Novel Drugs Targeting the c-Ring of the F1FO-ATP Synthase

Novel drugs targeting the *c*-ring of the F_1F_O-ATP synthase

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Abstract

Increasing evidence highlights the role of the ATP synthase/hydrolase, also known as F_1F_O-complex, as key molecular and enzymatic switch between cell life and death, thus increasing the enzyme attractiveness as drug target in pharmacology. Being inhibition of ATP production usually linked to antiproliferative properties, drugs targeting the enzyme complex have been mainly considered to fight pathogen parasites and cancer. In recent years, a number of natural macrolides, produced by bacterial fermentation and structurally related to the classical enzyme inhibitor oligomycin,, have been shown to bind to the membrane-embedded F_O sector and to inhibit the enzyme complex by an oligomycin-like mechanism, namely by interacting with the *c*-ring. Other than natural macrolide antibiotics, which display variegated inhibition power on different F_1F_O-complexes, synthetic compounds from the diarylquinoline and organotin families also target the *c*-ring and strongly inhibit the enzyme. Bioinformatic insights address drug design to target F_O subunits. Additionally, the possible modulation of the drug inhibition power, by amino acid substitutions or post-translational modifications of *c*-subunits, adds further interest to the target. The present survey on compounds targeting the *c*-ring and bi-directionally blocking the transmembrane proton flux which drives ATP synthesis/hydrolysis, discloses new therapeutic options to fight cancer and infections sustained by therapeutically recalcitrant microorganisms. Additionally, c-ring targeting compounds may constitute new tools to eradicate undesired biofilms and to address at the molecular level the therapy of mammalian diseases linked to mitochondrial dysfunctions. In summary, studies on the only partially known molecular interactions within the *c*-ring of the F_1F_O-complex may renew hope to counteract mammalian diseases.

Keywords: ATP synthase; *c*-ring; diarylquinolines;drug target; enzyme inhibition; macrolides; organotins

INTRODUCTION

The ATP synthase or F_1F_O-ATPase is the key enzyme in cell bioenergetics, due to its primary role to convert the transmembrane electrochemical gradient in ATP synthesis, the energy currency of all living organisms. While in the Animal Kingdom the F_1F_O-ATPase is inserted in the inner mitochondrial membrane by the F_O moiety and protrudes with its hydrophilic portion F_1 in the mitochondrial matrix, the bacterial homologue displays a similar subunit arrangement with F_O embedded in the plasma membrane and F_1 protruding in the cytoplasm [1]. In all organisms the membrane-bound enzyme complex stands at the interface between two aqueous environments with different electrochemical features and behaves in the same way, namely the membrane-embedded portion F_O allows proton flux across the membrane and the protruding hydrophilic F_1 acts as catalyst. The connection and coupling between the two portions are ensured by two stalks: a peripheral stalk and a inner stalk. A main role is played by the inner stalk, also named γ subunit, which in association with ε subunit [2], couples proton translocation within F_O to the catalytic events in F_1. Accordingly, F_1 undergoes conformational changes promoted by γ which allow the catalytic function. As unique bi-functional enzyme mechanism in biology, under physiological conditions the F_1F_O complex exploits the electrochemical gradient which channels protons from the positive to the negative side of the membrane to drive ATP synthesis from ADP and inorganic phosphate (Pi) and conversely it can use the free energy from ATP hydrolysis to pump protons and re-establish the electrochemical gradient [1]. The latter functioning mode, which leads to ATP hydrolysis and energy dissipation, may occur under physio-pathological conditions associated with oxygen lack, such as myocardial ischemia [3]. The equation "no-ATP synthase no life" has apparently no exceptions in aerobic organisms, both in eucaryotes and procaryotes. The enzyme is apparently the Achille's heel of multi-drug resistant microorganisms [4]. Accordingly, the inhibition of the ATP synthase in bacteria eventually decreases protein synthesis, DNA duplication and inhibits all energy-consuming processes required by cell proliferation. Additionally, the same enzyme complex, in its dimeric form, has recently been involved in cell death, by promoting the mitochondrial permeability transition [5] and leading to apoptosis. In this mechanism *c*-subunits play a critical role [5]. On these bases, compelling evidence highlights the role of this fascinating enzyme complex as key molecular and enzymatic switch between cell life and death and increases its attractiveness as pharmacological target. 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, or selectively kill abnormal cells [1, 7]. The crucial requirement for a drug relies is its capability to discriminate between eukaryotic and prokaryotic ATP synthases (and, preferentially, to act selectively on certain undesired prokaryotes), or 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) [8,9] and in some cancer types [10]. Pathological mutations often

involve changes in the Atp6p (or a) subunit of the ATP synthase, which contains most of the residues involved in proton translocation through F_O [10]. These mutations have also been associated with increased ROS production, suggesting that the enzyme inhibition or malfunctioning could cause electron leak from the respiratory chain [10] . The features and the subunit composition of the *c*-ring are described in Section 1. An appropriate molecular therapy could produce on the enzyme structure changes which somehow compensate for the aminoacid substitutions responsible for the pathological dysfunctions in the enzyme machinery.

For a long time, the mitochondrial ATP synthase has been thought as confined to the mitochondrial inner membrane of mitochondria. However, increasing literature proves the occurrence of F1Fo-ATP synthase or of its subunits, both nuclear and mitochondrially encoded [11], 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 [12,13] . Due to its expression at the cell surface, the adjective ectopic was assigned to this peculiar ATP synthase, whenever detected in extra-mitochondrial membranes. Interestingly, some ectopic ATP synthase subunits act as cell-surface receptors for various ligands, a feature not always shared with the mitochondrial enzyme. As far as we are aware, the origin and role of the ectopic ATP synthase are not elucidated yet. The F_1F_O complex, even if localized out of its typical mitochondrial site, maintains the oligomycin sensitivity [11] and the two opposite functions of ATP synthase and ATP hydrolase activities [14]. The overexpression of the ectopic 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 [14], cancerogenesis and metastatic progression. The ectopic ATP synthase is a typical "moonlighting protein", namely its (potential multiple) functions depend on variables such as localization, expression, ligand concentration and so on [15]. Thus, it may be a potential target for a wide variety of drugs to counteract cancer [16], dyslipidemia and cardiovascular diseases [14]. The potential exploitation of the ectopic ATP synthase as drug target to fight cancer is considered in Section 5. Accordingly, on considering drugs targeting the ATP synthase, their possible dual effect on the ectopic and on the mitochondrial enzyme complex should be taken into account.

