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Review

Structure and function of V-ATPases in osteoclasts: potential therapeutic targets for the treatment of osteolysis

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Summary. Excessive activity of osteoclasts becomes manifest in many common lytic bone disorders such as osteoporosis, Paget's disease, bone aseptic loosening and tumor-induced bone destruction. Vacuolar proton pump H^+-adenosine triphosphatases (V-ATPases), located on the bone-apposed plasma membrane of the osteoclast, are imperative for the function of osteoclasts, and thus are a potential molecular target for the development of novel anti-resorptive agents. To date, the V-ATPases core structure has been well modeled and consists of two distinct functional domains, the V_1 (A, B1, B2, C1, C2, D, E1, E2, F, G1, G2, G3, and H subunits) and V_0 (a1, a2, a3, a4, d1, d2, c, c' e1, e2 subunits) as well as the accessory subunits ac45 and M8-9. However, the exact configuration of osteoclast specific V-ATPases remains to be established. Inactivation of subunit a3 leads to osteopetrosis in both mice and man because of non-functional osteoclasts that are capable of acidifying the extracellular resorption lacuna. On the other hand, inactivation of subunits c, d1 and ac45 results in early embryonic lethality, indicating that certain subunits, such as a3, are more specific to osteoclast function than others. In osteoclasts, V-ATPases also cooperate with chloride channel protein CLC-7 to acidify the resorption lacuna. In addition, development of V-ATPases inhibitors such as bafilomycin A1, SB 242784 and FR167356 that selectively target osteoclast specific V-ATPases remains a challenge. Understanding the molecular and cellular mechanisms by which specific subunits of V-ATPase regulate osteoclast function might facilitate the development of novel and selective inhibitors for the treatment of lytic bone disorders. This review summarizes recent research developments in V-ATPases with particular emphasis on osteoclast biology.

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Introduction

Osteoclasts are the principal cells responsible for bone resorption and are derived from hematopoietic mononuclear cells (Teitelbaum, 2000). Many common lytic bone disorders such as osteoporosis, bone aseptic loosening and tumor-induced bone destruction are correlated to elevated osteoclast activity (Zheng et al., 2001; Phan et al., 2004). The main physiological function of osteoclasts is to degrade the mineralized bone matrix, a process involving the dissolution of the crystalline hydroxyapatite followed by the proteolytic cleavage of the collagen rich organic matrix (Vaananen et al., 2000). The dissolution of minerals is achieved by the targeted secretion of hydrochloric acid through the ruffled border into the resorption lacunae, which has been suggested to be the only process capable of solubilizing the hydroxyapatite crystals in the biological environment (Blair et al., 1989). As shown in Figure 1, osteoclastic bone resorption is a continuous and dynamic process characterized by four stages: 1) attachment of osteoclasts to the bone surface; 2) polarization of osteoclastic membrane and; 3) degradation and resorption of bone matrix; 4) Transportation of degraded bone matrix products via transcytotic carrier vesicles (TCV) from the ruffled border to the functional secretory domain (FSD) and released into the extracellular environment. During the process of polarization, osteoclasts form three discrete areas of plasma membrane. First, the basolateral membrane, which faces marrow space and is not in contact with bone; second, the tight sealing zone, which is closely opposed to the bone matrix; and third, the ruffled border, which is surrounded by the sealing zone. The culmination of these cytoskeletal and membrane changes results in the

formation of an isolated compartment between bone and the cytoplasmic protrusions of the osteoclastic ruffled border. This discrete compartment, surrounded by the sealing zone and known as the resorption lacuna, provides the acidic microenvironment for osteoclastic bone resorption. The generation of an acidic environment within the resorption lacuna by V-ATPases present in the ruffled border leads to the demineralisation and subsequent degradation of the bone matrix by cathepsin K and matrix metalloproteinases (MMPs) (Henriksen et al., 2006). Thus V-ATPases may potentially be a therapeutic target for the development of anti-resorptive agents.

The function and structure of V-ATPases in mammalian cells

The vacuolar H^+-adenosine triphosphatases (H^+-ATPase/V-ATPase) appear to be the most versatile proton pumps in nature (Nishi and Forgac, 2002). V-ATPases are primarily responsible for the acidification of intracellular compartments in all eukaryotic cells (Stevens and Forgac, 1997; Forgac, 1999). In addition, V-ATPases play an fundamental role in many cellular processes including endocytosis, intracellular membrane traffic, macromolecular processing and degradation, and ligand-coupled transport (Stevens and Forgac, 1997; Forgac, 1999). Recent studies have shown that V-ATPase interacts with small GTPase Arf6 (ADP-ribosylation factor 6) and its cognate GDP/GTP exchange factor ARNO (ADP-ribosylation factor nucleotide site opener) in early endosomes and regulates the protein degradation pathway, pointing to the role of V-ATPase as an essential component of the endosomal pH-sensing machinery (Hurtado-Lorenzo et al., 2006). In phagocytic cells such as macrophages, V-ATPases are known to mediate cytoplasmic pH homeostasis and acidification, both of which are crucial to phagocytosis and subsequent microbial degradation (Grinstein et al., 1992; Swallow et al., 1993). In renal intercalated cells, V-ATPases function in the acidification of urine (Smith et al., 2000), whilst in tumor cells, V-ATPases are targeted to the plasma membrane where they create an acidic extracellular environment that facilitates tumor metastasis (Martinez-Zaguilan et al., 1993). In osteoclasts, V-ATPases are required for the acidification of the space that is in contact with the bone surface, a biological process that is essential for bone resorption (Li et al., 1999; Frattini et al., 2000). It is clear that V-ATPases serve a wide variety of roles in mammalian cells.

