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(54) Title: VACUOLIN-1 AS AN INHIBITOR OF AUTOPHAGY AND ENDOSOMAL TRAFFICKING AND THE USE THEREOF FOR INHIBITING TUMOR PROGRESSION

(57) Abstract: Methods of treating autophagy related diseases, e. g. cancer and malaria, using novel autophagy inhibiting agents are described.

## DESCRIPTION

VACUOLIN-1 AS AN INHIBITOR OF AUTOPHAGY AND ENDOSOMAL TRAFFICKING  
AND THE USE THEREOF FOR INHIBITING TUMOR PROGRESSION

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## FIELD OF THE INVENTION

The present invention relates generally to the field of compositions and methods for treatment of autophagy related human diseases. More particularly, the present invention provides methods of using small chemicals to manipulate autophagy and to treat autophagy related human  
10 diseases, e.g. cancer.

## BACKGROUND

Autophagy is an evolutionarily conserved catabolic degradation cellular process in which misfolded proteins or damaged organelles are first sequestered by a double-membrane vesicle,  
15 called autophagosomes. Autophagosomes are then fused with lysosomes to digest and recycle the contents to maintain cellular homeostasis. Autophagy can also be markedly induced by a wide variety of stresses, e.g. nutrient starvation, infection, and aging, for cell survival. Dysfunctional autophagy has been associated with wide ranges of human diseases, such as, e.g., cancer, neurodegenerative diseases, heart disease, diabetes, and bacterial infection (Wirawan et  
20 al., 2012; Yang and Klionsky, 2010).

Autophagy is a double-edged sword for many cellular processes, depending upon the genetic background and microenvironment. For example, autophagy can act as a tumor suppressor by preventing oncogenic protein substrates, toxic misfolded proteins, damaged organelles, and reactive oxygen species (ROS) from accumulating to cause genome instability

and cancer initiation(Mathew et al., 2009; Yue et al., 2003). On the other hand, higher basal autophagic activity detected in established tumor cells functions to promote the survival and growth of tumors by maintaining energy production under increased metabolic consumption and a hypoxic microenvironment, thereby enabling tumors to escape chemotherapy and/or radiation(Amaravadi et al., 2007; Lock et al., 2011; Lum et al., 2005; Yang et al., 2011). Therefore, dissecting the molecular mechanisms in regulating autophagy and identifying specific autophagy inhibitors or inducers suitable for clinical application are necessary for specifically targeting autophagy to fight human disease.

A large number of chemicals have been found to either promote or inhibit autophagy. Some of these compounds have been widely used to dissect the mechanisms underlying autophagy(Baek et al., 2012). Popular autophagy inducers include mTOR kinase inhibitors, e.g., rapamycin and torin 1(Hanson et al., 2013), and chemicals inhibiting inositol monophosphatase, e.g., lithium and carbamazepine(Hidvegi et al., 2010). Notably, rapamycin is an immunosuppressant and has recently been used as an anticancer agent (Ravikumar et al., 2004). Lithium has also been used to treat Huntington's disease and other related neurodegenerative disorders (Sarkar et al., 2005). Commonly used autophagy inhibitors include chloroquine (CQ), 3-methyladenine, wortmannin, and bafilomycin (BAF)(Baek et al., 2012; Rote and Rechsteiner, 1983; Seglen and Gordon, 1982; Wu et al., 2013). Since established tumor cells normally activate autophagy to escape chemotherapy and/or radiation(Yang et al., 2011), numerous preclinical studies found that inhibition of autophagy by CQ restored chemosensitivity and promoted tumor cell death by diverse anticancer therapies (Kimura et al., 2013). Although CQ offers great promise for cancer therapy, CQ induces ocular toxicities and damages the renal system, and it is uncertain whether the tolerated doses of HCQ or CQ can be reached in human tumors to effectively inhibit autophagy(Kimura et al., 2013). Moreover, most of the available

autophagy inhibitors, like HCQ or CQ, also lack either specificity, potency, or antitumor activity(Janku et al., 2011). Thus, potent and specific inhibitors of autophagy are needed in order to provide a novel and powerful approach for future cancer therapy.

Recently, many new autophagy chemical modulators have been identified either by  
5 screens based on clearance of aggregates of mutant a-synuclein in cells or by image-based screens with GFP-LC3 transfected cells. Although these small chemicals are useful pharmacological tools to study autophagy and are potential therapeutic drugs for autophagy-related diseases, many of these compounds still lack either specificity or potency, or both(Rubinsztein et al., 2012). Therefore, the search for specific and potent autophagy chemical  
10 modulators must continue in order to gain deep insight into autophagy and provide potential therapeutic drugs.

By screening a Chembridge library (ChemBridge Corporation, San Diego) that contains around 10,000 drug-like or lead-like small chemicals, vacuolin-1 was originally found to induce homotypic fusion of endosomes or lysosomes, thereby forming large vacuoles. Yet, it does not  
15 alter other cell structures and membrane trafficking functions (Cerny et al., 2004; Huynh and Andrews, 2005; Shaik et al., 2009). It remains controversial whether vacuolin-1 blocks the  $Ca^{2+}$ -dependent exocytosis of lysosomes.

#### BRIEF SUMMARY

20 The present invention provides methods of using vacuolin-1 and its analogues to manipulate autophagy and to treat autophagy related human diseases, e.g. cancer. The present invention also provides application of vacuolin-1 and its analogues for inhibiting autophagy and endosomal trafficking.

In one aspect, methods for treatment of cancer, such as, but not limited to, lung carcinoma and nasopharyngeal carcinoma are provided. The methods comprise administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, alone or in combination with one or more chemotherapy drugs to a subject who has cancer. In at least one  
5 embodiment, the methods are used to treat cancers that are susceptible to treatment with an autophagy inhibiting agent.

In some embodiments, the methods result in inhibition of autophagy and/or endosomal traffic. In some embodiments, the methods result in activation of Rab5, which leads to maturation of endosomes and lysosomes and contributes to lysosomal pH increase. In some  
10 embodiments, the chemotherapy drugs can include taxol, 5-Fu, temsirolimus, and combinations thereof.

In other aspects of the invention, vacuolin-1 analogues are identified by virtual screening.

#### BRIEF DESCRIPTION OF DRAWINGS

15 Figure 1 shows structures of vacuolin-1 and structural analogues.

Figure 2 shows immunofluorescence staining analysis of RFP-GFP-LC3 expressing HeLa cells following treatment with bafilomycin, CQ, and vacuolin-1.

Figure 3 shows vacuolin-1 inhibits the fusion between autophagosomes and lysosomes in HeLa cells. Immunofluorescence staining analysis of RFP-GFP-LC3 expressing HeLa cells  
20 following treatment with bafilomycin, CQ, and vacuolin-1 is shown. Scale bar = 20  $\mu$ M. (A) Percentage of yellow LC3II puncta in RFP-GFP-LC3 expressing HeLa cells following induction by vacuolin-1. (B) Western blot analysis showing induction of accumulation of both LC3-II and p62 in HeLa cells by vacuolin-1 (1  $\mu$ M). (C) Immunofluorescence staining analysis of vacuolin-1 (1  $\mu$ M) induction of GFP-LC3-II puncta in HeLa cells, which are not colocalized with

RFP-Lamp1. Scale bar = 20  $\mu$ M. (D) Vacuolin-1 (1  $\mu$ M) induces the accumulation of autophagic vacuoles as shown in the electron micrographs and highlighted in areas D1 and D2.

Figure 4 shows (A) western blot analyses of LC3 and p62 in HeLa cells following treatment with vacuolin-1 or CQ at the indicated dose for 6 hours; (B) an MTT assay for cell viability in HeLa cells treated with vacuolin-1 or CQ for the indicated doses for 48 hours; (C) western blot analyses of LC3 and p62 in vacuolin-1 (1  $\mu$ M) treated HeLa cells; (D) microplate reader measurement of Lysosensor DND-189 stained HeLa cells following vacuolin-1 (1  $\mu$ M) induction; (E) microplate reader measurement of quantitative ratiometric LysoSensor Yellow/Blue DND-160 stained HeLa cells following vacuolin-1 (1  $\mu$ M) induction; and (F) Fura-2 loaded HeLa cells following vacuolin-1 (1  $\mu$ M) pretreatment for 5 hours followed by GPN (200  $\mu$ M) induced lysosomal  $Ca^{2+}$  release.

Figure 5 shows western blot analyses against LC3 and p62 in PC3 prostate cancer cells treated with vacuolin-1 or CQ at indicated dose for 6 hours.

Figure 6 shows line graphs of cell viability measured by MTT assays for PC3, HepG2, MCF7, and A549 cells treated with vacuolin-1 or CQ for the indicated doses for 48 hours.

Figure 7 shows (A) western blot analyses against LC3, p62, and cathepsin L in HeLa cells treated with or without vacuolin-1 (1  $\mu$ M) or bafilomycin (100 nM); (B) western blot analyses against EGFR in HeLa cells following an EGFR degradation assay (treatment of HeLa cells with EGF in the presence or absence of vacuolin-1); (C) immunofluorescence staining analysis for EGFR in HeLa cells treated with vacuolin-1 (1  $\mu$ M) (Scale bar = 20  $\mu$ M); and (D) flow cytometric analysis for DQ-BSA-green fluorescence in vacuolin-1 (1  $\mu$ M) treated and control HeLa cells.

Figure 8 shows (A) an *in vitro* V-ATPase assay showing V-ATPase activity in vacuolin-1 and bafilomycin treated cells; (B) results of a GST-tagged Rabaptin5 pull-down assay in HeLa

cells showing active Rab5 in cells treated with vacuolin-1 (1  $\mu$ M) or transfected with Rab5A-CA or Rab5A-DN; (C) western blot analysis against LC3-II and p62 in HeLa cells showing Rab5A knockdown blocks vacuolin-1 induced accumulation of LC3-II and p62; (D) expression of Rab5A-CA enhances vacuolin-1 induced autophagy arrest, whereas expression of Rab5A-DN blocks it in HeLa cells; (E) western blot analysis against LC3-II and p62 following expression of Rab5A-CA; and (F) immunostaining analysis against LC3-II and p62 following expression of Rab5A-CA (scale bar = 20  $\mu$ M).

Figure 9 shows expression of Rab5A-DN does not affect starvation induced LC3 puncta in HeLa cells.

Figure 10 shows Rab5A knockdown or expression of Rab5A-DN does not affect starvation induced LC3-II in HeLa cells.

Figure 11 shows an electron micrograph of enlarged lysosomes in HeLa cells following vacuolin-1 treatment (B) versus control (A).

Figure 12 shows vacuolin-1 analogues inhibit the fusion between autophagosomes and lysosomes in HeLa cells. Western blot analysis against LC3-I, LC3-II, and p62.

Figure 13 shows vacuolin-1 potently enhances the anti-tumor effects of chemotherapy drugs in human cancer cell lines. (A) Vacuolin-1 augments the anti-tumor effects of chemotherapy drugs (B) Vacuolin-1 in combination with chemotherapy drugs greatly decreases the colony formation efficiency cancer cells.

Figure 14 shows a virtual screening strategy for vacuolin-1 analogue identification using ChemDiv.

Figure 15 shows vacuolin-1 analogues.

Figure 16 shows vacuolin-1 markedly inhibited colony formation and migration of human nasopharyngeal carcinomas. (A) Vacuolin-1 treatment alone displayed little cell toxicity

in CNE-1 or HONE-1 cells. **(B)** Vacuolin-1 dramatically reduced the size and number of colonies generated from single CNE-1 or HONE-1 cell. **(C)** Vacuolin-1 efficiently suppressed the migration of both CNE-1 and HONE-1 cells as revealed by the observation that vacuolin-1 treatment induced a marked decrease in motility of tumor cell. **(D) and (E)** Vacuolin-1 markedly inhibited the migration **(D)** and invasiveness **(E)** of CNE-1 and HONE-1 cells.

Figure 17 shows vacuolin-1 markedly inhibited colony formation and migration of human lung cancer stem cells (LCSCs) in vitro. **(A)** Vacuolin-1 on autophagy inhibition in human lung cancer stem cells (hLCSCs) were 10 times less potent than those in other tumor cell lines. **(B)** Vacuolin-1 did not affect the cell proliferation of hLCSCs. **(C-F)** Vacuolin-1 (> 10  $\mu$ M) almost completely inhibited the colony formation **(C)**, migration **(D)**, invasion **(E)** and tumor sphere formation **(F)** of hLCSCs.

Figure 18 shows vacuolin-1 markedly suppressed tumor growth in nude mice. **(A)**. Intratumoral injection of vacuolin-1 (5 mg/kg) twice a week markedly inhibited tumor growth of LCSC xenograft in nude mice but had little effects on the weight gain. **(B)** vacuolin-1 treatment markedly inhibited autophagy in tumors. **(C)** Intraperitoneal injection of vacuolin-1 (250 mg/kg) in young adult mice exhibited little acute toxicity as the drug injected mice showed normal activity and weight gain as compared to control after two weeks.

20

#### DETAILED DESCRIPTION

The present invention describes vacuolin-1 and its analogues as potent and reversible inhibitors of autophagy and endosomal traffic and methods of using vacuolin-1 and its analogues alone or in combination with chemotherapeutic drugs, e.g. 5-FU, temsirolimus, and taxol, or

alone, to manipulate autophagy and to treat cancer, e.g. prostate, breast, and nasopharyngeal carcinoma, or malaria, respectively.

In one aspect, methods for treatment of cancer, such as but not limited to, colon carcinoma, prostate cancer, breast cancer, osteosarcoma and/or nasopharyngeal carcinoma, are provided. The methods comprise administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, either alone or in combination with one or more chemotherapy drugs to a subject who has cancer.

In another aspect, methods for treatment of malaria are provided. The methods comprise administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, to a subject who is infected with malaria.

In some embodiments, the methods result in inhibition of autophagy and/or endosomal traffic. In some embodiments, the methods result in activation of Rab5, which leads to maturation of endosomes and lysosomes and contributes to lysosomal pH increase. In some embodiments, the chemotherapy drugs can include, but are not limited to, taxol, 5-Fu, temirolimus, and combinations thereof.

In other aspects of the invention, additional vacuolin-1 analogues are identified by virtual screening.

The small chemical, vacuolin-1, and its analogues (Figure 1) potently and reversibly inhibit autophagy by blocking the fusion between autophagosomes and lysosomes in mammalian cells, thereby leading to the accumulation of autophagosomes (Figures 2-5, and 12). Vacuolins are less toxic than chloroquine (CQ) but are at least 10 folds more potent in inhibiting autophagy as compared to CQ (Figures 3-5). Vacuolin-1 and its analogues present a novel class of drug that can potently and reversibly modulate autophagy.

The present invention describes vacuolins as potent inhibitors of general endosomal traffic (Figure 7) and a novel class of Rab5 GTPase activators (Figure 8).

