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Review

Inhibitors of V-ATPases: old and new players

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Summary

V-ATPases constitute a ubiquitous family of heteromultimeric, proton translocating proteins. According to their localization in a multitude of eukaryotic endomembranes and plasma membranes, they energize many different transport processes. Currently, a handful of specific inhibitors of the V-ATPase are known, which represent valuable tools for the characterization of transport processes on the level of tissues, single cells or even purified proteins. 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. For this purpose, it appears absolutely essential to determine the exact inhibitor binding site in a target protein on the one side and to uncover the crucial structural elements of an inhibitor on the other side. 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 are elusive. The aim of this review is to summarize the recent advances for the old players in the inhibition game, the plecomacrolides bafilomycin and concanamycin, and to introduce some of the new players, the macrolacton archazolid, the benzolactone enamides salicylihalamide, lobatamide, apicularen, oximidine and cruentaren, and the indolyls.

Key words: H^+-translocating vacuolar-type ATPase, V-ATPase, antibiotic inhibitors, plecomacrolides, macrolactones, benzolactone enamides, indolyls.

Introduction

Vacuolar-type ATPases (V-ATPases) are ubiquitous proton pumps that occur in the endomembrane system of all eukaryotic cells and in plasma membranes of many animal cells. They have various functions including the energization of transport processes across membranes and the regulation of the intracellular or intraorganellar pH (Beyenbach and Wieczorek, 2006; Forgac, 2007). V-ATPases are heteromultimeric enzymes consisting of a cytosolically oriented catalytic V_1 complex, composed of the subunits $A_3B_3CDE_2FG_2H$ (numbers are indicating the putative stoichiometry of the subunits) and a membrane bound proton translocating V_O complex, composed of the subunits ac_nde with the possible c isoforms c′ and c″. Briefly, they convert the energy of ATP hydrolysis by the catalytic subunit A into a rotational movement of the central stalk subunits D, F and c resulting in a proton translocation through the interplay of two proton conducting half channels residing in subunit a and an acidic amino acid side chain residing in each of the c subunits.

In recent years, it became more and more evident that malfunction of the V-ATPase is correlated with an increasing number of diseases such as osteopetrosis, male infertility or renal acidosis (Hinton et al., 2007). Therefore, the V-ATPase got into the focus of biomedical research and was even considered to be an attractive target for cancer or osteoporosis drug therapy. In order to understand the development of these diseases and to design efficient drugs for their therapy it is necessary to uncover the mode of action of the enzyme and the inhibitors and to also search for novel and more sophisticated antibiotics that satisfy the specific therapeutic requirements.

The use of inhibitors for the characterization of enzymes has a long tradition and was often the starting point for the discovery of target proteins for a known or novel potent antibiotic. After the discovery of bafilomycin and concanamycin as specific V-ATPase inhibitors (Bowman et al., 1988; Dröse et al., 1993), the phenotype of the inhibitory effect was used as indication for the presence of a V-ATPase in special cells or tissues. Utilizing such a procedure, the importance of V-ATPases for many different functions in many different cellular and tissue locations has been unequivocally demonstrated (Wieczorek et al., 1999). A more basic approach is the analysis of the interaction of an inhibitor and its target protein in detail, to finally understand the mode of inhibition at the molecular level. For V-ATPase inhibitors we are far away from this final goal, however, for some of them we are getting closer step by step.