As shown in Fig. 2, the molecular structure of the various subunits of the V-ATPase pump is highly complex. Recent structural studies have revealed that the core structure of V-ATPase is essentially composed of two functional domains, denoted V_1 and V_0 (Stevens and Forgac, 1997; Forgac, 1999). The V_1 domain is responsible for hydrolysis of ATP and is a 570 kDa complex, consisting of eight different subunits, A-H, with a molecular mass between 14 to 70 kDa. On the other hand, the V_0 domain functions as the proton translocation unit located across the limiting membrane. The V_0 consists of a 260 kDa integral complex which is made of several distinct subunits of molecular weight 11 to 100 kDa (subunit a, d, and c), with six copies of the c subunits and single copies of a, c' and d subunits (Arai et al., 1988). In addition, accessory subunits Ac45 and M8-9 are also present in mammalian cells but absent in yeast (Supek et al., 1994; Ludwig et al., 1998; Demirci et al., 2001). Table 1 summarizes the systemic nomenclature of mouse homologues of various subunits of V-ATPases (Smith et al., 2003), their predicted function and observed knockout phenotypes.

Structure and Function of the V_1 subunits

The V_1 domain is the cytoplasmic portion of V-ATPase and comprises the catalytic nucleotide-binding subunit A and the non-catalytic nucleotide-binding subunit B in a stoichiometry of A_3:B_3 and the stalk subunits C-H in a proposed stoichiometry of C_1:D_1:E_1:F_1:G_2:H_1 (Forgac, 1999; Xu et al., 1999). The 70 kDa A and 60 kDa B subunits are arranged together to form a hexamer responsible for ATP hydrolysis.

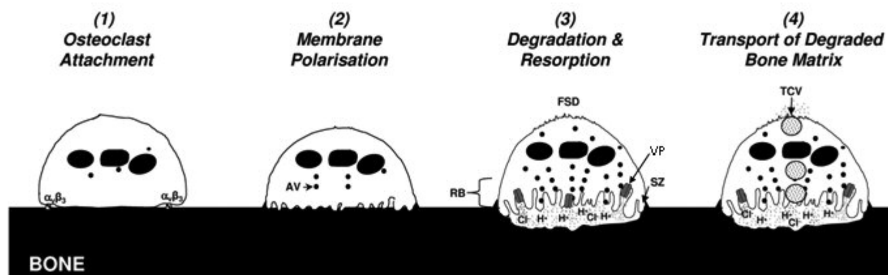

Fig. 1. Stages of osteoclastic bone resorption: (1) The osteoclast initiates resorption through attachment to calcified bone surfaces via actin microfilaments and $\alpha v_{–3}$ integrins, specific for bone matrix proteins possessing RGD sequences. (2) Upon attachment, osteoclasts undergo membrane polarisation through the formation of intracellular acidified vesicles (AV), which rapidly fuse to the bone-facing surface to create a ruffled membrane domain. The ruffled border (RB) is essentially, the "resorbing organ" and is enriched with v-ATPase proton pumps (VP). (3) During bone degradation and resorption, a sealing zone (SZ) seals off the acidic microenvironment generated by the release of protons (H^+) and chorlide ions (Cl^-) and electrochemical homeostasis is maintained through the basolateral domain. (4) Degraded bone matrix products are then endocytosed and transported via transcytotic carrier vesicles (TCV) from the ruffled border to the functional secretory domain (FSD) and released into the extracellular environment.

Mutational studies in yeast have revealed that the catalytic nucleotide binding sites located on the A subunit is critical for V-ATPase activity, whereas the non-catalytic sites on the B subunit might have a regulatory function (Liu et al., 1996; MacLeod et al., 1998; Vasilyeva et al., 2000).

The central rotor is composed of subunits D and F, whereas the peripheral stator is made of the C, E, G and H subunits (Xu et al., 1999; Arata et al., 2002). Based on cross-linking studies, the peripheral stator appears to connect the A/B hexamer to the V_0 complex (Arata et al., 2002; Lu et al., 2002). It has been shown that subunit D and F subunits exhibit ATP dependent rotation relative to the A/B hexamer (Imamura et al., 2003) as well as relative counter-clockwise rotation of the V_1 G and V_0 c subunits (Hirata et al., 2003). In addition, the D and F subunits have also been shown to be closely associated and exist as heterodimers independent of the V-ATPase complex (Arata et al., 2002; Nishi and Forgac, 2002). Collectively, ATP hydrolysis by the A/B hexamer drives

Table 1. Summary of mouse V-ATPase subunit genes.

