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Research Article

Synthesis and Evaluation of Cleistanthin A Derivatives as Potent Vacuolar H^+-ATPase Inhibitors

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Twelve new glycosides and alkane derivatives of cleistanthin A were designed and synthesized. Their *in vitro* antiproliferative activity was investigated against HCT-116, HepG2, A549, Hela tumor cell lines and HEK293 cell by MTT assay. Most of these compounds displayed antiproliferative effects on four cancer cells at submicromolar concentration, but they were less potent than cleistanthin A Moreover, they showed no antiproliferative effects on HEK293 cell at 200 nM. The most potent compounds, 3e and 4a, have been shown to inhibit the activity of vacuolar H^+-ATPase (V-ATPase) and neutralize the pH of lysosomes at submicromolar concentrations.

Key words: Cleistanthin A, inhibitor, synthesis, vacuolar H^+-ATPase

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The regulation of intracellular pH and modulation of intra-organellar acidity has been implicated in various physiological functions. The maintenance of intracellular pH is controlled by the vacuolar H^+-ATPases (V-ATPases), which are a class of ATP-driven multisubunit proton pumps found in all eukaryotic cells (1). Significantly, scientific evidence suggests that the acidic microenvironment is critical to managing cancer development, progression and metastasis. Thus, V-ATPases play a vital role in tumor invasion and metastasis development, which are responsible for microenvironment acidification (2,3). These results propose V-ATPases as a key target for new strategies in cancer treatment (4–8). Thus, inhibitors of V-ATPases could be promising novel therapeutics for cancer treatment. Most of the known V-ATPases inhibitors are natural compounds of microbial origin such as salicylihalamides and bafilomycin.

The natural lignan, diphyllin, has been proved to possess potent V-ATPase inhibitory activity with the IC_{50} value 17 nM (9). Recently, we have indentified two cytotoxic diphyllin glycosides, cleistanthin A and cleistanthoside A tetraacetate, as potent vacuolar H^+-ATPase (V-ATPase) inhibitors (10).

These results prompted us to design and synthesize a library of cleistanthin A derivatives to explore novel V-ATPase inhibitors with the hope of optimizing the anticancer activity of cleistanthin A. Herein, we report the syntheses, antiproliferative activity, V-ATPase inhibitory effects, and lysosomal pH analysis of these glycosides and alkyl ethers derivatives of cleistanthin A (Figure 1). Some of these derivatives showed strong potent V-ATPase inhibitory activity.

Methods and Materials

Chemistry

Materials and General Methods' Commercial grade reagents and solvents were used without further purification, except where noted. Flash chromatography was performed on silica gel. The purity of all compounds was judged by TLC analysis (single-spot/two-solvent systems) using a UV lamp. ^1H NMR and ^{13}C NMR spectra were taken on 400 MHz spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts were recorded in ppm values.

Typical synthetic procedure for synthesis of 3a, 3c, and 3e

A mixture of the cleistanthin A (0.10 mmol), glycosyl trichloroacetimidate (0.13 mmol), and powered 4 Å molecular sieves in anhydrous CH_2Cl_2 (3 mL) was stirred at −20 °C under N_2 for 10 min (Scheme 1). A solution of $BF_3{\cdot}Et_2O$ (0.13 mmol) in CH_2Cl_2 was added dropwise. After being stirred at room temperature for 4 h, the mixture was diluted with CH_2Cl_2 (3 × 10 mL), washed with saturated $NaHCO_3$ (1 × 10 mL), brine (2 × 20 mL), and the organic layer was dried on Na_2SO_4. The residue was purified by column chromatography on silica gel eluting with EtOAc/petroleum ether (1:2 v/v) to afford a solid.

