

Salicylhalamide A Inhibits the V_0 Sector of the V-ATPase through a Mechanism Distinct from Bafilomycin A_1 *

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The newly identified specific V-ATPase inhibitor, salicylhalamide A, is distinct from any previously identified V-ATPase inhibitors in that it inhibits only mammalian V-ATPases, but not those from yeast or other fungi (Boyd, M. R., Farina, C., Belfiore, P., Gagliardi, S., Kim, J. W., Hayakawa, Y., Beutler, J. A., McKee, T. C., Bowman, B. J., and Bowman, E. J. (2001) *J. Pharmacol. Exp. Ther.* 297, 114–120). In addition, salicylhalamide A does not compete with concanamycin or bafilomycin for binding to V-ATPase, indicating that it has a different binding site from those classic V-ATPase inhibitors (Huss, M., Ingenhorst, G., Konig, S., Gassel, M., Droese, S., Zeeck, A., Altendorf, K., and Wiczorek, H. (2002) *J. Biol. Chem.* 277, 40544–40548). By using purified bovine brain V-pump and its dissociated V_1 and V_0 sectors, we identified the recognition and binding site for salicylhalamide to be within the V_0 domain. Salicylhalamide does not inhibit the ATP hydrolysis activity of the dissociated V_1 -ATPase but inhibits the ATPase activity of the holoenzyme by inhibiting the V_0 domain. Salicylhalamide causes a dramatic redistribution of cytosolic V_1 from soluble to membrane-associated form, a change not observed in cells treated with either bafilomycin or NH_4Cl . By synthesizing and characterizing a series of salicylhalamide derivatives, we investigated the structural determinants of salicylhalamide inhibition in terms of potency and reversibility, and used this information to suggest a possible binding mechanism.

Acidification of intracellular compartments of eukaryotes is essential for many cellular processes, including receptor-mediated endocytosis, protein degradation in lysosomes, processing of hormones, uptake, and storage of neurotransmitters, and entry of many viruses into cells. The control of pH within these intracellular compartments is mediated by vacuolar H^+ -translocating ATPases, which acidify organelles of both constitutive and regulated secretory pathways (1–5). V-ATPases¹ are also

found in the plasma membrane of certain cells where they are responsible for cell type-specific processes, including urinary acidification and osteoclast-mediated bone resorption (6, 7).

The V-type ATPases are among the most widely distributed ATP-driven ion pumps in nature, present in all eukaryotic cells and in various bacteria. Within eukaryotic cells, the structure of these proton pumps is highly conserved from yeast to human, as a multiple subunit complex with a molecular mass exceeding 850 kDa. They contain at least 13 different subunits with various copy numbers, which are organized into two distinct domains, a peripheral V_1 domain that is the catalytic sector and a transmembrane V_0 domain that constitutes the proton channel.

V-pumps are regulated at various levels from transcription and protein synthesis to the regulation of its enzymatic activity through a variety of mechanisms. The most unique regulation mechanism for V-pumps is the reversible dissociation and association of V_1 and V_0 in response to energy demand, which has been extensively studied and clearly demonstrated in yeast and tobacco hornworm (8–10). However, whether this reversible dissociation and association of V-ATPase domains exists in mammals as a regulatory mechanism and, if it does, how this process is regulated, is not clear at the present.

During the past two decades, the importance of this class of ATPases for many critical cellular functions has become increasingly appreciated. Furthermore, the elucidation of the physiological role of V-pumps has revealed the important role these proteins play in a wide array of pathological processes, such as osteoporosis (6), certain renal diseases (7, 11), HIV infection (12), and tumor metastasis (5). The food vacuole of certain parasites is acidified by V-type proton pumps, and disruption of the acidification of this intracellular compartment results in death of the organism. Thus, the V-type pumps are potential targets for the development of pharmacological agents to treat a variety of diseases.

Because of the importance of V-ATPases as a potential therapeutic target, the mechanism by which inhibitors of V-ATPase interfere with pump function has become an area of great scientific interest. Over the past 15 years a few specific V-ATPase inhibitors have been identified, all of which are macrolides. These include bafilomycin A_1 and the closely related compound concanamycin A, both of which inhibit all V-ATPases at nanomolar concentrations and have become important tools for the detection and identification of V-ATPase activity (13). The site of bafilomycin inhibition has been localized to the V_0 proton channel (14, 15), and requires residues of subunit c (16, 17). However, progress in the development of clinically

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¹ The abbreviations used are: V-ATPases (V-pump), the vacuolar-type H^+ -translocating ATPases; V_1 , the peripheral, catalytic sector of V-pumps; V_0 , the membranous proton channel domain of V-pumps; 1799,

bis-(hexafluoroacetyl)acetone; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

useful inhibitors of V-pumps has been limited despite substantial efforts by both academic and pharmaceutical investigators due to problems associated with lack of tissue specificity. Moreover, the complicated chemical structure of the existing inhibitors has made chemical modification of these compounds very challenging.

Salicylhalamide A, originally identified as an anti-tumor agent, has recently been shown to be a specific inhibitor for V-ATPases (18). Salicylhalamide A is as potent a V-ATPase inhibitor as bafilomycin but does not compete with concanamycin or bafilomycin for binding to the pump (17), suggesting that this inhibitor has a different binding site. More importantly, salicylhalamide A is distinct from any previously identified V-ATPase inhibitors in that it inhibits only mammalian V-ATPases, but not the V-ATPases from yeast or other fungi (18). After completing the total synthesis of salicylhalamide A and a variety of its derivatives (20), we have used these reagents to characterize the inhibition of purified bovine brain V-ATPase and have confirmed that V-ATPase is the direct target for salicylhalamide A inhibition and that both ATPase and proton pumping activities are inhibited by salicylhalamide A at IC_{50} below 1 nM (21). Understanding why salicylhalamide A is so specific in its inhibition profile may provide insights into the structural basis for the inhibition of V-ATPases and may lay a foundation for the development of pharmaceutical agents that selectively inhibit subsets of V-ATPases.

In this article, we report (1) that the V_0 is the salicylhalamide binding and inhibited domain of the mammalian V-pump (2) the structural determinants of the potency and reversibility of salicylhalamide inhibition, and (3) that in cells salicylhalamide changes the distribution of the V_1 sector from mostly soluble to mostly membrane-bound.

EXPERIMENTAL PROCEDURES

Materials—Acridine orange was obtained from Eastman, [γ - ^{32}P]ATP from Amersham Biosciences, and all phospholipids were from Avanti Polar lipids, Inc. The proton ionophore 1799 was the generous gift of Dr. Peter Heytler (Dupont). The Lamp1 monoclonal antibody (H4A3) developed by J. T. August and J. E. K. Hildreth (The Johns Hopkins University) was obtained from the Developmental Hybridoma Bank (NICHD-University of Iowa). Polyclonal antibodies against subunits A and E of bovine brain V-ATPase, respectively, were generated using synthetic peptides, prepared in previous studies as described (22, 23). The chemiluminescence kit was from PerkinElmer Life Sciences (NEL103). All other reagents were obtained from Sigma. The synthesis of salicylhalamide A and analogs 1–16 was performed as previously described (20, 21). A tritium-labeled variant of compound 3 (14 Ci/mmol) was prepared by American Radiolabeled Chemicals, Inc, following the synthetic route for the synthesis of cold compound 3 described in Ref. 20.

