.

A "therapeutically effective amount" refers to a quantity of a specified agent sufficient to achieve a desired effect in a subject being treated with that agent. For example, a therapeutically effective amount may be an amount of an autophagy inducing agent that is sufficient to induce autophagy in at least one cell. A therapeutically effective amount may be an amount of an autophagy inhibiting agent that is sufficient to inhibit autophagy in at least one cell. Ideally, a therapeutically effective amount of an agent is an amount sufficient to inhibit or treat the disease or condition without causing a substantial cytotoxic effect in the subject. The therapeutically effective amount of an agent will be dependent on the subject being treated, the disease or condition being treated, the agent being used, and the amount of agent used.
treated, the severity of the affliction, and the manner of administration of the therapeutic composition.

"Treatment" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. As used herein, the term "ameliorating," with reference to a disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease. The phrase "treating a disease" is inclusive of inhibiting the full development of a disease or condition, for example, in a subject who is at risk for a disease, or who has a disease, such as cancer, particularly a metastatic cancer. "Preventing" a disease or condition refers to prophylactic administering a composition to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing a pathology or condition, or diminishing the severity of a pathology or condition.

Although not bound by any theory, the advantageous features of the combination therapies disclosed herein may be related to autophagy. It has now been found that a combination of an autophagy inducing agent and an autophagy inhibiting agent has a synergistic effect on the assayed cell lines with regard to cytotoxicity and autophagy blockade and subsequent apoptosis induction. In other words, the combination therapy simultaneously induces the cell survival response, autophagy, and blocks this cell survival response, through autophagy blockade, resulting in apoptotic cell death. These autophagy disrupting agents have not been previously examined with respect to the ability to synergize with autophagy inducing agents.

A substantial decrease in tumor growth was measured in both in vitro and in vivo pre-clinical cancer models treated with the combination therapy. The combined
effects of the agents was greater than predicted by the anti-tumor effects of either
agent used alone, suggesting that there is a synergistic therapeutic interaction
between the two agents. While the autophagy pathway is generally believed to be
protective, blockade of this pathway results in a dramatic level of tumor cell death
by programmed cell death, apoptosis.

For example, one embodiment disclosed herein is a method for treating
cancer by co-administering vinblastine and C₆-ceramide. It has been discovered that
in cancer cells (e.g., human hepatocarcinoma and human colon cancer), at
concentrations of C₆-ceramide and vinblastine that were nontoxic when administered
alone, the combination results in a dramatic, synergistic effect on cytotoxicity,
resulting in apoptotic cell death. Subsequent studies in murine xenograft models
support the in vitro observations, demonstrating a dramatic synergistic effect of C₆-
ceramide on vinblastine tumor growth suppression, without increasing toxicity.
Ceramide is believed to induce autophagy by nutrient deprivation (Peralta, ER,
treatment of ceramide with vinblastine, the ceramide-induced autophagy response is
blocked as a result of vinblastine's effect on the cellular cytoskeleton (Kochl R, Hu
XW, Cahn EY, Tooze, SA, Traffic. 2006 Feb;7(2): 129-45), leading to apoptotic cell
death. One mechanism that can explain the apoptotic cell death resulting from this
combination therapy is JNK activation, BCL-2 phosphorylation, and release of
proapoptotic factors Bax and Bak. In addition to nutrient deprivation, ceramide has
been shown to increase BCL-2 phosphorylation through JNK activation, resulting in
release of the pro-autophagic protein Beclin-1 (Pattingre et al., J Biol Chem. 2009
Jan 30; 284(5):27 19-28. Epub 2008 Nov 23). In addition to disruption of autophagy
though microtubule agglomeration, vinblastine has also been shown to increase JNK
activation and BCL-2 phosphorylation itself (Brantley-Finley et al., Biochem
Pharmacol. 2003 Aug 1; 66(3):459-69). We believe that this synergistic increase in
BCL-2 phosphorylation results in release of the proapoptotic factors Bax and Bak
from the BCL-2 complex, and initiates apoptosis (Wei Y, Sinha, S, Levine B,
The synergistic effect of the combination therapy may also enable lower dosage levels of the agents at more frequent, periodic doses ("metronomic dosing"). For example, administration of an autophagy inhibiting agent (e.g., a vinca alkaloid such as vinblastine) alone at therapeutic doses produces the common side effects of cancer chemotherapy, since these drugs are dosed to the limit of tolerability (maximum tolerated dose, MTD). By utilizing an autophagy inhibiting agent in combination with an autophagy inducing agent, lower doses at a fraction of the MTD of the autophagy inhibiting agent can be administered while maintaining therapeutic efficacy. This serves to decrease both costs and side effects, and also provides the option to use metronomic dosing to prevent cancer recurrence. Alternatively, a higher level of anti-tumor effects can be achieved by using standard autophagy inhibiting agent MTD dosing levels. All of the advantageous dosing scenarios are the product of the larger therapeutic window enabled by combination therapy described herein and will benefit cancer patients through improvements in survival and quality of life.

The autophagy inducing agent (also referred to herein as a pro-autophagy agent) may be any agent that induces autophagy in cancer cells. Inducing autophagy is inclusive of initiating an autophagic process in cancer cells and inducing cancer cells to commit to an autophagic process. There are several methods for identifying autophagy inducing agents (Tasdemir E, Galluzzi L, Maiuri MC, Criollo A, Vitale I, Hangen E, Modjtahedi N, Kroemer G. Methods Mol Biol. 2008; 445:29-76.). The most laborious is identifying an increase in autophagic vacuoles by electron microscopy in vitro or ex vivo. Recently, the autophagy biomarker, LC3 II, as a component of the autophagic vacuole, has been identified and can be measured by Western blot and transgenic techniques. Alternatively, a high throughput method to screen for autophagy utilizing an autolysosome specific dye has been developed (Neun BW, Stern ST. Methods Mol Biol. 2011; 697:207-12.). Illustrative autophagy inducing agents include a ceramide, an mTOR inhibitor, temozolomide, arsenic trioxide, an Akt inhibitor, a lithium salt, a BH3 mimetic, loperamide, amiodarone, niguldipine, pimoizide, nicardipine, penitrem A, fluspirilene, trifluoperazine, and therapeutic starvation. Examples of lithium salts are lithium chloride (LiCl) or any
other pharmaceutically acceptable salts thereof, including but not limited to; lithium carbonate, lithium citrate, lithium sulfate, lithium aspartate, lithium orotate. Examples of mTOR inhibitors include rapamycin (sirolimus), rapamycin derivatives, CI-779, everolimus (Certican™), ABT-578, tacrolimus (FK 506), ABT-578, AP-23675, BEZ-235, OSI-027, QLT-0447, ABI-009, BC-210, salirasib, TATA-93, deforolimus (AP-23573), AP-23841, and temsirolimus (Torisel™). An illustrative Akt inhibitor is perifosine. In certain embodiments, the autophagy inducing agent does not induce apoptosis in cancer cells when the autophagy inducing agent is administered as the only anticancer agent or is administered without the autophagy inhibiting agent.

The ceramide may be any N-acylsphingosine. Ceramides include sphingolipids in which the sphingosine is acylated with a fatty acid acyl CoA derivative to form an N-acylsphingosine. Ceramide may be either naturally occurring or chemically synthesized. Preferably, the carbon chain length is less than 18 carbons (typically 2-10 carbons). Examples include C₆-ceramide (N-hexanoyl-D-sphingosine), C₂-ceramide (N-acetyl-D-sphingosine), Cs-ceramide (N-octyl-D-sphingosine) and C₈- ceramide (N-palmitoyl-D-sphingosine). Other ceramides are known to one of skill in the art. In certain embodiments, the ceramide (which is lipid soluble) is water soluble or made water soluble to enable contact with the tumor cells in a subject. For example, ceramide (6%) may be solubilized initially in alcohol and then subsequently diluted in saline or a cremophore.

In other embodiments, the autophagy inducing agent is a short-chain ceramide enclosed in a lipid vessel (e.g., a nano-sized lipid vessel), or liposome (e.g., a nanoliposome). The nanoliposome overcomes the challenges of solubility and delivery of the ceramide by encasing the fatty acid compound in between two lipid layers. Examples of liposomal ceramides are described in US 2005/0025820, which is incorporated herein by reference.

The liposomal ceramides may be "pegylated" liposomes that are formulated to have one or more membranes comprised of a growth-arresting lipid-derived bioactive compound and/or a gene therapy agent and/or cholesterol. These pegylated liposomes have been formulated to contain PEG C₈ (pegylated cell-permeable
ceramide), ranging in size between 750-5000 MW and/or PEG DSPE (disteroylphosphatidylethanolamine) ranging in size between 2000-5000 MW. PEG C₈ is used to stabilize the lipid bilayer, allowing the liposome to contain high molar ratio (i.e., 30%) of free bioactive C₆ ceramide. In addition, the embodiment utilizes the PEG C₉ as an integral component of the liposome that contains the bioactive ceramide. Moreover, PEG-C₉ formulated liposomes ensures optimal intercalation and localization of the free ceramide into caveolin-rich lipid rafts, a prerequisite for membrane internalization and transfer to subcellular organelles including the mitochondria for subsequent induction of apoptosis or programmed cell death of the targeted tissue or tumor. The pegylated liposomes, also known as "stealth"
liposomes, are capable of evading clearance from the circulation by the reticuloendothelial system (RES), leading to improved circulation half life and tissue targeting. Targeting can be further achieved via the conjugation of particular targeting moieties, such as antibodies and/or receptor ligands, which will promote the targeted accumulation into specific cells or tissues of the body. Additional embodiments assert that lipid therapeutics can also be formulated into "cationic" liposomes comprised of cationic lipids, in the presence or absence of PEG-C₉, for effectively delivering negatively charged oligonucleotides; or as "fusogenic" liposomes, in the presence or absence of PEG-C₉, where the entire membrane of the liposome fuses with the cell membrane of the target site to deliver the constituents and contents of the liposome therein.

In another embodiment, resorbable nanoparticles having a calcium phosphor-silicate (CPS) shell are provided, in which the ceramide is loaded into the resorbable nanoparticles. The resorbable nanoparticles can deliver the ceramide systemically to living cells, which normally are not transportable through the circulation. A key feature of the synthesis of the resorbable nanoparticles is the proper dispersion (non-aggregation) of the nanoparticles in an aqueous liquid medium. One way to achieve dispersion is the use of size exclusion high performance liquid chromatography (SEC) modified specifically for the silicate-containing shell nanoparticles. Another way to achieve dispersion of the nanoparticles is to attach organic, inorganic or metal-organic dispersants to the outer CPS shell. Additionally, a carbodiimide-
mediated polyethylene glycol (PEG) coupling agent can be attached to the alkylamine silane or alkylcarboxylic acid coupling agent to further ensure the "dispersed" non-aggregating state of the nanoparticles in vivo and to provide a conjugation point for targeting moieties onto the PEG coupling agent, thus enabling the nanoparticles to target specific sites for intracellular drug delivery.

