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Discovery of a Novel Antitumor Benzolactone Enamide Class That Selectively Inhibits Mammalian Vacuolar-Type (H^+)-ATPases

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ABSTRACT

A series of naturally occurring compounds reported recently by multiple laboratories defines a new small-molecule class sharing a unique benzolactone enamide core structure and diverse biological actions, including inhibition of growth of tumor cells and oncogene-transformed cell lines. Here we show that representative members of this class, including salicylihalamide A, lobatamides A–F, and oximidines I and II inhibit mammalian vacuolar-type (H^+)-ATPases (V-ATPases) with unprecedented selectivity. Data derived from the NCI 60-cell antitumor screen critically predicted the V-ATPase molecular target, while specific biochemical assays provided confirmation and further illumination. The compounds potently blocked representative V-ATPases from human kidney, liver, and osteoclastic giant-cell tumor of bone but were essentially inactive against V-ATPases of *Neurospora crassa* and *Saccharomyces cerevisiae* and other membrane ATPases. Essential regulation of pH in cytoplasmic, intraorganellar, and local extracellular spaces is provided by V-ATPases, which are ubiquitously distributed among eukaryotic cells and tissues. The most potent and selective V-ATPase inhibitors heretofore known were the bafilomycins and concanamycins, which do not discriminate between mammalian and nonmammalian V-ATPases. Numerous physiological processes are mediated by V-ATPases, and aberrant V-ATPase functions are implicated in many different human diseases. Previous efforts to develop therapeutic pharmacological modulators of V-ATPases have been frustrated by a lack of synthetically tractable and biologically selective leads. Therefore, availability of the unique benzolactone enamide inhibitor class may enable further elucidation of functional and architectural features of mammalian versus nonmammalian V-ATPase isoforms and provide new opportunities for targeting V-ATPase-mediated processes implicated in diverse pathophysiological phenomena, including cancer.

V-ATPases are ubiquitous proton-translocating pumps of eukaryotic cells (Finbow and Harrison, 1997; Stevens and Forgac, 1997; Nelson and Harvey, 1999). The pumps are located on membranes of vacuoles, lysosomes, and other components of the endomembrane system, as well as on certain specialized plasma membranes. They are oriented such that protons are pumped out of the cytoplasm into the organelle or the extracellular space. Hydrolysis of adenosine triphosphate (ATP) generates an electrochemical potential across the membrane that drives the transport of ions and solutes. The regulation of intraorganellar acidity by V-ATPases within various mammalian cell types is required for a variety of physiological functions including membrane and organellar protein sorting, neurotransmitter uptake, cellular degradative processes, and receptor recycling. V-ATPase-mediated regulation of vacuolar pH has also been implicated in anticancer drug resistance (Martinez-Zaguilan et al., 1999). V-ATPases present on plasma membranes of kidney intercalated cells, osteoclasts, and sperm cells modulate urinary acidification, bone resorption, and fertility, respectively. Plasma membrane V-ATPases appear to be involved in other

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ABBREVIATIONS: V-ATPase, vacuolar-type (H^+)-ATPase; DMSO, dimethyl sulfoxide; hK, human kidney; hL, human liver; hOc, human osteoclasts; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; MES, 4-morpholineethanesulfonic acid; SAR, structure-activity relationship(s).

biological processes wherein local regulation of extracellular acidity is essential for cellular "invasiveness", as in angiogenesis, cellular proliferation, and tumor metastasis (Martinez-Zaguilan et al., 1998; Rojas et al., 2000). V-ATPases are also implicated in the regulation of apoptosis or programmed cell death (Ishisaki et al., 1999).

Given the plethora of events mediated by V-ATPases, it would seem that this enzyme system should provide a rich molecular target area for pharmaceutical research and development. Diverse diseases for which aberrant V-ATPase functions are implicated or suspected include abnormal secretion of degradative enzymes, diabetes, cardiovascular and clotting disorders, psychiatric disorders such as Alzheimer's disease, disorders involving abnormal receptor-mediated uptake processes, glaucoma, defective urinary acidification, osteoporosis, and cancer (Nelson, 1991; Keeling et al., 1997; Farina and Gagliardi, 1999). However, almost all V-ATPase-targeted drug discovery and development efforts thus far have failed, presumably due to the ubiquitous cellular and tissue distribution of V-ATPases and the lack of sufficiently selective pharmacological inhibitors. Nevertheless, continuing advances in understanding of the structure and function of V-ATPases, and recent evidence of preferential inhibition of tissue-specific V-ATPases, provide the basis for a more optimistic future perspective (Farina and Gagliardi, 1999).