Preparations—The bovine brain clathrin-coated vesicle V-ATPase was purified to a specific activity of 14–16 $\mu\text{mol of } P_i \times \text{mg}^{-1} \times \text{min}^{-1}$, as described (27). The uncoupled V-ATPase (subunit H-free V-ATPase preparation) (28), dissociated V_1 (29) and free V_0 (14) domains were prepared by separate procedures as described.

Measurement of ATPase Activities—ATPase activity was measured as the liberation of $^{32}P_i$ from [γ - ^{32}P]ATP (27). The purified holoenzyme of V-ATPase was assayed for Mg-ATPase activity, which requires phosphatidylserine (PS) for activation if not reconstituted into proteoliposomes. The V_1 sector has only Ca-ATPase activity and does not require phospholipids. The reactions were started by addition of 200 μl of ATPase assay solution (30 mM KCl, 50 mM Tris-MES, pH 7.0, 3 mM of either MgCl_2 or CaCl_2 , and 3 mM [γ - ^{32}P]ATP (400 cpm/nmol)) in the presence or absence of the inhibitors, and continued for 15 min at 37 °C (for soluble enzyme) or 25 °C (for reconstituted V-ATPase). The ATP hydrolysis reaction was terminated by adding 1.0 ml of 1.25 N perchloric acid, and the released $^{32}P_i$ was extracted and counted in a Beckman scintillation counter as described (32).

Reconstitution of V-pump and V_0 Proton Channel into Proteoliposomes—The bovine brain V-pump and the dissociated V_0 sector were reconstituted into proteoliposomes, which contain phosphatidylcholine (PC), phosphatidylethanolamine (PE), PS, and cholesterol at a weight ratio of 40:26.5:7.5:26, by the cholate dilution, freeze-thaw method, as

described (33). In brief, liposomes (200 μg) were added to 1 μg of V-ATPase or 0.5 μg of V_0 sector and were well mixed. Glycerol, sodium cholate (KCl/NaCl), and MgCl_2 were added to the protein-lipid mixture at final concentrations of 10% (v/v), 1%, 0.15 M, and 2.5 mM, respectively. The reconstitution mixtures were incubated at room temperature for 1 h, frozen in liquid N_2 for 1 min, and then thawed at room temperature. For ATPase and proton-pumping assays, the mixture was directly diluted with 0.2 ml of ATPase assay solution in a test tube or 1.5 ml of the proton-pumping assay buffer in a spectrophotometer cuvette, respectively, which allows for the formation of sealed and ready to assay proteoliposomes. For proton channel assays, the reconstitution mixture was first diluted 60-fold in dilution buffer (150 mM KCl, 20 mM sodium Tricine, pH 7.5, and 3 mM MgCl_2), and centrifuged to precipitate the sealed proteoliposomes. The sealed proteoliposomes were then suspended in a small volume of the dilution buffer. Alternatively, a dialysis step can be substituted for the dilution procedure.

Measurement of ATP-driven Proton Translocation—The assays were conducted in a SLM-Aminco DW2C dual wavelength spectrophotometer and the activity was registered as $\Delta A_{492-540}$. Generally, 5–10 μl of proteoliposomes were added to 1.5 ml of proton-pumping assay buffer containing 20 mM Tricine, pH 7.0, 6.7 μM acridine orange, 3 mM MgCl_2 , and 150 mM KCl. The reaction was initiated by addition of 1.3 mM ATP (pH 7.0), and 1 $\mu\text{g/ml}$ valinomycin, and was terminated by addition of the proton ionophore 1799.

Measurement of Proton Channel Activity—The proton channel activity of V_0 sector was performed as described. In brief, the reconstituted proteoliposomes of V_0 , sealed with 150 mM KCl inside as described above, were activated by incubating with 2 μl of 0.5 M MES (pH 3.4) per 5 μl of the proteoliposomes for 30 min at room temperature prior to assay. The membrane potential-driven proton translocation assay was also conducted in a SLM-Aminco DW2C dual wavelength spectrophotometer, and the activity was registered as $\Delta A_{492-540}$. The assay solution contained 150 mM NaCl, 30 mM Tricine, pH 7.5, 3 mM MgCl_2 , and 6 μM acridine orange. The reaction was initiated by addition of 1 μM valinomycin and finished by addition of 1799.

Glycerol Density Gradient Centrifugation—Glycerol density gradients from 15 to 30% was prepared in a solution containing 0.05% $C_{12}E_9$, 1 mM dithiothreitol, 0.5 mM EDTA, and 20 mM Tris-MES at pH 7.0. Protein samples are loaded onto the top of the gradient in a centrifuge tube for SW60 rotor and centrifuged at 60,000 rpm for 5 h at 4 °C. Fractions were collected by piercing the bottom of the tube and analyzed by SDS-PAGE and scintillation counting.

Distribution of V_1 in Cell Fractions—Sk-Mel-5 cells (from American Type Culture Collection, Manassas, VA) were cultured in MEM medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate in 10-cm dishes and used for experiments at 80–90% confluency. The cells were treated for 1 h with salicylhalamide A, bafilomycin, or ammonium chloride (NH_4Cl) at the indicated concentrations, then were washed twice with PBS, homogenized in sucrose buffer (0.2 M sucrose, 20 mM Hepes pH 7.3) and centrifuged at $960 \times g$ for 15 min. The supernatant was separated from the pellet and centrifuged at $128,000 \times g$ for 1 h. Equal proportions of the resulting high speed pellet (P2) and supernatant (S2), as well as the low speed pellet (P1), were resolved by SDS-PAGE and Western blotted against V_1 subunits A and E with rabbit antiserum or with monoclonal antibody recognizing the lysosomal membrane protein Lamp1. Immunoblots were treated for chemiluminescence and exposed to film. Images of the films were made by laser scanning densitometer (Molecular Dynamics, Sunnyvale CA).

RESULTS

The Inhibition Site of Salicylhalamide A Is Within the V_0 Domain—To determine which V-pump domain contains the site for salicylhalamide inhibition, we first examined the inhibition profile of salicylhalamide on reconstituted V-ATPase. When reconstituted into proteoliposomes, the ATPase activity of V-pump is largely inhibited due to the feedback inhibition by electrochemical potential. This inhibition can be partially lifted by addition of ionophores, which would reflect the portion of V-ATPase activity that is tightly coupled to proton translocation (28). As shown in Table IA, the strong inhibition of salicylhalamide was primarily on the coupled V-ATPase activity, which is more apparent when ionophores were added. We next compared the effect of salicylhalamide A on the ATPase activity of intact V-ATPase, the uncoupled V-ATPase that was prepared by removal of subunit H, and the dissociated V_1 sector,

TABLE I
Inhibition of bovine brain V-ATPase by salicylhalamide

The reconstitution of purified V-ATPase and the measurement of ATPase activity of both reconstituted and soluble V-ATPase preparations were performed as described under "Experimental Procedures." The amounts of protein used per assay were: intact V-ATPase and uncoupled V-ATPase, 0.5 μg ; V_1 preparation, 0.6 μg . The ionophores, valinomycin and 1799, were used at 1 μM , and salicylhalamide A was used at 0.1 μM . The reaction was started by adding the assay solution and was carried out for 15 minutes at 25 $^\circ\text{C}$ or 37 $^\circ\text{C}$ for the reconstituted or soluble enzyme preparations, respectively.

