Matrine induces V-ATPase-dependent cytoplasmic vacuolation derived from lysosome and inhibits lysosome function in leukemia cells

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Abstract

Matrine is a main component extracted from legumes and has extensive anti-cancer effects, but its molecular mechanism is unclear. In our study, we found that matrine can induce vacuolation of leukemia cells, whose occurrence was closely related to inhibition of cell proliferation. Interestingly, the vacuolization was reversed after the removal of matrine. Matrine-induced vacuoles are acidic in nature, Transmission Electron Microscope (TEM) revealed that these vacuoles mainly derived from the lysosome, the vacuoles acquire some characteristics of lysosome (LAMP1). RNA-seq showed that the expression of vacuolation-related genes and lysosomal-related genes were up-regulated, and the two parts highly overlapped. Western Blot and FACS confirmed that matrine inhibits the expression and activity of intracellular proteolytic enzymes. RT-qPCR and Western Blot showed that matrine significantly up-regulated the expression levels of V-ATPase subunit genes in cells, and V-ATPase inhibitor significantly reversed the occurrence of cell vacuoles. It is suggested that V-ATPase plays an important role in matrine-induced vacuoles. The molecular structure of matrine was further analyzed, and the protonation of matrine in lysosomes to activate V-ATPase may be the direct cause of the vacuoles, and it is also a new molecular mechanism of matrine against the proliferation of leukemia cells.

Introduction

Matrine is an alkaloid extracted from traditional Chinese medicine Sophora flavescens, Sophora alopecuroides and other legumes. It has various pharmacological effects [1]. Matrine inhibited growth and proliferation of various tumor cells [2–5]. For example, matrine inhibited the proliferation of leukemia cells by inhibiting the ERK/MAPK pathway mediated by the BCR/ABL fusion gene in chronic myeloid leukemia cells [6]. Matrine induced apoptosis of leukemia cells by inhibiting the phosphorylation level of PI3K/Akt/mTOR signaling pathway in leukemia cells [7]. Our team found that matrine down-regulates the expression of HK2 by inhibiting c-Myc, thereby inhibiting glycolysis and proliferation of leukemia cells [8]. Although the anti-tumor effect of matrine is well definite, the mechanism involved is still unclear and there are many unknowns to be explored.

Lysosome is a digestive organelle, and maintaining its normal function is crucial to the balance of the intracellular environment. It contains a large amount of cathepsin, which is a necessary structure for cell catabolism and phagocytosis of senescent cells [9]. The dysfunction of lysosome leads to cell death in various types [10–11]. After lysosomal damage, cathepsins are released into the cytoplasm to cleaves the BH3-interacting region death agonist BID into a pro-apoptotic tBID fragment, which promoted BAX oligomerization, and activated the caspase family, then induced apoptosis [12]. Lysosomal dysfunction leads to the release of cathepsin D into the cytoplasm, and the caspase-8 was cleavage, then promoted the activation of RIPK-1, so as to induce cell necrosis [13]. The decreased expression and activity of cathepsin affect the digestion and decomposition of lysosomes [14]. Some anti-tumor drugs alter the permeability of lysosomes, leading to the secretion of cathepsins from the lysosomes into the cytoplasm, which initiated the lysosomal apoptotic pathway and induced lysosome-dependent cell death [15]. When the lysosomal function was inhibited, the generation of intracellular iron and lysosomal reactive oxygen

species was affected, thereby induced ferroptosis [16]. Impaired lysosomal function also promoted the accumulation of RIPK1, RIPK3 (receptor-interacting protein kinases 1 and 3), and the necrosis effector protein MLKL, leading to cell necrosis [17]. Therefore, targeting lysosomes has become a potential target for the treatment of cancer in recent years.

While studying the inhibitory effect of matrine on leukemia cells, we found an interesting phenomenon that matrine can induce vacuoles in human leukemia cells. With the prolongation of time and the increase of concentration of matrine treatment, the vacuoles became more obvious. When matrine was withdrawn, the vacuoles were reversed. It has been reported that drug-induced vacuolation can promote cell death [18]. Vacuolization was caused by a variety of factors. The vacuolar structure of a monolayer membrane in the cytoplasm. The appearance of vacuolization is often accompanied by cell death, however, its mechanism in the process of cell death is still unclear [19]. Some lipophilic small molecule compounds with weak bases can enter the lysosome, causing the lysosome to absorb water and expands by changing the osmotic pressure, resulting in vacuoles [20]. Small molecule compounds inhibited the maturation of lysosomes by activating ERK kinases, leading to the formation of lysosomal vacuoles and promoting cell death [21]. Akebia saponin E(ASE) induced vacuolization of liver cancer cells, which in turn induced cell death [22].

Our results showed that matrine could induce vacuolation of leukemia cells, thereby inhibiting cell proliferation. Matrine-induced vacuoles are derived from lysosomes, which inhibit the function of lysosomes. Both expression and activity of lysosomal proteolytic enzymes were inhibited. The occurrence of vacuoles is determined by the chemical structure of matrine itself and the lysosomal membrane proton pump. This research provides a new scientific basis to elucidate the molecular mechanism of matrine anti-leukemia cells.

Methods

Cell Lines and Reagents

Human leukemia cell lines K562, U937 and HL60 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells are cultured in RPMI 1640 (Corning, USA) with 10% fetal bovine serum (Gibco, USA) at 37◦C, 5% CO2. Matrine was obtained from Shanghai Macklin Biochemical Technology Co. Ltd. (Macklin, Shanghai China), and its purity was 98%, A stock solution was prepared in double distilled water (ddH2O) at 40 mg/ml and stored at 4°C.

Cell Proliferation assay

K562, U937 and HL60 cells were treated with matrine, and cell proliferation was analyzed by Cell Counting Kit-8 kit (Dojindo Molecular Technologies, Japan). Cells were treated with various concentrations of matrine (0.2, 0.4 ,0.8, 1.6 mg/mL) for 24 h, 48 h and 72 h, respectively. The cell density is 1.5×10^5 /1×10^5 /0.75×10^5 per well into 96-well plates for 20 h, 44 h, or 68 h, then CCK-8 reagent

was added for 4 h before detection. Optical density (OD) was measured using a Microplate Reader (Bio-Rad, USA) at 450 nm.

Western blot and Antibodies

The harvested cells were lysed by RIPA Lysis Buffer (Beyotime, China) for the extraction of total protein. Protein concentration was measured with BCA Protein Assay Kit (Beyotime, China). Then, lysate protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membranes (Immobilon, USA). The membranes were blocked by 5% skimmed milk and incubated overnight with the following primary antibodies: β-Actin CTSB CTSK ATP6AP1 ATP6V1A ATP6V0D1 (Protein tech, USA), then incubating secondary antibodies (Cell Signaling Technology, USA). Protein expression was detected using an ECL kits (Beyotime, China). The protein bands were measured with image acquisition and analysis software (Bio-Rad, USA).