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 initial of the antibiotic oligomycin, which specifically inhibits both ATP synthesis and hydrolysis by blocking proton translocation through F_O. In recent years, natural compounds structurally related to oligomycin [17], have been shown to target F_O and to similarly inhibit the ATP synthase.

In drug design the possible use of the mitochondrial $F1F_O$-ATP synthase as a drug target has been extensively considered [1,8,18-21], especially to selectively kill noxious cells, namely pathogen and cancer cells [18].

The immediate link between block of ATP production and arrest of cell proliferation addressed most studies to inhibitors binding to the catalytic portion F_1 [8].

Among dietary phytochemicals targeting the enzyme complex and claimed as beneficial to fight cancer, only genistein was thought to bind to the F_O sector [22], due to its hydrophobicity and membrane penetration [23], but recent studies did not confirm this target. Other natural and synthetic compounds have been shown to inhibit the ATP synthase by specifically targeting F_O and particularly the *c*-ring, whose rotary motion is fundamental in the bi-functional F_1F_O machinery.

The possibility of exploiting F_O subunits as drug targets has been strongly supported by bioinformatic insights [24]. In detail, since amino acid residues in the a and c subunits of F_0 are specifically substituted in resistant bacterial strains, these subunits especially emerge as candidate targets for antibacterial drugs. Additionally, structural differences among bacteria in aminoacidic sequences of *c*-subunits and in the c-ring constitution may be exploited to selectively address drug action against pathogenic bacterial F_O. [24]. The possible modulation of the drug potency, with the aim of selectively addressing the pharmacological effect by modifying the drug structure and/or that of the *c*-ring through post-translational modifications, is an open challenge. In this perspective, the finding that *c*-subunits can undergo post-traslational oxidation of crucial cysteine thiols which make the enzyme less responsive to some drugs [17, 25] is especially intriguing. In theory, the redox state of these proteins can increase or decrease the enzyme susceptibility to pharmacological compounds, depending on how the drug binds to its target. The crucial requirement to produce a pharmacological effect lies in the molecular interactions between the drug and its binding site on the protein. This topic is now hot in drug discovery programmes and in the so-called mitochondrial pharmacology [26]. An improved knowledge of compounds targeting the *c*-ring and of the biochemical manipulation of their effect may broaden the spectrum of antibiotics and anticancer drugs. Moreover, the treatment of mitochondrial defects, at present mainly symptomatic [27], could be addressed to targeted therapies which would act directly on the enzyme structure and allow the recovery of the compromised function by chemically modifying the defective proteins.

1. FEATURES OF THE *c*-RING

The *c*-ring, the core and the main portion of $F_{O,}$ can be viewed as a sort of cylindric palisade in turn formed by lean cylindric monomers (*c* subunits) arranged as matches in a circle (Figure 1). The *c*-ring rotor can move clockwise or counter-clockwise (as shown from the outer membrane side in eucaryotes), thus transmitting the rotary motion through γ subunit to the catalytic portion F_1 and allowing ATP synthesis or hydrolysis according to the transmembrane gradient: protons move down through F_O from the intermembrane space to the matrix following the electrochemical gradient thus allowing ATP synthesis (Figure 1); conversely ATP hydrolysis drives proton transport against the electrochemical gradient from the matrix towards the intermembrane space.

Each *c* subunit is hairpin-shaped, protruding the N-terminal end towards the *c*-ring centre and the C-terminal end externally. The *c*-ring stoichiometry, namely the number of *c*-subunits, is species dependent and somehow related to the bioenergetic cost of ATP and organism requirements [28,29]. The cost of ATP, expressed as number of protons which must cross the membrane to build one ATP molecule, depends on the size of the c-ring. Generally large *c*-rings (11-15 subunits) as prokaryotic ones, imply high bioenergetic cost of ATP, while small *c*-rings, typical of eukaryotes and consisting of up to 10 subunits, usually 8 in mammals and 10 in yeasts, imply a low bioenergetic cost of ATP, namely less ions with respect to large *c*-rings are required to pass through to synthesize one ATP molecule. Evidently evolution favors small *c*-rings, namely more efficient rotors. The *c*-ring size directly affects proton flux since each *c* subunit has a single ion binding site. Approximately at the midpoint of each C-terminal transmembrane α-helix, a carboxylic residue from glutamate or aspartate switches between the protonated/deprotonated state, reversibly changing from the proton-locked conformation to the deprotonated open conformation, by binding and releasing an individual proton. Consequently, in this molecular machinery, the number of *c* subunits roughly corresponds to the number of protons bound which make the *c*-ring rotate. Other than stoichiometry, slight but crucial differences occur in the aminoacid composition of *c*-subunits which is 100% conserved among mammals and highly conserved in vertebrates and yeasts. In contrast, more striking differences emerge between eucaryotes and prokaryotes and, in this respect not all bacteria behave the same way. For instance, the highly conserved glutamate residue in the second transmembrane helix, essential for ion translocation, is replaced by aspartate in *Escherichia coli* but not in other bacteria [24]. *E. coli* has the most hydrophobic transmembrane helices, while other bacteria such as *Mycoplasma pneumoniae* show several hydrophilic residues in the transmembrane regions. Some pathogen strains contain one or even two (*Chlorobium tepidum*, *Thermotoga maritime*) additional acidic residues which could participate in ion translocation process. The differences in bacterial *c* subunit number and aminoacid residues provide the opportunity to develop selective antibiotics. Some aminoacid substitutions in the *a* and *c* subunits in antibiotic resistant bacterial strains shoulder the possible exploitation of *c* subunits as targets for antibacterial drugs [24]. Differences in *c*-subunit aminoacid sequences among vertebrates and bacteria strikingly emerge in the hydrophobic transmembrane α-elix region [8]. Accordingly, the cysteine residues of eukaryotes, are replaced by alanine, serine and asparagine in *E. coli*, *Ilyobacter tartaricus* and *Mycobacterium tubercolosis*, respectively [25].