Furthermore, methods to treat human cancers, e.g. colon carcinoma, osteosarcoma, and nasopharyngeal carcinoma, with vacuolins in combination with other chemotherapy drugs, e.g. 5-FU, temsirolimus, and taxol (Figures 13 and 14) are provided herein. It is also contemplated that vacuolins, and/or analogs thereof, can be utilized alone in the treatment of cancers. Furthermore, the present invention also provides methods to treat malaria with vacuolins (Figure 15).

In at least one aspect, vacuolins are used in combination with chemotherapeutic drugs, e.g. 5-FU, temsirolimus, and taxol, to synergistically kill a panel of tumor cell lines *in vitro* and suppress tumor growth in xenograft mouse models (Figures 13 and 14). It is expected that vacuolin-1 and its analogues provide a novel therapeutic strategy for fighting cancers. It is also expected that vacuolins sensitize tumor cells carrying wild type p53, not mutant p53, to chemotherapeutic drugs.

Vacuolins are cell-permeable and water soluble triazine based compounds. Compared to other popular autophagy modulators, such as CQ which has been applied in a dozen anti-cancer clinical trials, vacuolins are at least 10 fold more potent in inhibiting autophagy while exhibiting little toxicity in a wide variety of cancer cell lines (Figures 4 and 6). Besides inhibiting autophagy, vacuolins potently and reversibly inhibit endosomal traffic as well (Figure 7).

Vacuolins markedly activate Rab5 and expression of Rab5A-DN or Rab5A knockdown abolishes vacuolin-1 mediated autophagy inhibition (Figure 8). Rab5 is a master regulator for the biogenesis of the endolysosomal system, and no drugs, other than vacuolins, are found to modulate its activity thus far. Therefore, vacuolins are the first class of Rab5 agonists to be

identified and can be widely used in basic research related to both autophagy and intracellular traffic.

Vacuolins also modestly inhibit V-ATPase activity(30% at 1  $\mu$ M), which may be due to the fact that V-ATPase is sensitive to hydrophobic compounds in general (Figure 8). Thus, vacuolins are not specific V-ATPase inhibitors, but nonetheless at least partially lead to the increase of lysosomal pH, which may also contribute to autophagy inhibition. In addition, endosomal traffic is essential for the function and biogenesis of lysosome because lysosomes depend on the influx of new components. Without incoming endosomal traffic, lysosomes lose their intact morphology, contents of protons and other ions, and perinuclear localization(Huotari and Helenius, 2011; Spang, 2009). Thus, vacuolins constitutively activate Rab-5, and active Rab5 stops endosome maturation, which subsequently compromises the biogenesis and function of lysosomes. This should also partially contribute to the increase of lysosomal pH.

The present invention also provides toxicity studies of the vacuolins in combination with, or without, chemotherapy drugs in both tumor-free and tumor-bearing mice. Since vacuolins are much less toxic than CQ and its effects on cells are reversible, the toxicity of vacuolins on mice is much less than CQ.

The present invention further provides pharmacokinetic studies on vacuolins in normal mice or rats. The blood concentration of vacuolins after the intragastric (oral), intravenous, intramuscular, or subcutaneous administration is determined. In addition, the metabolites of vacuolins after incubating vacuolins with liver microsomes are determined. Compared to CQ, which exhibits high toxicity alone and its effective concentration against autophagy is hard to reach in an animal model, this invention shows vacuolin-1 and its analogues are better lead compounds for future clinical application against human cancer and malaria.

## MATERIALS AND METHODS

*Antibodies and reagents*- The antibodies used were as follows: LC3 (Novus; 1:1000 for the Western blotting analysis (WB)), p62 (Novus; 1:500 for the immunofluorescence analysis (IF) and 1:1000 WB), Cathepsin-L (BD Bioscience; 1:250 WB), EGFR (Santa Cruz; 1:250 WB),  
5 Rab5 (Cell Signaling; 1:1000 WB), GAPDH antibody (Sigma;1:5000 WB). Vacuolin-1 was purchased from Santa Cruz. Bafilomycin A1, Chloroquine (CQ), and Glycyl-L-phenyl-alanine- $\beta$ -naphthylamide (GPN) were purchased from Sigma-Aldrich. Fura-2 AM, lysosensor Green DND-189, and LysoSensorYellow/Blue DND-160 were purchased from Invitrogen.

10 *Cell culture*- HeLa, MCF-7, A549, and HepG2 cells (*ATCC*) were maintained in DMEM (*Invitrogen*) plus 10% fetal bovine serum (*Invitrogen*) and 100 units/ml of penicillin/streptomycin (*Invitrogen*) at 5% CO<sub>2</sub> and 37 °C. PC3 cells were maintained in RPMI (*Invitrogen*) plus 10% fetal bovine serum (*Invitrogen*) and 100 units/ml of penicillin/streptomycin (*Invitrogen*) at 5% CO<sub>2</sub> and 37 °C.

15 *shRNA and lentivirus production and infection*-Two optimal 21-mers were selected in the human Rab5A gene. One 21-mer was selected in the GFP gene as a control. These sequences were then cloned into the pLKO.1 vector for expressing shRNA. The production and infection were performed as described previously (Lu et al., 2013).

20 *Intracellular Ca<sup>2+</sup> measurement*- Cells were cultured in 24-well plates at the density of 7x10<sup>4</sup> cells/well in regular medium overnight and were labeled with 4 $\mu$ M Fura-2 AM (*Invitrogen*) in regular HBSS at room temperature for 30 min. The cells were then washed with calcium-free HBSS containing 2mM EGTA three times and incubated in the presence or absence of vacuolin-1 at room temperature for another 10 min. Cells were put on the stage of an Olympus inverted epifluorescence microscope and visualized using a 20x objective. Fluorescence images

were obtained by alternate excitation at 340 nm and 380 nm with emission set at 510 nm. Images were collected by a CCD camera every 3 or 6 seconds and analyzed by *Cell R* imaging software.

*Western blot and Immunofluorescence staining analyses*- Both assays were performed as described previously(Lu et al., 2013).

5 *Cell preparation for transmission electron microscopy (TEM)*-Cell preparation for TEM was performed as described previously (Mi et al., 2007).

*MTT cell proliferation assay*- Cells were treated in four replicates and seeded into 96-well plate. Following drug treatment, MTT solution of 20  $\mu$ l for every 100  $\mu$ l medium was added to wells and incubated for 2 hours, followed by the addition of 150  $\mu$ L of the DMSO  
10 solution to each well. The final reaction product, a purple formazan solution, was detected by a microplate reader (Techan infinite M200) for absorbance at a wavelength of 570 nm and a reference wavelength of 630 nm.

*Lysosomal pH measurement*- LysoSensor Green DND-189 is commonly used to measure the pH of acidic organelles, such as lysosomes, which become more fluorescent in acidic  
15 environments. Briefly, cells were loaded with 1 $\mu$ M LysoSensor Green DND-189 in pre-warmed regular medium for 20 min at 37°C. Then the cells were washed twice with PBS and immediately analyzed by flow cytometry (collecting FL1 fluorescence and 10,000 cells were collected for each sample) or in a microplate reader (excitation/emission = 485/530 nm).

Quantification of lysosomal pH was performed using a ratiometric lysosomal pH dye  
20 LysoSensor Yellow/Blue DND-160. The pH calibration curve was generated as described previously (Bankers-Fulbright et al., 2004). Briefly, cells were trypsinized and labeled with 2  $\mu$ M LysoSensor Yellow/Blue DND-160 for 30 min at 37°C in regular medium, and excessive dye was washed out using PBS. The labeled cells were treated for 10 min with 10  $\mu$ M monensin and 10  $\mu$ M nigericin in 25mM 2-(N-morpholino)ethanesulfonic acid (MES) calibration buffer, pH

3.5–6.0, containing 5 mM NaCl, 115 mM KCl and 1.2 mM MgSO<sub>4</sub>. Quantitative comparisons were performed in a 96-well plate, and the fluorescence was measured with a microplate reader at 37°C. Light emitted at 440 and 535nm in response to excitation at 340 and 380nm were measured, respectively. The ratio of light emitted with 340 and 380nm excitation was plotted  
5 against the pH values in MES buffer, and the pH calibration curve for the fluorescence probe was generated from the plot using Microsoft Excel.

*V-ATPase assay*- Fifth instar larvae of *M. sexta* (Lepidoptera, Sphingidae), weighing 6-8 g, were reared under long day conditions (16 h of light) at 27°C using the gypsy moth diet (MP Biomedicals, Germany). The purification of the V<sub>1</sub>V<sub>0</sub> holoenzyme was performed as previously  
10 described (Huss et al., 2002). Activity assays were performed in triplicate in a final volume of 160 µL and at a pH of 8.1. Assays contained 3 µg of purified V<sub>1</sub>V<sub>0</sub> holoenzyme, 50 mM Tris-Mops, 3 mM 2-mercaptoethanol, 1 mM MgCl<sub>2</sub>, 20 mM KCl, 0.003% C<sub>12</sub>E<sub>10</sub>, 20 mM NaCl, and 3 mM Tris-HCl. After 10 min of preincubation at 30 °C, with or without inhibitors, 1 mM Tris-ATP was added and after an incubation for 2 min at 30°C the reaction was stopped by  
15 freezing the samples in liquid nitrogen. Inorganic phosphate was determined as previously described (Wieczorek et al., 1990).

*Glutathione S-Transferase (GST) Pull-Down Assay*-Cells were collected and lysed in an ice-cold EBC lysis buffer described previously. Lysates were clarified by centrifugation at 13,000g for 10 min at 4°C, and equal amount of protein (500 µg) from each supernatant was  
20 incubated with 30 µl of GST-R5BD bound to the 30 µl glutathione-Sepharose beads for 1h at 4°C on a rotating mixer. The beads were subsequently washed and resuspended in the standard SDS-sample buffer, boiled and subjected to SDS-PAGE (15% gel), followed by immunoblot analysis with the anti-Rab5 mAb.

*Statistical analysis*- Data were presented as mean±S.E.M. The statistical significance of differences was estimated by one-way ANOVA or Student's t-test. P<0.05 was considered significant.

5

## EXAMPLES

Example 1. *The small chemical vacuolin-1 functions as an autophagy inhibitor by inhibiting the autophagosomal-lysosomal fusion in HeLa cells.*

Prompted by the fact that many available autophagy chemical modulators lack either potency or specificity (Kimura et al., 2013), a fluorescence image-based assay was set up to screen small molecules affecting autophagy. HeLa cells, an autophagy competent cell line, were infected with lentiviruses carrying expression cassettes that encode RFP-GFP-LC3 (tfLC3)(Kimura et al., 2007). Thus, the LC3-II positive autophagosomes are labeled with both GFP and RFP signals shown as yellow puncta, and after fusion with lysosomes, autolysosomes are shown as red only puncta because GFP loses its fluorescence in acidic pH. As shown in Figure 2, starvation greatly induced the increase of both yellow and red only puncta, yet treatment of cells with bafilomycin (BAF), an inhibitor of the vacuolar proton pump that blocks the fusion of autophagosomes with lysosomes(Yamamoto et al., 1998), or CQ, markedly induced the accumulation of yellow puncta only, indicating that the autophagy is arrested at autophagosomes. These data indicate that RFP-GFP-LC3 expressing HeLa cells can be used to monitor the progression of autophagy.

Next, a panel of small chemicals that are commercially available and have been previously shown to affect vesicle trafficking or organelle morphology were selected and screened for their effects on autophagy regulation in tfLC3 expressing HeLa cells (data not shown). One small chemical, vacuolin-1, potently induced LC3 yellow puncta, not red puncta

(Figure 3A). Similarly, western blot analyses confirmed that lipidated LC3-II was markedly increased in cells treated with vacuolin-1. p62, an autophagic substrate (Bjorkoy et al., 2006), was also accumulated in cells treated with vacuolin-1, suggesting that vacuolin-1 inhibits the fusion between autophagosome and lysosomes (Figure 3B). Indeed, GFP-LC3 puncta were greatly increased in vacuolin-1 treated cells and did not co-localize with Lamp1, which was similar to the cells treated with BAF (Figure 3C). Moreover, under the electron microscope, large numbers of autophagic vacuoles were observed in vacuolin-1 treated HeLa cells maintained in normal culture conditions (Figure 3D). Taken together, these data indicate that vacuolin-1, similar to BAF or CQ, blocks the fusion between autophagosomes and autolysosomes, thereby leading to accumulation of LC3-II positive autophagosomes to achieve its effect of autophagy inhibition.

*Example 2. Vacuolin-1 potently and reversibly inhibits autophagy but shows little cell toxicity.*

Vacuolin-1 is a cell-permeable and water soluble triazine based compound. It has been previously reported that vacuolin-1 induced rapid homotypic fusion of endosomes and lysosomes to form large and swollen structures, yet it did not disturb cell cytoskeletal network (Cerny et al., 2004; Huynh and Andrews, 2005; Shaik et al., 2009). Amazingly, vacuolin-1 was at least ten times more potent than CQ in suppressing autophagy (Figures 4A and 5), yet it exhibited much less cell toxicity than CQ in a wide variety of cell types (Figures 4B and 6). Interestingly, the fusion block between autophagosomes and lysosomes was completely relieved 3 hours after removal of vacuolin-1 from the medium (Figure 4C). Similarly, vacuolin-1 induced homotypic fusion between endosomes or lysosomes was recovered after removal of vacuolin-1 (data not shown). These data indicate that the effects of vacuolin-1 on autophagy inhibition or homotypic fusion are reversible.

Example 3. *Vacuolin-1 alkalinized lysosomal pH and decreased lysosomal Ca<sup>2+</sup> content.*

The mechanisms underlying vacuolin-1 induced autophagy arrest were explored. As lysosomal pH is essential for the fusion(Lu et al., 2013) and lysosomes were enlarged by  
5 vacuolin-1 treatment(Cerny et al., 2004; Huynh and Andrews, 2005; Shaik et al., 2009), it was examined whether vacuolin-1 affects lysosomal pH in these enlarged lysosomes. LysoSensor Green DND-189 (pK<sub>a</sub> = ~ 5.2) was first applied to qualitatively measure lysosomal pH(Davis-Kaplan et al., 2004). LysoSensor Green DND-189 permeates cell membranes and accumulates in acidic intracellular organelles, and its fluorescence increases or decreases in  
10 acidic or alkaline environments, respectively. As shown inFigure4D, vacuolin-1 treatment raised lysosomal pH in HeLa cells. Lysosomal pH was further quantified by a quantitative ratiometricLysoSensor Yellow/Blue DND-160 (DePedro and Urayama, 2009)stained cells and found that lysosomal pH in vacuolin-1 treated HeLa cells was increased from pH 4.7 in control cells to pH 5.2 (Figure 4E). Thus, it is clear that vacuolin-1 alkalinizes lysosomalpH.

