ABSTRACT

In eukaryotes, among the most important transport proteins designed to regulate pH is the vacuolar family of (H+)-ATPases. Specifically, V-ATPases are responsible for acidification of intracellular compartments and, for certain cell types, proton transport across the plasma membrane to the extracellular environment. Currently, a handful of inhibitors of V-ATPases are known, which represent valuable tools for the characterization of transport processes at tissue, single cells or even purified proteins level. The understanding of how these inhibitors function may provide a basis to develop new drugs for the benefit of patients suffering from diseases such as osteoporosis or cancer. However, even for some of the most popular and long known V-ATPase inhibitors, such as bafilomycin or concanamycin, the authentic structures of their binding sites were until recently elusive. In the past years, several studies aimed at a more detailed characterization of the binding of V-ATPase inhibitors to the proton pump made use of different biophysical methodologies and in this review we describe the different methodologies as well as the different information made available through them.
1. INTRODUCTION

Adenosine triphosphate (ATP) is used by living organisms as the source of energy for multiple biochemical processes. Direct hydrolysis of ATP into adenosine diphosphate and free phosphate is one of the main ways through which organisms can utilize ATP’s energy. In this reaction, ATP acts as a phosphoryl donor to a water molecule. These reactions are usually catalyzed by a class of enzymes termed ATPases. The energy thus produced is normally used for important physiological functions, including proton pumping through cell membranes [1]. pH is a critical parameter, carefully regulated in all biological systems. In eukaryotes, vacuolar (H+) ATPases are among the most important transporter enzymes. They translocate protons from the cytoplasm to the lumen or extracellular space, through ATP hydrolysis [2]. Because they were discovered and studied in vacuoles, they were originally named vacuolar ATPases (V-ATPases) [3]. However, they are found not only in vacuolar membranes in plant cells, but also in other cell membranes, including plasma membranes of more specialized cells [4].

Specifically, V-ATPases are responsible for acidification of intracellular compartments, and, for certain cell types, for the transport through the plasma membrane to the extracellular environment. Intracellular V-ATPases participate in endocytic and intracellular membrane traffic, in processing and degradation of macromolecules in the secretory and digesting compartments, in the co-transport of small molecules such as neurotransmitters and ATP, as well as in the entry of pathogens, including envelope virus and bacterial toxins. V-ATPases are also present in the plasma membranes of kidney cells, osteoclasts, macrophages, epididymal cells, and some tumor cells, in which they are important in urine acidification, bone resorption, pH homeostasis, sperm maturation and tumor cell invasion, respectively [4].

In this way, and taking into consideration their crucial role in cell function, V-ATPases have been implicated in the patophysiology of several human diseases, including osteoporosis and cancer. Therefore, in the last decades, V-ATPase inhibitors have been emerging as potential drugs for conditions in which the above mentioned processes are altered. However, this objective has been revealed as a great challenge for researchers in this field, because, firstly, knowledge about V-ATPase function in normal processes and (mainly) in disease is relatively incipient; Secondly, although there has been considerable advance in biophysical studies for determination of membrane protein
structures, complete structural characterization of V-ATPases is still lacking. This is a deterrent for the following steps, because in order to design efficient and specific inhibitors, it is necessary to know the structure and the function of the different pump protein subunits. The understanding of the mode of action of these inhibitors may provide the basis for development of new drugs for pathologies such as osteoporosis and cancer. To this purpose, exact determination of the inhibitor binding site in the protein is most probably essential, on the one hand, and unravelling of the crucial structural elements of the inhibitor candidates, on the other hand, seems to be essential. However, even for the first discovered inhibitors, like bafilomycin and concanamycin, the true structures of their binding sites are just beginning to be known.

This chapter briefly summarizes the structure and function of V-ATPases, before describing the different classes of inhibitors described so far in the literature, as well as the results of selected studies of their application in osteoporosis and cancer. Finally, the contribution of biophysical studies to the understanding of the inhibitor mode of action is reviewed.

2. VACULOAR (H⁺)-ATPASES (V-ATPASES)

2.1. Structure and mechanism of proton translocation

V-ATPases are large protein complexes, comprising 14 subunits organized in two subcomplexes. The transmembrane domain V0 is responsible for proton translocation, whereas the V1 cytoplasm domain catalyses ATP hydrolysis (Fig. 1). The former (260 kDa) has six distinct subunits, termed a, d, e, c, c' (absent in superior eukariotes) and c". Subunit c is present in 4 or 5 copies, while there is only one copy of each of the remaining subunits. c, c' and c" are highly hydrophobic peptides, and form a membrane-inserted ring. Subunit e is also highly hydrophobic, whereas subunit d is strongly bound to the cytoplasmic surface of the proteolipid ring, as well as to the structural subunits of the V1 domain. The peripheral latter subcomplex (640 kDa) is located in the cytoplasmic side of the membrane, and comprises eight subunits (A-H), with A3B3C1D1E2F1G2H1,2 stoichiometry (Fig. 1). Subunits A and B form an alternate hexameric arrangement, where the three ATP hydrolysis sites are located. The other subunits of this domain have structural and regulation functions [3].
Figure 1 - Schematic depiction of the V-ATPases. V-ATPase complex is composed of a peripherally located V1 domain and a integral membrane associated V0 domain. The V1 domain consists of eight different subunits (A–H) and is responsible for ATP hydrolysis. The V0 domain consists of four different subunits (a, c, d, and e) responsible for proton translocation. Furthermore two accessory subunits, Ac45 and M8-9, also exist but their structural location and association with other subunit remains to be determined. Reprinted from [5] with permission. Copyright 2007 Elsevier.