We define herein a novel V-ATPase inhibitor class that is unique in chemical structure and biological specificity in comparison with any previously known V-ATPase inhibitor. The new class has the key structural features depicted in Fig. 1. These are 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. Representative members of this benzolactone enamide class show unprecedented selectivity for mammalian versus nonmammalian V-ATPases. This contrasts sharply with members of the protypical bafilomycin/concanamycin class of V-ATPase inhibitor (Fig. 2), which indiscriminately block nonmammalian as well as mammalian V-ATPases and which

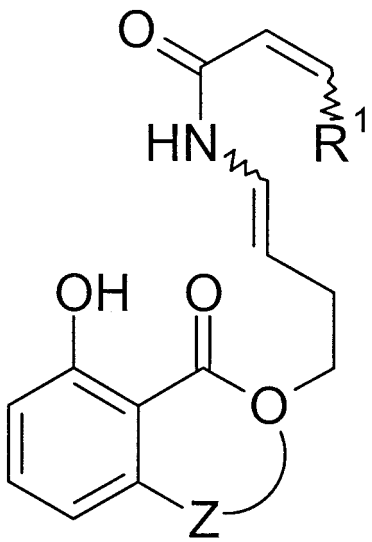

Fig. 1. Generic structure of the new benzolactone enamide V-ATPase inhibitor class, wherein Z represents a linker of variable length, composition, and stereochemistry; R^1 is a substituent of variable composition and geometric orientation; and geometric isomers are possible at the enamide linkage.

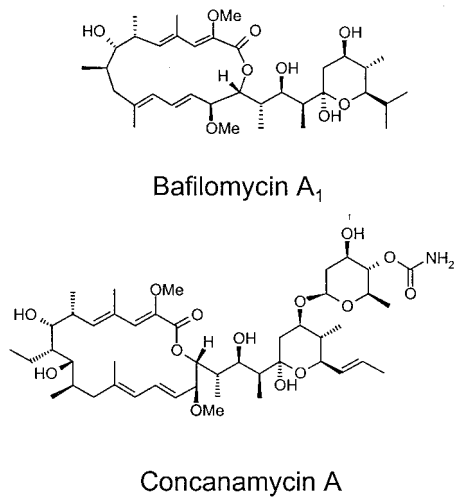

Fig. 2. Specific examples of compounds of the bafilomycin/concanamycin macrocyclic lactone class.

during the past decade have been used extensively in research of this complex enzyme system (Bowman et al., 1988; Dröse and Altendorf, 1997; Gagliardi et al., 1999).

Experimental Procedures

Materials. For mammalian V-ATPase assays, culture media and supplements were purchased from Life Technologies (Grand Island, NY), and phosphate-buffered saline from BioWhittaker (Walkersville, MD). All other reagents were purchased from Sigma (St. Louis, MO). For nonmammalian and bovine chromaffin granule V-ATPase assays and for other ATPase assays, most reagents were from Sigma, including the Na^+/K^+-ATPase from dog kidney (catalog no. A0142). Chromaffin granule membranes were the kind gift of Dr. David Apps (University of Edinburgh, Scotland, UK).

Compounds. The bafilomycin A_1 (Fig. 2) used in the mammalian V-ATPase assays was isolated and purified from a culture of *Streptomyces* sp. at SmithKline Beecham. The bafilomycin A_1 used in all other assays, as well as the concanamycin A (Fig. 2) standard used in all assays, were purchased from Kamiya Biomedical Company (Seattle, WA). Salicylihalamide A and lobatamides A–F (Fig. 3) were isolated and purified as described (Erickson et al., 1997; Galinis et al., 1997; McKee et al., 1998) in the National Cancer Institute (NCI) coauthors' laboratory. The oximidines I and II (Fig. 3) were isolated and purified as described (Kim et al., 1999) in the University of Tokyo coauthors' laboratory. It is noteworthy that both the bafilomycin A_1 and the concanamycin A standards exhibited significant instability and loss of inhibitory activity over time. Interestingly, the loss of potency appeared to stop at a level where the compound still remained a potent inhibitor of fungal V-ATPases (1–10 nM range rather than below 0.2 nM; E. J. Bowman, unpublished observations). A careful study of the bafilomycin A_1 stock solution in DMSO showed that it was most stable when stored at −80°C and thawed only once (P. Belfiore, unpublished observations).

Testing of Compounds in the NCI 60-Cell Screen. Compounds were tested in the NCI 60-cell screen (Boyd, 1997) as described previously (Boyd and Paull, 1995) in at least quadruplicate in each of two different concentration ranges (10^{-6} and 10^{-7} M upper limits) using five, 1 $\log_{10}$-spaced dilutions against the full 60-cell panel. Averaged mean-graphs were prepared from the appropriate data for each compound, and COMPARE correlation analyses were performed as described previously (Boyd and Paull, 1995).