15 Since lysosome is also a major intracellular Ca<sup>2+</sup> pool, and Ca<sup>2+</sup> and protons are tightly coupled in lysosomes (Morgan et al., 2011), it was also assessed whether vacuolin-1 treatment affects the lysosomal Ca<sup>2+</sup> content. Treatment of cells with glycyl-l-phenylalanine 2-naphthylamide (GPN) selectively disrupts the lysosomal membrane(Jadot et al., 2001), thereby releasing the lysosomal Ca<sup>2+</sup>(Srinivas et al., 2002).As shown in Figure 4F, pretreatment of cells  
20 with vacuolin-1 significantly lowered the ability of GPN induced lysosomal Ca<sup>2+</sup> release, suggesting that vacuolin-1 decreases lysosomal Ca<sup>2+</sup> levels as well.

Example 4. *Vacuolin-1 inhibited general endosomal-lysosomal degradation in HeLa cells.*

Since the increase of lysosomal pH normally compromises the lysosomal activity, it was assessed whether vacuolin-1 affects the general lysosomal functions to inhibit autophagy maturation. The processing of cathepsin L from the precursor form to its mature form has been commonly used as a marker for lysosomal activity, and it was found that the processing of cathepsin L into its mature form was not affected in vacuolin-1 treated HeLa cells, but vacuolin-1 did markedly increase the levels of immature form of cathepsin L (Figure 7A). Next, an epidermal growth factor receptor (EGFR) degradation assay was performed to examine whether vacuolin-1 affects the general endosomal-lysosomal pathway. In this assay, HeLa cells were treated with EGF in the presence or absence of vacuolin-1. After EGF binds to its receptors (EGFR), the receptor complex undergoes endocytosis and is targeted to lysosomes for degradation. As shown in Figure 7B, vacuolin-1 inhibited EGF-triggered EGFR degradation. Interestingly, EGFR was internalized normally but the endosomes containing EGFR failed to fuse with lysosomes; this explains the accumulation of EGFR in vacuolin-1 treated cells (Figure 7C). Similarly, a DQ-BSA-green degradation assay was applied to measure the general endosomal-lysosomal degradation. DQ-BSA-green is a BSA labeled with a self-quenching fluorescent dye. After DQ-BSA-green is delivered to lysosomes via endocytosis, it is hydrolyzed into single dye-labeled peptides by lysosomal proteases, thereby relieving self-quenching and the fluorescence can subsequently be monitored by flow cytometry. As shown in Figure 7D, vacuolin-1 markedly inhibited the degradation of BSA by lysosomes, yet DQ-BSA-Green was present in endosomes but failed to be delivered to lysosomes (data not shown). Collectively, these results support that vacuolin-1 inhibits the general endosomal-lysosomal degradation, which might be due to the alkalinized lysosomal pH arresting the fusion of endosomes with lysosomes.

Example 5. *Rab5 was required for vacuolin-1 induced autophagy arrest and endosomal fusion.*

Although vacuolin-1 increased lysosomal pH, vacuolin-1 had little effects on V-ATPase activity *in vitro* (Figure 8A). Vacuolin-1 also induced the homotypic fusion of endosomes or lysosomes, and Rab5, a small GTPase, is essential for endosome fusion(Zeigerer et al., 2012). Therefore, it was examined whether vacuolin-1 activates Rab5 to indirectly change lysosomal pH. Indeed, vacuolin-1 markedly activated Rab5, as shown by a GST-tagged Rabaptin5 pull-down assay, which specifically interacts with Rab5-GTP, not Rab5-GDP (Figures 8B)(Liu et al., 2007).Consistently, Rab5A knockdown markedly decreased vacuolin-1 induced accumulation of both LC3-II and p62 (Figure 8C). Similarly, overexpression of a dominant-negative form of Rab5A (Rab5A-DN) blocked vacuolin-1 induced autophagy arrest and homotypic fusion, whereas overexpression of a constitutive form of Rab5A (Rab5A-CA)(Bohdanowicz et al., 2012)augmented the ability of vacuolin-1 induced autophagy arrest and homotypic fusion (Figures 8D). Interestingly, Rab5A-CA overexpression alone also blocked the autophagosomal-lysosomal fusion, thereby inducing the accumulation of LC3-II and p62 (Figures8E and 8F). Notably, Rab5A-DN expression or Rab5A knockdown did not affect starvation-induced autophagy (Figures9 and 10). Taken together, these data indicate that Rab5A is required for vacuolin-1 induced autophagy arrest.

Example 6. *Vacuolin-1 markedly inhibited colony formation and migration of human nasopharyngeal carcinomas.*

Although CQ has already been applied to treat a wide spectrum of human cancers, CQ induces ocular or renal toxicity and easily triggers drug resistance(Kimura et al., 2013). We, thus, assessed whether vacuolin-1 exhibits any anti-tumor ability and whether it can, like CQ, potentiate the sensitivity of tumor cells to chemotherapeutic drugs. We first investigated the

anti-tumor effects of vacuolin-1 in two nasopharyngeal carcinoma cell lines, CNE-1 and HONE-1. As shown in **Figure 16A**, vacuolin-1 treatment alone displayed little cell toxicity in CNE-1 or HONE-1 cells. Surprisingly, vacuolin-1 also showed almost no synergistic effects on inhibiting tumor cell proliferation in combination with several clinical anti-tumor drugs, such as taxol, 5-FU, and temsirolimus (Fig. 16A and data not shown). Alternatively, colony formation assay was performed to assess whether vacuolin-1 affects the ability of a single tumor cell to grow into a colony. As shown in **Figure 16B**, vacuolin-1 dramatically reduced the size and number of colonies generated from single CNE-1 or HONE-1 cell. We subsequently performed wound healing assay to assess whether vacuolin-1 affects the migration of tumor cells. Strikingly, vacuolin-1 efficiently suppressed the migration of both CNE-1 and HONE-1 cells as revealed by the observation that vacuolin-1 treatment induced a marked decrease in motility of tumor cell (**Fig. 16C and data not shown**). Likewise, vacuolin-1 markedly inhibited the migration and invasiveness of CNE-1 and HONE-1 cells (**Fig. 16D, Fig. 16E, and data not shown**). Obviously, vacuolin-1 is an efficient anti-tumor agent in vitro.

15

*Example 7. Vacuolin-1 markedly inhibited colony formation and migration of human lung cancer stem cells (LCSCs) in vitro.*

Cancer stem cells (CSCs), a subpopulation of cells within tumors, actually drive tumor growth and recurrence. CSCs are resistant to many current cancer treatments, including chemotherapy and radiation therapy, thus they can survive these therapy to regenerate new tumors. CSCs have been shown to possess stem cell characteristics, such as self-renewal, stress and drug resistance, and enhanced migration, all of which have been implicated in disease recurrence and distant metastasis (Gupta et al., 2009). Therefore, it is of great interest to assess the anti-tumor effects of vacuolin-1 on CSCs. Indeed, vacuolin-1 on autophagy inhibition in human lung cancer stem cells

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(hLCSCs) were 10 times less potent than those in other tumor cell lines (**Fig. 17A**), and vacuolin-1 did not affect the cell proliferation of hLCSCs (**Fig. 17B**). Likewise, vacuolin-1 (> 10  $\mu$ M) almost completely inhibited the colony formation, migration, invasion and tumor sphere formation of hLCSCs (**Fig. 17C-17F**). Collectively, these data indicate that vacuolin-1 can efficiently suppress the tumorigenic potential of lung cancer stem cells in vitro.

*Example 8. Vacuolin-1 markedly suppressed tumor growth in nude mice.*

Promoted by the fact that vacuolin-1 potently inhibited the migration and colony formation ability of human nasopharyngeal carcinomas cells and human lung cancer stem cells in vitro, we examined the ability of vacuolin-1 to inhibit tumor progression in xenograft mouse model. We first assessed the ability of vacuolin-1 to suppress LCSC tumor xenograft in BALB/c nude mice. Briefly,  $1 \times 10^4$  lung cancer stem cells (LCSCs, 3<sup>rd</sup> generation) were suspended in Matrigel (BD Biosciences) at a ratio of 1:1, and 200  $\mu$ L of cells was subcutaneously injected into the back of nude mice. The tumor volume was measured every five days after injection and calculated from the formula: length  $\times$  width  $\times$  depth  $\times \pi /6$ . Mice were removed from the study when their tumor volumes exceed 2000 mm<sup>3</sup> (**Fig. 18A**). As shown in **Fig. 18A**, intra tumoral injection of vacuolin-1 (5 mg/kg) twice a week markedly inhibited tumor growth of LCSC xenograft in nude mice but had little effects on the weight gain. As expected, vacuolin-1 treatment markedly inhibited autophagy in tumors (**Fig. 18B**). Also, we tested the acute toxicity of vacuolin-1 in mice. Intraperitoneal injection of vacuolin-1 (250 mg/kg) in young adult mice exhibited little acute toxicity as the drug injected mice showed normal activity and weight gain as compared to control after two weeks (Fig. 18C). Taken together, these data indicate that vacuolin-1 is a promising anti-tumor agent in vivo with tolerable toxicity.

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- 35

## CLAIMS

We claim:

1. A method for treatment of human lung carcinoma, the method comprising administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, alone or in combination with one or more chemotherapy drugs to a subject who has colon carcinoma.

2. The method according to claim 1, wherein vacuolin-1 and its analogues inhibit autophagy.

3. The method according to claim 1, wherein vacuolin-1 and its analogues inhibit endosomal traffic.

4. The method according to claim 1, wherein vacuolin-1 and its analogues activate Rab5, which leads to maturation of endosomes and lysosomes and contributes to lysosomal pH increase.

5. The method according to claim 1, wherein the one or more chemotherapy drugs are selected from the group consisting of taxol, 5-Fu, temirolimus, and combinations thereof.

6. The method according to claim 1, wherein the vacuolin-1 analogues are identified by virtual screening.

7. A method for treatment of human lung carcinoma, the method comprising administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, alone or in combination with one or more chemotherapy drugs to a subject who has human lung carcinoma.

8. The method according to claim 6, wherein vacuolin-1 and its analogues inhibit autophagy.

9. The method according to claim 6, wherein vacuolin-1 and its analogues inhibit endosomal traffic.

10. The method according to claim 6, wherein vacuolin-1 and its analogues activate Rab5, which leads to maturation of endosomes and lysosomes and contributes to lysosomal pH increase.

11. The method according to claim 6, wherein the one or more chemotherapy drugs are selected from the group consisting of taxol, 5-Fu, temirolimus, and combinations thereof.

12. A method for treatment of nasopharyngeal carcinoma, the method comprising administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, alone or in combination with chemotherapy drugs to a subject who has nasopharyngeal carcinoma.

13. The method according to claim 11, wherein vacuolin-1 and its analogues inhibit autophagy.

14. The method according to claim 11, wherein vacuolin-1 and its analogues inhibit endosomal traffic.

15. The method according to claim 11, wherein vacuolin-1 and its analogues activate Rab5, which leads to maturation of endosomes and lysosomes, and contributes to lysosomal pH increase.

16. The method according to claim 11, wherein the one or more chemotherapy drugs are selected from the group consisting of taxol, 5-Fu, temirolimus, and combinations thereof.

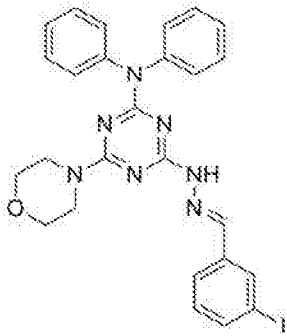
17. A method of treating malaria, the method comprising administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues, to a subject who is infected with malaria.

18. The method according to claim 17, wherein vacuolin-1 and its analogues inhibit autophagy.

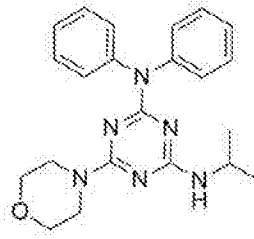
19. The method according to claim 17, wherein vacuolin-1 and its analogues inhibit endosomal traffic.

20. The method according to claim 17, wherein vacuolin-1 and its analogues activate Rab5, which leads to maturation of endosomes and lysosomes, and contributes to lysosomal pH increase.

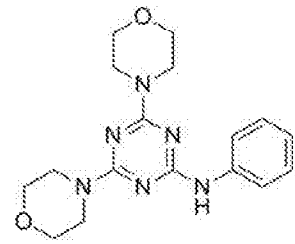
21. A method for treating cancer in a subject, wherein the cancer is susceptible to treatment with an autophagy inhibiting agent, the method comprising administering a therapeutically effective amount of vacuolin-1, and/or one or more analogues.



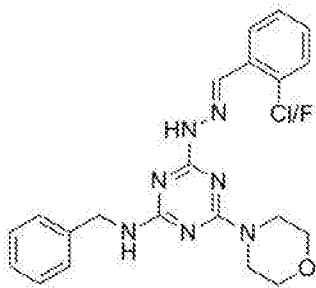
vacuolin-1



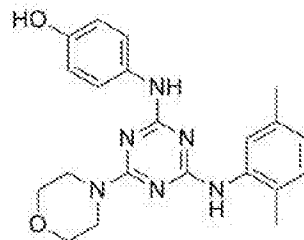
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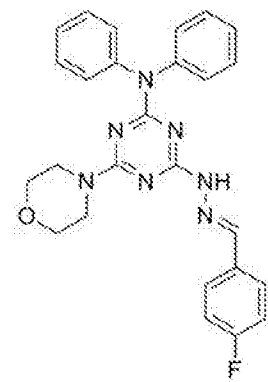
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Figure 1