The aim of this current review is to illustrate recent advances in the field, to present an update on the old players in the inhibition game, the plecomacrolides bafilomycin and concanamycin, and to introduce some of the new players, the macrolactone archazolid (Sasse et al., 2003), the benzolactone enamides salicylihalamide, lobatamide, apicularen, oximidine and cruentaren (Erickson et al., 1997; Galinis et al., 1997; Kim et al., 1999; Kunze et al., 1998; Kunze et al., 2006), and the indolyls (Gagliardi et al., 1998b; Nadler et al., 1998). Obviously this cast is not complete and, therefore, the specific V-ATPase inhibitors, which are not the focus of this review, are briefly mentioned here. (1) Destruxins are cyclic hexadepsipeptides isolated from fungi, e.g. *Metarhizium anisopliae*; for destruxin B, an IC_{50} value for the yeast V-ATPase of 5 μmol l^{-1} was reported (Muroi et al., 1994). (2) Prodigiosins (tripyrroles), which are produced by *Streptomyces hiroshimensis*, were originally reported to inhibit the proton pump activity of the V-ATPase in rat liver lysosomes in the nanomolar range (Kataoka et al., 1995) but later it was shown that the prodigiosins are

H^+/Cl^-symporters, which uncouple proton translocation (Sato et al., 1998). (3) Chondropsins are macrocyclic lactams purified from marine sponges, e.g. *Chondropsis* sp.; they exhibit IC_{50} values in the micromolar range for V-ATPases of chromaffine granules and *Neurospora crassa* (Bowman et al., 2003; Cantrell et al., 2000; Rashid et al., 2001). Application to *N. crassa* mutants with a lower sensitivity to bafilomycin suggests a similar mode of inhibition for both classes of antibiotics (Bowman et al., 2003). (4) Diphyllin, currently the latest specific V-ATPase inhibitor published, inhibits the V-ATPase activity in chromaffine granules and the acidification of lysosomes in human osteoclasts in the nanomolar range without having a cytotoxic effect on bone formation *in vitro* (Sørensen et al., 2007).

Old players: the plecomacrolides bafilomycin and concanamycin

The discovery of the plecomacrolide bafilomycin as the first specific and highly potent inhibitor of V-ATPases (Bowman et al., 1988) opened a new field of research by enabling the pharmacological identification of V-ATPases in a functional context. Bafilomycin and the other classical V-ATPase inhibitor concanamycin (Dröse et al., 1993) had already been identified and structurally described (Fig. 1) in the early 1980s (Kinashi et al., 1982; Kinashi et al., 1984; Werner et al., 1984). The structure–activity relationship for bafilomycin and concanamycin was investigated intensively (Dröse and Altendorf, 1997; Dröse et al., 1993; Dröse et al., 2001; Gagliardi et al., 1998a; Gagliardi et al., 1999). However, for more than a decade the location of the binding site in the enzyme was not known. Studies with the chromaffine granule V-ATPase suggested the membrane bound V_O complex to harbor the binding site (Hanada et al., 1990). This hypothesis was confirmed by two studies using the bovine clathrin coated vesicle V-ATPase. On one hand, acid-activated proton flux through the reconstituted V_O complex was inhibited by bafilomycin (Crider et al., 1994) and on the other hand, the addition of excess V_O complex restored the proton pump activity of coated vesicles (Zhang et al., 1994). As this latter effect was also observed after the addition of an excess of the V_O subunit a, the obvious conclusion was that this subunit contains the plecomacrolide binding site. On the contrary, there was a report that the V-ATPase could be purified by bafilomycin C affinity chromatography and that the affinity of the V-ATPase to the bafilomycin column decreased in the presence of the inhibitor DCCD, which binds to the V_O subunit c (Rautiala et al., 1993). Finally, two independent approaches revealed that the plecomacrolides indeed interact with the V_O subunit c. Firstly, amino acid exchanges in this subunit decreased the sensitivity of the V-ATPase from *N. crassa* to bafilomycin (Bowman and Bowman, 2002) and secondly, a radioactively labelled, semisynthetic diazyrinyl-derivative of concanamycin A (9-O-[p-(trifluoroethyldiazirinyl)-benzoyl]-21,23-dideoxy-23-[(125)I]iodo-concanolide A, in brief J-concanolide A) specifically labelled only this subunit in the V-ATPase of *Manduca sexta* by creating a covalent bond to the protein upon UV-irradiation (Huss et al., 2002). Since then, a plethora of mutations in *N. crassa* and in *Saccharomyces cerevisiae*, in combination with the recently available crystal structure of the bacterial Na^+–V_O ring from *Enterococcus hirae*, rounded up the picture and revealed a vivid model of the binding pocket (Bowman et al., 2006; Bowman et al., 2004; Murata et al., 2005). Meanwhile, a minor contribution of the V_O subunit a to the binding site could be proven because amino acid exchanges within this subunit also led to a slight decrease in plecomacrolide sensitivity (Wang et al., 2005). These findings may explain the early results cited above (Zhang et al., 1994).