Inhibition of Growth of Oncogene-Transformed Cell Lines. All 3Y1 cell lines (Shimura et al., 1990) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in Dulbecco's modified Eagle's medium supple-

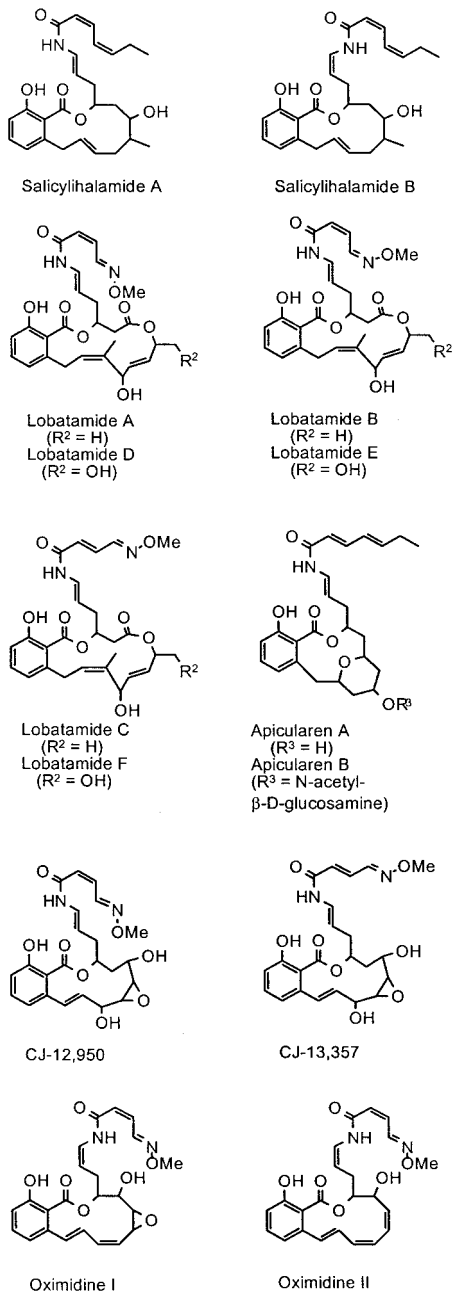

Fig. 3. Specific examples of compounds comprising the novel benzolactone enamide structural class.

mented with 10% heat-inactivated fetal calf serum and 0.1% glucose and grown at 37°C in a humidified atmosphere of 5% CO_2. Cells at 50% confluence were plated at one-tenth lower cell density and incubated for 3 days with various concentrations of samples. The growth was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 4 h at 37°C. Results were expressed as the mean IC_{50} values ± standard errors (S.E.) from triplicate determinations.

Purification of Osteoclastic Tumor Cells. Fresh samples of giant-cell tumors of bone (James et al., 1999) were incubated overnight in phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free, pH 7.2) at 37°C and 5% CO_2 before processing. The tissue was chopped with scissors in serum-free RPMI-1640 and stirred at 37°C for 1 h in RPMI supplemented with 0.3% (w/v) type I collagenase. The resulting material was then centrifuged at 1000*g* for 10 min, and the pellet was washed in RPMI supplemented with 10% of fetal calf serum to inactivate collagenase. Washing medium was then removed by centrifugation at 1000*g* for 5 min, and the pellet was resuspended in 10 mM Hepes buffer, pH 7.4, containing 0.2 M sucrose, 50 mM KC1, 1 mM EGTA, and 2 mM dithiothreitol. With consecutive low-speed sedimentations ($150g_{max}$ for 2 min) the cell suspension was enriched in giant cells (monitored by counting the cells microscopically) with a purity ratio of 1:2 (osteoclasts:contaminating cells).

Human Osteoclastic Tumor Cell Membrane Preparation. The aforementioned cell suspension was homogenized with a glass-Teflon homogenizer (1000 rpm × 20 strokes), and the material was centrifuged at 6000*g* for 20 min to remove debris, nuclei, lysosomes, and mitochondria. The supernatant was then centrifuged at 100,000*g* for 60 min to pellet the microsomal fraction. The pellet was resuspended in the medium (4 mg of protein/ml), frozen by liquid nitrogen immersion, and stored at −80°C.

Human Kidney Cortex and Liver Membrane Preparations. The renal membranes were prepared as described by Wang and Gluck (1990). The hepatic membranes were prepared as described by Yeh and Van Rossum (1991).

Endpoint Bafilomycin-Sensitive Mammalian ATPase Assay. The assays were performed in a 96-well microplate format with a 100 μl/well volume. The reaction medium contained 0.2 M sucrose; 50 mM KC1; 10 mM Hepes-Tris, pH 7.0; 1 mM CDTA; 3 mM ATP; 0.1 mM ammonium molybdate; 5 μM valinomycin; and 5 μM nigericin. Oligomycin (5 μg/ml) and vanadate (1 mM) were added to inhibit the F- and the P-type ATPase activity. The reaction was started with 5 mM $MgSO_4$. Anhydrous DMSO was used to dissolve and store all test compounds. The final concentration of DMSO in the assay was 10% (v/v), the highest concentration that did not influence the activity (data not shown). The presence in the assays of variable amounts of contaminating Mg^{2+}-dependent ATPase activities was overcome by testing both the controls (containing solely DMSO) and each concentration of the compounds in the presence and in the absence of 10 nM bafilomycin A_1. The bafilomycin-sensitive activity, calculated as difference of the ±bafilomycin treatments, was then expressed as percentage of inhibition versus control and used to draw the dose-response curves. A duplicate of each treatment was carried out. All assays were performed at 37°C. The incubation was for 30 min (human osteoclastic tumor and liver assays) or for 60 min (human kidney assay).

