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Covalent Modulators of the Vacuolar ATPase

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Supporting Information

ABSTRACT: The vacuolar H^+ ATPase (V-ATPase) is a complex multisubunit machine that regulates important cellular processes through controlling acidity of intracellular compartments in eukaryotes. Existing small-molecule modulators of V-ATPase either are restricted to targeting one membranous subunit of V-ATPase or have poorly understood mechanisms of action. Small molecules with novel and defined mechanisms of inhibition are thus needed to functionally characterize V-ATPase and to fully evaluate the therapeutic relevance of V-ATPase in human diseases. We have discovered electrophilic quinazolines that covalently modify a soluble catalytic subunit of V-ATPase with high potency and exquisite proteomic selectivity as revealed by fluorescence imaging and chemical proteomic activity-based profiling. The site of covalent modification was mapped to a cysteine residue located in a region of V-ATPase subunit A that is thought to regulate the dissociation of V-ATPase. We further demonstrate that a previously reported V-ATPase inhibitor, 3-bromopyruvate, also targets the same cysteine residue and that our electrophilic quinazolines modulate the function of V-ATPase in cells. With their well-defined mechanism of action and high proteomic specificity, the described quinazolines offer a powerful set of chemical probes to investigate the physiological and pathological roles of V-ATPase.

The vacuolar H^+ ATPase (V-ATPase) is a pivotal player in controlling intracellular pH to regulate various biological processes including membrane trafficking, endocytosis, protein degradation, bone resorption, and small-molecule uptake in eukaryotic cells.[1] Dysregulation of V-ATPase has been implicated in a number of diseases, including renal disease (renal tubular acidosis),[2] bone disease (osteoporosis),[3] and tumor metastasis.[4] V-ATPase is a large multisubunit protein complex that functions as a rotary molecular motor and is organized into two domains, V_0 and V_1. V_1 is located on the cytoplasmic side of the membrane and carries out ATP hydrolysis, whereas the V_0 domain is a membrane-embedded complex that is responsible for driving proton translocation across the membrane.[1,5] The V_1 domain is composed of eight different subunits (A, B, C, D, E, F, G, H) while the V_0 domain contains five different subunits (a, b, c, d, and e) in mammals. The core of the V_1 domain contains a hexamer of A and B subunits, which participates in ATP binding and hydrolysis.[6−8] A so-called nonhomologous region (NHR) in V-ATPase subunit A has been shown to be important for regulating the disassembly of the V_1-V_0 complex. This 90-amino acid insert in subunit A is highly conserved among V-ATPase A subunits from numerous species but is absent from the homologous β subunit of the F-ATPase.[9] Mutations in the NHR can alter *in vivo* dissociation of V-ATPase independent of its catalytic activity.[10]

A number of inhibitors of V-ATPase including both natural products and synthetic compounds have been identified and characterized previously.[11] Among these, the macrolides such as bafilomycin, concanamycin, and archazolid were all found to bind to the membranous subunit c and perturb rotation of the b/c-ring.[12−14] While diverse synthetic V-ATPase inhibitors such as benzolactone enamide,[15] indole derivatives,[16] and 3-bromopyruvate[17] have been reported recently, their binding site within V-ATPase and exact modes of action are often not known. Small molecules with new and defined modes of action against V-ATPase are needed to elucidate the working mechanism and validate the therapeutic relevance of V-ATPase. Herein we describe the discovery and characterization of novel quinazoline-based compounds that covalently target a cysteine (Cys) residue within the NHR of the A subunit with exquisite specificity across the human proteome and modulate the function of V-ATPase in cells.

