Identification of a New Chondropsin Class of Antitumor Compound That Selectively Inhibits V-ATPases*  

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We identify a new naturally occurring class of inhibitors of vacuolar H⁺-ATPases (V-ATPases) isolated from vacuolar membranes of Neurospora crassa and from chromaffin granule membranes of Bos taurus. To date, the new class includes six chondropsins and poccilastatin A, large polypeptide-derived macrolide lactams with 33–37 membered rings. In the National Cancer Institute’s 60-cell screen the chondropsin class showed a tumor cell growth inhibitory fingerprint essentially indistinguishable from that of the bafilomycin/concanamycin and the salicylihalamide/lobatamide classes of well-established V-ATPase inhibitors. Half-maximal inhibition of V-ATPase activity in vitro occurred at 0.04–0.7 μM for the fungal vacuolar V-ATPase and at 0.4 to >10 μM for the chromaffin granule V-ATPase. Thus, the new inhibitors are somewhat less potent than the other two classes, which typically have Kᵢ values of <10 nM for V-ATPases, and the new inhibitors differ from the other two classes in their specificity. The bafilomycin class inhibits all eucaryotic V-ATPases, the salicylihalamide class inhibits mammalian V-ATPases but not fungal V-ATPases, and the new chondropsin class inhibits the N. crassa V-ATPase better than the chromaffin granule V-ATPase. Two mutations in the N. crassa V-ATPase that affect the binding of bafilomycin had small but reproducible effects on the affinity of chondropsins for the V-ATPase, suggesting the possibility of a similar mechanism of inhibition.

Two classes of natural products act as specific and potent inhibitors of vacuolar H⁺-ATPases (V-ATPases)¹ (Fig. 1). The macrocyclic lactones, bafilomycin and concanamycin, were identified as inhibitors of eucaryotic V-ATPases from animals, plants, and fungi (1, 2). Subsequently tested in the National Cancer Institute’s (NCI) 60-cell antitumor screen, they showed a characteristic tumor cell growth inhibitory profile, particularly potent against melanoma cell lines. More recently, a large number of benzolactone enamides, including salicylihalamides and lobatamides, produced an inhibitory profile in the 60-cell screen nearly identical to that for bafilomycin/concanamycin. Subsequent analysis showed them to be excellent V-ATPase inhibitors. Surprisingly, this class preferentially inhibited V-ATPases from mammalian sources, with little effectiveness against V-ATPases from Neurospora crassa and Saccharomyces cerevisiae (3).

V-ATPases are abundant, ubiquitous ion pumps in eucaryotic cells (reviewed in Ref. 4). They regulate pH and generate an electrochemical gradient that drives the transport of molecules across many types of cellular membranes. A diverse collection of physiological processes depend on V-ATPases, including protein sorting, endocytosis, neurotransmitter uptake, apoptosis, and receptor recycling. The V-ATPase is a large, complex enzyme. The membrane-embedded sector, Vo, contains at least five different polypeptides (a, c, c’, c”, and d) and forms a proton-conducting pathway through the membrane. The peripheral sector, V1, is composed of at least eight different polypeptides (A–H) and contains the sites of ATP hydrolysis. Like the F-ATPase in mitochondria, chloroplasts, and bacteria (5), the V-ATPase functions as a molecular motor (6–8). The A and B subunits provide the driving force by hydrolysis of ATP. The D, F, c’, c, and c” subunits are tightly bound to each other and form the rotor. Other subunits, a, G, and H, form a stator, anchoring the A and B subunits to the membrane. The translocation of protons has been proposed to occur at the interface between the rotating ring of c subunits and the fixed a subunit (9).

Not surprisingly, given their widespread occurrence and involvement with so many cellular processes, V-ATPases play a role in many diseases, e.g. Alzheimer’s, osteoporosis, viral infections, diabetes, cardiovascular disorders, and cancer (4, 10–12). They are also implicated in tumor growth and resistance to anticancer agents (13–16). Because of the potential of V-ATPases as lead compounds to therapeutic drugs, several laboratories have undertaken and achieved the complete in vitro synthesis of bafilomycin, concanamycin, salicylihalamide, and lobatamide (17–22). Derivatives of bafilomycin have been generated and tested for effects on osteoporosis in rats; one gave encouraging results in preventing bone loss in ovariectomized animals (23).

In this report we introduce the chondropsins as a third class of natural products that exhibit the same NCI 60-cell antitumor fingerprint as bafilomycin, salicylihalamide, and related compounds and selectively inhibit V-ATPase activity in vitro. We use mutants from N. crassa that are resistant to bafilomycin to ask whether the new class of V-ATPase inhibitor may interact with the enzyme in a manner similar to the established inhibitors (24).