The release of phosphate was measured in a 96-well microplate reader (Molecular Devices, Menlo Park, CA) by the colorimetric malachite green assay previously described by Chan et al. (1986). Specific activities ± S.E. (nmol of phosphate/mg of protein/min; n = 6) were as follows: human kidney (hK), 40 ± 6; human liver (hL), 5.3 ±0.2; human osteoclasts (hOc), 40 ± 2. Each IC_{50} was evaluated by nonlinear regression fit of at least five concentration points, and the IC_{50} values were averaged from at least three separate experiments. The sigmoidal dose-response equation (three-parameter logistic) by GraphPad Prism software package (v2.01; GraphPad, San Diego, CA) was used.

Membrane Preparation and V-ATPase Activity Assays in Vacuolar Membranes of Fungi. Vacuolar membranes, plasma membranes, and mitochondria were isolated from *Neurospora crassa*, and V-ATPase activities were assayed as described previously (Bowman and Bowman, 1988). V-ATPase activity was measured as inorganic phosphate released in 20 min at 30°C in a reaction mixture containing 5 mM Na_2ATP, 5 mM $MgSO_4$, 10 mM NH_4Cl, 5 mM NaN_3, 0.1 mM Na_3VO_4, and 10 mM PIPES buffer, pH adjusted to 7.4 by the addition of Tris base. Vacuolar membrane vesicles of *Saccharomyces cerevisiae* were prepared and assayed as described previously (Uchida et al., 1985; Kane et al., 1989) for 30 min at 35°C in a mixture containing 5 mM Na_2ATP, 5 mM $MgCl_2$, 10 mM NH_4Cl, 25 mM MES buffer, and Tris base added to pH 6.9. V-ATPase activity in chromaffin granule membranes was assayed for 30 min at 37°C in the same reaction mixture used for the *N. crassa* enzyme. Where included, inhibitors were diluted directly into ATPase reaction mix-

ture from 1 mM concanamycin A or 10 mM (salicylihalamide A, lobatamides A–F, and oximidine I) stock solutions in DMSO.

Results

Testing in the NCI 60-Cell Screen Reveals That Representative Benzolactone Enamides Share the Same or Similar Molecular Target(s). We initially used the NCI 60-cell antitumor screen to establish a commonality of biological activity shared by available representative members of this novel compound class and to implicate V-ATPases as a molecular target. The origin, concept, rationale, technical details of operation of the 60-cell screen, and research applications thereof, such as the COMPARE algorithm, are reviewed elsewhere (Boyd and Paull, 1995; Boyd, 1997). The testing of salicylihalamide A, lobatamides A–F (Fig. 3), from the NCI coauthors' laboratory (Erickson et al., 1997; Galinis et al., 1997; McKee et al., 1998), and the oximidines I and II (Fig. 3) from the University of Tokyo coauthors' laboratory (Kim et al., 1999), gave essentially identical 60-cell screening profiles (e.g., see Fig. 4, B–D), implying that the compounds shared the same or similar molecular target(s). Furthermore, testing of salicylihalamide A and lobatamide A in the same biological assays and same laboratory (University of Tokyo) as used originally for characterizing the oximidines (Shimura et al., 1990; Kim et al., 1999) showed that they preferentially inhibited the growth of oncogene-transformed cell lines (Table 1), as had been reported earlier for the oximidines (Kim et al., 1999).

