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Original Article

Targeting v-ATPase to Inhibit Lysosomal Acidification: A Study on the Anti-Tumor Mechanisms of Alkaloid-Based Small Molecules

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ABSTRACT

Background: This study aims to identify small alkaloid molecules that inhibit lysosomal acidification and explore their anti-tumor mechanisms. Vacuolar adenosine triphosphatase (v-ATPase) regulates lysosomal acidification, affects cell growth, and inhibiting its function can intervene in tumor cell proliferation. **Materials and Methods:** Potential alkaloids were screened through molecular docking. Combined with cell transfection and fluorescence techniques, the inhibitory effects of nine alkaloids (Homharringtonine, Camptothecin, Berberine, Clolithecin, etc.,) on lysosomal acidification were verified. **Results:** These compounds significantly inhibited the activity of v-ATPase, affected the pH value of lysosomes, and further suppressed the proliferation of tumor cells, confirming that they exerted anti-tumor effects by regulating the acidification process. **Conclusion:** This study reveals that small alkaloid molecules serve as a new direction for anti-tumor treatment, providing a key basis for the development of therapies targeting lysosomal acidification.

Keywords: v-ATPase, Lysosomal Acidification, Alkaloid-Based Small Molecules, Anti-Tumor.

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INTRODUCTION

Cancer is the second leading cause of death globally, and the increasing cancer burden worldwide continues to pose a significant public health challenge. This is primarily due to uncontrolled tumor cell proliferation,[1] which results in tumorigenesis.[2] Given the high incidence and mortality rates of cancer, current cancer control measures and the pace of clinical research are insufficient to keep up with the rapid development of cancer. Therefore, it is crucial to update treatment strategies and prevent cancer progression.[3,4] Current cancer therapies, such as surgery, radiotherapy, chemotherapy, immunotherapy, and cell therapy, each have their advantages and limitations. These treatments can lead to complications such as drug resistance, immortalization of tumor cells, inflammatory responses, and the induction of new blood vessel formation.[5,6] For example, in patients with Chronic Lymphocytic Leukemia (CLL) and Acute Myeloid Leukemia (AML), repeated chemotherapy results in increased preventing apoptosis and causing immortalization of leukemic cells.[7-9] Additionally, in colon cancer patients receiving cetuximab treatment, mutations in downstream genes of the genetic tumor can induce the occurrence of resistance.[10,11] Therefore, there is a pressing need to identify more effective and less toxic therapies that can optimally control and eliminate cancer cells and to explore more efficient anti-cancer strategies.[12]

Lysosomes are limited, dynamic, and heterogeneous acidic organelles within cells, consisting of a single membrane structure.[13,14] They contain hundreds of membrane proteins, including various transporters and ion channels, playing a key role in the degradation and recycling of cellular materials.[15] Lysosomes are also central to cellular metabolism, protein degradation, and play a crucial role in the proliferation and survival of both normal and tumor cells.[16,17] Lysosomal hydrolases, which have widespread targets, are activated by the proton pump v-ATPase, enabling lysosomal acidification by lowering the pH

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to the required acidic range.[18] Studies have shown that lysosomal acidification is associated with various diseases, including viral infections, rheumatic diseases, tuberculosis, psoriasis, and fatty liver disease.[19-22] Moreover, lysosomal acidification significantly influences cancer progression. When lysosomal acidification is functioning normally, cells can grow, proliferate, and survive. However, when lysosomal acidification is disrupted, both normal and tumor cells are unable to proliferate and survive properly, which may exert anti-cancer effects.[23,24] Therefore, disrupting lysosomal acidification can hinder cancer cell proliferation and exhibit anti-cancer effects.

v-ATPase is a multimeric protein complex that functions as a proton pump, driven by ATP hydrolysis, responsible for activating lysosomal acidification.[25,26] During the lysosomal catabolic process, v-ATPase activation is essential for lysosomal acidification.[27] When unnecessary cellular material is transferred to lysosomes for degradation, v-ATPase maintains the acidic environment within lysosomes, allowing them to operate properly. This enables the normal functioning of the lysosomal system, processing unwanted cellular material via autophagy and endocytosis.[28] Furthermore, the activation of v-ATPase facilitates spatial acidification among cells. Numerous enzymes located within the lysosome become operational in this acidic environment, effectively decomposing substrates and digesting complex materials.[29] Thus, v-ATPase activation is essential for maintaining the acidic environment necessary for lysosomal function and hydrolytic enzyme activity.

Alkaloids are complex nitrogen-containing organic compounds that are natural products.[30] Many clinical anti-cancer drugs, such as paclitaxel and camptothecin analogs, are widely used to treat various cancers.[31,32] In recent years, alkaloid-based compounds have become a focal point in anti-tumor research. Studies by Liang Qing *et al.*, (2020) and Fan Yang *et al.*, (2022) showed that alkaloid small molecules induce cancer cell death through activation of the death receptor pathway.[33,34] Additionally, research by Wang Jinkang *et al.*, (2020) and Luo Yanming *et al.*, (2021) demonstrated that alkaloid small molecules regulate signaling pathways to enhance immune cell proliferation and immune functions, thereby eliminating cancer cells.[35,36] Furthermore, research by Yang Xu *et al.*, (2023) pointed out that alkaloid small molecules interact with growth-related activation proteins, inhibiting cancer cell growth and inducing cell death.[37,38] Most studies on alkaloid compounds in cancer research focus on promoting cell proliferation, enhancing immune responses, or directly activating cancer cell death pathways. However, there is still room for development in exploring how alkaloid compounds intervene in protein complexes to induce lysosomal acidification and exert anti-cancer effects.

Existing research has found that alkaloid compounds exert anti-tumor effects through mechanisms such as the death receptor pathway and immune function pathways, while very few studies have focused on the impact of alkaloid compounds on autophagy.[39-41] Therefore, in our study, we investigated various alkaloid compounds to evaluate their binding affinity with v-ATPase and explored their effects on lysosomal pH. The results revealed that alkaloid compounds can interact with v-ATPase, inhibiting the lysosomal acidification pathway, and ultimately leading to the effective suppression of cancer cell proliferation and survival. This finding highlights the potential of alkaloid compounds as v-ATPase enzyme inhibitors, which can impede cancer cell growth and proliferation by affecting lysosomal acidification function, thereby providing preclinical evidence for the development of new cancer treatment strategies.

MATERIALS AND METHODS

Alkaloid compounds exert antitumor effects by inhibiting lysosomal acidification

Cell culture and transfection

Hela cells and SKOV3 were cultured in DMEM medium (Gibco) contained 10% FBS (Vivacell) in a humidified atmosphere with 5% CO_2 at 37°C. Culture media were refreshed 24 hr post-transfection, and cells were imaged 48 hr post-transfection to allow sufficient recovery from transfection stress.

