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(54) **VACUOLE-INDUCING COMPOUNDS, METHODS OF MAKING AND USING THE SAME**

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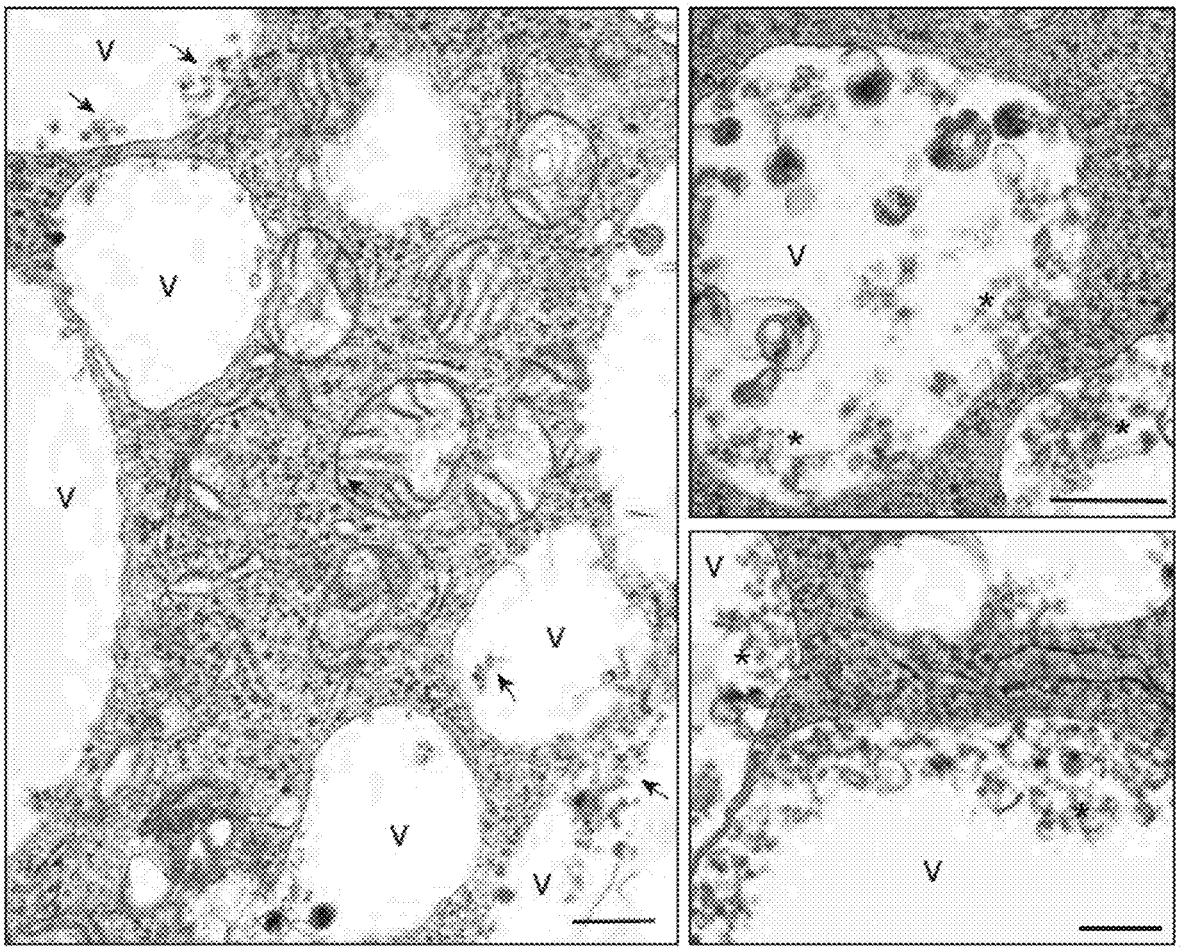
(52) **U.S. Cl.**

CPC ***A61K 31/4439*** (2013.01); ***A61K 31/5377*** (2013.01); ***A61K 31/53*** (2013.01)

(57) **ABSTRACT**

Vacuole- or exosome-inducing indole-based chalcone and substituted triazole-hydrazone compounds that induce endosomal vacuolization and increase exosome yield, but do not trigger growth arrest or cell death, and methods of making and using are described.