The autophagy inhibiting agent may be any agent that blocks autophagy flux in a cell undergoing autophagy in the presence of the agent compared to the level of autophagy flux in the absence of the agent. There are many methods to measure autophagy flux, such as measurement of proteins known to undergo autophagy-mediated degradation, or observing LC3 II levels with or without inclusion of agents that inhibit lysosomal degradation (e.g., protease inhibitors, alkalinizing agents) (Barth S, Glick D, Macleod KF. J Pathol. 2010 Jun; 221(2): 117-24. Review). Illustrative autophagy inhibiting agents include a vinca alkaloid, a quinoline compound (e.g., an aminoquinoline such as chloroquine), 3-methyladenine, hydroxychloroquine (Plaquenil™), nocodazole, bafilomycin A1, 5-amino-4-imidazole carboxamide riboside (AICAR), okadaic acid, autophagy-suppressive algal toxins which inhibit protein phosphatases of type 2A or type 1, analogs of cAMP, and drugs which elevate cAMP levels, adenosine, N6-mercaptopurine riboside, and wortmannin. In addition, antisense or siRNA that inhibits expression of proteins essential for autophagy, such as for example ATG5, may also be used. Illustrative vinca alkaloids include vinblastine, vincristine, vindesine, and vinorelbine.

In certain embodiments, the autophagy inducing agent and the autophagy inhibiting agent may be co-administered via an emulsion. The emulsion may be formulated utilizing hydrophobic ion pairing to incorporate a hydrophilic acid or base into an organic solvent phase (e.g. a lipid solvent). Thus, agents described herein (which may be an autophagy inducing agent or an autophagy inhibiting agent) may be administered to a subject in need thereof via a lipid-based delivery platform. For example, hydrophilic vinca alkaloids are basic and thus can be paired with an acidic hydrophobic ion pairing agent for incorporation into an organic phase of an emulsion.
Illustrative hydrophobic ion pairing agents include any pharmaceutically acceptable amphiphilic materials. The amphiphilic material may be any material with a hydrophobic portion and a hydrophilic portion. These materials are typically surfactants. The hydrophilic portion is preferably ionic, and more preferably anionic. The hydrophobic portion may be any hydrophobic group such as an alkyl, aryl or alkylaryl group. The hydrophobic portion is preferably a long chain alkyl or substituted alkyl. The amphiphilic material associates with the autophagy inducing agent or the autophagy inhibiting agent to form a hydrophobic ion pair which is soluble in the organic solvent phase of the emulsion. As used herein, amphiphilic material includes different salt forms of a material as well as ionic forms and dissociation products of a material, such as may be present in a solution.

Examples of anionic amphiphilic materials include sulfates, sulfonates, phosphates (including phospholipids), carboxylates, and sulfosuccinates. Some specific anionic amphiphilic materials include alkanoates (e.g., octanoate, decanoate), alkenoates (e.g., oleate), alkylphosphoric acids (e.g., mono-octylphosphoric acid, mono-decylphosphoric acid, mono-dodecylphosphoric acid), taurodeoxycholate, taurocholate, lauryl sulfate, octadecansulfonate, dioctylsulfosuccinate, 1-hydroxy-2-naphthoic acid, sodium dodecyl sulfate (SDS), bis-(2-ethylhexyl) sodium sulfosuccinate (AOT), cholesterol sulfate and sodium laurate. Examples of cationic amphiphilic materials include those having an ammonium group or a guadinium group, including substituted variations of those groups, alkylamines (e.g. diethyl amine), diethanolamine, quaternary ammonium ions (e.g. tetraalkylammonium), N-(2-hydroxylethyl) piperidine, amino acid esters (e.g. Phenylalanine methyl ester, histidine methyl ester). Preferred amphiphilic materials are those posing little or substantially no toxicological problem for the human or animal host.

The organic solvent may be any organic liquid in which the autophagy inducing agent or the autophagy inhibiting agent and the amphiphilic material, together, are soluble, such as in the form of a hydrophobic ion pair complex. The following is a non-limiting, representative list of some organic solvents, with specific exemplary solvents listed in parentheses: monohydric alcohols (methanol,
ethanol, 1-propanol, 2-propanol, 1-butanol, 1-hexanol, 1-octanol, trifluoroethanol); polyhydric alcohols (propylene glycol, PEG 400, 1,3-propanediol); ethers (tetrahydrofuran (THF), diethyl ether, diglyme); alkanes (decalin, isoctane, mineral oil); aromatics (benzene, toluene, chlorobenzene, pyridine); amides (n-methyl pyrrolidone (NMP), N,N-dimethylformamide (DMF)); esters (ethyl acetate, methyl acetate); chlorocarbons (CH.sub.2 Cl.sub.2, CHCl.sub.3, CCl.sub.4, 1,2-dichloroethane); saturated and/or unsaturated fatty acids and mixtures thereof (e.g., a vegetable oil such as soybean oil); others such as nitromethane, acetone, ethylene diamine, acetonitrile, and trimethyl phosphate; and mixtures thereof.

In certain embodiments, both the autophagy inducing agent and the autophagy inhibiting agent are included in the organic solvent (e.g., a lipid or oil) phase in an oil in water emulsion. Typically, parenteral/injectable emulsions are oil in water (o/w) with a submicron (nanoscale, 40-200 nm) droplet size to prevent pulmonary embolism. Additionally, nanoscale size, with narrow size distribution, has been shown to improve formulation stability. Desired tumor distribution of a predetermined ratio of an autophagy inducing agent:autophagy inhibiting agent may be obtained with both agents in the oil phase of the emulsion. For example, the ceramide:vinblastine ratio may range from 6:1 to 8:1. A method for making the emulsion is described below in Example 9.

The combination therapy may be administered as a unitary or singular pharmaceutical composition that includes (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell. The amount of the autophagy inducing agent and the autophagy inhibiting agent together should be effective to treat a target cancer. The ratio of the autophagy inducing agent to the autophagy inhibiting agent is dependent upon the potency of either agent in inducing the respective response in the target cancer. In certain embodiments, for example, the ratio of the amount of the autophagy inducing agent to the amount of the autophagy inhibiting agent may range from 250:1 to 1:1, more particularly 100:1 to 2:1, and most particularly 15:1 to 8:1, for instance 12:1.
The combination therapy disclosed herein may be useful for treating any type of neoplasm (e.g., cancer). Tumors or neoplasms include new growths of tissue in which the multiplication of cells is uncontrolled and progressive. Some such growths are benign, but others are termed "malignant," leading to death of the organism. Malignant neoplasms or "cancers" are distinguished from benign growths in that, in addition to exhibiting aggressive cellular proliferation, they invade surrounding tissues and metastasize. Moreover, malignant neoplasms are characterized in that they show a greater loss of differentiation (greater "dedifferentiation"), and of their organization relative to one another and their surrounding tissues. This property is also called "anaplasia."

The combination therapy is particularly effective for administering to subjects having a cancer susceptible to treatment with an autophagy mediating agent, especially those cancers that respond to an autophagy inducing agent. In certain embodiments, a subject is identified as having a cancer that may be responsive to an autophagy mediating agent, and the combination therapy disclosed herein is administered to the identified subject. To determine susceptibility to the combination therapy, cancers from patients could be biopsied, grown in culture and subjected to the combination therapy. Evaluation would include monitoring of autophagy and apoptotic responses to therapy, including such biomarkers as LC3 II and caspase 3/7 activity, respectively. Illustrative cancers include lymphoma, head and neck cancer, lung cancer, breast cancer, testicular cancer, liver cancer, colon cancer, brain cancer and leukemia. In certain embodiments, cancers susceptible to treatment with an autophagy mediating agent include liver cancer, colon cancer, and glioma.

Neoplasms treatable by the presently disclosed compounds include all solid tumors, i.e., carcinomas and sarcomas. Carcinomas include those malignant neoplasms derived from epithelial cells which tend to infiltrate (invade) the surrounding tissues and give rise to metastases. Adenocarcinomas are carcinomas derived from glandular tissue or in which the tumor cells form recognizable glandular structures. Sarcoma broadly includes tumors whose cells are embedded in a fibrillar or homogeneous substance like embryonic connective tissue.
A solid tumor can be malignant, e.g. tending to metastasize and being life threatening, or benign. Examples of solid tumors that can be treated according to a method of the present invention include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

Moreover, tumors comprising dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung. Thus, the presently disclosed methods provide for treatment of conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, Basic Pathology, 2d Ed., W . B . Saunders Co., Philadelphia, pp. 68-79). Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly
metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder. For a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J. B. Lippincott Co., Philadelphia.

In certain embodiments, the presently disclosed methods are directed to a method for inhibiting cancer growth, including processes of cellular proliferation, invasiveness, and metastasis in biological systems. Preferably, the method is employed to inhibit or reduce cancer cell proliferation, invasiveness, metastasis, or tumor incidence in living animals, such as mammals.

Also provided herein is a method of inducing cytotoxicity (cell killing) in cancer cells or reducing the viability of cancer cells. For example, the combination therapy can be used to induce cytotoxicity in cells of carcinomas of the prostate, breast, ovary, testis, lung, colon, or pancreas.

The killing of cancer cells can occur with less cytotoxicity to normal cells or tissues than is found with conventional cytotoxic therapeutics, preferably without substantial cytotoxicity to normal cells or tissues. For example, the combination therapy identified herein can induce cytotoxicity in cancer cells while producing little or substantially no cytotoxicity in normal cells. Thus, unlike conventional cytotoxic anticancer therapeutics, which typically kill all growing cells, the combination therapy can produce differential cytotoxicity: tumor cells are selectively killed whereas normal cells are spared. Thus, in another embodiment, there is disclosed a method for inducing differential cytotoxicity in cancer cells relative to normal cells or tissue.

As described above, the autophagy inducing agent and the autophagy inhibiting agent can be individually included in separate pharmaceutical compositions or they can both be included in the same pharmaceutical composition. In either embodiment, the pharmaceutical compositions for administration to a
subject can include at least one further pharmaceutically acceptable additive such as carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions can also include one or more additional active ingredients such as antimicrobial agents, anti-inflammatory agents, anesthetics, and the like. The pharmaceutically acceptable carriers useful for these formulations are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition (1995), describes compositions and formulations suitable for pharmaceutical delivery of the compounds herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually contain injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The agents disclosed herein can be administered to subjects by a variety of mucosal administration modes, including by oral, rectal, intranasal, intrapulmonary, or transdermal delivery, or by topical delivery to other surfaces. Optionally, the agents can be administered by non-mucosal routes, including by intramuscular, subcutaneous, intravenous, intra-arterial, intra-articular, intraperitoneal, intrathecal, intracerebroventricular, or parenteral routes. In other alternative embodiments, the agents can be administered ex vivo by direct exposure to cells, tissues or organs originating from a subject.