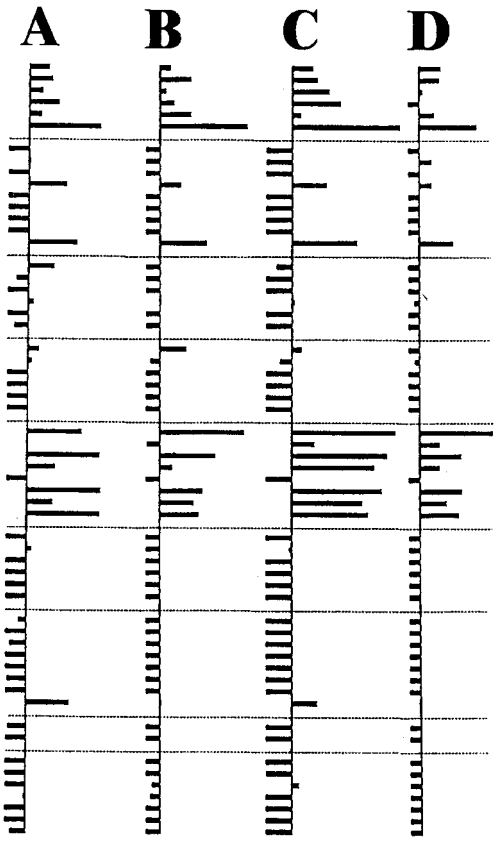

Fig. 4. Total growth inhibition (TGI)-based mean-graph profiles derived from testing of bafilomycin A_1 (A), salicylihalamide A (B), lobatamide A (C), and oximidine II (D) in the NCI 60-cell screen. Each horizontal bar represents the sensitivity of an individual cell line relative to the average sensitivity (represented by the central vertical line) of the full 60-cell panel. Bars projecting to the right represent relatively more sensitive cell lines and to the left the less sensitive lines. The dotted horizontal lines delineate the nine cell line subpanels, from top to bottom: leukemia, nonsmall-cell lung, colon, brain, melanoma, ovary, kidney, prostate, breast. Individual cell line identifiers have been omitted for clarity. Cell line identities and specific response values for each from a typical testing of salicylihalamide A can be found in Erickson et al. (1997).

COMPARE Analyses Implicate V-ATPase as a Molecular Target of Benzolactone Enamides. Using database analyses as described previously (Boyd and Paull, 1995), we found that the 60-cell profiles of the salicylihalamides and lobatamides gave consistently high correlations with the historical database profiles of bafilomycins and concanamycins (data not shown). Likewise, when the 60-cell profiles from our contemporary testing of the prototypical V-ATPase inhibitors bafilomycin A_1 or concanamycin A (Fig. 1C) were used as the seeds for COMPARE correlation analyses, the corresponding profiles of the representative benzolactone enamides showed extremely high correlations with the seeds (Table 2), and the visual identity of the 60-cell profiles was remarkable (Fig. 4, A–D). This led to our evaluation of the benzolactone enamides in V-ATPase assays.

Benzolactone Enamides Potently Inhibit Mammalian V-ATPases. All tested members of the new class were potent inhibitors of representative mammalian V-ATPases from human kidney, liver, and osteoclastic giant-cell tumor of bone (Table 3). The most potent was salicylihalamide A, which was almost equipotent to bafilomycin A_1 and concanamycin A when assayed against the osteoclastic V-ATPase, while it was about 10-fold less potent against the liver and kidney enzymes. Structural variations in the available lobatamides and oximidines did not affect significantly the potency of these compounds, all of which inhibited the human V-ATPases at low nanomolar levels.

Benzolactone Enamides Are Inactive against Fungal V-ATPases. The potent inhibition of V-ATPase by bafilomycins and concanamycins was first observed in *N. crassa* (Bowman et al., 1988; Dröse et al., 1993; Dröse and Altendorf, 1997), so we also tested the new compounds against the nonmammalian enzyme. In the present study, the positive control concanamycin A inhibited the *N. crassa* V-ATPase with a K_i of 0.1 nM (Fig. 5A), a value similar to that found for the same compound against the mammalian V-ATPases (Table 3). In contrast, salicylihalamide A, an excellent inhibitor of mammalian V-ATPases (Table 3), was found to be an extremely poor inhibitor of the fungal V-ATPase, showing little effect at concentrations up to 1.0 μM (Fig. 5, A and B). Oximidine I and lobatamides A–F were similarly ineffective as inhibitors of the V-ATPases of *N. crassa* V-ATPase, with

TABLE 1
Preferential inhibition of growth of oncogene-transformed cells by salicylihalamide A and lobatamide A
Values shown are averages from triplicate determinations.

Cell Line	Oncogene	IC_{50}	
		Salicylihalamide A	Lobatamide A
		nM (±S.E.)	
3Y1		770 (120)	150 (20)
SV-3Y1	SV40 large T	73 (12)	31 (18)
Ad12-3Y1	E1A, E1B	30 (8)	11 (5)
E1A-3Y1	E1A	32 (14)	13 (8)
HR-3Y1	v-H-*ras*	190 (40)	43 (9)
SR-3Y1	v-*src*	41 (12)	12 (7)

SV40, simian virus 40.

TABLE 2

COMPARE correlation coefficients, derived from testing of selected representative compounds in the NCI 60-cell screen

The COMPARE analyses used the TGI (total growth inhibition) mean-graph profiles of the named test compounds and seed compounds. The analyses were performed using averaged data from at least quadruplicate testing of each compound in the 60-cell screen.