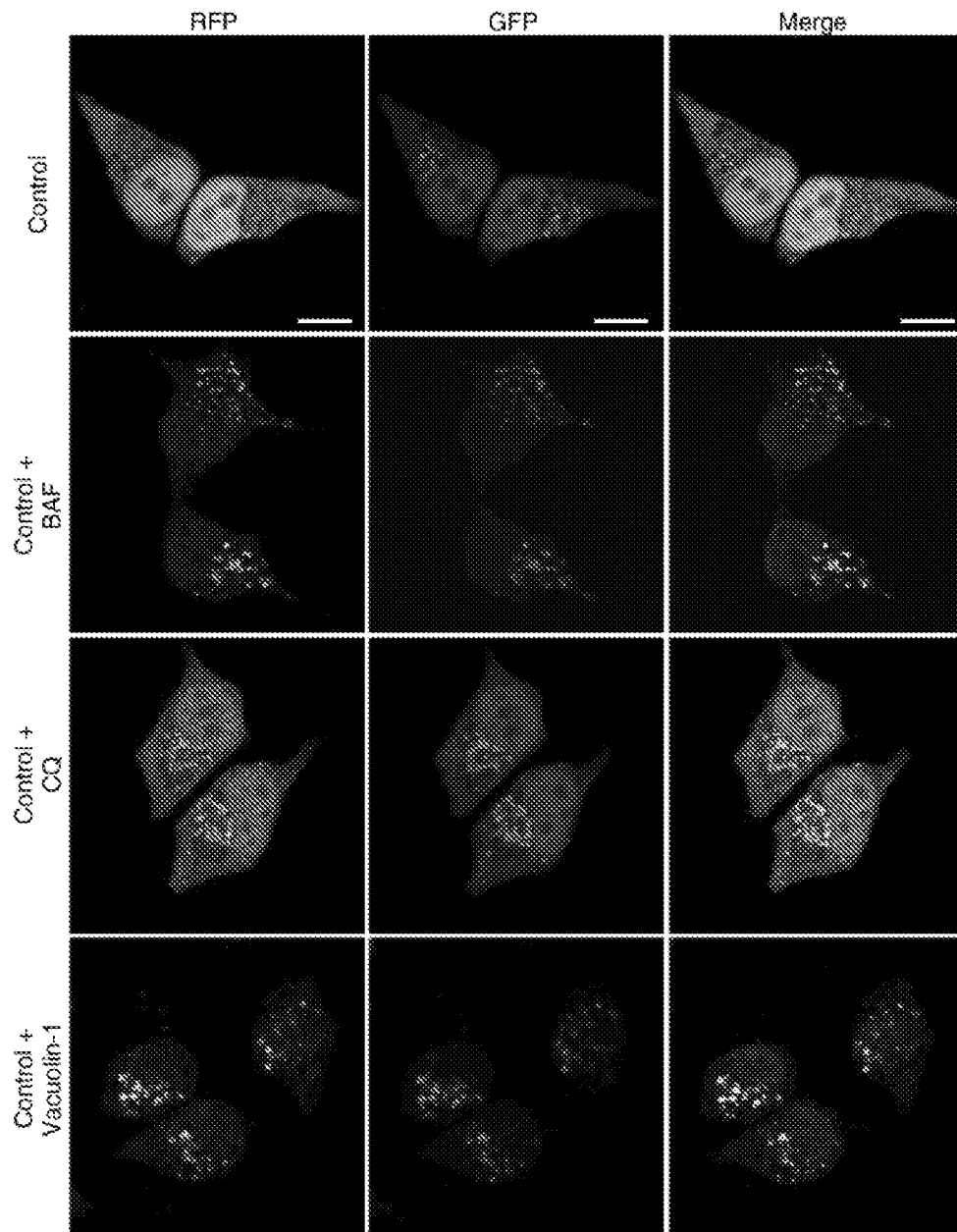


Figure 2

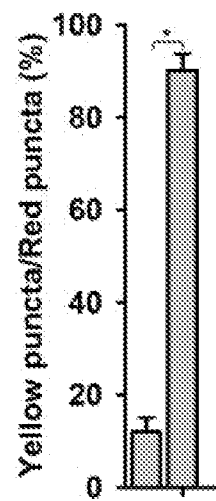
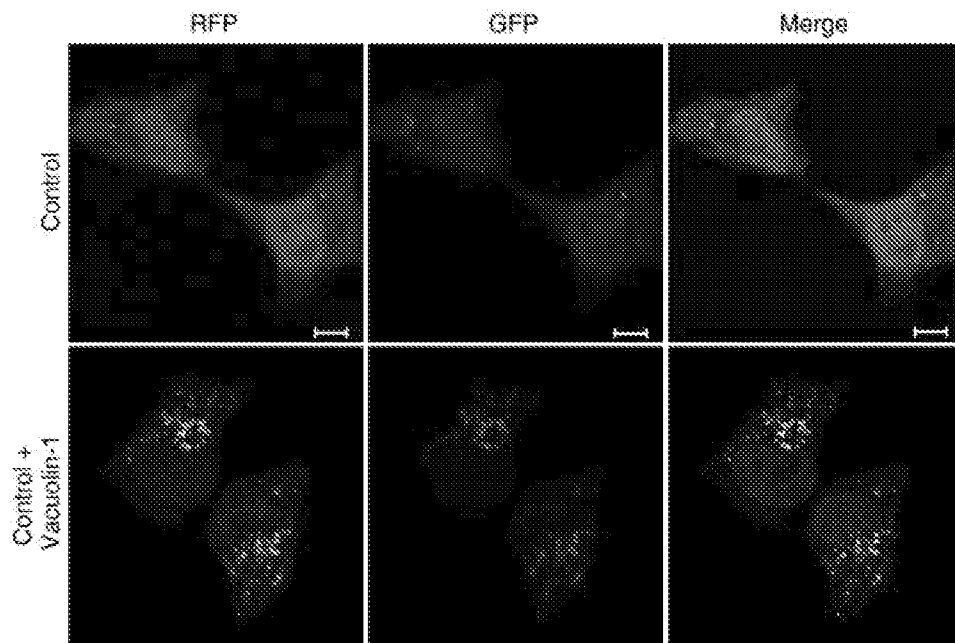


Figure 3A

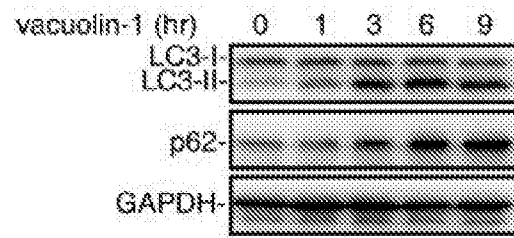


Figure 3B

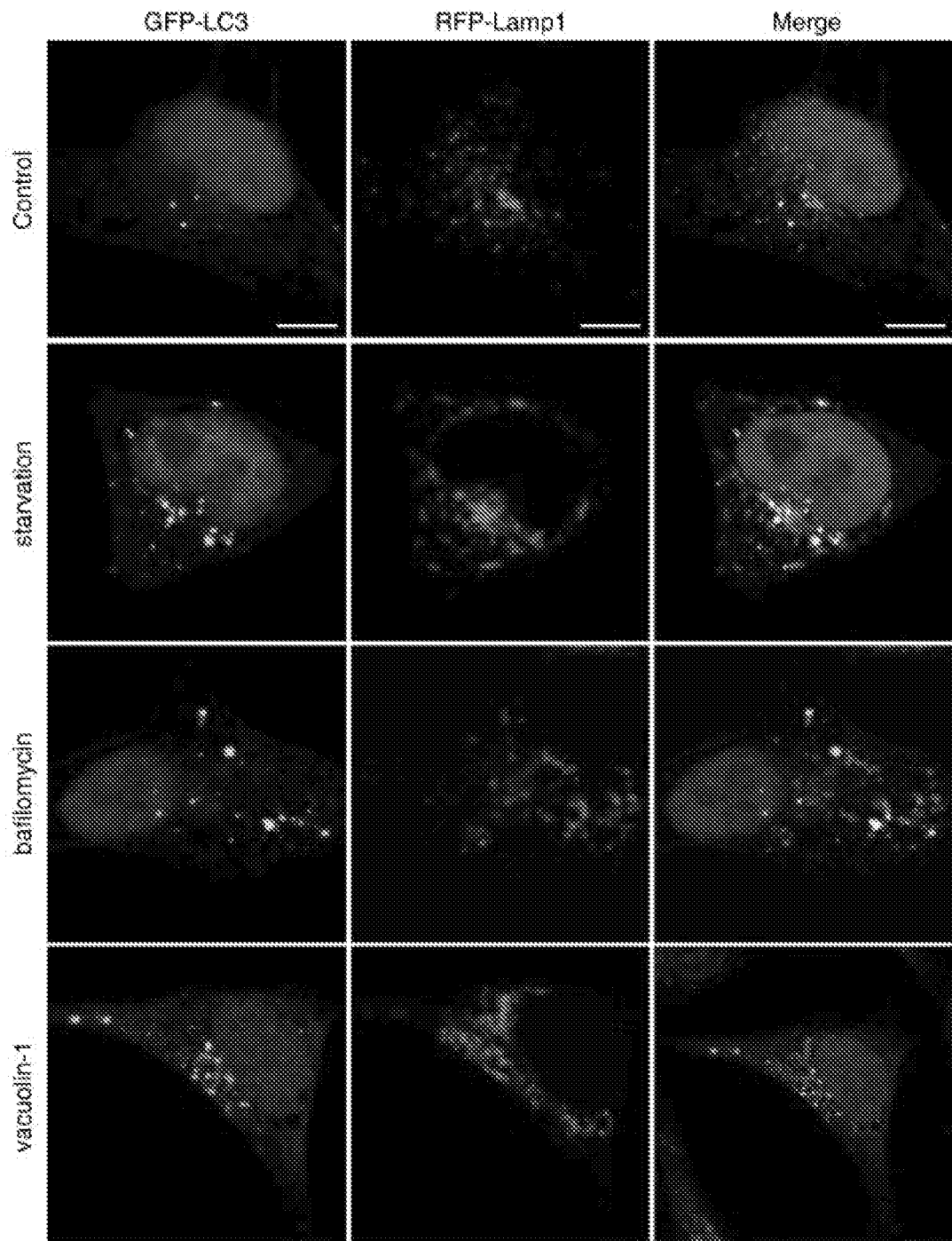
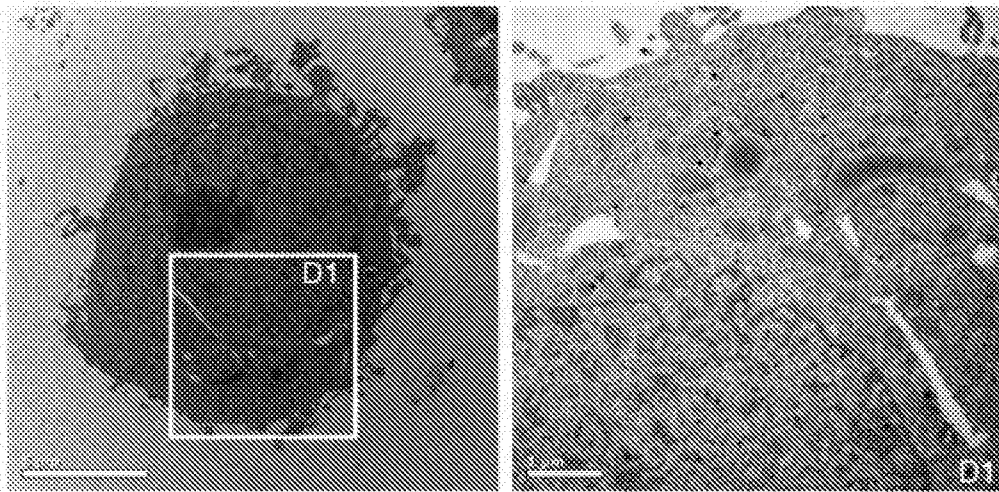
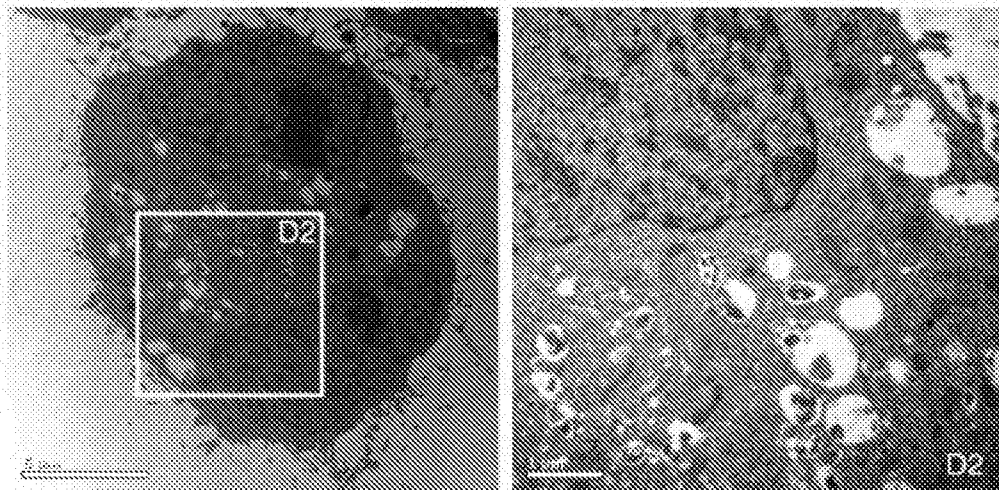