Changes in the *c*-subunits occur during the complex life cycle of the protozoon *Tripanosoma brucei,* agent of African trypanosomiasis (sleeping sickness) in humans and other animals. Three different *c* isoforms are expressed, all incorporated in the ATP synthase. Changes in the relative ratio of these isoforms, thought to differ in the intermembrane residues without altering the *c*-ring size, are likely to be related to the different life stages of the parasite and to the

different ATP synthase function [30]. These life-long differences as well as aminoacid substitutions which differentiate the mammalian host from the parasitic *c*-subunits can be potentially exploited in therapy..

Interestingly, ATP synthase *c* subunit expression is somehow related to some mammalian diseases: a puzzling *c*-subunit accumulation in lysosomes occurs in Batten disease (ceroid lipofuscinosis), a neurodegenerative fatal disease described in humans, goat, sheep, cattle, cats dogs and mice [31]. The mitochondrial function is apparently unaffected and no other mitochondrial component is stored. These *c* subunits are specifically stored in brain and other tissues in sheep and cattle, as well as in the human late infantile and juvenile diseases. This abnormal storage of *c* subunits is probably due to a deep alteration of the protein turnover pathway, most likely of protein degradation, after the normal insertion into the ATP synthase complex [32]. The ε-amino group of lys 43 is post-translationally trimethylated [33]; however this post translational modification is apparently unrelated to the disease and its role in the ATP synthase assembly is confined to higher organisms.

2. MACROLIDE DRUGS

Among bacteria, the genus *Streptomyces* shows an astonishing capability of synthesizing as secondary metabolites macrolide compounds, endowed with biological activity and displaying an amazing variability in chemical structure, depending on the species, the strain, the culture conditions and other only partially known variables. As soon as their bioactivity was recognized, such molecules were tested as drugs, even before their action mechanism was elucidated. Later, a number of these natural fermentation products of microorganisms was shown to target the ATP synthase and/or other F_1F_O complexes, according to their source.

The family of glycosides embraces a lot of bioactive compounds including macrolide compounds whose aglycone is a lactone ring of various size (most frequently 12-26 members), while the sugar moiety consists of one or more monosaccharides, or saccharide derivatives, namely deoxysugars and other modified monosaccharides. The polyketide backbone and the sugars enable these molecules to onset hydrogen bonds, while the lipophilic side-chains, associated with the capability of van del Waal's interactions, confer the properties of membrane-active modulators. Due to the variability in glycosylation patterns, the sugar moiety has been considered as not essential for the ATPase inhibition [34]. However the stereochemistry of sugars, in some cases still undefined [35], seems crucial for the interaction with membrane proteins.

The antiproliferative pharmacological properties of natural macrolides stimulated research to chemically synthesize these precious compounds. However, the synthesis of these biologically active compounds in the lab is laborious, due to their structural complexity and stereo-selective biological activities [36, 37]. Accordingly, most studies still exploit extracts from culture broths, often consisting in a mixture of structurally related compounds.

2.1. OLIGOMYCINS AND RELATED 26-MEMBERED MACROLIDE DRUGS

Oligomycins, produced as a mixture of related 26-membered macrolides by various *Streptomyces* strains, have been long considered as specific inhibitors of the ATP synthase, but only one half-century after the first oligomycin isolation [38], was the inhibitory mechanism understood [39], even if not completely [40, 41]. At present, several types of oligomycin are known, A, B, C, D (rutamycin A), E, F and rutamycin B, differing in the side chains localized between the sugar moiety and the macrolide ring [8] (Figure 2). Oligomycins at micromolar concentrations selectively target F_O, even if at high concentrations they can also bind to F_1 [40]. Oligomycin A, currently considered as one of the most powerful F_O inhibitors [43], leads to less stable interactions with F_O, while potentially oligomycin B has the most suitable structure to onset hydrogen bonds [44]. Most studies tested commercially available oligomycin mixtures, often a combination of A, B and C forms in variable proportions. Oligomycins were found to behave as mixed or non competitive inhibitors of the ATP synthase with respect to the ATP substrate [45, 46]. Oligomycin binding to F_O does not involve covalent bonds. The drug molecule forms hydrogen bonds between hydroxyl groups and the protein,, excluding the keto groups in the macrolide ring [47]. Additionally the drug establishes van der Waals interactions and intercalate between two adjacent α-helices. The onset of a critical hydrogen bond with the ion binding site accompanied by the interposition of a water molecule covers the crucial acidic residue and blocks ion translocation [39]. Accordingly, oligomycins, localized at the *c*-ring outer side, block both the ATP synthase and the hydrolase functions and are thus classified among non-selective inhibitors [3]. The oligomycin sensitivity of ATP hydrolysis is taken as a measure of the coupling between ATP hydrolysis and proton translocation through F_O [48]. Studies on oligomycin-resistant yeast mutants revealed a target site at the *a*/*c* interface, with an involvement of both Gly23 and Glu59 of the N- and C-terminal transmembrane helices of subunit *c* [8]. Interestingly, the oligomycin inhibition of F_O has been related to its antiapoptotic role [49], but the mechanisms involved are still unclear.

2.2. MACROLIDES WITH A SMALL RING (16-24 MEMBERS)

The antitumor drug ossamycin was first isolated from culture broth of *Streptomyces hygroscopicus var. ossamyceticus*. Its complex structure, only elucidated in 1996, thirty years after its isolation [35] shows a 24-membered macrolide ring and contains the aminodeoxysugar ossamine [50] (Figure 3).

Venturicidins, also isolated from crude extracts of *Streptomyces* strains, show a 20-membered macrolide ring. At present three different molecules are known, A, B and X, being venturicidin X an aglycone of venturicidin A or B. All venturicidins are fungicides [51]; venturicidin A displays antitrypanosomal activity *in vitro* [52]. The venturicidin binding region is thought to be in the middle of the membrane and to partially overlap with oligomycin binding sites within F_O [8].