4-O-[2‴, 3‴, 4‴, 6‴-O-tetra-O-acetyl-β-D-galactopyranosyl (1→2)-3″, 4″-di-O-methyl- β-D-xylopyranosyl]-diphyllin (3a)

white solid, yield 55%, m.p. 143–145 °C; $[\alpha]_{589}^{27}$: −29.7° ($CHCl_3$, *c* 1.0); ^1H NMR (400 MHz, $CDCl_3$): δ 7.85 (s, 1H,

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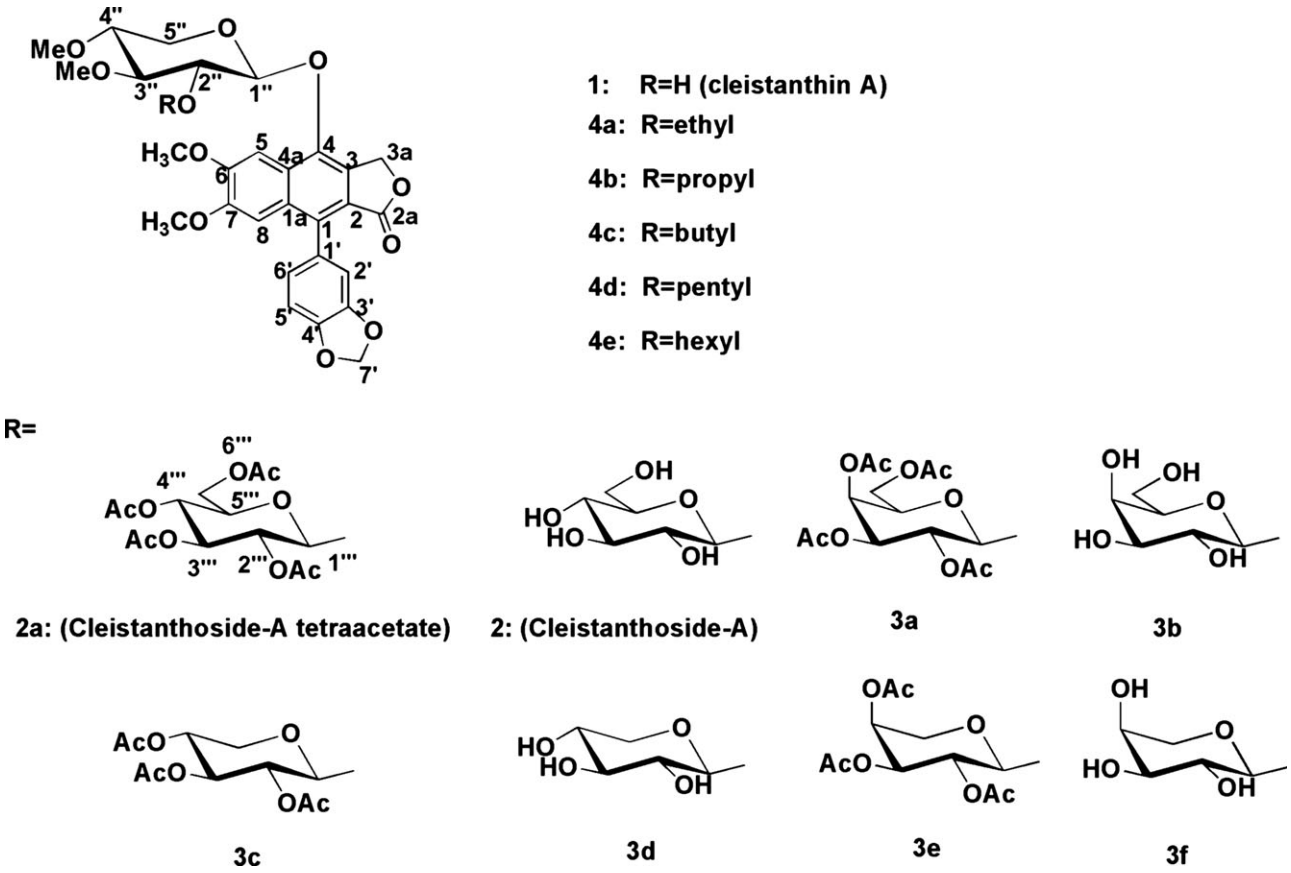


Figure 1: Cleistanthin A and its derivatives.