Figure 3C



control



Vacuolin-1

Figure 3D

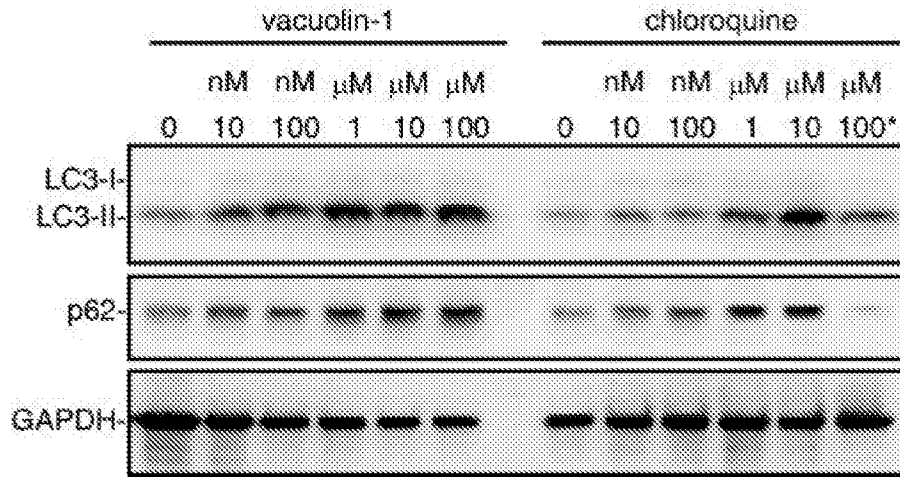


Figure 4A

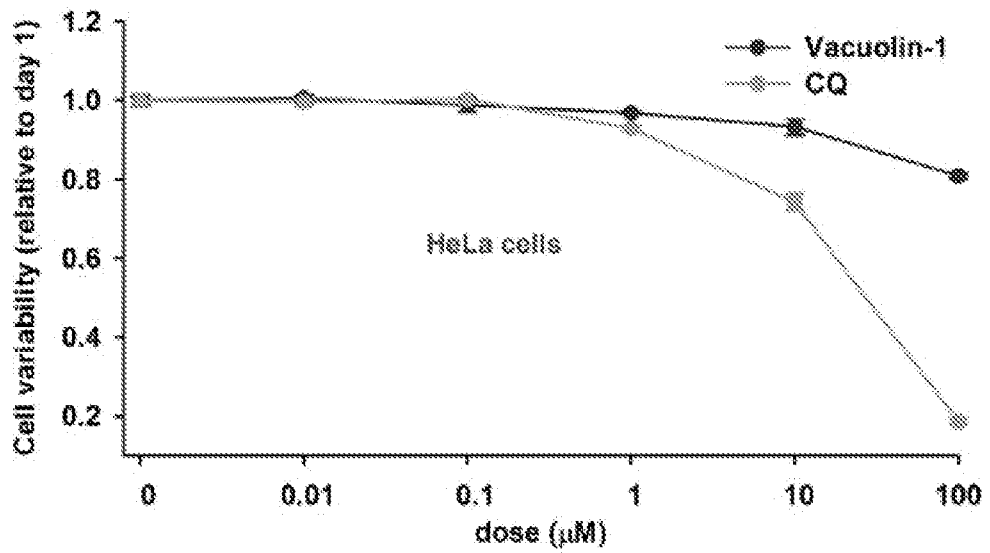


Figure 4B

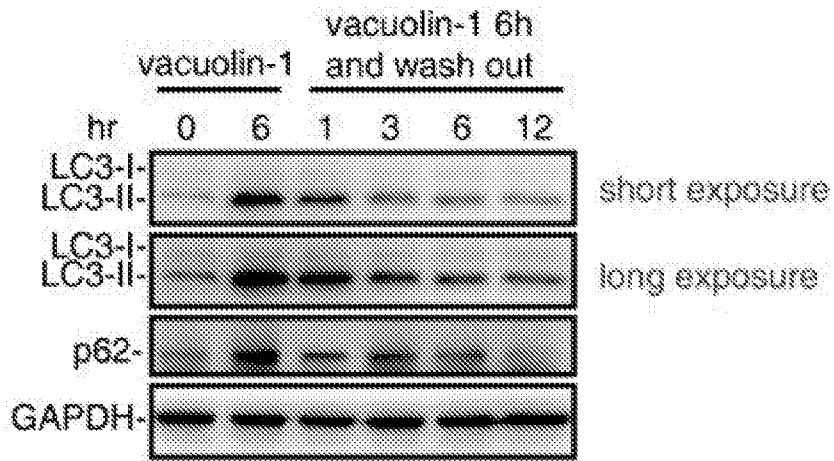


Figure 4C

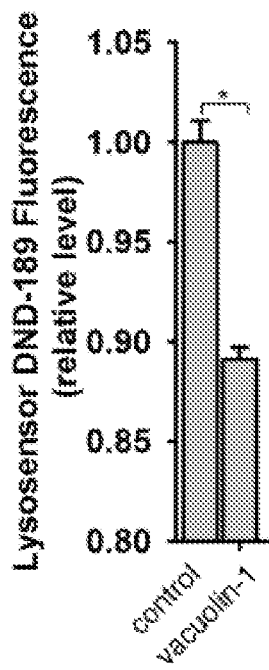


Figure 4D



Figure 4E

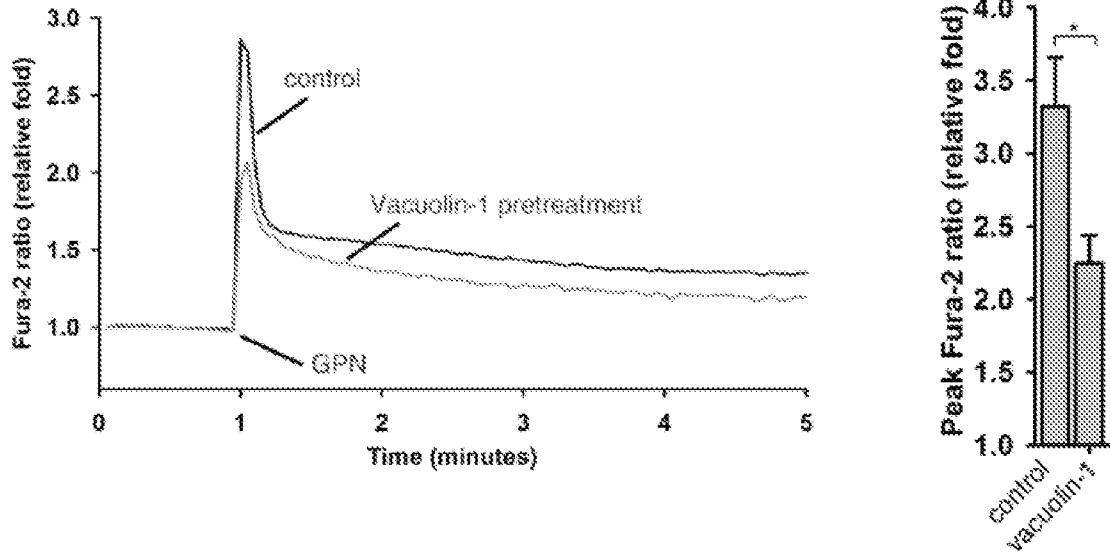


Figure 4F

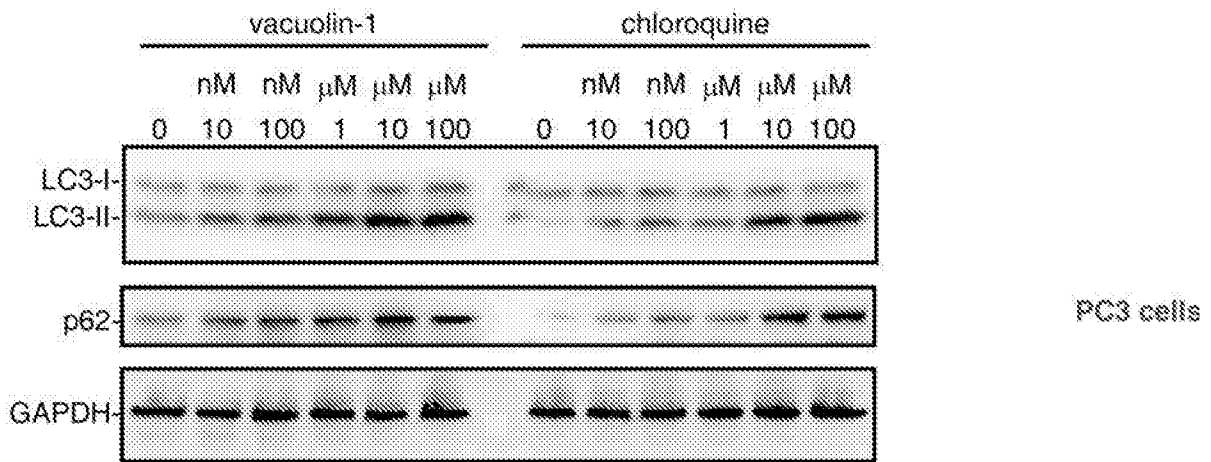


Figure 5

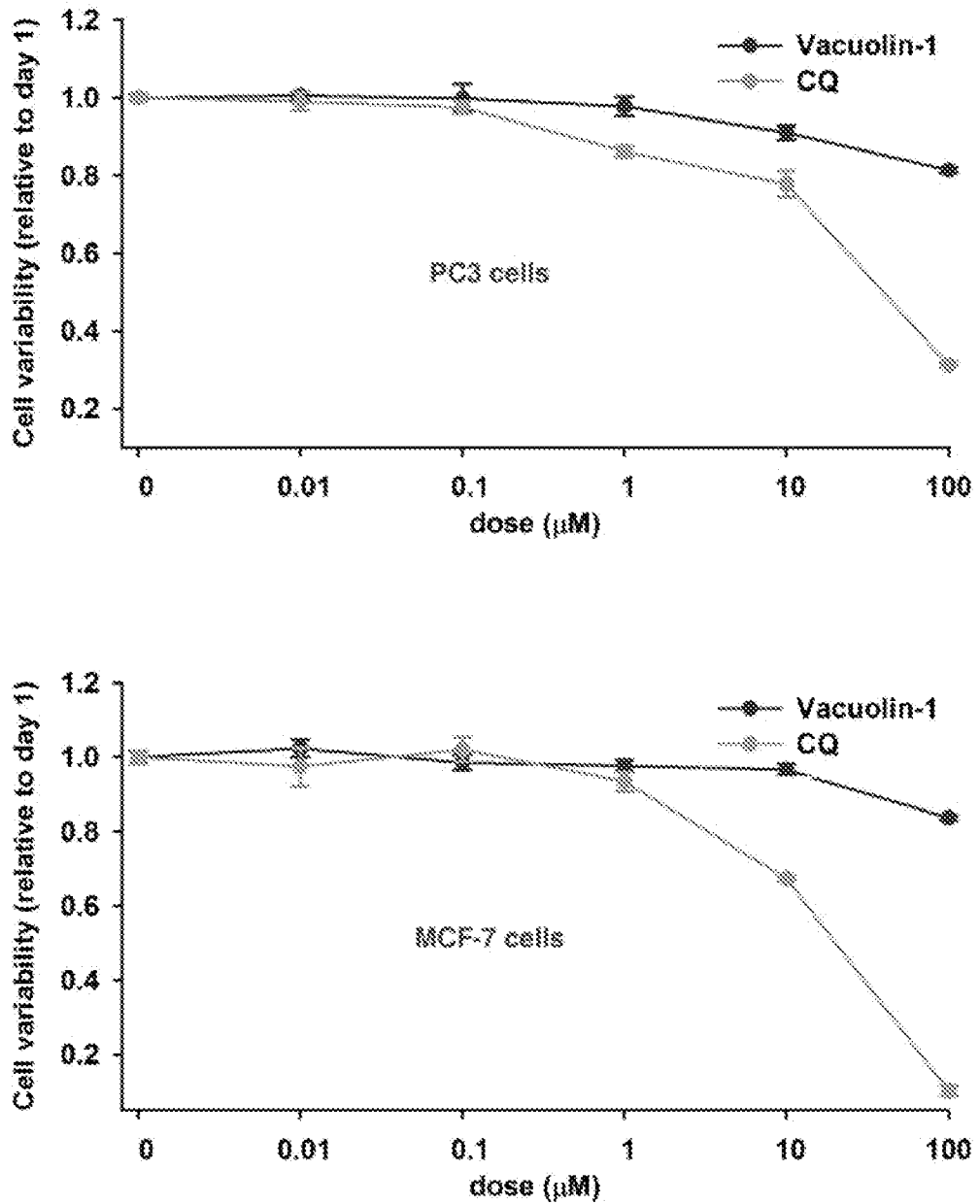


Figure 6

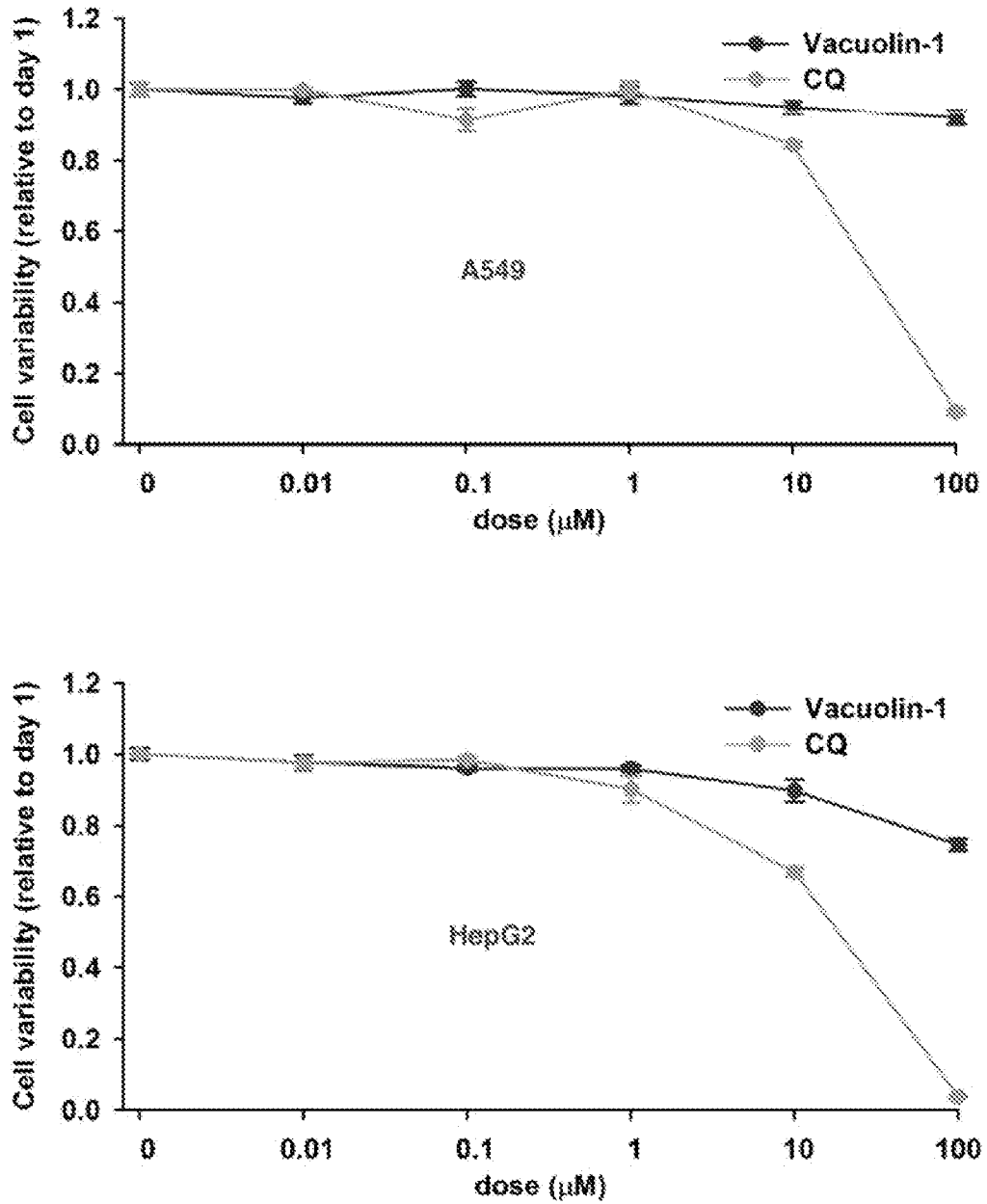


Figure 6 (continued)