EXPERIMENTAL PROCEDURES  

N. crassa Strains, Growth of Cells—Strain 74A of N. crassa was used as the wild type. The mutant strains, bfr53 and bfr65, were described previously (24). Briefly, they carry mutations in rna-3, the gene encoding subunit c of the V-ATPase, that allow them to grow in the presence of bafilomycin at alkaline pH and confer resistance to bafilomycin on
the V-ATPase \textit{in vitro}. The altered residues in subunit c are T32I and Y143H for strains bfr33 and bfr65, respectively. The strains are available at the Fungal Genetics Stock Center, Kansas City, KA. Strains were maintained on Vogel’s medium N (a minimal medium salt solution at pH 5.8) supplemented with 2% sucrose. For membrane isolations, cells were grown \( \text{at } 25 \, ^\circ \text{C} \) in 4 liters of Vogel’s medium inoculated with \( 10^6 \) conidia/ml (asexual spores) and aerated vigorously.

### Isolation of Membranes, Analysis of ATPase Activity, and Effects of Inhibitors

- Chromaffin granule membranes were prepared from bovine adrenal glands, obtained fresh from a local abattoir, as described (25). The membranes were stored in aliquots at \(-70 \, ^\circ \text{C}\). Vacular membranes, mitochondria, and plasma membranes were prepared from \textit{N. crassa} as described (26) and modified (27). Protein and ATPase activities were assayed as described (26), except that assays were typically done at \( 37 \, ^\circ \text{C} \). The chondropsins and poecillastrin A were added to assay mixtures from 5 or 10 mM stock solutions in dimethyl sulfoxide. When comparing the effects of inhibitors on different membranes, we ran the reactions at the same time in the same assay mix.

### Compounds

The macrolide lactams used in this work are illustrated in Fig. 2. They were isolated and purified from various marine sponges at the National Cancer Institute. Chondropsins A, B, and D were isolated from \textit{Chondropsin} sp (28, 29) and \textit{Ircina} sp (30) and poecillastrin A, from \textit{Poecillastra} sp (31). Dimethylchondropsin A was obtained by methylation of chondropsin A as described (28). All seven compounds were tested for their effects on V-ATPase activity in bovine chromaffin granule membranes and \textit{N. crassa} vacuolar membranes. Chondropsin B and 7-deoxychondropsin A were chosen for studies on inhibitor effects on other ATPases and on V-ATPases in mutant strains because they were available in larger quantities.

### RESULTS

**COMPARE Analyses Implicate V-ATPase as a Molecular Target of the Chondropsins**—We used the NCI 60-cell antitumor screen to look for biological activity of the chondropsins similar to compounds in the NCI databases (33). A dose-response curve was determined for each type of tumor cell, measuring cytotoxicity in microtiter plates after a 48-h exposure to the test compound. The most sensitive cell lines were killed at concentrations that were nearly 10,000-fold lower than the concentrations that affected the most resistant types of cells. Each cell line was compared with the mean effective dose for all cell lines, generating a “mean graph” that served as a profile of the response of the 60 cell lines to each test compound. This cellular response profile was compared with the response profiles of other test compounds using the COMPARE pattern recognition algorithm (33). We found that the 60-cell profiles of chondropsin A gave consistently high correlation with the data base profiles of lobatamide A, bafilomycin A1, salicylihalamide A, and concanamycin A (Table I). Because these four compounds are potent specific inhibitors of V-ATPases, this result prompted us to hypothesize that the new class of macrolide lactams might also target V-ATPases.

**Chondropsins and Poecillastrin A Inhibit V-ATPases**—The six chondropsins and poecillastrin A (see structures in Fig. 2) were tested for their effect on V-ATPases from bovine chromaffin granule membranes and from vacuolar membranes of

\[ \text{CONCANAMYCIN A} \]

\[ \text{SALICYLHALAMIDE A} \]

\[ \text{LOBATAMIDE A} \]

**Materials**—Concanamycin C was a gift from Dr. K. Altendorf (University of Osnabrück) and Dr. A. Zeeck (University of Göttingen). The Na+/K+ ATPase from dog kidney, ATP, sorbitol, phenylmethylsulfonyl fluoride, chymostatin, and most other chemicals were purchased from Sigma.

\[ \text{FIG. 1. Structures of bafilomycin A1, salicylihalamide A, concanamycin A, and lobatamide A.} \]


