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V-ATPases as Drug Targets

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V-ATPases are large, complex enzymes responsible for acidification of many internal compartments in eukaryotic cells. They also occur on plasma membranes of specialized cells, where they acidify the surrounding milieu. Numerous physiological processes depend on the activity of V-ATPases, and V-ATPases are implicated as a contributing factor in multiple diseases, including osteoporosis, deafness, and cancer. Three classes of natural products have been identified as potent inhibitors of V-ATPases. The bafilomycins and concanamycins, which inhibit all known eukaryotic V-ATPases, are the most extensively studied inhibitors. They bind the Vo subunit c and may inhibit the enzyme by preventing rotation of the c subunit ring. The salicylihalamides and lobatamides show remarkable specificity for animal V-ATPases. The chondropsins preferentially inhibit the fungal V-ATPase. Because of the variety of processes and diseases associated with V-ATPases and the possibility of designing selective inhibitors, the V-ATPases are attractive targets for development of therapeutic agents.

KEY WORDS: V-ATPase; vacuolar ATPase; proton pump; bafilomycin; concanamycin; salicylihalamide; lobatamide; chondropsin; *Neurospora crassa*.

INTRODUCTION

V-ATPases are abundant, ubiquitous proton pumps in eukaryotic cells. The pumps regulate pH and generate an electrochemical gradient that drives the transport of molecules across membranes of endosomes, Golgi, secretory vesicles, vacuoles or lysosomes, and other components of the endomembrane system. V-ATPases also occur on the plasma membranes of specialized cells such as osteoclasts and kidney intercalated cells, where they acidify the extracellular milieu. Not surprisingly, a diverse collection of physiological processes depend on V-ATPases, and a number of diseases have been associated with malfunction of these enzymes (Beutler and McKee, 2003; Kawasaki-Nishi *et al*., 2003; Nishi and Forgac, 2002).

In a few instances defects in V-ATPase genes cause a specific disease condition. Osteopetrosis (overproduction of bone) (Frattini *et al*., 2000), distal renal tubular acidosis, and sensorineural deafness (Karet *et al*., 1999) fall into this category. More common is evidence that bafilomycin and concanamycin, specific inhibitors of V-ATPases, affect development of the disease state in cultured cells. Osteoporosis (loss of bone mass) (Farina and Gagliardi, 2002) and cancer (Izumi *et al*., 2003) are the primary targets of these studies. However, the list of other diseases suspected of V-ATPase involvement is large and varied—diabetes (Rojas *et al*., 2004), Alzheimer's (Haass *et al*., 1995), Parkinson's (Nishiguchi *et al*., 2003), cardiovascular disorders (Otani *et al*., 2000), and glaucoma (Wax *et al*., 1997)—to name a few.

The research in our lab has concentrated on understanding the mechanism of V-ATPase inhibitors, compounds that might be used for development of drugs to target V-ATPases. We have identified three families of V-ATPase inhibitors.

CLASSES OF V-ATPASE INHIBITORS

The best studied and most frequently used V-ATPase inhibitors are the macrolide antibiotics with 16- or 18-membered lactone rings, the bafilomycins and concanamycins (Fig. 1) (Bowman *et al*., 1988; Dröse *et al*., 1993). Isolated from *Streptomyces sp*., these antibiotics