Most V-ATPase subunits form supporting elements, linking the two domains. The central support (subunits d, D and F) acts as rotor, coupling the release of energy from ATP hydrolysis in domain V1 to the rotation of domain V0. The peripheral support (formed by subunits C, E, G, H, and the N-terminal region of subunit a) acts as stator, preventing rotation of the A3B3 complex during pumping [3], and also takes part in V-ATPase regulation [6].

In superior organisms, V-ATPase subunits may exist in multiple isoforms. Most of them are expressed in a specific manner, depending on the organelle, cell and tissue. Whereas their function is still mostly unknown, it is thought that they may have regulating functions, e.g. controlling the efficiency and location of the pump [3].

Proton transport by V-ATPases occurs via a rotating mechanism (Fig. 1): ATP hydrolysis in the A3B3 complex induces conformational changes in subunit A, which produce a rotational force. Meanwhile, protons enter the V0 complex through one of the a subunit hemichannels, binding to one of the proteolipidic ring binding sites. As the latter rotates, protons cross through the lipid bilayer, ending up in the exit hemichannel of subunit a [7]. Because there are three ATP binding sites in the V1 domain and 6-10 sites available
for protonation in the V0 complex, the H\(^+\)/ATP stoichiometry lies approximately between 2:1 and 3.3:1 [3].

2.2. Cell functions

V-ATPases may be present in both intracellular and plasma membranes [4]. In the former case, these membranes define Golgi complex vesicles, endosomal and lysosomal vesicles, vacuoles (in plant cells) and secretory vesicles. In this way, V-ATPases participate in all the steps of normal cell membrane traffic, from the formation of early endosomes to the acidification of late endosomes and lysosomes (Fig. 2) [4]. Thus, V-ATPases play an essential role in receptor-mediated endocytosis, as they provide the acidic endosomal pH necessary to activate release of internalized ligands from their receptors. Besides these functions, intracellular V-ATPases also play an important part in the entry of some envelope viruses and toxins, which enter the cell via acidic endosomal compartments. Among the viruses to use this pathway are influenza virus, Semliki forest virus, and vesicular stomatitis virus. Regarding bacterial toxins, these include diphtheria toxin and anthrax toxin [4] (Fig. 2).
Figure 2 - Role of intracellular V-ATPases in normal and disease processes. (A) Role of intracellular V-ATPase in membrane trafficking, endocytosis, and secretion. Extracellular ligands are internalized by receptor-mediated endocytosis and trafficked to the sorting endosome. Acidification of the endosome by the V-ATPase allows for release of the ligand and the recycling of the receptor back to the membrane. Budding of endosomal carrier vesicles and multivesicular bodies are also dependent on the acidic environment. Lysosomal proteins are synthesized in the trans Golgi network and are trafficked to the lysosome via the mannose-6-phosphate receptor. Acidification of the late endosome allows for the release of the lysosomal proteins and recycling of the man-6-P receptor back to the Golgi. In the lysosome, acidification activates cathepsins and other degradative enzymes. The pH gradient created by the V-ATPase in secretory vesicles drives the uptake of neurotransmitters and other molecules to be secreted. The V0 domain has also been proposed to play an important role in membrane fusion. (B) Role of intracellular V-ATPase in the entry of envelope viruses and toxins. Envelope viruses and bacterial toxins such as diphtheria toxin enter the cell via endocytosis where they are trafficked to the sorting endosome. The low pH generated by the V-ATPase causes the viral coat to fuse with the endosomal membrane releasing the viral m-RNA into the cytoplasm. The acidic environment also induces the diphtheria toxin B chain (shown in green) to form a pore in the
membrane that facilitates the entry of the A chain (shown in red) into the cytoplasm. Reprinted from [8]. Copyright 2008 Elsevier.

V-ATPases also take part in important functions of the plasma membranes of cells [3]. By generating membrane potentials, they have the ability to provide energy for coupled transport processes, and are thus involved in entry and/or exit of a variety of molecules. V-ATPases also play a very important role in the regulation of cytoplasm pH, bone formation/resorption balance, and sperm maturation and storage in the epididymus and vas deferens. Finally, these proton pumps were recently implicated in cell mobility processes, such as cell migration during angiogenesis and tumor cell invasion, through the creation of an acidic extracellular pH, essential for degradation of the extracellular matrix (Fig. 3) [4].

**Figure 3** - Function of plasma membrane V-ATPases. (A) Function of V-ATPase in renal alpha-intercalated cells. To increase secretion of acid into the late distal tubule and collecting duct of the kidney, vesicles containing a high density of V-ATPases fuse with the apical membrane. These cells also absorb CO2, and release HCO3− into plasma through a Cl−/HCO3− exchanger, preventing the cytoplasm from becoming too alkaline. (B) During bone resorption V-ATPase are targeted to the membranes of osteoclasts that are in contact with bone, acidifying the extracellular matrix and activating degradative enzymes. (C) In epididymal clear cells, V-ATPases are targeted to the luminal membrane, providing the decreased pH of the lumen required for sperm maturation. (D) In neutrophils and in macrophages, plasma membrane V-ATPases are involved in pH homeostasis. (E) In insect midgut cells, V-ATPases are used to
drive a H+/2K+ antiporter resulting in the net secretion of K+ into the gut. (F) Some invasive tumor cells target V-ATPase to the plasma membrane where they create an acidic extracellular environment that aids invasion, likely through activation of cathepsins that breakdown matrix proteins. Reprinted from [8] Copyright 2008 Elsevier.