**Table I**

| Compound                  | TGI-COMPARE correlation coefficient | Mean-Panel GI50 | N. crassa | 10^(-6) M (z.S.D.) |
|---------------------------|------------------------------------|----------------|-----------|------------------|
| Lobatamide A              | 1.00                               | 0.56 (0.09)    |           |                  |
| Concanamycin A            | 0.94                               | 0.11 (0.03)    |           |                  |
| Bafilomycin A             | 0.92                               | 1.02 (0.71)    |           |                  |
| Salicylihalamide A        | 0.93                               | 4.97 (1.03)    |           |                  |
| Chondropsin A             | 0.92                               | 2.56 (0.77)    |           |                  |



**N. crassa.** As predicted, they inhibited V-ATPase activity in vitro. However, the specificity of their inhibition was different from that of either the bafilomycin/concannamycin class or the salicylihalamide/lobatamide class. Bafilomycin and its relatives act against all eucaryotic V-ATPases that have been tested (34). The salicylihalamide class shows a clear preference for V-ATPases from mammalian sources and is ineffective toward V-ATPases from fungi (3). To our knowledge, all animal V-ATPases tested to date are sensitive to this class of compounds.

By contrast, the new class of macrolide lactams inhibited V-ATPases from both bovine chromaffin granules and fungal vacuoles but was more potent against the fungal enzyme. For example, half-maximal inhibition of the chromaffin granule V-ATPase by chondropsin B and 73-deoxychondropsin A occurred at 5.8 and 2.9 μM, respectively, and half-maximal inhibition of the *N. crassa* V-ATPase by the same compounds was at 0.27 and 0.10 μM (Fig. 3, A and B). Data for effects of the seven macrolide lactams on the two V-ATPases are summarized in Table II. They showed a consistent pattern. All seven compounds were more potent inhibitors (8–30-fold) of the enzyme from *N. crassa* than from the enzyme from the animal.

The order of potency was similar for the two enzymes. Dimethylchondropsin A and chondropsin D were the strongest inhibitors, followed by chondropsin C and 73-deoxychondropsin A, and then poecillastrin A and chondropsin B; chondropsin A was the weakest inhibitor in this group.

The concentrations for half-maximal inhibition by the chondropsins ranged from 0.04 to 0.7 μM for the fungal enzyme and from 0.43 to >10 μM for the mammalian enzyme. Thus, although good inhibitors, they were not as potent as the previously characterized V-ATPase inhibitors, which typically have *K* values of 5 nM or less against their target enzymes when assayed in vitro (1–3). The relatively weak activity of chondropsin A against the chromaffin granule V-ATPase was unexpected. In the NCI 60-cell screen chondropsin A was ~2-fold more potent an inhibitor of tumor cell growth than salicylihalamide A (Table I), which inhibits the chromaffin granule enzyme *in vitro* with a *K* of 3 nM (data not shown). We speculate that the new class of compounds may target specific isoform(s) of mammalian V-ATPase yet to be defined.

The Chondropsins and Poecillastrin A Are Inactive Against Other Membrane ATPases—We previously demonstrated that the macroyclic lactone and the benzolactone enamide classes of V-ATPase inhibitors do not inhibit F-ATPases from mitochondrial or *Escherichia coli* or the plasma membrane H^-ATPase from *N. crassa* (1, 2). High concentrations of bafilomycin and concanamycin do inhibit mammalian P-type ATPases. Bafilomycin C1 inhibited the Na^+_/K^-/ ATPase of dog kidney with a *K* of 13 μM (35), whereas bafilomycin A1 inhibited the dog kidney enzyme with a *K* of 30 μM (1), 10,000 times greater than the *K* for V-ATPase inhibition. In the current study 0.1, 1.0, and 10.0 μM concentrations of chondropsin B and 73-deoxychondropsin A had no effect on the activity of the mitochondrial F-ATPase of *N. crassa*, the plasma membrane H^-ATPase of *N. crassa*, or the Na^+_/K^- ATPase from dog kidney (data not shown).