Real-Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and reversely transcribed into into cDNA using Prime Script RT reagent kit with gDNA remover (TOYOBO, Japan). cDNA was quantified by the real-time PCR with a SYBR Green qPCR kit (Bimake, USA). The relative expressions of every gene were assessed in comparison with β-actin. The sequences of the primers used were as Table S1.

Neutral red staining

Neutral red is a PH indicator that appears red when exposed to acidic conditions and yellow when exposed to PH 6.8-8.0. K562 and HL60 cells were treated with matrine for 24 h, U937 cells for 48 h, and stained with 1% neutral red (Solarbio, Beijing China) for 20 min. The supernatant was discarded by centrifugation, 1 mL of 1 × PBS was added, and the cells were mixed. Take 20 μL on a glass slide and observe the staining of cells under Optical Microscope (Olympus, Japan).

Transmission Electron Microscope analysis

Control and treatment groups of K562 cells were fixed with 2.5% glutaraldehyde, 1M phosphate buffer (PH7.4) washing 3 times, 15 min each time; 1% osmic acid 0.1M phosphate buffer (PH7.4) room temperature (20℃) fixed for 2 h; 0.1M phosphate buffer (PH7.4) washing 3 times, 15 min each time; 50%-70%-80%-90%-95%-100%-100% alcohol are dehydration, 15 min each time; Acetone: 812 packets Embedding agent = 1:1 mixed solution are infiltrated overnight; the sample was polymerized at 60℃ for 48 h; The prepared sample was cut 60–80 nm ultrathin and stained with 2% uranyl acetate saturated aqueous solution and lead citrate for 15 min to dry overnight at room temperature, and then observed under Transmission Electron Microscope(FEI Tecnai G20 TWIN,USA) and photographed.

RNA-sequencing experiment and analysis

RNA was extracted using Trizol reagent (Invitrogen, USA), according to the manufacturer's

protocol. Then use the sequencing reads and select differentially expressed genes (| log2(fold change | ≥1.8, Padj ≤ 0.05 and FPKM ≥ 1), between the two groups were analyzed using Top GO_CC. Gene Set Enrichment Analysis (GSEA) was used to analyzing the significant differentially expressed genes(DEGs)using the cluster Profiler R package.

Fluorescence Microscopy analysis

To confirm the specific location of vacuoles, a lysosomal membrane marker fused with green fluorescent protein (LAMP1-eGFP) was constructed onto a lentiviral vector (pRRLSIN-cPPT-SFFV-MCS-SV40-puromycin) specialized for suspension cells. Both vector construction and virus packaging were performed by Shanghai Genechem. The leukemic cells were infected with virus with a MOI of 30 to construct stable cell lines. After matrine treatment, the location distribution of green fluorescence in cells was observed by Fluorescence Microscope..

DQ-green BSA assay

DQ Green BSA is composed of the fluorescent dye BODIPY and bovine serum albumin derivatives. BODIPY has self-quenching properties. The two substances are coupled together and will not emit light. When protease digests and hydrolyzes BSA protein, the protein fragments produced will emit bright fluorescence, so DQ Green BSA monitor the proteolysis of BSA, and the stronger the hydrolase activity, the more fluorophores are released. General lysosomal protease activity was measured by DQ green BSA (Thermo Scientific, USA), 1×10^6/ml cells were incubated with 10μg/mL DQ green BSA for 3h at 37°C, 5% CO2, 0.8mg/mL matrine was added and cultured for 24h, Washing 3 times with PBS (Gibco, USA) for 5 min each, fluorescence intensity were assessed by Flow Cytometry (BD FACS Canto, USA).

Statistical Analysis

The data was analyzed with Graphpad Prism7 software and SPSS 25.0. The quantitative data were presented as means ± SD, in this study, t test was applied for comparison of the means of two groups, AVONA was applied fo multiple treatment groups were compared. Differences were determined statistically significant when *p*-value was < 0.05.

Results

Matrine induces vacuolation in human leukemia cells

Human leukemia cells were treated with different concentrations of matrine, and the morphological changes of the cells were observed by Optical Microscope. Matrine can obviously induced vacuoles in human leukemia cells. The higher the concentration of matrine, the larger the vacuole volume (Fig. 1A). Calculated percentages of the vacuolated cells, it was found that the higher the concentration of matrine, the more cells with vacuoles (Fig. 1B). K562 cells were treated with 0.8 mg/mL matrine for 24 h, and TEM confirmed the occurrence of vacuoles (Fig. 1C). It is suggested that matrine can induce vacuolation in different leukemia cells.

Vacuolation was closely related to cell proliferation

Different concentrations of matrine was used to treated cells for 24h, 48h and 72h, respectively. The results showed that matrine effectively inhibited the proliferation of human leukemia cells in a dose- and time-dependent manner (Fig. 2A). K562 cells were treated with 0.8 mg/mL matrine for 24 h, vacuoles occurred in the cells, the matrine was removed, the cells were cultured for 24 h, and the vacuoles disappeared and cell viability is restored (Fig. 2B,2C). K562 cells were treated with matrine for different times, and the changes in cell vacuoles were observed, and cell proliferation was measured. The higher the concentration and the longer the time of matrine treatment, the more vacuoles appeared in the cell and the lower the cell viability, until finally the cell burst and died (Fig. 2D). These data are accordant with cell viability assessment, implicating that vacuoles play an important role in matrine inhibiting the proliferation of human leukemia cells.

Matrine-induced vacuoles localize to lysosomes

To determine the origin of the vacuole, a neutral red stain was first used to determine whether it was acidic or alkaline. The results showed that the nuclei were stained ? in the control group, and the vacuoles were stained red in the matrine-treated group (Fig. 2A). The dates indicated that matrine-induced vacuoles are acidic. Subsequently, we observed the ultrastructure of cell vacuoles using TEM (Fig. 2B). As the time of matrine treatment was prolonged, the volume of the vacuoles became larger, and the vacuoles contained contents that were not completely decomposed and digested, which were different from the autophagosomes identified in the figure. The lysosomal membrane marker LAMP1-eGFP was used to fluorescently label lysosomes. The results showed that the fluorescence was punctately distributed in the cells before matrine treatment, and the fluorescence appeared in the periphery of the vacuoles after matrine treatment (Fig. 2C). It proved that matrine-induced vacuoles originated from lysosomes.

Matrine up-regulates vacuolation-related genes and lysosomal related genes

To explore the underlying mechanism of matrine-induced cytoplasmic vacuolization. K562, HL60 and U937 cells were treated with 0.8 mg/mL matrine for 24 h, and total cellular RNA was extracted for RNA-seq. Matrine-treated leukemia cells showed that 185 genes were significantly up-regulated and 241 genes were significantly down-regulated by Cell Component analysis. From the analysis of gene function, these up-regulated genes were related to vacuolization and lysosomal processes (Fig. 4A). 18 vacuolation-related genes and 15 lysosome-related genes were highly overlapped (Fig. 4B). The up-regulation of 15 overlapping genes in matrine-treated human leukemia cells were represented by a heat map (Fig. 4C). It was verified that matrine up-regulated the mRNA expression levels of vacuolation-related genes (marked in red in Fig. 4C), which was consistent with the RNA-seq results (Fig. 4D). This result indicated that matrine-induced vacuolation may be related to lysosome, and those overlapping genes may be the cause or result of matrine-induced vacuolation.