	Valinomycin	1799	Salicylhalamide	ATPase activity
				<i>nmol P_i/assay</i>
A. Reconstituted intact V-ATPase	–	–	–	10.3
	–	–	+	2.1
	+	–	–	12.5
	+	–	+	2.4
	+	+	–	39.2
	+	+	+	3.2
				<i>nmol P_i/assay</i>
Soluble enzyme preparation	Divalent cation	ATPase activity		
		Control	+ Salicylhalamide	
				<i>nmol P_i/assay</i>
B. Intact V-ATPase	Mg ²⁺	96.2		9.8
Uncoupled V-ATPase	Ca ²⁺	17.2		16.6
V_1	Ca ²⁺	26.5		24.2

all in soluble form. As previously reported, the uncoupled V-ATPase preparations, including dissociated V_1 sector and the subunit H-free V_1V_0 ATPase, have a minimal Mg-ATPase activity but a rather high Ca-ATPase activity that is not coupled to proton movement (28, 36, 37). As shown in Table IB nearly 90% of the activity of intact V-ATPase was inhibited by salicylhalamide, whereas the uncoupled V-ATPase and the V_1 ATPase activities were essentially insensitive to salicylhalamide. These results indicate that salicylhalamide inhibits only the coupled V-ATPase and suggest the possibility of an inhibition site within the V_0 domain.

To test this hypothesis and to further characterize the mechanism of salicylhalamide inhibition on V-ATPase, we next examined the effect of this inhibitor on the proton channel activity of the free V_0 . As previously reported, the free V_0 does not have proton channel activity *in vitro* unless it is activated by acidic pH treatment (14). In the proton channel assay, V_0 was reconstituted into proteoliposomes that were loaded with 150 mM KCl and opened by acidic pH treatment, and then assayed in a solution containing 150 mM NaCl instead of KCl, as described under "Experimental Procedures." The outward K^+ gradient generates a membrane potential in the presence of the K^+ ionophore valinomycin (interior negative), driving proton influx if a proton conductance mechanism, either a proton channel or a proton ionophore, is present. As shown in Fig. 1, salicylhalamide A inhibits the proton channel activity in a manner similar to inhibiting the proton pumping activity. These experiments demonstrate that the recognition and inhibition site(s) of salicylhalamide is within the V_0 domain and that the inhibition of mammalian V-ATPase activity by salicylhalamide is caused by blocking of the V_0 proton channel.

Structural Determinants of Salicylhalamide Inhibition—To gain more insight into the mechanism by which salicylhalamide inhibits the V-ATPase, we initiated detailed studies of the relationship of salicylhalamide structure to its function. Structurally, salicylhalamide A is composed of a salicylate-containing benzolactone decorated with a highly unsaturated *N*-acyl enamine side chain (Fig. 2, structure I). Initially, we focused on those modifications that were accessible from late stage synthetic intermediates to reveal suitable chemical linkages for introducing a radioactive or biotinylated probe, and to identify regions that would accommodate the resulting structural changes without compromising biological activity. The synthetic salicylhalamide analogs utilized in this study are shown in Fig. 2, and their ability to inhibit ATP-driven proton translocation of reconsti-

tuted bovine brain V-ATPase is presented in Table II. As can be seen from Table II, the ability of *N*-acyl modified analogs **2–6** to inhibit proton pumping activity at concentrations similar to the parent compound indicates that the hexadienyl fragment is not crucial for inhibitory activity and points to a potential site for introducing a reporter. Unfortunately, introduction of biotin in this region of the molecule (analog **9**) diminished inhibitory activity by 1000-fold. This loss of activity was probably not due to the polar nature of biotin because farnesyloxy and cholesteryloxy derivatives (analog **7** and **8**), designed to function as a potential lipophilic membrane anchor, were also devoid of activity. Also, modification of the C15-alcohol (*e.g.* **13**) or the phenol (*e.g.* **12**) led to a significant drop in inhibitory activity, thereby virtually eliminating the possibility of exploiting the most obvious functional handles to introduce a probe. The seriously compromised potency of derivatives **10**, **15**, and **16** demonstrates the importance of the *N*-acyl enamine functional group. Interestingly, saturation of the enamine (compound **11**) or replacement with an enone (compound **14**) had a less dramatic effect and only moderately affected their ability to inhibit proton-pumping activity.

Salicylhalamide Is a Functionally Irreversible Inhibitor of the V-ATPase—As will be argued in the discussion section, salicylhalamide can potentially form a covalent adduct with the V-ATPase. Since salicylhalamide A is a hydrophobic compound, and dilution or washing with an aqueous solution may not remove it effectively after binding to the V-ATPase, we developed a liposome dilution assay to determine if inhibition was reversible. First we incubated aliquots of the reconstituted proteoliposomes of V-ATPase with salicylhalamides at a concentration that would give a 60–75% inhibition. The inhibitor-treated proteoliposomes were then mixed with "empty" liposomes that contained a 3-fold higher lipid content to reconstitute proteoliposomes, or with buffer alone. Proton pumping was assayed after a 10-min incubation.

Typical traces of the reversibility assay are shown in Fig. 3. Dilution with liposomes had no detectable effect on the proton-pumping assay in the control setting where no inhibitor was added (*panel A*), did not alter the inhibition by the parent compound salicylhalamide A (*panel B*), but reversed the inhibition by analog **11** substantially (*panel C*). These results demonstrate that, under these experimental conditions, the inhibition of V-pump activity by analog **11** is reversible whereas that by the parental salicylhalamide A is not. To determine the structural characteristics required for irreversible inhibition, representative salicylhalamide derivatives and the classic V-ATPase inhib-

FIG. 1. Inhibition of salicylhalamide A on the proton-pumping activity of bovine brain V-ATPase and the proton channel activity of its V_0 sector. 2 pmol of purified bovine brain V-ATPase (*panel A*) or its isolated V_0 domain (*panel B*), was reconstituted into proteoliposomes and their respective activity measured as described under "Experimental Procedures." Proton-pumping activity was measured in the presence of valinomycin at 1 μ M, and the reaction was initiated by addition of ATP. Proton channel activity was initiated by addition of valinomycin. Both assays were conducted with or without addition of salicylhalamide A, at designated final concentration, or Me₂SO alone as a control. *Trace 1*, 10 nM; *trace 2*, 1 nM; *trace 3*, no inhibitor. 1799 was added at the end of both assays to ensure the proteoliposomes were sealed.