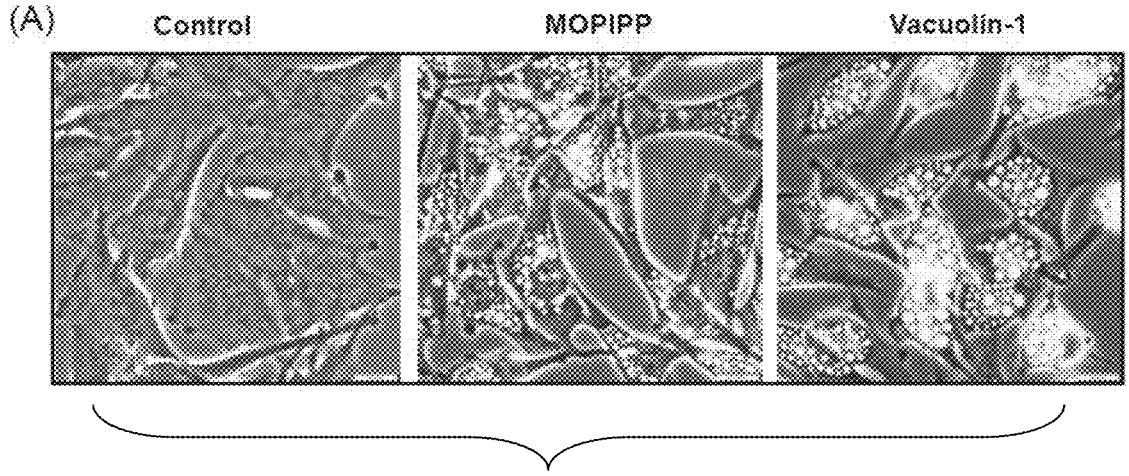


FIG. 1A

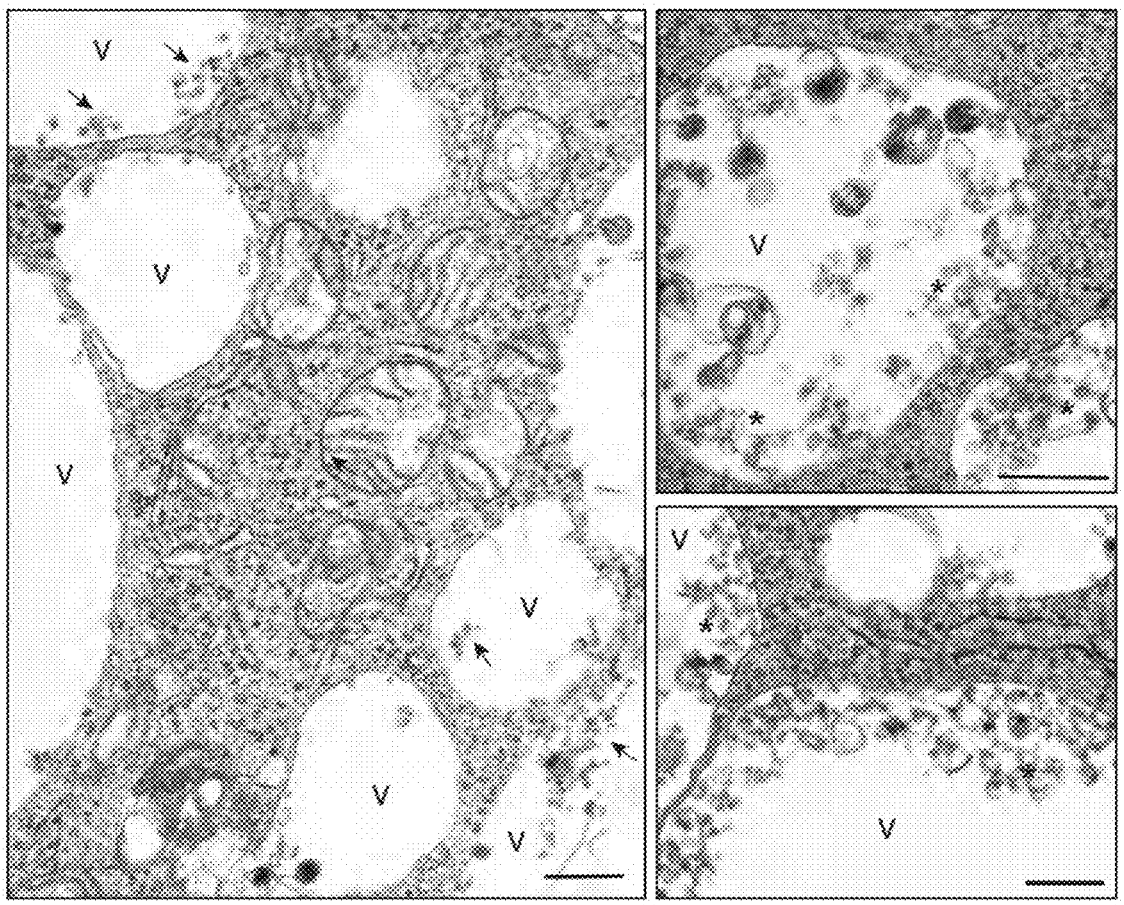


FIG. 1B

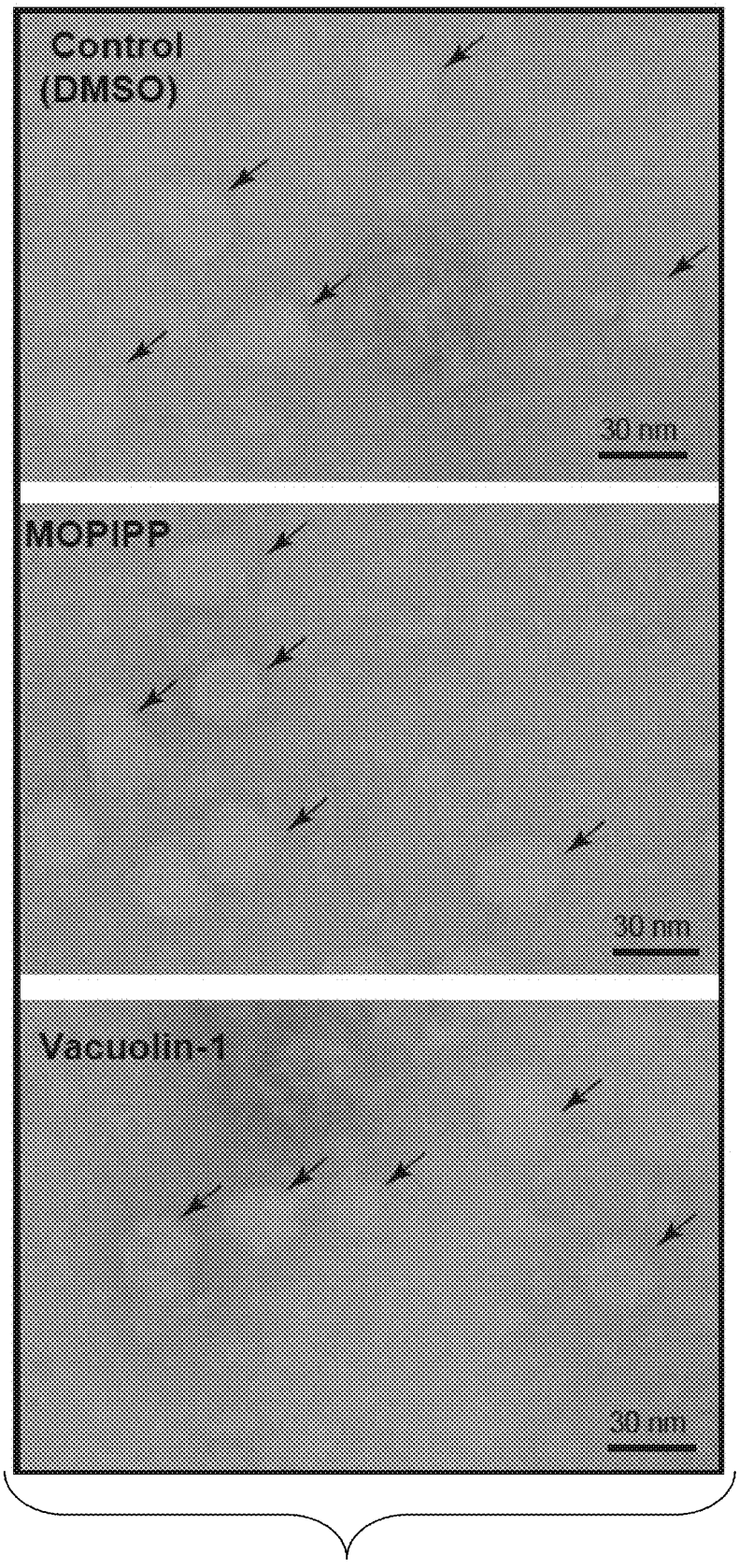


FIG. 1C

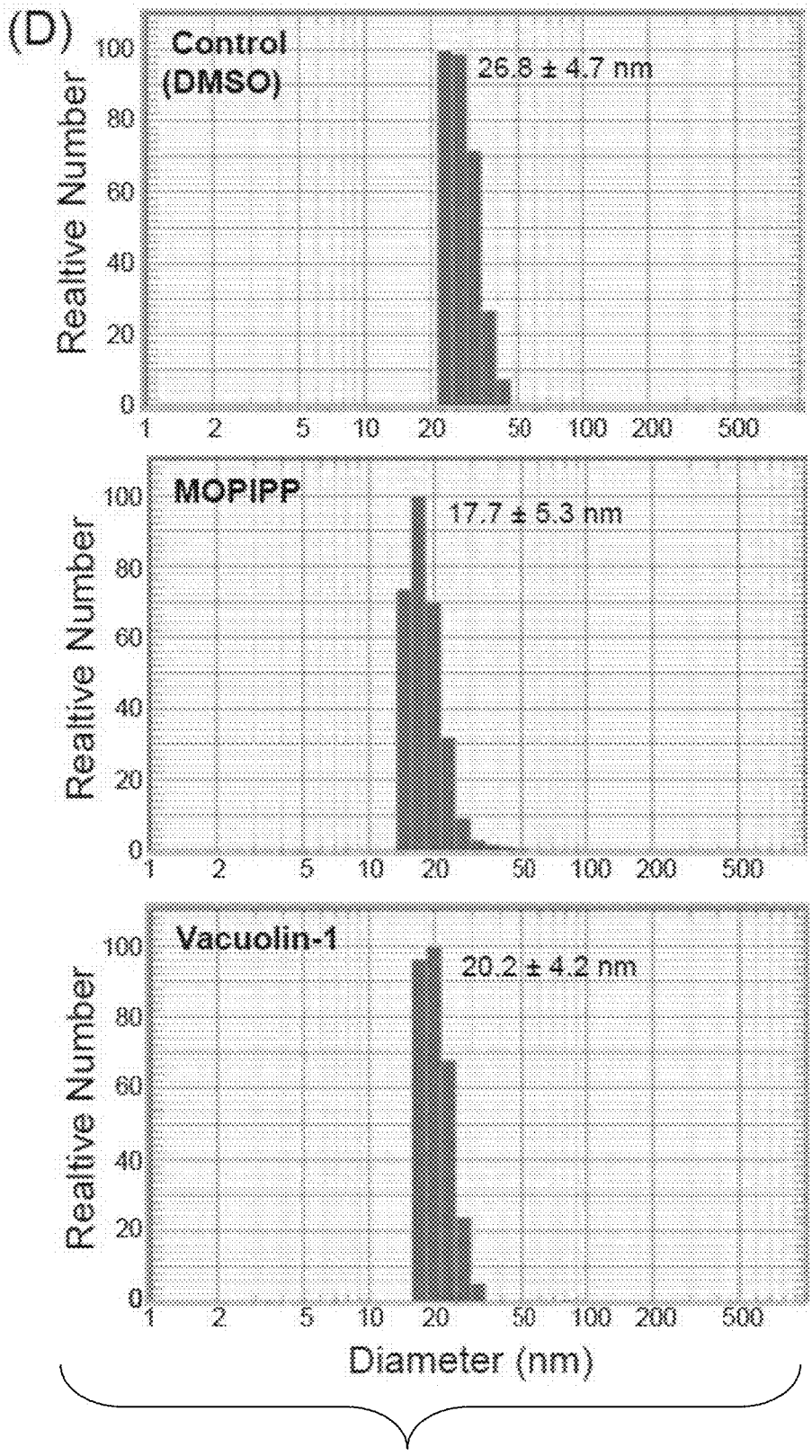

(D)
Control
(DMSO)
26.8 ± 4.7 nm
MOPIPP
17.7 ± 5.3 nm
Vacuolin-1
20.2 ± 4.2 nm
Realtive Number
0
20
40
60
80
100
1
2
5
10
20
50
100
200
500
Diameter (nm)

FIG. 1D

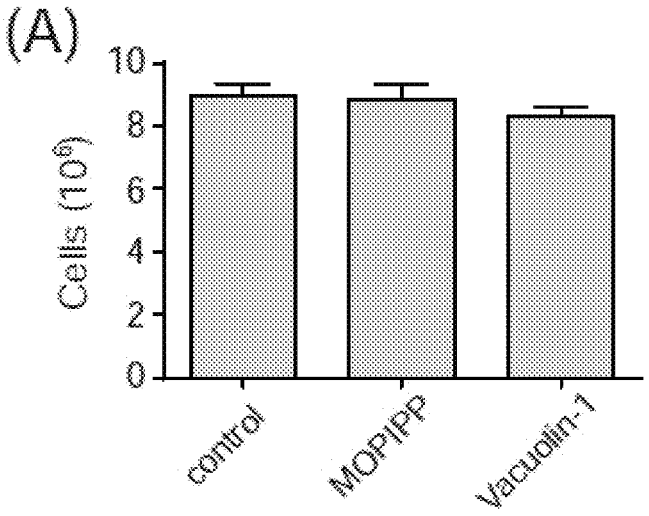

(A)
Cells (10^6)
10
8
6
4
2
0
control
MOPIPP
Vacuolin-1

FIG. 2A

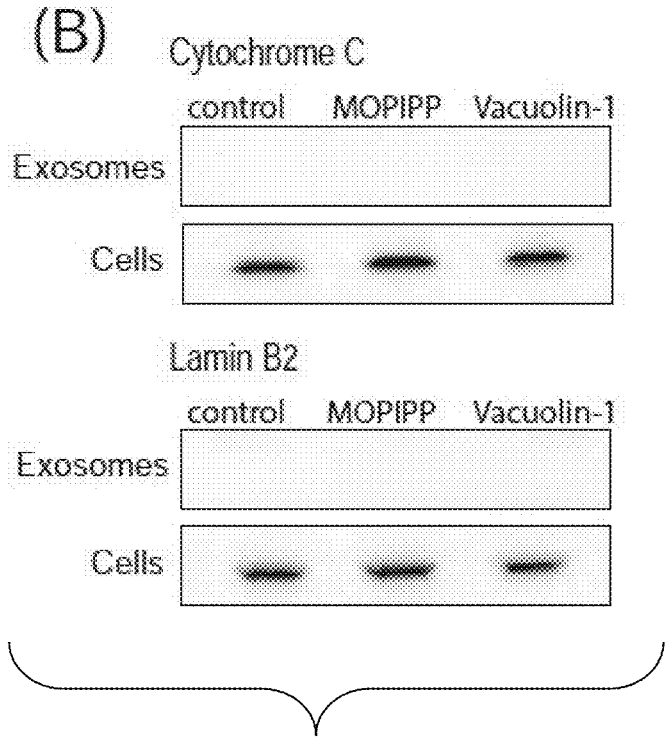

(B)
Cytochrome C
control
MOPIPP
Vacuolin-1
Exosomes
Cells
Lamin B2
control
MOPIPP
Vacuolin-1
Exosomes
Cells