Subunits	Gene Name	Synonyms	MW in kDa	Genbank No.	Yeast gene	Proposed Function/Location	Knock Out Phenotype
V_1 Peripheral Sector							
A	ATP6V1A	Atp6a2, VPP2 Atp6v1a1, VA68	70	NM_007508	VMA1	Catalytic ATP binding	
B1	ATP6V1B1	Vpp3,Vpp-3, Atp6b1	56	NM_134157	VMA2	Noncatalytic ATP binding	Normal hearing (Dou et al., 2003)
B2	ATP6V1B2	HO57, Atp6b2, R74844, U13839		NM_007509			
C1	ATP6V1C1	VATD	42	NM_025494	VMA5	Peripheral stator	
C2	ATP6V1C2	Vma8		NM_133699			
D	ATP6V1D	Atp6m, E2, P31 Vma4, Atp6e	34	NM_023721	VMA8	Central rotor	
E1	ATP6V1E1	Atp6e2, E1, Atp6e1 Atp6v1e	31	NM_007510	VMA4	Peripheral stator	
E2	ATP6V1E2			NM_029121			
F	ATP6V1F		14	NM_025381	VMA7	Central rotor	
G1	ATP6V1G1	VAG1, ATP6J, Vma10, Atp6g1	13	NM_024173	VMA10	Peripheral stator	
G2	ATP6V1G2	NG38, VAG2, Atp6g2		NM_023179			
G3	ATP6V1G3			NM_177397			
H	ATP6V1H	SFD, VMA13 CGI-11, SFDbeta	50	NM_133826	VMA13	Peripheral stator	
V_0 Membrane Sector							
a1	ATP6V0A1	Atp6n1, vpp1 Atp6n1a, ATP6a1	100-110	NM_016920	VPH1/STV1	Peripheral stator and H^+ Translocation	
a2	ATP6V0A2	Atp6n2, ATP6a2 Atp6n1d, Tj6, Stv1		NM_011596			
a3	TCIRG1	Oc116, atp6i		AB022322			Ostropetrosis (Li et al., 1999)
a4	ATP6V0A4	Atp6n1b		NM_080467			
d1	ATP6V0D1	Atp6d, p39 Ac39, VATX, Vma6	38-42	NM_013477	VMA6	Nonintegral membrane component	Embryonic development (Miura et al., 2003)
d2	ATP6V0D2			NM_175406			
c	ATP6V0C	Atp6l, Atp6c, Atp6c2 Atpl, PL16, Atpl-rd1 VATL, Vma3	16	NM_009729	VMA3	H^+ translocation	Early embryonic lethality (Inoue et al.,1999)
c'	ATP6V0B	Atp6f, VMA16	21	NM_033617	VMA16	H^+ translocation	
e1	ATP6V0E	M9.2, Atp6k	9	NM_025272	VMA21	Membrane sector-associated	
e2	ATP6V0E2	NM9.2		NM_133764		Membrane sector-associated	
Accessory Subunits							
ac45	ATP6AP1	Atpip1, XAP-3, ac45 VATPS1, Atp6s1, C7-1	45	NM_018794	None	Accessory subunit	Early embryonic development (Schoonderwoert et al., 2002)
M8-9	ATP6AP2	M8-9, Atp6ip2, ATP6M8-9, APT6M8-9	8-9	NM_027439	None	Accessory subunit	

Modified from Smith et al., 2003.

rotation of the central rotor and peripheral stalk of the V_1 domain, which in turns drives influx of protons into the cytoplasmic vesicle or extracellular space via the V_0 domain. Other structural features of the V_1 domain subunits with specific implications for function are highlighted in Table 1.

Recent studies have revealed that two V_1 subunits, B and C, bind to F-actin, suggesting that these subunits may function as an anchor protein regulating the linkage between V-ATPase and the actin-based cytoskeleton (Holliday et al., 2000; Vitavska et al., 2003). The E subunit have also been found to associate with aldolase (Lu et al., 2001). Aldolase colocalizes with V-ATPase in the osteoclast and is distributed at the ruffled border in activated osteoclasts. Deletion of the aldolase gene in yeast results in reduced cellular V-ATPase and dissociation between the V_1 and V_0 domains. This suggests that the E subunit's interaction with aldolase is important in V-ATPase activity and may exhibit regulatory roles. The E subunit has also been implicated in the regulation of the mSos1-dependent Rac1 signaling pathway involved in growth factor receptor-mediated cell growth control (Miura et al., 2001). Therefore identification of association between V-ATPase subunits and other cellular proteins may help to unravel novel regulatory pathways of V-ATPases.