New players, a growing team

Archazolid

One of the novel players is the recently discovered macrolactone archazolid, which is produced by the myxobacteria *Archangium gephyra* and *Cystobacter violaceus* (Menche et al., 2007c; Sasse et al., 2003). The major structural features of archazolid, as shown in Fig. 2, are 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 subnanomolar range (Sasse et al., 2003). A closer look into the cells revealed that archazolid led to the formation of vacuoles in the ER, a phenomenon that is typical for inhibitors of the V-ATPase (Sasse et al., 2003). Furthermore, archazolid prevented the acidification of lysosomes supporting the hypothesis that the V-ATPase was the possible target of the antibiotic (Huss et al., 2005). When tested on preparations of purified V-ATPase, Na^+/K^+-ATPase and mitochondrial F-ATPase,

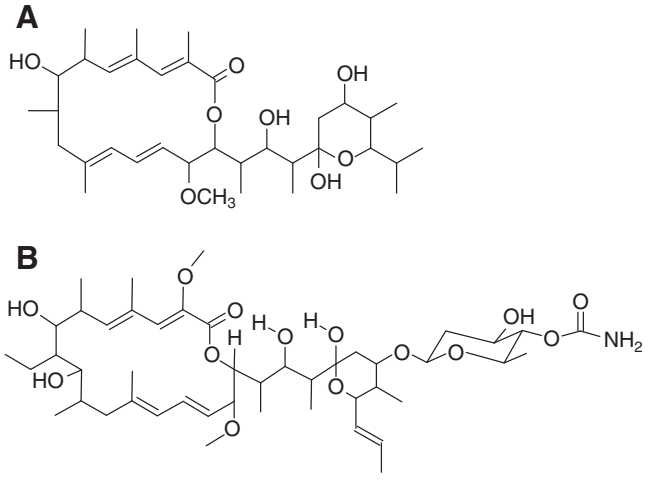

Fig. 1. Structures of bafilomycin A1 (A) and concanamycin A (B).

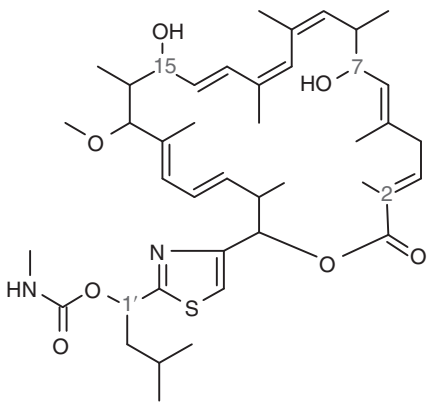

Fig. 2. Structure of archazolid A.

Table 1. Inhibition of the V-ATPase activity and cell growth by archazolids

Compound	V_1V_O holoenzyme *Manduca sexta* IC_{50} (μmol l^{-1})	Growth inhibition murine cell line L-929 IC_{50} (μmol l^{-1})
Archazolid A	0.02	0.001
Archazolid B	0.02	0.001
Archazolid C	5	2
Archazolid D	15	0.3
15-dehydro-archazolid A	0.3	>5
1′-descarbamoyl-archazolid A	0.1	0.01
7-*O*-*p*-Nitrobenzoate-archazolid A	6	0.3
7-*O*-TBS-archazolid A	4	0.9