Reagents

FDA-Approved Drug Library (Cat. No: HY-L022), LysoTracker™ Red DND-99 and CCK8 was obtained from MedChemExpress. Hoechst 33342 was from Thermo Fisher Scientific.

Cell proliferation assay

Cell inhibitory of HCC and human peripheral blood mononuclear cells was monitored by CCK8 assay. Cells were seeded in 96-well plates. After treatment, the culture medium was removed. Subsequently, CCK8 was added to each well and incubated for 2.5 hr after removal of the supernatant. The absorbance was recorded at 450 nm under a microplate reader (Bio-Tek Instruments, Highland Park., Winooski, VT, USA).

Inhibition rate (%) = [(A450, Control - A450, Sample)/(A450, Control -A450, Blank)]×100%.

Immunofluorescence assay method

Preparation of LysoTracker Red working solution: Allow the solution to return to room temperature and briefly centrifuge to concentrate it at the bottom of the tube. Dilute the 1 mM stock solution with culture medium or an appropriate buffer, such as PBS, to prepare the working solution, which should be 100 μM. Cell staining: For adherent cells, discard the cell culture medium, dissociate the cells with trypsin, and prepare a single-cell suspension. Centrifuge at 4°C, 1000 g for 3-5 min, discard the supernatant. Wash twice with PBS for 5 min each. Add 100 μL of LysoTracker Red working solution, and incubate at room

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temperature for 5-30 min. Centrifuge at 4°C, 400 g for 3-4 min, discard the supernatant. Wash twice with PBS for 5 min each. Resuspend the cells in serum-free cell culture medium or PBS and analyze using the Operetta CLS High Content Screening Imaging System.

Statistical analysis

Statistical analyses were conducted utilizing SPSS version 26.0 and GraphPad Prism version 9 software. Experimental data were expressed as mean as mean ± Standard Deviation (SD). Comparisons between two groups were analyzed using Student's t-test, while comparisons among multiple groups were conducted using one-way Analysis of Variance (ANOVA), followed by Tukey's multiple comparisons test. The experimental data were normalized. This experiment used fluorescent probe technology and was repeated three times.

RESULTS

The Anti-Tumor Effects of Alkaloid Compounds

Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride) and Topotecan (hydrochloride) can effectively inhibit the growth and proliferation of Hela cells and SKOV3 cells. When the dose of each drug reaches 10 μM, almost all of the two cancer cells die. According to the analysis results, it is evident that different concentrations of alkaloids have varying effects on cell viability. When compared to the control group (0 μM group), we can observe that each alkaloid exhibits an inhibitory effect on both Hela cells and SKOV3 cells. Furthermore, the impact of different concentrations on cell viability also varies; as the concentration of alkaloids increases, the viability of Hela cells and SKOV3 cells decreases, demonstrating significant differences ($*p<0.05$, $**p<0.01$, $***p<0.001$). The experimental results indicate that seven alkaloid compounds, namely Homoharringtonine, Camptothecin, Harringtonine, Theophylline, Reserpine (hydrochloride), Sanguinarine (chloride), and Topotecan (hydrochloride), exhibit significant anticancer effects, with higher concentrations leading to stronger anticancer activity (Figures 1a-1c).

The Inhibition of Lysosomal Acidification of Alkaloid Compounds

Compared with the 0 μM group, Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride) and Topotecan (hydrochloride) could significantly inhibit lysosomal acidification and increase the cellular pH. Immunofluorescence intensity analysis revealed significant differences in the relative protein expression levels of colchicine, camptothecin, berberine (chloride hydrate), vinblastine, theophylline, reserpine (hydrochloride), berberine (sulfate), sanguinarine (chloride), topotecan (hydrochloride), and the untreated control group, with $*p<0.05$, $**p<0.01$, $***p<0.001$. In the immunofluorescence intensity analysis chart, DND99 expression is higher in the control group, indicating low lysosomal pH. Upon the addition of alkaloid compounds, DND99 expression decreases, increasing lysosomal pH. Moreover, different alkaloid compounds exhibit varying effects, all showing significant differences compared to the control group (Figures 2a, 2b).

Alkaloid Compounds Targeting v-ATPase to Induce Lysosomal Acidification Defects and Exhibit Antitumor Effects

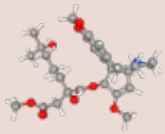
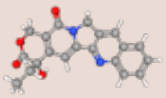
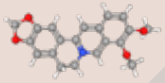
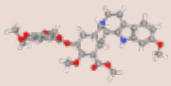
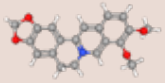
The results of this study indicate that alkaloid small molecules exert antitumor effects by inhibiting lysosomal acidification. v-ATPase is a key protein responsible for regulating the H^+ pump in the lysosome. To explore how these alkaloid compounds inhibit lysosomal acidification, we investigated their potential to target and inhibit v-ATPase, thereby inducing lysosomal acidification defects to exert antitumor effects.

Molecular docking was performed to analyze the regulatory effects of these small molecules on lysosomal v-ATPase. 3D structure files in SDF format for nine alkaloid compounds, including Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride), and Topotecan (hydrochloride), were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The 3D structure of v-ATPase was obtained from the Protein Data Bank (PDB) (http://www.rcsb.org/) (Table 1). Molecular docking was conducted using AutoDockTools 1.5.7, and the lowest binding energy results were selected for further analysis. The docking conformations were visualized using PyMOLWin and analyzed for interaction interfaces, hydrogen bonds, hydrophobic interactions, and other key interactions (Figure 3).

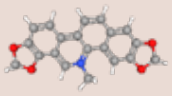
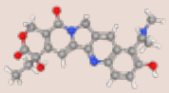
The molecular docking results showed that alkaloid compounds bind to v-ATPase, playing a role in the transport of protons (H+) across the endosomal membrane. During the inhibition of v-ATPase by these alkaloid compounds, the ATP in the V1 domain undergoes hydrolysis, converting ATP into ADP, which triggers the rotation of the central stalk (subunits D1 and F1), facilitating the transport of protons from the cytoplasm through the subunits a and c ring to the organelle lumen or extracellular space. When the c ring rotates, the alkaloid compounds provide steric hindrance, coupling the energy from ATP hydrolysis with the proton translocation process of the subunit half-channel, thereby preventing the interaction between the subunits a and c ring, thus blocking proton translocation. The docking sites of alkaloid compounds formed hydrogen bonds with specific protein residues on the V domain, such as LYS-24, ARG-236, and

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Table 1: **Source and Structure of Alkaloid Small Molecules.**

Name/Id	Constitution	Source	References
Homoharringtonine/285033		Cephalotaxus	42
Camptothecin/ 24360		The bark of the Camptotheca tree	43
Berberine (chloridehydrate)/ 155074		Quaternary ammonium alkaloids	44
Harringtonine/276389		Cephalotaxus	45
Theophylline/ 2153		Black tea	46
Reserpine(hydrochloride)/21155894		Serpentwood	47
Berberine (sulfate)/ 12457		Coptis, Phellodendron amurense	48

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Name/Id	Constitution	Source	References
Sanguinarine(chloride)/68635		Papaveraceae	49
Topotecan(hydrochloride)/60699		Camptothecin semisynthetic derivatives	50

ALA-272. These hydrogen bonds not only enhanced the stability of the alkaloid compounds binding to v-ATPase but also likely affected the conformational changes of v-ATPase domains.