Our lab has recently prepared a series of quinazolines featuring electrophiles at the 7 position, developed as covalent inhibitors of the receptor tyrosine kinase EphB3.[18] In an effort to monitor the target engagement of these electrophilic quinazolines, we derivatized one electrophilic quinazoline with a terminal alkyne group at the 3′ position of the anilino group as a "clickable" tag (Figure 1A). Treatment of HEK293H (293) cells with the resulting small-molecule probe **1** followed by cellular lysis, CuAAC (Cu(I)-catalyzed [3 + 2] azide−alkyne cycloaddtion)[19] with TAMRA-azide, SDS-PAGE, and in-gel fluorescence scanning revealed one major fluorescent band of ∼70 kDa across the tested concentration range for **1** (Figure 1B). A time-course experiment showed that this target protein

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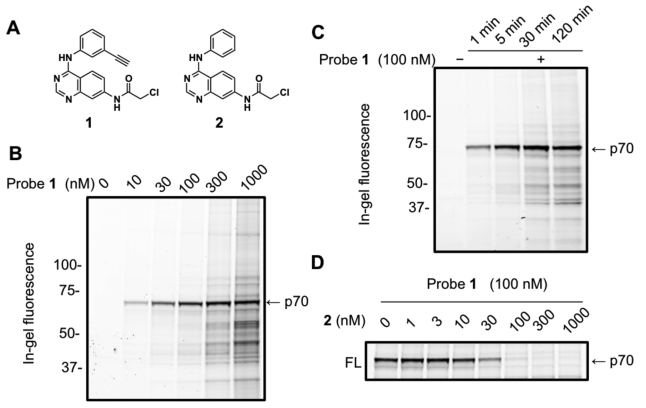
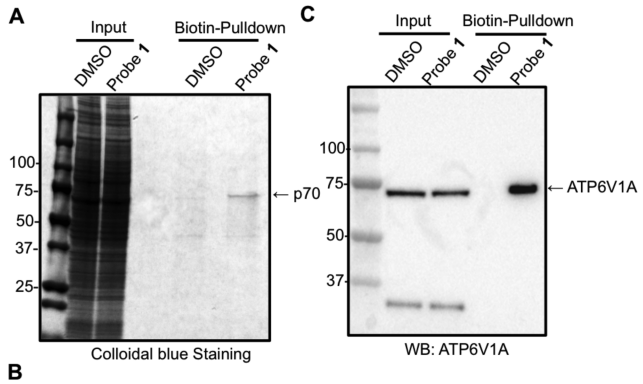


Figure 1. Potent, specific, and rapid labeling of a cellular protein by the electrophilic probe **1** in 293 cells. (A) Structures of an electrophilic quinazoline **2** and its clickable analog (**1**). (B) Dose response of live cell labeling revealed potent engagement of probe **1** to an unknown cellular target of ~70 kDa (p70). (C) Time-course experiment showed rapid engagement of **1** to p70. (D) Dose-dependent blockade of probe labeling by pretreatment of **2**.



B

Accession	Description	MW [kDa]	Area		Fold Enrichment
			DMSO	probe	
P38606	V-type proton ATPase catalytic subunit A	68.3	1.61E+08	6.43E+10	399

Figure 2. Identification of p70 as the vacuolar ATPase subunit A. (A) Streptavidin pull-down enriched p70. (B) LC-MS/MS analysis identified the target protein as ATP6V1A. (C) Immunoblot analysis using a house-made antibody confirmed the protein pulled down with probe **1** was ATP6V1A.

(dubbed p70) was near-completely labeled by **1** after 5 min (Figure 1C). Taken together, these data suggest that probe **1** can potently and selectively engage a major target protein of unknown identity in the human proteome. The p70 target appears to be present in many human cell types, as probe **1** exhibited a similar labeling pattern in other tested cell lines from different tissue origins (Figure S1).