Structure and function of the V_0 subunits

The integral membrane bound V_0 domain of the V-ATPase complex is connected to the V_1 domain by the central and peripheral stalks. In mammals, it consists of a, c, c" and d subunits. The more extensively studied yeast version of the V_0 domain contains a, c, c', c" and d subunits in the stoichiometric ratio of a_1:c_5:c'_1:c''_1:d_1 (Arata et al., 2002a,b; Wilkens and Forgac, 2001; Nishi and Forgac, 2002; Forgac, 1999). The largest subunit (subunit a) of 100 kDa of the V_0 is a transmembrane glycoprotein displaying characteristics of an N-terminal hydrophilic domain and a C-terminal hydrophobic domain with multiple potential transmembrane helices (Perin et al., 1991). The second largest single subunit (subunit d) of 38 kDa is a hydrophilic protein containing no membrane helices (Nelson, 1992) and has been found to be tightly associated with V_0 (Adachi et al., 1990). The smallest subunits (subunits c and c') are highly hydrophobic proteins of 17-19 kDa with characteristics of proteolipids (Forgac 1999). The V_0 domain contains the channel for proton translocation and it has been suggested that proton translocation occurs by rotation of the proteolipid c ring, relative to the A/B hexamer (Imamura et al., 2003; Yokoyama et al., 2003). The V_0 domain may also participate in membrane fusion (Peters et al., 2001; Bayer et al., 2003).

The a subunit, the largest of the V_0 subunits, is though to be a two-domain protein as previous reports have shown that the amino-terminal hydrophilic domain of the a subunit interacts with both subunit A and subunit H of the V_1 domain and thus been suggested to form part of the peripheral stator connecting V_1 and V_0 (Xu et al., 1999; Landolt-Marticorena et al., 2000). It is also predicted that the hydrophobic carboxyl-terminal component of the a subunit contains nine transmembrane segments (Leng et al., 1999) harbouring a number of charged residues that may important for its function. Arginine 735 of transmembrane segment 7 however, has been shown to be absolutely required for proton transport (Kawasaki-Nishi et al., 2001). Two a subunit isoforms exists in yeasts, Vph1p and Stv1p, and are associated with vacuolar and Golgi/endosomal membranes respectively (Manolson et al., 1994). In mammalian cells, four isoforms (a1, a2, a3 and a4) of

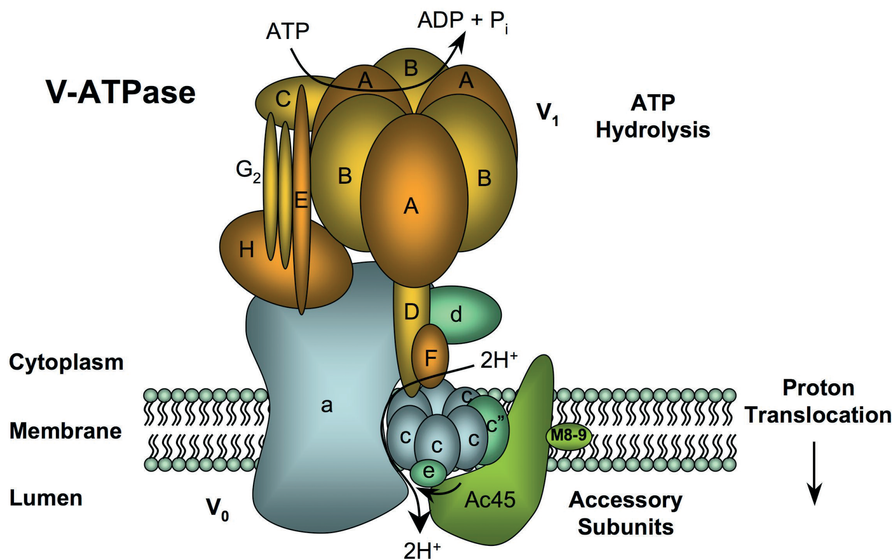

Fig. 2. Schematic depiction of the subunit structure of mammalian V-ATPases. The V-ATPase complex is composed of a peripherally located V_1 domain and a integral membrane associated V_0 domain. The V_1 domain consists of eight different subunits (A – H) and is responsible for ATP hydrolysis. In yeast the V_0 domain consists of five different subunits (a, c, c', c" and d) and functions in proton translocation. Mammalian V-ATPases lacks the c' subunit. Recently, another integral membrane subunit designated e was identified in both mammalian and yeast V-ATPase and is likely to be an important component of the V-ATPase complex in all eukaryotes. Furthermore two accessory subunits, Ac45 and M8-9 also exists but their structural location and association with other subunits remains to be determined. (Image modified from Arata et al., 2002).

the 100-kDa subunit have been identified (Nishi and Forgac, 2000; Toyomura et al., 2000) with each isoforms exhibiting specific organellar and cellular distributions and thus generating V-ATPases with possible differential functional properties. For example, the V-ATPase V_0 subunit a1 isoform was shown to localize specifically to nerve terminals and interacts with fusion proteins SNAREs in the fish Torpedo marmorata (Morel et al., 2003) and more recently found to be required for a late step in synaptic vesicle exocytosis in *Drosophila* (Hiesinger et al., 2005).