The docking results for these nine alkaloid compounds with v-ATPase showed different positions where hydrogen bonds were formed with protein residues (Table 2). Additionally, some overlapping binding sites were found for these alkaloids, such as Homoharringtonine, Camptothecin, Theophylline, and Sanguinarine (chloride), which all formed hydrogen bonds with the protein residue LYS. Similarly, Camptothecin, Berberine (chloride hydrate), Reserpine (hydrochloride), and Berberine (sulfate) all formed hydrogen bonds with the protein residue ASN, while Berberine (chloride hydrate) and Berberine (sulfate) both formed hydrogen bonds with ALA-272. Therefore, it can be inferred that the alkaloid compounds primarily target residues LYS, ASN, and ALA in v-ATPase.

The binding energy results obtained from the molecular docking (Table 3) indicated that when the binding energy was <-5.0 kJ/mol, the molecule exhibited good binding affinity with the target protein (e.g., Theophylline), while binding energies <-7.0 kJ/mol indicated stronger binding affinity (e.g., Camptothecin), and while the binding energy is greater than -5.0 kJ/mol, it indicates that the binding activity is relatively weak (e.g., Harringtonine). The higher the binding energy, the stronger the ability of the alkaloid compounds to bind with v-ATPase. At this point, v-ATPase cannot be properly driven by ATP hydrolysis, and the normal activation of lysosomal acidification functions is hindered. Consequently, the intercellular space cannot achieve the acidic environment required for signal transduction, thereby obstructing the signaling necessary for regulating tumor growth factors, effectively slowing down cell growth and proliferation. By comparing the binding energies, it can be determined that the binding energy of Camptothecin is -7.79 kJ/mol, demonstrating the strongest binding capability. This indicates that among the nine alkaloid compounds, camptothecin exerts the strongest inhibitory effect on lysosomal acidification and has the most potent anti-tumor effect, followed by Topotecan (hydrochloride). In contrast, Harringtonine, with a binding energy of -4.92 kJ/mol, exhibits the weakest binding ability to the target protein, resulting in a weaker inhibition of lysosomal acidification compared to the previous eight alkaloid compounds, thus having the least effect on suppressing the growth and proliferation of cancer cells.

DISCUSSION

The Relationship Between Lysosomal Acidification and Cancer

Mechanism of Lysosomal Acidification

The acidic lumen of the lysosome is primarily attributed to the activity of v-ATPase.[51,52] The formation of this acidic environment is mediated by the flow of cations and anions across the lysosomal membrane via ion transport proteins, which generate and maintain the acidic pH. v-ATPase is an electrochemical pump that creates an electrochemical gradient by utilizing ATP metabolism to pump protons (H^+) into the lysosomal lumen.[53] This proton influx occurs through the efflux of cations and/or the influx of anions, enabling continuous proton entry, which dynamically regulates the lysosomal pH and maintains an acidic environment.[54,55] The acidic pH stabilizes and activates lysosomal hydrolases, promoting the degradation of macromolecules and vesicular trafficking.[56,57] Moreover, v-ATPase uses the energy derived from ATP hydrolysis to actively transport H^+ ions into the lysosome, maintaining an acidic environment that is conducive to the optimal function of most enzymes (Figure 4).[58]

Mechanism and Potential Causes of Lysosomal Function Promoting Tumor Growth

The ability of tumor cells to proliferate depends on the function of acidic, active lysosomes.[59] Most lysosomal functions are contingent upon the acidic environment within the lysosomal lumen, as the majority of lysosomal enzymes perform optimally

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at a pH range of 4.5-5.[60] When lysosomal function is normal, an acidic environment is established, facilitating endocytosis to release products, followed by the uptake of transferrin-bound iron via transferrin receptors. This process alters mitochondrial metabolism and HIF signaling, ultimately promoting cell proliferation.[61] The potential cause for this is that lysosomes, acting as a signal hub for environmental adaptation, regulate transcription factors involved in tumor-related signaling pathways. This promotes the signaling of growth factors for cancer cells and, under conditions favorable for cell growth, activates proliferation-related factors, thus promoting the proliferation and growth of cancer cells.[61,62] When lysosomal acidification increases, it can enable cancer cells to survive under certain conditions and facilitate their migration.[63] This demonstrates that both normal and cancer cells can proliferate and renew themselves only when the lysosomal function is intact, maintaining an acidic environment.

Dysregulation of Lysosomal Acidification Leading to Tumor Cell or Cell Death

When lysosomal acidification is disrupted, it triggers a loss of iron in proliferating cells, and iron deficiency activates signaling in cultured primary neurons, leading to mitochondrial dysfunction and non-apoptotic cell death.[64] Inducing lysosomal swelling and inhibiting lysosomal acidification impairs lysosomal function, increases lysosomal membrane permeability, induces mitochondrial depolarization, disrupts cellular homeostasis, triggers inflammatory and cell death signals, and ultimately leads to cell death.[65,66] Thus, when lysosomal acidification is disrupted, the expression and functionality of related genes become