Matrine inhibited lysosomal function

To confirm whether matrine affects lysosome function, we detected the expression levels of lysosomal proteolytic enzymes, CTSB (cathepsin B) and CTSK (cathepsin K) in human leukemia cells. The protein levels of CTSB and CTSK decreased significantly after matrine treatment for 24h in K562, HL60 and U937 cells, indicating that matrine affects the expression of lysosomal proteolytic enzymes (Fig. 5A). Then we treated K562 cells with 0.8 mg/mL matrine for 24 h, and detected the total proteolytic enzyme activity of lysosomes. the fluorescence intensity of DQ-Green BSA was significantly weakened, and the total proteolytic enzyme activity of lysosomes decreased. (Fig. 5B). The date showed that matrine inhibits the expression level and activity of proteolytic enzymes, thereby inhibiting lysosomal function.

Matrine activates lysosomal proton pump V-ATPase

V-ATPase (vacuolar ATPase), as a proton pump in lysosomes [23, 24], was originally found in yeast and plant vacuoles. It is a complex protein composed of V1 and V0. V1 is located on the membrane surface, V0 is located in the membrane, and both V1 and V0 have different subunits [25].V-ATPase maintains the acidic environment of lysosomes, the activation of V-ATPase induced vacuoles [26, 27]. BafA1, V-ATPase inhibitor, is a kind of macrolide antibiotic isolated from Streptomyces sp. In a previous study, BafA1 was found to impair the formation of vesicular intermediates between early and late endosomes [28]. As another V-ATPase inhibitor, Concanamycin A (CMA) belongs to the macrolide class of antibiotics and acts by binding to the V0 domain of the proton pump V-ATPase [29]. V-ATPase inhibitors inhibit the activation of V-ATPase and stop the pumping of hydrogen into the lysosome. By analyzing the mRNA expression levels of V-ATPase subunits in RNA-seq, matrine up-regulated the gene expression levels V-ATPase subunits (Fig. 6A). RT-qPCR verified the expression of matrine on V-ATPase subunit, and the results confirmed that matrine up-regulated the mRNA level of V-ATPase subunit (Fig. 6B). HL60 cells were treated with 0.8 mg/mL matrine for 24 h, matrine up-regulated the protein expression level of V-ATPase subunit (Fig. 6C). Subsequently, leukemia cells were treated with V-ATPase inhibitor CMA or BafA1 in combination with matrine, and surprisingly few vacuoles were found, especially in K562 and HL60 cells (Fig. 6D). Calculated percentages of the vacuolated cells (Fig. 6E). V-ATPase inhibitors CMA or BafA1 alone treated leukemia cells, the cells of morphology were immutable. When used in combination with matrine, V-ATPase inhibitors effectively reduce the occurrence of vacuoles. These results suggested that matrine-induced vacuolization is related to the activation of V-ATPase.

Protonation of matrine may be the direct cause of vacuolation

Analysis of the structure of matrine and its analogues shows that oxymatrine adds an oxygen atom to the nitrogen atom at position 1, while matrine and sophoridine do not (Fig. 7A), which may prevent the protonation of nitrogen at position 1 of oxymatrine. Our results showed that matrine and sophoridine induced cell vacuolation, but oxymatrine did not (Fig. 7B-C), suggesting that the reason for vacuolation may be the protonation of nitrogen in position 1 of matrine and its analogens. In order to further explore the effect of matrine-induced cell vacuolization and cell proliferation under different pH conditions, we treated HL60 cells with matrine solutions of different pH for 24 h, It was found that matrine's ability to induce cell vacuole was weakened in PH 2.0 and PH 9.0 solvents, but not in PH 4.0 and PH 5.0 solvents

(Fig. 7D-E), while in PH 2.0 solvents, matrine's ability to inhibit cell proliferation was also significantly weakened (Fig. 7F). It is suggested that the ability of matrine to induce vacuoles may be related to PH of the solvent, and matrine may induce vacuole formation by protonation.

Discussion

Matrine induced vacuoles of human leukemia cells, and the formation of vacuoles is closely related to inhibition of cell proliferation. There is increasingly evidence that cytoplasmic vacuolization of human tumor cells by small molecules has a propulsive effect on inducing their death [30, 31]. Rac1 activated Arf6-GAP and GIT-1 proteins, to impair the function of Arf6-GTP protein, and leads to inactivation of Arf6 protein to induce vacuolation [32]. Doxycycline induced vacuolation in cancer cell lines DU145, MDA-MB-468 and A549 cells by activated H-Ras and increased Rac1 expression, the downregulation of Rac1 reversed cytoplasmic vacuolation and cell death [33]. In glioblastoma cells, Mitochondrial membrane potential and ATP levels increased by up-regulated H-Ras. These changes disrupt cellular metabolic function and prevent ATP-mediated fusion of late endosomes and lysosomes, and eventually the continuous accumulation of vacuoles destroys cell integrity and ultimately leads to cell death [34]. Similarly, matrine also inhibited cell proliferation by inducing vacuolation in leukemia cells.

By overexpressing the lysosomal labeled molecule LAMP1 fused with eGFP, a large amount of green fluorescence was observed at the cell vacuolar edge after matrine treatment by fluorescence microscope, The result confirmed that the vacuoles originated from lysosomes, but not mitochondria or Golgi bodies. Recent evidence (Zhang et al., 2010) [35] believed that matrine-induced were autophagic vacuoles in liver cancer cells. In this study, autophagosomes were considered to be spherical structures with double or multilayer membranes, containing cytoplasmic components with a diameter of 100-900nm. Matrine-induced autophagosomes were shown in Fig. 3C, which did inconsistent with the study. The researches (Wang et al., 2013) [36] have also confirmed that matrine enter the lysosome of tumor cells and increase the pH of the lysosome.

In addition, the result found that the 5.3 ± 0.7 nm gold NPs18, 29, 32 functionalized with positively charged N, N, N-trimethyl (11-mercaptoundecyl) ammonium chloride (TMA) and negatively charged 11-mer-captoundecanoic acid (MUA) ligands. After co-culture with tumor cells, and targeting the lysosome, nanoparticles aggregate in the lysosome to increase osmotic pressure, which makes the lysosome absorb water and expand, and finally the lysosome ruptures and leads to cell death, The nanoparticles TMA: MUA (4:1) of killing tumor cells, there is only cationic ligands of nanoparticles has cytotoxicity both cancer and healthy cells, only with anionic ligand nanoparticles because of not well attached on the negatively charged membrane,due to it is difficult to internalize and lost its effect on tumor destruction [37]. This result is similar to our study, matrine may be detained after entering the lysosome, inducing osmotic changes and lysosome swelling to forming vacuoles, and the production of vacuoles also inhibits lysosome function. With the further research, lysosomes have become an important target for anti-tumor therapy, and targeting lysosomes kill tumor cells is necessary [38, 39]. Combined with the current research results, we believe that the occurrence of vacuoles leads to down-regulation lysosome

proteolytic and autophagy related protein accumulation, which leads to lysosome dysfunction and affects the proliferation activity of human leukemia cells.

