A Therapeutic Role for the F1FO-ATP Synthase

A therapeutic role for the F_1F_O-ATP synthase

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Abstract

Recently the F_1F_O-ATP synthase, due to its dual role of life enzyme as main ATP maker and of death enzyme, as ATP dissipator and putative structural component of the mitochondrial permeability transition pore (mPTP), which triggers cell death, has been increasingly considered as drug target. Accordingly, the enzyme offers new strategies to counteract the increased antibiotic resistance. The challenge is to find or synthesize compounds able to discriminate between prokaryotic and mitochondrial F_1F_O-ATP synthase, exploiting subtle structural differences to kill pathogens without affecting the host. In this perspective, the eukaryotic enzyme could also be made refractory to macrolide antibiotics by chemically produced post-translational modifications. Moreover, since the mitochondrial F_1F_O-ATP ase activity stimulated by Ca^{2+} instead of by the natural modulator Mg^{2+} is most likely involved in mPTP formation, effectors preferentially targeting the Ca^{2+} activated enzyme may modulate the mPTP. If the enzyme involvement in the mPTP will be confirmed, Ca^{2+}-ATPase inhibitors may counteract conditions featured by an increased mPTP activity, such as neurodegenerative and cardiovascular diseases and the physiological aging. Conversely, mPTP opening could be pharmacologically stimulated to selectively kill unwanted cells. On the basis of recent literature and promising lab findings, the action mechanism of F_1 and F_O inhibitors is considered. These molecules may act as enzyme modifiers and constitute new drugs to kill pathogens, improve compromised enzyme functions and limit the deathly enzyme role in pathologies. The enzyme offers a wide spectrum of therapeutic strategies to fight at the molecular level diseases whose treatment is still insufficient or merely symptomatic.

Keywords

Mitochondria; F_1F_O-ATP synthase, drug binding sites; diseases; mitochondrial permeability transition pore.

Introduction

The F_1F_O-ATP synthase is a ubiquitous enzyme complex localized in the inner mitochondrial membrane and thylakoid membranes of eukaryotic cells and in bacterial plasma membrane, endowed with bifunctional properties of ATP synthesis and hydrolysis. Due to the F_1F_O-ATP synthase prominent feature of "enzyme of life", as key participant in the cell bioenergetic machinery in aerobiosis, it is quite obvious to imagine that enzyme inhibitors can be exploited as drugs to kill noxious cells. However, only in recent times this potentiality was considered. The main difficulty in the therapeutic exploitation most likely relies in the consideration that the enzyme function and mechanism are similar in all taxa, in spite of some structural divergences [1], while drugs should distinguish among targets. Basically, the F_1F_O-ATP synthase consists of an hydrophilic portion known as F_1, which hosts the catalytic sites, and a membrane-embedded F_O domain which translocates H^+ [2]. The hydrophilic F_1 domain protrudes in the mitochondrial matrix and is involved in the catalytic activity of ATP synthesis and hydrolysis. The embedded-membrane F_O domain channels H^+ driven by proton-motive force Δp and generates the torsion required for the F_1 catalytic activity. The chemo-mechanical coupling between the two domains is ensured by a peripheral stalk and a central stalk, which connect F_1 to F_O. The F_1F_O-ATP synthase synthesizes ATP by exploiting the electrochemical energy produced by the respiratory chain in the form of Mitchell's Δp across the inner mitochondrial membrane. Therefore ATP generation results from a chemo-mechanical coupling mechanism [3]. Moreover, under physio-pathological conditions the enzyme can work in *reverse* as ion pump. In this case it hydrolyzes ATP to generate the torque which provides the Δp required for H^+ uphill translocation [4]. All F_1F_O-ATP synthases have a conserved basic composition, which mirrors the relatively simple bacterial enzyme subunit stoichiometry of $(\alpha\beta)_3$, γ, δ, ε in the F_1 domain and of *a*, b_2, c_{10} subunits in the F_O domain. In mammals the hydrophilic F_1 domain consists of five different globular subunits with $(\alpha\beta)_3$, γ, δ, ε stoichiometry, while the hydrophobic F_O domain is formed from the subunits *a*, the c_8-ring, two membrane-inserted α-helices of *b* subunits and supernumeraries subunits, namely *e*, *f*, *g*, A6L, DAPIT and 6.8 kDa proteolipid, which are typical of mammalian mitochondria (Fig. 1). Mitochondrial δ and bacterial ε subunit are homologous, and mitochondrial OSCP subunit is homologous to bacterial δ subunit. Conversely, mitochondrial ε subunit has no homologue in prokaryotes [5]. In addition, the mitochondrial enzyme shows OSCP, *d*, F_6 extra subunits on the stator sub-complex joined to the extrinsic α-helices of *b* subunits [6]. The *a* and A6L subunits of the F_1F_O-ATP synthase are codified by the mitochondrial DNA, while the other subunits are from nuclear DNA. Moreover, the nuclear gene of the *c*-subunits has three isoforms. Indeed, these subtle structural differences between the same enzyme complex in mammalian mitochondria and in bacteria are crucial in the perspective of exploiting the ATP synthase as drug target to selectively fight pathogens without affecting the mammalian host.