To formulate the pharmaceutical compositions, the agents can be combined with various pharmaceutically acceptable additives, as well as a base or vehicle for dispersion of the compound. Desired additives include, but are not limited to, pH
control agents, such as arginine, sodium hydroxide, glycine, hydrochloric acid, citric acid, and the like. In addition, local anesthetics (for example, benzyl alcohol), isotonizing agents (for example, sodium chloride, mannitol, sorbitol), adsorption inhibitors (for example, Tween 80 or Miglyol 812), solubility enhancing agents (for example, cyclodextrins and derivatives thereof), stabilizers (for example, serum albumin), and reducing agents (for example, glutathione) can be included. Adjuvants, such as aluminum hydroxide (for example, Amphogel, Wyeth Laboratories, Madison, NJ), Freund's adjuvant, MPL™ (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, IN) and IL-12 (Genetics Institute, Cambridge, MA), among many other suitable adjuvants well known in the art, can be included in the compositions. When the composition is a liquid, the toxicity of the formulation, as measured with reference to the toxicity of 0.9% (w/v) physiological saline solution taken as unity, is typically adjusted to a value at which no substantial, irreversible tissue damage will be induced at the site of administration. Generally, the toxicity of the solution is adjusted to a value of about 0.3 to about 3.0, such as about 0.5 to about 2.0, or about 0.8 to about 1.7.

The agents can be dispersed in a base or vehicle, which can include a hydrophilic compound having a capacity to disperse the compound, and any desired additives. The base can be selected from a wide range of suitable compounds, including but not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (for example, maleic anhydride) with other monomers (for example, methyl (meth)acrylate, acrylic acid and the like), hydrophilic vinyl polymers, such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives, such as hydroxymethylcellulose, hydroxypropylcellulose and the like, and natural polymers, such as chitosan, collagen, sodium alginate, gelatin, hyaluronic acid, and nontoxic metal salts thereof. Often, a biodegradable polymer is selected as a base or vehicle, for example, polylactic acid, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer and mixtures thereof. Alternatively or additionally, synthetic fatty acid esters such as polyglycerin fatty acid esters, sucrose fatty acid esters and the like can be employed as vehicles. Hydrophilic polymers and other vehicles can be used alone or in
combination, and enhanced structural integrity can be imparted to the vehicle by partial crystallization, ionic bonding, cross-linking and the like. The vehicle can be provided in a variety of forms, including fluid or viscous solutions, gels, pastes, powders, microspheres and films for direct application to a mucosal surface.

The agents can be combined with the base or vehicle according to a variety of methods, and release of the agents can be by diffusion, disintegration of the vehicle, or associated formation of water channels. In some circumstances, the agent is dispersed in microcapsules (microspheres) or nanocapsules (nanospheres) prepared from a suitable polymer, for example, isobutyl 2-cyanoacrylate (see, for example, Michael et al., **J. Pharmacy Pharmacol.** 43:1-5, 1991), and dispersed in a biocompatible dispersing medium, which yields sustained delivery and biological activity over a protracted time.

The compositions of the disclosure can alternatively contain as pharmaceutically acceptable vehicles substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, and triethanolamine oleate. For solid compositions, conventional nontoxic pharmaceutically acceptable vehicles can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

Pharmaceutical compositions for administering the agents can also be formulated as a solution, microemulsion, or other ordered structure suitable for high concentration of active ingredients. The vehicle can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity for solutions can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of a desired particle size in the case of dispersible formulations, and by the use of surfactants. In many cases, it will be desirable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol and sorbitol, or sodium chloride in the composition. Prolonged absorption of
the compound can be brought about by including in the composition an agent which
delays absorption, for example, monostearate salts and gelatin.

In certain embodiments, the agents can be administered in a time release
formulation, for example in a composition which includes a slow release polymer.

5 These compositions can be prepared with vehicles that will protect against rapid
release, for example a controlled release vehicle such as a polymer, microencapsulated
delivery system or bioadhesive gel. Prolonged delivery in various compositions of the
disclosure can be brought about by including in the composition agents that delay
absorption, for example, aluminum monostearate hydrogels and gelatin. When
controlled release formulations are desired, controlled release binders suitable for use
in accordance with the disclosure include any biocompatible controlled release
material which is inert to the active agent and which is capable of incorporating the
compound and/or other biologically active agent. Numerous such materials are known
in the art. Useful controlled-release binders are materials that are metabolized slowly
under physiological conditions following their delivery (for example, at a mucosal
surface, or in the presence of bodily fluids). Appropriate binders include, but are not
limited to, biocompatible polymers and copolymers well known in the art for use in
sustained release formulations. Such biocompatible compounds are non-toxic and
inert to surrounding tissues, and do not trigger significant adverse side effects, such as
nasal irritation, immune response, inflammation, or the like. They are metabolized
into metabolic products that are also biocompatible and easily eliminated from the
body.

Exemplary polymeric materials for use in the present disclosure include, but
are not limited to, polymeric matrices derived from copolymeric and homopolymeric
polymers having hydrolyzable ester linkages. A number of these are known in the art
to be biodegradable and to lead to degradation products having no or low toxicity.
Exemplary polymers include polyglycolic acids and polylactic acids, poly(DL-lactic
acid-co-glycolic acid), poly(D-lactic acid-co-glycolic acid), and poly(L-lactic acid-co-
glycolic acid). Other useful biodegradable or bioerodible polymers include, but are
not limited to, such polymers as poly(epsilon-caprolactone), poly(epsilon-aprolactone-
CO-lactic acid), poly(epsilon-aprolactone-CO-glycolic acid), poly(beta-hydroxy
butyric acid), poly(alkyl-2-cyanoacrylate), hydrogels, such as poly(hydroxyethyl methacrylate), polyamides, poly(amino acids) (for example, L-leucine, glutamic acid, L-aspartic acid and the like), poly(ester urea), poly(2-hydroxyethyl DL-aspartamide), polyacetal polymers, polyoorthoesters, polycarbonate, polymaleamides, polysaccharides, and copolymers thereof. Many methods for preparing such formulations are well known to those skilled in the art (see, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978). Other useful formulations include controlled-release microcapsules (U.S. Patent Nos. 4,652,441 and 4,917,893), lactic acid-glycolic acid copolymers useful in making microcapsules and other formulations (U.S. Patent Nos. 4,677,191 and 4,728,721) and sustained-release compositions for water-soluble peptides (U.S. Patent No. 4,675,189).

The pharmaceutical compositions of the disclosure typically are sterile and stable under conditions of manufacture, storage and use. Sterile solutions can be prepared by incorporating the compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the compound and/or other biologically active agent into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated herein. In the case of sterile powders, methods of preparation include vacuum drying and freeze-drying which yields a powder of the compound plus any additional desired ingredient from a previously sterile-filtered solution thereof. The prevention of the action of microorganisms can be accomplished by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like.

In accordance with the various treatment methods of the disclosure, the agents can be delivered to a subject in a manner consistent with conventional methodologies associated with management of the disorder for which treatment or prevention is sought. In accordance with the disclosure herein, a prophylactically or therapeutically effective amount of the agent is administered to a subject in need of
such treatment for a time and under conditions sufficient to prevent, inhibit, and/or ameliorate a selected disease or condition or one or more symptom(s) thereof.

The administration of the agents can be for either prophylactic or therapeutic purpose. When provided prophylactically, the agents are provided in advance of any symptom. The prophylactic administration of the agents serves to prevent or ameliorate any subsequent disease process. When provided therapeutically, the compound is provided at (or shortly after) the onset of a symptom of disease or infection.

For prophylactic and therapeutic purposes, the agents can be administered to the subject by the oral route or in a single bolus delivery, via continuous delivery (for example, continuous transdermal, mucosal or intravenous delivery) over an extended time period, or in a repeated administration protocol (for example, by an hourly, daily or weekly, repeated administration protocol). The therapeutically effective dosage of the agent can be provided as repeated doses within a prolonged prophylaxis or treatment regimen that will yield clinically significant results to alleviate one or more symptoms or detectable conditions associated with a targeted disease or condition as set forth herein. Determination of effective dosages in this context is typically based on animal model studies followed up by human clinical trials and is guided by administration protocols that significantly reduce the occurrence or severity of targeted disease symptoms or conditions in the subject. Suitable models in this regard include, for example, murine, rat, avian, porcine, feline, non-human primate, and other accepted animal model subjects known in the art. Alternatively, effective dosages can be determined using in vitro models. Using such models, only ordinary calculations and adjustments are required to determine an appropriate concentration and dose to administer a therapeutically effective amount of the compound (for example, amounts that are effective to elicit a desired immune response or alleviate one or more symptoms of a targeted disease). In alternative embodiments, an effective amount or effective dose of the agents may simply inhibit or enhance one or more selected biological activities correlated with a disease or condition, as set forth herein, for either therapeutic or diagnostic purposes.
The actual dosage of the agents will vary according to factors such as the disease indication and particular status of the subject (for example, the subject's age, size, fitness, extent of symptoms, susceptibility factors, and the like), time and route of administration, other drugs or treatments being administered concurrently, as well as the specific pharmacology of the agent for eliciting the desired activity or biological response in the subject. Dosage regimens can be adjusted to provide an optimum prophylactic or therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental side effects of the agent is outweighed in clinical terms by therapeutically beneficial effects. A non-limiting range for a therapeutically effective amount of an agent within the methods and formulations of the disclosure is about 0.01 mg/kg body weight to about 20 mg/kg body weight, such as about 0.05 mg/kg to about 5 mg/kg body weight, or about 0.2 mg/kg to about 2 mg/kg body weight.

Dosage can be varied by the attending clinician to maintain a desired concentration at a target site (for example, the lungs or systemic circulation). Higher or lower concentrations can be selected based on the mode of delivery, for example, trans-epidermal, rectal, oral, pulmonary, or intranasal delivery versus intravenous or subcutaneous delivery. Dosage can also be adjusted based on the release rate of the administered formulation, for example, of an intrapulmonary spray versus powder, sustained release oral versus injected particulate or transdermal delivery formulations, and so forth.

Example 1 - c 6-Ceramide (NCL 170)/Vinblastine Cytotoxicity Assay in Human Hepatocarcinoma and Colon Cancer Cells

Design and Methods
Cytotoxicity was determined as described in the NCL method, Hep G2 Human Hepatocarcinoma Cytotoxicity Assay (GTA-2). Briefly, vinblastine was diluted to 0.008-1 µM in cell culture media, alone or with 0, 12, 23, or 47 µM c 6-ceramide nanoliposome (NCL 170), or non-drug loaded ghost liposome (NCL 169), co-treatment. The HepG2 (human hepatocarcinoma) or LS174T Cells (human colon
cancer) were plated in 96-well, microtiter plate format. Cells were preincubated for 24h prior to test material addition, reaching an approximate confluence of 80%. Cells were then exposed to test material, or media control, for 24h, and 48h in the dark, and cytotoxicity was determined using the MTT cell viability assay.

