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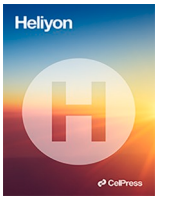

Review article

An overview of ATP synthase, inhibitors, and their toxicity

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ABSTRACT

Mitochondrial complex V (ATP synthase) is a remarkable molecular motor crucial in generating ATP and sustaining mitochondrial function. Its importance in cellular metabolism cannot be overstated, as malfunction of ATP synthase has been linked to various pathological conditions. Both natural and synthetic ATP synthase inhibitors have been extensively studied, revealing their inhibitory sites and modes of action. These findings have opened exciting avenues for developing new therapeutics and discovering new pesticides and herbicides to safeguard global food supplies. However, it is essential to remember that these compounds can also adversely affect human and animal health, impacting vital organs such as the nervous system, heart, and kidneys. This review aims to provide a comprehensive overview of mitochondrial ATP synthase, its structural and functional features, and the most common inhibitors and their potential toxicities.

1. Introduction

ATP synthases are protein complexes that are multi-subunit and membrane-associated. They use an electrochemical proton motive force across the membrane to convert adenosine diphosphate (ADP) and inorganic phosphate (Pi) into adenosine triphosphate (ATP) [1]. This complex protein is found in the bacterial cytoplasmic membranes, the thylakoid membranes of chloroplasts, the inner membrane of mitochondria, and the cytoplasmic membranes of various cell kinds, including endothelial cells, keratinocytes, and adipocytes [2].

Mitochondria are crucial for bioenergetics and cell physiology in eukaryotes by producing most of the ATP. They have an outer and inner membrane, folded into deep invaginations called cristae. These cristae increase the inner membrane's surface area to support numerous respiratory chain complexes and ATP synthase. The transfer of electrons through the respiratory chain is tied to proton movement from the matrix to the cristae lumen, resulting in an electrochemical proton gradient that powers ATP production by the ATP synthase [3].

The formation of dimers by the mitochondrial ATP synthase is crucial for mitochondria's proper structure and function. These dimers associate into rows along the high-curvature membrane regions of the cristae, contributing to the membrane's bending and the cristae's formation [4]. Studies have shown that the loss of ATP synthase dimers leads to abnormal cristae morphology, indicating their essential role in maintaining the integrity of the mitochondria [5].

ATP synthases/ATPases (commonly referred to as F1/F0 ATPase, F-ATPase, or complex V) belong to the family of rotary ATPases. F1/Fo ATPase structures share a common architecture, consisting of two main subcomplexes: the F1, which is hydrophilic, and the Fo, which is hydrophobic. Nonetheless, mutations or defects in this enzyme can lead to various human diseases, including cardiovascular diseases [6], obesity [7], type II diabetes [7], neurodegenerative disorders [8], and cancer [9]. It is essential to understand the

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importance of maintaining this critical enzyme's health and function for our bodies' overall well-being.

Studies have shown that inhibiting ATP synthase in rat cardiac myocytes led to elevated oxidative stress and calcium levels, ultimately leading to cell death [10]. Similarly, mutations in ATP synthase genes have been linked to various human diseases, from cardiomyopathy to hearing loss [11]. Extensive *in-vitro/in-vivo* studies indicate that any malfunction of ATP synthase can potentially cause numerous health complications. However, the exact poisonous effects of such dysfunction on humans remain unclear.

Subunit (a) mutations have been found to correlate with various pathological forms, including Leigh syndrome and NARP. *Leigh syndrome* is a fatal neurological condition that results from a significant impairment in ATP production, which causes severe neurodegeneration. The typical life expectancy for individuals with Leigh syndrome is two to three years [12]. NARP, conversely, is characterized by neurogenic muscle weakness, ataxia, and retinitis pigmentosa and is caused by abnormalities of mitochondrial energy generation due to mutations in subunit (a) [8]. In conditions such as neuronal ceroid lipofuscinoses or Kufs' disease, the stored material comprises subunit c of ATP synthase [13]. Low expression of the β subunit and cytoplasmic buildup of the ATP synthase α subunit have been noted in Alzheimer's disease [14]. Furthermore, F6 of ATP synthase functions as an endogenous vasoconstrictor by inhibiting cytosolic phospholipase A2 and can increase blood pressure when present at high plasma levels [15]. However, the presence of ATP synthase on the cellular membrane of endothelial cells is significant in facilitating the process of angiogenesis, which is essential for the development of tumors. This enzyme generates extracellular ATP, which then permeates back into the cell, serving as a supplementary reservoir of ATP [16]. According to Das et al. [17], the β subunit of ATP synthase has been identified as a protein target for the innate antitumor cytotoxicity executed by natural killer and interleukin 2-activated killer cells.

The power source for most of the cell's energy needs comes from the remarkable work of mitochondrial ATP synthase through oxidative phosphorylation under normal conditions. Significant changes in oxidative phosphorylation are observed in high-energy-metabolizing tissues such as the brain, heart, and skeletal muscles [6–9]. This highlights the vast potential of the enzyme as a drug target for multiple disease conditions and energy metabolism regulation.

ATP synthase has been found to interact selectively with some drugs. For example, Apoptolidin is a selective cytotoxic agent that induces apoptosis in cancer cell lines. It works independently of p53 status, is inhibited by BCL-2, and depends on caspase-9 action. The cell death signal induced by apoptolidin involves a mitochondria-dependent pathway. Additionally, apoptolidin is a potent inhibitor of F0F1-ATPase activity. It targets the (α) subunit and inhibits its activity [18,19]. The immunomodulatory benzodiazepine Bz-423 induces apoptosis in pathogenic lymphocytes via the mitochondrial pathway in a selective manner. This is done by generating superoxide within the mitochondria. Superoxide is one of the reactive oxygen species (ROS) that signals the initiation of apoptosis. Bz-423 binds to a portion of the mitochondrial F1F0-ATPase called the oligomycin sensitivity conferring protein (OSCP) subunit [20,21]. This attachment causes a conformational rearrangement of helices in OSCP and could change the way OSCP interacts with F1 in an allosteric manner, as noted by Stelzer et al., in 2010 [22].