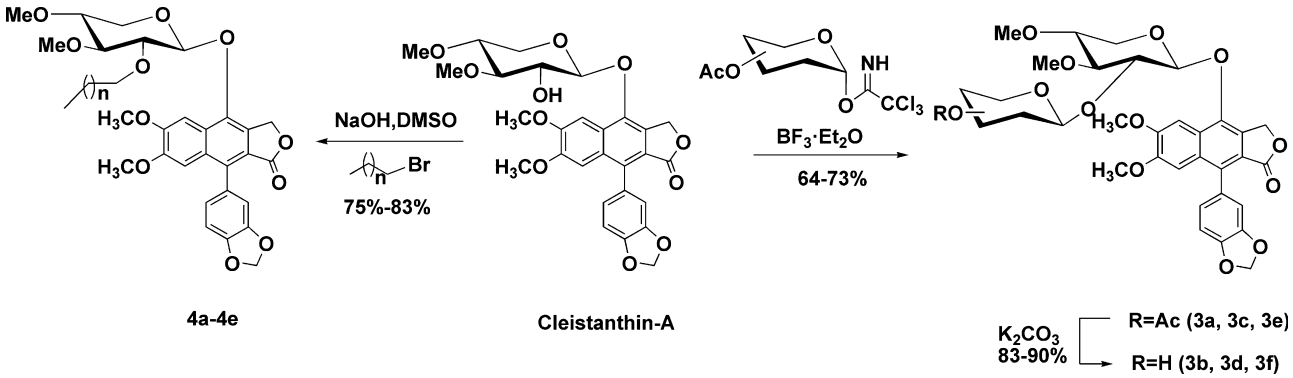
5-ArH), 7.08 (s, 1H, 8-ArH), 6.98(d, J = 8.4 Hz, 1H, 6′-ArH), 6.86–6.79 (m, 2H, 2′, 5′-ArH), 6.10 (d, J = 20.8 Hz, 2H, 7′-CH_2-), 5.58–5.50 (m, 2H, 3a-CH_2-), 5.46 (d, J = 2.4 Hz, 1H, 3‴-CH-), 5.31 (t, J = 8.4 Hz, 1H, 4‴-CH-), 5.10–5.05 (m, 2H, 1‴,2‴-CH-), 4.86 (d, J = 7.2 Hz, 1H, 1″-CH-), 4.16 (s, 3H, 6-OMe), 4.10–4.01 (m, 3H, 6‴–CH_2- × 2, 5″-CH_2-), 3.97–3.89 (m, 2H, 2″, 5‴-CH-), 3.82 (s, 3H, 7-OMe), 3.63 (s, 3H, 3″-OMe), 3.48 (s, 3H, 4″-OMe), 3.44-3.41 (q, J = 4.4, 8.4 Hz, 1H, 4″-CH-), 3.29 (t, J = 8.4 Hz, 1H, 3″-CH-), 3.14 (t, J = 9.6 Hz, 1H, 5″-CH-), 2.15 (s, 3H, 2‴-OCOMe), 2.12 (s, 3H, 3‴-OCOMe), 2.02 (s, 3H, 4‴-OCOMe), 1.87(s, 3H, 6‴-OCOMe); ^{13}C NMR ($CDCl_3$): δ 170.2, 169.4, 151.7, 150.1, 147.5, 147.4, 136.3, 131.1, 130.7, 128.4, 127.2, 123.6, 119.2, 110.7, 108.2, 106.0, 103.5, 102.0, 101.3, 85.0, 80.0, 79.7, 71.0, 70.7, 69.1, 67.4, 67.0, 62.9, 60.9, 60.6, 58.3, 56.5, 55.8, 20.9, 20.7, 20.4; HRMS (ESI-TOF) (m/z) $[M + Na]^+$ calcd for $C_{42}H_{46}O_{20}Na$ 893.24801, found 893.24920.

Typical synthetic procedure for synthesis of 3b, 3d, and 3f

The solid of peracetyl glycoside (**3a**, **3c**, **3e**) (0.1 mmol) was dissolved in MeOH (5 mL), and then, K_2CO_3 (0.25 mmol) was added. After stirring at room temperature for 60 min, the solution was neutralized with 1 M HCl and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with CH_2Cl_2/MeOH (30:1 v/v) to afford a solid.

