Salicylihalamide A Inhibits the \( V_0 \) Sector of the V-ATPase through a Mechanism Distinct from Bafilomycin A1*

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The newly identified specific V-ATPase inhibitor, salicylihalamide 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, salicylihalamide 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., Drose, S., Zeec, A., Altendorf, K., and Wieczorek, 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 salicylihalamide to be within the \( V_0 \) domain. Salicylihalamide 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. Salicylihalamide 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 NH4Cl.

By synthesizing and characterizing a series of salicylihalamide derivatives, we investigated the structural determinants of salicylihalamide 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-ATPases\(^1\) 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).

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 \( V_0 \) sectors, 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 \( A_2 \), 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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§ The abbreviations used are: V-ATPases (V-pump), the vacuolar \( H^+ \)-translocating ATPases; \( V_1 \), the peripheral, catalytic sector of V-pumps; \( V_0 \), the membranous proton channel domain of V-pumps; 1799, bis-(hexafluoroacetonyle)lacetone; MES, 2-\((\text{-morpholino})\text{ethanesulfonic acid; Tricine, }N\text{-}[2\text{-hydroxy-1,1-bis(hydroxymethyl)ethyl}]\text{l}y\text{cine.}

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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.

Salicylihalamide A, originally identified as an anti-tumor agent, has recently been shown to be a specific inhibitor for V-ATPases (18). Salicylihalamide A is as potent a V-ATPase inhibitor as bafilomycin but does not compete with concanamycin or bafilomycin for binding to the V0 sector (17), suggesting that this inhibitor has a different binding site. More importantly, salicylihalamide 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 salicylihalamide 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 salicylihalamide A inhibition and that both ATPase and proton pumping activities are inhibited by salicylihalamide A at IC50 below 1 mM (21). Understanding why salicylihalamide 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 V0 is the salicylihalamide binding and inhibited domain of the mammalian V-pump (2) the structural determinants of the potency and reversibility of salicylihalamide inhibition, and (3) that in cells salicylihalamide changes the distribution of the V1 sector from mostly soluble to mostly membrane-bound.

**EXPERIMENTAL PROCEDURES**

Materials—Acridine orange was obtained from Eastman, [γ-32P]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 Lambda 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 salicylihalamide 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 μmol of P2 + mg -1 x min -1, as described (27). The uncoupled V-ATPase (subunit H-free V-ATPase preparation) (28), dissociated V1 (29) and free V0 (14) domains were prepared by separate procedures as described.

**Measurement of ATP-driven Proton Translocation**—The assays were conducted in a SLM-Aminco DW2C dual wavelength spectrophotometer and the activity was registered as ΔA250-450. 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 MgCl2, and 150 mM KCl. The reaction was initiated by addition of 1.3 mM ATP (pH 7.0), and 1 μg/ml valinomycin, and was terminated by addition of the proton ionophore 1799.

**Measurement of Proton Channel Activity**—The proton channel activity of the V0 sector was performed as described. In brief, the proteoliposomes of V0 being sealed with 150 mM KCl inside as described above, were activated by incubating with 2 μl of 0.5 mM 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 ΔA250-450. The assay solution contained 150 mM NaCl, 30 mM Tricine, pH 7.5, 3 mM MgCl2, 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% were prepared in a solution containing 0.5% C12E6, 1 mM Na2-β-glycerophosphate, 0.5 mM ATP, 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 V1 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 salicylihalamide A, bafilomycin, or ammonium chloride (NH4Cl) at the indicated concentrations, then were washed twice with PBS, homogenized in sucrose buffer (0.2 M sucrose, 20 mM Hepes pH 7.5, 1 mM EDTA, and centrifuged at 960 × g for 15 min. The supernatant was separated from the pellet and centrifuged at 128,000 × 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 V1 subunits A and E with rabbit antisera 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 Salicylihalamide A Is Within the V0 Domain**—To determine which V-pump domain contains the site for salicylihalamide inhibition, we first examined the inhibition profile of salicylihalamide 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 salicylihalamide was primarily on the coupled V-ATPase activity, which is more apparent when ionophores were added. We next compared the effect of salicylihalamide A on the ATPase activity of intact V-ATPase, the uncoupled V-ATPase that was prepared by removal of subunit H, and the dissociated V1 sector,
all in soluble form. As previously reported, the uncoupled V-ATPase preparations, including dissociated V$_1$ sector and the subunit H-free V$_0$ V$_0$ ATPase, have a minimal Mg$^{2+}$-ATPase activity but a rather high Ca$^{2+}$-ATPase activity that is not coupled to proton movement (28, 36, 37). As shown in Table I B nearly 90% of the activity of intact V-ATPase was inhibited by salicylihalamide, whereas the uncoupled V-ATPase and the V$_1$ ATPase activities were essentially insensitive to salicylihalamide. These results indicate that salicylihalamide 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 salicylihalamide 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 ionophore, is present. As shown in Fig. 1, salicylihalamide inhibits the proton channel activity in 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.

**Structural Determinants of Salicylihalamide Inhibition**—To gain more insight into the mechanism by which salicylihalamide inhibits the V-ATPase, we initiated detailed studies of the relationship of salicylihalamide structure to its function. Structurally, salicylihalamide 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 salicylihalamide analogs utilized in this study are shown in Fig. 2, and their ability to inhibit ATP-driven proton translocation of reconstituted 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 functionalities 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.