The c, c' and c" subunits are termed proteolipids because of their high hydrophobic nature (Hirata et al., 1997). The yeast c and c' subunits are both 16 kDa and contain four putative transmembrane helices (TM1 to TM4) with a crucial Glu residue in TM4 essential for proton transport. The c" subunit is a 23 kDa subunit containing five transmembrane segments with the crucial acidic residue in TM3 (Hirata et al., 1997). Together these units make up a proteolipid ring, although the exact configuration of this ring is uncertain. In yeast, there are likely to be five copies of the c subunit, one copy of the c' and c" subunits (Arata et al., 2002; Nishi and Forgac, 2002). However, the arrangement of c and c" subunits in mammalian cells is unclear. The yeast c ring differs to the mammalian ring, in that the mammalian c ring is not known to contain a c' subunit (Arata et al., 2002; Nishi and Forgac, 2002). The significance of this remains to be determined. In addition to proton transport, the c subunit has been implicated in cell-cell communication as a component of gap junctions as well as in neurotransmitter release as a component of the mediatophore (Finbow et al., 1992). Furthermore, recent studies have shown that the proteolipid ring in conjunction with other V_0 subunits forms a homotypic end-to-end associations between two fusing membranes, and is thought to be the fusion pore first detected as a transient high-conductance channel (Peters et al., 2001; Bayer et al., 2003; Hiesinger et al., 2005). Gene knock-out studies has shown that the targeted disruption of the gene encoding the proteolipid subunits of the mouse vacuolar H(+)-ATPase leads to early embryonic lethality (Inoue et al., 1999).

The interaction between a subunit and c/c" subunits in mammalian V-ATPase is complex and remains to be fully unraveled. Most studies are based on interactions in lower order eukaryotes and comparative modeling based on a similar group of proton transporters, the ATP synthases (F-ATPase). Comparison with F-ATPase led to the proposal of a rotary mechanism. In this model hydrolysis of ATP is thought to cause rotation of the c ring relative to the a subunit (Forgac, 1999; Yokoyama et al., 2003). The a subunit is held stationary through its association with the peripheral stalk and A/B hexamer. This rotation opens two hemi-channels allowing protons to access and be released from buried carboxyl groups in the c subunits. One hemi-channel is exposed on the cytoplasmic side and the other is exposed on the luminal or extracellular side. This rotation mechanism has recently been confirmed in two studies using similar methods (Hirata et al., 2003; Yokoyama et al., 2003). Yokoyama et al. fixed the A/B hexamer to specifically prepared glass and used streptavidin to couple a bead to the c ring. It was observed that V-ATPase activity causes rotation of the c ring and thus rotation of the visible bead (Yokoyama et al., 2003).

The function of d subunit is not well documented but has been suggested to play a role in coupling proton transport and ATP hydrolysis (Nishi et al., 2003), and in embryonic development (Miura et al., 2003). Disruption of the yeast homologue VMA6 results in the inability of the yeast cells to acidify intracellular compartments due to an impairment in the assembly of the intact V_1V_0 complex as well as the V_0 domain itself (Bauerle et al., 1993). Studies have shown that the subunit d is a hydrophilic, non-membrane bound protein that is connected to the V_0 domain by contact with other V_0 subunits (Adachi et al., 1990). A secondary isoform of d, designated d2, has been identified and exhibits specific tissue distribution in comparison to the ubiquitously expressed d isoform (Smith et al., 2002). Together with the a subunit, the d subunit forms a cuff around the hydrophobic c/c" subunits (Arata et al., 2002).

The e subunit has been identified as a 9.2kDa subunit in bovine studies (Ludwig et al., 1998). The e subunit bears relatively close similarity to the yeast V-ATPase assembly protein Vma21p, sharing 45% similarity and 19% identity. However, the yeast Vma21p protein only contributes to V-ATPase assembly and is not actually associated with V-ATPase. On the other hand, the e subunit was found to be associated with both the V_0 and V_1V_0 complex providing evidence that this subunit is integrated into the final V-ATPase complex (Ludwig et al., 1998). Most recent studies using genome-wide screening lead to the identification of Vma9p, the yeast homologue of the mammalian e subunit. Vma9p is an integral membrane V_0 subunit of the yeast V-ATPase and is required for the stable assembly of the V_0 domain as well as assembly of the V_1V_0 complex (Sambade and Kane, 2004; Compton et al., 2006). Furthermore, the fus-1 gene encoding the e subunit in *C. elegans* was shown to be essential for the repression of epidermal cell fusion and the loss of this subunit as well as other V-ATPase subunits causes widespread hyperfusion indicating possible roles for V-ATPase subunits in cellular fusion (Kontani et al., 2005).

Structure and function of the accessory subunits

In addition to the V_1 and V_0 subunits, accessory subunits have been discovered in mammalian cells. Ac45 was initially identified from bovine adrenal chromaffin granular V-ATPase as an accessory subunit associated with the V_0 domain (Supek et al., 1994) and currently no homologue counterpart have been identified in yeast (Stevens and Forgac, 1997). Structural analysis has shown that the C-terminus of Ac45 carries a 26 residue-cytoplasmic tail that contains autonomous

internalization signals that are necessary for the regulation of essential routing information (Jansen et al., 1998). Recent knockout studies in mice have found that the Ac45 null mutation impairs normal development of the blastocyst, indicating an essential role of Ac45 in early embryonic development (Schoonderwoert and Martens, 2002). Another membrane sector-associated protein M8-9 was identified from the vacuolar proton-translocating ATPase of bovine chromaffin granules, but the role of this M8-9 protein remains to be elucidated (Ludwig et al., 1998; Demirci et al., 2001).