Other 20-membered macrolides targeting the ATP synthase [18, 34] are produced by *Nocardiopsis sp*. These compounds were named apoptolidins, from their apoptotic capability in selected cell types [8]. Apoptolidins contain the aglycone apoptolidinone [36], which shows strong similarities to that of oligomycins and bafilomycins [18] and differ in side chains, in the chiral centers and in the carbohydrate units [53]. Apoptolidin A_1 (Fig.3), the major product, being apoptolidins B and C minor metabolites [36], shows antifungal activity and also sectively kills some cancer cell lines. Drugs that can selectively sensitize cancer cells to apoptosis are likely to play a vital role in cancer therapy [18]. The cell selectivity may be related to the strong pH dependence of the molecule, being acidic pHs decrease the Ki for the enzyme, and to the induction of the mitochondrial pathway of apoptosis of targeted cells [18]. Apart from the inhibition of the ATP synthase, other mechanisms such as the induction of genes related to mitochondrial functions can mediate the selective cytotoxicity by increasing the level of target proteins [18]. The sugar portion of apoptolidin seemed not crucial for the binding to F_O [37]. However the acetylation of some hydroxyl groups strikingly raised the Ki value of yeast ATP synthase, thus suggesting that free hydroxyl groups of sugars are somehow required for the binding to the enzyme structure [54].

Isoapoptolidins, also produced by *Nocardiopsis sp*, are 21-membrered macrolides (Fig.3). Their structure is probably due to a intramolecular rearrangement which enlarges the ring with respect to that of apoptolidins and concomitantly lessens the inhibition of yeast ATP synthase [54].

Concanamycins are 18-membered macrolides, produced in different forms by *Streptomyces neyagawaensis* and other *Streptomyces* sp. Concanamycin A (Fig. 3), also named folimycin, is used as agricultural antifungal antibiotic.

Bafilomycins, primarily known as V-ATPase inhibitors, contain a 16-membered lactone ring [55]. Bafilomycins can be produced by *Streptomyces spp* in different forms, A_1, B_1, C_1, and D, all structurally related to concanamycins [55],

2.3. MACROLIDE TARGETS AND MUTUAL INTERACTIONS

Macrolides targeting F_O (*e.g*. oligomycins, venturicidins, ossamycins, apoptolidins) differ in the macrolide ring size and mainly in the sugar portion [34]. Interestingly, oligomycins, ossamycin and apoptolidin target specifically mitochondrial ATP synthases, while venturicidin also strongly inhibits bacterial ATP synthases [56]. The smaller macrolides bafilomycin and concanamycins are apparently less specific because they target V-type ATPases [57], but can also inhibit F-type ATPases [55].

In mammalian mitochondria venturicidin, oligomycin and bafilomycin were shown to bind to specific binding sites on the *c*-ring pinpointed in a common drug binding region [17], thus confirming the hypothesis of a highly conserved antibiotic binding site within F_O [55]. The *c*-ring drug binding region likely embraces amino acid residues from two adjacent membrane-embedded *c* subunits: each macrolide would accommodate within the shared binding region

according to its own combination of interactions within F_O. In binary mixtures, each macrolide acts as uncompetitive inhibitor of the mitochondrial F_1F_O-ATPase. Interestingly, bafilomycin apparently strengthens the inhibition produced by the other co-occurring macrolide [17]. Each macrolide displays different inhibitory power among different taxa. Accordingly, the enzyme affinity for the inhibitor is ruled by the strength of drug-enzyme attractions, in turn depending both on the aminoacid sequence of the *c* subunits and on the macrolide structure. The macrolide side chain of the drug may address the target, while the inhibitory potency may depend on the occurrence of crucial aminoacids in the targeted protein which allow specific interactions with the drug.

3. DIARYLQUINOLINES

Diarylquinolines are a family of small molecules containing a diarylquinoline scaffold, namely two fused aromatic rings, benzene and pyiridine, linked to other two aryl groups (Figure 4). Disubstituted quinolines are long known to have antimycobacterial properties [58]. A new bromine-containing quinoline, bedaquiline, also known as TMC207 or R207910, structurally different from fluoroquinolone antibiotics and related quinolines [59], looks as an innovative drug to fight multi-drug resistant tuberculosis. Bedaquiline selectively targets the ATP synthase of the microorganism *M. tuberculosis* which shows 20,000 times higher affinity for the drug than the human mitochondrial enzyme counterpart [60]. Bedaquiline, most likely in the protonated form [61], targets the mycobacterial *c* subunits and blocks ATP synthesis. In spite of metabolic adjustments, mycobacteria undergo delayed death [62]. Host and other bacterial ATP synthases are apparently unaffected. Studies on mycobacterial resistant mutants revealed that the mutated residues, with a tyrosine specifically conserved at position 64, define a cleft motif between two adjacent c subunits in the *c* ring. This cleft, which encompasses the proton-binding site (Glu61), can bind the bromoquinoline moiety of the drug. Any substitution in the aminoacids which define the cleft motif between two adjacent *c* subunits will produce bedaquiline-resistant mutants of *M. tuberculosis* [63]. The complex net built by the different interactions which tightly anchor bedaquiline to *c*-subunits has been recently defined [64]. Interestingly, the overall interaction pattern is very similar in the two mycobacterial species *M. tubercolosis* and *M. phley*, but the position of the aminoacids involved is slightly different in the two species. In both cases a wide spectrum of interactions embracing ionic, hydrogen, and halogen bonds is established between bedaquiline and the aminoacid residues of the C-terminal α-helices of two *c* subunits, namely the protomer c_H, which hosts the proton binding site blocked by the drug, and the adjacent protomer c_A. In detail, the quinolone ring of bedaquiline binds to Gly58 (Gly62 in *M. phley*), Glu61 (Glu65 in *M. phley*), Ala62 (Ala66 in *M. phley*) and Phe65 (Phe69 in *M. phley*) of *c*H. In addition, Glu61 (Glu65 in *M. phley*) of *c*H forms a specific hydrogen bond with the dimethylamino moiety of the drug. In this way bedaquiline penetrates between the two adjacent *c*-subunits and, through its hydroxyl group, can establish water molecule bridges between the oxygen of