To evaluate the structure–activity relationship of the quinazoline-p70 interaction, we applied a competitive gel-based profiling strategy, screening our library of quinazolines (Figure S2) for a blockade of p70 labeling by probe **1**. Out of 8 quinazolines, only the chloroacetamide-containing compound **2** successfully blocked p70 target labeling by probe **1**. By treating 293 cells with various concentrations of **2** for 30 min before addition of 100 nM of probe **1**, we calculated an apparent IC_{50} value of ~30 nM for **2** labeling of p70 *in situ* (Figure 1D). Consistent with the *in situ* labeling, we also found that the p70 target was labeled by probe **1** *in vitro* in 293 cellular lysates and that pretreatment with **2** blocked this labeling event with similar potency to that observed in live cells (Figure S3). Taken together, these results suggest that the p70 protein target potently and selectively reacts with chloroacetamide quinazolines both *in vitro* and in cells.

We next set out to identify the p70 protein by chemical proteomics. We treated 293 cells with 100 nM of probe **1** for 30 min, followed by lysis and CuAAC-mediated conjugation to a cleavable biotin-azo-azide tag. Biotin-linked target proteins were then captured by streptavidin agarose beads, eluted with sodium dithionite, and resolved by SDS-PAGE. Protein bands were excised, trypsinized, and subjected to LC-MS/MS analysis (Figure 2A).[20] Using spectral counting to quantify the fold-enrichment for proteins derived from probe-treated compared to DMSO-treated cells, we identified the vacuolar ATPase catalytic subunit A (ATP6V1A) as the likely target of **1** (Figure 2B). ATP6V1A has a predicted molecular size of 68.3 kDa, which matches the major fluorescent band observed in our proteomic labeling experiments. Western blot analysis, using an ATP6V1A antibody[21] to label the proteins enriched during the biotin pulldown, revealed an intense band in the probe-treated sample but not in the mock-treated control, further confirming ATP6V1A as the target of probe **1** (Figure 2C). Finally, transfection of 293 cells with 3×FLAG-tagged human ATP6V1A followed by treatment with probe **1** led to the appearance of a new labeled band of slightly retarded mobility compared to the original band (Figure 3B, arrow). Thus, multiple lines of evidence converged to support ATP6V1A as the target of probe **1** in cells.

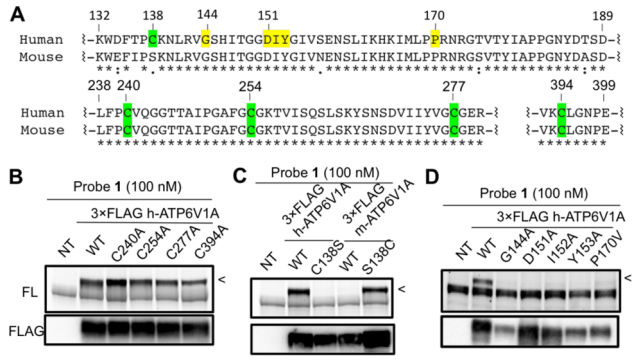


Figure 3. Identification of the probe-labeling site in ATP6V1A. (A) Sequence alignment of human and mouse ATP6V1A highlighting the mutated sites. (B) Mutation of four conserved Cys residues failed to significantly affect probe labeling of 3×FLAG-tagged ectopic ATP6V1A (<). (C) Mutation of C138 abolished probe labeling of human ATP6V1A while the converse mutation of S138C enabled labeling of the mouse protein. (D) Five mutations in distinct regions of NHR all diminished probe binding substantially. The top panels in B–D are in-gel fluorescence images (FL), and the bottom are Western blots showing levels of FLAG-tagged ATP6V1A. NT: nontransfected.