The role of V-ATPase subunits in osteoclast function

As previously indicated, osteoclasts are capable of generating an acidic microenvironment necessary for bone resorption by utilizing V-ATPases to pump protons into the resorption lacuna. This acidification process is mediated by the clustering of many V-ATPase proton pumps towards the bone apposed plasma membrane or ruffled border of active osteoclasts during bone resorption (Baron et al., 1985; Blair et al., 1989; Baron, 1989). There is increasing evidence to suggest that the acidification of the millieu near the ruffled membrane border as well as within several intracellular endocytic/lysosomal organelles is vital for the endocytotic and transcytotic transport in osteoclasts (Baron et al., 1985; Baron, 1989; Vaananen et al., 2000). Using the specific V-ATPase inhibitor bafilomycin A_1, V-ATPases have been implicated to play a role in osteoclast apoptosis and endocytosis (Xu et al., 2003). In addition to its role in bone degradation in mature osteoclasts, evidence has been presented that expression of V-ATPases is involved in osteoclastogenesis. Antisense RNA to the V-ATPase V_1 subunits have been shown to abolish the generation of osteoclast-like cells (Laitala-Leinonen et al., 1999).

The V_1 subunit B2 isoform has been shown to be expressed at the ruffled border of osteoclasts (Lee et al., 1996), and has been suggested to be translocated from the cell interior to a special domain of the ruffled membrane close to the sealing zone (Mattsson et al., 1997). Both isoforms of the B subunit share an actin microfilament binding site at their amino termini (Chen et al., 2004). Interaction with actin via the B subunit could be an important mechanism for the intracellular trafficking and localization of the V-ATPase complex to various subcellular compartments and membranes. This is supported by the observation that the introduction of cytochalasin D, an actin depolymerizer, inhibits localization of V-ATPase to the plasma membrane in osteoclasts (Holliday et al., 2000; Chen et al., 2004). Therefore it is likely that binding of V-ATPase to the actin cytoskeleton is an important process for the trafficking of the V-ATPase complex from subcellular membrane compartments towards the plasma membrane into the ruffled border of osteoclasts during bone degradation. On the other hand, human mutations in the gene encoding B1 subunit of H^+-ATPase has been linked with renal tubular acidosis and sensorineural deafness (Karet et al., 1999; Stover et al., 2002). However, recent studies have found that mice lacking the B1 subunit of H^+-ATPase appears to have normal hearing, and B1 is not required for normal inner ear development or normal inner ear function in mice (Dou et al., 2003).

The crucial role of V-ATPase in osteoclastic bone resorption is perhaps exemplified by targeted disruption studies in mice. Disruption of V_0 subunit ATP6I gene, encoding the a3 subunit of the V_0 sub-complex, (also termed OC-116kD) in mice results in severe osteopetrosis. Osteoclasts derived from a3 null mice have impaired extracellular acidification but are able retain the ability to maintain intracellular pH homeostasis (Li et al., 1999). Interestingly, osteoclasts with 10 or more nuclei have been shown to exhibit higher expression of the a3 subunit as compared with osteoclasts with five or fewer nuclei (Manolson et al., 2003), suggesting the importance of the a3 subunit in osteoclast maturation. Mutations in the human ATP6I gene have been shown to be responsible for a subset of human malignant infantile osteopetrosis, a genetically heterogeneous autosomal recessive disorder of bone metabolism (Sobacchi et al., 2001), further highlighting the importance of V-ATPases in osteoclast formation and bone resorption.

Increasing data regarding the expression, regulation, and role of individual V-ATPase subunits in osteoclastogenesis and bone resorption are emerging. Recent work in our laboratory has identified abundant gene transcripts of V_0 subunits c, c', d1, d2, e1, e2 and Ac45 in osteoclasts (Xu, et al., unpublished data), indicating their potential involvement in osteoclast function.

The V-ATPase proton pump of the osteoclast ruffled membrane is suggested to be tightly coupled to a passive chloride channel (Blair et al., 1989). More interestingly, mice deficient for the ubiquitously expressed chloride channel protein ClC-7 have been found to exhibit severe osteopetrosis and retinal degeneration (Kornak et al., 2001; Kasper et al., 2005; Lange et al., 2006). Furthermore, mutations in the gene encoding ClC-7 have been identified in patients with human infantile malignant osteopetrosis (Kornak et al., 2001; Henriksen et al., 2004), indicating that the chloride conductance is required for efficient proton pumping by V-ATPases at the osteoclast ruffled membrane (Kornak et al., 2001). More recently, ClC-7 was found to form complexes with OSTM1, which acts as a beta-subunit of ClC-7, and mutations in the Ostm1 gene lead to severe osteopetrosis, resembling the ClC-7-deficient mice (Lange et al., 2006). It remains to be seen whether chloride channels serve as a potential drug target for anti-resorptive agents.