In the process of vacuolization, V-ATPase as a hydrogen donor in the acidic environment of lysosomes, and its role is to pump hydrogen ions from the lysosomal membrane into the lysosome, it keeps a high hydrogen ion concentration in the lysosome, and maintains a significant difference in the hydrogen ion concentration inside and outside the lysosome, thereby maintaining the acidic environment in the lysosome. Our study found that among the structural analogs of matrine- sophoridine had a significant effect of inducing vacuolation, while oxymatrine did not this action. After analyzing the molecular structure of matrine, it was found that matrine and sophoridine contain free amine groups that can bind hydrogen ions, while oxymatrine does not have this structure. In addition, matrine-induced cell vacuolization can be affected by changing the acid-base environment of matrine. These evidences suggest that the occurrence of cellular vacuolation is related to the protonation of matrine. In the early years, Christian de Duve also proposed the mechanism of the accumulation of lipophilic amine small molecules in the acidic environment of lysosomes: amine-containing weak base drugs and uncharged drugs enter cells by simple diffusion, they are not only transported into the cytoplasm, but also are also transported into organelles. When these molecules diffuse into acidic organelles and protonate with hydrogen ions, they are charged and trapped in acidic organelles. the higher the osmotic pressure in the organelle, and the more organelle swells, vacuoles were observed by optical microscope. With the prolongation of drug time and the increase of concentration, the vacuoles gradually become larger and eventually rupture and died [40, 41]. Therefore, we believe that the protonation of matrine may be an important mechanism for its induction of vacuoles, the inhibition of lysosomal function by matrine-induced vacuolization is one of the ways that matrine exerts its anti-leukemia effect.

As mentioned above, matrine enters the lysosome, On the one hand, the acidic environment of the lysosome makes matrine protonated in the lysosome, resulting in an increase in the osmotic pressure in the lysosome, and local vacuoles absorb water and swelling to lead to cytoplasmic vacuolization. On the other hand, matrine is protonated in the lysosome to consume hydrogen ions, and the lysosome activates V-ATPase in order to maintain its acidic environment. In this study, it was found for the first time that matrine induced vacuolation of leukemia cells, and vacuolation originated from lysosome and inhibited lysosome function, which provided important reference value for matrine targeting lysosome therapy of leukemia cells with strong innovation. Based on current research results, in the future study, we should focus on matrine cause key molecular mechanism of cytoplasm vacuoles, in order to further elucidate the pharmacological effects of matrine, variety of matrine development and provide targeted lysosomes to treat cancer cell with meaningful reference, and increase the research of Chinese medicine to promote the development of traditional Chinese medicine.

Declarations

Conflicts of interest

The authors declare no conflict of interest.

Author Contributions

FY and WZ perform and analyze all the experiments. JC, JT and BL drafted the work for important intellectual content. LM wrote the manuscript and designed the study.

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Supplementary Tables

Table S1 is not available with this version

Figures

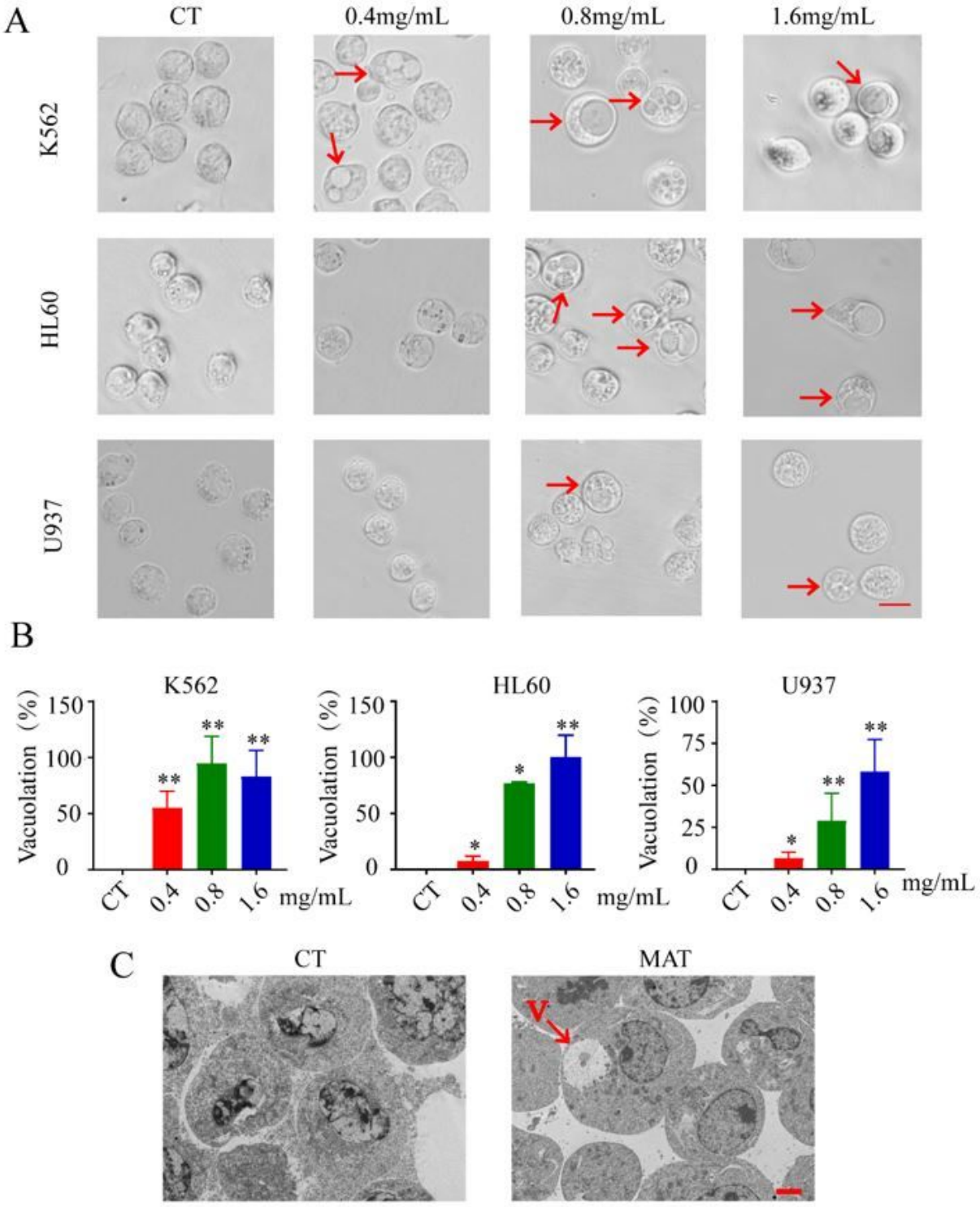


Figure 1

Matrine induces vacuolation in human leukemia cells. K562 and HL60 cells were treated with different concentrations (0.4mg/mL, 0.8mg/mL, 1.6mg/mL) of matrine for 24h, and U937 cells were treated for 48h. The cell morphology was observed by Optical Microscope, Bar = 20μm (A); Calculated percentages of the vacuolated cells = the number of cells with vacuoles in 15 fields/the total number of cells in 15

fields × 100% (B).K562 cells were treated with 0.8mg/mL matrine for 24 h, and cell vacuoles were observed by TEM, Bar = 20 μm (C); *$p < 0.05$, **$p < 0.01$.