5

Results and Conclusion

The maximum concentrations tested in the Hep G2 and LS174T cytotoxicity study was 1 µM vinblastine, with 0, 12, 23, or 47 µM C₆-ceramide nanoliposome (NCL 170), or ghost nanoliposome co-treatment. Treatment of cells with these test materials resulted in a dose- and time-responsive loss of cell viability (see FIG. 1A, B, FIG. 2A, B, FIG. 3A, 3B, and FIG. 4A, 4B), as measured by the MTT assay. C₆-ceramide nanoliposome/vinblastine co-treatment resulted in a dramatic synergistic loss of cell viability.

15 Example 2- C₆-Ceramide (NCL 170)/Vinblastine-Induced Apoptosis Measured by Caspase 3/7 activation assay in Human Hepatocarcinoma cells

Activation of Caspase 3/7 levels, a marker of apoptosis, was measured by Apo-One Homogeneous Caspase 3/7 Assay (Promega). HepG2 cells were plated at 2 x 10⁴ cells/well into 96-well microtiter plate format and were allowed to grow for an additional 24 hr prior to treatment, reaching an approximate 80% of confluence. In an initial experiment, cells were treated with 11, 23, and 45 µM of C6 ceramide liposomes (NCL170) and caspase 3/7 enzyme activity was evaluated following incubation for 24 and 48 h. In the combination agent studies, Hep G2 cells were co-treated with 12 µM of C₆-ceramide liposomes (NCL170) in the presence of varying concentrations of vinblastine (0.004 - 1 µM), and compared to vinblastine treatment alone. The positive and negative controls for this assay were 25 mM acetaminophen and cell culture media, respectively. All the treatments were performed in triplicate. Following incubation in the dark for 24 and 48 h at 37°C, cells were washed with the media and incubated for an hour with kit reagents. Caspase 3/7 enzyme activity was measured by spectrophotometer at excitation 499 nm and emission 521 nm. Data are presented as percent control caspase 3/7 activity from triplicate samples.
Results and conclusion

Activation of caspase 3/7 was used as a measure of apoptosis. Treatment of Hep G2 cells with 11, 23, and 45 uM of C₆-ceramide liposomes (NCL170) resulted in no significant increase in the activation of caspase 3/7 at 24 and 48 h (FIG. 5A). Cells treated with 25 mM acetaminophen (positive control) had increased caspase 3/7 activity at 24 and 48 h, to 1000x control. The caspase 3/7 activity was analyzed to evaluate whether autophagy dysfunction induced by the C₆-ceramide liposomes (NCL170) and vinblastine co-treatment in the Hep G2 cells lead to increased cell death (FIG. 5B). Co-treatment with C₆-ceramide liposomes (NCL170) and vinblastine resulted in a dramatic increase in caspase 3/7 activity as compared with the vinblastine treatment alone at 24 and 48 h. Note: As this was a homogenous assay that did not normalize to viable cell number, the decrease in caspase 3/7 activity at high vinblastine concentrations most likely reflects a decreased number of viable cells contributing to the caspase 3/7 activity. In conclusion, cytotoxicity induced by the co-treatment in Hep G2 cells involves the activation of caspase 3/7.

Example 3- C₆-Ceramide (NCL 170)/Vinblastine Autophagy Induction Measured by LC3 immunoblot assay in Human Hepatocarcinoma cells

This method measures micro tubule-associated protein (MAP) LC3-I and the lipilated form, MAP LC3-II, by an immunoblot analysis. The lipilated form of MAP LC3-II is commonly used as a marker of autophagy. LC3 immunoblot was performed according to Stern ST, Zolnik BS, McLeod CB, Clogston J, Zheng J, McNeil SE. Toxicol Sci. 2008 Nov; 106(1): 140-52. In brief, Hep G2 cells were treated in duplicate for 24 h in T-75 flasks with negative control (media control), 47 uM C₆-ceramide liposomes alone, 0.2 uM vinblastine alone, or co-treatment with 47 uM C₆-ceramide liposomes and 0.2 uM vinblastine. Following treatment, cells were washed three-times with ice-cold PBS and the cell pellet was lysed with 200 ul of Invitrogen Cell Extraction Buffer containing protease inhibitors. A comparable amount of denatured protein from each sample was separated by 4-20% tris-glycine
gels and transferred to a PVDF membrane. The membrane was probed with specific antibodies (LC-3, p62, and B-actin) and developed using ECL Plus reagent.

**Results and conclusion**

During the formation of autophagosomes, LC3-I is converted to LC3-II, which is an established marker for autophagy. Cell lysates isolated from co-treatment of C₆-ceramide liposomes (NCL170) and vinblastine for 24 h synergistically increased LC3-II conversion to a much greater degree than either of the treatment alone (FIG. 6). The increase in the autophagy marker LC3-II may occur due to an increased formation of autophagosomes and/or a blockage in the maturation and degradation of the autophagosomes. The synergistic increase in LC3-II upon co-treatment is consistent with ceramide induction of autophagy and vinblastine blockade of autophagosome degradation (flux). The lack of LC3-II induction upon ceramide treatment alone most likely reflects the high turnover of LC3-II in the absence of autophagy blockade. P62 protein is a marker for autophagic flux as this protein is normally degraded by the autophagy pathway. The increase in p62 protein levels in the C₆-ceramide liposomes (NCL170) and vinblastine co-treatment, together with the increase in autophagy marker LC3-II, suggests blockade of autophagosome degradation (FIG. 6).

**Example 4** - C₆-ceramide (NCL 170)/Vinblastine Autophagy Induction Measured by Transmission Electron Microscopy in Human Hepatocarcinoma cells

Hep G2 cells (Human heptocellular carcinoma cell line) were plated in 6-well chambers and were allowed to grow for 24 h prior to initiation of treatment. Cells were treated for 24 h with complete media (negative control), 47 uM of C₆-ceramide liposomes (NCL170), 0.2 uM vinblastine, or co-treatment with 47 uM C₆-ceramide liposomes (NCL170) and 0.2 uM vinblastine in duplicate. Following 24 h treatment, cells were washed twice with media prior to fixing in TEM fixative solution (4% formaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer) and uranyl acetate (0.5% uranyl acetate in 0.1 M cacodylate buffer). Post-fixation of cells were
done in osmium tetroxide (1% osmium tetroxide in 0.1 M cacodylate buffer) and uranyl acetate (0.5% uranyl acetate in 0.1 M cacodylate buffer). Following post-fixation, cells were dehydrated stepwise in ethanol and embedded in embed-182 epoxy resin. Thin sections of 70-90 nm were trimmed using an ultramicrotome and were transferred onto formvar-copper mesh grids. Sections were stained with 3% uranyl acetate and lead citrate. Stained sections were carbon coated, and placed into a Hitachi H7600 microscope running at 80 kV voltage to acquire TEM images.

Results and conclusion

Electron microscopy revealed a significant increase in the formation of autophagic vacuoles in the Hep G2 cells co-treated with C₆ ceramide liposomes and vinblastine as compared to either of the treatment alone (Fig 7). Cells treated with media control (negative control) exhibited low basal amounts of autophagic vacuoles.

Example 5 - In vivo efficacy (ADME TOX 53)

Design and Methods

Objective

The study objective was to evaluate antitumor activity of C₆ ceramide nanoliposome (NCL170-4), or control blank nanoliposome (NCL169-2) in combination with vinblastine. Antitumor activity was evaluated in a single intravenous dose efficacy experiment in a human colon cancer (LS 174T) xenograft model.

Summary

Combination treatment with a single intravenous injection of NCL170-4 + vinblastine (20 mg/kg) resulted in significant decrease in tumor growth compared to saline + vinblastine (20 mg/kg).

Co-treatment with NCL170-4 + vinblastine demonstrated a dose-responsive decrease in tumor growth and positive effect on animal survival compared to NCL169-2 + vinblastine. The effects on tumor growth by NCL169-2 and NCL170-4 alone were comparable to saline treatment. Treatment with vinblastine alone resulted in an initial decrease in body weight loss with no further decrease in the combination...
treatment groups (FIG. 10). Combination treatment did not increase toxicity as measured by body weight loss and organ weight, hematology, and clinical chemistry parameters.

Husbandry
Animals were acclimated to the study environment for two weeks prior to study initiation. Animal rooms were kept at 50% relative humidity, 68-72 °F with 12 h light/dark cycles. Female athymic NCr-nu/nu mice 7 weeks-old were housed by treatment group, with 5 animals/cage, with ¼” corncob bedding. Animals were allowed ad libitum access to Purina 18% NIH Block and chlorinated tap water. General procedures for animal care and housing were in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council.

Test Article
NCL169-2 (ghost nanoliposomes), NCL170-4 (C₆-ceramide nanoliposomes), and clinical grade vinblastine® were suspended in physiological saline (0.9% NaCl). The test compounds were prepared fresh each time prior to dosing the animals. NCL169-2 is the ghost nanoliposome without C₆ ceramide and was dosed at an equivalent volume of NCL170-4. The intensity weighted hydrodynamic size of NCL169-2 and NCL170-4 in saline are 122 nm and 131 nm, respectively by DLS. Both the nanoliposomes nanoparticles are neutral in charge. An HPLC assay was performed to determine the concentration of C₆-ceramide nanoliposome in NCL170-4, which was 2.0 mg C₆ ceramide/mL. The route of administration for nanoliposomes (NCL169-2 and NCL170-4) and vinblastine was tail vein (i.v.). The injection volume for nanoliposomes (NCL169-2 and NCL170-4) and vinblastine were 10 mL/kg and 20 mL/kg of mouse body weight, respectively (Table 1).
Tumor Cell Culture
LS 174T, a human colon carcinoma cell line was maintained in RPMI 1640 medium and 10% heat-inactivated fetal bovine serum. Tumor cells were cultured in a humidified incubator at 37°C and 5% CO2. Cells were harvested by trypsinization with 0.05% trypsin/EDTA, resuspended in RPMI 1640 medium, centrifuged, and counted with a hemocytometer.

Tumor Cell Implantation
Tumor cells were inoculated into nude mice by subcutaneous injection into the left flank of 6 x 10^6 LS 174T cells in 0.1 mL Hanks Balanced Salt Solution. Four days after cell implantation, each animal had an ear tag inserted for identification. Tumors were allowed to grow for 7 days post-implantation, or until tumors reached approximately 5 mm in longest diameter, at which time chemotherapy treatment was initiated. Animals were not sorted either based on body weight or tumor volume.