backbone carbonyl and the carboxyl group of Glu61. The dimethylamino moiety of bedaquiline is also bound with Tyr64 (Tyr68 in *M. phley*) of *c*H, Ala62 (Ala66 in *M. phley*) and Ala63 (A67 in *M. phley*) of *c*A. Additionally, Its naphtyl group establishes hydrophobic interactions among Tyr64 (Tyr68 in *M. phley*), Leu68 (Leu72 in *M. phley*) of *c*H and Ile66 (Ile70 in *M. phley*) of *c*A. Finally, Phe65 (Phe69 in *M. phley*) of *c*H establishes van der Waals interactions with the bedaquiline phenyl group [64]. All together, these specific molecular interactions, which cannot be established in other prokaryotes and in eukaryotes, generate an extraordinary high affinity of the mycobacterial *c*-ring for bedaquiline. Moreover, the proton barrier of the positively charged guanidinium group of Arg of the *a* subunit [65] affects the ion binding site during the putative intermediate rotation steps of the *c*-ring [66]. Such interaction, which is somehow mimicked by the intermolecular bond between Glu61 and the dimethylamino moiety of bedaquiline in the surrounding membrane, would trap H^+ within F_O. Accordingly, the drug bound to the *c*-ring could not reach the polar *a*/*c* interface of the F_1F_O-ATPase. A single molecule of bedaquiline is sufficient to completely block the torque generation in the F_1F_O-ATPase [64].

Recently, since bedaquiline was also found to bind to ε subunit, a new binding model has been proposed. The drug would form a wedge between the ε subunit of the central stalk and the c-ring by interacting with the aromatic residues of Trp15 of ε and Phe50 of the *c*-ring, respectively, whileThr19 and Arg37 of ε would provide polar interactions with the drug molecule [67].

Novel diarylquinolines, differing from bedaquiline in the lateral chains and also targeting bacterial *c*-subunits, in recent years emerged as promising therapeutic agents to fight resistant strains of Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus pneumoniae* [4]. It seems likely that the antibacterial spectrum of this chemical class of compounds can be even broadened, maintaining the same molecular target, by providing slight changes in their molecular structure [58] to optimize their selective binding to the *c*-ring.

4. ORGANOTIN COMPOUNDS

Tin derivatives constitute a separate class of organometallic compounds. Some organotins are highly cytotoxic [68] and pharmacologically exploited as antitumor [69] and antimicrobic drugs, as well as biocides in agriculture and various industrial uses [68, 70]. The biological activity highly depends on tin chemistry, which allows various geometries and coordination numbers, mainly up to tetra-coordinated complexes, which can be exploited in chemotherapy [71]. Higher coordination numbers come from inter- and/or intra-molecular interactions, especially in complexes where tin bonds to electronegative atoms, such as oxygen, nitrogen and sulfur. The chemical versatility of tin is the basis to build a wide spectrum of organotin structures and to obtain molecules endowed with variegated biological activities.

Trisubstituted organotins such as tributyltin (TBT) [72] and triphenyltin [73] target $F_{O..}$ TBT covalently binds to a low affinity thiol-containing site, most likely on adjacent *c*-subunits (Figure 5). These cysteines are oxidized by tin binding

and such oxidation in turn desensitizes the enzyme to other inhibitors, including macrolide antibiotics [17]. However, as far as we are aware, the therapeutic use of organotins is hampered by their overall toxicity [8].

5. THERAPEUTIC STRATEGIES

Any difference in the *c*-ring between normal and pathological as well between host and pathogen forms can be potentially exploited in therapy. This goal is especially intriguing to fight diseases when the classical pharmacological treatment is ineffective, such as infections sustained by antibiotic-resistant microorganisms. Additionally, .recent advances consider the ATP synthase as pharmacological target to fight cancer [7,16] and cardiovascular diseases [3, 14]. Macrolide inhibitors of the mitochondrial F_0F_1-ATP synthase selectively kill metabolically active tumor cells that do not exhibit the Warburg effect, namely the increase in glycolysis under conditions of oxygen availability which is a typical metabolic shift of some cancer cell types [10, 18]. Anticancer drugs targeting the ATP synthase should be designed on considering two features of tumor cells: the occurrence of mutated subunits and the expression of ectopic ATP synthase. Mutations in mitochondrially encoded subunits of the ATP synthase have been found in thyroid, pancreatic and prostate cancer [10], as well as in several neurodegenerative diseases [9]. Thus, in theory, any post translational modification able to locally counteract the aminoacid substitutions so as to allow the function or to improve the activity of the enzyme complex may be exploited. As far as we are aware the link between mutations in enzyme subunits and cancerogenesis is not clear. Mutations in F_O subunits are often associated with reduced apoptosis, consistently with the ATP synthase involvement in cell death [5,6] or increased ROS production, due to electron leak from the respiratory chain because of the ATP synthase dysfunction [10]. Interestingly, the loss of natural inhibitor IF_1 which only blocks ATP hydrolysis and binds to the the dimeric form of the enzyme complex which has been involved in apoptosis [5, 74], may protect against cell death. The ectopic ATP synthase, which is especially abundant in the plasma membrane of highly proliferating eukatyotic cells including tumor cells, seems an intriguing target for anti-cancer therapy, since its inhibition results in cytotoxicity and in inhibition of tumor cell proliferation [15, 16]. Since the inhibition of the mitochondrial ATP synthase may trigger the mitochondrial pathway of apoptosis, the challenge is to develop an ideal drug which only targets the ectopic ATP synthase without affecting the mitochondrial enzyme of normal cells.

6. CONCLUSION

Increasing evidence points out the ATP synthase as crucial enzyme for energy metabolism even in resting and non-growing bacteria, namely under conditions requiring a minimal level of energy conversion within the cell. On these bases, drugs targeting the *c*-ring, thus bi-directionally blocking the transmembrane proton flux, may represent a therapeutic approach to fight chronic infections refractory to classical antibiotics, such as that sustained by quiescent

mycoplasma, as well as innovative strategies to eradicate undesired biofilms and planktonic bacteria [4]. Thus, studies on the *c*-ring constitution, in the perspective of exploiting this membrane-embedded structure as drug target, may renew hope to counteract chronic infections sustained by therapeutically recalcitrant bacterial strains as well as to cure mammalian diseases in which mitochondrial dysfunctions play a prominent role.