2.3. Regulation

Figure 4 - Regulation of the V-ATPase. V-ATPases are regulated by a number of mechanisms including: A, reversible disulfide bond formation between conserved cysteine residues that prevent the catalytic site from cycling between the open and closed conformations required by the binding change mechanism; B, changes in pump density through fusion of vesicles containing a high number of V-ATPase; C, changes in either Cl− or H+ conductance through distinct channels; D, reversible dissociation into inactive V1 and V0 domains; and E, changes in coupling efficiency. Reprinted from [7]. Copyright 2008 Elsevier.
Given their importance at the cellular level, it is unsurprising that V-ATPases are tightly regulated by a variety of mechanisms (Fig. 4). One of the most important control processes is the reversible dissociation of the V0 and V1 domains. This mechanism is triggered by nutrient depletion and pH decrease, presumably as a way of conserving cellular stores of ATP. Another type of regulation involves changes in the efficiency of coupling between the two domains, that is, between ATP hydrolysis and proton transport. A variety of alterations in cell physiology (e.g., high ATP concentrations), as well as some mutations in specific subunits, may change the efficiency of proton transport. The pump may also be reversibly inactivated through the formation of covalent bonds between residues in the catalytic site of subunit A of domain V1. Control of V-ATPase density in the plasma membrane is another critical form of regulation. In the latter case, modulation is achieved by reversible fusion of intracellular vesicles with the plasma membrane [7].

3. V-ATPASE INHIBITORS IN CANCER AND OSTEOPOROSIS

Because of the involvement of V-ATPases in several human diseases, and taking into account that many protein subunits are specifically expressed in different organelles, cells and tissues, it is pertinent to consider V-ATPases as potential therapeutic targets in the treatment of several pathologies [9]. For example, there are four different isoforms of subunit α, numbered from α1 to α4. Isoform α1 is ubiquitously expressed, as is associated to targeting of V-ATPase to endosomes and lysosomes [10]. In renal alpha intercalated cells and epididymal clear cells, it was demonstrated that isoform α2 targets V-ATPase to apical endosomes, whereas isoform α4 targets V-ATPase to the apical membrane [11]. In osteoclasts, it has been verified that isoform α3 is present in lysosomes and the plasma membrane, suggesting translocation of V-ATPase following cell activation [5,12].

Osteoporosis leads to a decrease in bone mineral density, rendering bone structure fragile to the point of susceptibility to spontaneous fracture. Bone-resorbing osteoclasts and bone-forming osteoblasts are central in skeleton development and bone mass regulation. A difference in the activity of these bone cells may trigger osteoporosis. During bone resorption, V-ATPases secrete protons in the bone resorption lacuna, a compartment formed between the plasma membrane and the bone surface. This compartment is rich in proteases and an acidic lumen is essential for mineral dissolution.
and matrix protein degradation [9]. One of the possible deregulations identified in osteoporosis is that of V-ATPase over-function, which renders bone tissue more acidic, leading to its demineralization [13,14].

As mentioned above, isoform a3 of V-ATPases is specifically located in the plasma membrane of osteoclasts. In accordance with this finding, mutations in this isoform result in deficient bone resorption and osteopetrosis in humans and mice [15,16,17]. These results are corroborated by the study of Li et al. [18] in which a3 knockout mice showed pronounced osteopetrosis due to osteoclast-mediated inhibition of extracellular acidification. Therefore, because of the predominant role of a3 isoform in bone resorption by osteoclasts, inhibitors specific to this isoform may be efficient in controlling osteoporosis-related bone loss [5]. It is noteworthy that the drugs currently available for bone loss reduction have only a temporary effect, and novel highly specific therapies, with prolonged effect, are necessary [19].

On the other hand, in the preceding years it has been verified that V-ATPases, by providing an acidic tumoral microenvironment, may be the key to control cancers and metastasis [20]. Firstly, it should be mentioned that again in this case, specificity of the different isoforms of subunit a is important. In breast cancer cell lines, a different expression of a isoforms between highly and lowly metastatic cells has been verified. For example, breast cancer MB231 cell line is considered as highly metastatic and has shown high levels of a3- and a4-encoding mRNA, in comparison with the lowly metastatic MCF7 cell line [2].

Uncontrolled cell growth and increased metabolic activity are characteristic features of the tumoral phenotype. Abnormal glucose metabolism and deficient perfusion due to weak vascularisation cause a total dependence on glycolysis for energy production [21]. As a result, high levels of lactic acid are produced, which may decrease intracellular pH. To control the latter, cells use several proton transporters, including V-ATPases. V-ATPases play an active role in tumor progression and metastasis. Plasma membrane V-ATPases can acidify the environment of several tumor cell lines, including breast cancer and melanoma [22]. Particularly, V-ATPases play a major part in the development of tumor metastasis, because many tumor cells secret lysosome enzymes that participate in the degradation of the extracellular matrix necessary for metastatic invasion. It was shown that cathepsins are secreted from lysosomes in a variety of cancer cells that require an acidic environment in order to function [10,23]. Cathepsins are proteases that, when activated, have the ability of degradating the extracellular matrix and to
activate metaloproteases, that can also contribute to this effect. Without the extracellular matrix, separation of tumor cells from the primary tumor and transport of the former through the blood to distant organs is much facilitated [24,25]. Plasma membrane V-ATPases may help activate proteases by acidifying the extracellular environment. Additionally, V-ATPases may help in the traffic of proteases from lysosomes to the extracellular matrix, through an increase in vesicle recycling. Among the different mechanisms that regulate the tumoral microenvironment, V-ATPases are especially relevant as they can be inhibited by proton pump inhibitors [20].