**Salicylihalamide Is a Functionally Irreversible Inhibitor of the V-ATPase**—As will be argued in the discussion section, salicylihalamide can potentially form a covalent adduct with the V-ATPase. Since salicylihalamide 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 salicylihalamides 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 salicylihalamide 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 salicylihalamide A is not. To determine the structural characteristics required for irreversible inhibition, representative salicylihalamide derivatives and the classic V-ATPase inhib-
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 Salicylihalamide—We next sought to identify the V-pump subunit(s) that binds to salicylihalamide, taking advantage of the irreversible inhibition by certain salicylihalamide derivatives. Based on the above described structure-function data, and taking into account issues related to ease of synthesis, we selected salicylihalamide derivative 3 as a candidate to introduce radioactivity. Introduction of a tritiated pentyloxy 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

![Diagram of salicylihalamide A and analogs](image-url)
The effect of the structural alteration of salicylihalamide 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 salicylihalamide and analogs, labeled as 1 through 16, are shown in Fig. 2. IC_{50} 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 |
|----------|---------|-----------------|----------|---------|-----------------|
| 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        | 14      | 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 salicylihalamide 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 salicylihalamide 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 salicylihalamide 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-salicylihalamide complex from the free inhibitor. In this experiment, 220 μg of purified V-ATPase in a volume of 250 μl for each sample was incubated without any inhibitor (control) or with either the tritium-la

beled irreversible salicylihalamide 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 quantify 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 salicylihalamide derivatives but also indicated that the salicylihalamide derivative 8/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 salicylihalamide 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.

Salicylihalamide and Bafilomycin Differ in Their Effects on the Distribution of the V_{1} Sector in Cells—Yeast cells maintain an equilibrium between assembled and disassembled V_{1} and V_{0} 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 V_{1} 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 salicylihalamide A, salicylihalamide 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...
probed with antibodies to the V₁ 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 V₁ sector between cytosolic S2 or membrane P2 fractions. In cells treated with salicylihalamide A, however, the majority of the E (Fig. 5) and A (not shown) proteins were found in the P2 fraction, indicating that more V₁ 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-
ATPase in SK-Mel-5 cells, did not show the shift of these $V_1$ subunit proteins from the cytosolic to the membranous fractions. Interestingly, the effect of the reversible inhibitor compound 14 on the distribution of $V_1$ 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 $V_1$ shift at the higher dose, more similar to the irreversible inhibitor salicylihalamide A. The dose-response of Sali A for redistributing $V_1$ 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 Sali A required for this effect (~100-fold) cannot be explained solely by the reduced potency of this analog versus salicylihalamide 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 salicylihalamide 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 salicylihalamide, but not bafilomycin, induces a dramatic redistribution of a cytosolic pool of $V_1$-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 salicylihalamide. For example, the protein sequence identity/similarity between yeast and human in $V_0$ subunits $a$, $d$, $c'$, and $c$ are only 40%/55%, 44%/63%, 55%/70%, and 71%/85%, respectively. In addition, the yeast $V_0$ subunit $c'$ has not been found in mammals and the mammalian $V_0$ 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 salicylihalamide binds to one of the other subunits. By using a reconstituted purified bovine brain V-ATPase, we have shown that salicylihalamide inhibits the proton pumping and coupled ATPase activities of the enzyme through interaction with the $V_0$ domain, the proton channel (Fig. 1). These results further confirmed that the mammalian V-pump is a direct target of salicylihalamide. While the $V_0$ sector is also the domain targeted by bafilomycin, it has been reported that salicylihalamide does not compete for the same

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**FIG. 5.** Salicylihalamide, but not analog 14, bafilomycin or ammonium chloride, recruits $V_1$ subunits A and E to a lysosome-containing membrane fraction. A, SK-MEL-5 cells treated for 1 h with salicylihalamide A (Sali A), bafilomycin A (Baf A), ammonium chloride ($NH_4Cl$), 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 pellet (P2) and supernatant (S2), as well as the low speed pellet (P1), were resolved by SDS-PAGE and immunoblotted for the $V_1$ 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.
binding site in the tobacco hornworm *Manduca sexta* V-ATPase (17).

To map more specifically the site(s) within the V₀ domain to which salicylihalamide binds, we needed a functionally irreversible salicylihalamide-based reporter construct (radioactive or biotinylated). We had speculated that salicylihalamide 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 salicylihalamide 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 ω,ω-unsaturated carbonyl functional group in the hexadienoyl side chain of salicylihalamide 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. 6, 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 → B), followed by capture with a nucleophilic amino acid side chain (B → C) (24). Consistent with this conjecture, V-ATPase inhibition with compound 11 (IC₅₀ = 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, a,ω-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 (IC₅₀ = 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 → B in Fig. 6A) under denaturing conditions. It is known that, whereas thiol adducts (X = S; Cys) are stable, carboxylate (X = O₂C; 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 cannot 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 \((B \rightarrow C, \text{Fig. 6B})\). The corresponding N-acyl aminial 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-acylaminial complex \(C\) (18%) and covalent non-labeled imine complex \(D\) (38%). The mechanism of non-proteinogenic N-acylaminial 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 is known that a mild acidic pH facilitates the assembly of the V-pump from separated V1 and V0 sectors. However, it is unlikely that the observed re-distribution of V1 complete blocking of dissociation despite possessing more than normal dissociation in response to glucose deprivation (35), with previous observation that inhibition of V-ATPase activity been demonstrated alone. Although the salicylihalamide effect reminiscent of salicylihalamide A, but behaved more like bafilomycin, suggests that the unique response with salicylihalamide might be related to its unique inhibition characteristics. An intriguing possibility is that the irreversible inhibitor salicylihalamide 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 V1 sector, the differences in inhibition characteristics between salicylihalamide and the prototypical V-ATPase inhibitor bafilomycin has a functional consequence in cells. As such, it endows salicylihalamide 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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Salicylihalamide A Inhibits the $V_0$ Sector of the V-ATPase through a Mechanism Distinct from Bafilomycin A₁

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