4-O-[β-D-galactopyranosyl (1→2)-3″, 4″-di-O-methyl-β-D-xylopyranosyl]-diphyllin (3b)

white solid, yield 90%, m.p. 239–242 °C; $[\alpha]_{589}^{27}$: −45° (MeOH, c 1.0); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.86 (s, 1H, 5-ArH), 7.05 (d, J = 8 Hz, 1H, 8-ArH), 6.99 (s, 1H, 6′-ArH), 6.93 (d, J = 5.6 Hz, 1H, 2′-ArH), 6.81 (d, J = 8 Hz, 1H, 5′-ArH), 6.13 (s, 2H, 7′-CH_2-), 5.58 (dd, J = 15.2, 31.6 Hz, 2H, 3a-CH_2-), 5.20 (t, J = 6.4 Hz, 1H, 1‴-CH-), 4.80–4.76 (m, 2H, 5″,6‴-CH_2-), 4.64 (d, J = 7.6 Hz, 1H, 1″-CH-), 4.48 (t, J = 4.8 Hz, 2H, 2″, 5‴-CH-), 4.07–4.01 (m, 2H, 5″-CH_2-, 2‴-CH-), 3.98 (s, 3H, 6-OMe), 4.10–4.01 (m, 3H, 6‴-CH_2- × 2, 5″-CH_2-), 3.97–3.89 (m, 2H, 2″, 5‴-CH-), 3.57 (s, 3H, 7-OMe), 3.53–3.40 (m, 5H, 3″, 4″, 3‴, 4‴-CH-, 6‴-CH_2-), 3.38 (s, 3H, 3″-OMe), 3.33 (s, 3H, 4″-OMe); ^{13}C NMR ($CDCl_3$): δ 169.4, 151.9, 150.4, 147.4, 144.4, 135.0, 130.1, 129.1, 128.9, 128.7, 126.9, 124.0,119.2, 111.3, 108.4, 105.9, 104.5, 102.6, 101.8, 101.5, 83.5, 79.6, 78.9, 77.7, 75.8, 73.8, 71.5, 68.3, 67.5, 60.6, 57.7; HRMS (ESI-TOF) (m/z) $[M + Na]^+$ calcd for $C_{34}H_{38}O_{16}Na$ 725.20575, found 725.20724.



Scheme 1: Synthesis of cleistanthin A derivatives.

Typical synthetic procedure for synthesis of 4a–4e

To a stirring solution of cleistanthin A (0.22 mmol) in DMSO (5 mL) was added NaOH (1.76 mmol) (Scheme 1). After stirring at room temperature for 10 min, bromoethane (4.4 mmol) was added. The reaction mixture was stirred for 5 h at room temperature and then was diluted with CH_2Cl_2 (50 mL), washed with water (2 × 10 mL), brine (2 × 20 mL). The organic layer was dried on Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel eluting with EtOAc/petroleum ether (1:2 v/v) to afford a solid.

4-O-[2″-O-ethyl-3″, 4″-Di-O-methyl-β-D-xylopyranosyl]-diphyllin (4a)

white solid, yield 83%, m.p. 137–140 °C; $[\alpha]_{589}^{27}$: −59.7° ($CHCl_3$, *c* 1.0); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (s, 1H, 5-ArH-), 7.09 (s, 1H, 8-ArH-), 6.97 (d, *J* = 9.2 Hz, 1H, 6′-ArH-), 6.85–6.79 (m, 2H, 2′, 5′-ArH-), 6.10 (s, 1H, 7′-CH_2-), 6.05 (s, 1H, 7′-CH_2-), 5.51–5.41 (m, 2H, 3a-CH_2-), 4.83 (d, *J* = 7.6 Hz, 1H, 1″-CH-), 4.14–4.06 (m, 4H, 5″-CH-, 6-OMe), 4.03–3.96 (m, 2H, 2″, 5″-CH-), 3.81 (s, 3H, 7-OMe), 3.69 (s, 3H, 3″-OMe), 3.50 (s, 4H, 3″-CH, 4″-OMe), 3.41–3.52 (m, 1H, -CH_2-), 3.25 (t, *J* = 8.8 Hz, 1H, -CH_2-), 3.08 (t, *J* = 11.6 Hz, 1H, 4″-CH-), 1.36 (t, *J* = 6.8 Hz, 3H, -CH_3); ^{13}C NMR ($CDCl_3$): δ 169.8, 151.8, 150.9, 147.5, 144.2, 136.2, 130.8, 130.7, 128.3, 126.9, 123.6, 119.2, 110.7, 108.2, 106.2, 104.7, 101.2, 100.8, 85.6, 81.5, 79.3, 69.0, 67.3, 63.5, 61.0, 58.8, 56.3, 55.8, 15.8; HRMS (ESI-TOF) (*m/z*) $[M + Na]^+$ calcd for $C_{30}H_{32}O_{11}Na$ 591.1842, found 591.1853.