Undoubtedly, the *c*-ring, with its wide variety of structural differences in subunit number and amino acid sequences in the Animal Kindgom, is an intriguing target for cytotoxic drugs, and, on the other hand, it constitutes a plastic matter which can undergo mutations and post-traslational modifications of recognized role in health and disease. In this perspective, selected compounds targeting the *c*-ring may act as post-translational modifiers to compensate for aminoacid substitutions which decrease the efficiency of the whole enzyme machinery. Viewed from another standpoint, some exogenous compounds may be fruitfully exploited to produce the so-called acquired drug resistance. Unfortunately, in spite of the wealth of studies, the target, namely the c-ring, is only partially known. Accordingly, future pharmacological research is stimulated to develop towards two directions: the broadening of knowledge on the structure and function of *c*-subunits and, concomitantly, the synthesis and isolation of compounds targeting the *c*-ring.

List of abbreviations

ATP, adenosinotriphosphate; Pi, inorganic phosphate; MILS, maternally inherited Leigh's syndrome; NARP, neuropathy, ataxia, retinitis pigmentosa; TBT, tributyltin.

Conflict of Interest

The authors declare no conflict of interest

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Figures

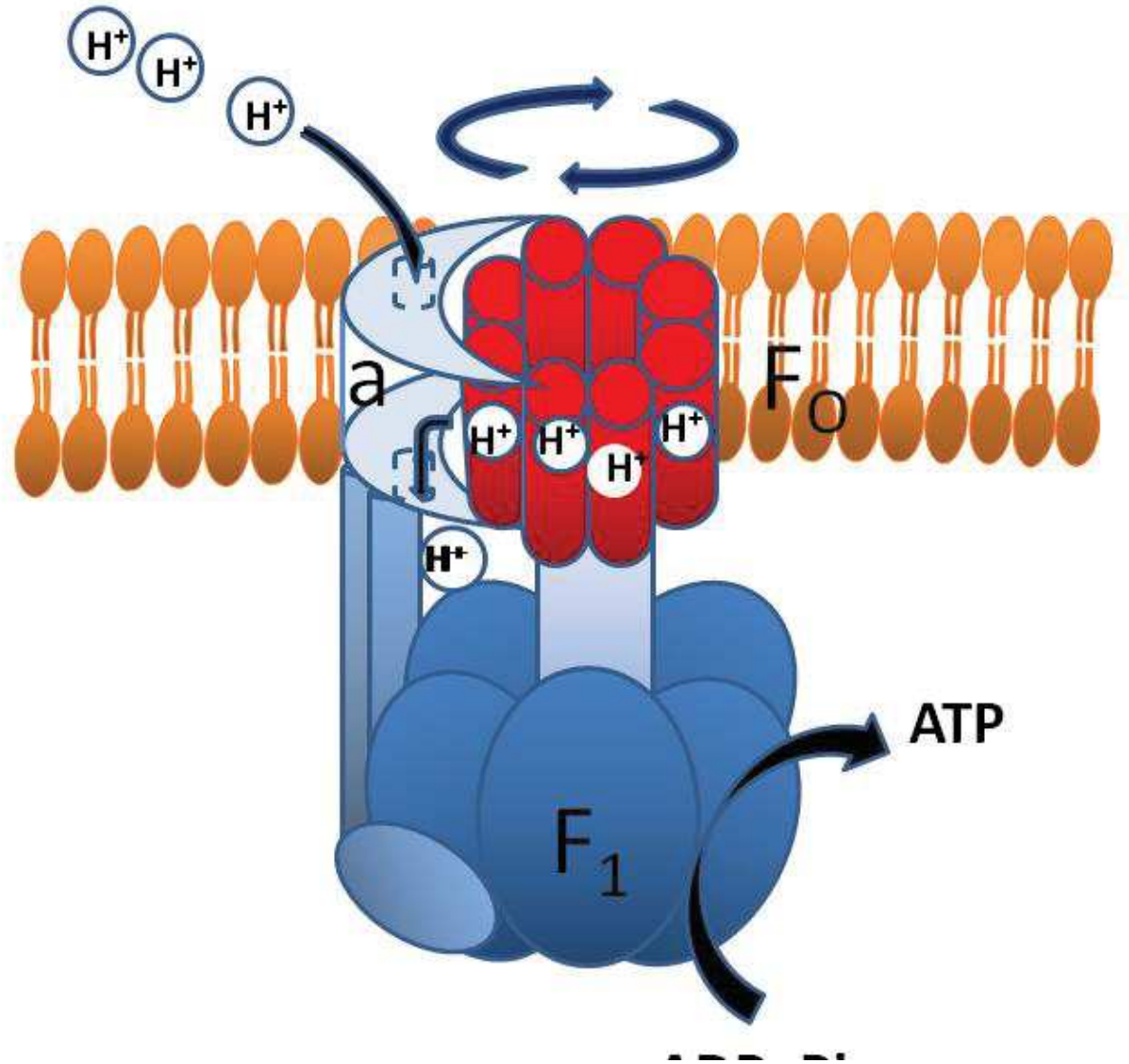

Figure 1. The mitochondrial *c*-ring and its function. The clockwise rotation of the *c*-ring (in red), seen from the membrane side and illustrated by the arrows, combined with downhill H^+ transport across the inner mitochondrial membrane, allows ATP synthesis from ADP and Pi by the hexameric catalytic portion F_1.