It's great to know that bedaquiline (R207910) is one of the effective drugs developed for combating drug-resistant strains of Mycobacterium tuberculosis. The drug has a novel mechanism of action that targets subunit c, thus blocking bacterial ATP synthases and preventing ATP synthesis [23]. Resveratrol, a phytopolyphenol, is also an inhibitor of mitochondrial ATP synthase and is known to bind between the C-terminal part of the γ subunit and the βTP subunit [24]. In addition, resveratrol has been found to increase basal energy expenditure and thermogenesis, with multiple signaling pathways converging on the mitochondria [25].

According to research conducted by Bianchi et al. [26], blocking ATP synthase could potentially hinder the production of extracellular ATP and result in antiangiogenic and antitumorigenic effects [27]. reported that ATP synthase inhibitors can interact with the non-mitochondrial ATP synthase present in endothelial cells, which can significantly impede the cells' ability to migrate and proliferate. However, this inhibition had a minimal impact on intracellular ATP levels.

The development of over 300 compounds from natural and synthetic sources has led to the targeting of this molecular machine with great success. These inhibitors have proven effective in disrupting the energy metabolism of microbes and plants and have found widespread application as antibiotics, herbicides, and fungicides. However, it is essential to note that these compounds can also harm several human and animal organs, including the nervous system, heart, and kidneys [28–32]. Therefore, our present review aims to provide verified information on ATP synthase natural and synthetic inhibitors, summarizing their known or proposed modes of action and toxicity.

2. Structure and function

ATP synthase is an extraordinarily prevalent protein found in all living things and is responsible for generating ATP. The catalytic β-subunit of this protein exhibits remarkable conservation across bacteria, plants, and mammals, as more than 60 % of its amino acid residues have withstood evolutionary pressure [33]. There are various rotary ATP synthases, such as those found in archaea and certain extremophilic bacteria, the Phosphorylation Factor (F), eukaryotic vacuolar (V-type) ATPases, and A-type ATPases. While each enzyme executes a distinct function, they all strive to facilitate ATP synthesis and/or hydrolysis.

The protein complex known as the human mitochondrial ATP synthase consists of multiple subunits and has an estimated molecular weight of 550 kDa. As the fifth element in the oxidative phosphorylation chain, it is critical in facilitating ATP production [34]. As the most diminutive biological nanomotor, ATP synthase converts the energy derived from the electrochemical gradient into ATP through the phosphorylation of ADP [35].

Primarily responsible for ATP synthesis, the mitochondrion is an organelle. The electron transport chain (ETC), proton gradient, and H^+ chemiosmosis is utilized in this procedure [36]. In contrast, prokaryotic organisms produce ATP via photophosphorylation and chemiosmosis due to the absence of mitochondria.

Complexes facilitate the asymmetric transfer of electrons from NADH, FADH, and other oxidizable substrates across the inner

membrane of mitochondria. The movement of electrons induces the conveyance of protons (H^+) across the membrane, thereby generating an electric gradient (ΔΨ) and a chemical gradient (ΔpH). The energy accumulation caused by the difference in proton concentration and charge separation across the interior mitochondrial membrane generates the proton motive force (PMF). The PMF is accountable for facilitating ATP synthesis. Using the proton-specific channels (Fo) component of ATP synthase, protons return to the matrix, thus meeting Mitchell's primary criterion for chemiosmotic coupling [37]. The interior mitochondrial membrane must be impermeable to protons to satisfy this criterion. As a result, protons are compelled to re-enter the matrix via F0, whereas F1 facilitates the enzymatic reaction of ATP synthesis [38].

Despite minor structural variations, ATP synthases are structurally and functionally identical across bacteria, chloroplasts, and mitochondria. An example of this can be seen in the eight subunits of the E. coli ATPase/synthase, the two isoforms of the chloroplast ATPase, and the seven to nine additional subunits of the mitochondrial ATPase [14]. ATP synthase, Complex V, comprises two crucial subcomplexes, Fo and F1. Proton translocation is the function of the Fo, which is associated with the inner mitochondrial membrane; the F1, located in the mitochondrial matrix, is the water-soluble catalytic domain. Initially identified and isolated from bovine heart mitochondria, F1 is a protein that participates in oxidative phosphorylation. "Fraction 1″ was its designated designation. Fo, conversely, obtained its nomenclature due to its ability to impart oligomycin sensitivity to soluble F1 [39].

The F1Fo-ATPases found in mammalian mitochondria are composed of two main domains-a soluble F1 head domain (consisting of α3β3 subunits) that is responsible for ATP synthesis and a membrane Fo domain (consisting of c8-ring, ATP6, or a, ATP8 or A6L, e, f, g, DAPIT, and 6.8 PL) that are responsible for proton translocation. These two domains are connected by a central stalk (consisting of γ, δ, and ε subunits) that rotates inside the F1 region and a peripheral stalk (consisting of F6, b, d, and OSCP subunits) that remains stationary [40,41]. The human ATP synthase comprises 29 polypeptide chains of 18 subunits (including a regulatory protein IF1) and a combined molecular mass of 592 kDa (Fig. 1 (A-B)) [40].