Due to its function of main ATP maker, the F_1F_O-ATP synthase inhibition is incompatible with life under aerobic conditions. However, the recent implication of the F_1F_O-ATP synthase in cell death extends the potential exploitation of the enzyme as drug target to kill unwanted cells or, by blocking its lethal role, to prevent cell death associated with some still poorly treatable pathologies. Accordingly, under patho-physiological conditions the enzyme complex can favour or even directly produce an increase in permeability of the inner mitochondrial membrane, an event which dissipates Δp and ion homeostasis and ultimately leads to programmed cell death [7]. Interestingly, cell death in diseases featured by mitochondrial dysfunctions and bioenergetic failure is accompanied by a common event, namely the formation of the mitochondrial permeability transition pore (mPTP) [8]. The mPTP is an open high-conductance channel in the inner mitochondrial membrane which opens

when Ca^{2+} concentration rises in the mitochondrial matrix [9,10]. Recent works strongly suggest that the F_1F_O-ATP synthase, in its dimeric state or by exploiting the *c*-ring [11,12], could form the mPTP.

On considering the emerging double role of this intriguing enzyme complex, as aerobic builder of ATP, the main energy currency molecule, and as energy-dissipating engine, the perspective of exploiting it as drug target (Table 1) is not remote and some promising attempts have already been described [13]. New-generation inhibitors may be addressed to selectively kill bacterial pathogens, overcoming the increasing threat of antibiotic resistance, while mPTP modulators targeting the F_1F_O-ATP synthase may constitute innovative therapeutic interventions [14–16] to prevent cell death in diseases associated with mitochondrial dysfunctions or, conversely to initiate the deathly pathway to lessen the proliferation of malignant cells. While the possibility of exploiting F_O subunits as drug targets has been strongly supported by bioinformatic insights [17], the possible modulation of the drug potency, on the one side by acting on drug design or structural modifications of natural compounds [13,18] and, on the other, through aminoacid substitutions or post-translational modifications on both F_1 and F_O domains [19], still poses some technical difficulties and interrogatives.

The F_1F_O-ATP synthase as target of new antimicrobial drugs

The use of the mitochondrial F_1F_O-ATP synthase as a drug target has been extensively considered in drug design [19–22], especially to selectively kill noxious cells [23], due to the immediate link between ATP deprivation and cell death. The possibility of exploiting the F_1F_O-ATP synthase as target of innovative drugs to kill pathogens is based on the prerequisite that drug molecules can selectively bind and inhibit the bacterial enzyme and do not affect the mammalian F_1F_O-ATP synthase. In the worrying context of increased antibiotic resistance, luckily the enzyme looks as the Achille's heel of multi-drug resistant microorganisms [24]. To fight pathogens, slight structural differences between microbial and mammalian enzymes are crucial. Inhibitors of the F_1F_O-ATP synthase bind to the enzyme and often produce post-translational modifications of enzyme proteins, namely they chemically modify amino acid residues which are essential for the enzyme catalysis or, indirectly, for driving protons across the inner mitochondrial membrane [19,25]. Moreover, the role of the F_1F_O-ATP synthase as key molecular and enzymatic switch between cell life and death [21,26] increases its attractiveness in pharmacology. Any natural or synthetic compound which targets the F_1F_O complex and/or modulates its catalytic activity can potentially be used in therapy to counteract pathogens [15,27], assumed that it is able to discriminate between eukaryotic and prokaryotic F_1F_O-ATP ATP synthases. The tight connection between antibiotics and F_1F_O-ATP synthase is long known. Accordingly, the membrane-embedded rotor F_O takes its name from the antibiotic oligomycin, which specifically inhibits both F_1F_O-ATP synthesis and hydrolysis by blocking proton translocation through F_O. In the last decades many natural compounds produced by microorganisms, insects and amphibians were shown to bind to and inhibit the F_1F_O-ATP synthase. Some of them exhibit multiple properties, namely antimicrobial/anticancer activities.

Drugs targeting F_1

The immediate connection between block of ATP production and arrest of cell proliferation addressed most studies to inhibitors which bind to the catalytic portion F_1 [28]. Many phytochemicals with antimicrobial properties inhibit bacterial F_1F_O-ATP synthase by interacting with a phytochemical-binding region on F_1 [13] (Fig. 2). Most of them have a polyphenolic or polycyclic structure. Thymoquinone, safranal, piceatannol and baicalein 100% inhibit the *E. coli* wild-type F_1F_O-ATP synthase. The inhibition power depends on the type and positioning of the functional groups of these

molecules and offer helpful hints in drug design. Accordingly, the addition, deletion, and rearrangement of functional groups can enhance the degree of inhibition. Natural resveratrol causes about 40% inhibition of F_1F_O-ATP synthase with IC_{50} at about 94 μM but its chemical modification by removal, addition, or repositioning of its functional groups can result in much more powerful drugs [29]. Another phytochemical, hydroxytyrosol from olives, caused about 60% inhibition of *E. coli* membrane-bound F_1F_O-ATP synthase, but the repositioning of its -OH groups resulted in almost complete enzyme inhibition [13]. Some venom peptides [18] from wasp, spider, bee and scorpion target the F_1F_O-ATP synthase also block bacterial proliferation. These peptides bind to the βDELSEED-motif residues of F_1. Modified venom peptides with C-terminal amide (-NH_2) groups enhanced both the enzyme inhibition and *E. coli* cell death. These peptides show different degrees of hydrophobicity and hydrophilicity and interact with different aminoacid residues in the common region of F_1.