We reasoned that the covalent interaction between ATP6V1A and probe **1** was likely mediated by a nucleophilic attack of a Cys thiol group in ATP6V1A at the chloroacetamide group of **1**. To identify the residue being modified by the probe, we tested the probe-labeling sensitivity of 3×FLAG-tagged ATP6V1A alleles in which select Cys residues were individually mutated to serine (Ser) or alanine. Sequence analysis revealed that human ATP6V1A contained a total of seven Cys residues, out of which six are conserved in the mouse orthologue (Figure 3A). Excluding two predicted buried Cys residues, we first

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mutated the other four conserved Cys residues and examined their effects on probe labeling. However, mutation of these four Cys residues had little effect on probe labeling of the 3×FLAG-tagged ATP6V1A (Figure 3B). We then considered the possibility that the nonconserved Cys (C138) was the modification site (Figure 3A). Mutation of C138 to Ser, the corresponding residue in mouse ATP6V1A, indeed abolished probe labeling. The converse mutation (S138C) conferred the mouse ATP6V1A with a strong sensitivity to probe labeling (Figure 3C). It is interesting to note that the sequence surrounding position 138 is highly conserved between human and mouse ATP6V1A. We suspect that C138 might have been evolved to serve as a redox sensor in the human protein. C138 is located at the beginning of the ATP6V1A NHR, which has been shown to be important for regulation of complex assembly and dissociation.[10] We introduced several mutations in this region and observed a consequent loss of probe labeling for five distinct residues (Figure 3D). Taken together these results imply that probe **1** binds to a pocket that is likely formed by the NHR and covalently targets C138. Consistent with the remoteness of NHR from the ATPase active site, up to millimolar concentrations of ATP failed to block ATP6V1A labeling by probe **1** *in vitro* (Figure S4).

The gel-based profiles of probe **1** reactivity suggested high specificity for ATP6V1A, but we recognized that additional, lower-abundance protein targets of **1** may also exist in human cells. We addressed this question by using the quantitative chemical proteomic method isoTOP-ABPP (isotopic tandem orthogonal proteolysis-activity-based protein profiling) following previously described protocols.[22] MDA-MB-231 cell lysates were pretreated with DMSO or **2**, followed by treatment with the general Cys-reactive probe, iodoacetamide (IA)-alkyne, followed by quantitative isoTOP-ABPP analysis. C138 of ATP6V1A was fully protected from IA-alkyne labeling by **2** at 500 nM (competition R value of 20, reflecting 95% or greater protection); in contrast, none of the other >2900 cysteines quantified in these experiments were substantially affected by **2** (competition R values <4), including another Cys (C277) in ATP6V1A (Figure 4A, B). These chemical proteomic data indicate that **2** reacts with C138 of ATP6V1A with remarkable selectivity in human cells.

3-Bromopyruvate (3-BP) was previously found to affect intracellular pH by inhibiting V-ATPase activity putatively through modification of an unidentified cysteine.[17] To test if 3-BP covalently targets C138 of ATP6V1A, we pretreated 293-cell lysate with various concentrations of 3-BP before labeling with probe **1**. Pretreatment of 3-BP completely abolished ATP6V1A labeling by probe **1** (Figure 4C). These results support covalent modification of C138 in ATP6V1A as a plausible mechanism of 3-BP's inhibition of V-ATPase.

To test if our covalent ATP6V1A probes affect V-ATPase function, we treated HeLa cells with probe **1** or compound **2** at 10 μM for 2 h, followed by labeling with the lysosomotropic red-fluorescent dye LysoTracker Red DND-99 to report V-ATPase-driven intravesicular acidification as described before.[23] Under these conditions, there was no significant difference between untreated cells and those treated with **1** or **2** (Figure 4D). We had previously developed a more sensitive assay to study V-ATPase inhibition in cells that involves pretreatment with bafilomycin and measuring vesicle reacidification after it has been washed off.[23] Using this more sensitive assay, we determined the functional consequences of our electrophilic probes on V-ATPase-dependent vesicle acidification after