Paul Boyer postulated that the "Binding Change Mechanism" is an essential constituent of the catalytic mechanism, which comprises rotational motion and steady-state catalysis [42]. The transportation and catalytic functions of the ATP synthase complex are dependent on the activity of its subunits. A complete 360° turnover at each catalytic site requires three conformations to be achieved and changed, and a cycle concludes with a 120° rotation at a separate catalytic site. ATP becomes firmly bonded to a nucleotide when it connects to ATP. Following this, an additional conformational change results in the liberation of ATP. The enzyme's inner core rotates

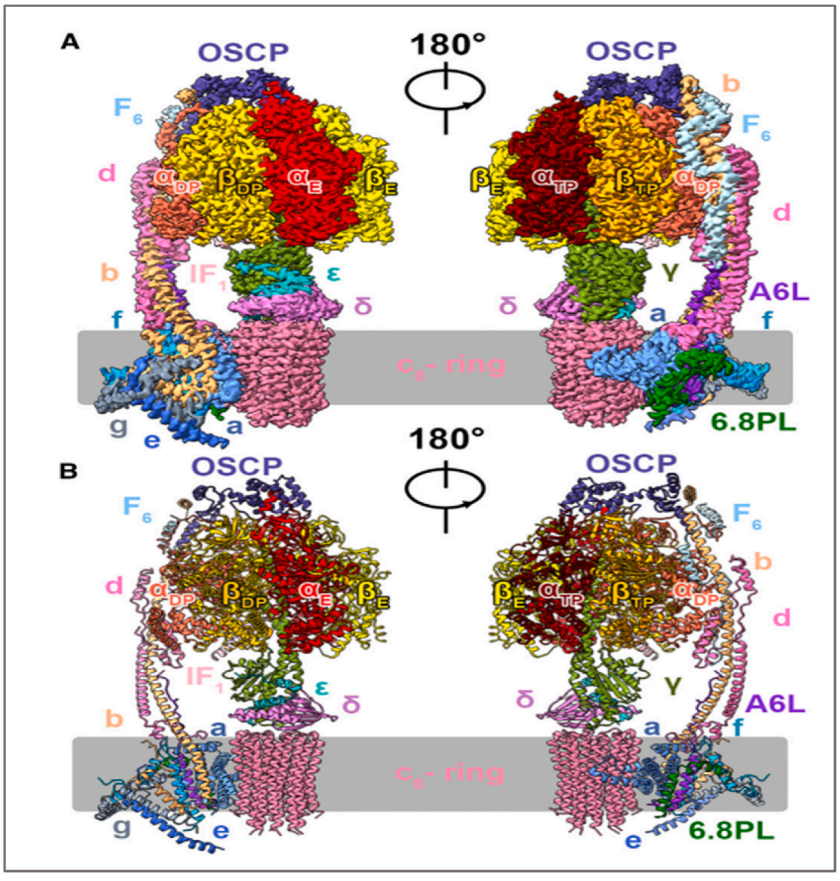

Fig. 1. The architecture Human mitochondrial ATP synthase based on Lai et al., [40].
(A) Compositional map of the rotational state of human ATP synthase. (B) Cartoon illustration of the subunits of the enzyme.

to accomplish these conformational changes; this rotation is driven by the proton motive force generated by protons traversing the mitochondrial membrane. Alternating positions of 3αs and 3βs, each with a distinct conformation and function, are observed in a circular pattern [43]. Although the α subunit binds to ATP, it remains stationary and does not contribute to the reaction.

In contrast, the β subunits possess three catalytic sites whose nucleotide-binding states vary. During ATP synthesis, these sites cooperatively transition between distinct conformations: βE (empty or open), βDP (bound to ADP or loose), and βTP (bound to ATP or tight). Each 360° rotation results in the formation and subsequent release of three Mg2+-ATP molecules in an alternative conformation [44]. ATP hydrolysis utilizes an identical pathway, albeit in the opposite orientation. The c-ring and β subunit rotate in the opposite direction during the transition between βE, βTP, and βDP states, resulting in the formation of a proton gradient. In this gradient, the concentration of protons is higher in the mitochondrial matrix. To facilitate the translocation of protons to the opposite side of the membrane, the F1 motor forces Fo motor to rotate the c-ring in reverse direction [45].

ATP synthase exhibits dual functionality by producing ATP and inducing cellular apoptosis. Although necessary for ATP production, it can also create the mitochondrial permeability transition pore (mPTP) [4]. The mPTP opening can trigger programmed cell death by disturbing the electron transport chain (ETC). This disruption interferes with ATP synthesis and leads to depolarization, depletion of pyridine nucleotides, release of calcium, inhibition of respiration, and swelling in the mitochondrial matrix. This swelling can release proapoptotic proteins such as cytochrome *c*, endonuclease G, and AIF [46]. On the contrary, recent research indicates that mPTP opening might serve a physiological purpose, as it remains open in the embryonic mouse heart and can temporarily open to modulate mitochondrial signaling in mature cells [47].

Research has indicated that, despite possessing enzymatic activity, ATP synthase monomers tend to aggregate into ribbons of even-numbered oligomers and dimers in vivo [5]. It has been discovered that this oligomerization process shapes the cristae membranes, which may provide a physiological benefit [4]. Oligomerization of ATP synthase is critical for enhancing its activity and yielding energy by establishing and preserving the local proton charge and mitochondrial membrane potential [48]. This is particularly important given that the mitochondrial ATP synthase harbors the PTP.

The mitochondrial permeability transition pore (mPTP) was initially noticed as a sudden swelling and uncoupling of mitochondria in response to high calcium levels, phosphate, oxidative stress, or other factors; this phenomenon has been studied for almost 50 years [49]. Haworth and Hunter coined the term "permeability transition" and additionally discovered the pharmacological properties of the PTP [50]. Subsequent research has confirmed that the permeability transition entails opening a large pore/ion channel in the inner mitochondrial membrane (IMM), which allows the passage of molecules up to 1.5 kDa.

It has been observed that the number of PTP-related paradigms has significantly increased, with ischemia-reperfusion injury, muscular dystrophies, and neurological disorders being the most well-known. PTP is viewed as a promising cardio-protection target in ischemia-reperfusion [51]. Mitochondrial dysfunction occurs faster in the heart when blood flow is restored after prolonged ischemia.