Test Compound	Seed Compound	
	Bafilomycin A_1	Concanamycin A
Bafilomycin A_1	1.00	0.96
Concanamycin A	0.96	1.00
Salicylihalamide A	0.88	0.86
Lobatamide A	0.92	0.94
Lobatamide B	0.92	0.89
Lobatamide C	0.93	0.89
Lobatamide D	0.94	0.92
Lobatamide E	0.92	0.89
Lobatamide F	0.89	0.95
Oximidine I	0.87	0.85
Oximidine II	0.86	0.87

TABLE 3

Inhibition of mammalian V-ATPases by selected representative compounds

V-ATPases tested were from hK, hL, and osteoclastic giant-cell tumors of bone (hOc). Values shown are averages from at least triplicate determinations.

Compound	IC_{50}		
	hK	hL	hOc
	nM (±S.E.)		
Benzolactone enamides			
Salicylihalamide A	0.58 (0.20)	0.62 (0.19)	0.40 (0.02)
Lobatamide A	1.4 (0.2)	6.2 (2.9)	1.8 (0.8)
Lobatamide B	0.68 (0.05)	2.3 (0.6)	0.71 (0.11)
Lobatamide C	1.8 (0.2)	2.8 (0.4)	1.9 (0.2)
Lobatamide D	1.2 (0.1)	2.5 (0.6)	0.85 (0.07)
Lobatamide E	0.89 (0.09)	3.1 (0.4)	1.1 (0.3)
Lobatamide F	6.9 (1.3)	14.0 (2.0)	9.8 (0.9)
Oximidine I	2.9 (0.3)	5.6 (0.9)	3.3 (0.4)
Oximidine II	1.3 (0.5)	1.9 (0.5)	0.91 (0.22)
Reference compounds			
Bafilomycin A_1	0.04 (0.01)	0.36 (0.05)	0.06 (0.01)
Concanamycin A	0.06 (0.01)	0.32 (0.02)	0.03 (0.01)

10 μM concentrations inhibiting activity by only 10 to 30% (Fig. 5B). To see if the lack of inhibition by the new class could be generalized to V-ATPases from other fungi, we used vacuolar membrane vesicles from the yeast, *S. cerevisiae*. The yeast enzyme responded like the *N. crassa* enzyme; while 1 μM concanamycin A inhibited the yeast enzyme by 77%, 1 μM salicylihalamide A, lobatamide A, and oximidine I had no inhibitory effect (data not shown; the absence of inhibitors of F- and P-type ATPases in the assay mixture likely explains the lack of complete inhibition by concanamycin A). To further verify this unexpected specificity of inhibition, we used identical assay mixtures to concurrently test the effects of concanamycin A and salicylihalamide A against the *N. crassa* V-ATPase and the bovine chromaffin granule V-ATPase. In this experiment, concanamycin A (10 nM) inhibited both enzymes approximately 90%, while salicylihalamide A (10 nM) inhibited the mammalian enzyme 76% but had no effect on the fungal enzyme. These results confirmed that fungal and mammalian V-ATPases differed dramatically in their sensitivities to the new inhibitor class.

Benzolactone Enamides Are Inactive Against Other Membrane ATPases. The initial characterization (Bowman et al., 1988) of the effects of bafilomycins on membrane ATPases revealed that the macrocyclic antibiotics did not inhibit