Spectroscopic data of **3c**, **3e**, **3d**, **3f**, **4b**, **4c**, **4e** can be found in Supporting information.

In vitro *antiproliferative assays*

Human hepatocellular carcinoma (HepG2), human non-small-cell lung tumor (A549), human colon carcinoma (HCT-116), human embryonic kidney 293 cell line (HEK293), and human cervical carcinoma (HeLa) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in a 37 °C incubator with a 5% CO_2 environment. Compounds were dissolved in DMSO with a concentration of 10 mM. Cells were seeded into 96-well plates at a concentration of 2×10^3 per well, cultured for 24 h, exposed to the compounds at various concentrations for cancer cell viability experiments, cells were cultured for 72 h, and viability was determined through the use of the MTT assay.

Activity of V-ATPase assays

The human liver hepatocellular carcinoma cell line (HepG2) was used for V-ATPase activity assay. Briefly, cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HepG2 cells were exposed to the compounds at various concentrations, cultured for 72 h, and collected. To evaluate V-ATPase activity, the release of phosphate was measured in spectrophotometer by colorimetric assay according to the manufacturer's instruction. Absorption (340 nm) of samples at 0 min, the 5th min, and the 10th min after beginning of the reaction was measured in reaction medium of malachite green to determine the V-ATPase activity. The experiment was repeated three times.

Lysosomal pH analysis

HepG2 cells were grown on coverslips in six-well plates and loaded with 100 nM LysoTracker Red and treated with different concentration of compounds diphyllin, **3e** and **4a** for 3 h. Coverslips were washed with PBS, fixed for 10 min with 1% formaldehyde, washed again with PBS, and mounted on slides for fluorescence or DIC imaging using a Leica DMRXA2 confocal microscope and Simple PCI software.

Statistical analysis

Data were presented as means ± SD and analyzed by SPSS software (SPSS 19.0; SPSS Inc., Chicago, IL, USA).

Pictures were processed with Photoshop software. Mean values were obtained from at least three independent experiments.

Results and Discussion

Chemistry

Previously, we have reported the synthesis of cleistanthin A from diphyllin. Therefore, we could use the synthesized cleistanthin A as staring material in this study. We also found that the glycosylation of cleistanthin A could be achieved effectively by $BF_3{\cdot}Et_2O$ catalyzed Schmidt glycosylation (10). In the first part of this study, six diglycosides **3a**–**3f** bearing different glycosyl moities were synthesized employing Schmidt glycosylation from cleistanthin A. Briefly, the glycosylation of cleistanthin A with glycosyl trichloroacetimidate was performed under the catalysis of $BF_3{\cdot}Et_2O$ (1.5 equiv) in the presence of 4 Å MS in CH_2Cl_2 at −20 °C to give per-acetylated glycosides (**3a**, **3c**, **3e**) in 55–75% yield (11,12). The following deacetylation with K_2CO_3 in methanol was performed smoothly to produce glycosides (**3b**, **3d**, **3f**) in 90–98% yields (Scheme 1). The assignments of ^1H NMR signals were made on the basis of the comparison of naturally occurring diphyllin glycosides and synthesized diphyllin glycosides in our laboratory.

Secondly, the etherification reaction of cleistanthin A and alkyl bromide in the presence of NaOH afforded the alkylation products **4a**–**4e** in 77–83% yields.

Antiproliferation activity

With twelve cleistanthin A derivatives in hand, four cancer cell lines from various solid tumors, HCT-116, HepG2, A549, Hela, and a normal cell HEK293, were used to determine their antiproliferation activity employing a MTT assay. The average inhibitory concentrations of 50% were shown in Table 1.

Most of these compounds displayed antiproliferative effects on four cancer cells at submicromolar concentration, but they were less potent than cleistanthin A. These derivatives showed antiproliferative selectively for HCT-116 cell line, with a potency of over several times that of other three cell lines. Moreover, they showed no antiproliferative effects on normal HEK293 cell at 200 nM. In the glycosides derivatives, **3b**, **3d**, and **3f** showed weak antiproliferative activity, while peracetyl glycosides **3a**, **3c**, and **3e** possessed significant effects, which suggested the hydrophobic glycosyl moieties were critical to their antiproliferative activity. In particular, the glycoside **3e** having an acetylated α-L-arabinose exhibited the best inhibitory effect on these four cancer cells. This result prompted us to further design a series of compounds with hydrophobic terminals by introducing hydrophobic C2-C6 alkane chains. We found all these five alkane derivatives **4a**–**4e** possessed strong antiproliferation activity similar to the control drug paclitaxel. Moreover, these derivatives showed comparatively better activity against HCT-116 cell line than other three cell lines.