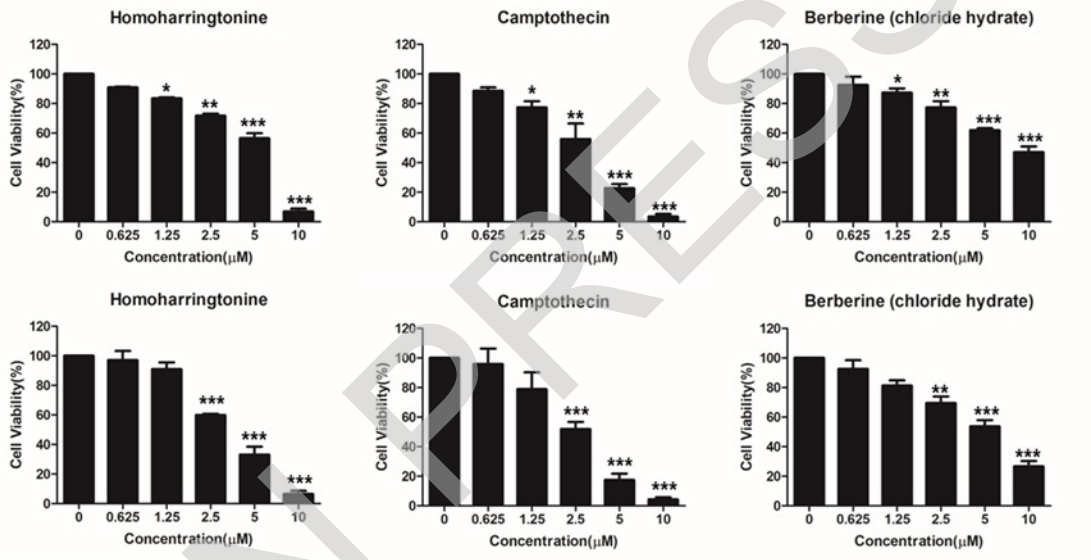


Figure 1a

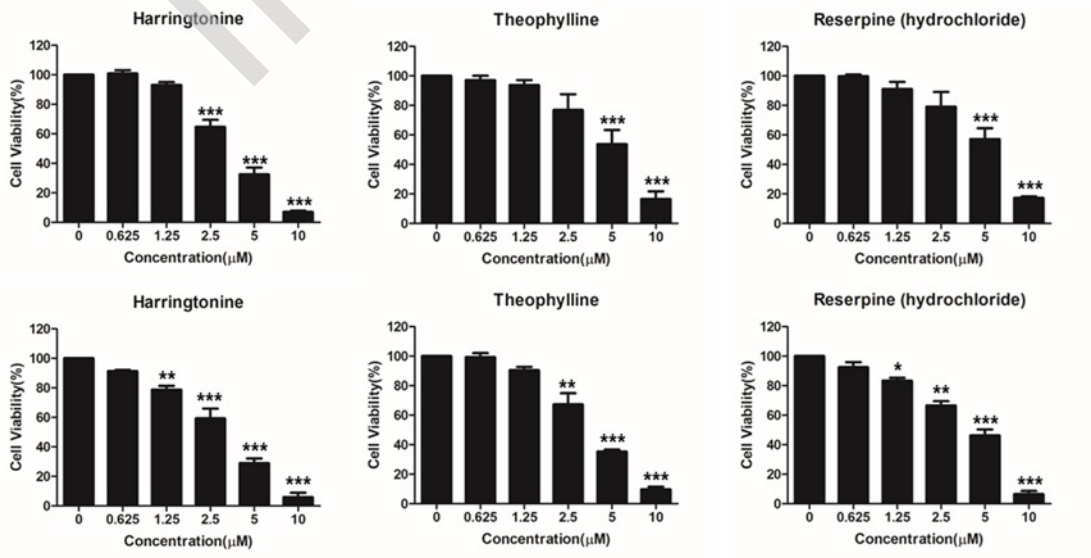


Figure 1b

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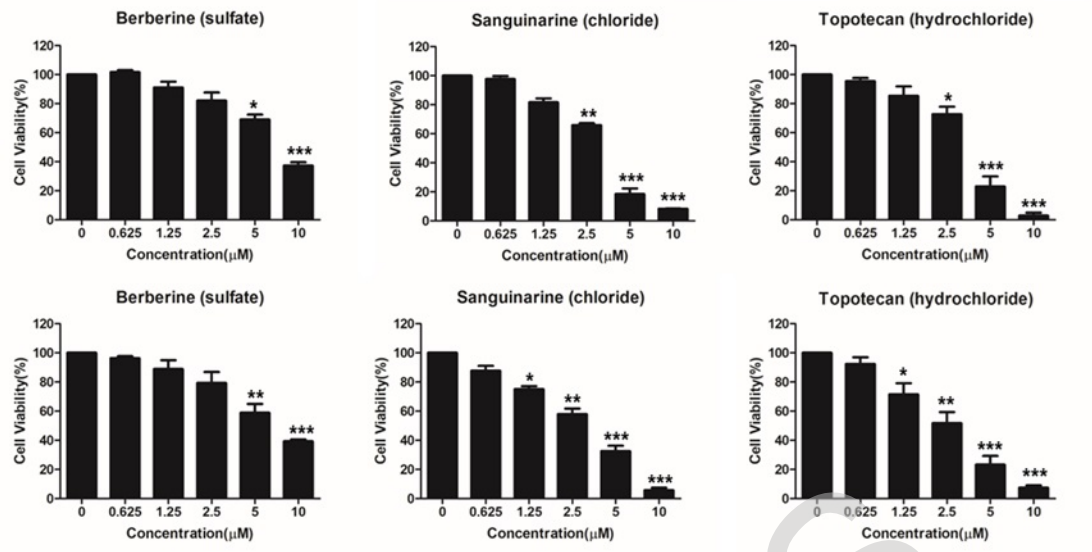


Figure 1c

Figure 1: (a-c) Homoharringtonine, Camptothecin,Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride) and Topotecan (hydrochloride) were all administered for 24 hr. The top row shows Hela cells, and the bottom row shows SKOV3 cells. Compared with the 0μM group,*p<0.05.**p<0.01.*** p<0.001.

Table 2: Locations of Hydrogen Bond Interactions Between Alkaloid Small Molecules (Ligands) and v-ATPase.

Alkaloid (Ligand)	Position of Hydrogen Bonds
Homoharringtonine	LYS-320; ILE-319
Camptothecin	LYS- 84; GLU-74; ASN-5; LEU-7
Berberine (chloride hydrate)	ALA-272; ASN-176
Harringtonine	ASP-352
Theophylline	LYS-24; ARG-236; VAL234
Reserpine (hydrochloride)	ASP-177; TYR-260; ASN-175
Berberine (sulfate)	ALA-272; ASN-176
Sanguinarine (chloride)	LYS-84
Topotecan (hydrochloride)	ASP-257; CYS-256

disordered and damaged, ultimately causing the cessation of growth or even death of both normal and tumor cells.