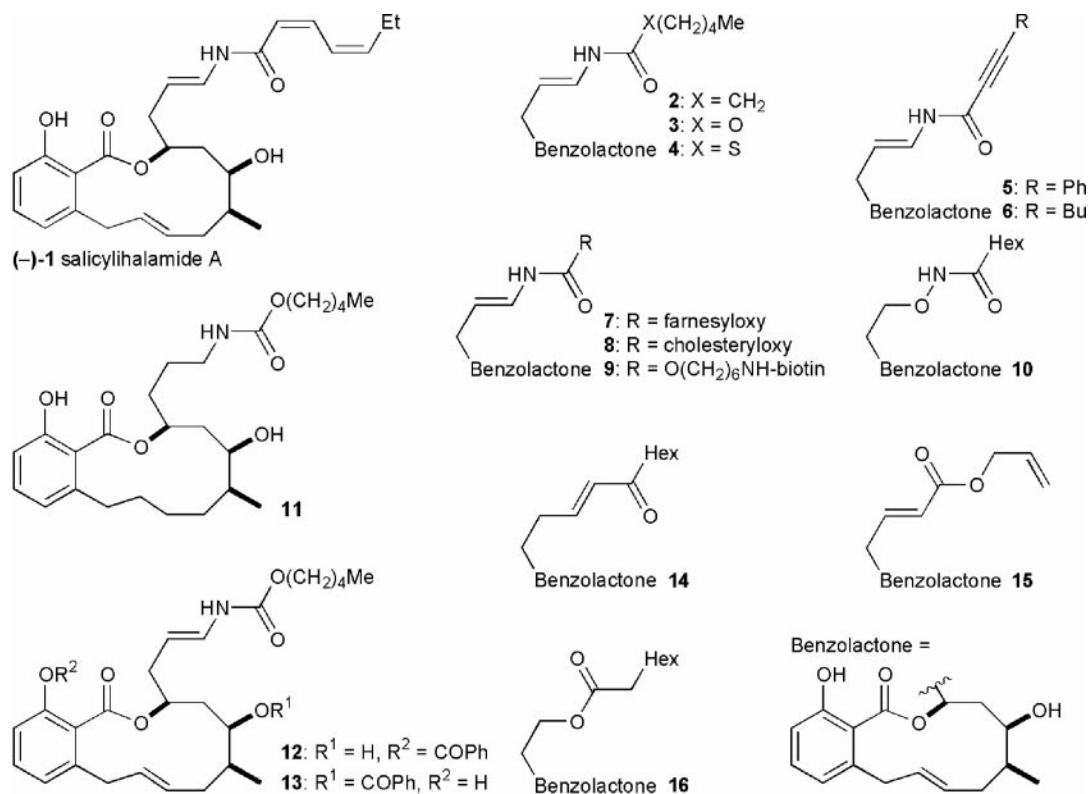
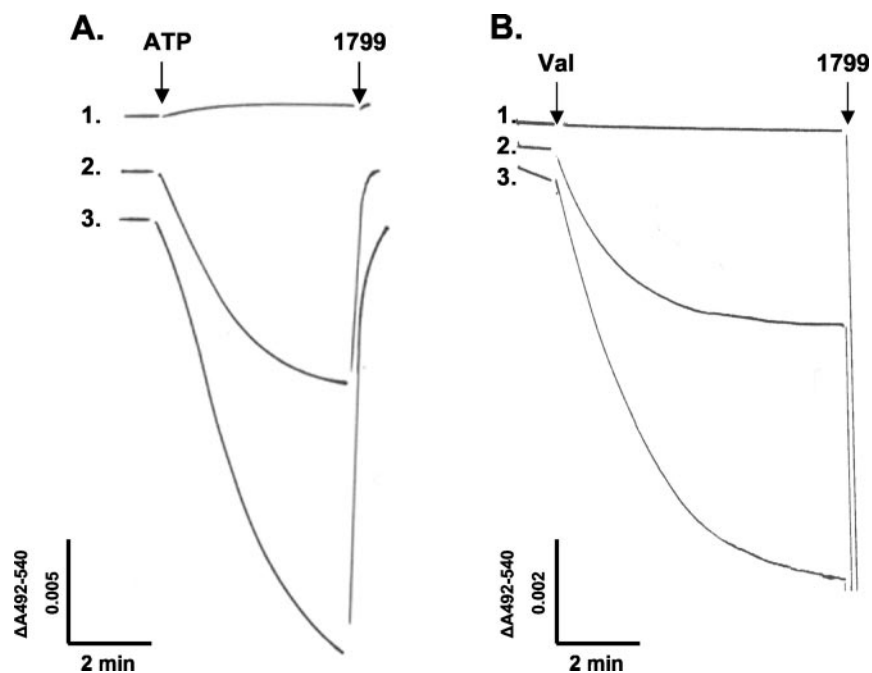


FIG. 2. The structure of salicylhalamide A and a series of salicylhalamide analogs used in this study.

itor bafilomycin were tested in the liposome dilution assay and compared in Fig. 3, *panel D*. Whereas inhibition by bafilomycin was reversible under these assay conditions, proton-pumping activity could not be recovered for those tested derivatives that retain the *N*-acyl enamine functionality (compounds **1–6** and **12**). In contrast, the inhibition by derivatives **11** and **14** was reversed significantly by liposome dilution, further revealing the functional importance of the *N*-acyl enamine.

Attempts to Map the Binding Site of Salicylhalamide—We next sought to identify the V-pump subunit(s) that binds to salicylhalamide, taking advantage of the irreversible inhibition

by certain salicylhalamide derivatives. Based on the above described structure-function data, and taking into account issues related to ease of synthesis, we selected salicylhalamide derivative **3** as a candidate to introduce radioactivity. Introduction of a tritiated pentaenoxy side-chain during the final step of the synthesis provided a tritium labeled version of compound **3** with a specific activity of 14 Ci/mmol. Surprisingly, when purified bovine brain V-ATPase was incubated with this radioactive derivative, we failed to detect any specific binding by SDS-PAGE and autoradiography (data not shown). A possible interpretation of these results was that the binding of this inhibitor, although

TABLE II

The effect of the structural alteration of salicylhalamide on the inhibition potency and reversibility of proton-pumping activity of V-ATPase

The reconstitution of purified bovine brain V-ATPase and the proton-pumping assay were performed as described under "Experimental Procedures." The structures of salicylhalamide and analogs, labeled as **1** through **16**, are shown in Fig. 2. IC₅₀ was obtained by a titration of each compound. The inhibition reversibility for selected compounds was determined by the liposome-dilution assay as described in the legend to Fig. 3.