FIG. 2B

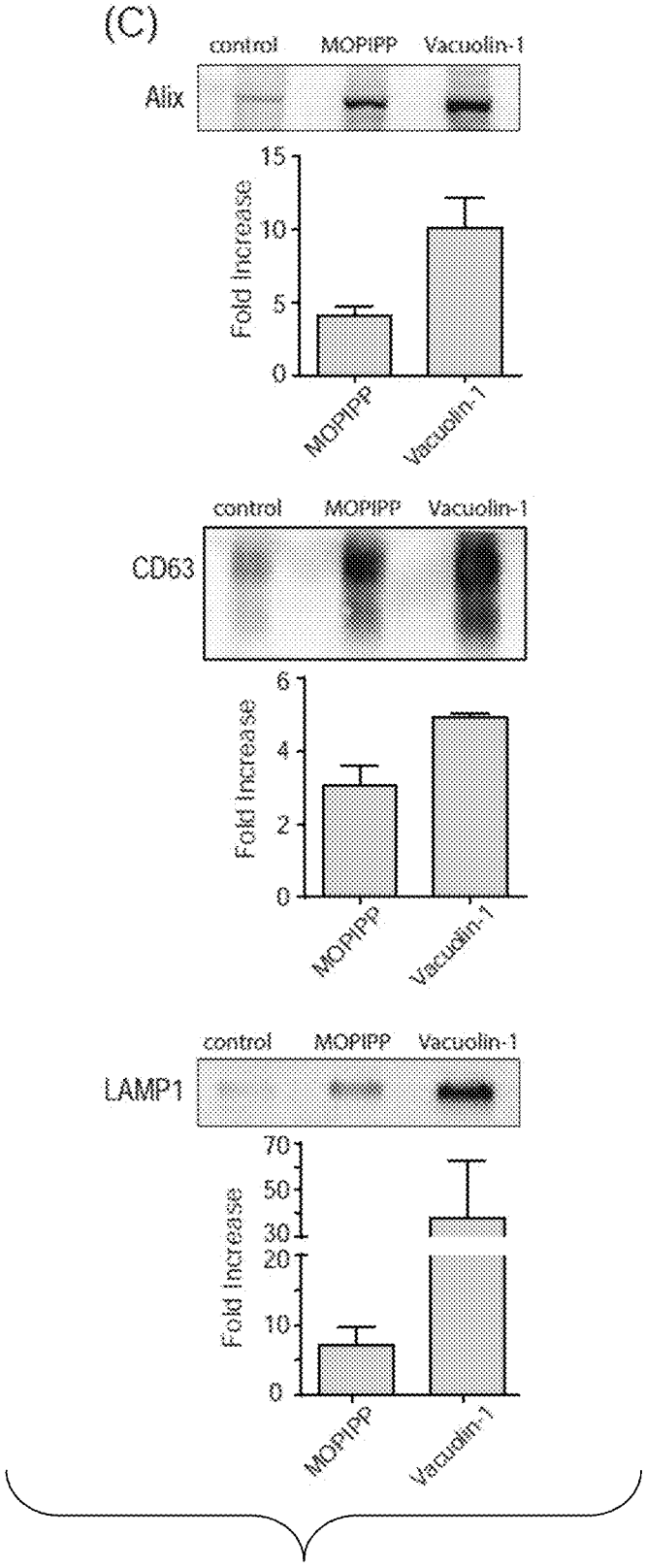

(C)
control
MOPIPP
Vacuolin-1
Alix
Fold Increase
15
10
5
0
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1
CD63
Fold Increase
6
4
2
0
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1
LAMP1
Fold Increase
70
50
30
20
10
0
MOPIPP
Vacuolin-1

FIG. 2C

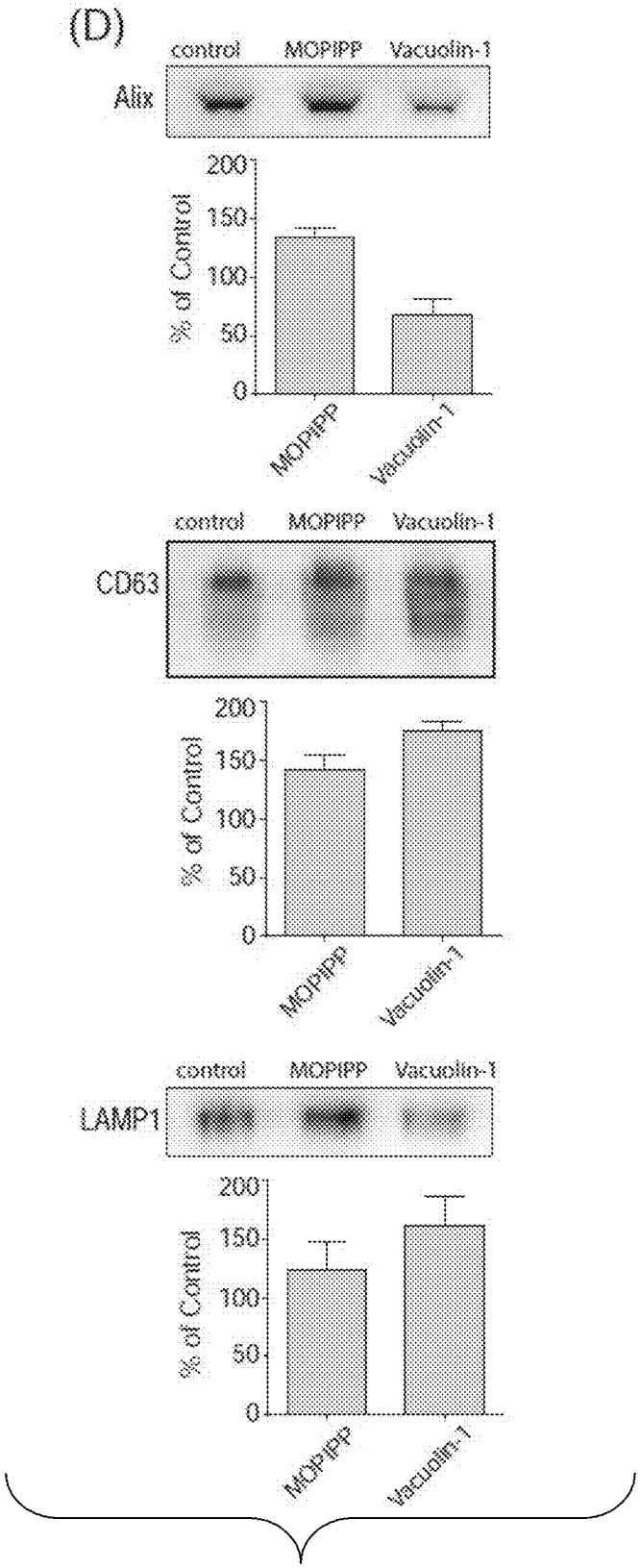

(D)
control
MOPIPP
Vacuolin-1
Alix
200
150
100
50
0
% of Control
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1
CD63
200
150
100
50
0
% of Control
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1
LAMP1
200
150
100
50
0
% of Control
MOPIPP
Vacuolin-1