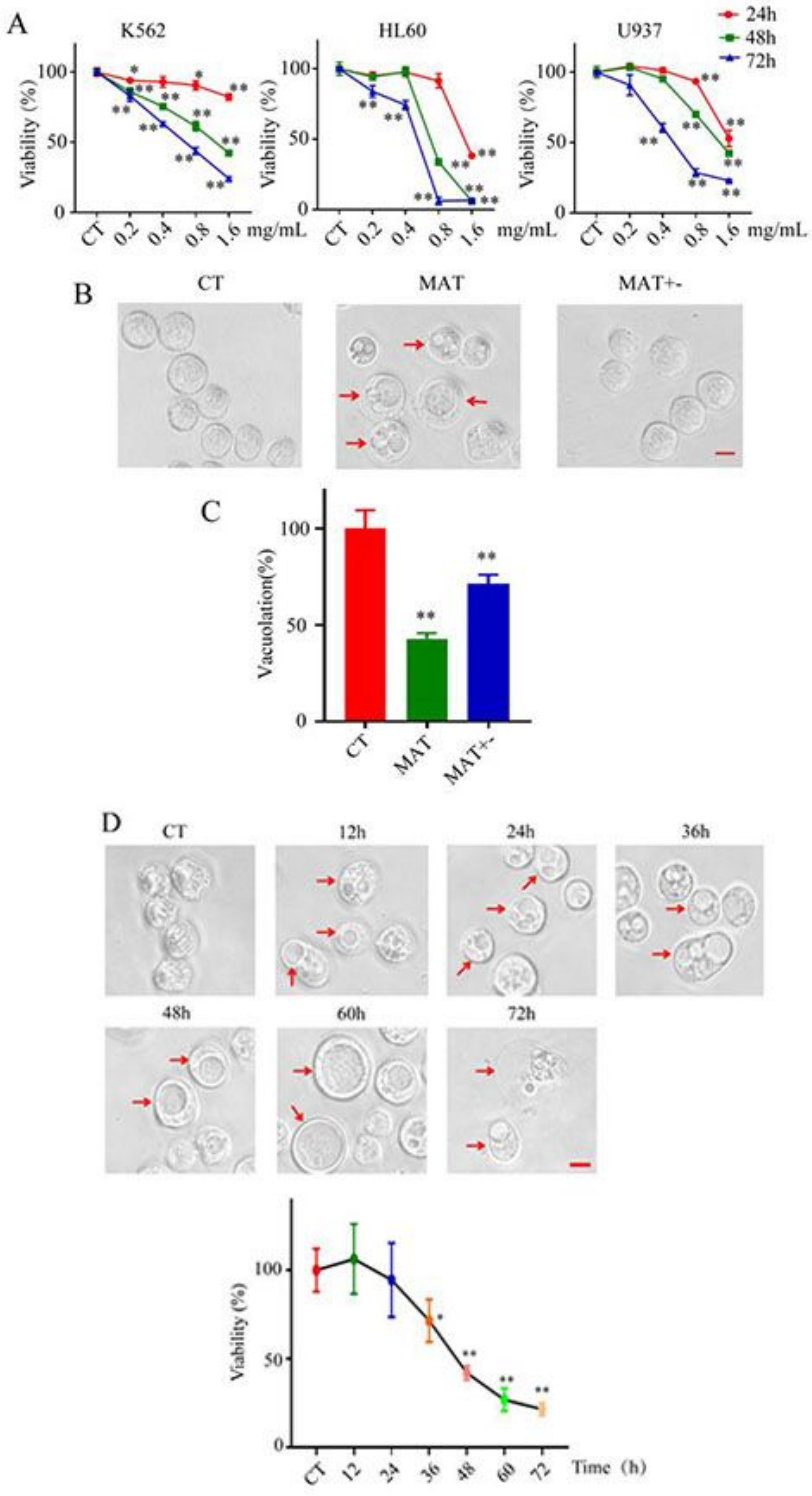


Figure 2

The more obvious the vacuole, the lower the cell viability. 0.2mg/mL, 0.4mg/mL, 0.8mg/mL and 1.6mg/mL of matrine were used to treated leukemia cell lines K562, HL60 and U937 cells for 24h, 48h

and 72h, and the cell proliferation activity was detected by CCK8 Assay (A). K562 cells were treated with 0.8 mg/mL matrine for 48 h, compared with 0.8 mg/mL matrine for 24 h, and the drug was removed to culture for 24 h. The cell morphology was observed by Optical Microscope, and the cell proliferation activity was detected by CCK8 Assay, Bar = 20 μm (B, C). K562 cells were treated with 0.8mg/mL matrine for different time (12h, 24h, 36h, 48h, 60h, 70h) to observe the changes of vacuoles, and cell proliferation activity was detected by CCK8 Assay, Bar=20μm (D). * $p < 0.05$, ** $p < 0.01$