V-ATPase inhibitors for osteoclast function

In light of the fact that osteolysis occurs in several systemic and local skeletal and joint diseases, and the

importance of V-ATPase-mediated acidification during osteoclastic bone resorption, therapeutic interventions have been proposed that specifically target inhibition of the osteoclast proton pump. Modulation of osteoclastic V-ATPase activity has been considered to be a suitable therapy for the treatment of osteoporosis, Paget's disease, and various manifestations of skeletal cancer (Farina and Gagliardi, 1999). Table 2 lists a number of V-ATPase inhibitors with their potential targets and various chemical structures (Fig. 3). It remains a challenge to identify specific V-ATPase inhibitors for osteoclast function.

Initial steps in developing inhibitors for V-ATPases involve the structural analysis of bafilomycin A_1 (Werner et al., 1984). Bafilomycin A_1 is a macrolide antibiotic with potent inhibitory effect on all V-ATPases in vitro and in vivo. Bafilomycin A_1 is able to inhibit bone resorption both in vitro (Sundquist et al., 1990; Chatterjee et al., 1992) and in vivo (Sundquist and Marks, 1994) as well as exerting potent inhibitory effect on endocytosis and apoptosis of osteoclasts and their precursor cells (Xu et al., 2003). However, being a non-specific inhibitor of all V-ATPases, bafilomycin could exhibit unacceptable levels of systemic toxicity, thus making its use as anti-resorptive agent unfeasible. Therefore, for the treatment of excess bone resorption, it is necessary to modify the structure of bafilomycin to confer higher selectivity for the osteoclast V-ATPase enzyme (Farina and Gagliardi 1999).

In addition to bafilomycin A_1, concanamycin A is another potent macrolide V-ATPase inhibitor that is structurally related to bafilomycin A_1(Kinashi et al., 1982, 1984). Previous data has strongly implicated subunit c of the V-ATPase V_0 in binding of the specific

Table 2. Summary of V-ATPase inhibitors.

V-ATPase Inhibitors	Acting targets	Sources	Reference
Bafilomycin A1	Vo a subunit	Streptomices species	Werner et al., 1984
Concanamycin A	Vo c subunit	Streptomices species	Kinashi et al., 1984
SB 242784	Osteoclast V-ATPase	Bafilomycin derivatives	Gagliardi et al., 1998
FR167356	Osteoclast V-ATPase	Novel chemical structure	Niikura et al., 2004
Salicylihalamide A	V-ATPase	Benzolactone enamide core structure	Snider and Song 2000
Lobatamides	V-ATPase	Benzolactone enamide core structure	Boyd et al., 2001,
Oximidines	V-ATPase	Benzolactone enamide core structure	Boyd et al., 2001
Destruxin B	V-ATPase	Fungus Metarhisium anisopliae	Muroi et al., 1994
Omeprazole	Gastric H^+/K^+-ATPase	Thiol reagents	Fellenius et al., 1981

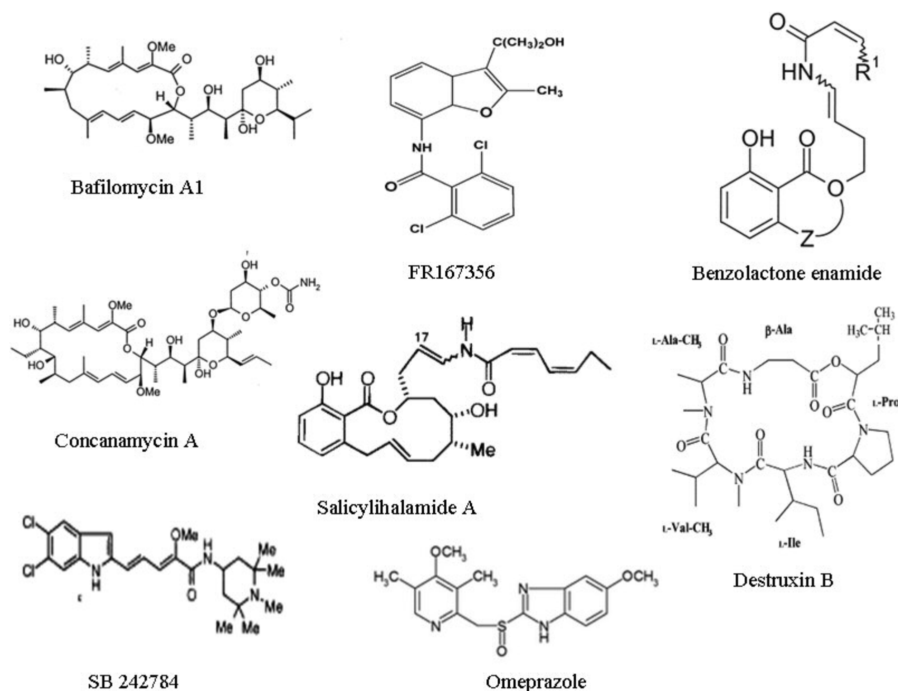

Fig. 3. Chemical structures of V-ATPases inhibitors.