FIG. 2D

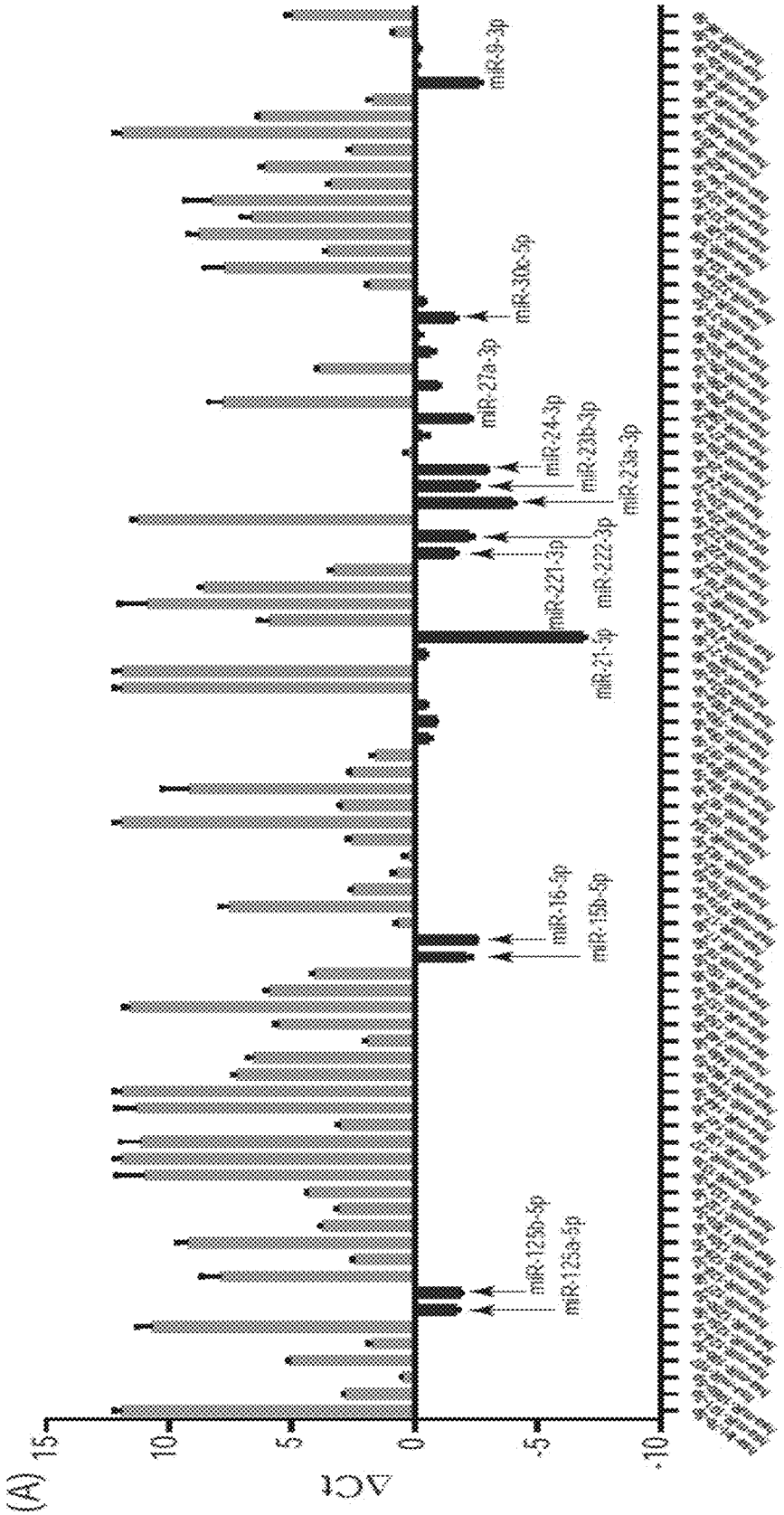

(A)
ΔCt
15
10
5
0
-5
-10
miR-9-3p
miR-30c-5p
miR-27a-3p
miR-24-3p
miR-22b-3p
miR-23a-3p
miR-222-3p
miR-221-3p
miR-21-3p
miR-16-2p
miR-15b-5p
miR-125b-5p
miR-125a-5p

FIG. 3A

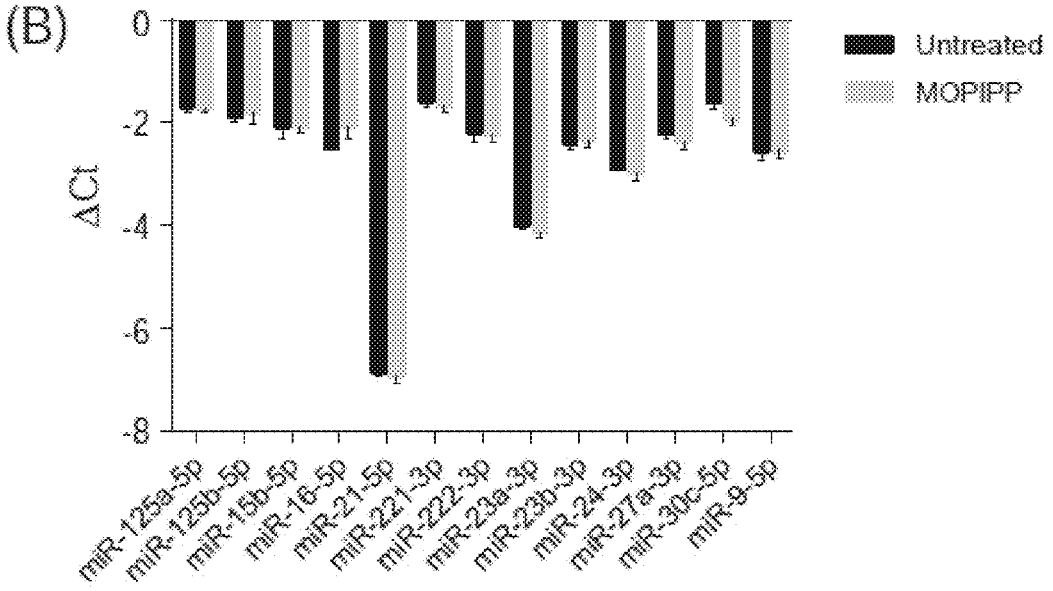

(B)
0
-2
-4
-6
-8
ΔCt
Untreated
MOPIPP
miR-125a-5p
miR-125b-5p
miR-16b-5p
miR-16-5p
miR-21-5p
miR-221-3p
miR-222-3p
miR-23a-3p
miR-23b-3p
miR-24-3p
miR-27a-3p
miR-30c-5p
miR-9-5p

FIG. 3B

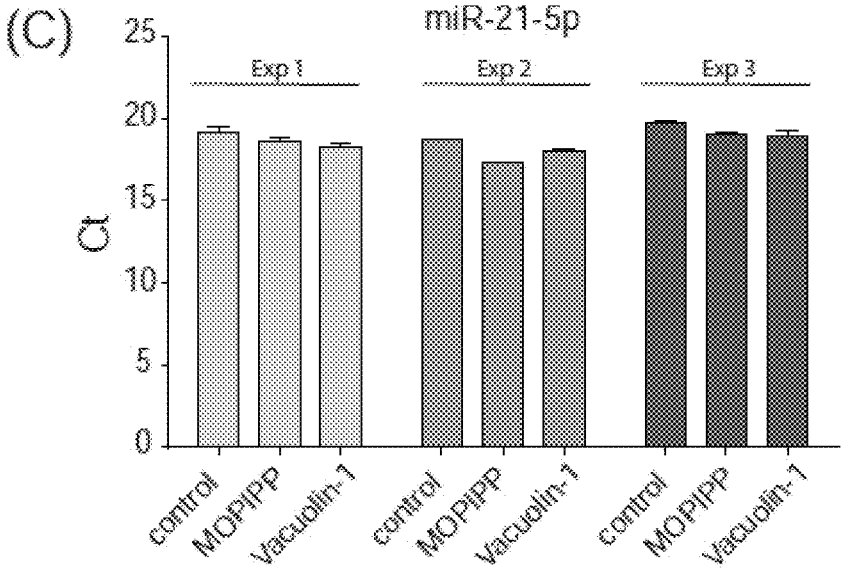

(C)
miR-21-5p
25
20
15
10
5
0
Ct
Exp 1
Exp 2
Exp 3
control
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1
control
MOPIPP
Vacuolin-1

FIG. 3C

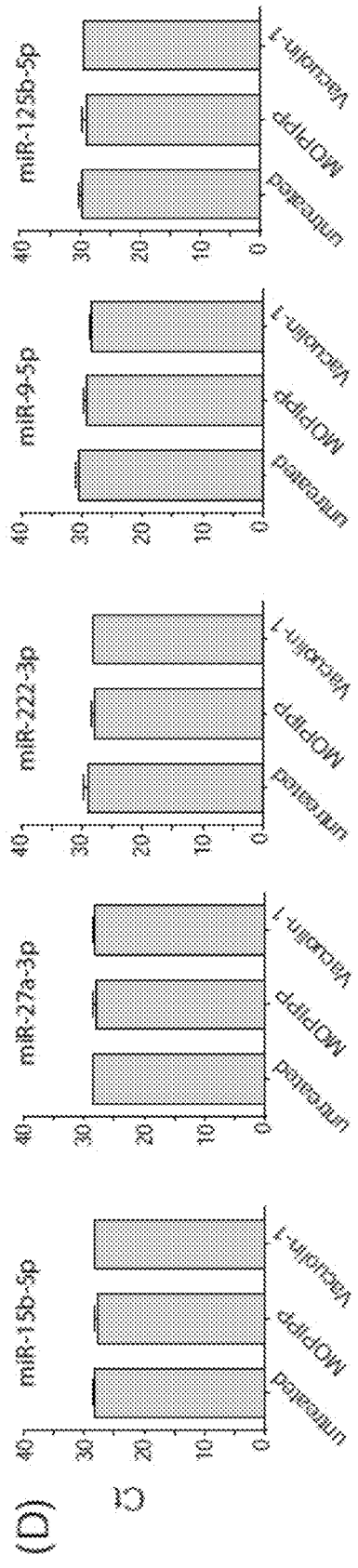

(D)
miR-15b-5p
miR-27a-3p
miR-222-3p
miR-9-5p
miR-126b-5p
0
10
20
30
40
untreated
MDPPP
Vasculotin-1

FIG. 3D

VACUOLE-INDUCING COMPOUNDS, METHODS OF MAKING AND USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Ser. No. 62/458,660 filed Feb. 14, 2017, the entire disclosure of which is expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] The invention was made with U.S. Government support under grant number R01 CA114595 awarded by the National Institutes of Health. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Exosomes are vesicles with diameters in the range of 30-120 nm, which are released from many types of cells. Exosomes originate as intralumenal vesicles (ILVs) within multivesicular late endosomes (MVEs). The vesicles are released into the extracellular environment upon fusion of MVEs with the plasma membrane. MVEs are commonly viewed as occupying an intermediate position between early endosomes and lysosomes in the endolysosomal degradative pathway of eukaryotic cells. Several distinct multiprotein ESCRTs (endosomal sorting complexes required for transport) function in MVE biogenesis. Proteins displaying monoubiquitin signals interact with the ESCRTs and are sorted into the intralumenal vesicles, which are ultimately degraded when the MVEs merge with lysosomes. While exosomes contain proteins typically found in ILVs, there is some evidence that they may be derived from functionally distinct subpopulations of MVEs that are routed to the plasma membrane instead of the lysosomes. The mechanisms that control the trafficking of MVEs to the cell surface and the release of exosomes are not well understood. Nevertheless, several common factors appear to influence this process, including ceramide levels, intracellular calcium, macroenvironmental pH and specific Rab GTPases.

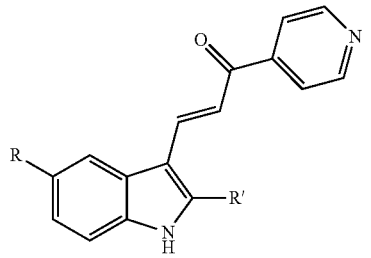
[0004] Despite intense interest in the potential diagnostic and therapeutic applications of exosomes, small molecules that can either inhibit or stimulate exosome production without affecting cell growth or viability are lacking. Many compounds that perturb endolysosomal vesicle trafficking and induce vacuolization of late endosomal compartments have been identified. However, little is known about the possible effects of such compounds on exosome biogenesis because many of them are cytotoxic.