Experimental Procedure
Following tumor cell inoculation, mice were randomly assigned to control or the treatment groups. Dose levels for the treatment groups were 10 and 20 mg vinblastine/kg, and 20 mg liposomal C6-ceramide/kg of NCL170-4, or equivalent volume of blank nanoliposome (NCL169). Control groups consisted of saline and NCL169-2 (ghost nanoliposome). Study drugs were given as a single dose by i.v injection. Vinblastine was dosed 15 min post nanoliposome dose. Animals were monitored daily for mortality and signs of pharmacologic or toxicologic effects. Body weights and tumor growth were measured on alternate days until the study was terminated on study day 29. Tumor measurement for each mouse was recorded using vernier calipers and their volumes calculated. The tumor volume, in mm^3, was calculated according to the formula: (width^2 x length)/2, where width is always the
smaller of the two measurements. Mean tumor volumes for all the groups were calculated by averaging together all the individual animal tumor volumes per measurement date. The neoplasia-related endpoint criteria were ulcerated tumor or tumor diameter >2 cm, at which point animals were euthanized. The morbidity criteria for euthanization included loss of greater than 20% of initial body weight and immobility. All remaining animals were euthanized at study termination on day 29. The animals were euthanized by CO2 asphyxiation, and necropsy consisted of tumor sizing, organ weights, gross organ description, hematology and clinical chemistry, and histopathology of all organs identified with gross lesions.

Table 1. Experimental Design for LS 174T Efficacy Experiment
Treatment groups, dose volumes and animal numbers are displayed in the table below.

<table>
<thead>
<tr>
<th>Treatment Group (mg/kg BW)</th>
<th>Volume (mL/kg BW)</th>
<th>Number of Animals (Female Athymic nu/nu)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>NCL169-2</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>NCL170-4</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>10 mg/kg Vinblastine®</td>
<td></td>
</tr>
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<td>Saline</td>
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<tr>
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</tr>
<tr>
<td>NCL170-4</td>
<td>20 mg/kg Vinblastine®</td>
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</tr>
</tbody>
</table>
Statistical Methods

Statistical differences in the tumor volume and body weight (parametric data) values between the treatment and vehicle groups were determined using ANOVA or MANOVA, with post-hoc comparisons by Dunnett's T test (StatSoft, Inc., Tulsa, OK).

Nonparametric data are analyzed by the Kruskal-Wallis ANOVA with multiple comparisons test. Time to endpoint animal survival data were analyzed by the Kaplan-Meier survival analysis using Graphpad statistical software version 9.6.0 (Graphpad Software Inc., La Jolla, CA).

Results

In-life results:

Mouse survival: Treatment with combination or a single agent did not result in any mortality. Termination of animals from the study were either due to tumor ulceration, tumor diameter exceeding 2 cm (neoplasia related endpoints), or upon study completion on day 29 (Table 2).

Survival analysis using Kaplan-Meier survival curves was performed to generate survival plots. Vinblastine treated animals had a statistically significant increase in animal survival compared to saline control. Treatment with NCL170-4 + vinblastine (20 mg/kg) resulted in survival of two animals with tumor volume less than 2 cm on day 29 (study end date). Treatment with NCL169-2 + saline and NCL170-4 + saline did not result in significant differences in animal survival compared to saline control (FIG. 8).

Table 2. Time to Endpoint Survival Plot

The table displays the days of survival, criteria for study drop-out and remission for Kaplan-Meier survival analysis. Drop-out criteria were tumor ulceration and tumor diameter exceeding 2 cm. The remission codes were "1" for animals reaching the neoplasia-related endpoint, and "0" for the animals not reaching the neoplasia-related endpoint either due to study termination on day 29 (study end date).
<table>
<thead>
<tr>
<th>Treatment groups (animal number)</th>
<th>Survival (Days)</th>
<th>Dropout</th>
<th>Remission</th>
</tr>
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<tr>
<td>Saline + Saline</td>
<td>2</td>
<td>&gt;2 cm</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
<td>&gt;2 cm</td>
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<td>12</td>
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<td>&gt;2 cm</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>11</td>
<td>&gt;2 cm</td>
</tr>
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<td>11</td>
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<td>Drop-out</td>
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<table>
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<tr>
<th>Treatment groups (animal number)</th>
<th>Survival (Days)</th>
<th>Drop-out</th>
<th>Remission</th>
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<td><strong>NCL169-2 + vinblastine</strong> (20 mg/kg)</td>
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<tr>
<td>62</td>
<td>11</td>
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<tr>
<td>64</td>
<td>13</td>
<td>&gt;2 cm</td>
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</tr>
<tr>
<td>66</td>
<td>25</td>
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<td>22</td>
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<td>1</td>
</tr>
<tr>
<td>70</td>
<td>15</td>
<td>&gt;2 cm</td>
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<tr>
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<tr>
<td>72</td>
<td>22</td>
<td>&gt;2 cm</td>
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</tr>
<tr>
<td>74</td>
<td>25</td>
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<td>76</td>
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<td>1</td>
</tr>
<tr>
<td>80</td>
<td>20</td>
<td>ulcer</td>
<td>1</td>
</tr>
<tr>
<td><strong>NCL170-4 + vinblastine</strong> (20 mg/kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
<td>88</td>
<td>29</td>
<td>survived</td>
<td>0</td>
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</table>
Relative tumor response to the drug treatment: A single i.v. dose of NCL170-4, followed 15 min later with a vinblastine dose (20 mg/kg), resulted in a synergistic decrease in tumor growth compared to NCL 169-2 + vinblastine (20 mg/kg) and saline + vinblastine (FIG. 9). Treatment with NCL170-4 + vinblastine (20 mg/kg) resulted in a sustained effect on tumor growth that was not observed in any other treatment combination. Vinblastine treatment in combination with either the NCL169-2 or saline, resulted in a comparable effect on tumor growth. Treatment with NCL169-2 + saline and NCL170-4 + saline had no significant effect on tumor growth and were comparable to saline control.

Clinical observations: Drug treatment did not result in any clinical abnormalities, aside from those associated with tumor progression. Animals were terminated from the study due to neoplasia-related endpoints (ulcerated tumor or tumor diameter over 2 cm).

Body weight: Treatment with vinblastine as a single agent, or in combination with NCL169-2 or NCL170-4, resulted in an initial decrease in body weight (<10% of initial body weight) from which the animals recovered (FIG. 10). The NCL169-2 and NCL170-4 alone had no significant effect on body weight and was comparable to saline control.

Necropsy results:
Organ weight: Treatment with NCL170-4 + vinblastine (10 mg/kg) resulted in significant decrease in spleen weight (absolute weight, % brain weight) in comparison to saline control. This change in organ weight was not considered biologically significant. No other significant changes in the remaining organ weights were noted, for either the combination or a single agent treatment groups.

Hematology Parameters: Combination treatment with NCL170-4 + vinblastine (20 mg/kg) resulted in a modest, but statistically significant, increase in red blood cell
distribution width (RDW) as compared to saline control. This change in clinical chemistry parameters was not considered biologically significant. Combination or single agent treatment did not affect any other hematology parameters.

Clinical Chemistry Parameters: Treatment of animals with the NCL 169-2 + saline significantly decreased the levels of electrolytes (calcium, potassium, and sodium), compared to the saline control animals. Treatment with NCL170-4 + saline resulted in an increase in creatinine levels compared to saline. These changes in clinical chemistry parameters was not considered biologically significant. Combination or a single agent treatment did not affect any of the remaining clinical chemistry parameters.

Gross pathology Findings: No treatment-related lesions were noted in any of the organs examined. Discoloration of lung, pancreas, and spleen was the result of hemorrhage at the time of necropsy. Spleen and lymph node enlargement were due to extramedullary hematopoiesis that commonly occurs in xenograft-implanted animals.

Comparative Example - C6-Ceramide (NCL 170)/Paclitaxel Cytotoxicity Assay in Human Hepatocarcinoma Cells

The objective of this study was to determine if C6-ceramide nanoliposome/vinblastine or C6-ceramide nanoliposome/paclitaxel co-treatment results in greater cytotoxicity than either component alone, in a human hepatocarcinoma (Hep G2). Paclitaxel has the same cellular target as vinblastine, the microtubule cytoskeleton, but paclitaxel stabilizes the non-acetylated microtubule cytoskeleton and does not appreciably inhibit autophagy flux (meaning that paclitaxel is not an autophagy inhibitor agent as used herein). In contrast, vinblastine disrupts both the acetylated and non-acetylated microtubule skeleton and dramatically blocks autophagy flux. Recent data confirms that vinblastine, due to

Design and Methods

Cytotoxicity was determined as described in the NCL method, Hep G2 Human Hepatocarcinoma Cytotoxicity Assay (GTA-2). Briefly, vinblastine was diluted to 0.008-1 µM in cell culture media, and paclitaxel was diluted to 3.9-500 µM in cell culture media, alone or with 0, 12, 23, or 47 uM nanoliposomal C₆-ceramide (NCL 170) co-treatment. The HepG2 cells were plated in 96-well, microtiter plate format. Cells were preincubated for 24h prior to test material addition, reaching an approximate confluence of 80%. Cells were then exposed to test material, or media control, for 24h and 48h in the dark, and cytotoxicity was determined using the MTT cell viability assay. Synergy was assessed by calculating the combination index (CI), using the equation CI = (([C₆-ceramide]/IC₅₀C₆-Ceramide) + ([paclitaxel or vinblastine]/IC₅₀Paclitaxel or vinblastine)). As evident from the combination index calculation, CI < 1 Synergistic, CI = 1 Additive, CI > 1 Antagonistic.

Results and Conclusion

The maximum concentrations tested in the Hep G2 cytotoxicity study was 1 µM vinblastine or 500 µM paclitaxel, alone or with 0, 12, 23, or 47 uM nanoliposomal C₆-ceramide (NCL 170-3) co-treatment. Treatment of cells with these test materials resulted in a dose- and time-responsive loss of cell viability (FIG. 1 and FIG. 12), as measured by the MTT assay. C₆-Ceramide nanoliposome/vinblastine co-treatment resulted in a dramatic synergistic loss of cell viability (FIG. 11), while C₆-ceramide nanoliposome/paclitaxel co-treatment resulted in a less than additive response (FIG. 13).

Example 6 - Induction of caspase 3/7 activity by C₆-ceramide/vinblastine

The study objective was to evaluate activation of caspase 3/7 enzymes by C₆-ceramide/vinblastine combination treatment in comparison to vinblastine alone.
Design and Methods

Activation of caspase enzymes, a marker of apoptosis, was measured by a method that utilizes the Apo-One Homogeneous Caspase 3/7 Assay kit (Promega, Madison, WI), a high throughput screening method to evaluate activation of caspase 3/7 enzymes. This method does not involve isolation of cell lysates and the caspase 3/7 levels are not normalized to total protein. LS174T cells were plated at 4 x 10^4 cells/well into 96-well plate format and were allowed to grow for additional 24 h reaching an approximate 80% of confluence. C6-ceramide nanoliposomes (24 µM) were co-treated in the presence of varying concentrations of vinblastine (0.004 - 1 µM). All the treatments were performed in triplicate. Following incubation in the dark for 24 h at 37°C, cells were washed with the media and incubated for an hour with kit reagents. Caspase 3/7 enzyme activity was measured by spectrophotometer at excitation 499 nm and emission 521 nm. Data are presented as percentage control caspase 3/7 activity.