4. CLASSES OF INHIBITORS

4.1. Plecomacrolide antibiotics

The discovery of the plecomacrolide bafilomycin, the first highly potent and specific V-ATPase inhibitor [26] opened up a new research direction, allowing pharmacological identification of V-ATPases in a functional context. Bafilomycin and the other classic V-ATPase inhibitor, concanamycin [27] were identified and structurally described in the early 1980s [28,29,30,31,32] (Fig. 5).

Figure 5 - Structures of bafilomycin A1 (left) and concanamycin (B). Modified from [20].

Bafilomycins and concanamycins are closely related compounds. Both belong to the unusual macrolide group, characterized by a macrocyclic lactone ring of 10 to 48 carbons, synthesized by bacteria. Together with related, highly unsaturated macrolides produced by Streptomyces species, bafilomycins and concanamycins encompass the group of plecomacrolides [33].

Interestingly, the discovery of bafilomycins and concanamycins resulted from a screening procedure totally unrelated to V-ATPases. Henses et al. [34] were searching for microbial secondary metabolites that would have effects similar to those of the cardiac glycosides ouabain and digitoxin. The macrolide antibiotic L681,110A1, later
reclassified as bafilomycin C1, isolated from the culture medium of *Streptomyces sp.* MA-5038, inhibited the target enzyme of the cardiac glycosides, the Na\(^+\)/K\(^+\)-ATPase (Ptype), with an inhibition constant of 11 mmol L\(^{-1}\). However, the compound lacked cardiotonic activity *in vivo* [35]. Further studies revealed that the antibiotic showed antihelmintic activity against *Caenorhabditis elegans*, was active against ticks and tapeworms and also stimulated the release of \(\gamma\)-aminobutyric acid (GABA) from synaptosomes of rat brain [34]. Independently, Werner et al. [31,32] isolated several naturally occurring bafilomycin derivatives from the mycelium of *Streptomyces griseus*, including bafilomycin A1 (Fig. 5). These derivatives exhibited antibiotic activity against several fungi, yeast and Gram-positive bacteria. Another group of related macrolides was found in a screening for immunosuppressive compounds [28,29]. These antibiotics, which were isolated from the culture medium of *Streptomyces diastatochromogenes*, were named concanamycins because they inhibited the proliferation of concanavalin-A-stimulated T-cells [30]. Further studies revealed that this group of antibiotics exhibited activity against fungi, but not against bacteria. The latter observation can be explained by the additional sugar moiety (Fig. 5), since glycosylation of bafilomycins also abolishes their bactericidal activity [33].

When used at low concentrations, bafilomycin and concanamycin are highly specific for V-ATPases, inhibiting all V-ATPases both *in vitro* and *in vivo* [5]. In fact, bafilomycins and concanamycins are able to inhibit bone resorption, both *in vitro* [36, 37,38] and *in vivo* [39], exerting additional inhibiting effects on endocytosis and apoptosis of osteoclasts and their precursor cells, enabling them to control osteoporosis [5,40]. Sundquist et al. [36] verified that, in cell culture, bafilomycin A1 inhibits bone resorption by osteoclasts in nM concentrations.

Additionally, these macrolides, both isolated and combined with anti-neoplastic agents, showed a beneficial effect regarding invasion and migration, as well as tumor cell resistance against cytotoxic drugs [8,41]. For example, bafilomycin was able to inhibit the invasion and migration of the highly invasive human breast cancer cell line MB-231 [8].

The structure–activity relationship of these two 18-carbon lactone ring macrolide antibiotics was intensely investigated [27,33,42,43,44]. The enzyme binding site remained unknown for more than a decade, although studies of V-ATPases in chromaffin granules suggested subunit a of the V0 domain as the binding site for plecomacrolides [20]. Nowadays, it is known that plecomacrolides also interact strongly
with subunit c of the V0 domain [5,45, 46]. Namely, these compounds inhibit rotation of
the latter subunit [5,47].

Even though this group of antibiotics exhibited additional interesting effects, as
insecticides [48] in the inhibition of bone resorption [36], and in tumor progression and
metastasis [8], the high toxicity of these compounds was prohibitive for clinical
applications. For example, mice die after intraperitoneal injection of 1 mg of
concanamycin A per kilogram body mass [27,30,33]. Therefore, the universal action of
pleomacrolides on V-ATPases also limits their application, because they inhibit all cell
V-ATPases, affecting all processes that depend on acidification by these enzymes [33].
Structural alterations are thus required for high selectivity for osteoclast and cancer cell
V-ATPases [49].