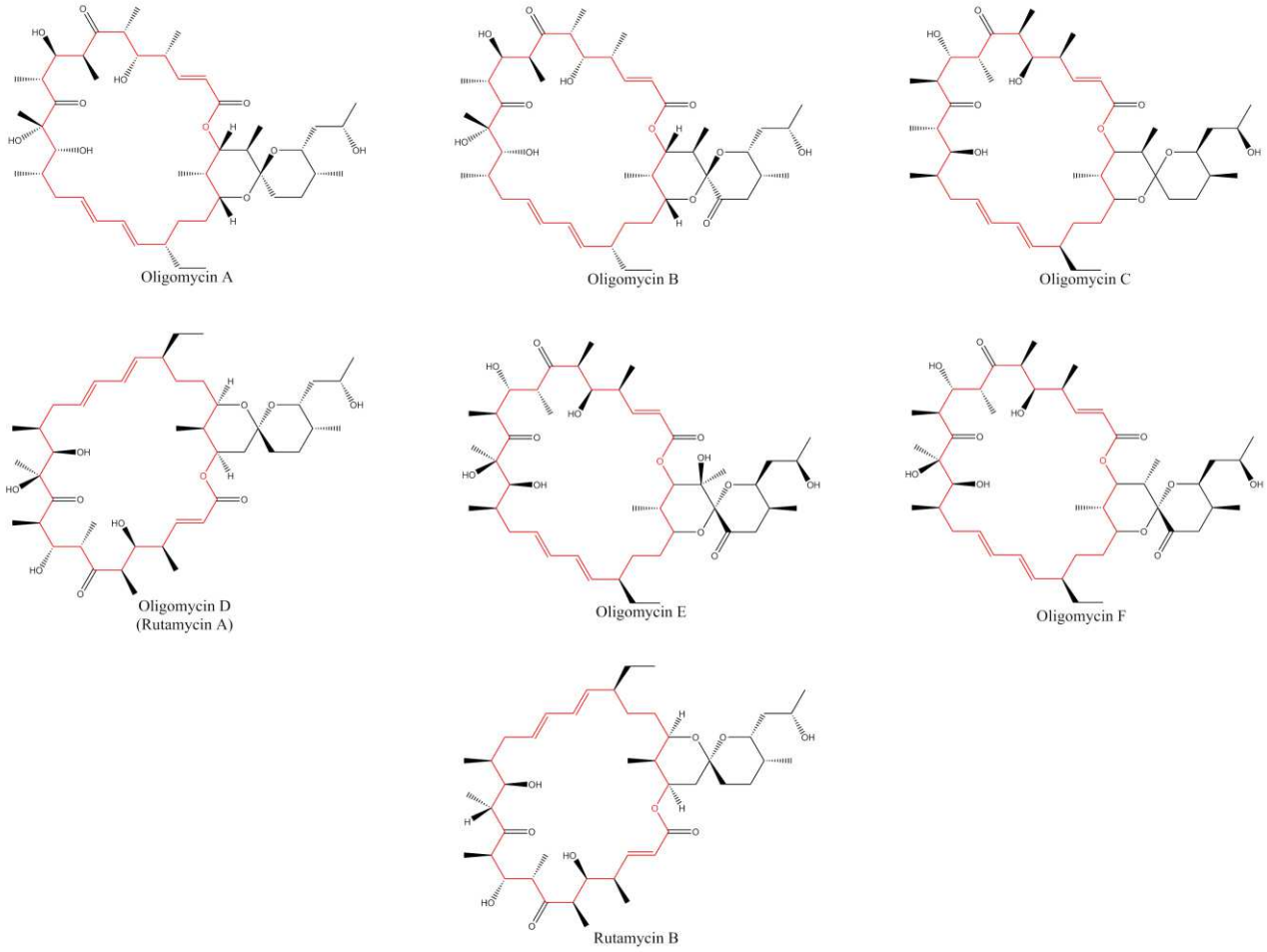

Figure 2. Structures of oligomycins and related 26-membered macrolides. The macrolide ring is highlighted in red.