The Protein Structure of v-ATPase and Its Impact on Lysosomal Acidification

Protein Structure of v-ATPase (PDB)

v-ATPase shares the same structural characteristics as the F-ATPases found in mitochondria and chloroplasts, retaining its function as a proton pump.[67,68] v-ATPase is composed of a peripheral domain (V1) responsible for ATP hydrolysis and a membrane-integral domain (Vo) that transports protons across membranes or organellar membranes. The V1 domain contains eight subunits (A3, B3, C1, D1, E3, F1, G3, and H1), while the Vo domain consists of six subunits (a1, d1, e, and the c-ring, which is composed of three subunits: c, c' and c") (Figures 2a, 2b).[69,70] The A3 and B3 subunits form a hexamer that provides ATP hydrolysis sites.[71,72] The peripheral stalk is composed of the E3, G3, c, and H1 subunits, while the central stalk is formed by the D1, F1, and d1 subunits.[73,74] Upon ATP hydrolysis at the A3-B3 subunit interface, the central stalk is driven to rotate. The A3 and B3 subunits in the v-ATPase structure form three pairs of AB heterodimers, which are involved in ATP binding and hydrolysis.[75,76] During the ATP hydrolysis cycle, the C-terminal domains of the A and B subunits undergo conformational changes due to the binding of ATP and ADP, ultimately driving the rotation of the central stalk (D1 and F1 subunits) and facilitating the transport of protons from the cytoplasm to the organelle lumen or extracellular space, resulting in ATP synthesis or hydrolysis.[77-79] The c-ring couples the energy from ATP hydrolysis to the proton translocation process across the subunit half-channels.[80-82]

Mechanism by Which v-ATPase Affects Lysosomal Acidification

The core mechanism regulating lysosomal acidity is v-ATPase, which functions as an ATP-driven proton pump. Its activity is controlled by regulating the assembly of the v-ATPase complex or through dynamic modulation of v-ATPase expression on the membrane surface.[83] v-ATPase uses the energy generated by ATP hydrolysis to actively transport H^+ ions into the lysosomal lumen, thereby acidifying the organelle.[84] The V1 domain of v-ATPase can rapidly and reversibly shuttle between the lysosome containing the Vo domain, recruiting the V1 domain to the Vo domain on the lysosomal membrane for active v-ATPase assembly, which then acidifies the lysosomal lumen.[85-87] v-ATPase, along with anti-ion transport proteins, plays a crucial role in maintaining the steady-state pH inside the lysosome. The lysosome generates and maintains a pH gradient through the activity of the proton pump v-ATPase. The enzyme uses ATP-derived metabolic energy

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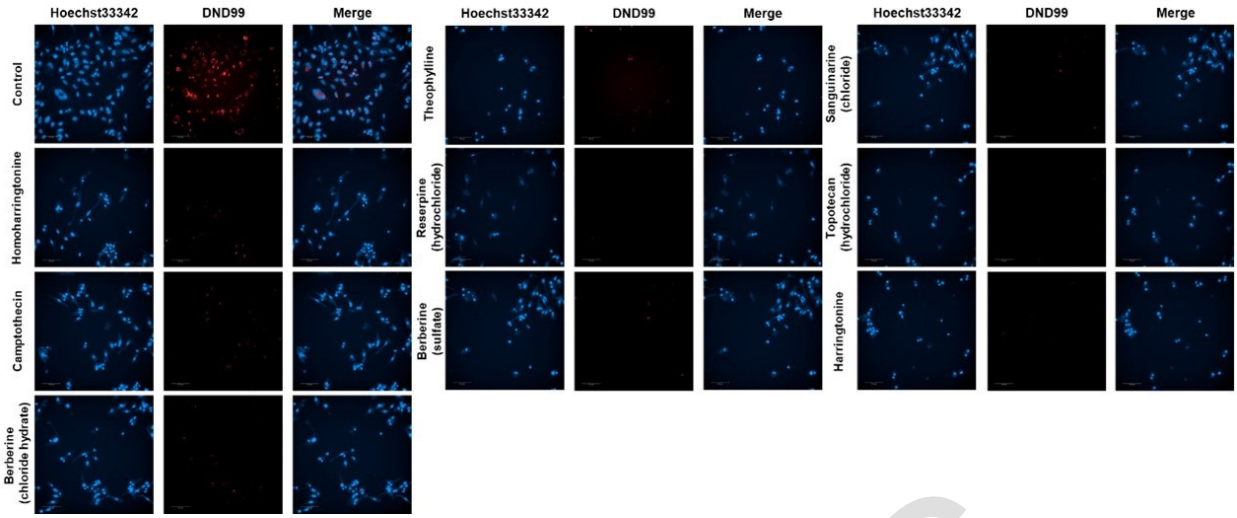


Figure 2a

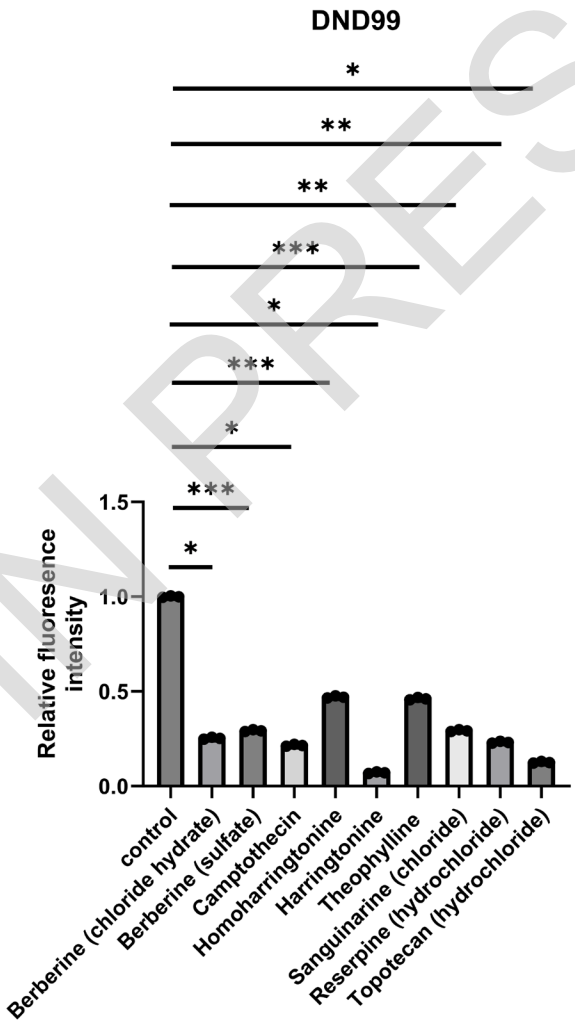


Figure 2b

Figure 2: Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride), Topotecan (hydrochloride) and untreated control were all administered for 24 hr (a). Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride), Topotecan (hydrochloride) and untreated control Protein relative expression levels ; Significant immunofluorescence intensity analysis, compared with the control group: *p<0.05, **p<0.01, ***p<0.001 。(b).