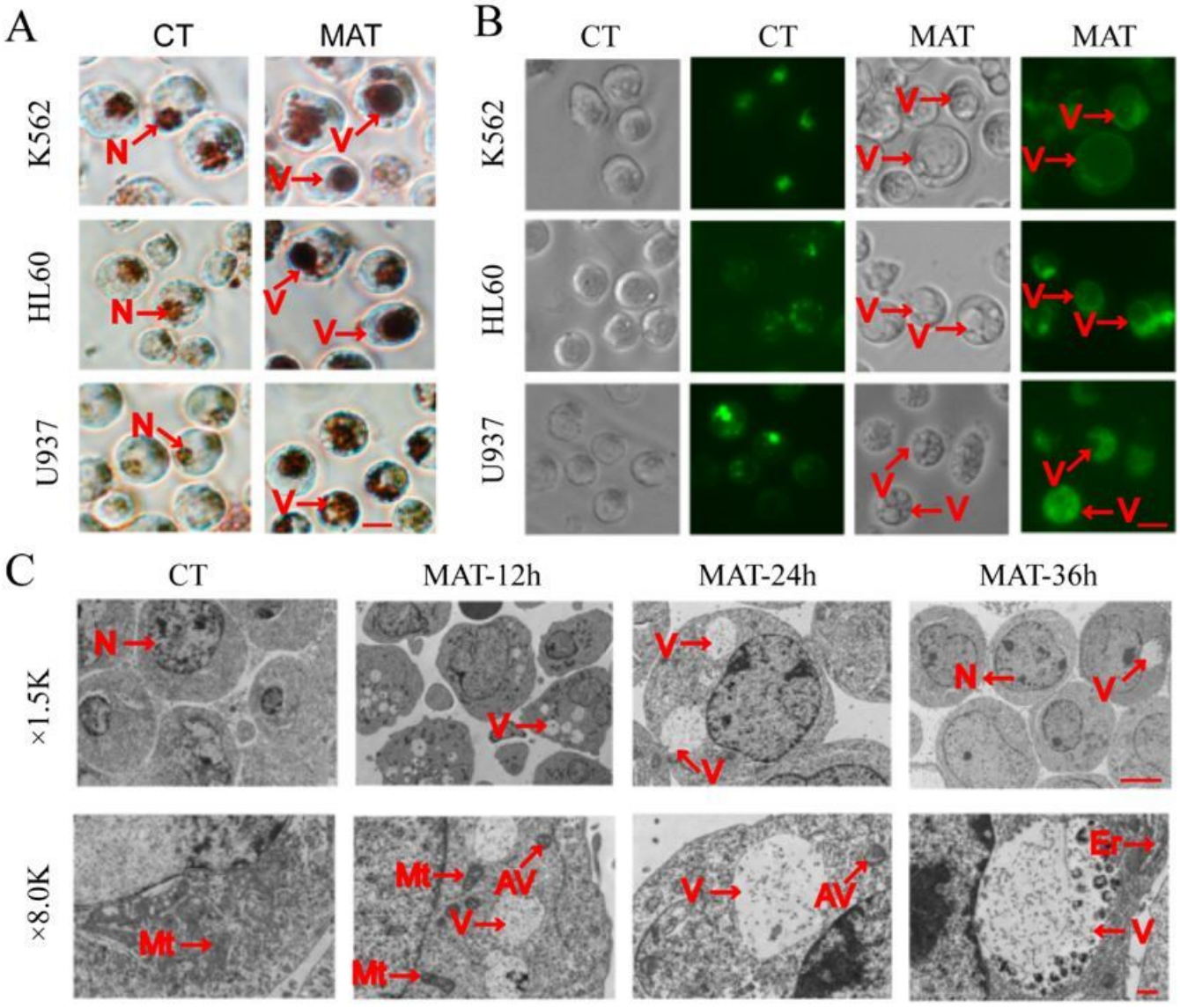


Figure 3

Matrine-induced vacuoles originate from lysosomes. K562 and HL60 cells were treated with 0.8mg/mL matrine for 48h, and 1.6mg/mL matrine was treated with U937 cells for 24h, stained with 1% neutral red for 20min, and the vacuoles staining was observed by Optical microscope, Bar = 10μm (A). K562 and HL60 cells overexpressing LAMP1-eGFP were treated with 0.8 mg/mL matrine for 24 h, and 1.6 mg/mL matrine was treated with U937 cells overexpressing LAMP1-eGFP for 48 h. The fluorescence distribution

was observed by Fluorescence Microscope, Bar = 20 μm (B). K562 cells were treated with matrine for 12h, 24h, and 36h, and vacuoles were observed by TEM, Bar = 10 μm(×1.5K), Bar = 1 μm(×1.5K) (C). V: vacuoles, N: nucleus, Er: endoplasmic reticulum, Mt: mitochondria, AV: autophagosome.

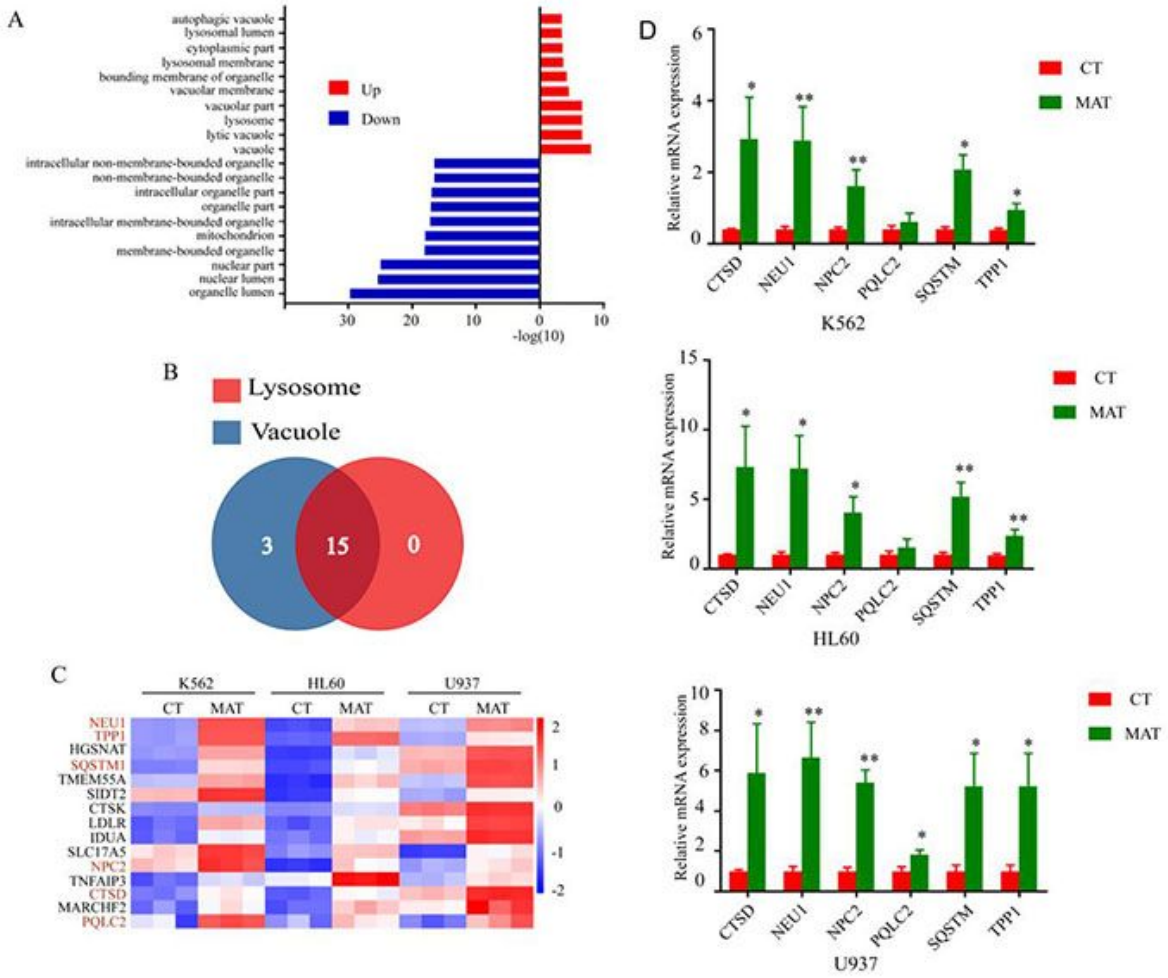
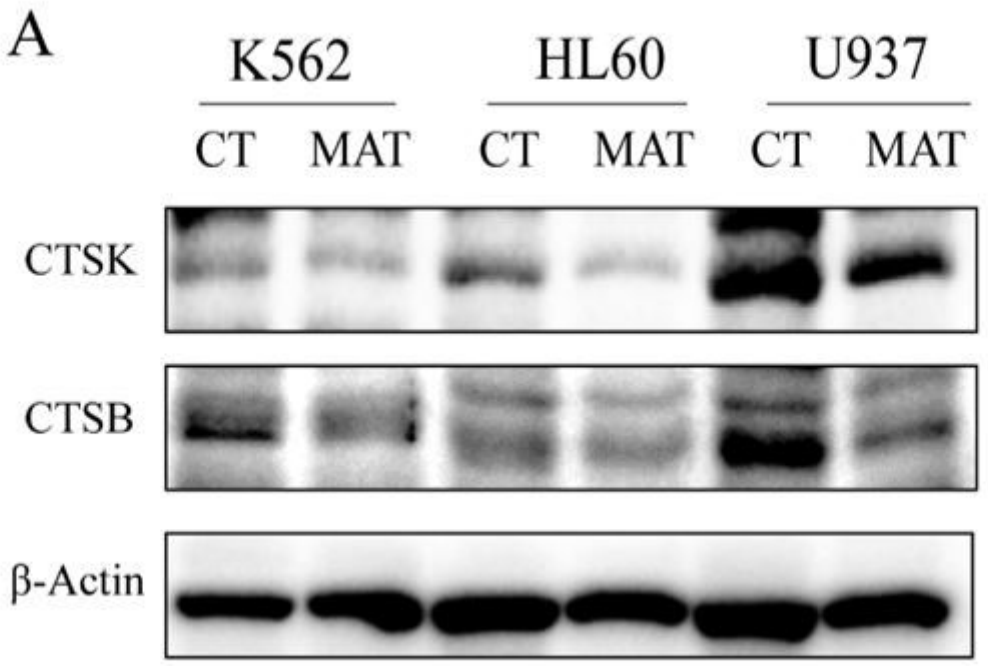