Drugs targeting F_O

More recently, the membrane-embedded portion F_O revealed its attractiveness as target of lipophilic drugs [15,16,30]. Some natural compounds structurally related to oligomycin [31], have been shown to target F_O and to similarly inhibit the ATP synthase. Moreover, the susceptibility of *c*-subunits to post-translational cysteine (Cys) thiol oxidation [31,32] discloses a potential capability to modulate the enzyme susceptibility to inhibitors. The sulfur-containing Cys is especially susceptive to post-translational modifications of its thiol group which may act as chemical switch according to the cell redox state [33]. Interestingly, bacterial F_1F_O-ATP synthase contain less Cys than the mitochondrial enzyme and these Cys are apparently not essential for the enzyme functions. Accordingly, the enzyme function in *Escherichia coli* is fully maintained when all the 21 native Cys are replaced by alanines [34]. Post-translational modifications are poorly documented in bacteria and probably confined to some proteins and species [35]. Conversely, the mitochondrial F_1F_O-ATP synthase is a hot spot for oxidative post-translational modifications involving thiols. Interestingly, some thiols of F_O are essential to bind the antibiotic oligomycin, which blocks the H^+ flux within F_O. In detail, oligomycin binds to the *c*-ring by interacting with the aminoacid side chains of two adjacent *c* subunits (in general identified as *c1* and *c2*) and covers the H^+ binding site of Glu residue on *c1* [36] which is involved in ion translocation [37]. If these thiols are oxidized, the F_1F_O-ATP synthase becomes refractory to oligomycin and other macrolide antibiotics such as venturicidin, apoptolidin and bafilomycin [31] (Fig. 2). The proteolipid subunits of the *c*-ring frame a common "drug-binding region", which bind closely related macrolide antibiotics in distinct sites which share a common region [16,31,36]. Therefore, an intriguing hypothesized antibacterial strategy may be based on the potential desensitization of the mammalian enzyme to these antibiotics by exploiting a chemical treatment which oxidizes these crucial thiols. In this case the so-called drug-binding region of the F_1F_O-ATP synthase will become an effective target for antibiotics [31,36] which will leave the host enzyme unaffected [16]. Working in this direction, unfortunately up to now the antibiotic-desensitizing properties were only found for the highly toxic organotin compounds [32,38,39], which can bind to the F_O domain [40] (Fig. 2) and whose biocidal effects prevent their use in therapy.

Tuberculosis is a major health problem and still a great threat for humans, due to the increasing resistance to traditional anti-mycobacterial drugs which make the need for new therapeutic tools really pressing [41]. *Mycobacterium tuberculosis* is usually fought by mixtures of drugs which act on different targets [42]. The peculiar structure of the *c*-ring of mycobacterial F_1F_O-ATP synthase, which only catalyzes ATP synthesis and cannot operate in the reverse ATP hydrolysis mode, offers new therapeutic options to fight tuberculosis. The new diarylquinoline drug, also known as bedaquiline

(BDQ) trade name Sirturo, code names TMC207 and R207910 [43] binds to the *c*-ring [44] and also interacts with the mycobacterial ε subunit [45] by inhibiting the F_1F_O-ATP synthase, which is essential for *Mycobacterium* growth. The BDQ molecule approach the *c*-ring and it binds the region of the proton-binding sites (Fig. 2) by resembling the DCCD and oligomycin mode of inhibition of F_1F_O [44]. BDQ would also act as uncoupler by elecroneutral H^+/K^+ antiporter [46]. According to the Nath's model, BDQ binds H^+ from outside the mycobacterial cell and translocates H^+ inside along the electrochemical gradient. The transport is electroneutral because simultaneously K^+ is transported outside [46]. However, differently from classical uncouplers, BDQ only dissipates ΔpH and does not interfere with the membrane potential ($\Delta\psi$) which would be maintained by the negatively charged monoanion succinate translocation from outside to inside through F_O or by the electrically equivalent K^+ transfer from inside to outside. The futile cycle created by BDQ accelerates respiration. The preserved $\Delta\psi$ would impair succinate homeostasis and could be the ultimate cause of mycobacterial death. Accordingly, succinate could be translocated outwards coupled with H^+ by the redox reactions, but could not re-enter due to the $\Delta\psi$. According to this model, the depletion of internal succinate required for Krebs cycle and oxidative phosphorylation would kill mycobacteria [47]. In spite of some aspects which remain to be fully understood, this recent model, sustained by $\Delta\psi$ measurements, provides new insights in the BDQ bactericidal mechanism.. Even if unfortunately BDQ-resistant strains are increasing [48] and BDQ cardiovascular side-effects of this drug are still a matter of concern [49], the assessment of BDQ properties opens the way to the design of new antimycobacterial drugs targeting the F_1F_O-ATP synthase. Accordingly, novel compounds named 5228485 and 5220832 selectively targeting the F_1F_O-ATP synthase of *M. tuberculosis* and showing excellent bactericidal activity *in vitro* have been identified [50]. These compounds, which show low toxicity to mammalian mitochondria, interact with mycobacterial *c*-subunits by binding to different aminoacids with respect to BDQ.