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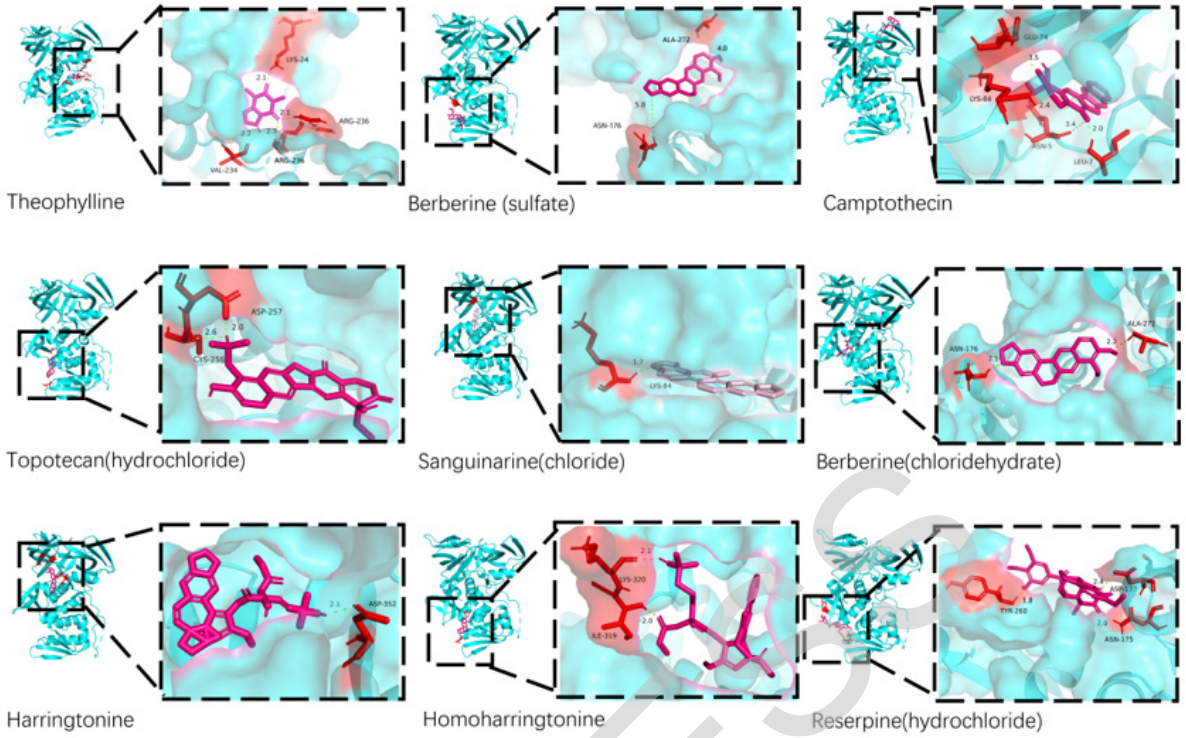


Figure 3: Schematic Representation of Molecular Docking Results for v-ATPase with Homoharringtonine, Camptothecin, Berberine (chloride hydrate), Harringtonine, Theophylline, Reserpine (hydrochloride), Berberine (sulfate), Sanguinarine (chloride), and Topotecan (hydrochloride) (b).

Table 3: Binding Energy Results of Alkaloid Small Molecules with v-ATPase.

Alkaloid (Ligand)	ID	Binding energy (kJ/mol)
Homoharringtonine	285033	-4.92
Camptothecin	260	-7.79
Berberine (chloride hydrate)	155074	-5.84
Harringtonine	276389	-4.31
Theophylline	2153	-5.08
Reserpine (hydrochloride)	21155894	-5.39
Berberine (sulfate)	12457	-5.80
Sanguinarine (chloride)	68635	-6.03
Topotecan(hydrochloride)	60699	-6.74

to pump protons into the lysosomal lumen, resulting in charge separation (Figures 5a-5c) and generating a transmembrane voltage. To dissipate this voltage and enable net pumping, another ion must move. This anti-ion can be a cation (moving out of the lysosome) or an anion (moving into the lysosome).[88,89] In this manner, v-ATPase ensures the proper functioning of lysosomal acidification.

Introduction to v-ATPase Inhibitors

Numerous studies have shown that v-ATPase is expressed on the plasma membrane of invasive cancer cells, including breast cancer, pancreatic cancer, liver cancer, gastric cancer, prostate cancer, and others.[90-93] This suggests that v-ATPase may support cancer cell survival and growth by regulating the cytosolic pH through its effect on lysosomal acidification.[94] Therefore, inhibiting v-ATPase can limit cancer cell growth, migration, and invasion.[95] v-ATPase inhibitors have membrane permeability, which allows them to suppress all v-ATPase activity within cells. The use of these inhibitors has been shown to effectively reduce the invasion and migration of metastatic cells.[96] When v-ATPase inhibitors are applied, v-ATPase signaling is disrupted, causing the separation of the V1 and Vo regions, downregulating ATP hydrolysis and proton transport, and inhibiting v-ATPase activity, resulting in the closure of the Vo complex to protons.[97-100]

When using v-ATPase inhibitors, they inhibit lysosomal acidification by affecting soluble cellular structures or membrane sites, reducing the drop in extracellular pH, thus slowing the growth and invasion of cancer cells or increasing cancer cell death.[101,102] Additionally, in the tumor microenvironment, the intercellular environment is acidic. v-ATPase inhibitors can also inhibit cancer cell growth and induce their death by reducing the volume and acidity of lysosomes within cells, thereby increasing the pH of the extracellular environment and lysosomes.[103-105] At the same time, v-ATPase not only affects cancer progression through changes in lysosomal acidity but can also control the changes in cancer cells through immunotherapy. It involves looking for relevant proteomics data of v-ATPase inhibitors and using

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specific peptide segments to competitively bind to the binding sites, further regulating immune cells to combat the growth of cancer cells.[106] The activity of v-ATPase can also be inhibited to reduce the capture of immune cells, thereby maintaining the efficacy of immunotherapy in slowing the proliferation of cancer cells.[107]

The literature review indicates that numerous v-ATPase inhibitors exert anti-cancer effects through lysosomal acidification or immune regulation. For instance, the macrolide antibiotic bafilomycin A1 targets v-ATPase to inhibit lysosomal acidification, thereby exhibiting anti-cancer activity.[28] Achiol A is a v-ATPase inhibitor from actinomycetes that limits cancer cell growth by inhibiting lysosomal acidification.[108] The v-ATPase inhibitor bafilomycin A induces apoptosis in cancer cells by inhibiting lysosomal acidification.[109] Concanamycin A is also an inhibitor of v-ATPase; after its action, immune cells can come into close contact with and kill cancer cells.[110] Additionally, novel inhibitors such as benzofuran-one amides or chondroitin sulfate have been identified, although their binding sites have not yet been fully characterized.[111-114] In addition to influencing the lysosomal acidification pathway through the action of v-ATPase, which ultimately inhibits the proliferation and survival of cancer cells, we have also discovered other pathways that do not participate in the regulation of lysosomal acidification. For instance, the use of alkaloid inhibitors to block DDX3-dependent v-ATPase activity further modulates mitochondrial homeostasis for the treatment of breast cancer.[115] RUVBL1/2 alkaloid inhibitors target RUVBL1/2 to prevent YTHDF1 from inducing information translation and signal transduction in cancer cells, achieving an anticancer effect through targeted therapy.[116]