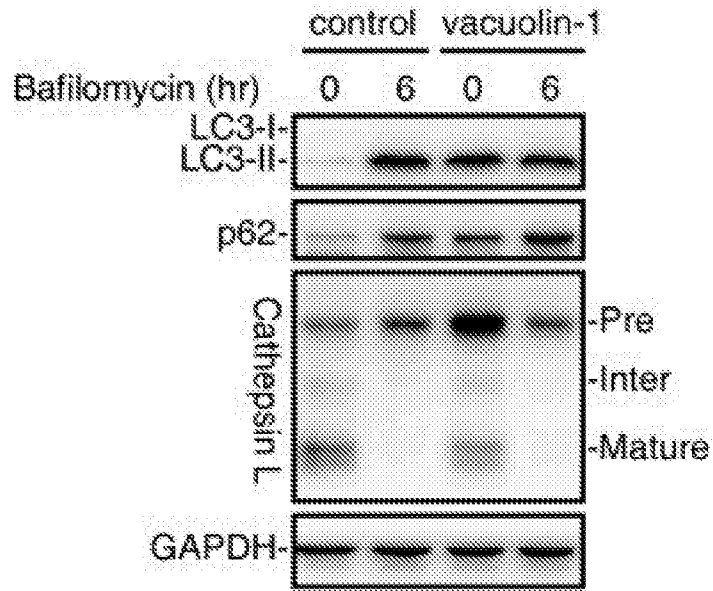


Figure 7A

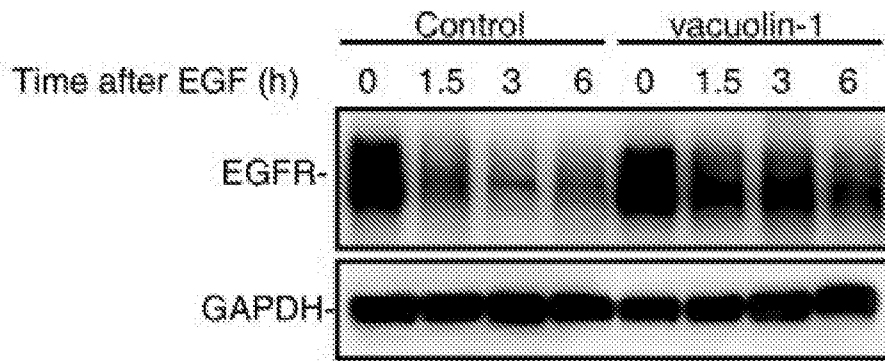


Figure 7B

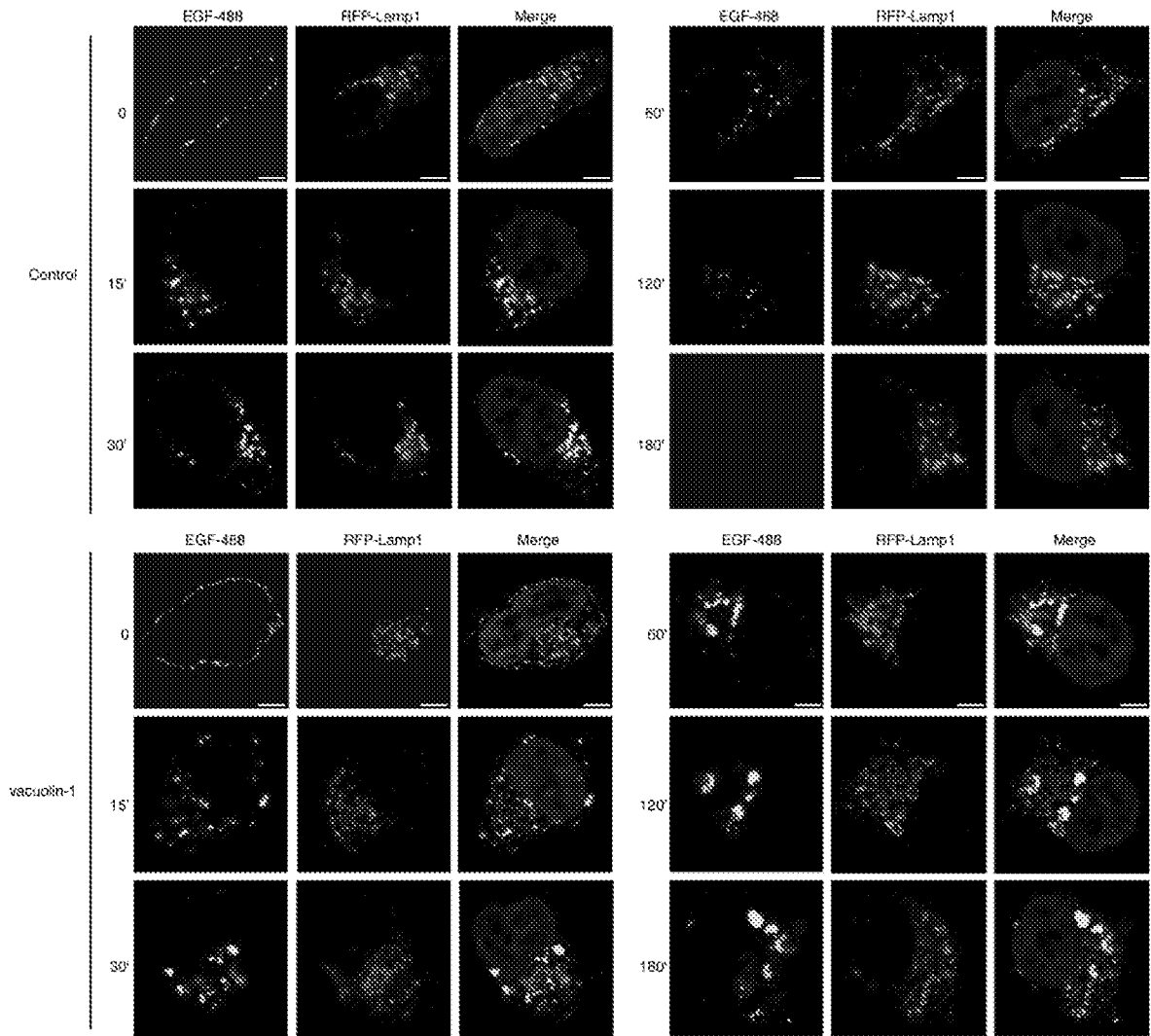


Figure 7C

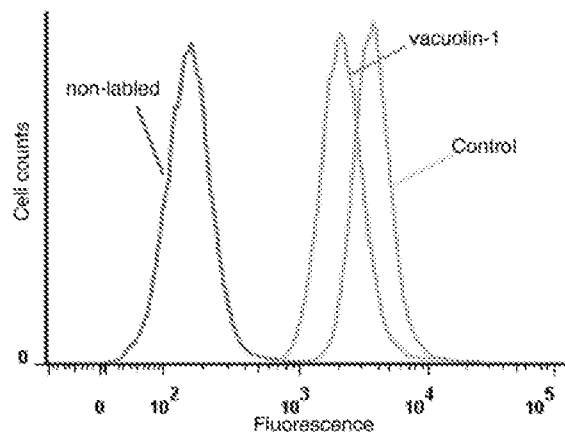


Figure 7D

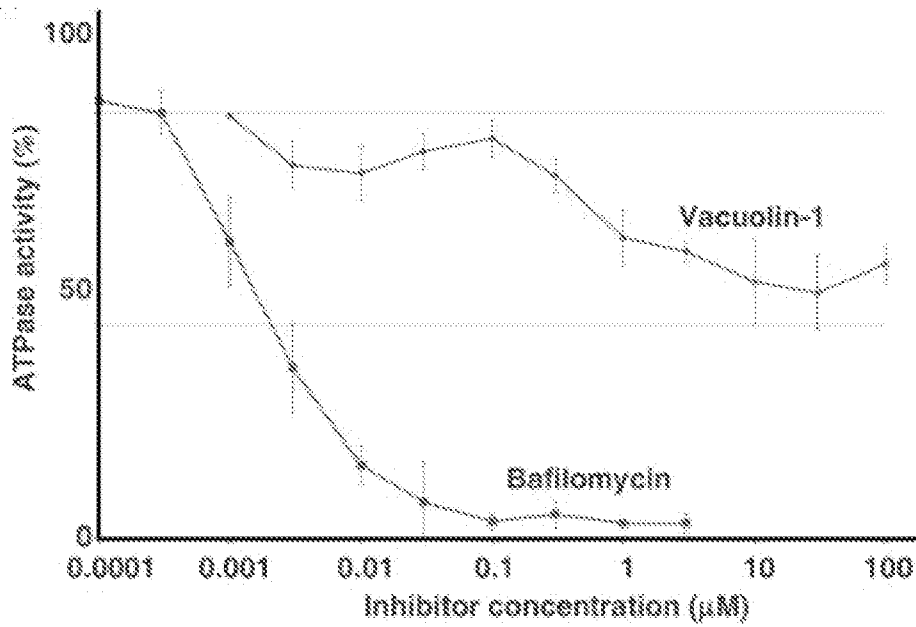


Figure 8A

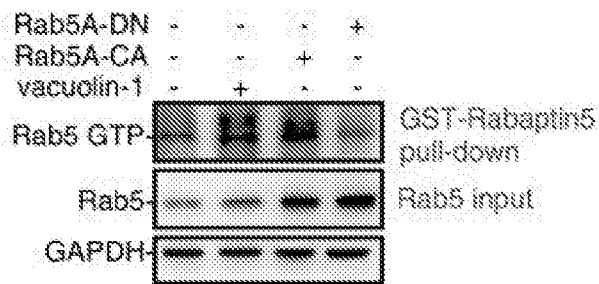


Figure 8B

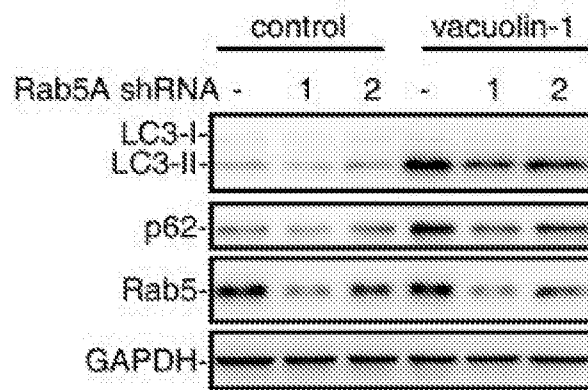
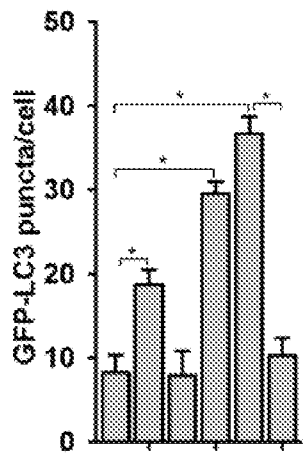
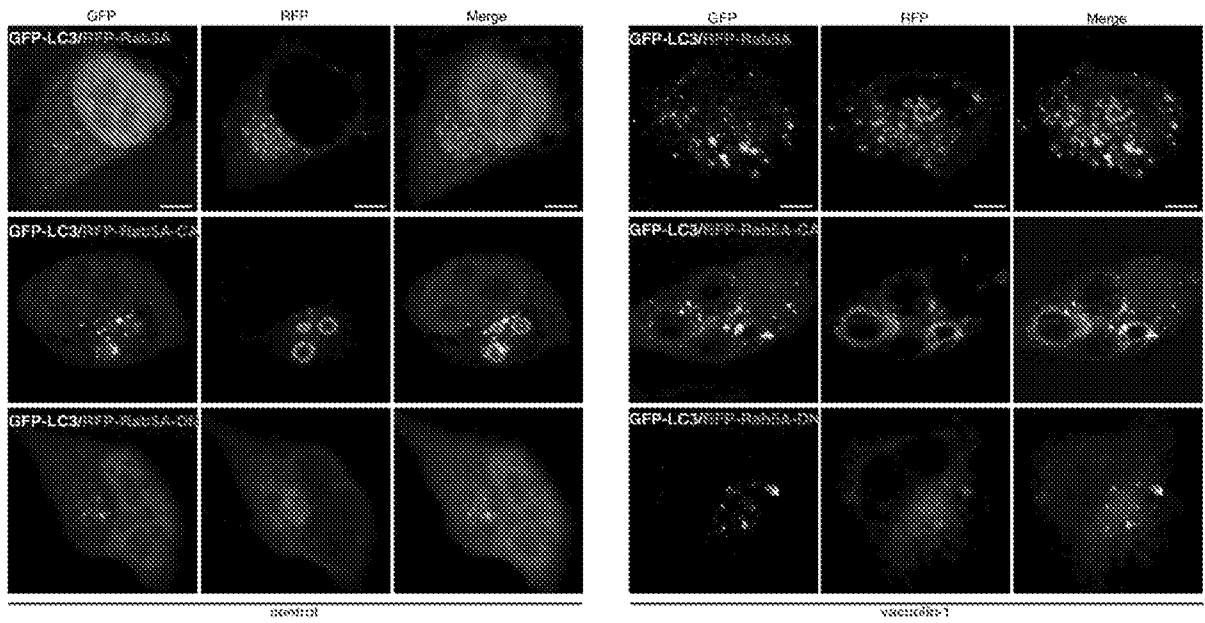


Figure 8C



Rab5A	+	-	-	+	-	-
Rab5A-CA	-	+	-	-	+	-
Rab5A-DN	-	-	+	-	-	+
vacuolin-1	-	-	-	+	+	+