Data taken from Huss et al. (Huss et al., 2005) and Menche et al. (Menche et al., 2007a; Menche et al., 2007b; Menche et al., 2007c).

respectively, archazolid appeared to be an exclusive and highly potent inhibitor of V-ATPases with IC_{50} values in the nanomolar range (Huss et al., 2005). Although archazolid is one of the most recent V-ATPase inhibitors, at least a part of its binding site has already been identified. It obviously interacts with the V_O subunit c as it competes with concanamycin for its binding site within this subunit. This was shown by cross-linking studies using the radioactively labelled J-concanolide A mentioned above (Huss et al., 2005). After pre-incubation with an excess of archazolid, radioactive labelling of the V_O subunit c was prevented. Additional structure-activity relationship studies with natural and semi-synthetic derivatives of archazolid revealed first insights which parts of the pharmacophore play a crucial role for the inhibitory properties (Table 1). Alterations at the side chains of the carbon atoms 2, 15 or 1′, which are present in archazolid B, 15-dehydro-archazolid A or 1′-descarbamoyl-archazolid A, respectively, had either no or only minor effects and resulted in an increase of the IC_{50} values maximally up to factor of 20 (Huss et al., 2005; Menche et al., 2007a). By contrast, modifications at the carbon atom 7 had a more drastic effect. For instance, the linkage to a sugar residue in the natural derivatives archazolid C and D or the synthetic alterations to a *para*-nitrobenzoate or sylil-ether increased the IC_{50} values more than 1000-fold (Menche et al., 2007b). Attempts to elucidate the biologically active conformation of archazolid by NMR and to investigate its binding site in detail by site directed mutagenesis of subunit c are in the focus of current projects (Farès et al., 2008; Hassfeld et al., 2006) (S. Bockelmann, M.H. and H.W., unpublished results).