LIMITATIONS

Despite the promising results observed in the targeting of v-ATPase by alkaloid compounds to inhibit lysosomal acidification for anti-cancer effects, several limitations should be acknowledged. First, in our data selection, the alkaloid compounds we chose did not timely consider that despite being the same alkaloid, they may exhibit multiple conformations and protonation states. In our research methods, we employed cell proliferation assays and molecular docking analyses, but we did not conduct *in vivo* experiments through animal models or clinical evaluations to confirm the anti-tumor effects. We solely focused on preclinical anti-tumor findings without timely translation into clinically applicable research. Furthermore, in studying alkaloid compounds targeting v-ATPase, there may be off-target effects due to the structural similarities of alkaloids, multi-target actions, and metabolic Interferences, potentially resulting in the targeting of other proteins or pathways. In the molecular docking studies, we overlooked the conformational changes of the receptor, as different conformations could lead to varying predictive outcomes. Additionally, employing flexible or semi-flexible docking may result in discrepancies as well. To

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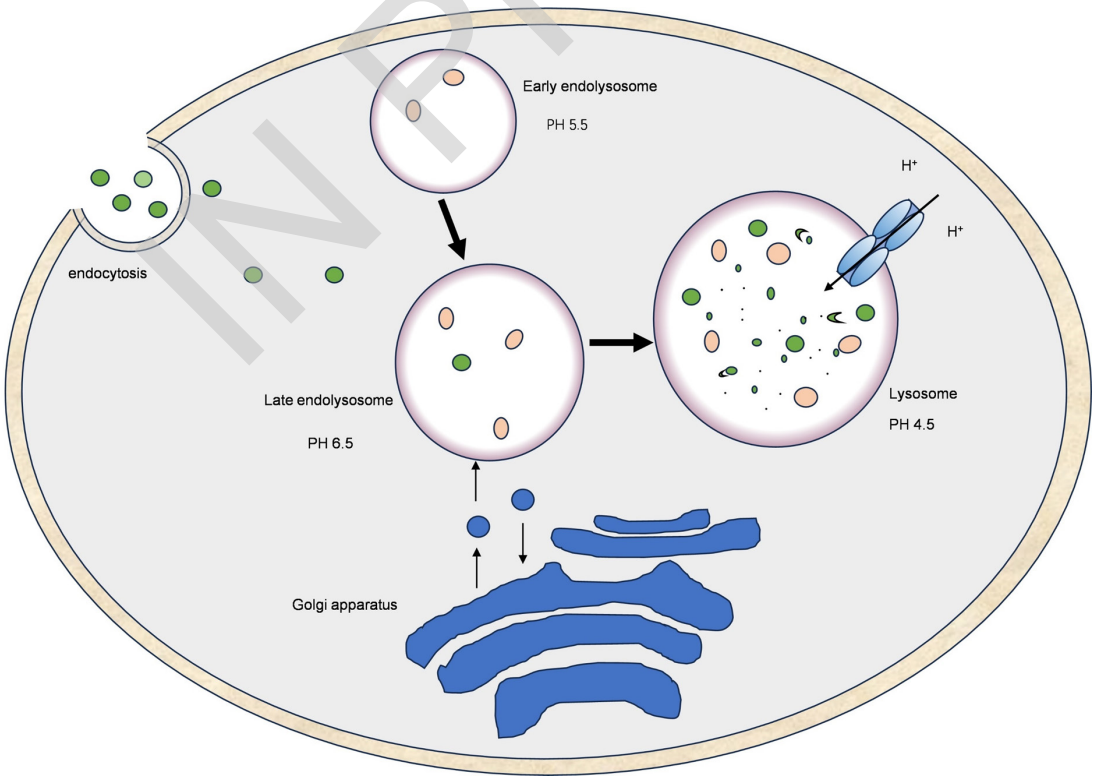


Figure 4: Mechanism of lysosomal regulation of intracellular pH: The changes in lysosomal pH during the transformation from early to late endosomes; the v-ATPase on the lysosomal membrane actively transports H^+ ions into the lysosome, thereby establishing and maintaining the acidic environment, which allows extracellular material to enter the cell via endocytosis and undergo degradation in the acidic lysosome.

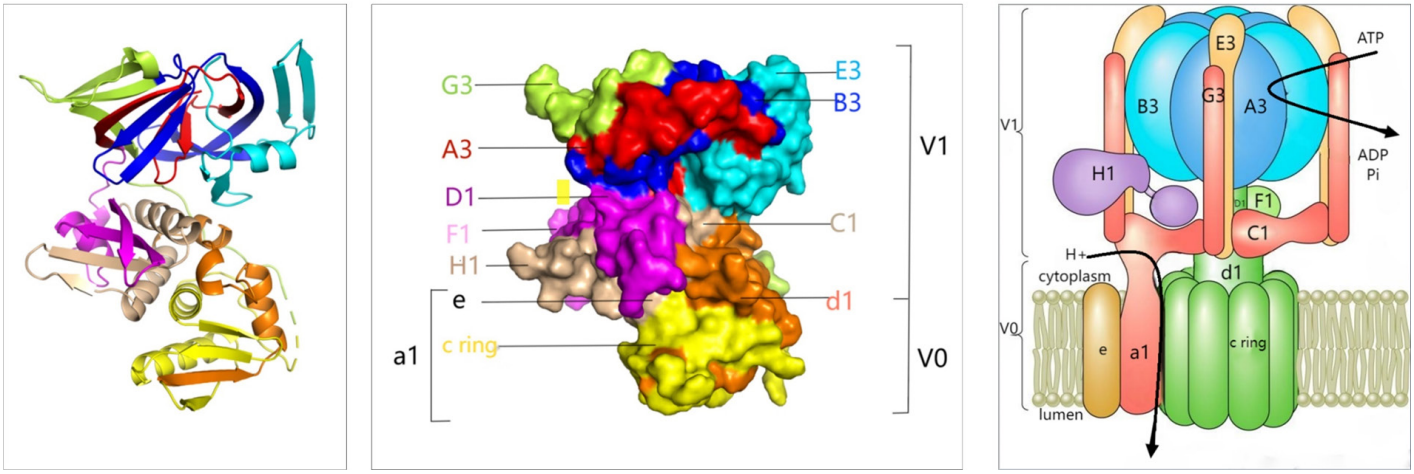


Figure 5: Structural Diagram of v-ATPase (a/b); Mechanism of v-ATPase Involvement in pH Regulation (c).

address these limitations, we will consider exploring whether the anti-cancer effects of alkaloid compounds differ under various conditions, and conduct *in vivo* validation experiments to provide stronger evidence for the anti-cancer effects of alkaloids targeting v-ATPase in inhibiting lysosomal acidification. This would help translate our preclinical findings into clinically applicable research. Furthermore, we will adopt optimization schemes to enhance target selectivity using different target validation methods and multi-omics analyses to better avoid off-target effects and the occurrence of targeting other proteins or pathways. Future research will incorporate methods such as pull-down assays to rule out non-specific interactions, thereby addressing the inherent flaws in molecular docking studies.