The F_1F_O-ATP synthase as target of cardioprotectants

The F_1F_O-ATP synthase susceptibility to oxidative stress suggests that it can be adequately modulated by exogenous compounds which modify the cell redox state. Many cardiovascular diseases are featured by oxidative stress [51], which, among diffused damage to biomolecules, causes post-translational modifications on the enzyme. Accordingly, pharmacological manipulations of oxidative and nitrosative pathways are known to be beneficial in patients with heart failure [52,53]. Moreover, oxidative stress in cardiovascular diseases often stems from mitochondrial dysfunctions. In recent years pharmacological research has been focused on the development of compounds which specifically target mitochondrial components to treat cardiovascular pathologies. Unfortunately, up to now no drugs specifically conceived to modulate mitochondrial functions are currently available [54]. However, many hints suggest that the beneficial effects of some currently used cardioprotectants are due to their chemical effects on the F_1F_O-ATP synthase. During heart failure the α subunit of F_1 forms disulfide bonds between Cys294 on neighboring α subunits as well as between Cys294 and Cys103 on γ-subunit. The same Cys294 can also be *S*-glutathionylated and *S*-nitrosylated. The formation of disulfide bonds and the glutathionylation (RS-SG) at these regulatory sites inhibits the F-ATPase activity, thus suggesting that these bonds modify the enzyme conformation and prevent catalysis [19]. Some heart malfunctions featured by compromised electric activities are increasingly treated by the resynchronisation therapy (RST), namely by electrode insertions in specific heart areas to coordinate the contraction of heart chambers, namely to "resynchronize" the heart. Among the RST effects, some chemical modifications are especially interesting. An adequate RST in heart failure patients affects the mitochondrial subproteome by modifying some proteins involved in cellular redox

control and oxidative phosphorylation pathways [55]. The RST reverses the disulfide bond formation and replaces it by *S*-nitrosylation accompanied by a recovery of the F-ATPase activity. It seems likely that, the cardiac RST therapy may also stimulate the mitochondrial antioxidant defense systems [56] or enhance the reducing status in the molecular environment. Reversible Cys oxidations may protect against permanent oxidative damage to the F_1F_O-ATP synthase which would decrease ATP production [56]. On the other hand, during heart failure, the F_1F_O-ATP synthase inhibition would have the physiological meaning to limit ATP consumption, contributing to ATP homeostasis, reducing the $\Delta\psi$ and consequently the driving force for Ca^{2+} uptake [57]. It seems likely that Cys294 in the F_1F_O-ATP synthase α subunit, localized on the enzyme surface and surrounded by several basic amino acids residues, would act as a redox switch. Under physiological conditions it is probably deprotonated and susceptive to oxidants. Most likely, at first Cys294 is oxidized to sulfenic acid, thus causing conformational changes which in turn expose other side chains such as Cys103 of γ subunit. When *S*-glutathionylated or involved in disulfide bonds between two Cys in adjacent α subunits as well as between Cys294 and Cys103 of the γ subunit, these cross-links block the enzyme rotation and consequently ATP production. The RST would stimulate the cellular antioxidant efficiency, break the disulfide bonds and favor *S*-nitrosylation, a post-translational modification which is compatible with the catalytic activity [56]. Thus, this single modifiable Cys in the F_1F_O complex would act as a redox modulator of cellular ATP concentration [58]. However, since an increase in *S*-nitrosylation of α subunits of F_1 causes a dose-dependent decrease of the enzyme activity and *S*-nitrosylation inhibits the F-ATPase activity during ischemia/reperfusion [53], most likely the effects on the catalytic activity of the F_1F_O complex may depend on the targeted Cys and still poorly defined variables. It is not clear if, at least under some conditions, *S*-nitrosylation can also lead to *S*-glutathionylation [51].

In this molecular context, the beneficial effects of nitrite, abundant in many vegetables and now recommended to prevent cardiovascular diseases, most likely could depend on post-translational modifications on the F_1F_O-ATP synthase [52]. During hypoxia/reoxygenation cycles in cardiac mitochondria a sudden loss of membrane potential is accompanied by an increase in reactive oxygen species (ROS) [59]. Upon oxidative stress, nitrite would generate the radical $\cdot NO_2$, which in turn would promote the formation of tyrosyl radicals from protein tyrosine residues [60]. By binding to the enzyme-ATP complex (ES), consistently with the uncompetitive inhibition mechanism [52], nitrite would produce a two-step post-translational modification: the first step generates tyrosyl radicals while the subsequent step yields the ES complex and dityrosine. Dityrosine formation would be favored by the conformational change of the catalytic sites during ATP hydrolysis, which, by making closer two adjacent aromatic radicals, would make them bind together. Nitrite does not form nitrotyrosines, the most common post-translational pathological modification of tyrosine [61], but dityrosine. Consistently with this radical mechanism, dityrosine formation promoted by nitrite and the F_1F_O inhibition are enhanced by oxidative stress [52]. Consistently, the benefits of nitrite could be at least partially ascribed to the decreased ATP dissipation [62].

The F_1F_O-ATP synthase as target of anticancer drugs

The inhibition of the mitochondrial ATP production could be an efficient antiproliferative weapon, to kill malignant cells. Even if cancer cells mainly rely on glycolysis for ATP production (Warburg effect) [63], many natural phytochemicals which target the F_1F_O complex were claimed as beneficial to fight cancer and prevent metastatic dissemination. Most studies were addressed to the catalytic portion F_1 that binds various natural compounds. The F_1F_O-ATP synthase has about twelve discrete inhibitor binding sites including peptides and other inhibitors located at the interface of α/β subunits