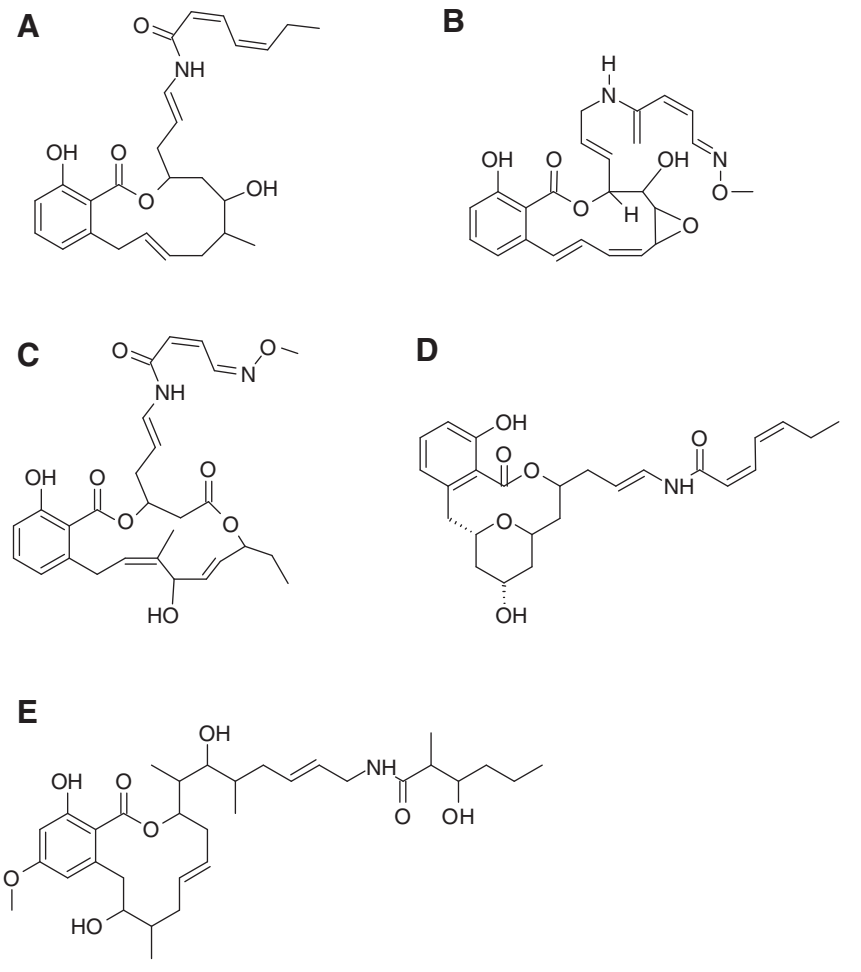

Fig. 3. Structures of salicylihalamide A (A), oximidine I (B), lobatamide A (C), apicularen A (D) and cruentaren (E).

Benzolactone enamides

In the late nineties of the last century a new class of highly cytotoxic compounds, which all share a benzolactone enamide core structure as a common feature, entered the field (Erickson et al., 1997; Galinis et al., 1997; Kim et al., 1999; Kunze et al., 1998). They had been 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 (Fig. 3). The benzolactone enamides isolated from sponges and tunicates are assumed to be metabolites of microbial symbionts (Boyd et al., 2001). In cell tests, all of these substances exhibited IC_{50} values in the nanomolar range (Erickson et al., 1997; Huss et al., 2005; Kim et al., 1999; Kunze et al., 1998; McKee et al., 1998) and in the NCI 60-Cell screen, all of them revealed a V-ATPase inhibitor pattern similar to that of the plecomacrolides (Boyd et al., 2001) (Brigitte Kunze, personal communication). Assays of their inhibitory specificity for the V-ATPase carried out with either membrane preparations of human kidney, liver, osteoclast or bovine chromaffin granules (Boyd et al., 2001; Shen et al., 2002), or with purified V-ATPase from insect midgut (Huss et al., 2002; Huss et al., 2005) or bovine clathrin coated vesicles (Xie et al., 2004) clearly showed that the benzolactone enamides are indeed a novel family of highly specific V-ATPase inhibitors, with IC_{50} values in the nanomolar range. One of the most astonishing and interesting features of the benzolacton enamides is that they do not inhibit V-ATPases from fungal sources, as was shown so far for salicylihalamide, lobatamides and oximidines with preparations of vacuolar membranes from *N. crassa* and *Streptomyces cerevisiae* (Boyd et al., 2001), and for apicularen with preparations of vacuolar membranes from *S. cerevisiae* (S. Bockelmann, M.H., B. Kunze and H.W., unpublished results). Therefore, the benzolactone enamides are the first class of V-ATPase inhibitors that discriminate for unknown reasons between the V-ATPase from different species. Another unknown feature is the binding site for the benzolactone enamides; for salicylihalamide and apicularen it had been shown by competition experiments with the radioactive and UV-inducible crosslinker J-concanolide A that their binding site is different from that for the plecomacrolides as both compounds did not prevent labelling of subunit c with J-concanolide A (Huss et al., 2002; Huss et al., 2005). Nevertheless, Xie and coworkers showed that the binding site should reside within the V_O complex as salicylihalamide inhibited not only the proton pumping activity of the reconstituted V_1V_O holoenzyme from bovine clathrin coated vesicles but also the H^+-channel activity of the reconstituted V_O complex (Xie et al., 2004).