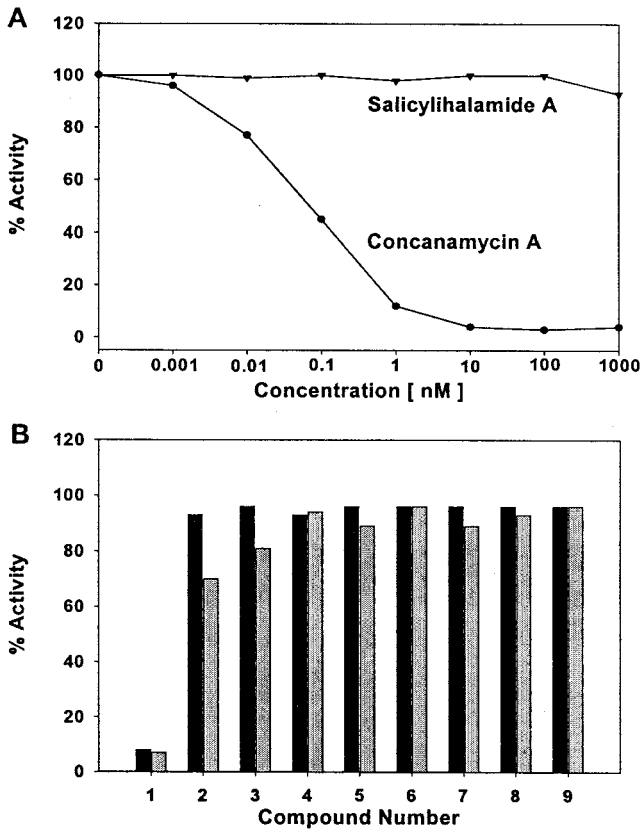

Fig. 5. The new benzolactone enamide inhibitor class has no effect on the fungal V-ATPase. A, V-ATPase activity in vacuolar membranes of *N. crassa* was assayed with 2 μg of membrane protein/reaction in the presence of 0 to 1000 nM concanamycin A (●) or salicylihalamide A (▼). Control specific activity (no inhibitor added) was 1.7 μmol/min/mg of protein. Multiple repeat experiments consistently confirmed essentially complete inhibition by concanamycin A at 1 and 10 μM but essentially no inhibition by 1 μM salicylihalamide A. B, V-ATPase activity was assayed with 2 μg of membrane protein/reaction in the presence of high concentrations of the new inhibitor class. Control specific activity was 2.7 μmol/min/mg of protein. Concanamycin A (1) was tested at 0.1 and 1.0 μM concentrations (black bar and gray bar, respectively). Salicylihalamide A (2), oximidine I (3), and lobatamides A–F (4–9) were tested at 1.0 and 10.0 μM concentrations (black bars and gray bars, respectively). The experiment was repeated, and essentially identical results were obtained (less than 6% difference for each point).

F-ATPases from mitochondria or *Escherichia coli* or the plasma membrane (H^+)-ATPase from *N. crassa*. However, at very high concentrations (about 10 μM), bafilomycins inhibited P-type ATPases from mammalian cells (Na^+/K^+-ATPase and Ca^{2+}-ATPase) and from *E. coli* (Kdp-ATPase) (Bowman et al., 1988). Accordingly, we tested effects of the new class on these other types of membrane ATPases. High (10 μM) concentrations of concanamycin A, salicylihalamide A, lobatamides A–F, and oximidine I had no effect on activity of the H^+-plasma membrane ATPase or of the F-type mitochondrial ATPase from *N. crassa*. Likewise, 10 μM concentrations of salicylihalamide A, lobatamides A–C, and oximidine I had no effect on the activity of the Na^+/K^+-ATPase from dog kidney. As expected, 1 μM concanamycin A showed a small (27%) inhibitory effect on this enzyme.

Discussion

Although definitive demonstrations of biochemically distinct mammalian or nonmammalian V-ATPase isoforms

have not yet been achieved, there is ample evidence of structural and functional variation among known V-ATPases to suggest the possibility for selective pharmacological modulation. Current V-ATPase structural models, composited from studies of both mammalian and nonmammalian V-ATPases, typically consist of a heteromultimeric protein complex configured in a "ball and stalk" architecture comprising at least 12 to 13 subunits (Finbow and Harrison, 1997; Stevens and Forgac, 1997; Nelson and Harvey, 1999). The "ball" part (V_1) is oriented into the cytoplasm and has the ATP-binding catalytic site; it is connected via the "stalk" to the integral membrane part (V_o) that comprises the proton-conducting channel. Structural features and evolutionary considerations suggest that the V-ATPases translocate protons by a rotational mechanism similar to that proposed for mitochondrial F-ATPases (Boekema et al., 1999). Notwithstanding the extant, generalized model for V-ATPases from fungi, plants, insects, and animals, there is evidence of considerable heterogeneity among V-ATPases, with substantial *inter*- and *intra*-species-specific differences in sequences of V-ATPase subunits. For example, recent reports describe three distinct isoforms of a murine 100-kDa V-ATPase subunit, multiple alternatively spliced variants of two of the isoforms, and tissue-specific expression of these isoforms (Nishi and Forgac, 2000; Toyomura et al., 2000). Given such heterogeneities and the multiplicity of ways that V-ATPase activity may be regulated (Finbow and Harrison, 1997; Stevens and Forgac, 1997; Nelson and Harvey, 1999), it is reasonable to expect that selective chemical modulation of V-ATPase functions should be feasible. However, most chemical inhibitors of V-ATPases thus far characterized are nonspecific for or among V-ATPases. The most common inhibitors that have been studied are nonselective alkylating or thiol reagents that can bind and inhibit many different enzymes (Farina and Gagliardi, 1999).

The first selective V-ATPase inhibitors described were the bafilomycins (Bowman et al., 1988). In that instance, selectivity was defined by the potent inhibitory activity against both mammalian and nonmammalian V-ATPases but relatively little or no inhibitory activity against Na^+/K^+-type or F-type ATPases. The structurally related concanamycins were subsequently described as V-ATPase inhibitors with selectivity similar to that of the bafilomycins (Dröse et al., 1993). During the past 10 years, compounds of the bafilomycin/concanamycin class have been used extensively as experimental probes of V-ATPase structure and function (Dröse and Altendorf, 1997; Gagliardi et al., 1999). There have also been some efforts to develop certain members of that class as anticancer agents; however, the compounds are highly toxic, presumably due to indiscriminate inhibition of V-ATPases (Farina and Gagliardi, 1999). Furthermore, the bafilomycin/concanamycin macrocyclic lactone class is structurally rather complex, thus compromising synthetic efforts to explore structure-activity relationships (SAR) or to identify smaller, less toxic, and potentially more selective derivatives. In spite of the synthetic intractability, remarkable progress has been reported recently on elucidation of SAR requirements for bafilomycin-like V-ATPase inhibition and the synthesis of small-molecule mimetics with promising selectivity for the human osteoclast V-ATPase (Gagliardi et al., 1998; Keeling et al., 1998; Farina and Gagliardi, 1999). The latter progress provided additional evidence of existence of a functionally distinct, differentially responsive mammalian V-ATPase isoform and is especially promising for further research and development of new treatments for osteoporosis. It also encourages a broader expansion of research focusing more generally on V-ATPase as a potential therapeutic molecular target for many different diseases, including cancer. A key to accelerating further advancements in this nascent field will be the identification of new, small-molecule V-ATPase inhibitor leads that are tractable for synthesis and that have greater inherent selectivity characteristics than previously known.