Figure 8D

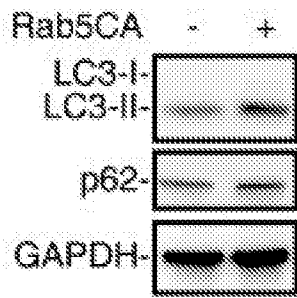


Figure 8E

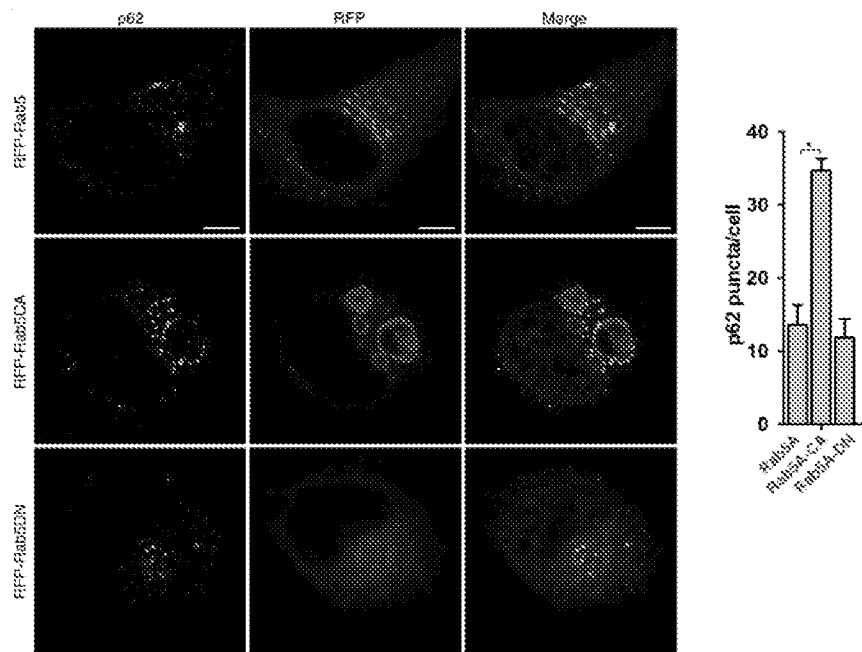


Figure 8F

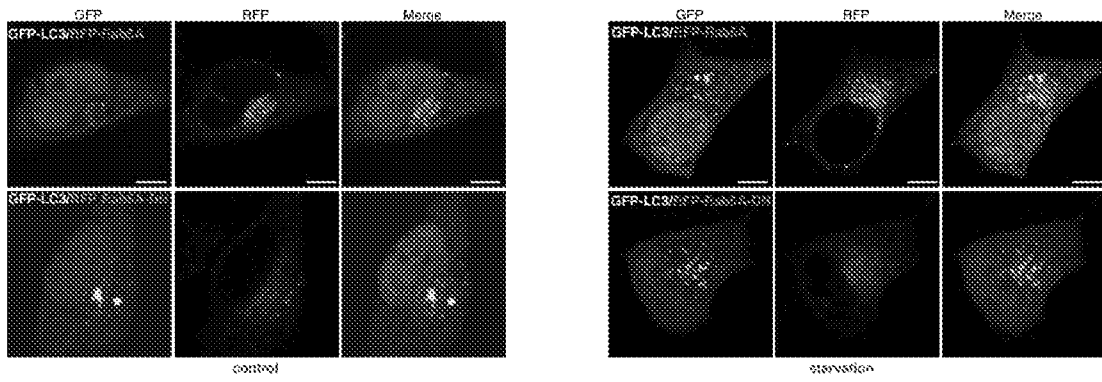


Figure 9

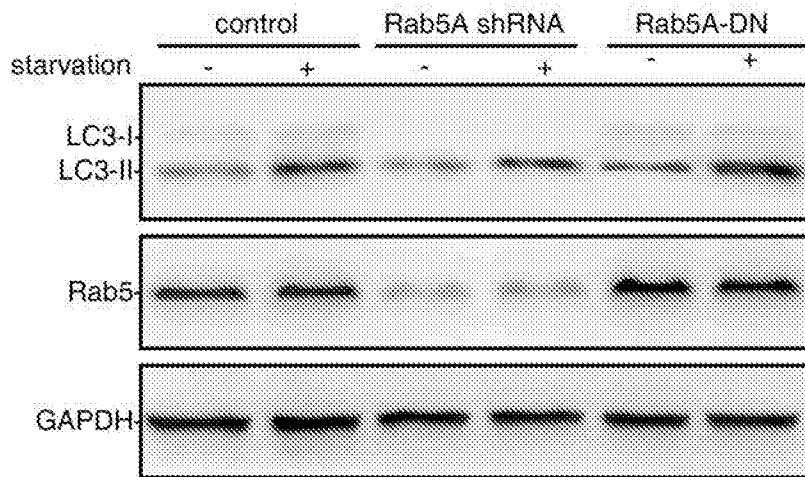
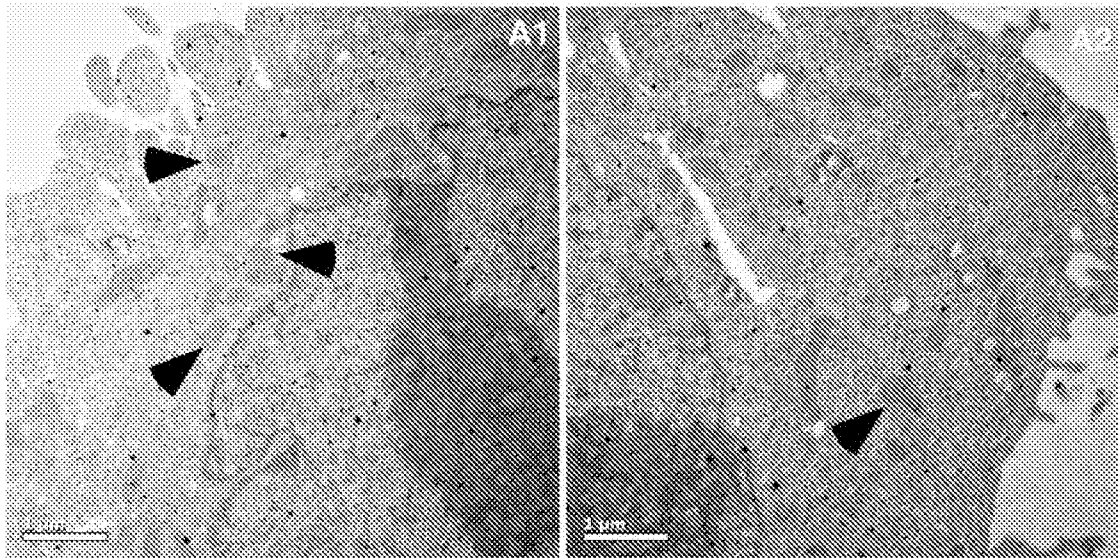
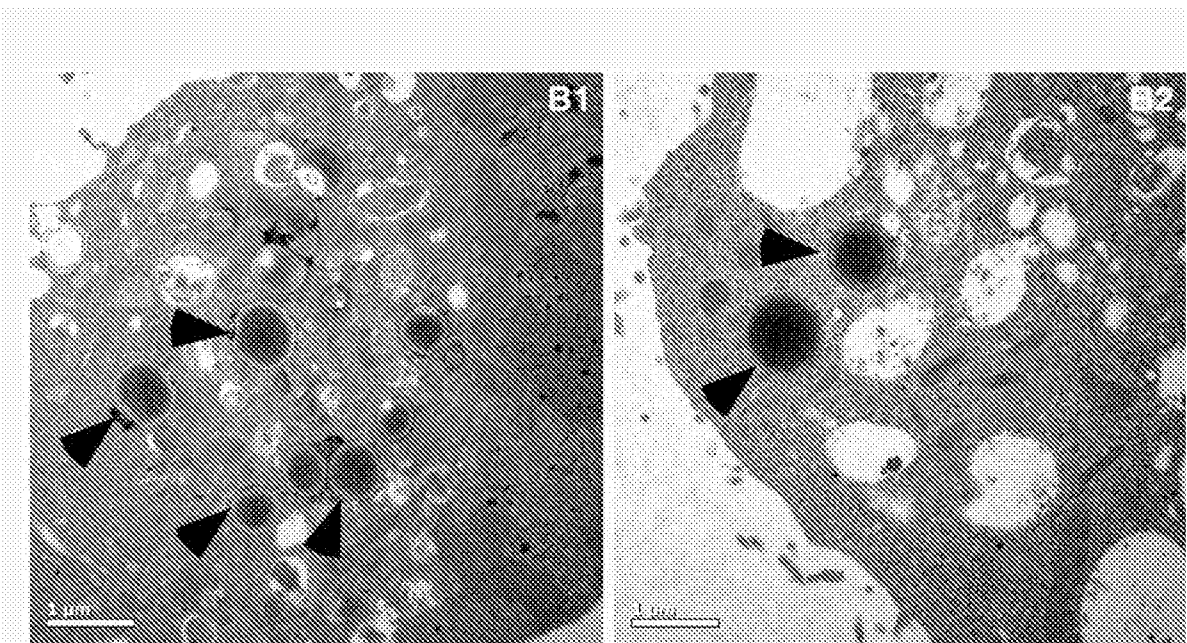