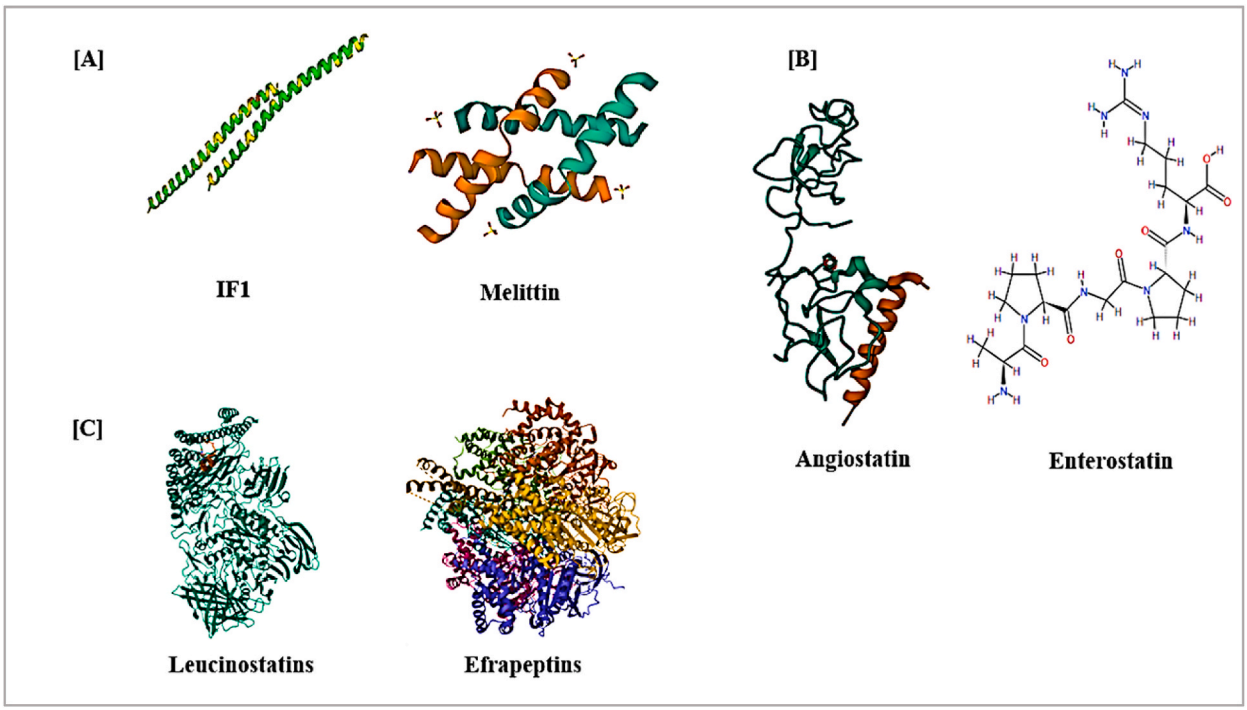

Fig. 2. Structures of peptide inhibitors. (A) α-Helical basic peptide inhibitors. IF1 (PDB ID: 1GMJ), melittin (PDB ID: 2MLT). (B) Angiostatin (PDB ID: 2DOH) and enterostatin (ID:3082883). (C) Leucinostatins (PDB ID: 8A19) and efrapeptins (PDB ID: 1EFR). The figures were drawn with the program PDB and Chem4Word.

However, the ischemic condition does not cause PTP opening, likely due to the protective effects of intracellular acidosis [52]. PTP opening is facilitated during the reperfusion phase. Several experimental models, including isolated cardiomyocytes, perfused hearts, and living animals have provided evidence that PTP plays a role in reperfusion injury [53]. PTP activation has also been associated with excitotoxicity, neuronal cell death, and mitochondrial alterations in diseases such as Reye's syndrome, multiple sclerosis, amyotrophic lateral sclerosis, and Alzheimer's [54,55]. PTP-dependent mitochondrial dysfunction has also been shown to play a crucial role in muscular dystrophy [56].

Recent studies on the electric field within ATP synthase, the enzyme responsible for energy production, suggest that it has exceptional enzymatic efficiency. Molecular electrostatic potential calculations have revealed that alterations in the electric field support proton movement and ATP formation, demonstrating that the enzyme operates beyond its biological catalytic role [57]. In addition to its primary function of catalyzing ATP formation, the enzyme's newly proposed free energy terms reveal two more vital functions. Firstly, the potential difference between proton entry and exit enhances the electrochemical gradient of the inner mitochondrial membrane, offsetting the energy required to dissipate the proton selection and modifying the free energy of proton translocation. The potential spike at proton entry acts as a kinetic barrier, indicating that ATP synthase influences proton migration. These revolutionary discoveries support previous estimates that ATP synthase operates with a remarkable efficiency rate of approximately 90 % [57].

3. FoF1-ATP synthase as a target for drug development

FoF1 ATP synthase has emerged as a promising molecular target for developing new drug therapies for disease conditions and energy metabolism. Over 300 natural and synthetic inhibitors have been identified, each with reported inhibitory sites and suggested modes of action [24].

3.1. Peptide inhibitors

The inhibitors in this group bind to F1 and inhibit ATPase activity. Their inhibitory activity is related to α-helical structures containing basic residues. This group includes α-helical basic peptide inhibitors such as inhibitory Factor 1 (IF1) and melittin (Fig. 2 (A)).

3.1.1. α-Helical basic peptide inhibitors

IF1, also known as Inhibitory Factor 1, is a protein that naturally inhibits mitochondrial ATP synthase. It comprises 56–87 residues and binds tightly to the F1 catalytic domain, with a 1:1 stoichiometric ratio. The N-terminal domain of the IF1 protein binds to the interface between αDP and βDP subunits, contacting βTP386, αE355, and the γ subunit [58]. This binding inhibits the hydrolysis of ATP but does not affect its synthesis. IF1's inhibitory activity is reversible and non-competitive, and it exclusively binds to F1 when ATP and Mg^{2+} are present [59]. It has been shown that IF1 is more potent against the ATP synthase complex than when used alone [60]. Recent research has revealed that F1 inhibited by IF1 is only activated when rotated in the clockwise direction, which is the direction for ATP synthesis, but not in the counterclockwise direction [61].