4.2. Benzolactone enamides

During the 1990s a new class of highly cytotoxic compounds emerged, possessing a
benzolactone enamide central structure as common feature [50,51,52,53]. Benzolactone
enamides contain a salicylic acid residue and a lateral enamide chain joined by a
variable bond to form a lactone ring [20]. The benzolactone core is responsible for these
compounds' high cytotoxicity [51]. They were extracted from different natural sources
varying from marine macroorganisms such as the sponge Haliclona sp. or the tunicate
Aplidium lobatum, to microorganisms such as the gram negative bacterium
Pseudomonas sp. or the myxobacterium Chondromyces sp., and were referred to as
salicylihalamides, lobatamides, oximidines and apicularens, respectively [54] (Fig. 6).
In cell tests, all of these substances exhibited IC$_{50}$ values in the nM range
[50,52,53,55,56]. Assays of their inhibitory specificity for the VATPase carried out with
either membrane preparations of human kidney, liver, osteoclast or bovine chromaffin
granules [57,58] or with purified V-ATPase from insect midgut [45,545] or bovine
clathrin coated vesicles [59] clearly showed that the benzolactone enamides are indeed a
novel family of highly specific V-ATPase inhibitors, with IC$_{50}$ values in the nM range.
Moreover, the toxicity profile in NIC-60 cancer cells was similar to that of bafilomycin.
For these reasons, benzolactone enamides are being intensely studied by several organic
synthesis groups [57,60].
One of the most surprising and interesting features of benzolactone enamides is the fact that they do not inhibit V-ATPases from fungi, for reasons yet unknown [57], inhibiting solely animal V-ATPases, in nM concentrations [57,59]. Therefore, they are selective inhibitors of V-ATPases of mammals, differing from bafilomycins and concanamycins in this respect. For this reason, and also because of their potent anti-tumoral activity and simple structure, they show promise as anti-cancer drugs [20].

The enzyme binding site of benzolactone enamides is still not known. It was demonstrated that salicylihamide and apicularen have a different binding site to that of the plecomacrolides [54,55]. However, Xie and coworkers showed that the binding site must reside within the V0 complex, given that salicylihamide inhibited not only the activity of the proton pump of V1V0 holoenzyme reconstituted from bovine clathrin coated vesicles, but also the activity of the H+ channel of the reconstituted V0 complex [59].

Recently, a potentially very useful application for the benzolactone enamides has been reported [61]. The authors detected a strikingly synergistic effect of the chemotherapeutic drug paclitaxel, better known as taxol, and RTA 203, a derivative of salicylihamalamide, when applied simultaneously to the cancer cell line H1155 even at doses where the single compounds had only a minor effect. Additionally, oximidines, introduced by Kim et al. as antitumor macrolides originating from Pseudomonas sp.,
were shown to selectively inhibit the growth of 3Y1 cells transformed with E1A, ras, and src oncogenes [52].

During a screening for biologically active metabolites from myxobacterium *Byssovorax cruenta*, Kunze et al. [62] discovered a novel macrolide, the benzolactone cruentaren A from, which exhibits a high cytotoxicity for mammalian and fungal cells. Because of its structural relationship to the benzolactone enamides, with apicularen as the closest relative, it was initially assumed that cruentaren might also be a specific V-ATPase inhibitor. But surprisingly, cruentaren had no effect on V-ATPases; instead it inhibited the evolutionarily related mitochondrial F-ATPases at nanomolar concentrations [62,63]. Additionally, the interaction site of cruentaren resides in the F1 complex, unlike the benzolactone enamides, which, as mentioned above, operate via the membrane bound V0 complex [54].

4.3. Archazolid

Archazolid is a recently discovered compound produced by myxobacteria *Archangium gephyra* and *Cystobacter violaceus* [64,65]. The main structure, as illustrated in Fig. 7, is composed of a macrocyclic lactone ring with a thiazole side chain. The biological activity of archazolid was discovered by screening for novel antibiotics produced by myxobacteria and was shown to exhibit a high activity against a large set of mammalian cell lines with IC$_{50}$ values in the sub-nM range. Cell inspection revealed that archazolid caused formation of vacuoles in the endoplasmic reticulum, which is typical for V-ATPase inhibitors [65]. Furthermore, archazolid inhibited lysosome acidification, corroborating the hypothesis that V-ATPase was the target of the antibiotic [55]. When tested in purified preparations of V-ATPase, Na$^+$/K$^+$-ATPase and mitochondrial F-ATPase, archazolid seems to be a highly potent inhibitor, specific for V-ATPases, with IC$_{50}$ values in the nM range [55].
Archazolid A: R = CH₃
Archazolid B: R = H

Figure 7 – Structure of archazolid A and B. Modified from [20].

Although archazolid is one of the most recent V-ATPase inhibitors, at least a part of its binding site has already been identified from cross-linking studies as the subunit c of the V0 domain [54]. Additional structure-activity relationship studies with natural and semisynthetic derivatives of archazolid revealed first insights on which parts of the pharmacophore play a crucial role for the inhibitory properties [54,66].

4.4. Indolyls

Structural studies on bafilomycin and concanamycin have revealed the main structural elements necessary for V-ATPase inhibitors' biological activity, thus leading to the design and synthesis of structurally simpler new inhibitors, such as indolyls [44,54]. (Fig. 8).

Figure 8 – Structures of indolyl V-ATPase inhibitors NiK-12192 (left) and INDOL0 or SB 242784 (right). Modified from [54,66].

The most potent known inhibitor of this family, with an IC₅₀ value of 30 nM for chicken osteoclasts, is (2Z,4E)-5-(5,6-dichloro-2-indolyl)-2-methoxy-N-(1,2,2,6,6-pentamethylpiperidin-4-yl)-2,4-pentadienamide (Fig. 4), also referred to as INDOL0 or SB 242784 [67]. The protecting effect of this inhibitor in the prevention of bone mass
loss was comparable to that of an optimal dose of oestrogen [68]. Furthermore, it is 1000 times more selective for osteoclast V-ATPases compared to those of endothelial cells, and has no effect upon other V-ATPases in the cell [68]. Another inhibitor, NiK-12192, increases the antitumoral activity of chemotherapeutic agent camptothecin in two cell lines of colon carcinoma and in non-small lung cancer cells [69,70]. The binding mechanism of these novel inhibitors is not completely clarified, and the molecular mechanism of SB 242784 selectivity remains to be elucidated [5]. However, it was shown that SB 242784 interacts with the transmembrane segments of subunit c, which indicates that it may have a mode of action similar to that of bafilomycin [46,71,72]. Furthermore, mutations in that subunit that confer lower sensitivity to bafilomycin, also lead to decreased sensitivity to SB 242784, suggesting a similar inhibition mechanism for the two compounds [54]. Additionally, it seems that this potent V-ATPase inhibitor does not interact with the 7th transmembrane domain of subunit a [73].