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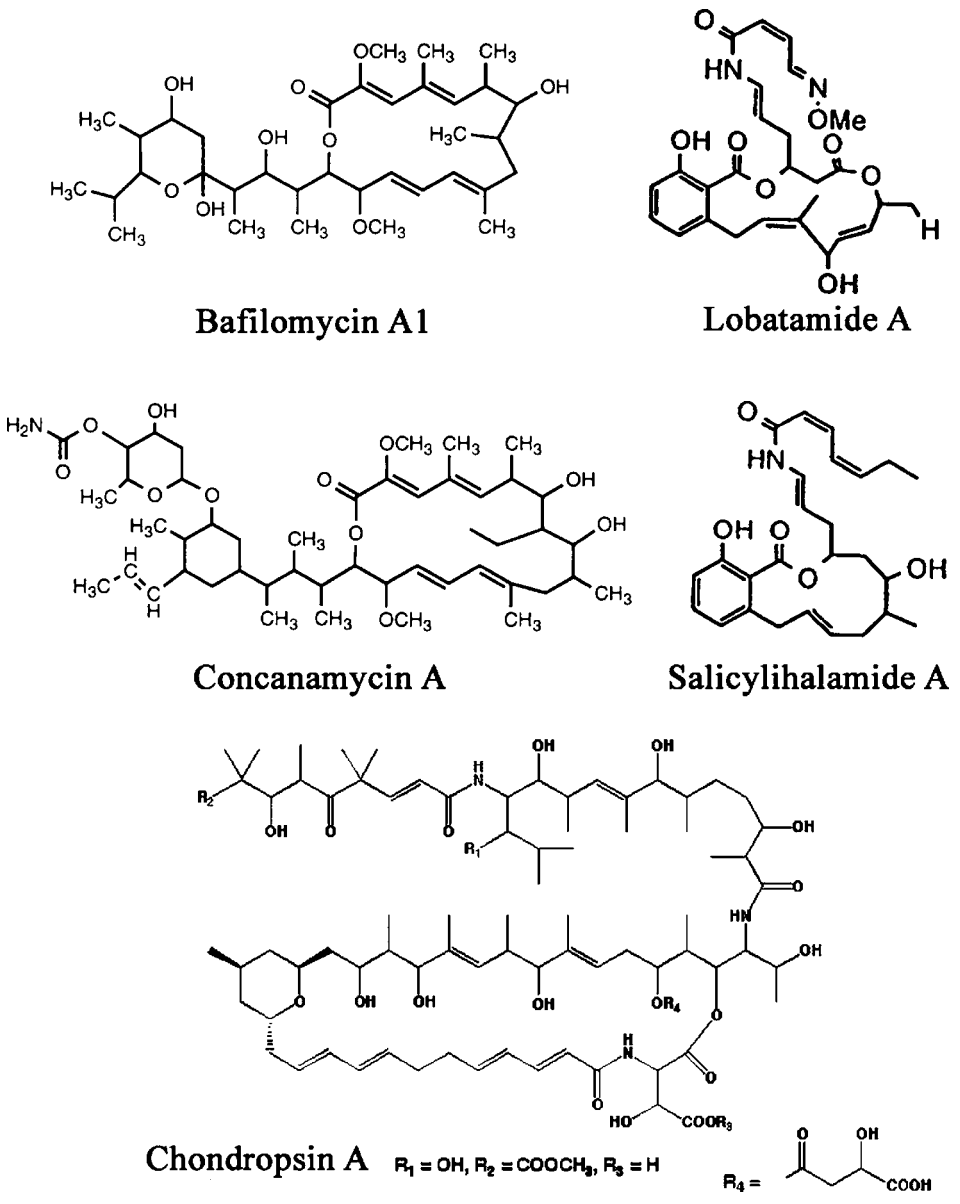

Fig. 1. Structures of bafilomycin A1, concanamycin A, salicylihalamide A, lobatamide A, and chondropsin A.

were originally thought to be inhibitors of P-type ATPases. We fortuitously found them to be far more potent against V-ATPases. Thus, half-maximal inhibition of eukaryotic V-ATPases from plants, animals, and fungi is achieved by concentrations in the low nM range. Some, but not all, P-type ATPases are inhibited at approx. 10,000-fold greater concentrations, and F-type ATPases are not inhibited by concentrations as high as 1.0 mM. When used at low concentrations, bafilomycin and concanamycin are highly specific for V-ATPases. For example, the inhibitors strongly inhibit the growth of wild-type *Neurospora crassa* but have no effect on strains with an inactivated V-ATPase, indicating that no additional enzymes are affected (Bowman *et al.*, 2000; Bowman and Bowman, 2000). Bafilomycin and concanamycin are available commercially from several sources, and several labs have developed procedures to synthesize them *in vitro* (Scheidt *et al.*, 2002 and references therein).