Compound	IC ₅₀	Inhibition mode	Compound	IC ₅₀	Inhibition mode
	<i>nM</i>			<i>nM</i>	
1	<1	Irreversible	9	>1000	Not assayed
2	<1	Irreversible	10	>5000	Reversible
3	1.6	Irreversible	11	75	Reversible
4	1.8	Not assayed	12	130	Irreversible
5	<1	Irreversible	13	180	Not assayed
6	1	Not assayed	14	7.5	Reversible
7	>2500	Not assayed	15	230	Not assayed
8	1800	Not assayed	16	>1000	Not assayed

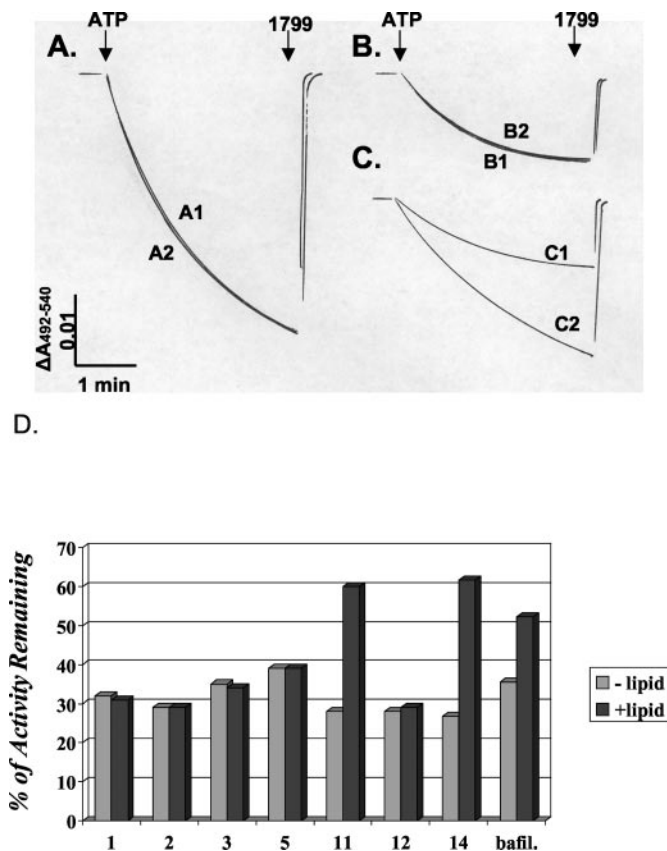


FIG. 3. Inhibition reversibility of salicylhalamide derivatives. The experiments were conducted as outlined in the text, and the proton-pumping assay was performed as described in the legend to Fig. 1. Panels A, B, and C show the acidification tracings of bovine brain V-ATPase proteoliposomes, either without inhibitor (panel A) or treated with salicylhalamide A (panel B) or analog **11** (panel C), respectively. Traces A1, B1, and C1 are controls that were diluted with buffer only, and traces A2, B2, and C2 were diluted with empty liposomes. Panel D tabulates and compares the reversibility of inhibition for representative salicylhalamide derivatives.

irreversible under non-denaturing conditions, may not be stable when the protein is denatured by SDS, or other denaturants such as urea and guanidine chloride.

To further address this issue, we used a non-denaturing condition to separate the V-ATPase-salicylhalamide complex from the free inhibitor. In this experiment, 220 μ g of purified V-ATPase in a volume of 250 μ l for each sample was incubated without any inhibitor (control) or with either the tritium-labeled irreversible salicylhalamide derivative **3** (4400 cpm/pmol) or the reversible derivative **14** at a molar ratio of 4:1 and 25:1 (inhibitor/protein), respectively, for 10 min, which resulted in complete inhibition of the proton pumping activity of both

samples. The control and the samples with inhibitor were subjected to glycerol density gradient centrifugation for 5 h as described under "Experimental Procedures." Fractions were collected from the bottom, and aliquots of each fraction were analyzed by SDS-PAGE (to identify the position and structural integrity of the enzyme), by scintillation counting (to quantitate the bound radioactivity), and by proton-pumping assay after reconstitution into proteoliposomes. As shown in Fig. 4A, only 4.5% of the radioactivity came down with the V-ATPase, which translates to 18% of V-ATPase molecules remaining labeled by radioactive compound **3** after the 5 h centrifugation. Comparing to the control, the proton pumping activity of V-ATPase treated with **3** recovered from 0 to 44%, whereas that of the sample treated with reversible derivative **14** recovered to 96% (Fig. 4B). In light of this result, the irreversible inhibition by analog **3** has to be defined as under the conditions used in the liposome-dilution assay, which differs from the glycerol gradient experiment in several aspects. To confirm that the radioactivity associated to the V-ATPase peak in the glycerol gradient is specific, the experiment was repeated with an 8-fold excess of unlabeled inhibitor. A proportional decrease of the radioactivity in the glycerol gradient fraction that contains V-ATPase was observed with the distribution pattern otherwise identical to that of using lower concentration of the unlabeled inhibitor (data not shown).

These results again demonstrated the difference in inhibition reversibility between salicylhalamide derivatives but also indicated that the salicylhalamide derivative **3**/V-ATPase complex is fragile, at least under the non-denaturing conditions employed for the glycerol density gradient centrifugation. Furthermore, since the 18% of V-ATPase that was radiolabeled does not correspond to the observed 56% inhibition of proton pumping activity, it becomes apparent that the binding and inhibition mechanism of salicylhalamide is more complicated than we originally thought. In the discussion section, we will postulate a hypothesis based on the chemistry of *N*-acyl enamines that is consistent with our observations.

Salicylhalamide and Bafilomycin Differ in Their Effects on the Distribution of the V₁ Sector in Cells—Yeast cells maintain an equilibrium between assembled and disassembled V₁ and V₀ sectors and assembly of the active pump is regulated in both yeast and insect cells (38). Furthermore, a mutant yeast V-ATPase that can assemble but cannot hydrolyze ATP or pump protons fails to disassemble (19). Here we demonstrate that, like yeast cells, SK-Mel-5 cells maintain membrane-bound and cytosolic pools of V₁ sectors (Fig. 5). To investigate the effect of blocking proton translocation and ATP hydrolysis by V-ATPase in mammalian cells, SK-Mel-5 cells were treated for an hour with salicylhalamide A, salicylhalamide analog **14**, or bafilomycin A, and the cells were fractionated into a low speed pellet (P1), a cytosolic supernatant (S2), and high speed pellet (P2). Equal amounts of these fractions were analyzed by PAGE and

A.