4.5. Other V-ATPase inhibitors

Besides the inhibitor families described above, other compounds with inhibitory action upon V-ATPase activity have been more recently discovered. Among these there are the FR inhibitors (FR202126, FR177995 and FR167356) and the KM91104 inhibitor, which were developed by Nikura and coworkers [74,75,76] and Kartner et al. [77], respectively. Their structures are depicted in Fig. 9.

![Figure 9](https://example.com/figure9.png)

**Figure 9**- Structures of (A) FR167356, (B) FR202126, (C) FR177995, and (D) KM91104. Modified from [74]; [75]; [76]; and [77], respectively.
In 2007, Nikura et al. demonstrated the efficiency of FR202126, a specific inhibitor of osteoclast V-AATPase, in the reduction of osteolysis induced by metastatic lung cancer [75]. Other recent inhibitors apparently do not affect cancer, but do prevent bone destruction in induced arthritis by inhibiting osteoclasts, as in the case of FR177995 [76]. Other inhibitors, such as FR167356, may even discriminate between osteoclast V-ATPases and lysosomal V-ATPases, a fact that has potential therapeutic implications [74].

Kartner and co-workers [77], in a study of protein-protein interaction, verified that V-AATPase subunits a and B interact. Specifically, these researchers verified that isoform a3 (the isoform of subunit a predominantly expressed in osteoclasts) interacts with isoform B2, which is also highly expressed in osteoclasts. Solid-phase binding assays were subsequently used to screen a chemical library for inhibitors of the a3-B2 interaction. A small benzohydrazide derivative (3,4-dihydroxy-N’-(2-hydroxybenzyliden)benzohydrazide, termed KM91104) was thus found. This compound inhibited osteoclast resorption with an IC$_{50}$ of ca. 1.2 µM, without significantly affecting osteoclast viability and differentiation. Thus, this molecule may reduce bone resorption by osteoclasts without significantly affecting osteoclastogenesis and preserving osteoclast-osteoblast communication. These results confirm that the a3-B2 interaction is biologically important, and the identification of inhibitors targeted to this interaction constitutes a viable approach. Even though KM91104 may not have shown enough specificity, this study hints at the possibility that a wider screening procedure may produce the required “magic bullet”.

5. BIOPHYSICAL STUDIES WITH V-ATPase INHIBITORS IN MODEL SYSTEMS

Most of the information regarding the structure of V-ATPases was obtained from electron microscopy, and only a few soluble subunits have been characterized by X-ray crystallography. In the absence of high-resolution structures, information on the mechanism of action from V-ATPase inhibitors has been generally obtained through random and site-directed mutagenesis studies which are able to identify the aminoacids which are in some way relevant for the inhibitory process. Bowman and co-workers [46,78] identified several aminoacids relevant for bafilomycin and concanamycin...
action. Given that a large cluster of relevant amino acids were found in the interface between the second and the fourth transmembrane (TM) segment of subunit c, the authors proposed that this interface contained the binding sites for the two inhibitors. Since the effects of mutations in the activity of one antibiotic were generally different from the effect observed with the other one, the authors also anticipated that the binding sites, although overlapping, were not completely identical. Nevertheless, different mutations in other sites of the subunit c were also found to influence bafilomycin and concanamycin activity adding complexity to the problem. Additionally, a similar study performed on subunit a of V-ATPase identified several mutations that significantly affected bafilomycin inhibitory activity [79]. In this way, the information gained from determining the activity of mutants, although valuable, can be somewhat ambiguous regarding the identification of a binding site, as there is no way to distinguish effects from changes in protein conformation to changes in the direct interaction with inhibitor molecules.

These studies have been complemented with techniques that are able to directly monitor the interactions between the molecules such as spectroscopic methods. These methods are much less ambiguous in the sense that they are very sensitive to direct interactions between molecules. For that purpose, purified subunits of V-ATPase, or selected segments of the protein, were labelled with electron paramagnetic resonance (EPR) or fluorescent labels and reconstituted in model membrane systems. These studies recovered relevant information on this system and are described in greater detail below.

Model membrane systems are generally used for these biophysical studies. They are extremely valuable tools in the study of ligand-receptor docking since they provide a simplified and completely controlled mimetic of complex cellular membranes. The application of membrane model systems also allows for the possibility of detecting and quantifying interactions under much lower background signal, and since the composition of these membranes is easily modulated, it is possible to investigate the exact role of each membrane component on ligand-receptor interaction. In addition, the study of direct interactions of ligands with membrane model systems is also of great relevance since in many cases, biological membranes act as concentrators of ligands in the vicinity of the receptor, promoting the rate of ligand-receptor binding up to $10^5$ times [80]. Membranes are also able to create constraints in ligand position and orientation inside the bilayer, further promoting optimal conditions for receptor binding. Studies aimed to quantify ligand binding to lipid membranes and characterize the
properties of biologically relevant molecules in the lipid bilayers can contribute for the
development of more effective analogs which could make full use of the catalytical
potential of lipid membranes.