**V-ATPase Mutations That Confer Resistance to Bafilomycin Cause Small but Reproducible Changes in Inhibition by Chondropsin**—We have isolated bafilomycin-resistant mutant strains of *N. crassa* that are altered in subunit c of the V-ATPase. Assayed *in vitro*, the mutant enzymes show 20–60-fold resistance to bafilomycin (24). Three of the mutants consistently exhibited a 3-fold resistance to concanamycin as well. We reasoned that if the chondropsins bind the V-ATPase at the same sites as bafilomycin and concanamycin, they should have a changed affinity for the mutant enzymes as compared with the wild type enzyme. We tested the effects of chondropsin B and 73-deoxychondropsin A on the V-ATPases from the wild type strain 74A and from two mutant strains, bfr33 (T32D) and bfr65 (Y143H). The two chondropsins had similar effects on the mutant enzymes, giving a 2.5-fold increase in *K* for the bfr33 enzyme and a 2-fold decrease in *K* for the bfr65 enzyme as compared with the wild type control (Fig. 4, A and B, Table III). The experiment was done three times. We assayed two different preparations of vacuolar membranes from each mutant strain and three from the wild type. The increase in *K* for the bfr33 enzyme with the two chondropsins ranged from 2.2–3.3-fold, and the decrease in *K* for the bfr65 enzyme ranged from 1.4–2.3-fold. Thus, although small, the effects were reproducible. These results can be interpreted as suggesting that the chondropsins interact with the V-ATPase in a similar manner to bafilomycin. Alternatively, the small changes in the *K* for chondropsin could be due to indirect effects; a conformational change in subunit c could alter chondropsin binding at another site in the enzyme.

**DISCUSSION**

V-ATPases are the target of a variety of antibiotics that have been isolated in screens of natural products. Three classes stand out by their characteristic structures (Figs. 1 and 2). The family of bafilomycins and concanamycins, macrolide antibiotics with 16- or 18-membered lactone rings, comes from *Streptomyces* sp.; they inhibited growth of bacteria and fungi in a disc diffusion assay. The first potent specific inhibitors of V-ATPases to be identified, bafilomycin and concanamycin became important aids in characterizing V-ATPases in new locations and in probing the role of V-ATPases in a number of physiological processes (34). The family of salicylihalamides, lobatamides, oximidines, and apicularens are benzolactone enamides described by three structural features, (a) a salicylic acid residue, (b) an enamide side chain, and (c) a linker of variable length, composition, and stereochemistry that joins a and b, forming a lactone ring. Originally isolated from marine sponges and ascidians, these compounds exhibited potent tumor growth inhibition in the NCI 60-cell screen. The cellular response profiles from this screen matched the profiles of bafilomycin and concanamycin in the NCI data base, prompting us to test them for inhibitory activity against V-ATPases. Confirmed as excellent V-ATPase inhibitors, the benzolactone enamide class had the unprecedented property of discriminating between mammalian and fungal V-ATPases (3). They are currently under study by several laboratories as potential therapeutic leads (36).

In this report we identify a third class of natural product as V-ATPase inhibitors. Once again, the class members were isolated from marine sponges and suspected to act as V-ATPase inhibitors because of their distinctive pattern of cellular growth inhibition and cytotoxicity in the NCI 60-cell screen. A mean-graph COMPARE analysis (33) revealed a high correlation between the 60-cell profiles of the chondropsins and the other known inhibitors of V-ATPase (Table I). Compounds that correlate highly with one another can be expected to share a
common molecular target or biological mechanism of action, even if they differ significantly in structure. The new class, presently composed of six chondropsins and poecillastrin A, are polyketide-derived macrolide lactams with 33–37 members in the macrocyclic ring (Fig. 2) (28–31). The compounds selectively inhibit V-ATPases (Fig. 3, A and B, Table II) and have no inhibitory activity on membrane ATPases from the F- and P-ATPase families.

The new class of V-ATPase inhibitor differs from the other classes in two ways: it is less potent, and it preferentially inhibits V-ATPases.
inhibits the fungal enzyme as compared with the mammalian enzyme. These properties give rise to a question: if chondropsin A is a weaker inhibitor of V-ATPase activity than salicylihalamide and the other inhibitors in Table I, why was it equally as effective in preventing growth of tumor cells? The answer is likely to be found in the complexity of mammalian V-ATPases, which exist in a multitude of forms that may vary in sensitivity to these drugs. Isoforms of genes encoding most of the V-ATPase subunits and examples of alternative splicing have been reported (37). For example, two laboratories found evidence for three distinct isoforms of the 100-kDa subunit α, multiple alternatively spliced variants of two of the isoforms, and tissue-specific expression of these isoforms in the mouse (38, 39). We speculate that chondropsin A is effective against tumor cells because it targets V-ATPases that may subtly differ in structure from the chromaffin granule enzyme tested in our experiments.

The enhanced sensitivity of the fungal V-ATPase relative to the chromaffin granule V-ATPase to inhibition by chondropsins suggests that it may be possible to design molecules that inhibit specific V-ATPases. Although not so dramatic as the all or none response seen with the salicylihalamide family and the animal versus fungal V-ATPases (3), it is conceivable that de-
Novel V-ATPase Inhibitor Class

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