on the F_1. Antitumor peptides bearing α-helical structure interact with the β DEELSEED site of F_1 [64]. These peptides are derived from various animal taxa, including insects, yeasts and amphibians. Some of them also display antimicrobial activity, due to a shared antiproliferative mechanism. The anticancer activity of some polyphenols may be at least partially ascribed to their binding to the F_1F_O-ATP synthase[64]. Among phytochemicals, only genistein binds to the F_O sector [29], due to its hydrophobicity and membrane penetration, but recent studies did not confirm this target. Other natural and synthetic compounds such as apoptolidin have been shown to inhibit the ATP synthase by specifically targeting F_O and particularly the *c*-ring. The natural macrolide apoptolidin, which selectively leads transformed cells to apoptosis, revealed significant similarity between the apoptolidin aglycone and oligomycin. Accordingly, apoptolidin binds to the macrolide binding region of F_O [22] (Fig. 2). Other natural and synthetic polyketide macrolides (mandelalides) are highly cytotoxic especially on cells with an oxidative phenotype. Some of these compounds bind to the F_1F_O-ATP synthase and stimulate caspase-dependent apoptosis in HeLa cells after inhibition of glycolysis by D-deoxyglucose [65]. However not always the F_1F_O-ATP synthase inhibition by apoptolidin and analogues correlates with the induction of cell death [66]. Most likely, the antiproliferative properties of these compounds are only partially referable to the F_1F_O-ATP synthase inhibition and alterations in the signaling pathways are involved. However, the properties of these compounds suggest that relatively small molecules binding to the F_1F_O-ATP synthase can be exploited to modulate the apoptotic pathway. An alternative approach to kill unwanted cells by driving them to apoptosis may be to favor the lethal activity of the F_1F_O-ATP synthase, namely to stimulate the formation of the mPTP, as considered below.

The F_1F_O-ATP synthase as target of pore-modulators

Compounds able to prevent the mitochondrial permeability transition, the master player in apoptosis and necrosis, are increasingly considered as beneficial tools in cytoprotection. Since the mitochondrial permeability transition is ascribed to the mPTP, these compounds should be pore-shutters. Assumed that, as strongly suggested by recent advances, the mPTP formation involves the F_1F_O-ATP synthase [11,67], compounds acting on the enzyme complex may modulate the mPTP. The 1,4-benzodiazepine (Bz-423), inhibitor of F_1F_O-ATP synthase, binds to the OSCP subunit at the same site as cyclophilin D (CyPD), the only known protein modulator of mPTP [68]. Bz-423 induced apoptosis by sensitizing the mPTP to Ca^{2+} [11] (Fig. 2). CyPD interactions with the OSCP subunit decrease the hydrolysis of MgATP while cyclosporin A (CsA) increases the Mg-ATPase activity by displacing CyPD from its binding site [69]. Moreover, CsA has long been known to inhibit the mPTP [70]. The CsA-dependent mPTP inhibition is responsible for the immunosuppressive action by calcineurin. The mPTP opening as end stage event of mitochondrial demise contributes to cellular damage in spinal cord injury [71]. The non-immunosuppressive CsA analog, NIM811, improves function recovery in spinal cord injury. Therephore, in the central nervous system the mitochondrial function can be potentially exploited as drug target [71].

Recent evidence indicates that both Ca^{2+} and Mg^{2+} elicit F_1F_O-ATP synthase activities are referable to the same F_1F_O-complex [72],and, when Ca^{2+} rises in mitochondria, the enzyme complex would switch to the Ca^{2+}-activated mode, the inner mitochondrial membrane is depolarized [59] and the F_1F_O-ATP synthase works "in reverse", namely it hydrolyzes ATP. According to an intriguing model, when the enzyme complex is activated by Ca^{2+} instead of by the natural cofactor Mg^{2+}, the higher steric hindrance of Ca^{2+}, which would insert in the catalytic sites of F_1 in replacement of Mg^{2+}, would trigger conformational changes [73]. These changes, once transmitted to F_O, would detach the two joined

monomers of the dimer, thus opening the mPTP [26]. In this case the F_1F_O-ATP synthase would act as "death enzyme" leading to cascade events which ultimately cause cell death [74]. So, any compound which preferentially inhibits the Ca^{2+}-activated F_1F_O-ATP synthase with respect to the Mg^{2+}-activated F_1F_O-ATP synthase can be potentially thought as pore-shutter and be used to limit or prevent cell death. This property can be exploited as an efficient molecular tool to treat mPTP related-mitochondrial dysfunctions which are a common feature not only of mitochondrial disorders but also of a variety of human pathologies. However, to prevent mPTP formation and be used in therapy, the pore-shutter should discriminate between normal and mutated or modified (pathological) enzyme complexes, such as those found in some neurodegenerative human diseases, namely maternally inherited Leigh's syndrome (MILS) and neuropathy, ataxia, retinitis pigmentosa (NARP) [75]. Pathogenic mutations often involve changes in the Atp6p (or *a*) subunit of the F_1F_O-ATP synthase, which contains most of the residues involved in proton translocation through F_O [37]. Other pathologies are featured by increased mPTP formation. These diseases are often associated with oxidative stress, which in turn favors mPTP opening. Drug design could be addressed to different directions. A first approach could aim at obtaining molecules which selectively bind to the mutated aminoacid residues in the genetic diseases so as to chemically modify their properties and allow/improve the mitochondrial F_1F_O-ATP synthase function.

Some suggestions are provided by known antiapoptotic proteins. Bcl-X_L is an anti-apoptotic protein which acts at the outer mitochondrial membrane [76], but can also target the mitochondrial inner membrane. When Bcl-X_L is co-localized with the β subunit of the F_1F_O-ATP synthase [77], it acts as an anti-apoptotic regulator by blocking mPTP opening. The chemotherapeutic agent ABT-737, which mimicks BH3, the only protein which bind to Bcl-X_L, reverses the Bcl-X_L binding to the β subunit and increases the membrane leak conductance [78].