Figure 4

Matrine up-regulated vacuolation-related genes and lysosome-related genes, and the two highly overlapped. K562, HL60 and U937 cells were treated with 0.8 mg/mL matrine for 24 h, then RNA-seq was performed. Differential genes were screened according to |log2|≥1.8, Padj≤0.05, and FPKM≥1, and the up-regulated genes and down-regulated genes were enriched (A). Venn diagram showing the intersection of 18 vacuolation-related genes and 15 lysosome-related genes in human leukemia cells treated with matrine (B). Drawing a heat map representing the expression levels of vacuolation-related genes in matrine-treated human leukemia cells (C). RT-qPCR confirmed that matrine up-regulated vacuolation-related genes in RNA-seq (D). * $p < 0.05$, ** $p < 0.01$



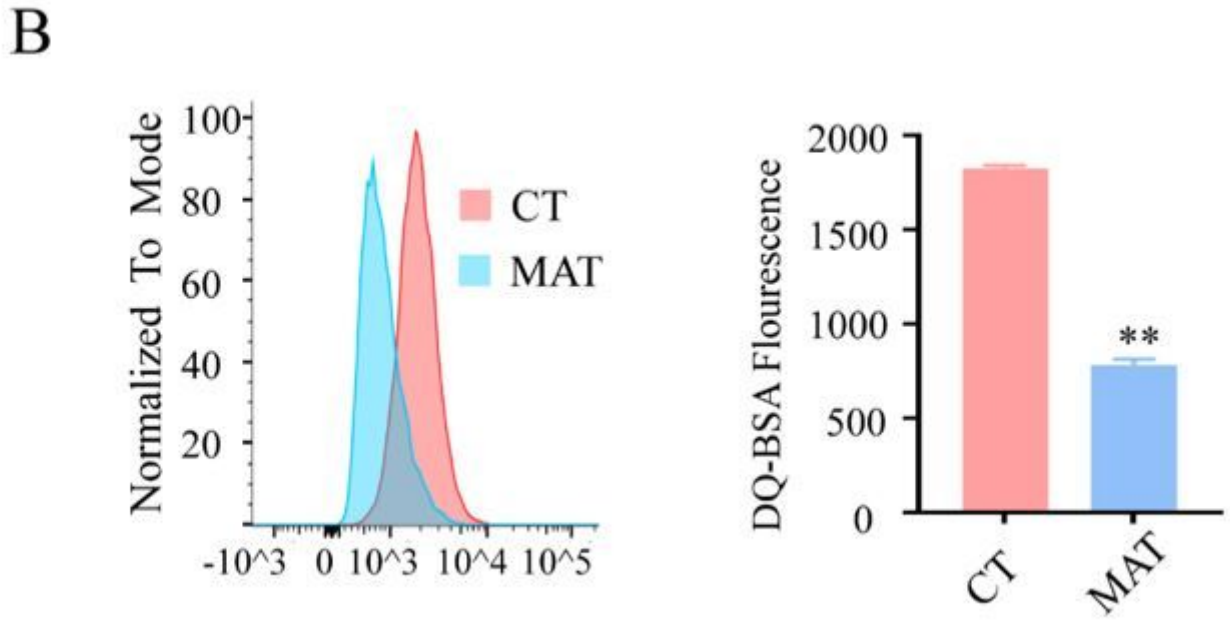


Figure 5

Matrine inhibited lysosomal function. K562 and HL60 cells were treated with 0.8 mg/mL matrine for 24 h, and 1.6 mg/mL matrine was treated with U937 cells for 24 h. The protein expression levels of CTSB and CTSK were detected by Western Blot (A). K562 cells were treated with 0.8 mg/mL matrine for 24 h, and the intracellular DQ-Green BSA fluorescence intensity was detected by FACS (B), ** $p < 0.01$.

A

Gene	FC		
	K562	HL60	U937
ATP6V0A2	1.56	<1.50	<1.50
ATP6V0A1	1.57	1.83	<1.50
ATP6V1E1	1.74	<1.50	<1.50
ATP6V1D	2.59	<1.50	1.70
ATP6V1B2	2.08	1.68	<1.50
ATP6AP1	2.13	1.57	<1.50
ATP6V0D1	2.29	<1.50	1.58
ATP6V0E1	1.57	<1.50	<1.50
ATP6AP1L	1.62	2.11	2.06
ATP6V0D2	<1.50	<1.50	2.37