Melittin is a 26-residue polypeptide that is cationic and amphiphilic. It has a helix-turn-helix structural motif [62] and is the primary active component of honeybee venom (*Apis mellifera*). Melittin is known to have potent anti-inflammatory and antimicrobial effects. Studies have shown that it can inhibit the ATPase activity of F1 in both mitochondrial and chloroplast ATP synthases. Additionally, melittin binds to F1 at the exact location of IF1 [63].

3.1.2. Angiostatin and enterostatin

A proteolytic fragment called angiostatin is derived from plasminogen and has a triangular shape with three to five contiguous kringle domains (Fig. 2(B)). This peptide is an effective angiogenesis inhibitor. It can suppress the growth and spread of tumors by stopping the proliferation and movement of endothelial cells [64]. As per findings, it can bind to the alpha and beta subunits of ATP synthase and impede ATP hydrolysis [65]. Moreover, studies have shown that angiostatin can also hinder ATP generation in human vascular endothelial cells by inhibiting nonmitochondrial ATP synthase [66].

Enterostatin, a pentapeptide, is derived from the N-terminal cleavage of pancreatic procolipase during fat absorption in the small intestine (Fig. 2(B)). It has been found to bind to the β subunit in ATP synthase and inhibit ATP synthesis. A reduction of around 31 % in ATP production, coupled with elevated thermogenesis and oxygen consumption, was documented in a study involving insulinoma cells (INS-1), wherein the binding of Enterostatin to the mitochondrial ATP synthase was observed [67]. Earlier research by Erlanson-Albertsson and coworkers suggested that the mitochondrial F1-ATPase β-subunit could be a potential receptor for Enterostatin. Their study involved purifying F1-ATPase β-subunit from rat brains with Enterostatin affinity chromatography and demonstrating the binding of a labeled Enterostatin analog YGGAPGPR with purified bovine F1-ATPase β-subunit [68].

3.1.3. Leucinostatins and efrapeptins

Lipopeptide antibiotics called Leucinostatins [A to D, H, and K] are produced by various species of *Paecilomyces* fungi [69] (Fig. 2 (C)). These antibiotics have a specific α-helical conformation and contain nine amino acid residues, including unique ones like α-aminoisobutyric acid (AIB) [70]. Additionally, Leucinostatins can bind to the Fo component of ATP synthases, inhibiting oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts [71]. Leucinostatins have no inhibitory activity on isolated F1-ATPase [72,73] showed that antibiotics A20668 A, B, and C (synonyms for Leucinostatins A, B, C] inhibit phosphorylation of

ADP, Mg^{2+}-ATPase, and the ATP-driven transhydrogenase of rat liver submitochondrial particles, but not the purified F1 ATPase.

Efrapeptins, synthesized by *Tolypocladium* species (Fig. 2 (C)), is a potent group of lipophilic antibiotics comprising efrapeptins C to G. These antibiotics exhibit their efficacy by preventing ATP hydrolysis and synthesis reactions in a wide range of organisms, including chloroplasts, mitochondria, photosynthetic bacteria, and non-photosynthetic bacteria. During ATP synthesis, efrapeptin bonds to the F1 catalytic domain, outcompeting ADP, and phosphate [59]. It also hinders extracellular ATP generation by attaching to endothelial cells' nonmitochondrial ATP synthase [63]. Assumedly, efrapeptin hinders ATP synthase activity by preventing the transition of the βE subunit into a nucleotide-binding conformation.

3.2. Polyphenolic phytochemicals

Phytochemicals are bioactive compounds derived from plants linked to a reduced risk of multiple diseases. They have various biological activities and can bind to different molecular targets in the body [74]. Polyphenolics are a group of phytochemicals that inhibit mitochondrial ATP synthase. The phenolic structures of polyphenolic phytochemicals are crucial to their inhibitory powers [15].

3.2.1. Stilbenes

A diverse group of natural polyphenols is found in various plant species, known as stilbene compounds. They consist of two phenolic rings connected by a double-bond spacer. Resveratrol is one of the most popular stilbenes, commonly found in grapes and peanuts (Fig. 3(A)). Resveratrol can hinder the ATPase function of mitochondrial ATP synthase by attaching to a hydrophobic cavity located between the hydrophobic end in the C-terminal section of the γ subunit and the hydrophobic interior of a ring-like structure provided by the βTP subunit [75,76]. Hydrogen bonds and hydrophobic interactions stabilize the binding, and it appears to obstruct the rotation of the γ subunit, inhibiting both ATP hydrolysis and synthesis. Furthermore, resveratrol binds solely to a unique binding site in F1, and there are no comparable sites between the γ subunit and the βDP or βE subunits.

3.2.2. Flavones and isoflavones

Flavonoids are a group of phenolic compounds found in plants, with flavones and isoflavones being two classes of flavonoids. The difference between these two classes lies in the location of a phenyl group on the backbone (2-phenyl-1-benzopyran-4-one). While flavones are produced in various plants, beans predominantly have isoflavones.

Several flavones, including quercetin, kaempferol, morin, and apigenin (Fig. 3(B)), have been found to inhibit ATP hydrolysis. Quercetin has been shown to inhibit the ATPase activities of mitochondrial F1 and FoF1 [77], while kaempferol and morin have a similar inhibitory effect on the ATPase activity of mitochondrial FoF1. Apigenin, however, has about half the inhibitory potency of these compounds [77].