inhibitors bafilomycin and concanamycin which act by preventing the rotation of the c subunits (Huss et al., 2002; Bowman et al., 2004). Subunit a of V-ATPase participates along with subunit c in binding bafilomycin (Zhang et al., 1994; Bowman and Bowman, 2002; Wang et al., 2005), but the contribution of the c ring to the bafilomycin binding site is quantitatively greater than the contribution of subunit a (Wang et al., 2005). Mutation studies indicate that residues in subunit a (or subunit c) that are not involved in binding of bafilomycin are likely to participate in binding of concanamycin, and that the concanamycin binding site has unique determinants that are not altered by changes in the bafilomycin site (Wang et al., 2005).

The majority of V-ATPase inhibitors that selectively target osteoclast V-ATPase have been developed from bafilomycin A_1. The observation that subunit a3 is expressed specifically in osteoclasts led to investigation of subunit a as a potential target for osteoclast specific inhibitors. Bafilomycin is able to bind the a subunit, so it would appear a logical starting point for such drugs. The most significant advance thus far, is the development of SB242784, ((2Z,4E)-5-(5,6-dichloroindolyl)-2-methoxy-N-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide), which shows a high degree of selectivity and potency in inhibiting osteoclast V-ATPase (Gagliardi et al., 1998;Visentin et al., 2000). SB242784 has been shown to completely prevent retinoid-induced hypercalcaemia in thyroparathyroidectomised rats (Visentin et al., 2000). The protective effect of SB 242784 on preventing bone loss was comparable to an optimal dose of estrogen (Visentin et al., 2000). In addition, SB 242784 had a greater than 1000-fold selectivity for the osteoclast V-ATPase compared with those measured in the kidney, liver, spleen, stomach, brain or endothelial cells and has no effect on other cellular ATPases (Visentin et al., 2000). Evaluation of SB 242784 toxicity in ovariectomised rats during a 6-month treatment program recorded no overt toxic effects on urinary acid excretion (Visentin et al., 2000). Since V-ATPases located on the plasma membrane in kidney tubules participates in urinary acidification, this finding confirms in vivo selectivity. The efficacy of this compound has been shown to be exceptional via measurements of BMD, biochemical markers of bone resorption and histomorphometry (Visentin et al., 2000). However, the molecular mechanism of SB 242784 selectivity remains to be elucidated.

FR167356, or 2,6-dichloro-N-[3-(1-hydroxy-1-methylethyl)-2-methyl-7-benzofuranyl]benzamide, is a compound that was obtained from random screening using osteoclast microsomes and chemical modifications of a parental hit compound imidazopyridine to benzofuranyl. FR167356 inhibits H^+ transport in plasma membrane vesicles of murine osteoclasts (IC50 170 nM) and chicken osteoclast microsomes (IC50 220 nM), but exerts no effect on H^+ transport of mitochondrial ATPase (F-ATPase) or gastric H^+,K^+-ATPase (P-ATPase) (Niikura et al., 2004). Further studies have shown that FR167356 prevents bone resorption in ovariectomized rats and alveolar bone destruction in experimental periodontitis models (Niikura et al., 2005a,b).

The salicylihalamides, lobotamides and oximidines are three groups of V-ATPase inhibitors that are able to inhibit all mammalian V-ATPases, but show little or no activity against other species (Farina and Gagliardi, 2002; Wu et al., 2002). These compounds share a benzolactone enamide core, which may explain there unusual selectivity (Snider and Song, 2000; Boyd et al., 2001). Salicylihalamide A inhibits the V_0 sector of the V-ATPase through a mechanism distinct from bafilomycin A_1 (Xie et al., 2004).

Other naturally occurring inhibitors of V-ATPase include Destruxin B, which was originally isolated from the fungus *Metarhisium anisopliae* (Muroi et al., 1994). In addition, thiol reagents represent another group of general inhibitors of V-ATPase; these include omeprazole, nitrobenzothiazole, nitrobenzimidazole, methobenzoxazole. It has been shown that omeprazole inhibits gastric H^+/K^+-ATPase at low concentrations, and V-ATPase at higher concentrations (Fellenius et al., 1981; Elander et al., 1986), suggesting that these compounds are not selective for osteoclast V-ATPase.

Taken together, the essential role of V-ATPase in bone resorption provides a prospect for pharmacological intervention in the treatment of osteolytic disorders. The emergence of novel V-ATPase inhibitors may ultimately lead to the development of selective V-ATPase inhibitors targeting osteoclasts.

Conclusion

This review summarizes recent research developments in V-ATPase biology and highlights the importance of V-ATPase as a potential prime target for anti-resorptive agents. With the advance of molecular and biological techniques such as knockout mouse models, the body of knowledge regarding its structure and function in osteoclasts is growing and this might facilitate the search of selective inhibitors that target osteoclast specific V-ATPase structure for the treatment of bone resorption disorders, such as osteoporosis, aseptic loosening and tumor-induced bone loss.

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