Another approach, which can be applied to treat all (genetic and non-genetic) mPTP-related pathologies, is to find appropriate pore-shutters. Other than in mitochondrial dysfunctions of variegated ethiology, selective inhibitors of the Ca^{2+}-activated F_1F_O-ATP synthase, such as nitrite, could shut the mPTP, prevent cell death and play a main role in cytoprotection in different body districts [62]. When Ca^{2+} rises in mitochondria, the enzyme complex activated by Ca^{2+} hydrolyzes ATP. In this case the enzyme inhibition by nitrite would lessen ATP dissipation [52]. Accordingly, high nitrite concentrations may efficiently block the F-ATPase activity sustained by Ca^{2+} without affecting that activated by Mg^{2+}. The enhanced enzyme inhibition by nitrite under oxidative stress, which features many diseases, spanning from diabetes to cancer and neurodegenerative diseases, may be helpful to limit cellular injury under pathological conditions. Nitrite constitutes a good representative of small molecule which directly acts on the F_1F_O-ATP synthase. The list of mPTP-related pathologies on one hand and, on the other, of mPTP blockers is fated to increase with the knowledge in this field. The advantage of small molecules over complex compounds as drugs targeting mitochondria relies in their easier incorporation in mitochondria without requiring to be vehiculated [79]. Since the mPTP opening plays a key role in the progression of myocardial cell death secondary to reperfusion in myocardial infarction a new pharmacological approach using small-molecule based on a 1,3,8-triazaspiro[4.5]decane targets the *c* subunit of the F_1F_O-ATP synthase and inhibits the mPTP activity by decreasing apoptotic rate during reperfusion [80].

The F_1F_O-ATP synthase, aging and neurodegenerative disorders

Many diseases share with aging some basic mechanisms of cell death. In turn, the incidence of some severe pathologies is tightly linked to an increased life span and increases with age. Since the mPTP

formation is one of these age-related mechanisms, it is clear that the pore-shutters may have multiple therapeutic roles and also display anti-aging properties. However, if pore-modulators targeting the F_1F_O-ATP synthase can be effective in the treatment of neurodegenerative disorders and other mPTP-related diseases, alternative therapeutic approaches, also based on the F_1F_O-ATP synthase modulation, have been considered. Recently the synthetic compound J147 emerged as promising therapeutic molecule since it rescues the severe cognitive deficits in aged, transgenic mice. J147 targets the mitochondrial α-F_1-ATP synthase (ATP5A) (Fig. 2) and causes a modest enzyme inhibition which drives intracellular Ca^{2+} to activate AMP-activated protein kinase to inhibit mammalian rapamycin target, a known mechanism which extends lifespan from worms to mammals [48]. Developing treatments for amyotrophic lateral sclerosis aim at maintaining the mitochondrial function [81] and often reveal a close parallelism between mPTP formation and the activity of the F_1F_O-ATP synthase. The increase in ion conductance due to the mPTP formation and recorded within the *c*-subunit of the F_1F_O-ATP synthase [12] is inhibited by the neuroprotective drug dexpramipexole, which induces conformational changes in the F_1F_O complex [82]. Therefore the mutually linked modulation of the F_1F_O-ATP synthase and of the mPTP strongly supports the ongoing development of drugs targeting the ATP synthase to treat neurodegenerative disorders.

The ectopic F_1F_O-ATP synthase: a new target for drugs?

For a long time in eukaryotes, the F_1F_O-ATP synthase has been confined to the mitochondrial inner membrane. However, the occurrence of F_1F_O-ATP synthase or of its subunits, both nuclear and mitochondrially encoded [83], in extra-mitochondrial membranes such as the plasma membrane of tumor and normal cells, namely endothelial cells, hepatocytes and adipocytes and also in the endoplasmic reticulum, cannot be neglected. This peculiar F_1F_O-ATP synthase was defined “ectopic”, since it is found in extra-mitochondrial membranes. Interestingly, some ectopic F_1F_O-ATP synthase subunits act as cell-surface receptors for various ligands, a feature not always shared with the mitochondrial enzyme which extends its potentiality as putative drug target. The origin and biological function of the ectopic F_1F_O-ATP synthase, which shows oligomycin sensitivity and both the ATP synthase and ATP hydrolase activities [84], are still a matter of debate. The overexpression of ectopic F_1F_O-ATP synthase under peculiar physiopathological conditions and its receptor-like behavior shoulder its involvement in cell signaling for the regulation of vascular tone, cholesterol metabolism, cancerogenesis and metastatic progression [23,85]. The ectopic F_1F_O-ATP synthase is a typical “moonlighting protein”, whose function apparently depends on many variables such as localization, expression, ligand concentration and so on [85]. Thus, the ectopic enzyme may be a potential target for a wide variety of drugs. However, before setting out therapies targeting the ectopic F_1F_O-ATP synthase, its role must be clarified. Moreover, on considering drugs targeting the F_1F_O-ATP synthase, their possible dual effect on the ectopic and on the mitochondrial enzyme complex should be evaluated. Recently, the interaction of some phytophenols and natural peptides with the ectopic F_1 was considered as putative mechanism which significantly contributes to the anticancer properties of these compounds [64].