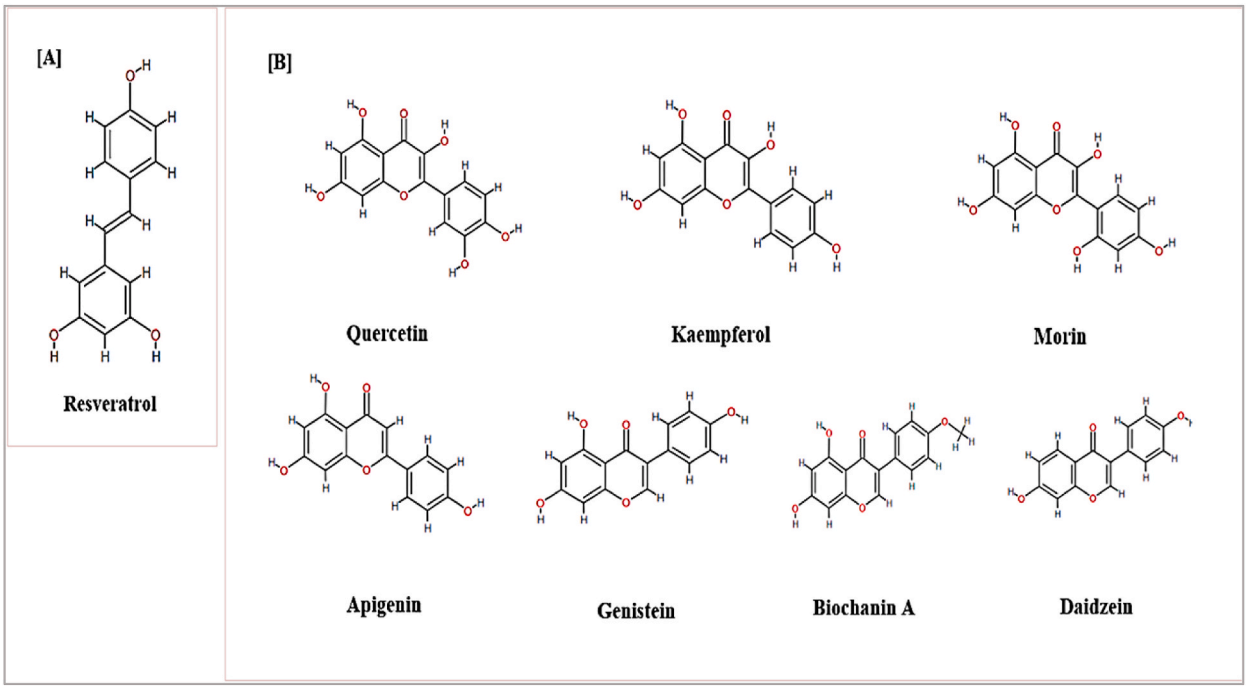

Fig. 3. Structures of polyphenolic phytochemicals. (A) Stilbenes. Resveratrol (B) Flavones and isoflavones. The figures were drawn with the program Chem4Word.

On the other hand, isoflavones such as genistein, biochanin A, and daidzein (Fig. 3(B)) are phytoalexins found in soybeans. Genistein has been found to inhibit both ATP hydrolysis and ATP synthesis activities of mitochondrial ATP synthase, primarily targeting Fo [63]. This inhibition is noncompetitive. Similarly, biochanin A inhibits the ATPase activity of mitochondrial FoF1, while daidzein has about half the inhibitory potency of the other two compounds [60].

3.3. Polyketide inhibitors

Polyketides are bioactive secondary metabolites found in various microorganisms, such as bacteria (used as antibiotics), fungi (used as aflatoxins), and some plants (used as flavoring and pigment agents). These compounds comprise two-carbon ketide units, which are synthesized by polyketide synthases. Macrolides are a type of polyketide that contains a large macrocyclic lactone ring to which one or more deoxy sugars can be attached, typically cladinose and desosamine, are attached [75]. Streptomyces sp. produces oligomycin, a class of 26-membered macrolactones (Fig. 4). It inhibits oxidative phosphorylation in mitochondria by blocking ATP synthesis through the oligomycin-binding site on subunit-c of the Fo portion of the ATP synthase [78]. Venturicidin is a glycosylated macrolide with 20 members (Fig. 4) produced by *Streptomyces* spp. This compound binds to the c-subunit of the ATP synthase, inhibiting both proton translocation and membrane-bound ATPase activities from mitochondria [63]; on the other hand, it inhibits the oxidative phosphorylation activities of mitochondrial ATP synthase [60]. Finally, Apoptolidin A (Fig. 4), a 20-membered *Nocardiopsis* sp. macrolide, inhibits the membrane-bound mitochondrial ATP synthase [75].

3.4. Organotin compounds and structural Relatives

Tin-containing organic compounds (organotin compounds (OTs)) are commonly used in producing PVC plastics and as agents for agriculture, marine antifouling, and wood preservation [79]. These compounds are classified as R4Sn, R3SnX, R2SnX2, and RSnX3 (Fig. 5). The studies demonstrated that organotin compounds could lead to the inhibition of F1Fo ATP synthase. This enzyme is responsible for ATP hydrolysis and cell synthesis [80]. The mechanism of inhibition involves the non-covalent interaction of these compounds with the enzyme, primarily within the ion channel of subunit-a. Additionally, this inhibition is reversible, with the organotin combinations competing with Na^+ or H^+ for the same binding site as ATP. Notably, these compounds do not affect the ATPase activity of isolated F1 [80].

3.5. The polyenic α -pyrone derivatives

The α-pyrone (or 2-pyrone) group is a structural characteristic of various biologically active metabolites. It is a six-membered cyclic unsaturated ester commonly found in natural products and some mycotoxins that contain α-pyrone, like aurovertin, citreoviridin, and asteltoxin (Fig. 6). These mycotoxins inhibit ATP synthase by targeting F1.