To understand the structural key features of the benzolactone enamides for their V-ATPase inhibition capacity, so far, structure activity relationship studies with natural and synthetic variations have been carried out for apicularen, salicylihalamide and lobatamide. For example, studies with apicularen point out the importance of analyzing the inhibitory efficacy of an antibiotic and its derivatives both on the isolated enzyme and in the cellular context, because the results may differ to a great extent (Table 2). In the case of apicularen, the natural glycoside apicularen B, which has *N*-acetylglucosamine linked to the hydroxyl group at C11, revealed a 1000-fold increase of the IC_{50} value when applied to whole cells but only a 3-fold IC_{50} increase when tested on the purified V-ATPase (Huss et al., 2005) (see also Table 2). Similar effects were observed for the semisynthetic derivatives open-, *N*-methyl- and oxime-apicularen A, where in five cell lines the mean IC_{50} values increased 80-, 150- and 500-fold, respectively, as compared with apicularen A (Petri et al., 2005). By contrast, the IC_{50} values for the purified enzyme did not change for open-apicularen A, and for *N*-methyl- and oxime-apicularen A only a 10-fold IC_{50} increase was observed (Table 2). Nevertheless, these studies revealed that modifications at the enamide side chain represented by enyne-, oxime- and *N*-methyl-apicularen A are critical for inhibitory efficacy whereas modifications at the macrolactone ring, e.g. a deoxylation or a *N*-acetylglucosamine at C11 or an internal opening of the ring, are accepted.

For salicylihalamide, it was shown that *N*-acyl modifications had no effect on the inhibition properties and therefore Xie and coworkers concluded that the hexadienyl could be omitted (Xie et al., 2004). However, attempts to functionalize this position by the insertion of a biotin for detection or farnesyloxy and cholesteryloxy for membrane anchoring failed as this led to an increase of the IC_{50} values by a factor of 1000 (Xie et al., 2004). Surprisingly, the oxidation of the enamide or its substitution by an enone had only little effect on the inhibitory properties.

The lobatamides were carefully analyzed by Shen and colleagues, who found out that the enamide NH, the salicylate phenol and ortho-substitution of the salicylate ester are key features for the inhibition of the V-ATPase (Shen et al., 2003). Furthermore, they tried to synthesize simplified and photoactivatable analogues of this compound; the details of their results would go far beyond the scope of this review and we therefore refer to their original publications (Shen et al., 2005; Shen et al., 2002; Shen et al., 2003).

Recently, a potentially very useful application for the benzolactone enamides has been reported (Whitehurst et al., 2007). The authors detected a strikingly synergistic effect of the chemotherapeutic drug paclitaxel, better known as taxol, and RTA 203, a derivative of salicylihalamide, when applied simultaneously to the cancer cell line H1155 even at doses where the single compounds had only a minor effect.

During the screening for biologically active metabolites from myxobacteria, Kunze and coworkers discovered a novel macrolide, the benzolactone cruentaren A from *Byssovorax cruenta*, (Fig. 3),

Table 2. Inhibition of the V-ATPase activity and cell growth by apicularen

Compound	V_1V_O holoenzyme *Manduca sexta*[a] IC_{50} ($\mu mol\,l^{-1}$)	Growth inhibition Murine cell line L-929[b] IC_{50} ($\mu mol\,l^{-1}$)
Apicularen A	0.02	0.005
Apicularen B	0.06	0.6
N-methyl-apicularen	0.2	0.8
11-deoxy-apicularen	0.02	0.02
Open apicularen	0.02	0.2
Oxime apicularen	0.2	2
Enyne apicularen	1	5

Data taken from [a]Huss et al. (Huss et al., 2005), M.H., M. E. Maier and H.W. (unpublished observations), and [b]Petri et al. (Petri et al., 2005).

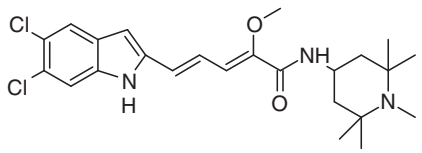

Fig. 4. Structure of INDOL0.

which exhibits a high cytotoxity for mammalian and fungal cells (Kunze et al., 2006). Because of its structural relationship to the benzolactone enamides, with apicularen as the closest relative, it was initially assumed that cruentaren may 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 (Kunze et al., 2006; Kunze et al., 2007). Beyond that the interaction site of cruentaren resides in the F_1 complex whereas the benzolactone enamides, as mentioned above, operate *via* the membrane bound V_O complex.