Conclusions

Due to its unique properties of crucial enzyme complex able to drive the opposite conditions of life and death in cells, the F_1F_O offers great opportunities as drug target in therapy. Accordingly, compounds able to bind to the F_1F_O and to affect the enzyme function may be used as pharmacological

tools to selectively address unwanted cells to death or, on the contrary, to prevent the cell lethal fate, not only due to mitochondrial impairment or dysfunctions but also to mPTP opening, in which most likely the Ca^{2+}-activated enzyme is involved [75]. The main challenge in the perspective of exploit new molecules as antibiotics to overcome the antibiotic resistance, is to block the enzyme vital role of ATP maker only in unwanted (pathogen) cells without affecting the host enzyme. To this aim, slight structural differences between prokaryotic and eukaryotic F_1F_O complex can be exploited. Moreover, as recent advances suggest, the mammalian enzyme could be made refractory to known antibiotics by post-translational modifications, induced by chemicals and unable to affect the prokaryotic F_1F_O-ATP synthase.

Assumed that the Ca^{2+}-ATPase is involved in the mitochondrial pathway of apoptosis, some compounds targeting the enzyme complex could constitute antiproliferative drugs or conversely delay or lessen cell death under severe pathological conditions. mPTP modulators which target the Ca^{2+}-activated F_1F_O-ATP synthase and favour or block the mPTP formation and opening, may expand the range of therapeutic options. Accordingly, the symptoms of some severe neurodegenerative and cardiovascular disorders, as well as some age-related diseases, all conditions featured by an increased mPTP formation, could be attenuated by the so-called pore shutters, such as nitrite, which would block the conformational transmission mechanism which opens the mPTP. On the other hand the mPTP activation by compounds which stimulate the Ca^{2+}-ATPase activity could be exploited to kill malignant cells. In this fascinating perspective, novel drugs could be found among natural or synthesized compounds which selectively target the Ca^{2+}-activated F_1F_O, namely the putative deathly engine of mitochondria.

Molecular therapies based on the F_1F_O modulation may offer new strategies, also in combination with the existing therapies, to counteract insufficiently treated human pathologies. Accordingly, drugs targeting the F_1F_O may help to attenuate structural and/or functional mitochondrial defects, to overcome the antimicrobial resistance and also to improve the prognosis of mPTP-related diseases.

The F_1F_O complex attractiveness as promising drug target is strongly substantiated by the recent knowledge. Further studies may also expand the ongoing perspectives, define the *pros* and the *cons* for a potential use of the enzyme in pharmacology, toxicology, clinics and personalized medicine.

Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table 1. Main known inhibitors of the F_1F_O-ATP synthase

Compound or compound group (alphabetical order)	Target	Reference
5228485 and 5220832 antimycobacterial drugs	F_O	50
Bedaquiline	F_O	43,44
4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)	F_1	27
Dicyclohexylcarbodiimide (DCCD)	F_O	1,86
Insect venom peptides	F_1	18
J147	F_1	48
Oligomycin	F_O	36
other macrolide antibiotics (apoptolidin, bafilomycin, venturicidin)	F_O	31
Nitrite*	F_1	60,62
Polyphenolic phytochemicals	F_1	13,29
1,3,8-triazospiro [4,5] decane-derived small molecules	F_O	83
Trisubstituted organotins (tributyltin)	F_O	40

*The asterisk indicates that the compound especially inhibits the enzyme when it is activated by Ca^{2+} instead of by the natural cofactor Mg^{2+}

Figure Legends

Figure 1. Eukaryotic and prokaryotic structures of the F_1F_O-ATP synthase. Protein subunits are drawn as ribbon representations obtained from modified PDB ID codes: 6B8H of yeast mitochondrial F_1F_O ATP synthase monomer and 5T4O of bacteria F_1F_O ATP synthase. In the eucaryotic structure the 6.8 kDa proteolipid (*i*/*j* subunit in yeast), the truncated DAPIT (*k* subunit in yeast), *e* and *g* subunit are not represented.

Figure 2. Binding sites of F_1F_O-ATP synthase inhibitors. The inhibitor structures are in ball and stick mode, while the enzyme is drawn as ribbon (modified PDB ID codes: 6B8H).