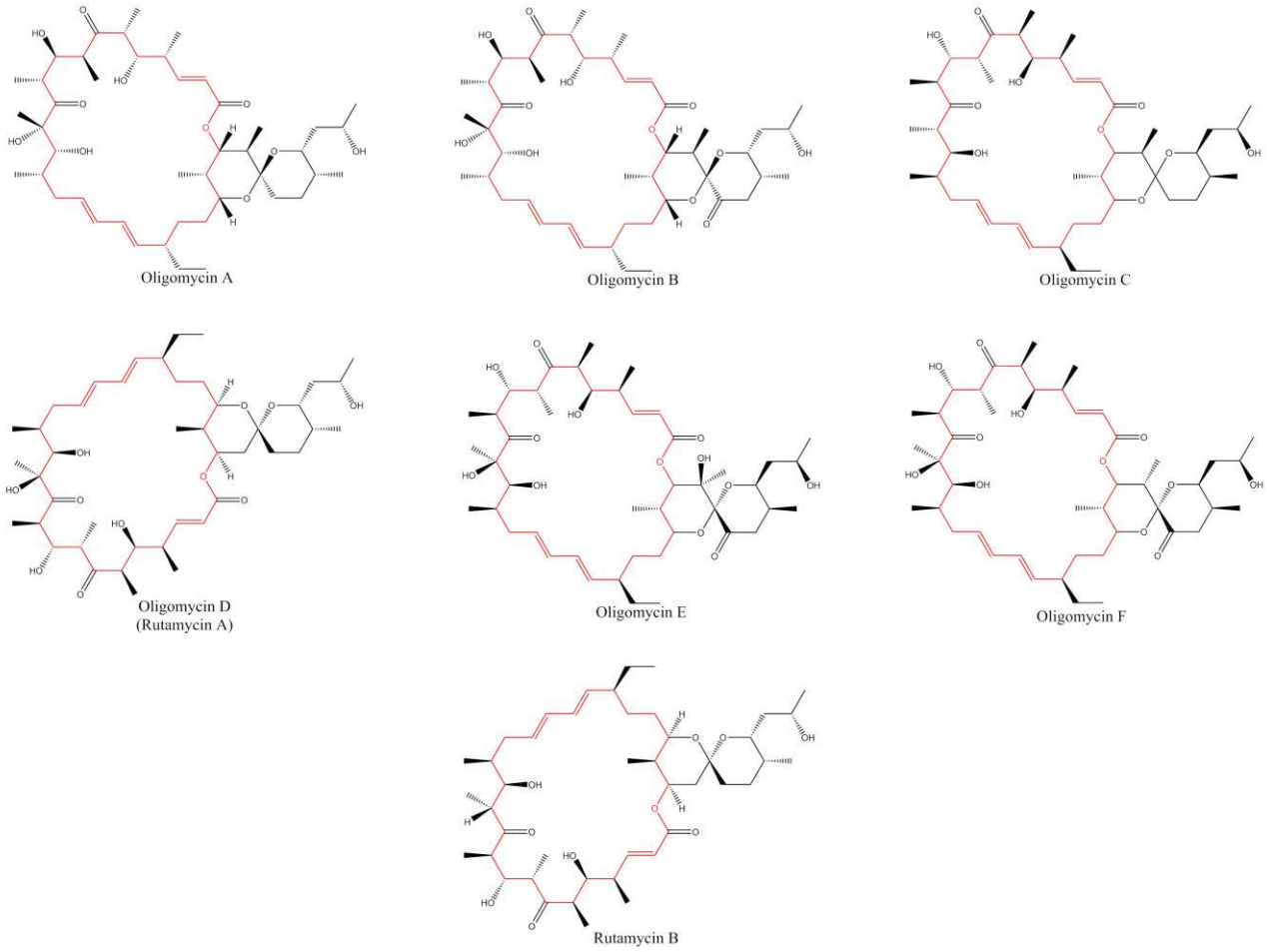

Figure 3. Structures of the main representatives of 16-24-membered macrolides. The macrolide ring is highlighted in red.

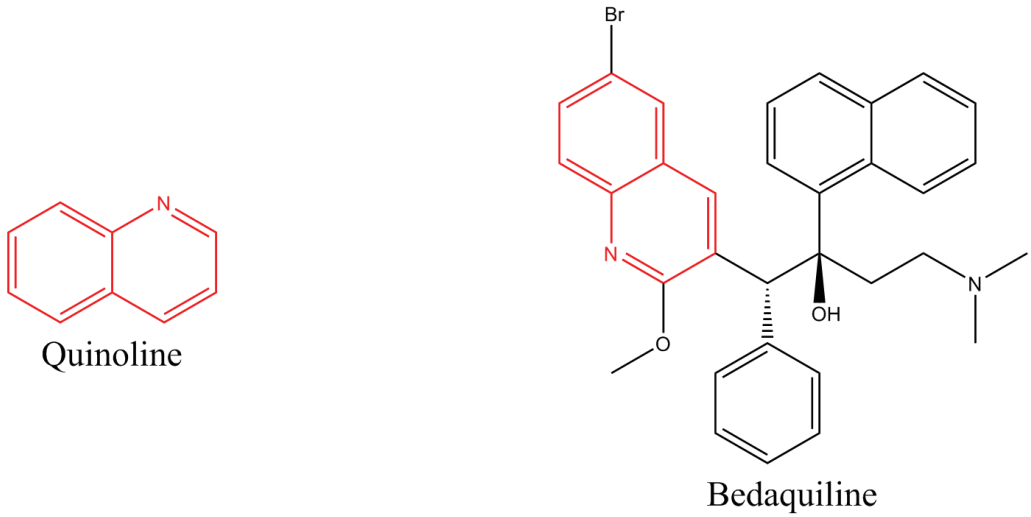

Figure 4. Structures of quinoline and bedaquiline (TMC207). The two condensed rings of quinoline are highlighted in red. The numbers indicate the possible substitution sites exploited in antimycobacterial drug design (modified after [58])

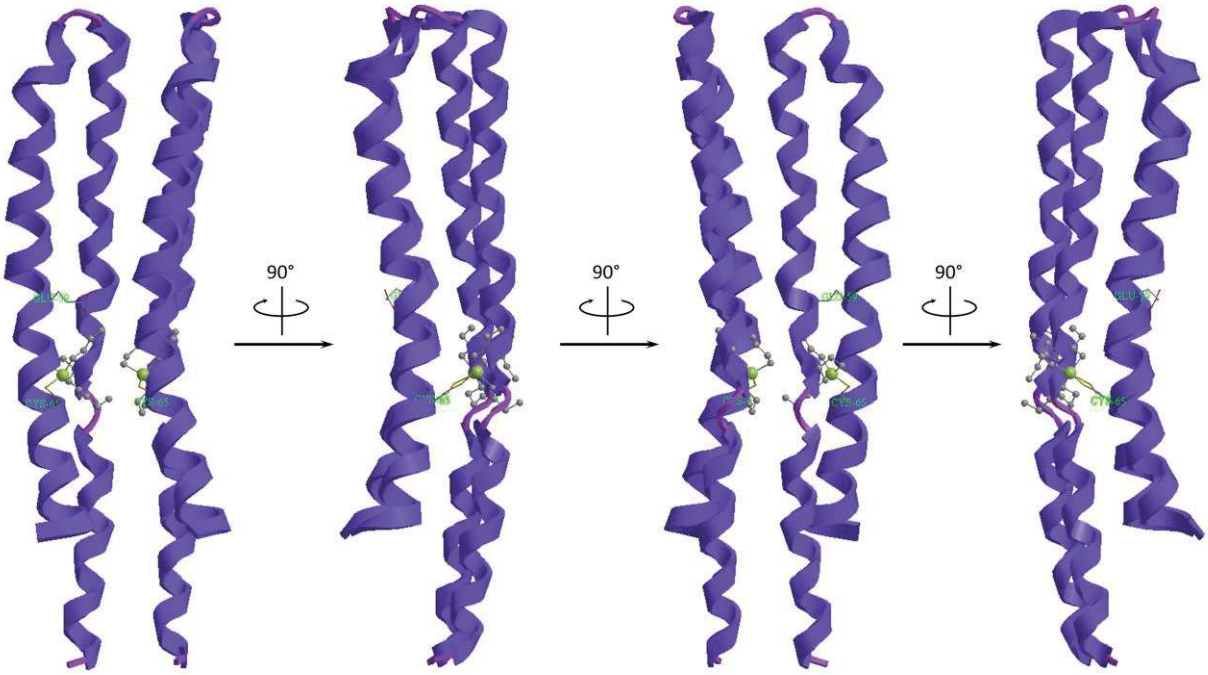

Figure 5. Model of TBT binding to adjacent *c*-subunits viewed from four different perspectives. The putative cysteine thiols involved in the binding to tin are highlighted in green. Glu represents the proton binding site.