Results and conclusion

The caspase 3/7 activity, a marker of caspase-dependent apoptosis, was analyzed in human colon cancer LS174T cell line to evaluate whether the blockade of autophagy flux by co-treatment of C6-ceramide nanoliposome and vinblastine results in enhanced apoptotic cell death. Indeed, co-treatment of C6-ceramide nanoliposome and vinblastine led to a dramatic and statistically significant increase in caspase 3/7 activity as compared with the vinblastine treatment alone at 24 h. The results suggest that C6-ceramide nanoliposome synergistically enhanced vinblastine-induced caspase-dependent apoptosis in LS174T cells.

Example 7 - Induction of autophagic marker LC3-II by C6-ceramide/vinblastine

The study objective was to monitor autophagy by measuring the conversion of LC3-I to LC3-II by Western blot analysis in LS174T cell lines. LC3-II is
recruited to the membrane of autophagosomes and hence makes an excellent marker for evaluating autophagy induction.

**Design and Method**

In brief, LS174T cells were treated with 12 μM C6-ceramide nanoliposomes, alone or in combination with 0.2 μM vinblastine for 24 h. Media treatment alone was used as negative control. Total protein was isolated using Invitrogen Cell Extraction Buffer containing protease inhibitors (Sigma, St. Louis, MO). We loaded 20 μg of total protein lysates from each treatment, separated on 4-20% tris-glycine SDS-PAGE gels and transferred onto PVDF nylon membranes for immunoblotting.

The protein-transferred membrane was probed with primary antibody specific to anti-LC3 (1:200 dilution in Starting Block blocking buffer) or anti-P62 (1:1000 dilution in 5% BSA blocking buffer) for 2 h at room temperature. The membrane was washed twice with tris-buffered saline containing 0.01% Tween-20 for 15 min each, followed by incubation with the secondary donkey anti-mouse IgG-HRP conjugate (1:50,000 dilution in StartingBlock blocking buffer) for 1 h at room temperature. The membrane was then washed twice with tris-buffered saline containing 0.01% Tween-20 for 15 min each. The washed membrane was incubated with ECL peroxidase substrate solution and the immunoblot was developed using Hyperfilm ECL. The membrane was incubated with stripping buffer followed by re-probing with anti-P-actin antibodies.

**Results and conclusion**

Induction of autophagy by the combination treatment was analyzed by an immunoblot analysis for LC3. During the formation of double membrane autophagosomes, cytosolic LC3-I is cleaved and lipidated to form LC3-II, and is recruited to the membrane of autophagosomes, making LC3-II an excellent marker for evaluating autophagy induction. The cell lysates isolated from combination treatment of C6-ceramide nanoliposome and vinblastine showed a synergistic increase in the levels of LC3-II compared to either of the agents alone in LS174T cells. The increase in LC3-II levels can occur due to an enhanced formation of
autophagosomes and/or a blockage in the maturation and degradation of the autophagosomes. The synergistic increase in LC3-II conversion following combination treatment is consistent with autophagy induction by C₆-ceramide and blockade of autophagosome degradation by vinblastine.

To further confirm the increase in LC3-II levels in both the cell lines by the combination treatment is due to the blockade in autophagosome degradation, we examined P62 protein levels. P62 protein is a marker for autophagic flux, as the autophagy pathway normally degrades this protein. In agreement with our autophagy blockade hypothesis, we observed increased P62 protein levels in response to the combination treatment. This, taken together with the synergistic increases in LC3-II, is indicative of a blockade of autophagosome degradation.

Example 8 - Induction of autophagic vacuoles by C₆-ceramide/vinblastine

The study objective was to morphologically assess an increase in the formation of autophagic vacuoles by treatment using transmission electron microscopy (TEM).

Design and Method

LS174T cells were plated in 6-well chambers and were allowed to grow for 24 h prior to initiation of treatment. Cells were treated for 24 h with media (negative control), or 12 µM C₆-ceramide nanoliposomes, alone or in combination with 0.2 µM vinblastine, or co-treatment with 12 µM C₆-ceramide nanoliposomes and 0.2 µM vinblastine, in duplicate. Following 24 h treatment, cells were washed twice with media prior to fixing in TEM fixative solution (4% formaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer). Post-fixation of cells were performed in osmium tetroxide (1% osmium tetroxide in 0.1 M cacodylate buffer) and uranyl acetate (0.5% uranyl acetate in 0.1 M cacodylate buffer). Following the post-fixation step, cells were dehydrated stepwise in ethanol and embedded in embed-182 epoxy resin. Thin sections of 70-90 nm were trimmed using an ultramicrotome and were transferred onto formvar-copper mesh grids. Sections were stained with 3% uranyl acetate and lead citrate. Stained sections were carbon coated,
and placed into a Hitachi H7600 microscope running at 80 kV voltage to acquire TEM images.

**Results and conclusion**

The accumulation of autophagic vacuoles was assessed by transmission electron microscopy. Electron microscopy is considered the gold standard for evaluating ultrastructural features of cells undergoing autophagy. Combination treatment resulted in a significant increase in autophagic vacuoles in comparison to either of the treatments alone or media control.

**Example 9 - Emulsion**

**Incorporation of hydrophobic ion paired vinblastine oleate:** Due to its hydrophilicity, the commercially available salt form of vinblastine (vinblastine sulfate) is not suitable for loading into the oil droplets of the nanoemulsion formulation. Therefore, a stabilizer, sodium oleate, has been utilized to make a hydrophobic ion paired vinblastine oleate to facilitate its incorporation into oil droplets.

**Incorporation of C₆ Ceramide:** C₆ Ceramide hydrophobicity requires formulation in a drug delivery vehicle. C₆ ceramide is incorporated into the lipophilic oil droplets of the nanoemulsion formulation for the coadministration with vinblastine.

**Reagents**

Soybean oil (Cat # S7381 Sigma Aldrich, St. Louis, MO, USA)
Glycerol (Cat # G5516, Sigma Aldrich, St. Louis, MO, USA)
Vinblastine sulfate (Cat # V 1377, Sigma Aldrich, St. Louis, MO, USA)
Sodium Oleate (Cat # 07501, Sigma Aldrich, St. Louis, MO, USA)
Egg phosphatidylcholine (Egg PC) (Cat # 840051, Avanti Polar Lipids, Alabaster, Alabama, USA)
C₆-Ceramide (Cat # 860506P, Avanti Polar Lipids, Alabaster, Alabama, USA)
Sodium hydroxide (Cat # 12419-0010, ACROS Organic, Geel, Belgium)
Chloroform (Cat # 650498, Sigma Aldrich, St. Louis, MO, USA)
Millipore water

Methods

5 Formulation

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<th>Amount</th>
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<tr>
<td>Glycerol</td>
<td>175 mg</td>
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<tr>
<td>Vinblastine sulfate</td>
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<tr>
<td>( C_6 ) ceramide</td>
<td>35 mg</td>
</tr>
<tr>
<td>Sodium oleate</td>
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<tr>
<td>Sodium hydroxide (as required to adjust the pH)</td>
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</tr>
<tr>
<td>Millipore water</td>
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</table>

Compounding Nanoemulsion:

1. Weigh out 35 mg of \( C_6 \)-Ceramide in a screw cap vial (4 mL) and QS to 750 \( \mu L \) with chloroform. Reserve 10 \( \mu L \) of \( C_6 \) ceramide solution for HPLC quantification. Add 1.32 g soybean oil to a 20 mL vial, and then add the \( C_6 \)-ceramide chloroform solution to the soybean oil and vortex to mix two liquids completely. Completely evaporate the chloroform using rotavap under reduced pressure (~ 350) at 60 °C for 40 minutes. This portion constitutes the oil phase for nanoemulsion formulation.

2. Weigh out 10 mg of sodium oleate and 175 mg of glycerol in a 20 mL amber vial. Add 5 mL of millipore water and vortex for complete dissolution of sodium oleate. Incubate the solution in water bath for 5 minutes at 65 °C and add the egg PC (210 mg), vortex for 2 minutes and disperse completely under stirring for about 30 minutes. This portion constitutes the aqueous phase for nanoemulsion formulation.

3. Weigh out 7 mg of vinblastine sulfate in an amber glass vial (4 ml), QS to 750 \( \mu L \) with millipore water and store the vinblastine sulfate solution in refrigerator.
wrapped in aluminum foil to protect from light. Reserve, 20 µL of sample in an eppendorf tube for HPLC quantification.

4. Heat both aqueous and oil phase in a water bath at 65 °C for 10 minutes. Add the hot oil phase to the sodium oleate-glycerol solution from step 2 all at once with the aid of a glass pasteur pipet and quickly vortex the mixture for 2 minutes.

5. Homogenize the coarse emulsion mixture from step 4 using a polytron inside the water bath at 63 °C at 8000 rpm for 2 minutes.

6. Add the vinblastine sulfate solution (730 µL) from step 3 to the coarse emulsion from step 5, vortex for 2 minutes and again homogenize for 2-3 minutes until a primary emulsion forms. Hydrophobic ion paired vinblastine oleate is expected to localize into oil phase. Reserve, 100 µL of this primary emulsion sample in an eppendorf tube for HPLC quantification of C₆ ceramide and vinblastine.

7. Process 6 mL of the primary emulsion from step 6 using a 10 mL inlet plastic syringe with a microfluidizer (LV1, Microfluidics MA) at ascending high pressure strokes once at 10 K, twice at 15 K, once 20K and twice at 25 K psi to achieve a final nanosized emulsion. There should not be any air entrapment inside the syringe during feeding of the emulsion to the microfluidizer. Before feeding emulsion, feed millipore water (RT) several times to get rid of the storage liquid (70% ethanol) and finally, feed warm millipore water (-50 °C) 3 times to make the inside chamber worm.

8. Following microfluidization of the nanoemulsion, clean the microfluidizer with 70% ethanol until the outlet reservoir syringe is free of any white emulsion ingredients (collected ethanol must be clear).
9. Measure the nanoemulsion pH from step 7 and readjust to 7.5 using NaOH (0.1 N) or HCl (0.1 N) solution if necessary. Reserve, 50 μl of nanoemulsion sample in an eppendorf tube for HPLC quantification of C6 ceramide and vinblastine.

10. Check the prepared nanoemulsion from step 7 for size distribution and zeta potential using a Malvern zetasizer.

11. Filter the prepared Nanoemulsion with a PTFE filter (0.2 μm) under the biosafety hood and store in a sterile amber glass vial. Reserve, 500 μl of filtered nanoemulsion sample in an eppendorf tube for HPLC quantification of C6 ceramide and vinblastine. Also, assesses the size and zeta potential again from the sample after filtration.