[0005] It would be useful to have small molecules that can either inhibit or stimulate exosome production without affecting cell growth or viability.

SUMMARY OF THE INVENTION

[0006] Provided herein a method for increasing exosome production in a cell by exposing the cell to an indole-pyridinyl-propenone compound that induces endosomal vacuolization but does not trigger growth arrest or cell death.

[0007] In certain embodiments, the indole-pyridinyl-propenone compound has the following chemical structure:



[0008] where R═OH, OCH_3, OCH_2CH_3, $OCH(CH_3)_2$, $NHCOCH_3$ or $NHCO_2C(CH_3)_3$; and

[0009] R'═H, CH_3, $CH_2CH_2CH_3$, $CH(CH_3)_2$, or $CH_2CH(CH_3)_2$,

[0010] excluding where R═OCH_3 and R'═CH_3.

[0011] In certain embodiments, the indole-pyridinyl-propenone compound has the formula where: R═OCH_3; and, R'═H, $CH_2CH_2CH_3$, $CH(CH_3)_2$, or $CH_2CH(CH_3)_2$.

[0012] In certain embodiments, the indole-pyridinyl-propenone compound has the formula where: R═OCH_3, and R'═$CH_2CH_2CH_3$ (i.e., 3-(5-methoxy-2-propyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (MOPIPP).

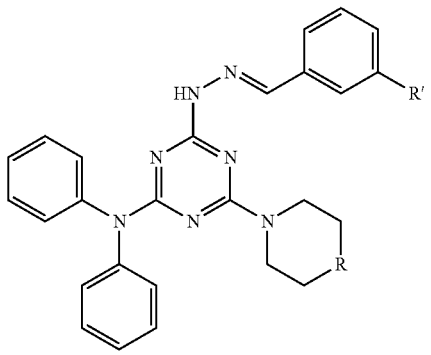
[0013] In certain embodiments, the indole-pyridinyl-propenone compound has the formula where: R═OH, OCH_2CH_3, $OCH(CH_3)_2$, $NHCOCH_3$ or $NHCO_2C(CH_3)_3$; and, R'═CH_3.

[0014] In certain embodiments, the indole-pyridinyl-propenone compound has the formula where: R═OCH_2CH_3; and, R'═CH_3.

[0015] In certain embodiments, the indole-pyridinyl-propenone compound has the formula where: R═OCH_2CH_3; and R'═$CH_2CH_2CH_3$.

[0016] In another aspect, there is provided a method for increasing exosome production in a cell culture, comprising: exposing the culture to a substituted triazole-hydrazone compound that induces endosomal vacuolization but does not trigger cell death or attenuate cell growth.

[0017] In certain embodiments, such substituted triazole-hydrazone compound has the following chemical structure:



[0018] wherein R═CH_2, NH, O; and R'═an electron withdrawing group. Non-limiting examples of electron withdrawing groups include: halides (F, Cl, Br and I), trifluoromethyl (CF_3), nitro (NO_2), acetyl (—$COCH_3$) and

trimethylammonium ($N(CH_3)_3$ wherein this becomes positively charged when further attached as a substituent. Also, in certain embodiments, the substituted triazole-hydrazone compound has the formula where R═O; and R'=Iodo.

[0019] Also described herein is a method where exosomes are collected exosomes by one or more of the following: ultracentrifugation; column chromatography; size exclusion; and filtration through a device containing an affinity matrix that is selective toward exosomes.

[0020] Also described is a method using such exosomes by harvesting the exosomes for use as nanocarriers for packaging and delivery of a therapeutic material to a subject in need thereof.

[0021] In certain embodiments, the therapeutic material comprises one or more of: small molecules, mRNAs, and proteins.

[0022] In certain embodiments, the cell comprises one or more of: multipotent mesenchymal stromal cells; stem cells; and, cancer cells. Further, in certain embodiments, the cell is a mammalian cell.

[0023] Also described herein is a method of producing transiently expressed miRNA containing cells, comprising: transfecting a cell population of cells capable of producing exosomes produced according to the method described herein with one or more plasmids encoding miRNA; harvesting cells from the cell population after transfection; and, confirming the presence of the miRNA in one or more of the harvested cells.

[0024] Also described herein is a method of treating a subject in need thereof with modified exosomes, comprising: transfecting exosome-producing cells produced by the method described herein, with one or more carriers encoding a therapeutic material; harvesting the exosomes from the cell population or media containing same after transfection; confirming the presence of the therapeutic material in one or more of the harvested exosomes; and, administering to the subject one or more of the harvested exosomes in a pharmaceutically effective amount to treat the subject.

[0025] Other systems, methods, features, and advantages of the present invention will be or will become apparent to one with skill in the art upon examination of the following drawings and detailed description. It is intended that all such additional systems, methods, features, and advantages be included within this description, be within the scope of the present invention, and be protected by the accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The patent or application file may contain one or more drawings executed in color and/or one or more photographs. Copies of this patent or patent application publication with color drawing(s) and/or photograph(s) will be provided by the Patent Office upon request and payment of the necessary fee.

[0027] FIGS. **1**A-**1**D—Comparison of cell and exosome morphologies in cultures treated with MOPIPP and vacuolin-1. (FIG. **1**A) U251 cells were examined by phase contrast microscopy after 24 h treatment with 10 μM MOPIPP, 1 μM vacuolin-1 or an equivalent volume of DMSO (control). Scale bars=20 μm. (FIG. **1**B) Electron micrographs of U251 cells after 24 h treatment with MOPIPP show many large vacuoles (v), with some containing clusters of ILVs (asterisks). Scale bars=500 nm. (FIG. **1**C and FIG. **1**D) Exosomes isolated from cells treated for 24 h with the indicated compounds were characterized by electron microscopy (FIG. **1**C) or DLS (FIG. **1**D).

[0028] FIGS. **2**A-**2**D—MOPIPP and vacuolin-1 increase the amounts of exosomal marker proteins in vesicle fractions recovered from conditioned medium. In three separate experiments, U251 cells were treated for 24 h with 10 μM MOPIPP, 1 μM vacuolin-1 or an equivalent volume of DMSO vehicle. The cells from each experiment were counted (mean±SEM) (FIG. **2**A), and the medium from the same cultures was used to prepare exosomes with the Exo-spin™ Purification method. Equal aliquots of the final exosome preparations were subjected to western blot analysis for the indicated proteins (FIG. **2**B and FIG. **2**C). In FIG. **2**C, representative blots are shown, and the fold-increase in the treated cells relative to the controls is graphed below each blot (mean±SEM). The cells from the same experiments (see FIG. **2**A), were subjected to western blot analysis for the indicated proteins, with equal amounts of protein loaded on each lane (FIG. **2**B and FIG. **2**D). In FIG. **2**D, representative blots are shown, with signals for the proteins in the treated cells expressed as percent of the corresponding DMSO controls in the graphs below each blot (mean±SEM).

[0029] FIGS. **3**A-**3**D—MOPIPP or vacuolin-1 do not have major effects on the profile of miRNAs represented in exosomes. (FIG. **3**A) Cellular miRNAs expressed in untreated U251 cells were profiled using the Human Brain Cancer miScript® arrays (n=3) and ΔCt values were determined using the six snoRNA/snRNA miScript PCR controls included on each array. The specific miRNAs selected for further study are indicted with arrows. (FIG. **3**B) U251 cells were treated with 10 μM MOPIPP or an equivalent volume of DMSO for 24 h and cellular expression of each of the indicated miRNAs was determined by RT-PCR (n=3). (FIG. **3**C) In three separate experiments, exosomes were isolated from U251 cells treated with MOPIPP, vacuolin-1 or DMSO and equal amounts of exosomal RNA were subjected to reverse transcription and RT-PCR to assess miR-21-5p. (FIG. **3**D) RT-PCR comparisons of five additional miRNAs, starting with equal amounts of exosomal RNA from cells treated with MOPIPP, vacuolin-1 or DMSO.

DETAILED DESCRIPTION

[0030] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

[0031] Definitions

[0032] In general, the terms and phrases used herein have their art-recognized meaning, which can be found by reference to texts, journal references and contexts known to those skilled in the art.

[0033] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and equivalents thereof known to those skilled in the art, and so forth. As well, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. The expression "of any of claims XX-YY" (wherein XX and YY refer to claim numbers) is intended to

provide a multiple dependent claim in the alternative form, and in some embodiments is interchangeable with the expression "as in any one of claims XX-YY."

[0034] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0035] Every formulation or combination of components described or exemplified herein can be used to practice the invention, unless otherwise stated.

[0036] When a group of substituents is disclosed herein, it is understood that all individual members of that group and all subgroups, including any isomers and enantiomers of the group members, are disclosed separately. When a Markush group or other grouping is used herein, all individual members of the group and all combinations and subcombinations possible of the group are intended to be individually included in the disclosure. It is intended that any one or more members of any Markush group or listing provided in the specification can be excluded from the invention if desired. When a compound is described herein such that a particular isomer or enantiomer of the compound is not specified, for example, in a formula or in a chemical name, that description is intended to include each isomers and enantiomer of the compound described individual or in any combination. Additionally, unless otherwise specified, all isotopic variants of compounds disclosed herein are intended to be encompassed by the disclosure. For example, it will be understood that any one or more hydrogens in a molecule disclosed can be replaced with deuterium or tritium. Isotopic variants of a molecule are generally useful as standards in assays for the molecule and in chemical and biological research related to the molecule or its use. Specific names of compounds are intended to be exemplary, as it is known that one of ordinary skill in the art can name the same compounds differently.

[0037] Every formulation or combination of components described or exemplified herein can be used to practice the invention, unless otherwise stated.

[0038] All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing components that are described in the publications that might be used in connection with the presently described invention.

[0039] The definitions are provided to clarify their specific use in the context of the invention.