The benzolactone enamides (Fig. 1), which contain a salicylic acid residue and an enamide side chain joined by a variable linker to form a lactone ring, are interesting because they inhibit animal V-ATPases at low nM

concentrations yet have essentially no effect on fungal V-ATPases (Boyd *et al*., 2001). Such specificity is unprecedented. Members of this family have been isolated from marine sponges, ascidians, and bacteria. Originally suspected to be V-ATPase inhibitors because their pattern of toxicity in the NCI 60-cell cancer screen was similar to the pattern for bafilomycin, they are now under active investigation by several organic synthesis labs. Salicylihalamide A (Wu *et al*., 2000), lobatamide C (Shen *et al*., 2003), and apicularen A (Lewis *et al*., 2001) have been synthesized *in vitro* (see Beutler and McKee, 2003 for other references). A derivative of salicylihalamide is already under investigation at Reata Pharmaceuticals, Inc. as an encouraging lead compound for treatment of cancer (http://www.reatapharma.com/rta203.asp).

A third family of V-ATPase inhibitor we have identified contains the chondropsins, polyketide-derived macrolide lactams with 33–37 members in the macrocyclic ring (Fig. 1). Isolated from marine sponges, the chondropsins are distinct from the other two families by being less potent and by preferring the fungal over the mammalian enzyme in *in vitro* assays (Bowman *et al*., 2003). The supply of these compounds in natural collections is almost exhausted, and we are unaware of anyone trying to synthesize them *in vitro*.

MECHANISM OF ACTION

To understand how bafilomycin and concanamycin act on the V-ATPase we have taken a genetic approach, using the filamentous fungus *Neurospora crassa* (Bowman and Bowman, 2000). A prominent compartment of fungi is the vacuole, which stores large quantities of basic amino acids, polyphosphate, Ca^{++} and other ions, and proteases and other degradative enzymes. The vacuolar membrane is thickly lined with V-ATPases, which pump protons into the interior, acidifying the interior and facilitating hydrolytic reactions. Like other V-ATPases, the *N. crassa* enzyme is a large complex of 14 different types of polypeptides, arranged into a removable headpiece where ATP is hydrolyzed and a membrane-embedded sector through which protons pass (Kawasaki-Nishi *et al*., 2003; Margolles-Clark *et al*., 1999; Sambade and Kane, 2004). The headpiece consists of eight kinds of subunits, named A–H; the membrane sector contains six kinds of polypeptides, named a, c, c′, c″, d, and e. The enzyme functions as a rotary motor. During proton translocation through the membrane multiple copies of the 16 kDa subunit c, together with single copies of c′ and c″, rotate relative to subunit a, the 100 kDa protein forming part of the stator.

A hallmark of fungal strains with an inactivated V-ATPase gene is the inability to grow at alkaline pH (Nelson and Nelson, 1990). Similarly, wild-type strains cannot grow on medium buffered to pH 7.5 and containing bafilomycin or concanamycin (typically 0.2–1.0 μM) (Bowman *et al*., 1997; Bowman and Bowman, 2000). We mutagenized *N. crassa* conidia (asexual spores) with ultraviolet light and selected mutant strains that could grow on this medium. With concanamycin as the selective agent, we obtained many strains that grew weakly and exhibited abnormal hyphal growth in the presence of the antibiotic; none of them was altered in a V-ATPase gene. Most were altered in *pma-1,* the gene encoding the H^+-plasma membrane ATPase, which is not sensitive to the drug. In these mutant strains, the affinity of the H^+-plasma membrane ATPase for ATP was increased approx. 10-fold, from 2.0 to 0.2 mM. We hypothesized that resistance to the drug was a secondary effect. The cells were more efficient at exporting toxic levels of ions that accumulate in the cytoplasm of cells with inhibited V-ATPases (Bowman *et al*., 1997).