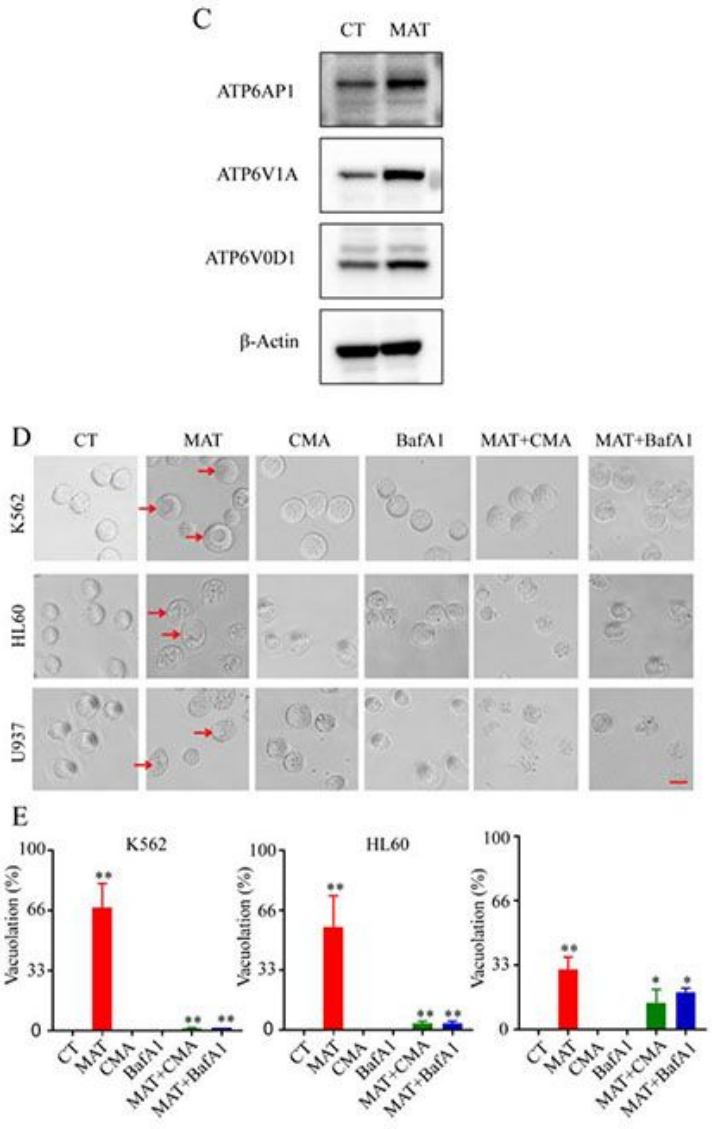


Figure 6

Matrine up-regulated the expression level of V-ATPase. RNA-seq data analysis of V-ATPase subunit mRNA expression level (A); K562, HL60 and U937 cells were treated with 0.8 mg/mL matrine for 24 h, and the V-ATPase subunit mRNA level was detected by RT-qPCR (B); HL60 cells were treated with 0.8 mg/mL matrine for 24 h, and the protein levels of V-ATPase subunit was detected by Western Blot (C); K562 and HL60 cells were treated with 0.8 mg/mL matrine for 24 h and U937 cells were treated with 1.6 mg/mL matrine for 48 h, combined with CMA (1nM for K562, 0.4nM for HL60, 0.4nM for U937) or BafA1 (K562, HL60 for 10 nM , U937 for 20 nM), cell morphology was observed by Optical microscope, Bar = 20 μm (D); Calculated percentages of the vacuolated cells (E). * $p < 0.05$, ** $p < 0.01$.

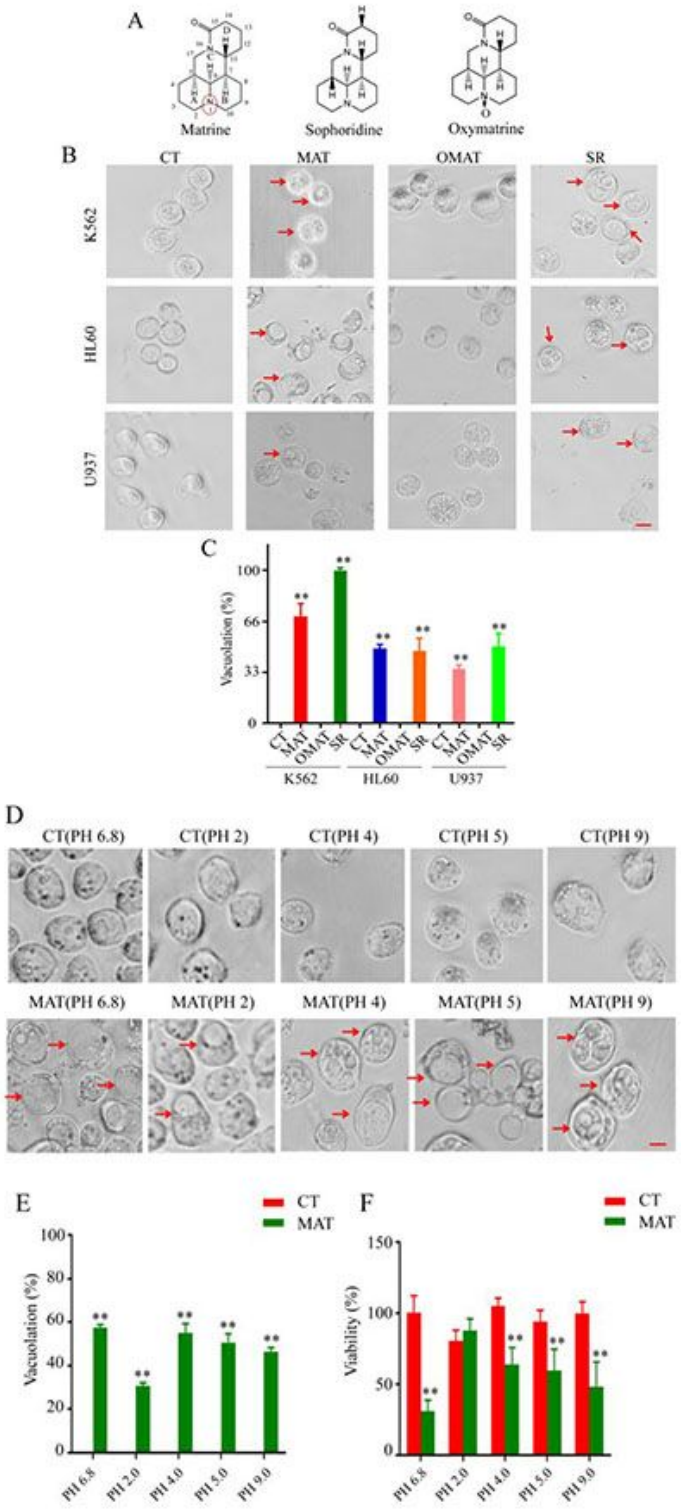


Figure 7

Matrine-induced vacuolation is related to the amine group at position 1. Molecular structural formula of matrine, sophoridine and oxymatrine (A). K562 and HL60 cells were treated with 0.8mg/mL matrine analogs for 24 h, and 1.6 mg/mL matrine analogs were treated with U937 cells for 48 h. The cell morphology was observed by Optical Microscope, Bar=20 μm (B); Calculated percentages of the vacuolated cells (C). HL60 cells were treated with 0.8 mg/mL matrine at different pH (pH 2.0, pH 4.0, pH

5.0 and pH 9.0) for 24 h, and the changes of cell vacuoles were observed by Optical Microscope, Bar = 20 μm (D), Calculated percentages of the vacuolated cells (E). HL60 cells were treated with 0.8 mg/mL matrine at different pH (pH 2.0, pH 4.0, pH 5.0 and pH 9.0) for 48 h, and cell proliferation activity was detected by CCK8 Assay (E). * $p < 0.05$, ** $p < 0.01$.