[0040] Additionally, described herein are pharmaceutically acceptable derivatives of the inventive compounds, and methods of treating a subject using these compounds, pharmaceutical compositions thereof, or either of these in combination with one or more additional therapeutic agents. The phrase, "pharmaceutically acceptable derivative", as used herein, denotes any pharmaceutically acceptable salt, ester, or salt of such ester, of such compound, or any other adduct or derivative which, upon administration to a patient, is capable of providing (directly or indirectly) a compound as otherwise described herein, or a metabolite or residue thereof.

[0041] The term "stable", as used herein, preferably refers to compounds which possess stability sufficient to allow manufacture and which maintain the integrity of the compound for a sufficient period of time to be detected and preferably for a sufficient period of time to be useful for the purposes detailed herein.

[0042] As used herein, the terms "patient" and "subject" are intended to include living organisms in which certain conditions as described herein can occur. Examples include humans, monkeys, cows, sheep, goats, dogs, cats, mice, rats, and transgenic species thereof. In a preferred embodiment, the patient is a primate. In an even more preferred embodiment, the primate is a human. Other examples of subjects include experimental animals such as mice, rats, dogs, guinea pigs, cats, goats, sheep, pigs, and cows. The experimental animal can be an animal model for a disorder, e.g., a transgenic mouse with cancer.

[0043] As used herein, the term "IC_{50}" refers to an inhibitory dose which is 50% of the maximum response obtained.

[0044] General Description

[0045] Various indolyl pyridinyl propenones are described in Maltese et al. U.S. Pat. No. 9,023,871 issued May 5, 2015; Maltese et al. U.S. Pat. No. 9,028,796 issued May 12, 2015; Maltese et al. U.S. Pat. No. 9,061,994 issued Jun. 23, 2015; Trabbic et al., "*Differential Induction of Cytoplasmic Vacuolization and Methuosis by Novel 2-Indolyl-Substituted Pyridinylpropenones,*" ACS Med. Chem. Lett., 5 (2014) 73-77; and, Trabbic et al., "*Synthesis and biological evaluation of indolyl-pyridinyl-propenones having either methuosis or microtubule disruption activity,*" J. Med. Chem., 58 (2015) 2489-2512, the entire contents of which are explicitly incorporated herein.

[0046] Various indolyl pyridinyl propenone compounds induce the formation of cytoplasmic vacuoles in cultured human cells without causing appreciable cytotoxicity or growth arrest.

[0047] Described herein are methods using the vacuole-inducing analogs of the indolyl pyridinyl propenone chemical family to stimulate an increase in exosome production in cultured cells without altering the cargo carried by these exosomes.

[0048] Also described herein are methods using the vacuole-inducing analogs of the indolyl pyridinyl propenone chemical family to stimulate an increase in exosome release by cultured cells without altering the cargo carried by these exosomes.

[0049] Also described herein are therapeutic applications using such exosomes; for example, using these exosomes as nanocarriers for packaging and delivery of a desired cargo to the cell. In certain embodiments, the cargo can include small molecules (e.g., anti-cancer drugs), miRNAs, and proteins.

[0050] These exosomes are useful in that they may incorporate various cargoes in producer cells.

[0051] The methods of production of these exosomes overcomes one of the major impediments to more widespread development of exosome-based therapies; that is, the low yield of exosomes.

[0052] For example, one compound 3-(5-methoxy-2-propyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (MOPIPP), and related molecules are useful as non-cytotoxic pharmacological agents to boost exosome production.

[0053] MOPIPP and related molecules are useful in enhancing large-scale commercial production of exosomes from cultured cells, particularly for therapeutic purposes.

[0054] The following examples show the use of cultured human glioblastoma cells to assess the effects of MOPIPP and vacuolin-1 on exosome production. The results show that both compounds may have utility as non-toxic agents to enhance the cellular release of exosomes.

EXAMPLES

[0055] The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. All publications, including patents and non-patent literature, referred to in this specification are expressly incorporated by reference. The following examples are intended to illustrate certain preferred embodiments of the invention and should not be interpreted to limit the scope of the invention as defined in the claims, unless so specified.

[0056] It is to be understood, that throughout the specification and claims herein, the terms "vacuole," "vesicle" and "exosome" generally refer to a space within the cytoplasm of a cell, enclosed by a membrane and typically containing fluid, which may also be released from the cell. For example, exosomes are generally described as small vesicles created and released from the plasma membrane of various types of cells, especially immune cells, and capable of inducing antigen-specific immune responses.

[0057] Materials and Methods

[0058] Cell Culture

[0059] U251 human glioblastoma cells were purchased from the DCT Tumor Repository (National Cancer Institute, Frederick, Md.). Stock cultures were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (JR Scientific, Woodland, Calif.) at 37° C. with an atmosphere of 5% CO_2 in air. Cultures were passaged for less than 6 months and were monitored periodically for Mycoplasma contamination.

[0060] Isolation of Exosomes

[0061] Prior to beginning studies, cells were seeded in 10 cm diameter culture dishes at 500,000 cells/dish and maintained for 24 h in DMEM supplemented with 10% Gibco™ exosome-depleted FBS (ThermoFisher Scientific, Waltham, Mass.). The medium was then replaced with fresh medium containing either 10 μM MOPIPP (synthesized as described in Trabbic et al., "Differential Induction of Cytoplasmic Vacuolization and Methuosis by Novel 2-Indolyl-Substituted Pyridinylpropenones, ACS Med. Chem. Lett., 5 (2014) 73-77) or 1 μM vacuolin-1 (Santa Cruz Biotechnology, Santa Cruz, Calif.) dissolved in DMSO. Control cultures contain an equivalent volume of the DMSO vehicle (0.1%) in the medium. After 24 h, the medium was collected from the dishes (typically 10-12 dishes per condition) and the attached cells were pooled and counted with a Coulter Counter (model Z1). Exosomes were isolated from medium using the Exo-spin™ Exosome Purification system (Cell Guidance Systems, St. Louis, Mo.). The medium was precleared by centrifugation at 4° C., first at 300×g for 10 min and then at 16,000×g for 30 min. Then a volume of Buffer A equal to half the volume of medium was added and the mixture was incubated overnight at 4° C. The precipitate, enriched with exosomes, was collected by centrifugation at 16,000×g for 1 h, and the pellet was re-suspended in 100 μl of Dulbecco's phosphate-buffered saline (PBS), pH 7.4. The material was applied to an Exo-Spin column equilibrated with PBS, and the purified exosomes were eluted in 200 μl of PBS.

[0062] Electron Microscopy

[0063] Aliquots of purified exosomes obtained from control and drug-treated cell cultures were fixed with 4% paraformaldehyde and stained with 2% uranyl acetate on Formvar carbon-coated electron microscopy grids. Vesicles were visualized using a Hitachi HD-2300 transmission electron microscope at an accelerating voltage of 200 kV. Electron microscopy of vacuolated cells was carried out (as described in Johnson et al. "Gene silencing reveals a specific function of hVps34 phosphatidylinositol 3-kinase in late versus early endosomes, J. Cell Sci., 119 (2006) 1219-1232).

[0064] Dynamic Light Scattering (DLS)

[0065] Exosomes suspended in PBS were subjected to DLS using a Nicomp 380 ZLS instrument (Particle Sizing Systems, Port Richey, Fla.). Samples were placed in the path of a helium neon laser of wavelength 658 nm at 23° C., and data were collected at a scattering angle of 90°. For each sample, three measurements of 8 min each were performed, and the particle size distribution (number-weighted diameter) was displayed.

[0066] Immunoblot Analysis

[0067] The cells in the dishes used for collection of exosomes were washed three times with PBS and lysed in SDS sample buffer (Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227 (1970) 680-685). The protein concentration was determined by colorimetric assay using Bio-Rad reagent (Bio-Rad, Richmond, Calif.). Samples containing equal amounts of total cell protein were subjected to SDS-PAGE and immunoblot analysis (as described in Maltese et al., "Retention of the Alzheimer's amyloid precursor fragment C99 in the endoplasmic reticulum prevents formation of amyloid beta-peptide, J. Biol. Chem., 276 (2001) 20267-20279). For analysis of proteins in exosomes, equal volumes of purified exosomes isolated from control or treated cells were mixed with ⅕ volume of 5×SDS sample buffer prior to SDS-PAGE. Monoclonal antibodies against CD63 and LAMP-1 were obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). Antibodies against Alix and cytochrome c were from Santa Cruz Biotechnology (Santa Cruz, Calif.), and the antibody against Lamin B_2 was from Cell Signaling Technology (Danvers, Mass.). HRP-coupled goat anti-mouse IgG was from BD Biosciences (San Jose, Calif.). Chemiluminescent signals on the membranes were quantified using an Alpha Innotech FluorChem HD2 imaging system with Alpha View software.

[0068] Analysis of miRNAs

[0069] Total RNA was extracted and purified from cultured cells or exosomes using QIAzol™ lysis reagent followed by RNAeasy Mini spin-columns, according to the manufacturer's protocol (SA Biosciences/Qiagen, Germantown, Md.). For each sample, cDNA was generated by reverse transcription of 150 ng of total RNA, using the miScript II RT kit (Qiagen). RNA and cDNA were quantified and checked for purity (OD 260/280) using a Nano-Drop -1000 spectrophotometer (ThermoFisher). For initial profiling of the miRNAs expressed in control or MOPIPP-treated

cells, equal amounts of cDNA were applied to Human Brain Cancer miScript® miRNA PCR arrays (MIHS-108Z) (SA Biosciences/Qiagen), and real-time PCR reactions were carried in an Applied Biosystems StepOne Plus™ system using SYBR Green master mix. Raw Ct values for the individual miRNAs were normalized to the average Ct value for six snoRNA/snRNA miScript PCR controls included on each array, yielding ΔCt values. For comparisons of the miRNA contents of exosomes collected from the medium of cells treated with MOPIPP, vacuolin-1 or vehicle (DMSO), individual miScript primers were purchased for six of the most highly expressed miRNAs detected in U251 cells (SA Biosciences/Qiagen). The primers were reconstituted in SYBR green master mix and combined with cDNAs derived from equal amounts of exosomal RNA (150 ng). RT-PCR reactions were carried out in triplicate to obtain Ct values for each miRNA.