12. Store the prepared sterile nanoemulsion in the refrigerator wrapped in aluminum foil to protect from light.

Characterization of a C6 ceramide (C6 Cer)-vinblastine (VB) oleate Nanoemulsion

1. Purpose- To characterize the size, size distribution, surface charge and loading of a C6 ceramide (C6 Cer)-vinblastine (VB) oleate nanoemulsion

2. Methods-

2.1 LC-UV Analysis of VB
Nanoemulsions were diluted 1:10 in acetonitrile (20uL of nanoemulsion + 180 μl of acetonitrile) in an eppendorf tube, vortexed for 1 minute and centrifuged at 14K rpm for 7 minutes at 4 °C. 150 μl of the supernatant was then transferred to another eppendorf tube and 350 μl of ammonium acetate (20 mM in millipore water) was added to reconstitute, final acetonitrile: ammonium acetate (30:70). The sample was then transferred to an amber screw cap HPLC vial for analysis.
The standard curve of VB base was made in acetonitrile: ammonium acetate (20 mM in millipore water) mixture (30:70 v/v). Standards were 0.01, 0.025, 0.05, 0.1, 0.2 and 0.3 mg/mL. A 10 mg/mL stock of VB sulfate (500 µL) was prepared in acetonitrile and further diluted to 1 mg/mL (1 mL) with acetonitrile. This 1 mg/mL stock was used to prepare standards (0.01, 0.025, 0.05, 0.1, 0.2 and 0.3 mg/mL) by diluting with acetonitrile and ammonium acetate (30:70).

The LC system consisted of a LC-20AT pump, SPD-20AC auto injector, and C-R3A integrator (Shimadzu Scientific Instruments, Inc.) and SPD-M20A UV/Vis detector. The analysis conditions were a flow rate of 0.3 mL/min, 10uL injection volume, with 2.1 x 100 mm-3.5-micron flow Instruments, 20AT with millipore water). The cap for methanol) Nanoemulsions 2.2 The min, min, nm. The acetonitrile/ammonium acetate gradient utilized was: 30% acetonitrile at 0 min, 75% acetonitrile 0-30 min, 75% acetonitrile 30-40 min, 30% acetonitrile 40-45 min, 10 min column re-equilibration time. The VB retention time was: 19.6 min. The standard curve is provided in the results section.

2.2 LC-UV Analysis of C6 Cer in nanoemulsion formulation
Nanoemulsions were diluted 1:20 in methanol (20µL of nanoemulsion + 380 µL of methanol) in an eppendorf tube, vortexed for 1 minute and centrifuged at 14K rpm for 7 minutes at 4 °C. 200 µL of the supernatant was then transferred to another eppendorf tube and 300 µL of acetonitrile: millipore water (70:30) (0.1% TFA) was added to reconstitute. The sample (500 µL) was then transferred to an amber screw cap HPLC vial for analysis.

The standard curve of C6 Cer was made in methanol (0.1% TFA) and acetonitrile: millipore water (70:30) (0.1% TFA) mixture at 40:60 ratio. The standards were 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 mg/mL of C6-Cer. A stock solution of 10 mg/mL (500 µL) of C6-Cer in methanol (0.1 % TFA) was prepared and then diluted to 1 mg/mL with methanol (0.1 % TFA). This 1 mg/mL of solution was used to make the standards with methanol and acetonitrile: water (70:30) at 40:60 ratio to prepare the standards (0.025, 0.05, 0.1, 0.2, 0.3, 0.4 mg/mL). The LC system consisted of a LC-20AT pump, SPD-20AC auto injector, and C-R3A integrator (Shimadzu Scientific Instruments, Inc.) and SPD-M20A UV/Vis detector. The analysis conditions were a flow rate of 0.3 mL/min, 10uL injection volume, with 2.1 x 100 mm- 3.5-micron
Zorbax SB-C18 column, UV detection at $\lambda_{\text{max}}$ 204 nm. Methanol (0.1% TFA) and acetonitrile: water (70:30) (0.1% TFA) gradient utilized was: 50% methanol at 0 min, 60% methanol in 0-20 min, 60% methanol was held for 20-25 min, 50% methanol 25-27 min, 8 min column re-equilibration time. The C6 Cer retention time was: 6.5 min. The standard curve is provided in the results section (Fig 19B).

2.3 Measurement of size and surface charge

2.3.1 size measurement

5 $\mu$L of prepared nanoemulsion was diluted (2000x) in millipore water to make a final volume of 1 mL. Size measurement was performed at a scattering angle of 90° and measurement was made at 25 °C using a low volume disposable cuvette in a photon correlation spectrophotometer (Zetasizer, Nano S90, Malvern instruments, Worcestershire, UK). The size distribution data is an average of 12 measurements (Table 4).

2.3.2 Zeta ($\zeta$) potential Measurement

The surface charge ($\zeta$-potential) measurements were carried out using 10mM saline in disposable zeta cell. 10 $\mu$L of the prepared nanoemulsion was diluted (1000x) in 10 mM saline solution to make a final volume of 1 mL. $\zeta$-potential measurement was performed at 25 °C and viscosity of 0.911 cps using a Malvern Zeta sizer (Nano ZS, Malvern instruments, Worcestershire, UK). The $\zeta$ potential data is average of 5 measurements (Table 4).
Table 3 - Summary of C6 Cer and VB loading for NE 004-1 (C6Cer + VB Oleate) and NE 005-1 (VB Oleate)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>C6 Cer (mg/mL)</th>
<th>VB (mg/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>± SD</td>
<td>± SD</td>
</tr>
<tr>
<td>NE 004-1 (C6Cer +</td>
<td>3.63 ± 0.11</td>
<td>0.52 ± 0.0005</td>
</tr>
<tr>
<td>VB Oleate)</td>
<td></td>
<td></td>
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<tr>
<td>NE 005-1 (VB Oleate)</td>
<td>NA</td>
<td>0.45 ± 0.05</td>
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Table 4 Summary of the hydrodynamic size and ζ potential for NE 004-1 (C6Cer + VB Oleate) and NE 005-1 (VB Oleate)

<table>
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<tr>
<th>Formulation</th>
<th>Z (d.nm) ±</th>
<th>PDI ± SD</th>
<th>Int-Peak %</th>
<th>Vol-Peak %</th>
<th>ζ potential (mV ± SD)</th>
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<tbody>
<tr>
<td>C6 Cer + VB oleate</td>
<td>147.0 ±</td>
<td>0.100</td>
<td>162.8 ±</td>
<td>134.4 ±</td>
<td>-26.1 ±</td>
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<tr>
<td></td>
<td>1.1 ±</td>
<td>0.02</td>
<td>4.3</td>
<td>2.2</td>
<td>1.1</td>
</tr>
<tr>
<td>VB Oleate</td>
<td>161.6 ±</td>
<td>0.097</td>
<td>177.1 ±</td>
<td>153.8 ±</td>
<td>-22.1 ±</td>
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<tr>
<td></td>
<td>1.9 ±</td>
<td>0.03</td>
<td>4.5</td>
<td>3.0</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Note: Results are the average of at least 12 measurements for hydrodynamic size and 5 measurements for ζ potential. Standard deviations are given in parentheses. Z-Avg is the intensity-weighted average. Pdl is the polydispersity index. Int-Peak is the intensity-weighted average over the primary peak. % Int is the percentage of the intensity spectra occupied by the primary peak. Vol-Peak is the volume-weighted average over the primary peak. % Vol is the percentage of the volume spectra occupied by the primary peak.
Conclusion: Both the 004-1 (C6Cer + VB Oleate) and NE 005-1 (VB Oleate) nanoemulsions had high drug loading, nanoscale size, narrow size distributions and negative zeta potentials. C6 ceramide incorporation resulted in a nanoemulsion with a smaller size, 147.0 ± 1.1 vs. 161.6 ± 1.9, for NE 004-1 (C6Cer + VB Oleate) and NE 005-1 (VB Oleate), respectively.

Aspects of the compositions and methods disclosed herein are described below in the following numbered paragraphs:

1. A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer.

2. A method for treating cancer in a subject, comprising administering to a subject having a cancer susceptible to treatment with an autophagy mediating agent a therapeutically effective amount of a composition comprising an autophagy inducing agent and an autophagy inhibiting agent, thereby treating the cancer.

3. The method of paragraph 1 or 2, wherein the autophagy inducing agent is a ceramide and the autophagy inhibiting agent is a vinca alkaloid.

4. The method of paragraph 3, wherein the vinca alkaloid is vinblastine.

5. The method of paragraph 3 or 4, wherein the ceramide is a liposomal C6-ceramide.

6. The method of paragraph 1 or 2, wherein the autophagy inducing agent is selected from a ceramide, an mTOR inhibitor, temozolomide, arsenic trioxide, an Akt inhibitor, a lithium salt, a BH3 mimetic, loperamide, amiodarone, niguldipine,
pimozide, nicardipine, penitrem A, fluspirilene, trifluoperazine, or any combination thereof.

7. The method of any one of paragraph 1, 2 or 6, wherein the autophagy inhibiting agent is selected from a vinca alkaloid, a quinoline compound, 3-methyladenine, nocodazole, bafilomycin Al, 5-amino-4-imidazole carboxamide riboside (AICAR), okadaic acid, autophagy-suppressive algal toxins which inhibit protein phosphatases of type 2A or type 1, analogs of cAMP, and drugs which elevate cAMP levels, adenosine, N6-mercaptopurine riboside, wortmannin, or any combination thereof.

8. The method of any one of paragraphs 1 to 7, further comprising selecting for treatment a subject having a cancer susceptible to treatment with an autophagy mediating agent.

9. The method of paragraph 8, wherein the cancer responds to a combination of an autophagy inducing agent and an autophagy inhibiting agent.

10. The method of any one of paragraphs 1 to 9, wherein the cancer is hepatocarcinoma or colon cancer.

11. A method of inhibiting growth of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.

12. A method for inducing cytotoxicity of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.
13. A composition comprising (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell.

14. The composition of paragraph 13, wherein the autophagy inducing agent is a ceramide and the autophagy inhibiting agent is a vinca alkaloid.

15. The composition of paragraph 14, wherein the vinca alkaloid is vinblastine.

16. The composition of paragraph 14 or 15, wherein the ceramide is a liposomal C₆-ceramide.

17. The composition of paragraph 13, wherein the autophagy inducing agent is selected from a ceramide, an mTOR inhibitor, temozolomide, arsenic trioxide, an Akt inhibitor, a lithium salt, a BH₃ mimetic, loperamide, amiodarone, nigulidine, pimozone, nicardipine, penitrem A, fluspirilene, trifluoperazine, or any combination thereof.

18. The composition of paragraph 13 or 17, wherein the autophagy inhibiting agent is selected from a vinca alkaloid, a quinoline compound, 3-methyladenine, nocodazole, bafilomycin A₁, 5-amino-4-imidazole carboxamide riboside (AICAR), okadaic acid, autophagy-suppressive algal toxins which inhibit protein phosphatases of type 2A or type 1, analogs of cAMP, and drugs which elevate cAMP levels, adenosine, N6-mercaptopurine riboside, wortmannin, or any combination thereof.