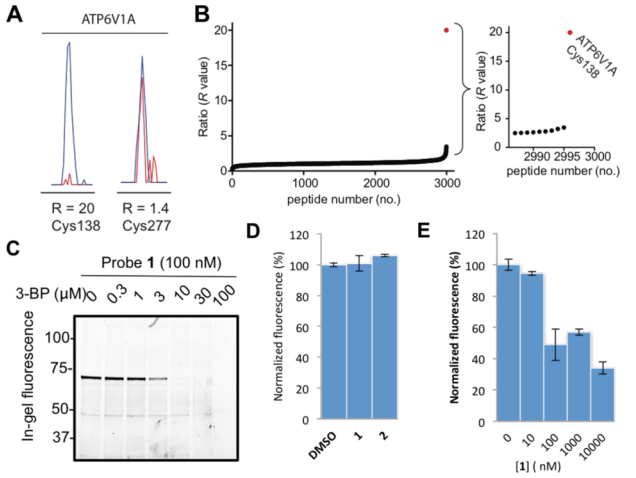

Figure 4. Chloroacetamide quinazolines exhibit exquisite proteomic selectivity, expose the mechanism of action of 3-bromopyruvate, and inhibit vesicle reacidification. (A) Quantitative isoTOP-ABPP experiments[22] revealed that pretreatment with **2** *in vitro* fully protected C138 from iodoacetamide alkyne labeling while iodoacetamide alkyne labeling of a second Cys (C277) in ATP6V1A was insensitive to **2**. Samples were treated with either **2** (Light-Red) or DMSO (Heavy-Blue) and the ratio of MS1 chromatographic peaks compared (R = 20; > 95% decrease in MS1 peak intensity). (B) C138 exhibits a maximal competition R value of 20 while all other Cys have R values less than 4. (C) 3-Bromopyruvate (3-BP) caused a dose-dependent blockade of ATP6V1A labeling by probe **1**. (D) The two quinazolines at 10 μM had no significant effect on steady-state vesicle pH. (E) Probe **1** caused dose-dependent inhibition of vesicle reacidification.

exposing cells to bafilomycin (Figure S5). Immediately after bafilomycin washout, cells were treated with probe **1** at various concentrations, labeled with DND-99, followed by lysis and fluorescence measurement after 3 h. Probe **1** inhibited vesicle reacidification with an apparent IC_{50} value of 30 nM (Figure 4E), which aligns with the potency of its target occupancy measured by in-gel fluorescence (Figure 1B). These results suggest that our electrophilic probes suppress the recovery of V-ATPase activity after its inhibition with bafilomycin.

In summary, we report the first covalent small-molecule modulators of V-ATPase. These quinazoline-based electrophilic probes specifically targeted a Cys residue in the catalytic subunit of V-ATPase, provided novel mechanistic insight into the actions of a previously reported inhibitor of V-ATPase, and modulated the function of V-ATPase in cells. With their well-defined mechanism of action and high proteomic specificity, the described quinazolines offer a powerful set of chemical probes to investigate the physiological and pathological roles of V-ATPase.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b12511.

Supplementary figures, experimental protocol, synthetic scheme and compound characterization (PDF)