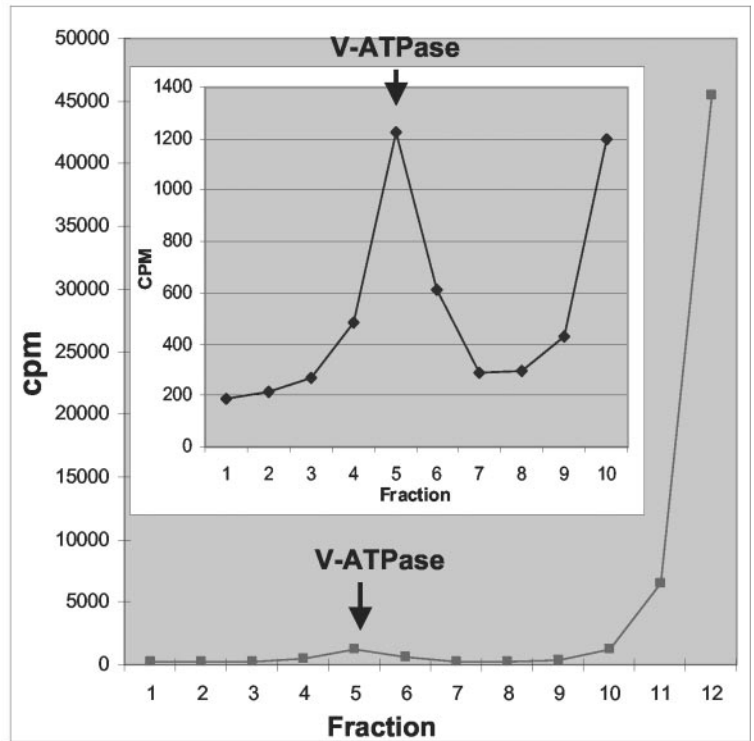
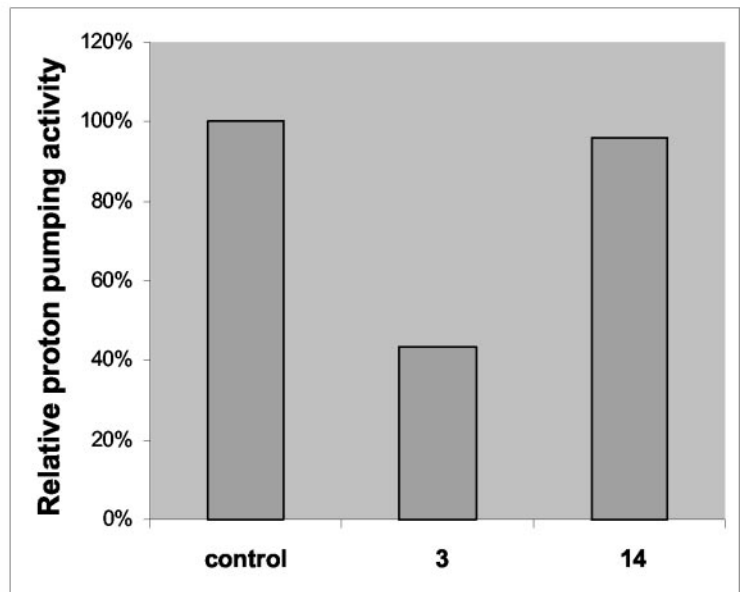


FIG. 4. Analysis of the binding of salicylhalamide analogs to V-ATPase by glycerol density gradient centrifugation. The experiment was performed as outlined in the text and all assays were conducted as described under "Experimental Procedures."

B.



probed with antibodies to the V_1 subunits A and E, or to the lysosomal membrane protein Lamp1. As a control for effects resulting from the collapse of the intracellular pH gradient independent from V-ATPase inhibition, cells were treated with concentrations of ammonium chloride that raise lysosomal pH to ~ 7.0 . Under all conditions of this experiment, the majority of Lamp1 was detected in the membrane P2 fraction, indicating the location of lysosomes. Collapsing intracellular pH gradient

by ammonium chloride had no effect on the distribution of the V_1 sector between cytosolic S2 or membrane P2 fractions. In cells treated with salicylhalamide A, however, the majority of the E (Fig. 5) and A (not shown) proteins were found in the P2 fraction, indicating that more V_1 sector was associated with membranes when the cells were treated with this compound. In contrast, cells treated with identical concentrations of bafilomycin A, which has an equivalent potency for inhibiting V-

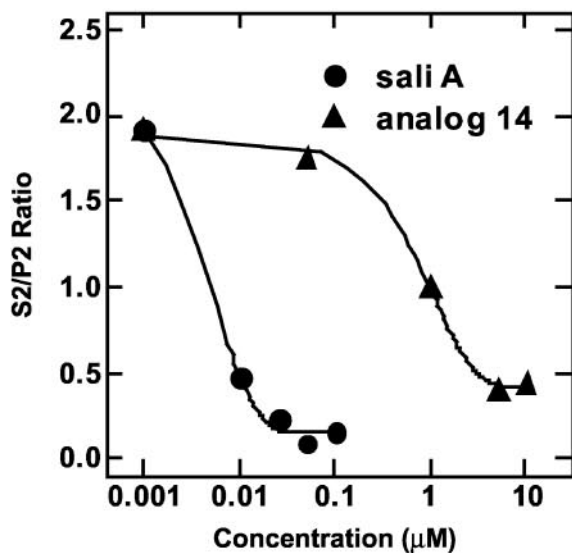
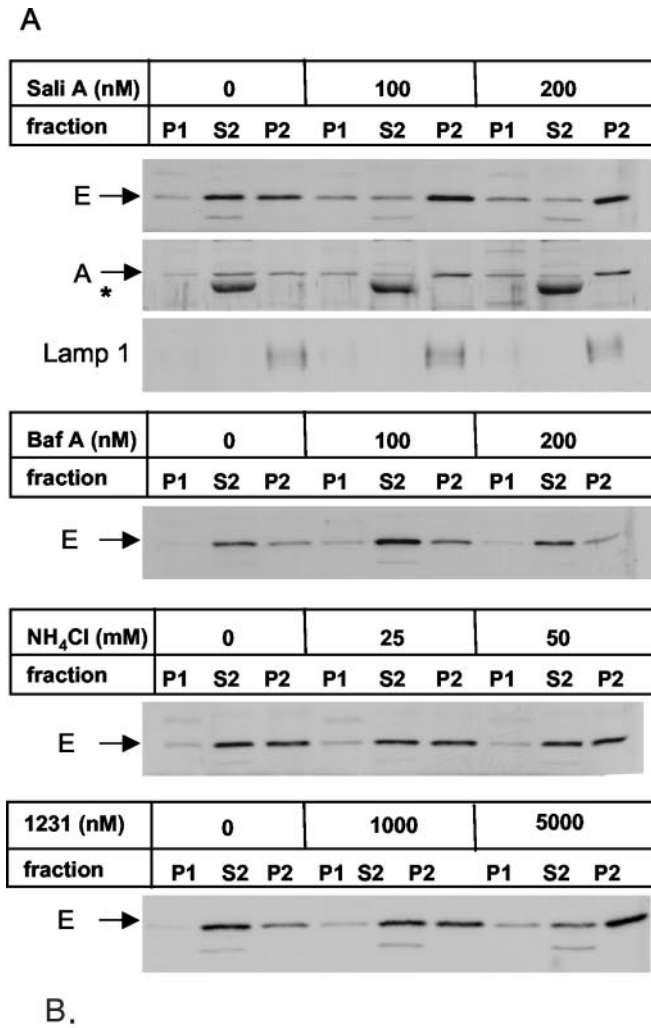