5.1 Electron Paramagnetic Resonance (EPR) Spectroscopy

EPR has been widely used as a method to detect interaction of membrane proteins with
other membrane components. The reduced mobility experienced by ligands upon
protein interaction is often detected in the EPR spectra, and parameters such as protein-
ligand stoichiometry, affinity or exchange rates can be recovered.
The interaction of macrolide and indolyl type inhibitors with model membrane systems
was addressed through EPR studies while making use of spin labelled lipids. In these
lipids, a nitroxide group is present at different positions along the $sn$-2 chain. From the
maximum outer hyperfine splitting ($A_{\text{max}}$), taken from the EPR spectra of these lipids, it
is possible to have sensitivity to amplitude of motion in the membrane plane
correspondent to the position where the nitroxide label is along the $sn$-2 chain. Concanamycin
A at a 1:1 inhibitor:lipid ratio was shown to increase the $A_{\text{max}}$ associated

with a phosphatidylcholine spin-labelled at the 5$^{th}$ position along the $sn$-2 chain in a
dimyristoylphosphatidylcholine (DMPC) membrane [81]. This indicated that the
macrolide inhibitor inserted between phospholipid chains and disturbed membrane
dynamics to some extent. Disturbances in the EPR spectra were also found for lipids
spin labelled at positions closer to the bilayer center. SB242784 induced an even greater
disturbance in the EPR spectra of spin-labelled lipids, suggesting lipid packing was
severely disturbed with this inhibitor at a 1:1 inhibitor:lipid ratio.
The same authors also synthesized spin-labelled derivatives of the indolyl inhibitor
SB242784, and studied the EPR properties of these molecules in a DMPC bilayer [82].
Spectral line shapes were used to deduce the mode of insertion of the inhibitors in the
membrane. These molecules were shown to intercalate between the lipid chains while
one of the spin-labelled inhibitors (INDOL5) exhibited significantly more segmental
motion. This offered the opportunity of detecting specific ligand–protein interactions
with INDOL5, through the resolution of a second immobilised EPR spectral component.
Chemical reduction by aqueous ascorbate was also used to determine the position of the
molecules in the membrane. The nitroxide groups from the spin-labelled inhibitors were
found to be in the hydrocarbon interior of the membrane but located close to the polar–
apolar interface. The spin-labelled indolyl inhibitors were subsequently used to identify binding to membranous preparation containing the 16-kDa proteolipid analog of the V-ATPase c-subunit [71]. The EPR spectra of INDOL5 presented an additional motionally restricted component in the presence of the protein that suggests binding. Experiments on the interaction of macrolide and indolyl inhibitors with the 16-kDa proteolipid analogue of subunit c were also conducted with spin-labeled protein [72]. The protein was labeled either at the unique cysteine C54 with a nitroxyl maleimide or on the functionally essential glutamate E140 with a nitroxyl analogue of dicyclohexyl-carbodiimide (DCCD). The inhibitors were consistently found to induce disturbance on the EPR spectra of spin-labels in both positions, with immobilization on both the nanosecond and microsecond time scales. The two labeled residues in the c-subunit are expected to reside in close proximity on adjacent helices (TM2 and TM4), and these results suggest that the binding site for these inhibitors is in fact composed at least in part by the second and the fourth transmembrane helices as suggested by the mutational studies. The authors also suggested that the reduction in the rotational mobility was related with the inhibitory mechanism, with the indolyl inhibitor acting through the interruption of the ATP-driven rotation of the proteolipid assembly that powers proton transport, similarly to the mechanism of macrolide inhibitors [83].

5.2 Fluorescence Spectroscopy

The main advantage of fluorescence spectroscopy is the inherent sensitivity associated with it. Additionally, it provides greater flexibility in working temperatures. Protein-ligand interaction can be followed through changes in the fluorescence quantum yield, spectral changes, and in the case of fluorescent ligands, it is possible to detect binding through a change in ligand fluorescence anisotropy. Fluorescence static or collisional quenching methods can provide structural type of information similar to EPR as it provides information on the environment immediately around the fluorophore, while Förster resonance energy transfer (FRET), is particularly powerful, since it is sensitive to distances up to 100 Å.

SB 242784 is intrinsically fluorescent and a detailed structural study of the interaction of this inhibitor with the lipidic environment has been conducted [84]. The authors studied both SB 242784, corresponding to a high efficient indolyl V-ATPase inhibitor, and a low efficiency analog, INH-1. The quantum yield of both molecules increased
several times upon membrane interaction, allowing for a precise quantification of inhibitor binding through steady-state fluorescence intensity measurements. It was found that both molecules displayed similar partition coefficients to dioleoylphosphatidylcholine (DOPC) membranes, and that the differences in inhibitory efficiencies were not related to lipid membrane affinities, as membrane effective concentrations of the two molecules are expected to be equal. Using spin labeled fatty-acids with the nitroxide group present at different positions along the acyl, the authors induced fluorescence quenching of both indolyl inhibitors. From the application of the parallax method of differential quenching [85] to the fluorescence quenching efficiencies obtained from each quencher, it was possible to calculate an approximate location of the molecules in these membranes. The two molecules were also shown to be at similar positions in the bilayer, around the polar-apolar interface. This was in agreement with the position of spin-labeled SB 242784 in the membrane [81]. The advantage of the fluorescence methodology relative to EPR in this case was that the former only reported for the position of the paramagnetic group inside the molecule while from fluorescence, the fluorophore composing the whole axis of the molecule was assessed. From linear dichroism studies, the orientation of SB 242784 and INH-1 were also found to be identical, further supporting the notion that the lower IC$_{50}$ observed for SB 242784 for V-ATPase inhibition is solely dictated by specific ligand-receptor recognition processes.