Values for isoTOP-ABPP experiments of MDA-MB-231 (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Forgac, M. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 917−929.
(2) Karet, F. E.; Finberg, K. E.; Nelson, R. D.; Nayir, A.; Mocan, H.; Sanjad, S. A.; Rodriguez-Soriano, J.; Santos, F.; Cremers, C. W. R. J.; Di Pietro, A.; Hoffbrand, B. I.; Winiarski, J.; Bakkaloglu, A.; Ozen, S.; Dusunsel, R.; Goodyer, P.; Hulton, S. A.; Wu, D. K.; Skvorak, A. B.; Morton, C. C.; Cunningham, M. J.; Jha, V.; Lifton, R. P. *Nat. Genet.* **1999**, *21*, 84−90.
(3) Toyomura, T.; Murata, Y.; Yamamoto, A.; Oka, T.; Sun-Wada, G. H.; Wada, Y.; Futai, M. *J. Biol. Chem.* **2003**, *278*, 22023−22030.
(4) Sennoune, S. R.; Bakunts, K.; Martinez, G. M.; Chua-Tuan, J. L.; Kebir, Y.; Attaya, M. N.; Martinez-Zaguilan, R. *Am. J. Physiol. Cell Physiol.* **2004**, *286*, C1443−C1452.
(5) Maxson, M. E.; Grinstein, S. *J. Cell Sci.* **2014**, *127*, 4987−4993.
(6) Liu, Q.; Kane, P. M.; Newman, P. R.; Forgac, M. *J. Biol. Chem.* **1996**, *271*, 2018−2022.
(7) Liu, Q.; Leng, X. H.; Newman, P. R.; Vasilyeva, E.; Kane, P. M.; Forgac, M. *J. Biol. Chem.* **1997**, *272*, 11750−11756.
(8) MacLeod, K. J.; Vasilyeva, E.; Baleja, J. D.; Forgac, M. *J. Biol. Chem.* **1998**, *273*, 150−156.
(9) Zimniak, L.; Dittrich, P.; Gogarten, J. P.; Kibak, H.; Taiz, L. *J. Biol. Chem.* **1988**, *263*, 9102−9112.
(10) Shao, E.; Nishi, T.; Kawasaki-Nishi, S.; Forgac, M. *J. Biol. Chem.* **2003**, *278*, 12985−12991.
(11) Huss, M.; Wieczorek, H. *J. Exp. Biol.* **2009**, *212*, 341−346.
(12) Bowman, B. J.; Bowman, E. J. *J. Biol. Chem.* **2002**, *277*, 3965−3972.
(13) Huss, M.; Ingenhorst, G.; Konig, S.; Gassel, M.; Drose, S.; Zeeck, A.; Altendorf, K.; Wieczorek, H. *J. Biol. Chem.* **2002**, *277*, 40544−40548.
(14) Huss, M.; Sasse, F.; Kunze, B.; Jansen, R.; Steinmetz, H.; Ingenhorst, G.; Zeeck, A.; Wieczorek, H. *BMC Biochem.* **2005**, *6*, 13.
(15) Whitehurst, A. W.; Bodemann, B. O.; Cardenas, J.; Ferguson, D.; Girard, L.; Peyton, M.; Minna, J. D.; Michnoff, C.; Hao, W.; Roth, M. G.; Xie, X. J.; White, M. A. *Nature* **2007**, *446*, 815−819.
(16) Petrangolini, G.; Supino, R.; Pratesi, G.; Dal, B. L.; Tortoreto, M.; Croce, A. C.; Misiano, P.; Belfiore, P.; Farina, C.; Zunino, F. *J. Pharmacol. Exp. Ther.* **2006**, *318*, 939−946.
(17) Dell'Antone, P. *Life Sci.* **2006**, *79*, 2049−2055.
(18) Kung, A.; Chen, Y. C.; Schimpl, M.; Ni, F.; Zhu, J.; Turner, M.; Molina, H.; Overman, R.; Zhang, C. *J. Am. Chem. Soc.* **2016**, *138*, 10554−10560.
(19) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599.
(20) Chen, Y. C.; Zhang, C. *Genes Cancer* **2016**, *7*, 148−153.
(21) Hurtado-Lorenzo, A.; Skinner, M.; El Annan, J.; Futai, M.; Sun-Wada, G. H.; Bourgoin, S.; Casanova, J.; Wildeman, A.; Bechoua, S.; Ausiello, D. A.; Brown, D.; Marshansky, V. *Nat. Cell Biol.* **2006**, *8*, 124−136.
(22) Backus, K. M.; Correia, B. E.; Lum, K. M.; Forli, S.; Horning, B. D.; Gonzalez-Paez, G. E.; Chatterjee, S.; Lanning, B. R.; Teijaro, J. R.; Olson, A. J.; Wolan, D. W.; Cravatt, B. F. *Nature* **2016**, *534*, 570−574.
(23) Merkulova, M.; Paunescu, T. G.; Azroyan, A.; Marshansky, V.; Breton, S.; Brown, D. *Sci. Rep.* **2015**, *5*, 14827.

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