FIG. 5. Salicylhalamide, but not analog 14, bafilomycin or ammonium chloride, recruits V1 subunits A and E to a lysosome-containing membrane fraction. A, SK-MEL-5 cells treated for 1 h with salicylhalamide A (*Sali A*), bafilomycin A (*Baf A*), ammonium chloride (*NH₄Cl*), or analog 14 at the indicated concentrations were homogenized and separated by differential centrifugation into a low speed pellet, a high speed supernatant (cytosol), and a high speed pellet (microsomes) fractions. Equal proportions of the resulting high speed

ATPase in SK-Mel-5 cells, did not show the shift of these V₁ subunit proteins from the cytosolic to the membranous fractions. Interestingly, the effect of the reversible inhibitor compound 14 on the distribution of V₁ subunit proteins in SK-MEL-5 cells at the lower dose was more similar to the effect seen by the reversible inhibitor bafilomycin, but did induce a significant cytosolic V₁ shift at the higher dose, more similar to the irreversible inhibitor salicylhalamide A. The dose-response of Sali A for redistributing V₁ subunit E (Fig. 5B) further demonstrated that the concentration of Sali A required for this effect *in vivo* correlated well with the concentration required to inhibit the ATPase *in vitro*. On the other hand, the much higher concentrations of analog 14 than SaliA required for this effect (~100-fold) cannot be explained solely by the reduced potency of this analog *versus* salicylhalamide A (~10-fold) but is likely also contributed by its highly reversible inhibition of the V-ATPase *in vitro*.

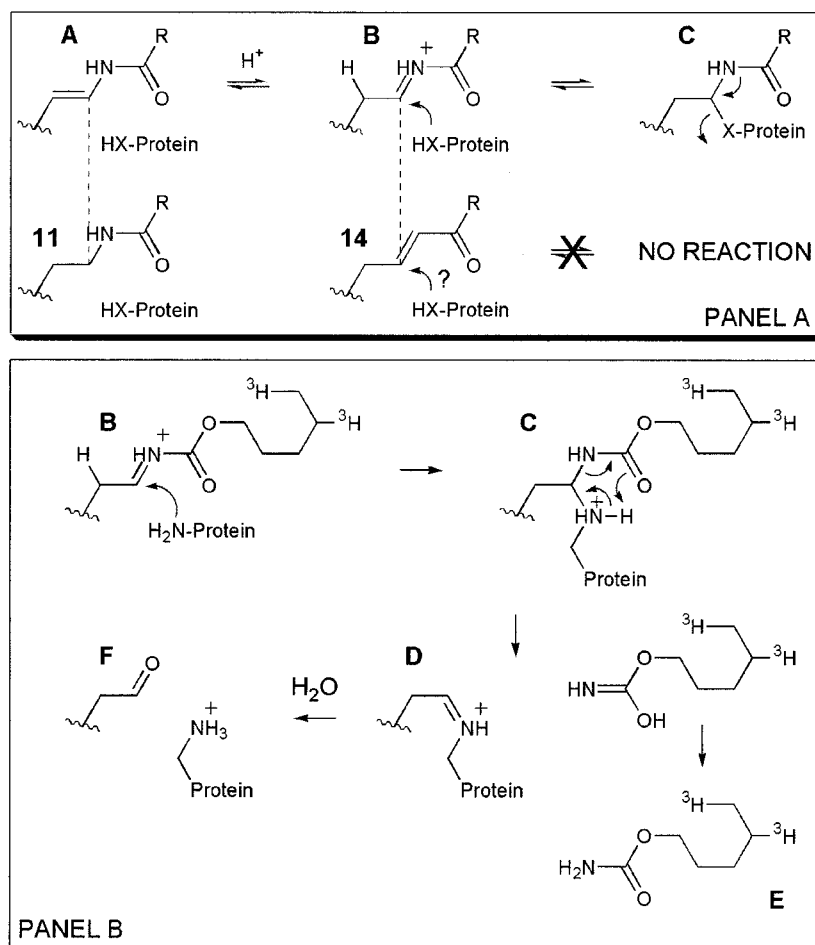
DISCUSSION

Although the plecomacrolides bafilomycin and concanamycin have emerged as powerful selective pharmacological inhibitors of V-type ATPases, they do not discriminate between V-ATPases of different origins. In 2001, Boyd *et al.* (18) reported that the marine-derived metabolite salicylhalamide A and related benzolactone enamides inhibit V-ATPase activity in membrane preparations of mammalian cells, but not the V-ATPases from yeast and other fungi; an observation that distinguishes them from any previously identified V-ATPase inhibitors. In addition, our study demonstrates that salicylhalamide, but not bafilomycin, induces a dramatic redistribution of a cytosolic pool of V₁-domains in mammalian cells (Fig. 5). Understanding the molecular basis for this differential pharmacological behavior constitutes a fundamental step toward developing inhibitors that also could discriminate between tissue and organelle specific isoforms of V-ATPase. This would be advantageous for exploiting V-ATPase as a therapeutic target for many diseases, including osteoporosis and cancer.

There is significant diversity in the primary structure of mammalian and yeast V-ATPase subunits, any of which may provide a structural basis for the discriminating action of salicylhalamide. For example, the protein sequence identity/similarity between yeast and human in V₀ subunits a, d, c', and c are only 40%/55%, 44%/63%, 55%/70%, and 71%/85%, respectively. In addition, the yeast V₀ subunit c' has not been found in mammals and the mammalian V₀ subunit Ac45 is not present in yeast. It may not be a coincidence that bafilomycin, which inhibits all pumps, binds to the most conserved c subunit (17). Therefore, it is possible that salicylhalamide binds to one of the other subunits. By using a reconstituted purified bovine brain V-ATPase, we have shown that salicylhalamide inhibits the proton pumping and coupled ATPase activities of the enzyme through interaction with the V₀ domain, the proton channel (Fig. 1). These results further confirmed that the mammalian V-pump is a direct target of salicylhalamide. While the V₀ sector is also the domain targeted by bafilomycin, it has been reported that salicylhalamide does not compete for the same

pellet (P2) and supernatant (S2), as well as the low speed pellet (P1), were resolved by SDS-PAGE and immunoblotted for the V₁ subunit E and the lysosomal membrane protein Lamp 1. The Lamp 1 blot indicates that the majority of lysosomes fractionated in P2. In the absence of any drug, the E subunit is mainly divided between cytosol and microsomes. Treatment with Sali A, but not with Baf A, NH₄Cl, or analog 14 (at lower dose), causes redistribution of the E subunit from the cytosol to microsomal fractions. B, Western blots of the dose-dependent effect of Sali A and analog 14 on E subunit redistribution were quantified by densitometry and plotted as S2/P2 ratios. The figures shown are representative of three experiments.

FIG. 6. Delineation of postulated mechanisms for the inhibition reversibility of salicylilhalamide analogs.



binding site in the tobacco hornworm *Manduca sexta* V-ATPase (17).