V-ATPase inhibitory activity

Based on the above MTT results, **3e** and **4a** were used for V-ATPase activity assay (Figure 2). The human liver hepatocellular carcinoma (HepG2) was treated with **3e** and **4a** in a range of concentrations (0, 25, 50, 100 nM, respectively), and then, the activity of V-ATPase was examined by V-ATPase activity assay kit (10). Previously,

Table 1: *In vitro* antiproliferative activity of these compounds

	$IC_{50} \pm SD$ (nM)				
Compounds	HepG2	HCT-116	A549	Hela	HEK293
1	7.9 ± 1.2	0.9 ± 0.5	4.4 ± 0.7	19.2 ± 2.3	>200
2a	77.5 ± 2.3	95.0 ± 1.2	128.1 ± 0.2	56.1 ± 0.1	>200
2	>200	>200	>200	>200	>200
3a	30.3 ± 2.6	10.9 ± 2.4	15.4 ± 0.5	60.2 ± 0.6	>200
3b	>200	>200	>200	>200	>200
3c	25.2 ± 1.6	7.7 ± 1.2	18.9 ± 0.2	78.3 ± 2.2	>200
3d	>200	>200	>200	>200	>200
3e	13.9 ± 0.6	0.8 ± 0.5	5.2 ± 0.8	25.4 ± 4.2	>200
3f	>200	>200	>200	>200	>200
4a	26.3 ± 2.1	3.5 ± 1.6	5.8 ± 1.5	46.2 ± 2.5	>200
4b	23.5 ± 0.7	0.8 ± 0.3	4.2 ± 0.5	36.3 ± 0.2	>200
4c	57.5 ± 2.2	6.0 ± 0.9	23.9 ± 4.8	80.7 ± 4.6	>200
4d	30.0 ± 1.2	6.2 ± 0.4	18.6 ± 0.6	67.6 ± 3.2	>200
4e	27.7 ± 2.4	7.8 ± 0.6	12.4 ± 0.2	44.5 ± 2.5	>200
Paclitaxel	13.6 ± 0.7	1.1 ± 1.3	4.3 ± 1.6	32.2 ± 3.2	5.5 ± 1.0

NT, not detect.
All experiments were independently performed at least three times.

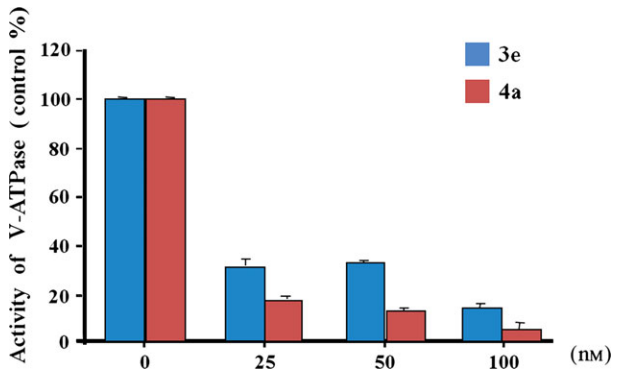


Figure 2: Inhibition of V-ATPase activity by **3e** and **4a**.

we measured the inhibitory of V-ATPase activity of cleistanthin A Treatment of HepG2, it decreased <10% the percentage of activity of V-ATPase at 25 nM (10). While treatment of HepG2 with **3e** and **4a** at 25 nM significantly decreased the percentage of activity of V-ATPase to 34% and 18%, respectively, which proved they were more potent than cleistanthin A. But we found their V-ATPase inhibitory activity did not matched well with their antiproliferation activity. We deduced that the inhibition of activity of V-ATPase was not the only mode of action of their antiproliferation activity, and they could also act on both TopoII and tubulin. Therefore, we believe that diphyllin glycosides are multitargeted antitumoral natural compounds.