CONCLUSION

As the "digestive workshop" of the cell, the lysosome contains various hydrolytic enzymes that provide the degradation capability necessary for the breakdown of proteins, lipids, and polysaccharides. The activity of these enzymes, as well as the movement and maturation of the lysosome, is closely related to the pH level within the lysosomal lumen. When the lysosomal pH is regulated between 4 and 5, an acidic environment is established, activating most of the hydrolytic enzymes required for the breakdown of nutrients that support the growth and proliferation of both normal and cancer cells. For lysosomes to maintain their acidifying function, regulation via v-ATPase is essential. The energy generated by ATP hydrolysis into ADP and Pi facilitates the translocation of protons (H^+) across membranes, driving the proton accumulation cycle. This process, in conjunction with ion transport pathways (i.e., cytoplasmic anion influx and organelle or lumen cation efflux), helps maintain the voltage difference across organelle membranes, acting as an electrostatic shunt to create an acidic environment that promotes cell proliferation.

Modern studies have revealed that natural alkaloids possess unique chemical structures and biological activities. These compounds provide a material foundation for further research into biological activity through total synthesis and serve as lead compounds with medicinal value through structural modifications, making them a hot topic in the field of anti-tumor drug development.[114] Alkaloid compounds can directly modify cancer cell genes, degrade structural proteins in cancer cells, induce cell cycle arrest in the S phase, exert cytotoxic activity to inactivate and kill cancer cells, inhibit cancer cell growth and proliferation by affecting DNA synthesis, or induce apoptosis in a dose-dependent manner.[117]

For example, Dioxyreserpine inhibits the expression of the NALM-6 gene in the e. B-ALL cell line, directly suppressing cancer cell growth.[118] The 3m compound in bis-β-carboline alkaloids effectively inhibits the activity of Top1 and Top2, leading to DNA damage accumulation and subsequent cancer cell death.[119,120] The compound 91 in 10-hydroxycamptothecin binds with β-glucuronidase, degrading the proteoglycans in the cancer cell basement membrane to limit tumor progression.[121-123] In the presence of Camptothecin (CPT), cancer cells experience cell cycle arrest during the S phase, eventually leading to cancer cell death.[2,124] Quinoline alkaloid compounds 34 and 36-38 exhibit varying cytotoxic activity against specific human cancer cell lines, such as BC1, causing cancer cell inactivation and death.[125,126]

However, many studies have not explored the impact of alkaloid small molecules targeting v-ATPase to induce lysosomal acidification in tumor cells. The effects of lysosomal acidification remain unknown. Therefore, this study finds that alkaloid compounds, as small molecules, can target v-ATPase to inhibit its activity, thereby suppressing lysosomal acidification and ultimately exerting anti-tumor effects.

Current research has found that alkaloid small molecules can regulate the expression of cancer cell proteins by directly targeting binding sites within the cell cycle, thereby inhibiting cyclin expression and increasing the expression of pro-apoptotic proteins. These molecules can also selectively induce cytotoxic effects in cancer cells. Additionally, alkaloid small molecules act as inhibitors of v-ATPase, blocking its activation and affecting lysosomal acidification, which in turn inhibits the expression

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of certain abnormal genes linked to disease onset, including inflammation, Parkinson's disease, Alzheimer's disease, Down syndrome, and liver cancer. Our study further confirms the antitumor effects of alkaloid small molecules. However, unlike most existing studies on the anticancer mechanisms of alkaloid compounds, our research reveals that these molecules do not directly influence the cancer cell cycle to inhibit tumor progression. Instead, we discovered that they affect the growth environment required by cancer cells, hindering their proliferation by disrupting lysosomal acidification. Specifically, alkaloid small molecules activate v-ATPase through molecular docking, leading to lysosomal acidification dysfunction and exerting antitumor effects.

Through this study, we further determined that alkaloid small molecules do not directly act on cancer cell gene expression to produce antitumor effects. Rather, they target active sites on v-ATPase, which were identified through molecular docking analysis. The high binding energy values obtained indicate that these molecules bind effectively to v-ATPase, influencing its function in maintaining the pH homeostasis of the lysosome. By inhibiting lysosomal acidification, the acidic environment required for cancer cell growth is not maintained, ultimately preventing cancer cells from meeting their proliferative needs, and leading to their gradual apoptosis.

Based on our molecular docking results, we observed that the majority of alkaloid small molecules bind to v-ATPase at hydrogen-bonding sites on LYS and ASN residues. This suggests that the binding sites of most alkaloid small molecules overlap at the same locations. Furthermore, the molecular docking binding energy results demonstrate a strong binding affinity, suggesting that alkaloid small molecules can inhibit lysosomal acidification by binding to v-ATPase, thereby exerting antitumor effects. Of course, the molecular docking experiments in this study have certain limitations, as various factors may influence the results, leading to experimental errors. Future studies should expand the number of alkaloid small molecules tested and employ other research methods to further explore the mechanisms by which these molecules target v-ATPase activation, identify specific binding sites, and understand their effects.

The use of alkaloid small molecules to target and activate v-ATPase, thereby inhibiting lysosomal acidification and achieving antitumor effects, provides a new perspective and approach for cancer treatment. Alkaloid small molecules are an emerging focus of research, and their potential to treat various diseases by affecting v-ATPase is a topic of great interest. Since the lysosome is central to cellular catabolism, the activity of v-ATPase significantly influences its function. The acidification process is crucial for the growth and apoptosis of both normal and cancer cells. This study, based on current progress in the research on antitumor bioactive compounds and lysosomal acidification, explores the potential of alkaloid small molecules in targeting v-ATPase to inhibit lysosomal acidification as an anticancer strategy. It is hoped that this research will provide valuable insights for the development of new antitumor drugs.

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ABBREVIATIONS

v-ATPase: Vacuolar-type adenosine triphosphatases; **CLL:** Chronic lymphocytic leukemia; **AML:** Acute myeloid leukemia; **Bcl-2:** B-cell lymphoma-2.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Molecular docking experiment, Formal analysis draft, and Writing-original were performed by Xueyi Chen. Immunofluorescence test and Validation were performed by Xueting Lin. Cell experiment and Visualization were performed by Lingchen Liao. Supervision and Investigation were performed by Yuzhou Pang. Investigation and Funding acquisition were performed by Gang Fang. Review, Editing, Supervision, Investigation, Funding acquisition and Conceptualization were performed by Zhiyong Cao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

ETHICAL CONSIDERATION

Confirm that the database license terms adopted by the public are compliant.

DATA AVAILABILITY

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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