[0070] Results

[0071] Characterization of Exosomes

[0072] Treatment of cultured U251 glioblastoma cells with MOPIPP resulted in vacuolization of endocytic compartments. The accumulation of numerous vacuoles was readily detected by phase contrast microscopy (FIG. **1**A). Treatment with vacuolin-1 induced a very similar phenotype (FIG. **1**A). Despite the extreme vacuolization, the cells treated with both compounds remained attached to the culture dishes and continued to proliferate. Electron microcopy revealed that most of the vacuoles in the cells treated with MOPIPP were surrounded by a single membrane and were largely devoid of intralumenal contents (FIG. **1**B). However, a distinct subpopulation of vacuoles (approximately 20-30%) contained clusters of heterogeneous vesicles, many of which were of a size (<50 nm) consistent with ILVs and exosomes (examples shown in FIG. **1**B).

[0073] When 24-h conditioned media were collected from control and drug-treated cultures and subjected to a procedure designed to yield purified exosomes, electron microscopy showed that the final exosome fraction consisted mainly of vesicles with diameters of approximately 25-30 nm (FIG. **1**C), matching the lower end of the size range reported for exosomes. There were no discernable differences in the morphologies of the vesicles obtained from the treated cells compared to the control. Particle analysis by DLS confirmed that the purified vesicle populations had unimodal distributions with mean diameters in the range of 18-27 nm. Differences between control and treated cells were not statistically significant (FIG. **1**D).

[0074] Quantification of Exosome Marker Proteins

[0075] To determine if treatment with MOPIPP and/or vacuolin-1 might alter the production of exosomes, conditioned medium was pooled from 10-12 control or treated cultures and exosomes were isolated as described above. The cells from the same cultures were also harvested and pooled. FIG. **2**A shows that comparable numbers of cells were present in the control and drug-treated cultures, consistent with the lack of growth inhibition by MOPIPP and vacuolin-1. The immunoblots in FIG. **2**B demonstrate that cytochrome c and lamin B_2, markers for mitochondria and nuclear envelope respectively, were not detectable in the purified exosome populations, confirming that the latter were not contaminated with intracellular organelles released via cell lysis. In FIG. **2**C and FIG. **2**D, the isolated exosomes and cells were probed for three proteins commonly enriched in MVEs and exosomes: CD63, a member of tetraspanin protein family, Alix, a protein involved in the biogenesis of endosomal ILVs, and LAMP-1, an abundant membrane glycoprotein in lysosomes and late endosomes.

[0076] The results show that the relative amounts of all three proteins were increased by several fold in the extracellular vesicle preparations from cultures treated with MOPIPP (FIG. **2**C). An even greater increase was observed in the cultures treated with vacuolin-1 (FIG. **2**C). In contrast, changes in expression of the same marker proteins in the corresponding cell populations were comparatively modest (FIG. **2**D). Since the exosomes were isolated from nearly identical numbers of cells in the control and treated cultures (FIG. **2**A), the results show that MOPIPP and vacuolin-1 promote an increase in the release of exosomes into the extracellular environment.

[0077] Comparison of miRNA Profiles

[0078] miRNA cargoes carried by exosomes play important roles in intercellular communication. As shown herein, it was then determined whether, in addition to increasing exosome output, MOPIPP and vacuolin-1 would alter the miRNA composition of the exosomes. miRNAs expressed in untreated U251 glioblastoma cells were profiled, using a RT-PCR array that detects 84 miRNAs commonly associated with human brain cancers. Based on their ΔCt values relative to a panel of six normalization controls (FIG. **3**A), 13 miRNAs with high expression were selected for further comparison between control and MOPIPP-treated cells.

[0079] As shown in FIG. **3**B, treatment with MOPIPP did not cause major changes in the cellular expression of any of the selected miRNAs. Among the miRNAs profiled on the array, miR-21-5p was clearly the most abundant. RT-PCR reactions were carried out with miR-21-5p primers to assess the relative amounts of miR-21-5p in exosomes collected from cells treated with MOPIPP or vacuolin-1, compared to untreated controls. Since the selection of a definitive RT-PCR standard for normalization of exosomal miRNA is controversial, triplicate reactions were set up with equal amounts of total exosomal RNA from control and treated cells and directly compared the raw Ct values (FIG. **3**C).

[0080] Using this approach for three independent experiments, there were no observations of any changes in the relative abundance of miR-21-5-p in exosomal RNA collected from cells treated with MOPIPP or vacuolin-1. Similar results were obtained when the analysis was extended to five additional miRNAs (FIG. **3**D).

[0081] Discussion

[0082] The results show that vacuolization of late endosomal compartments induced by two distinct small molecules, MOPIPP and vacuolin-1, is accompanied by a several-fold increase in exosomal marker proteins in extracellular vesicles prepared from comparable numbers of glioblastoma cells. Cell proliferation and viability were not markedly impaired by these compounds, and the characteristics of the exosomes from treated cells were similar to the controls. Since the intracellular amounts of the marker proteins were only modestly affected by the compounds, the results are indicative of an increase in exosome biogenesis and/or secretion in cells treated with MOPIPP and vacuolin-1.

[0083] Small molecules that promote vacuolization of late endosomal compartments have the potential to affect exosome production in several ways. In one scenario, vacuolization of endosomes could disrupt the molecular machinery for the formation of ILVs, so that any MVEs that

subsequently fuse with the plasma membrane release fewer exosomes. In a second scenario, formation of ILVs might remain unaffected, but trafficking of enlarged MVEs to the cell surface might be disrupted, also resulting in diminished exosome secretion. Finally, in a third scenario, drug-induced vacuoles derived from late endosomes could retain the ability to generate ILVs, but be unable to merge with lysosomes where the vesicles are degraded. In that case, enlarged MVEs might accumulate and deliver more ILVs to the extracellular environment, provided that they remain competent to fuse with the plasma membrane. It is now believed that the third scenario explains the increase in exosome secretion (FIG. **2**), considering that many vacuoles in MOPIPP-treated cells contained numerous ILVs (FIG. **1**B).

[0084] The miRNA cargo carried by exosomes may differ from the miRNA profile of the producer cell, implying that segregation of miRNAs into exosomes is not random. It is now shown herein that extensive vacuolization of endosomal compartments did not affect the expression of several common miRNAs in glioblastoma cells. Furthermore, the vacuole-inducing compounds did not substantially alter the relative concentrations of several miRNAs represented in the total RNA in the secreted exosomes. Thus, while the analysis of exosomal proteins shows that MOPIPP and vacuolin-1 induce large increases in exosome secretion, the exosomes appear to be qualitatively similar to those collected from untreated cells in terms of their miRNA cargo.

[0085] Therapeutic Uses

[0086] As described herein, inventive vacuole-inducing molecules induce vacuolization in cells without cell death. In this regard, the present vacuole-inducing molecules are useful in a number of pathological applications. Certain applications are mentioned below; others will be apparent to those of ordinary skill in the art.

[0087] Therefore, vacuole-inducing molecules can be used to induce vacuolization without cell death of cancer cells. For example, the present are useful in treating cancers of the brain, lung, bladder, liver, spleen, pancreas, bone, colon, stomach, breast, prostate, ovary, central nervous system and skin. For example, glioblastoma and breast carcinoma can be treated.

[0088] Further non-limiting examples of therapeutic applications of exosomes include using these vesicles as nanocarriers for packaging and delivery of small molecules (e.g., anti-cancer drugs), miRNAs, and proteins.

[0089] Small molecules like MOPIPP are useful as non-cytotoxic pharmacological agents to boost exosome production.

[0090] Research Uses

[0091] In addition to the various pharmaceutical uses described above, vacuole-inducing compounds have utility in a variety of research applications, e.g., in vitro assays, including, for example, as chemical probes. Those of ordinary skill in the art will appreciate that the field of chemical genetics attempts to identify chemical agents with definable effects on biological events, pathways, or products so that these agents can be used as tools to analyze the relevant biological events, pathways, or products. Vacuole-inducing molecules described herein are particularly well suited for such studies. Accordingly, such uses also include assays, e.g., in vitro assays, utilizing the vacuole-inducing molecules to analyze vacuolization, intracellular trafficking, antigen presentation, membrane fusion events, and related cellular processes. Furthermore, the vacuole-inducing molecules can also be used in screening assays to identify second generation vacuole-inducing molecules, e.g., molecules having modified chemical structures which function as vacuole-inducing molecules. Azide, propargyl or other forms of the vacuole-inducing molecules can be used in a variety of cell-based or molecular screening assays to identify specific protein targets that bind to such molecules.

[0092] Formulations

[0093] As described herein, vacuole-inducing compounds can be utilized in any of a variety of contexts, and can be formulated appropriately according to known principles and technologies.

[0094] For example, the vacuole-inducing molecules can be provided in substantially pure form, in an organic solvent such as DMSO. Alternatively or additionally, vacuole-inducing molecules can be formulated as a pharmaceutical composition, for example being combined with a pharmaceutically acceptable carrier. It will also be appreciated that certain of the vacuole-inducing compounds can exist in free form for treatment, or where appropriate, as a pharmaceutically acceptable derivative thereof. A pharmaceutically acceptable derivative includes, but is not limited to, pharmaceutically acceptable salts, esters, salts of such esters, or a prodrug or other adduct or derivative of a compound which upon administration to a patient in need is capable of providing, directly or indirectly, a compound as otherwise described herein, or a metabolite or residue thereof.

[0095] As used herein, the term "pharmaceutically acceptable salt" refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals with little or no undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts of amines, carboxylic acids, and other types of compounds, are well known in the art (see, for example, Berge, et al., 3.1. J. Pharmaceutical Sciences, 66:1-19, 1977, incorporated herein by reference).