Selections with bafilomycin were more successful. We found bafilomycin-resistant (bfr) strains that were altered in subunit c of the V-ATPase. These strains were more strongly resistant than the concanamycin-resistant strains, and the mutant hyphae growing on bafilomycin had normal morphology. The mutant V-ATPases, assayed *in vitro*, showed a 4- to 70-fold increase in resistance to bafilomycin and a modest 1- to 3-fold increase in resistance to concanamycin (Bowman and Bowman, 2002). By mutagenizing a bafilomycin-resistant strain and selecting for resistance to concanamycin we could obtain strains resistant to both antibiotics. The bcr strains (bafilomycin- and concanamycin-resistant) had second mutations in subunit c. In multiple screening attempts we never obtained mutants with changes in a second V-ATPase gene. Mutation of a second residue in subunit c resulted in increased resistance of the V-ATPase to both bafilomycin and concanamycin *in vitro*. The V-ATPase of the strongest bcr mutant was 325-fold more resistant to bafilomycin and 39-fold more resistant to concanamycin than the wild-type control (Bowman *et al*., 2004).

We have identified nine residues that affect binding of the antibiotics to subunit c. The subunit has four membrane spanning helices. In a secondary structural diagram five residues cluster along one face of helix 4, opposite the position of the proton-binding E138 residue, and four others lie in the upper part of helices 1 and 2. No mutant sites conferring resistance are on helix 3. A helical wheel model of mutant sites on subunit c predicts a binding pocket formed by helices 1, 2, and 4. Our working hypothesis is that bafilomycin and concanamycin may differ

in specific contact points but that they bind in a similar region of subunit c and may inhibit by preventing rotation of the c-subunit ring (Bowman *et al*., 2004; Bowman and Bowman, 2002). We are testing this hypothesis with site-directed mutants and improved modeling, made possible by the recent publication of the crystal structure of the K-subunit (homolog of subunit c) ring of *Enterococcus hirae* (Murata *et al*., 2005).

Where the benzolactone enamides bind the V-ATPase is not yet known. Because these compounds were isolated and identified from collections in natural products labs, quantities were limited. An early goal of investigators was to synthesize the inhibitors and to identify derivatives as potential leads for drug development or enzyme labeling studies. Because the benzolactone enamides are specific for V-ATPases in animals, a good genetic system for mutant selection is not available. Limiting amounts of material are a further challenge. The published procedures for *in vitro* synthesis of lobatamide (Shen *et al*., 2003) and salicylihalamide (Wu *et al*., 2000) should help. The benzolactone enamides may bind the membrane sector of the enzyme at a site different from bafilomycin and concanamycin. Xie *et al*. (2004) reported that salicylihalamide interacted with the Vo sector of the bovine coated vesicle V-ATPase, but attempts to identify the target subunit by reaction with a radioactive derivative did not succeed. Huss *et al*. (2002) found that salicylihalamide inhibited activity of the V-ATPase from *Manduca sexta* but did not compete for binding of a radiolabeled derivative of concanamycin to subunit c.

Even less is known about the site of chondropsin binding to the V-ATPase. We tested two bafilomycin-resistant V-ATPases from *N. crassa* for resistance to two chondropsins. The mutant enzymes showed a small but reproducible increase in resistance, 2- and 2.5-fold. It is possible that the chondropsins also act on the rotor mechanism of the V-ATPase (Bowman *et al*., 2003).

CONCLUSIONS

V-ATPases play a role in a growing number of diseases. We have identified three classes of compounds that are potent, specific inhibitors of these complex enzymes. Bafilomycins and concanamycins inhibit all known eukaryotic V-ATPases. They bind subunit c and may act by preventing the rotary motion of the enzyme. Because the subunit c sequence is so highly conserved, it may not be an ideal target for a drug. Perhaps the best use of these inhibitors is to demonstrate the importance of V-ATPase activity in diseases. The benzolactones, which exhibit specificity for animal V-ATPases and for selected cancer cell lines, are more attractive for drug development. The intriguing question for these compounds is, how do they inhibit the enzyme? The chondropsins could lead to antifungal agents. Many investigators with different expertise and interests are now entering the field to study V-ATPases and their potential as drug targets.

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