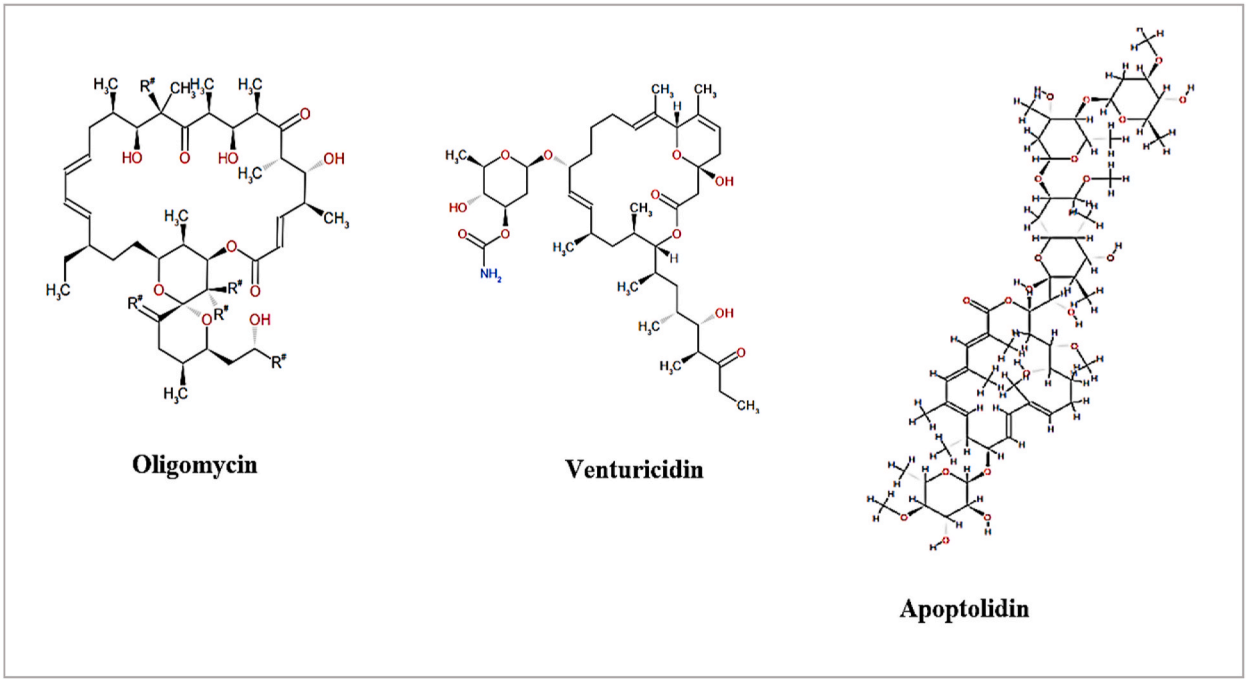

Fig. 4. Structures of some polyketide inhibitors (oligomycin, venturicidin, and apoptolidin). The figures were drawn with the program Chem4Word.

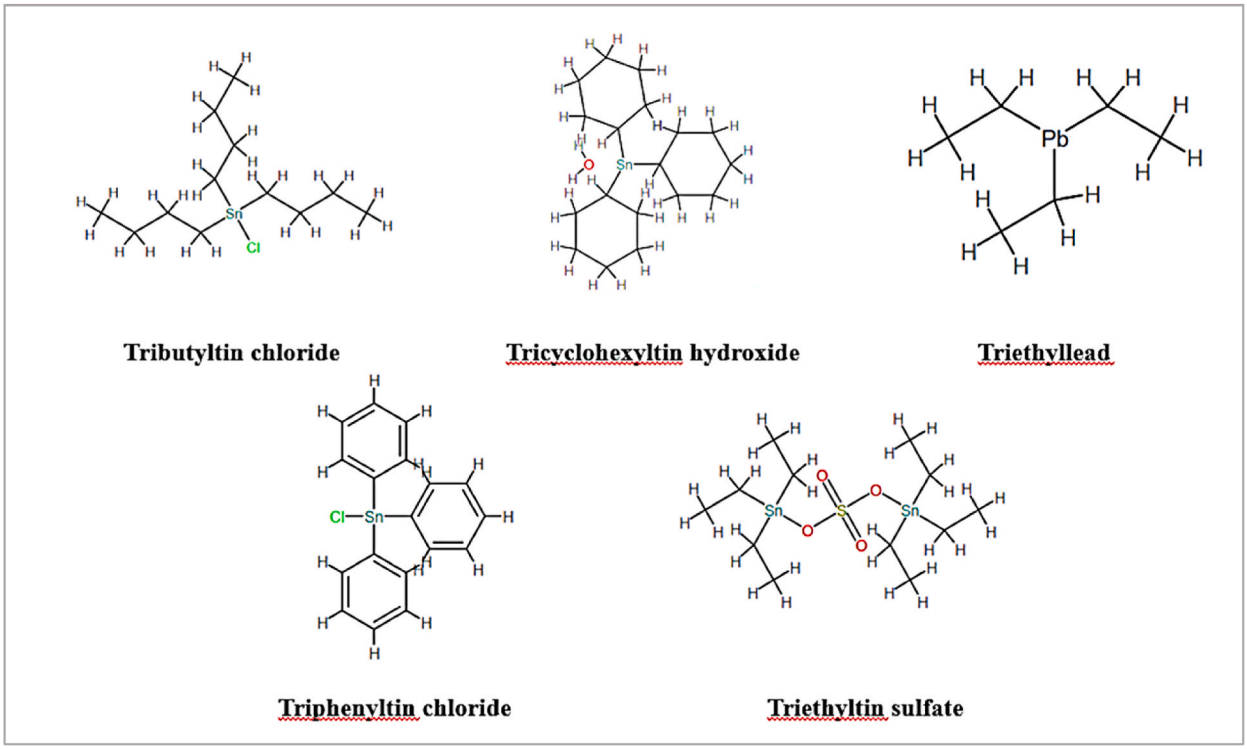

Fig. 5. Some Organotin (OTn) compounds and their associated structures. The figures were drawn with the program Chem4Word.