The lysosome acidity was examined in HepG2 cells at various concentrations of diphyllin, **3e** and **4a** (Figure 3). HepG2 cells were stained with a pH-sensitive fluorescence dye (LysoTracker Red) and sequential loaded with diphyllin, **3e** and **4a**. As shown in Figure 3, the amount of red fluorescence decreased in cells treated with diphyllin, **3e** and **4a** compared with untreated cells. The V-ATPase was completely suppressed at the concentrations of 60 nM **3e** or **4a** and 3 μM diphyllin, which indicated they were more potent than diphyllin. The results also implied that the modulation of lysosomes acidity by **3e** and **4a** was consistent with their inhibitory effect on V-ATPase activity.

Conclusion

In summary, we have designed and synthesized a new series of cleistanthin A derivatives bearing glycosyl moieties or chain alkanes as potential V-ATPase inhibitors. Most of these compounds displayed antiproliferative effects on four cancer cells at submicromolar concentration, but they were less potent than cleistanthin A. The acetylated α-L-arabinoside **3e** and C2 alkane **4a** possessed the best antiprolifera-

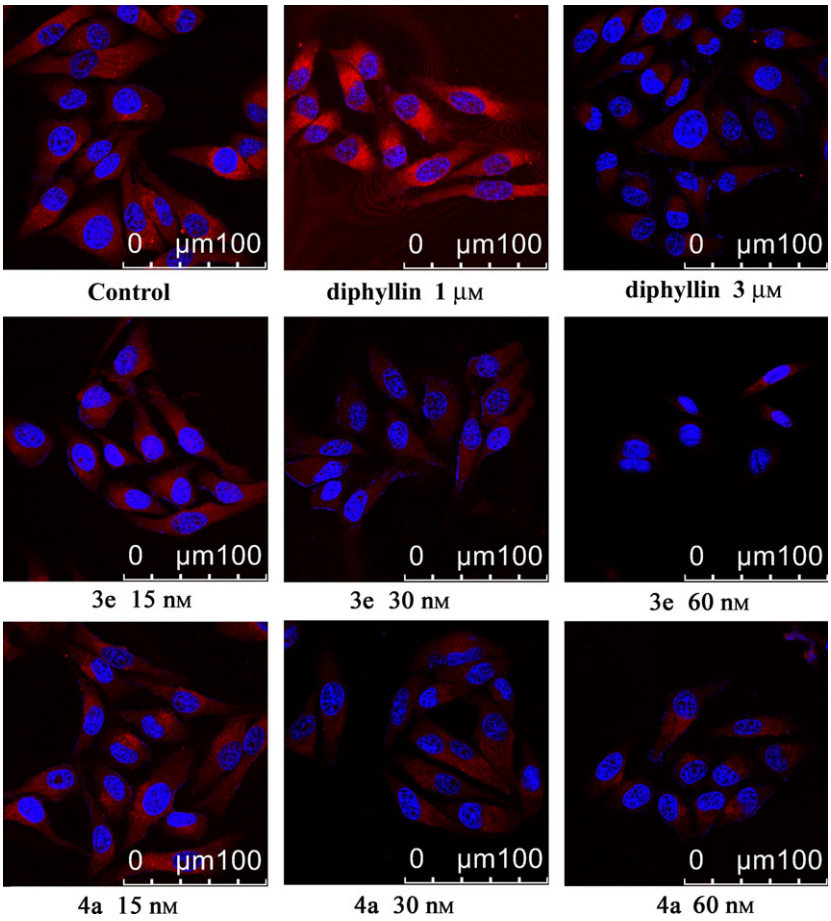


Figure 3: Lysosomal pH analysis of HepG2 treated with diphyllin, **3e** and **4a** for 48 h. Acidity of lysosomes was visualized using a pH-dependent LysoTracker and confocal microscopy. One representative picture of three independent sets is shown.

tive activity *in vitro* against four cancer cell lines. Moreover, these derivatives showed no antiproliferative effects on normal HEK293 cell at 200 nM. They also displayed potent inhibitory activity against V-ATPase. Lysosomal pH analysis illuminated that they inhibited the HepG2 lysosomal acidification at submicromolar concentrations. Compounds **3e** and **4a** possessed promising V-ATPase inhibitory activity, which need to be studied further.

Acknowledgments

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Conflict of interest

All authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Characterization for all the synthesized compounds.