To map more specifically the site(s) within the V_0 domain to which salicylilhalamide binds, we needed a functionally irreversible salicylilhalamide-based reporter construct (radioactive or biotinylated). We had speculated that salicylilhalamide could form a covalent complex with the V-ATPase, which would eliminate the need to introduce a photoactivatable functional group. Concurrent with this hypothesis, inhibition of V-ATPase by salicylilhalamide could not be reversed in our liposome-dilution assay, whereas inhibition by bafilomycin was reversible under the same assay conditions (Fig. 3). Adduct formation via protein thiol addition to the α,β -unsaturated carbonyl functional group in the hexadienoyl side chain of salicylilhalamide was ruled out based on the observation that derivatives **2** and **3** lack this functionality but retain the irreversible inhibition characteristics. Instead, we anticipated a specific role for the *N*-acyl enamine functional group. It is known that electrophilic *N*-acyliminium ions can be generated from *N*-acyl enamines under acidic conditions (30). As delineated in Fig. 6A, this suggested a mechanism for the irreversible inhibition of the V-ATPase by compounds **1–3**, **5**, and **12** via *N*-acyliminium ion formation within the confines of the binding site (A \rightarrow B), followed by capture with a nucleophilic amino acid side chain (B \rightarrow C) (24). Consistent with this conjecture, V-ATPase inhibition with compound **11** ($IC_{50} = 75$ nM), a reduced version of irreversible inhibitor **3** and incapable of covalent bond formation (Fig. 6A), was readily reversed by liposome dilution. To further probe the mechanism of inhibition, α,β -unsaturated enone **14** was evaluated as a potential Michael-acceptor mimic of the *N*-acyliminium ion **B** (Fig. 6A). However, the reversible

inhibition profile with this analog ($IC_{50} = 7.5$ nM) indicated that a cysteine residue was probably not the putative active site nucleophile.

The question remains as to why we have not been able to identify or characterize a covalent adduct so far. Our results with a tritium-labeled variant of irreversible inhibitor **3** provided some clues. Our failure to detect any specific binding with this radioactive derivative by SDS-PAGE and autoradiography could be rationalized by assuming a rupture of the small molecule-protein adduct (*i.e.* the reverse of adduct formation, C \rightarrow B in Fig. 6A) under denaturing conditions. It is known that, whereas thiol adducts (X = S; Cys) are stable, carboxylate (X = O_2C ; Asp, Glu) and alkoxy (X = O; Thr, Ser, Tyr) adducts can generate *N*-acyliminium ions by fragmentation under neutral or acidic conditions (30). Expecting that adduct C would be more stable within the confines of the folded protein, we purified the putative covalent complex under non-denaturing conditions (glycerol density gradient centrifugation). Under these conditions, we did detect incorporation of radioactivity into the V-ATPase complex, but only to the extent of 18% (Fig. 4A). This partial labeling probably did not solely result from a reversible release of the radioactive inhibitor since proton-pumping activity was inhibited by 56% (Fig. 4B). Although we can not rule out the possibility that the reversible binding (covalent or not) of derivative **3**, and/or the glycerol density gradient centrifugation conditions, could have caused an irreversible conformational change that affects proton pumping activity, we deemed this unlikely because proton pumping activity could be fully recovered after centrifugation of both the non-treated control and the non-covalent inhibitor **14**/V-ATPase complex (Fig. 4B). All the available experimental data can be reconciled with the

covalent modification mechanism, if one considers capture of the transient *N*-acyliminium ion by an active site lysine residue (B → C, Fig. 6B). The corresponding *N*-acyl aminal adduct C is poised to fragment into a covalent protein/small molecule imine complex D with release of the amide side chain E that coincidentally contained the tritium reporter (Fig. 6B). As such, the fraction of proton pumping activity that was inhibited after the glycerol density gradient centrifugation (56%) can be accounted for by covalent radiolabeled *N*-acylaminal complex C (18%) and covalent non-labeled imine complex D (38%). The mechanism of non-proteinogenic *N*-acylaminal breakdown has been studied in detail by Loudon *et al.* (26) and a half-life for the non-enzymatically catalyzed reaction at neutral pH can be estimated to be below 5–6 h. The recovered enzymatically active fraction (44%) would be the result of a further hydrolysis of the imine complex D to the non-modified protein and a small molecule aldehyde F. The aqueous conditions employed during the glycerol density gradient centrifugation conditions could promote this hydrolysis, whereas the imine complex formed within the V-ATPase proteoliposomes is shielded from water. This notion is in agreement with our observation that proton-pumping activity could not be recovered in the liposome-dilution assay and partially recovered (44%) with the glycerol density gradient centrifugation assay. Our current efforts are directed toward characterizing the putative imine complex D.

Although at this moment, the detailed mechanistic aspects underlying this characteristic salicylhalamide inhibition mode remain to be determined, the possibility to control the mode of inhibition with functionally reversible and irreversible salicylhalamide derivatives provides special benefits with potential biochemical/cell biological implications downstream of the inhibition of the V-pump. In this context, the observation that salicylhalamide induced cytosolic V₁ to bind to membranes is very interesting. First, it is possible that the shift of soluble V₁ to a membrane-associated form represents the dynamic process of the association of V₁ sector to the membranous V₀ sector. It is known that a mild acidic pH facilitates the assembly of V-pump from separated V₁ and V₀ sectors *in vitro*, which is the essential condition in our reassembly protocol (37) and has been demonstrated *in vivo* in the yeast system as well (31). However, it is unlikely that the observed re-distribution of V₁ in cells treated with salicylhalamide is a consequence of a cytosolic acidification resulting from impaired proton transport by the pump, because this effect was not observed when the cells were treated with the V-ATPase inhibitor bafilomycin or NH₄Cl, which collapses all intracellular proton gradients. Therefore the *in vivo* V₁ re-distribution that was induced by salicylhalamide cannot be explained by a simple pH effect alone. Although the salicylhalamide effect *in vivo* is consistent with previous observation that inhibition of V-ATPase activity in yeast through mutagenesis blocks dissociation (19, 34), the correlation between the activity of V-ATPase and its reversible dissociation has not been clearly established. Mutagenesis studies with subunit D showed that a mutant with only 6% of wild-type level of proton pumping activity still demonstrated normal dissociation in response to glucose deprivation (35), whereas some mutations in subunit A resulted in a partial or complete blocking of dissociation despite possessing more than 30% of wild-type levels of activity (25). In this context, our observation that reversible salicylhalamide analog **14** at the lower dose did not induce the cellular V₁ redistribution response reminiscent of salicylhalamide A, but behaved more

like bafilomycin, suggests that the unique response with salicylhalamide might be related to its unique inhibition characteristics. An intriguing possibility is that the irreversible inhibitor salicylhalamide structurally stabilizes the holoenzyme complex (*i.e.* prevents the disassembly of the V₁ and V₀ subunits), perhaps allosterically or through formation of a covalent bond to the V₁ sector.

The implication of our observations is important as it demonstrates that, despite binding to the same V₀ sector, the differences in inhibition characteristics between salicylhalamide and the prototypical V-ATPase inhibitor bafilomycin has a functional consequence in cells. As such, it endows salicylhalamide with unique characteristics that should promote its use as an important pharmacological tool to enhance our understanding of mammalian V-ATPase function and regulation.

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