[0096] The salts can be prepared in situ during the final isolation and purification of the compounds, or separately by reacting a free base or free acid function with a suitable reagent, as described generally below. For example, a free base function can be reacted with a suitable acid. Furthermore, where the compounds carry an acidic moiety, suitable pharmaceutically acceptable salts thereof can, include metal salts such as alkali metal salts, e.g., sodium or potassium salts; and alkaline earth metal salts, e.g., calcium or magnesium salts. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hernisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamo-

ate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, loweralkyl sulfonate and aryl sulfonate.

[0097] Additionally, as used herein, the term "pharmaceutically acceptable ester" refers to esters that hydrolyze in vivo and include those that break down readily in the human body to leave the parent compound or a salt thereof. Suitable ester groups include, for example, those derived from pharmaceutically acceptable aliphatic carboxylic acids, particularly alkanoic, alkenoic, cycloalkanoic and alkanedioic acids, in which each alkyl or alkenyl moiety advantageously has not more than 6 carbon atoms. Examples of particular esters include formates, acetates, propionates, butyrates, acrylates and ethylsuccinates.

[0098] The pharmaceutical compositions can additionally comprise a pharmaceutically acceptable carrier, which, as used herein, includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional carrier medium is incompatible with the compounds, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatine; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil, sesame oil; olive oil; corn oil and soybean oil; glycols; such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

[0099] The vacuole-inducing molecules disclosed herein can be formulated or administered together with any other known agent having a complementary biological effect. For example, vacuole-inducing molecules can be combined with steroids or other immunomodulating agents in order to regulate immunological events as described herein.

[0100] It will be appreciated that the compounds and compositions can be administered using any effective amount and any effective route of administration. Thus, the expression "effective amount" as used herein, refers to a sufficient amount of agent to result in vacuolization and/or inhibition of compartment trafficking as described herein. The exact amount required can vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the infection, the particular therapeutic agent, its mode of administration, and the like. The compounds are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of therapeutic agent appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the compounds and compositions will be decided by the attending physician within the scope of sound medical judgment.

[0101] The specific therapeutically effective dose level for any particular patient or organism can depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, gender and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[0102] Dosing

[0103] The actual dosage amount of a composition administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount can vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0104] In certain embodiments, pharmaceutical compositions can comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound can comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition can be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens can be desirable.

[0105] In other non-limiting examples, a dose can also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500

microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

[0106] Furthermore, after formulation with an appropriate pharmaceutically acceptable carrier in a desired dosage, the pharmaceutical compositions can be administered to humans and other animals orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, or drops), bucally, as an oral or nasal spray, or the like, depending on the severity of the infection being treated. In certain embodiments, the compounds can be administered at dosage levels of about 0.001 mg/kg to about 50 mg/kg, from about 0.01 mg/kg to about 25 mg/kg, or from about 0.1 mg/kg to about 10 mg/kg of subject body weight per day, one or more times a day, to obtain the desired therapeutic effect. It will also be appreciated that dosages smaller than 0.001 mg/kg or greater than 50 mg/kg (for example 50-100 mg/kg) can be administered to a subject. In certain embodiments, compounds are administered orally or parenterally.

[0107] Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms can contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

[0108] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution, suspension or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, U.S.R and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables.

[0109] The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0110] In order to prolong the effect of a drug, it is often desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This can be accomplished by the use of a liquid suspension or crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution that, in turn, can depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include (poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

[0111] Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the compounds with suitable nonirritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active compound.

[0112] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form can also comprise buffering agents.

[0113] Solid compositions of a similar type can also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They can optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. Solid compositions of a similar type can also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as

lactose or milk sugar as well as high molecular weight polethylene glycols and the like.

[0114] The active compounds can also be in micro-encapsulated form with one or more excipients as noted above. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art.

[0115] In such solid dosage forms the active compound can be admixed with at least one inert diluent such as sucrose, lactose and starch. Such dosage forms can also comprise, as in normal practice, additional substances other than inert diluents, e.g., tableting lubricants and other tableting aids such as magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms can also comprise buffering agents. They can optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

[0116] Dosage forms for topical or transdermal administration of a compound include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants or patches. The active component is admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as can be required. Ophthalmic formulation, ear drops, and eye drops are also contemplated as being within the scope of this invention. Additionally, the present invention contemplates the use of transdermal patches, which have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms are made by dissolving or dispensing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the compound in a polymer matrix or gel.

[0117] Kits

[0118] In other embodiments, the compounds, or compositions containing them are packaged into a kit for conveniently and effectively carrying out the methods described herein. In general, the pharmaceutical pack or kit comprises one or more containers filled with one or more of the ingredients of the pharmaceutical compositions. Such kits are especially suited for the delivery of solid oral forms such as tablets or capsules. Such a kit preferably includes a number of unit dosages, and can also include a card having the dosages oriented in the order of their intended use. If desired, a memory aid can be provided, for example in the form of numbers, letters, or other markings or with a calendar insert, designating the days in the treatment schedule in which the dosages can be administered. Alternatively, placebo dosages, or calcium dietary supplements, either in a form similar to or distinct from the dosages of the pharmaceutical compositions, can be included to provide a kit in which a dosage is taken every day. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0119] While the invention has been described with reference to various and preferred embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof.

[0120] Therefore, it is intended that the invention not be limited to the particular embodiment disclosed herein contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the claims.

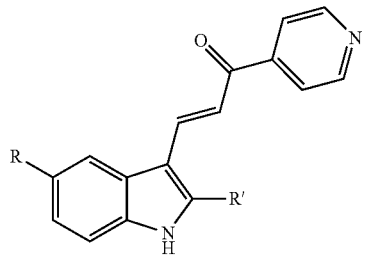
[0121] The publication and other material used herein to illuminate the invention or provide additional details respecting the practice of the invention, are incorporated by reference herein, and for convenience are provided in the following bibliography.

[0122] Citation of any of the documents recited herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

What is claimed is:

1. A method for increasing exosome production in a cell, comprising: exposing the cell to an indole-pyridinyl-propenone compound that induces endosomal vacuolization but does not trigger growth arrest or cell death.

2. The method of claim **1** wherein, the compound has the following chemical structure:



R═OH, OCH_3, OCH_2CH_3, $OCH(CH_3)_2$, $NHCOCH_3$ or $NHCO_2C(CH_3)_3$; and

R'═H, CH_3, $CH_2CH_2CH_3$, $CH(CH_3)_2$, or $CH_2CH(CH_3)_2$;

excluding where R═OCH_3 and R'═CH_3.

3. The method of claim **2**, wherein R═OCH_3; and, R'═H, $CH_2CH_2CH_3$, $CH(CH_3)_2$, or $CH_2CH(CH_3)_2$.

4. The method of claim **3**, wherein R═OCH_3, and R'═$CH_2CH_2CH_3$.

5. The method of claim **2**, wherein R═OH, OCH_2CH_3, $OCH(CH_3)_2$, $NHCOCH_3$ or $NHCO_2C(CH_3)_3$; and, R'═CH_3.

6. The method of claim **5**, wherein R═OCH_2CH_3; and, R'═CH_3.

7. The method of claim **2**, wherein R═OCH_2CH_3; and R'═$CH_2CH_2CH_3$.

8. The method of claim **1**, wherein exosomes are collected by one or more of the following: ultracentrifugation; column

chromatography; size exclusion; and filtration through a device containing an affinity matrix that is selective toward exosomes.

9. A method using exosomes produced by claim **1**, comprising harvesting the exosomes for use as nanocarriers for packaging and delivery of a therapeutic material to a subject in need thereof.

10. The method of claim **9**, wherein the therapeutic material comprises one or more of: small molecules, miRNAs, and proteins.

11. The method of claim **1**, wherein the cell comprises one or more of: multipotent mesenchymal stromal cells; stem cells; and, cancer cells.

12. The method of claim **1**, wherein the cell is a mammalian cell.

13. A method of producing transiently expressed miRNA containing cells, comprising:

transfecting a cell population of cells capable of producing exosomes produced according to the method of claim **1** with one or more plasmids encoding miRNA;

harvesting cells from the cell population after transfection; and,

confirming the presence of the miRNA in one or more of the harvested cells.

14. A method of treating a subject in need thereof with modified exosomes, comprising:

transfecting exosome-producing cells produced by the method of claim **1**, with one or more carriers encoding a therapeutic material;

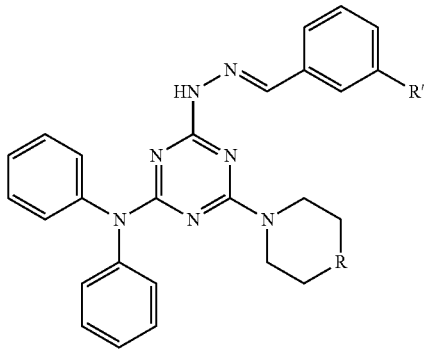
harvesting the exosomes from the cell population or media containing same after transfection;

confirming the presence of the therapeutic material in one or more of the harvested exosomes; and,

administering to the subject one or more of the harvested exosomes in a pharmaceutically effective amount to treat the subject.

15. A method for increasing exosome production in a cell culture, comprising: exposing the culture to a substituted triazole-hydrazone compound that induces endosomal vacuolization but does not trigger cell death or attenuate cell growth.

16. The method of claim **15**, wherein the compound has the following chemical structure:



wherein R=CH_2, NH, O; and R'=an electron withdrawing group.

17. The method of claim **16**, wherein R=O; and R'=Iodo.

18. The method of claim **16**, wherein the electron withdrawing groups are selected from: halides (F, Cl, Br and I), trifluoromethyl (CF_3), nitro (NO_2), acetyl (—$COCH_3$) and trimethylammonium ($N(CH_3)_3$.

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