A fluorescence quenching assay of a pyrene labeled V-ATPase showed significant pyrene quenching in the presence on nanomolar concentrations of both concanamycin and SB 242784, indicating direct binding of concanamycin and SB 242784 with similar affinities [86]. Concanamycin and SB 242784 were also found to bind to the subunit c in a competitive manner further supporting the hypothesis of an overlapped binding site, while binding was not observed for protein mutants with resistance to these inhibitors. As already discussed, SB 242784 presents 1000 times more selectivity for osteoclast V-ATPases, which only differ from other V-ATPases in their subunit a isoform, strongly suggesting a role for this subunit in binding. However, fluorescence quenching of pyrene-labeled subunit c by SB 242784 was observed in the presence or absence of subunit a. The authors concluded despite the relevance of subunit a for the inhibitory mechanism, subunit c contained the major proportion of the structural determinants for inhibitor binding.
A different strategy to evaluate binding of ligands to a specific binding site in the protein relies on using isolated representative peptides from the protein sequence. In this way it is possible to delineate the relevance of a particular aminoacid sequence for the creation of the binding pocket. Binding of V-ATPase inhibitors to isolated α-helical fragments of V-ATPase subunits (TM7 of subunit a, and TM 4 of subunit c) was also measured through Förster resonance energy transfer (FRET) from either tryptophan or tyrosine residues from the peptides (acting as FRET donors) to SB 242784 or to the macrolide inhibitor bafilomycin A1 (acting as FRET acceptors) [45,73]. SB 242784 was found not to bind TM7 of subunit a or TM4 of subunit c, up to an inhibitor:lipid ratio of 2%. Since FRET is sensitive to the presence of FRET acceptors at a distance, low concentrations of ligands (FRET acceptors) had to be used in order to avoid that the signal from interacting species had been overwhelmed by the presence of non-interacting molecules. In this way, FRET is ideal to identify high affinity interactions, but presents lower sensitivity for low affinity binding pairs. On the other hand, bafilomycin A1 was shown to bind TM4 alone, although the measured binding efficiency was many times smaller than binding efficiencies measured with intact c-subunit. It was concluded then that the binding site of macrolide and indolyl inhibitors in V-ATPase likely comprises at least TM2 and TM4 of subunit c, and that interactions with TM4 alone are not able to drive significant immobilization of the inhibitor.

5.3 Infrared Spectroscopy

The use of selected segments of V-ATPase to identify the inhibitor binding site was further extended by infrared spectroscopy studies [87]. In this study, polarized attenuated total reflectance infrared spectra of α-helical peptides corresponding to TM4 of subunit c, and TM7 of subunit a, incorporated in DOPC bilayers at a low lipid:protein ratio, were acquired. The order parameters of the α-helices were found to be significantly increased by the presence of concanamycin but only TM4 of subunit c was marginally sensitive to SB 242784. Apparently, upon concanamycin binding to V-ATPase some interactions with subunit a may occur in addition to TM2 and TM4 of subunit c, but once more, no evidence was found for interaction of SB 242784 with subunit a. As discussed above, an interaction between TM4 of subunit c and SB 242784 had not been detected through FRET [45]. Possibly, this was related to the inhibitor concentrations used in the study, as the FRET experiments were performed at
inhibitor:lipid ratios below 2%, while the infrared measurements were conducted at a ratio of 5%, allowing for the detection of a marginal degree of interaction. In this way, different methodologies are sensitive to different protein-ligand affinities.

6. CONCLUSIONS

There are two interdependent major challenges in V-ATPase inhibitor research. The first one is to discover how and where (binding site) a potential inhibitor may interact with V-ATPase, and to understand its potential effect. The second one is to develop drugs useful in the treatment of diseases such as osteoporosis and cancer.

Regarding the first point, it appears that the development of biophysical approaches such as EPR, infrared spectroscopy and FRET, among others, may allow detection and detailed characterization of inhibitor-V-ATPase interaction. From these studies it is clear that the binding pockets in V-ATPase for macrolide and indolyl inhibitors overlap but are not identical. This site is apparently located between TM2 and TM4 of subunit c, as TM4 alone is not sufficient to drive significant inhibitor immobilization on the protein surface. In addition, TM7 from subunit a does not establish strong interactions with the inhibitor, indicating that subunit a may play an important role in the inhibitory mechanism, but is probably not required for inhibitor binding. Still, it must be noted that these approaches would benefit immensely from the unravelling of a high-resolution structure of V-ATPase.

As for the second point, as illustrated above, numerous promising results have been obtained mostly in recent years. However, a long path must be travelled in order to take these molecules from out of the laboratory bench and into clinical practice. Despite the increasing number of known V-ATPase inhibitors, their utility in clinical practice is a matter of discussion, especially as the mode of action of most of these molecules is not yet clear (which brings us back to the first point). The potential future use of these molecules in the treatment of cancer and osteoporosis is largely dependent on a thorough assessment of the overexpression of V-ATPase subunit isoforms specific to tumors and osteoclasts, respectively. This could enable the development of inhibitors specific to the overexpressed isoforms, increasingly specific treatments and minimization of adverse effects.

6. ACKNOWLEDGEMENTS

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