Figure 10



control

Figure 11A



Vacuolin-1

Figure 11B

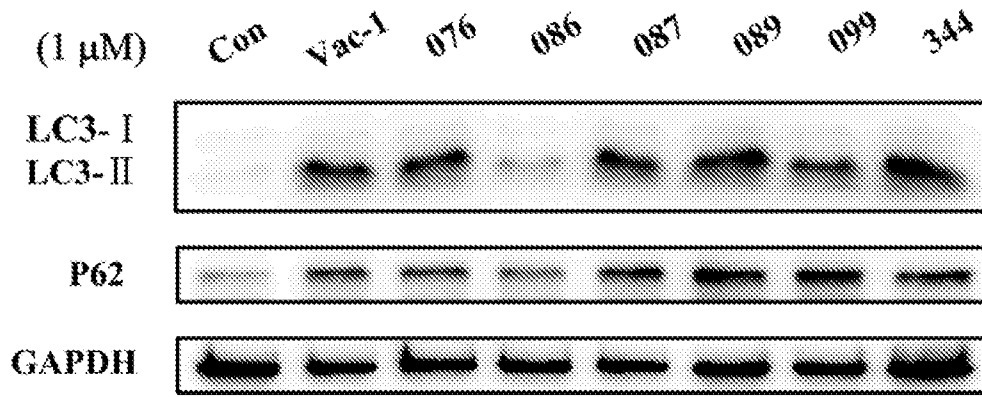


Figure 12

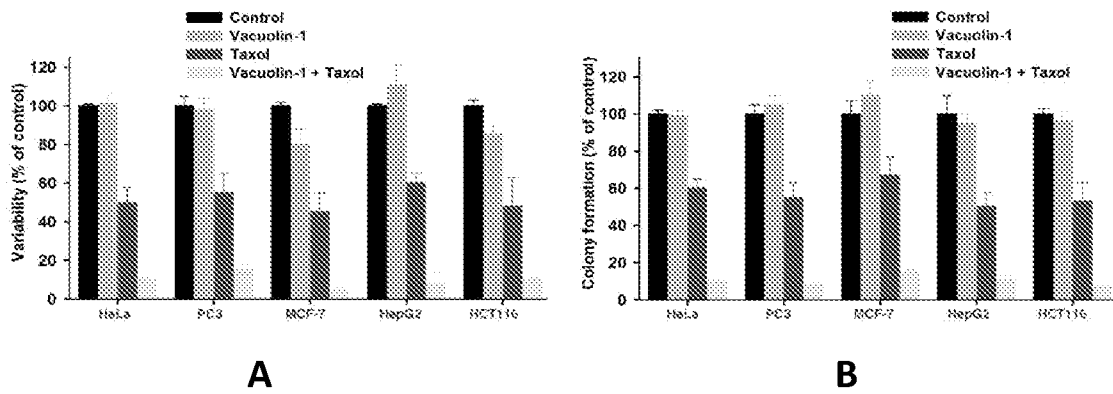


Figure 13

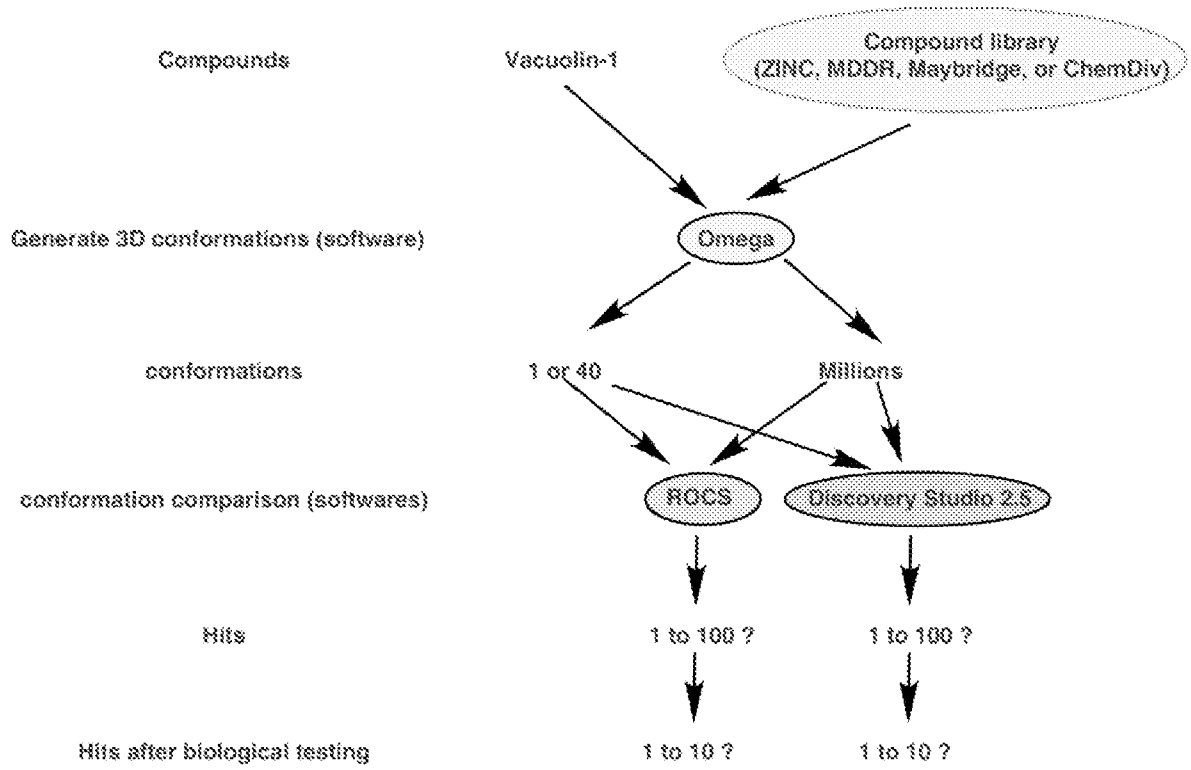


Figure 14

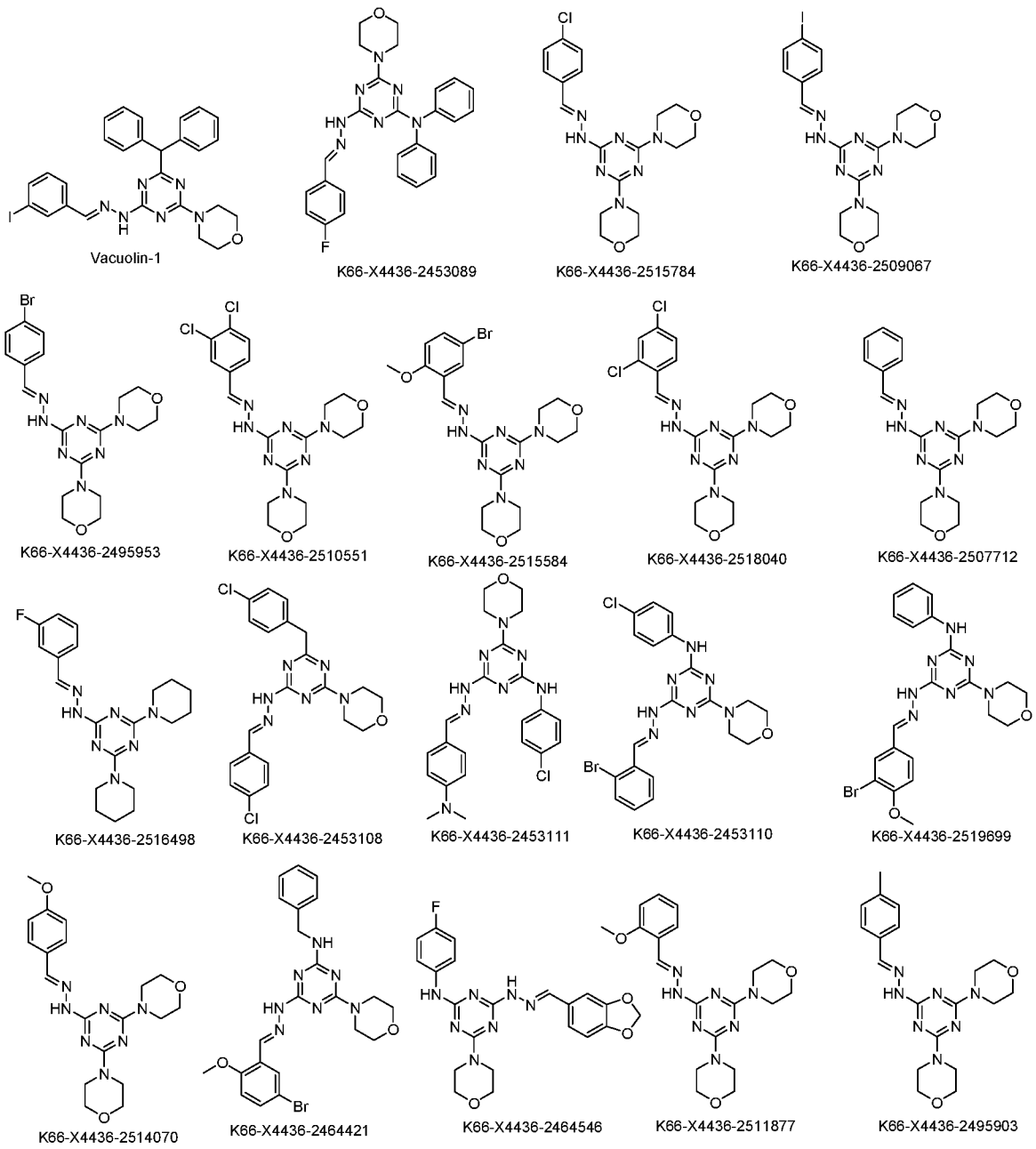


Figure 15

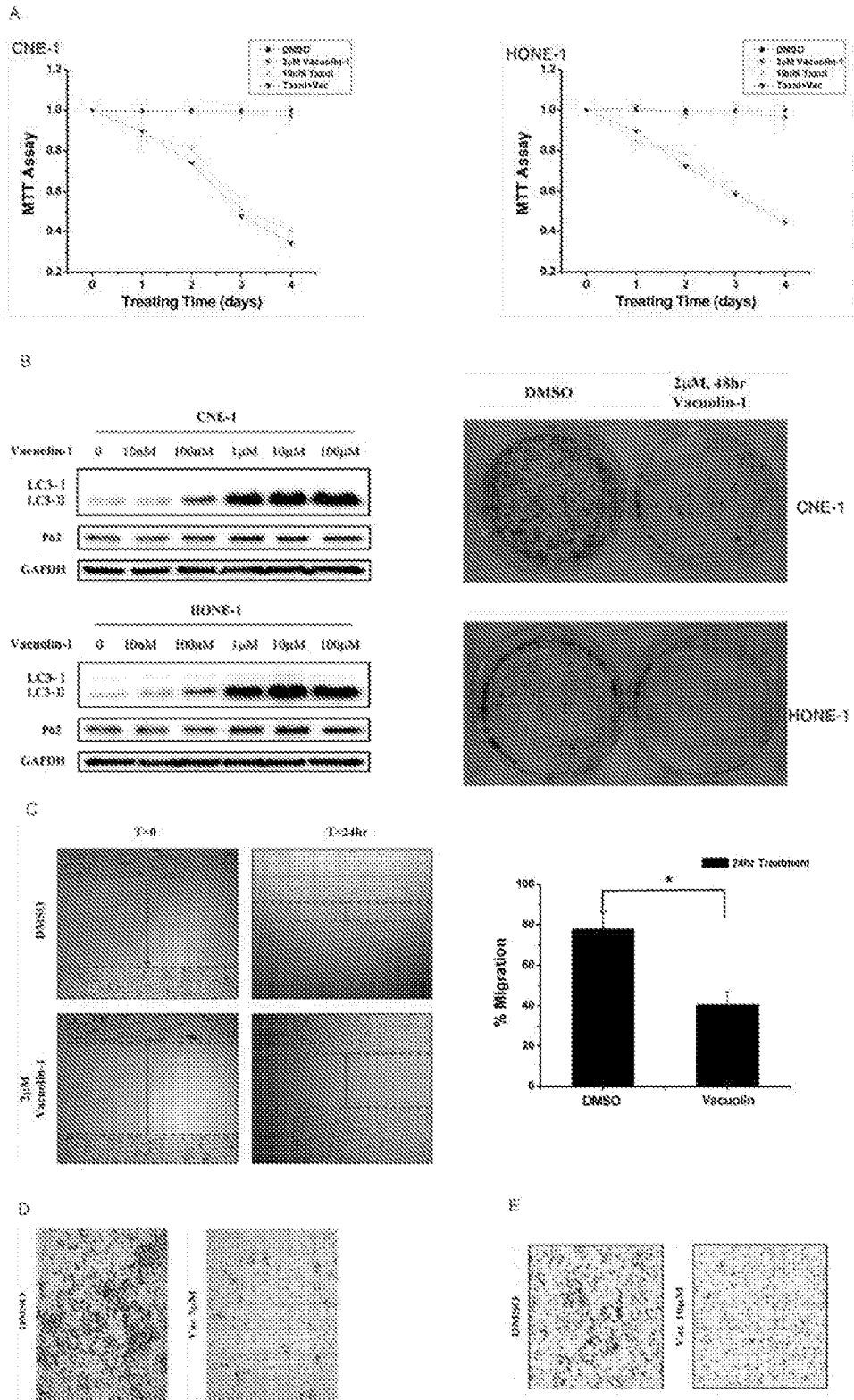


Figure 16

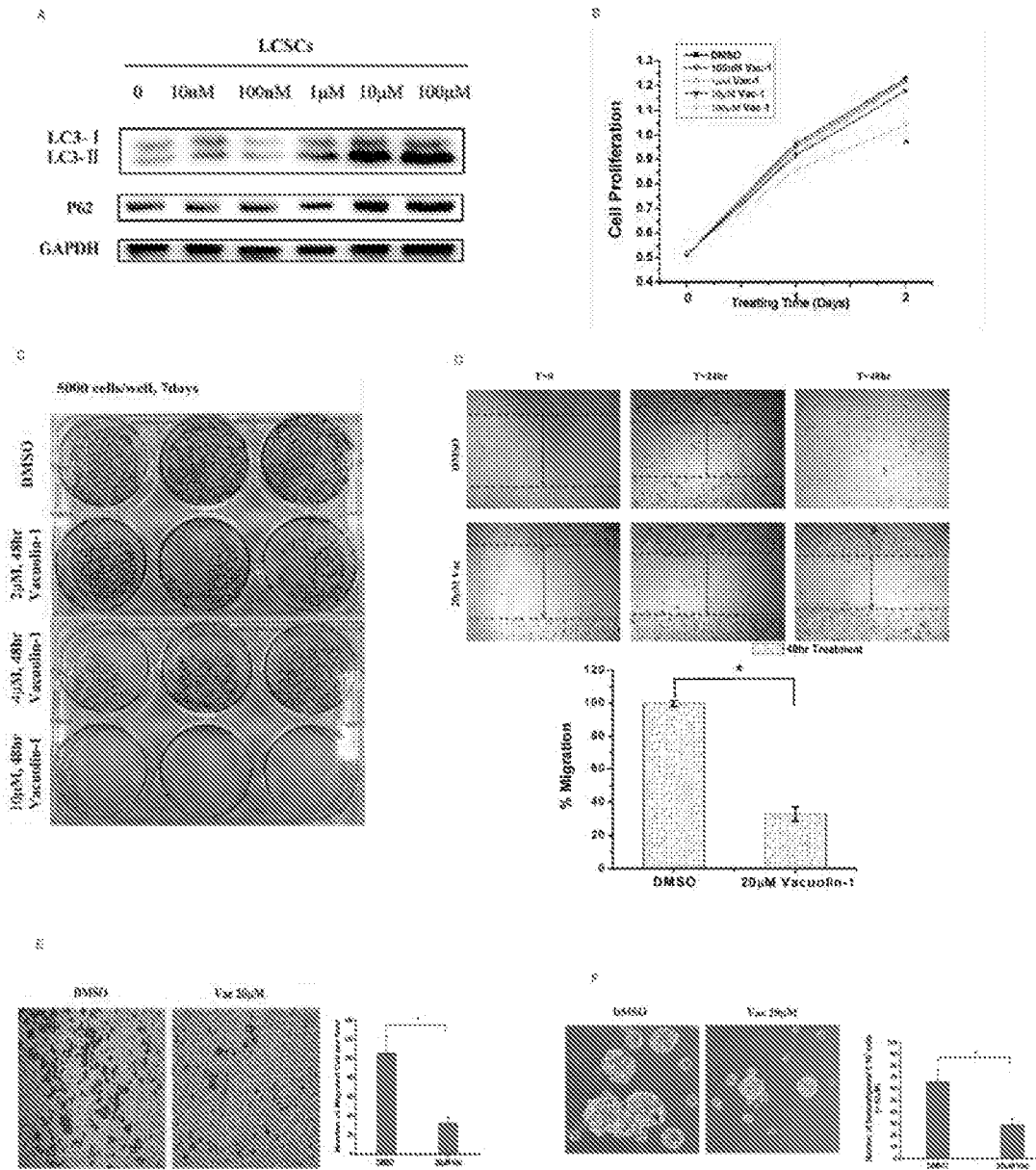


Figure 17

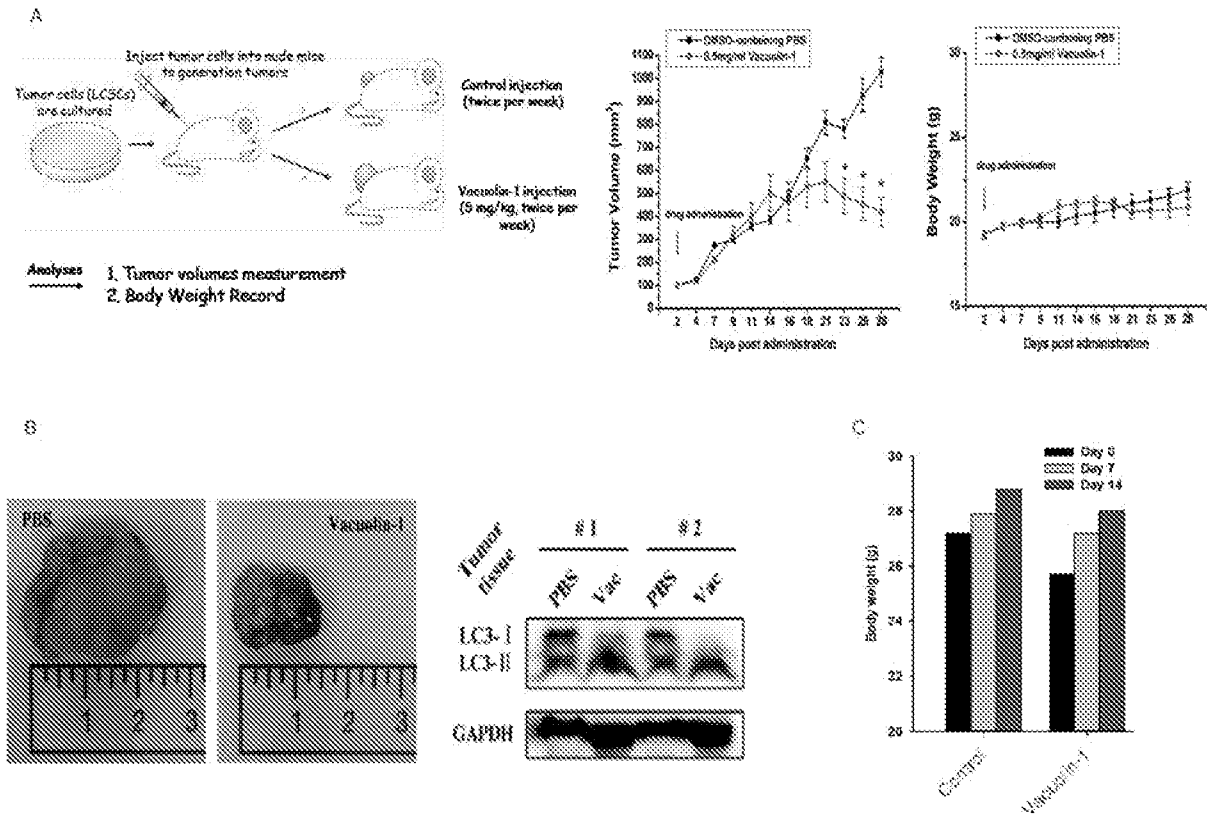


Figure 18

## INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CN2015/073255****A. CLASSIFICATION OF SUBJECT MATTER**

A61K 31/5377(2006.01)i; A61P 35/00(2006.01)i; A61P 33/06(2006.01)i; A61K 31/337(2006.01)i; A61K 31/436(2006.01)i; A61K 31/513(2006.01)i

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K; A61P

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)

WPI, EPODOC, CNPAT(CN), CNKI(CN), Chinese Pharmaceutical Abstract (CN), CHEMICAL ABSTRACTS(US), EMBASE, STN: vacuolin+, lung, cancer, tumour, tumor, carcinoma, neoplasms, autophagy, endosomal traffic, malaria, taxol, fluorouracil, 5-fu, temirolimus, temisrolimus, combin+

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20060019951 A1 (CBR INSTITUTE FOR BIOMEDICAL RESEARCH) 26 January 2006 (2006-01-26) see the whole document	1-21

 Further documents are listed in the continuation of Box C. See patent family annex.

\* Special categories of cited documents:

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

"E" earlier 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

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

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

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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

**19 May 2015**

Date of mailing of the international search report

**04 June 2015**

Name and mailing address of the ISA/CN

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Authorized officer

**XIU, Wen**

Facsimile No. (86-10)62019451

Telephone No. (86-10)62411205

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2015/073255

**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.: **1-21**  
because they relate to subject matter not required to be searched by this Authority, namely:  
[1] The claims relate to methods of treating disorders (PCT R39.1(iv)), but the search has been carried out and based on the reasonably anticipated subjects, i.e. the use of vacuolin-1, and/or one or more analogues, alone or in combination with one or more chemotherapy drugs in manufacture of medicaments for treating disorders.
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:

- [1] Claims 1-16, 21 relate to methods of treating carcinoma or cancer, claims 17-20 relate to methods of treating malaria. The same technical feature between above two groups of claims is vacuolin-1. The prior art has been identified as document US 20060019951 A1 and discloses compound vacuolins. It follows that the technical feature said above of claims 1-16, 21 and claims 17-20 does not make a contribution over the prior art and can not be considered as a special technical feature within the meaning of Rule 13.2 PCT.

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 additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- 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.

**INTERNATIONAL SEARCH REPORT**  
**Information on patent family members**

International application No.

**PCT/CN2015/073255**

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
US 20060019951 A1	26 January 2006	WO 2004001018 A2	31 December 2003
		AU 2003253735 A1	06 January 2004
		AU 2003253735 A8	03 November 2005
		WO 2004001018 A3	01 July 2004