Figure 1

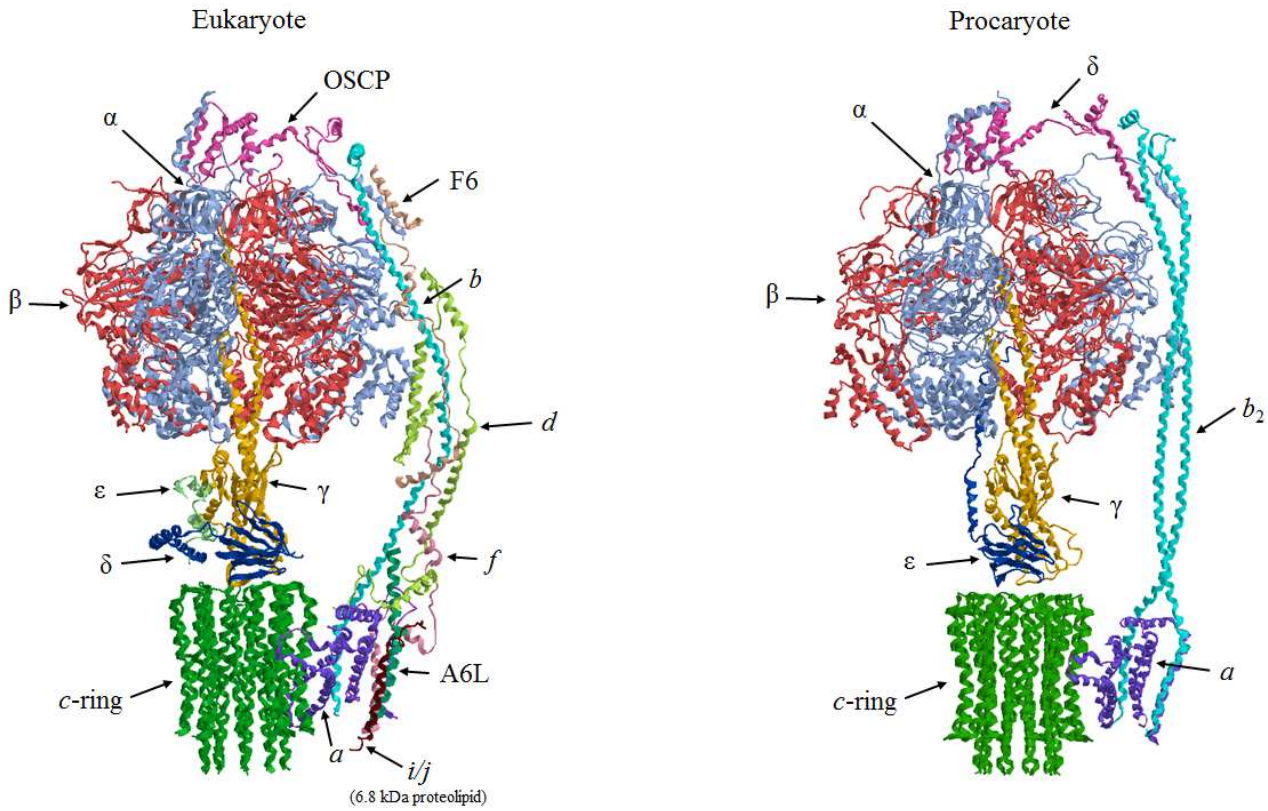
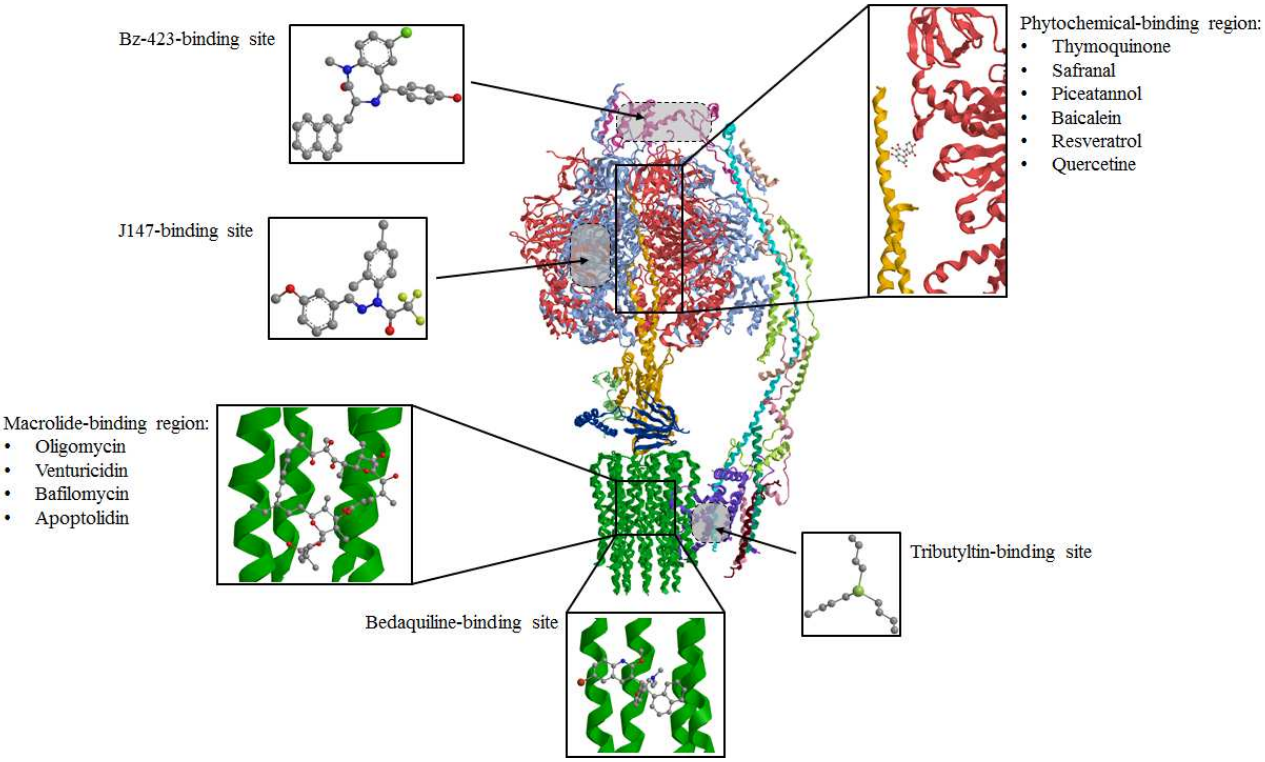

Eukaryote
OSCP
α
F6
β
b
d
ε
γ
δ
f
A6L
c-ring
a
i/j
(6.8 kDa proteolipid)
Procaryote
δ
α
β
b_2
γ
ε
a
c-ring

Figure 2


Bz-423-binding site
J147-binding site
Macrolide-binding region:
• Oligomycin
• Venturicidin
• Bafilomycin
• Apoptolidin
Bedaquiline-binding site
Tributyltin-binding site
Phytochemical-binding region:
• Thymoquinone
• Safranal
• Piceatannol
• Baicalein
• Resveratrol
• Quercetine