Indolyls

Bafilomycin-based structure activity studies with different V-ATPase containing membrane preparations led to the identification of several key structural elements for biological activity (Gagliardi et al., 1998a). This allowed the design and synthesis of novel indole derivatives as structurally simpler V-ATPase inhibitors (Gagliardi et al., 1998b). The most potent inhibitor, with an IC_{50} of 30 nmol l^{-1} for the chicken osteoclast V-ATPase, was (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 (Nadler et al., 1998). This compound enabled new approaches in V-ATPase inhibitor research by the incorporation of spin labelled derivatives of INDOL0 into the lipid membrane, the V-ATPase holoenzyme or parts of it and by applying biophysical techniques such as electron paramagnetic resonance (EPR), fluorescence spectroscopy or fluorescence resonance energy transfer (FRET) (Dixon et al., 2003; Dixon et al., 2004; Dixon et al., 2008; Fernandes et al., 2006a; Fernandes et al., 2006b; Pali et al., 2004a; Pali et al., 2004b; Whyteside et al., 2005). In this way, by using either a spin labelled subunit c or nitroxide spin-labeled derivatives of INDOL0 for instance, it was shown that INDOL0 strongly interacts with the transmembrane segments of subunit c (Dixon et al., 2008; Pali et al., 2004b). Furthermore it was shown that mutations in subunit c, which led to a lower sensitivity to bafilomycin, were also less sensitive to INDOL0, implying a similar mode of inhibition for both bafilomycin and INDOL0.

Perspectives

There are two major challenges in V-ATPase inhibitor research. The first is to uncover where exactly and how an inhibitor interacts with the protein, in order to understand the nature of the inhibitory effect. The second venture is to develop applicable drugs for the treatment of diseases like osteoporosis or cancer.

Regarding the first aspect, a lot is to be expected from the above mentioned biophysical approaches such as EPR, NMR or FRET as they allow the interaction between inhibitors or their derivatives with the V-ATPase to be measured, which itself may be altered by the exchange of amino acids within the putative inhibitor binding site by mutagenesis. For those inhibitors where the binding site is less understood or completely unknown, new techniques are emerging such as designing cross-linkable derivatives of the antibiotics to make responsible subunits detectable (Bender et al., 2007; Biasotti et al., 2003; Mayer and Maier, 2007; Shen et al., 2005). But as long as a high resolution structure of the complete V-ATPase or at least the V_O complex is not available, a major part of this interaction will remain enigmatic.

Regarding the second aspect, there were a handful of promising results during the past years. In the case of cancer treatment, the discovery of the synergistic effect of the synthetic benzolactone enamide RTA 203 together with taxol is very interesting and immediately implies the question of what could be achieved when other inhibitors are applied in varying combinations. In addition, the so far unique feature of the benzolactone enamides to strictly discriminate between the V-ATPases from different sources, may open up new opportunities to develop tissue specific drugs. On the other side, a high tissue specificity may be negligible provided that the drug is efficiently delivered to target cells, which may be achieved by targeting *via* antibody or ligand conjugation (Hilgenbrink and Low, 2005; Schrama et al., 2006). Moreover, as documented by the rising number of recently discovered inhibitors as a result of extensive screening for novel and specialised antibiotics from all different natural sources, the chance to find the 'inhibitor of choice' for a given task is increasing.

An important footnote may be added. A recently discovered surprising property of bafilomycin A_1 is that it also represents a carrier type K^+ ionophore (Teplova et al., 2007). Therefore, bafilomycin A_1 not only inhibits the V-ATPase but it may also have additional effects in complex tissues. This will make it necessary to revisit old findings and may be a warning not to simply rely on what is called inhibitor specificity.

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