19. A composition for treating cancer in a subject, comprising (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell.
20. The use of an autophagy inducing agent and an autophagy inhibiting agent in the manufacture of a medicament(s) for treating cancer in a subject.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention.
What is claimed is:

1. A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer.

2. A method for treating cancer in a subject, comprising administering to a subject having a cancer susceptible to treatment with an autophagy mediating agent a therapeutically effective amount of a composition comprising an autophagy inducing agent and an autophagy inhibiting agent, thereby treating the cancer.

3. The method of claim 1 or 2, wherein the autophagy inducing agent is a ceramide and the autophagy inhibiting agent is a vinca alkaloid.

4. The method of claim 3, wherein the vinca alkaloid is vinblastine.

5. The method of claim 3 or 4, wherein the ceramide is a liposomal C₆⁻ceramide.

6. The method of claim 1 or 2, wherein the autophagy inducing agent is selected from a ceramide, an mTOR inhibitor, temozolomide, arsenic trioxide, an Akt inhibitor, a lithium salt, a BH3 mimetic, loperamide, amiodarone, niguldipine, pimozide, nicardipine, penitrem A, fluspirilene, trifluoperazine, or any combination thereof.

7. The method of any one of claim 1, 2 or 6, wherein the autophagy inhibiting agent is selected from a vinca alkaloid, a quinoline compound, 3-methyladenine, nocodazole, bafilomycin Al, 5-amino-4-imidazole carboxamide riboside (AICAR), okadaic acid, autophagy-suppressive algal toxins which inhibit protein phosphatases.
of type 2A or type 1, analogs of cAMP, and drugs which elevate cAMP levels, adenosine, N6-mercaptopurine riboside, wortmannin, or any combination thereof.

8. The method of any one of claims 1 to 7, further comprising selecting for treatment a subject having a cancer susceptible to treatment with an autophagy mediating agent.

9. The method of claim 8, wherein the cancer responds to a combination of an autophagy inducing agent and an autophagy inhibiting agent.

10. The method of any one of claims 1 to 9, wherein the cancer is hepatocarcinoma or colon cancer.

11. A method of inhibiting growth of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.

12. A method for inducing cytotoxicity of cancer cells, comprising co-administering to the cancer cells a therapeutically effective amount of a ceramide sufficient to induce autophagy in the cancer cells and a therapeutically effective amount of a vinca alkaloid sufficient to inhibit autophagy in the cancer cells.

13. A composition comprising (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell.

14. The composition of claim 13, wherein the autophagy inducing agent is a ceramide and the autophagy inhibiting agent is a vinca alkaloid.
15. The composition of claim 14, wherein the vinca alkaloid is vinblastine.

16. The composition of claim 14 or 15, wherein the ceramide is a liposomal C\textsubscript{6} - ceramide.

5

17. The composition of claim 13, wherein the autophagy inducing agent is selected from a ceramide, an mTOR inhibitor, temozolomide, arsenic trioxide, an Akt inhibitor, a lithium salt, a BH3 mimetic, loperamide, amiodarone, niguldipine, pimozide, nicardipine, penitrem A, fluspirilene, trifluoperazine, or any combination thereof.

18. The composition of claim 13 or 17, wherein the autophagy inhibiting agent is selected from a vinca alkaloid, a quinoline compound, 3-methyladenine, nocodazole, bafilomycin A1, 5-amino-4-imidazole carboxamide riboside (AICAR), okadaic acid, autophagy-suppressive algal toxins which inhibit protein phosphatases of type 2A or type 1, analogs of cAMP, and drugs which elevate cAMP levels, adenosine, N6-m mercaptopurine riboside, wortmannin, or any combination thereof.

19. A composition for treating cancer in a subject, comprising (i) a therapeutically effective amount of an autophagy inducing agent sufficient to induce autophagy in a cancer cell and (ii) a therapeutically effective amount of an autophagy inhibiting agent sufficient to inhibit autophagy in the cancer cell.

20. The use of an autophagy inducing agent and an autophagy inhibiting agent in the manufacture of a medicament(s) for treating cancer in a subject.

21. A composition comprising an emulsion comprising:
   (i) a vinca alkaloid and a pharmaceutically acceptable amphiphilic agent associated together in the form of a hydrophobic ion pairing; and
   (ii) a ceramide.
22. The composition of claim 21, wherein component (i) and component (ii) are both in an organic phase of the emulsion.

23. The composition of claim 21 or 22, wherein the emulsion is an oil in water emulsion.

24. The composition of any one of claims 21 to 23, wherein the amphiphilic agent is selected from an alkanoate, an alkenoate, an alkylphosphoric acid, taurodeoxycholate, taurocholate, lauryl sulfate, octadecansulfonate, dioctylsulfosuccinate, 1-hydroxy-2-naphthoic acid, sodium dodecyl sulfate (SDS), bis-(2-ethylhexyl) sodium sulfosuccinate (AOT), cholesterol sulfate, sodium laurate, or a mixture thereof.

25. The composition of any one of claims 21 to 24, wherein the vinca alkaloid is vinblastine and the ceramide is a C₆ ceramide.

26. The composition of any one of claims 21 to 25 for use in treating cancer.

27. The composition of any one of claims 21 to 26, wherein the vinca alkaloid and the ceramide are each present in a therapeutically effective amount to treat cancer.

28. A method for treating cancer in a subject, comprising administering to a subject in need thereof a therapeutically effective amount of the composition of any one of claims 21 to 25.
FIG. 5A

Acetaminophen (APAP) – Positive control
FIG. 6

NC  NCL170  Vinblastine  NCL170 + Vin

LC3-I (16kDa)  
LC3-II (14kDa)  
P62  
B-actin

PC – serum-starvation
NC – complete media
FIG. 14

- Vinblastine 24 h
- C6-ceramide + vinblastine 24 h

% Control (Caspase 3/7 activity)

500 450 400 350 300 250 200 150 100 50 0

0.002 0.004 0.008 0.015 0.031 0.062 0.12 0.25 0.5 1
B) NE 005-1 (VB Oleate)
FIG. 19A

\[ Y = 4E+07 \times -44310 \]

\[ R^2 = 0.9999 \]

Area

0

1000000
2000000
3000000
4000000
5000000
6000000
7000000
8000000
9000000
10000000

VB base (mg/mL)

0

0.1
0.2
0.3
FIG. 19B

Y = 2E+07x + 66228
R² = 0.9984

C6-Ceramide Std...

Area

0 0.05 0.1 0.15 0.2 0.25

C6-Cer conc. (mg/mL)
## Classification of Subject Matter


## A. Classification of Subject Matter

- INV. A61K31/164
- INV. A61K31/475
- INV. A61K45/06
- INV. A61K8/14
- INV. A61K9/10
- INV. A61K9/107

## B. Fields Searched

Minimum documentation searched (classification system followed by classification symbols)

- A61K

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 practicable, search terms used)

- EPO-Internal
- BIOSIS
- EMBASE
- WPI Data

## C. Documents Considered to be Relevant

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>X</td>
<td>wo 00/59517 AI (ROGER WILLIAMS HOSPITAL [US]; WANEBO HAROLD J [US]; MEHTA SHASHI KANT []) 12 October 2000 (2000-10-12) page 64, lines 25-29</td>
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<tr>
<td>Y</td>
<td>page 11, paragraph 4 page 10, paragraph 4</td>
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- Further documents are listed in the continuation of Box C.
- See patent family annex.

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "B" earlier application or patent 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

**Date of the actual completion of the international search**

18 July 2012

**Date of mailing of the international search report**

24/07/2012

**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

Strack, Eberhard
INTERNATIONAL SEARCH REPORT

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.:

   21-28(completely) ; 1-20(partially)

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. :

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.
### DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>WO 01/22937 A1 (SONUS PHARMA INC [US]; CONSTANTINIDES PANAYIOTIS [US]; LAMBERT KAREL [:] 5 April 2001 (2001-04-05) claims 1,8-10, 18,25,34-36 page 3, paragraphs 3,4</td>
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<td>KR 100 891 278 B1 (KOREA INST SCI &amp; TECHNOLOGY) 6 April 2009 (2009-04-06) paragraphs [0001], [0078], [0081]</td>
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Form PCT/ISA/210 (patent family annex) (April 2008)
This International Search Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inducing agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is a ceramide

---

2. Claims: 1, 2, 6-10, 13, 17-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inducing agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is an mTOR inhibitor

---

3. Claims: 1, 2, 6-10, 13, 17-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inducing agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is temozolomide

---

4. Claims: 1, 2, 6-10, 13, 17-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inducing agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is arsenic trioxide

---

5. Claims: 1, 2, 6-10, 13, 17-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inducing agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is...
an Akt inhibitor

6. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is a Li salt

7. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is a BH3 mimetic

8. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is loperamide, pimozone, fluspiridine

9. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is amiodarone

10. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is
11. claims: 1, 2, 6-10, 13, 17-20 (all 1 partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administration to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is a pentretem A

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12. claims: 1, 2, 6-10, 13, 17-20 (all 1 partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administration to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inducing agent is a trifluoperazine

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13. claims: 1-20 (partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administration to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is a vinca alkaloid

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14. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administration to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is a quinoline

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15. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administration to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent
16. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is nocodazole ---

17. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is bafilomycin ---

18. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is 5-amino-4-imidazole carboxamide riboside ---

19. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is okadaic acid ---

20. claims: 1, 2, 6-10, 13, 17-20 (all partially)

A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy mediating agent, the method comprising co-administering to the subject an autophagy inducing agent and an autophagy inhibiting agent in amounts that together are effective in treating the cancer, wherein the autophagy inhibiting agent is 3-methyl adenosine, analogs of cAMP, drugs which elevate cAMP levels, adenosine, N6-mercaptopurine riboside ---
treati ng the cancer, wherei n the autophagy i nhibi ti ng agent
i s a protei n phosphatase of type 2A or type 1

21. c laims: 1, 2, 6-10, 13, 17-20(all p arti al ly)

A method for treati ng cancer i n a subject, wherei n the
cancer i s suscepti ble to treatment wi th an autophagy
medi ati ng agent, the method compri si ng co-admi ni steri ng to
the subject an autophagy induci ng agent and an autophagy
i nhibi ti ng agent i n amounts that together are effecti ve i n
treati ng the cancer, wherei n the autophagy i nhibi ti ng agent
i s wortmanni n

22. c laims: 21-28

A composi ti on compri si ng an emul si on compri si ng: (i) a vi nca
alkaloi d and a pharmaceuti cal ly acceptabl e amphi phi li c agent
associ ated together i n the form of a hydrophobi c
pair i ng; and (ii) a cerami de.