Thus far, 14 examples (Fig. 3) having the novel benzolactone enamide structure that characterizes the new V-ATPase inhibitor class (Fig. 1) have appeared in the literature. The first members of the class, isolated in the NCI coauthors' laboratory, were the salicylihalamides from the marine sponge *Haliclona* sp. (Erickson et al., 1997) and the lobatamides from the tunicate *Aplidium lobatum* (Galinis et al., 1997; McKee et al., 1998). A compound named YM-75518, identical to lobatamide A, was subsequently reported from the fermentation broth of a *Pseudomonas* sp. (Suzumura et al., 1997). Next, the compounds CJ-12,950 and CJ-13,357, which were potent inducers of the human low-density lipoprotein receptor gene, were described from the zygomycete *Mortierella verticillata* (Dekker et al., 1998). The cytostatic apicularens A and B were reported from culture extracts of the myxobacterium *Chondromyces robustus* (Kunze et al., 1998; Jansen et al., 2000). And most recently the oximidines I and II, which were first reported as selective inhibitors of oncogene-transformed cell lines, were isolated by the University of Tokyo coauthors from another *Pseudomonas* sp. (Kim et al., 1999). The diversity of sources of these natural products is remarkable. Although the salicylihalamides and lobatamides were isolated from marine macroorganisms, it seems probable that they are products of microbial inhabitants thereof. The natural functions or selective pressures leading to microbial biosynthesis of compounds of this class are unknown.

Our original hypothesis that the benzolactone enamides might represent a novel V-ATPase inhibitor class was based on our analyses of data from the NCI 60-cell screen (Boyd and Paull, 1995; Boyd, 1997). On this basis, we also correctly predicted that salicylihalamide A and lobatamide A, and likely other members of the class, shared with the oximidines the ability to inhibit selectively the growth of oncogene-transformed cell lines (e.g., Table 1). Furthermore, although neither the related CJ compounds nor the apicularens (Fig. 3) have yet been tested specifically against these oncogene-transformed cell lines or against V-ATPases, our initial testing of apicularen A (kindly provided by Prof. H. Reichenbach, G. Höfle, and colleagues, GBF, Braunschweig, Germany) in the 60-cell screen revealed a mean-graph profile essentially indistinguishable (Jansen et al., 2000) from those shown in Fig. 2, A through D, thus predicting these compounds are also V-ATPase inhibitors.

In conclusion, we have defined a new class of V-ATPase inhibitor having chemical structural features as well as V-ATPase inhibitory characteristics distinctly different from any previously known V-ATPase inhibitor family. The relative simplicity and likely synthetic tractability of the essential core structure (Fig. 1) bode well for identifying and optimizing V-ATPase isoform-selective inhibition and other

desirable pharmacological attributes and minimizing undesirable properties for drug development. Reinforcing this feasibility are recent reports of total synthesis of salicylihalamide A (Wu et al., 2000) and progress toward total synthesis of lobatamides (Shen and Porco, 2000) and the oximidines (Kuramochi et al., 2000). Synthetic access to diverse compounds of this class, including novel research probes containing affinity labels, radiolabels, and the like, will also facilitate exploration of functional and architectural features of mammalian versus nonmammalian V-ATPases. Future molecular modeling studies and synthetic modifications will help further clarify the minimal essential requirements for V-ATPase-inhibitory activity and will enable systematic exploration of potential isoform selectivity thereof. Already the importance of the enamide side chain is indicated by the fact that zearalenone, a macrocyclic lactone structurally similar to salicylihalamide A but lacking the enamide, was devoid of V-ATPase-inhibitory activity (data not shown). The simplest starting point of interest for modeling and SAR investigations of the core structure (Fig. 1) may be a compound wherein Z = 0 (i.e., the linker therefore comprising a direct bond, forming a five-membered ring) and R^1 = H. Finally, although the present data do not necessarily imply a direct causal relationship between the V-ATPase-inhibitory activity and antitumor properties of the benzolactone enamide class, availability of these compounds will facilitate further exploration of the validity of V-ATPase as a molecular target for cancer therapeutics.

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