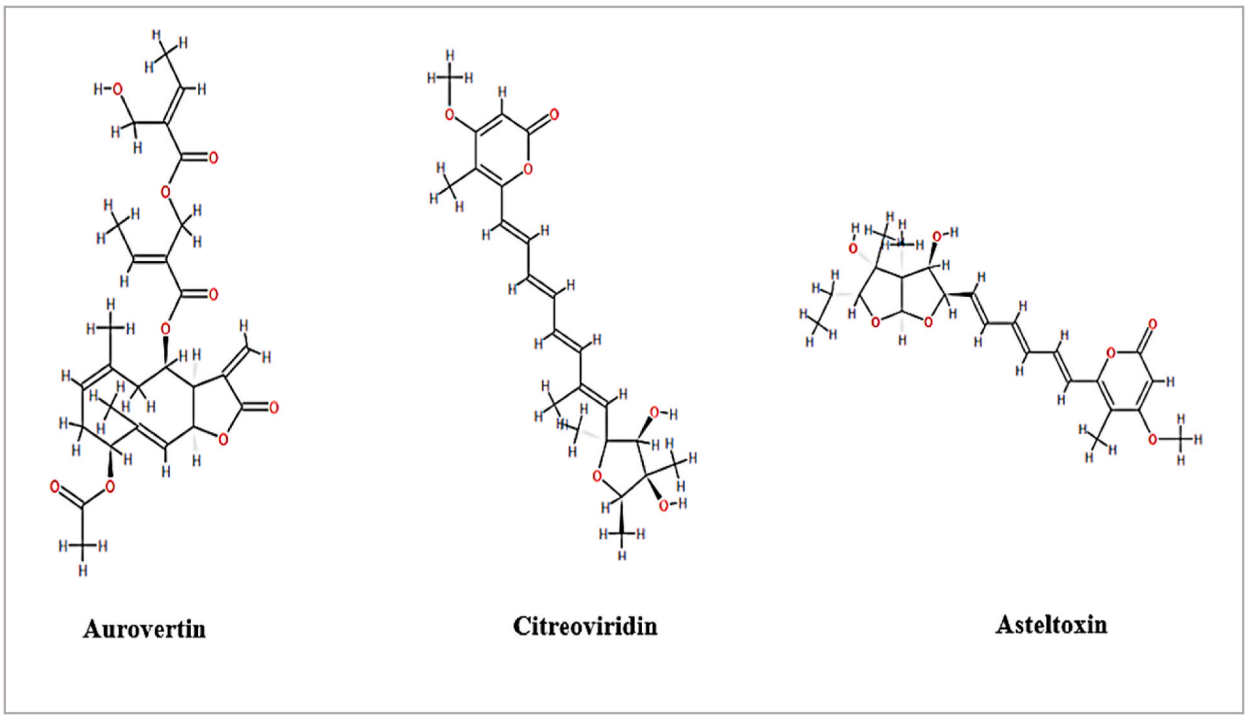

Fig. 6. Structures of some polyenic α-pyrone derivatives. The figures were drawn with the program Chem4Word.

Aurovertin is an antibiotic that is produced by a fungus named *Calcarisporium arbuscula*. There are five distinct types of aurovertin, namely A, B, C, D, and E. Aurovertin has been found to inhibit the ATPase activity of F1 in both mitochondria and mesophilic bacteria, as reported in previous study [81]. However, it does not affect thermophilic TF1 [82]. Aurovertin binds to the ATP synthase β subunit and acts as an uncompetitive inhibitor of its ATPase activity [81].

Citreoviridin is a mycotoxin produced by some genera of *Penicillium* and *Aspergillus* molds. It inhibits the mitochondrial ATPase activity by binding to the β subunit at a different site than aurovertin [65]. Citreoviridin binding to F1 or its isolated β subunit is non-competitive with aurovertin [83].

Asteltoxin is produced by the fungi *Aspergillus stellatus Curzi* and *Emericella variecolor*. It has a distinct 2,8-dioxabicyclooctane ring structure and functions as a mitochondrial F1 inhibitor in a 1:1 proportion in the presence of ADP. It binds to the same binding site as aurovertin [84].

3.6. Cationic inhibitors

Certain dyes, called amphiphilic cationic dyes, contain both a primary amine group and a lipophilic portion. These dyes have been found to inhibit the ATPase activities of F1 and FoF1. Most of these dyes have a more significant inhibitory effect on the ATPase activity of FoF1 than they do on F1 [85]. Rhodamines are a specific group of these dyes, which include rhodamine B, rhodamine 123, and rhodamine 6G (Fig. 7(A)). Interestingly, both rhodamine B and rhodamine 123 bind to F1 at multiple sites and inhibit the ATPase activity of MF1 from the bovine heart in a parabolic, non-competitive manner. In contrast, rhodamine 6G binds to at least two sites at high concentrations, and its mode of inhibition is mixed [86].

Another group of dyes, called brilliant green dyes, which includes rosaniline and malachite (Fig. 7(A)), also inhibits MF1 in a parabolic mixed fashion, suggesting the existence of at least two binding sites at high concentrations [87]. Safranin O (Fig. 7(A)) has been found to inhibit the ATPase activities of membrane-bound FoF1 from bovine heart mitochondria and soluble MF1 but with weaker inhibitory potency [87]. Lastly, Nile blue A (Fig. 7(A)) has been shown to inhibit the ATPase activity of the Fo portion from mitochondria but has no inhibitory effect on isolated F1 [87].

On the other hand, Mitochondria in endotherms operate at a higher temperature than body temperature due to the impedance of heat transfer across bioenergetic membranes. The exact temperature difference has been controversial. Chrétien and his team conducted experiments that revealed that mitochondria are significantly hotter than their surrounding environment by approximately 10 °C. However, theoretical calculations based on steady-state conditions suggest a maximum temperature difference of only 10-5 °C. This vast difference, a million-fold, is known as the "hot mitochondrion paradox" [88].

As mitochondria break down respiratory substrates, they create a proton gradient across their inner membrane, producing a pH gradient and a membrane potential known as Δψmit [89]. The researchers propose that every proton moved through ATP synthase

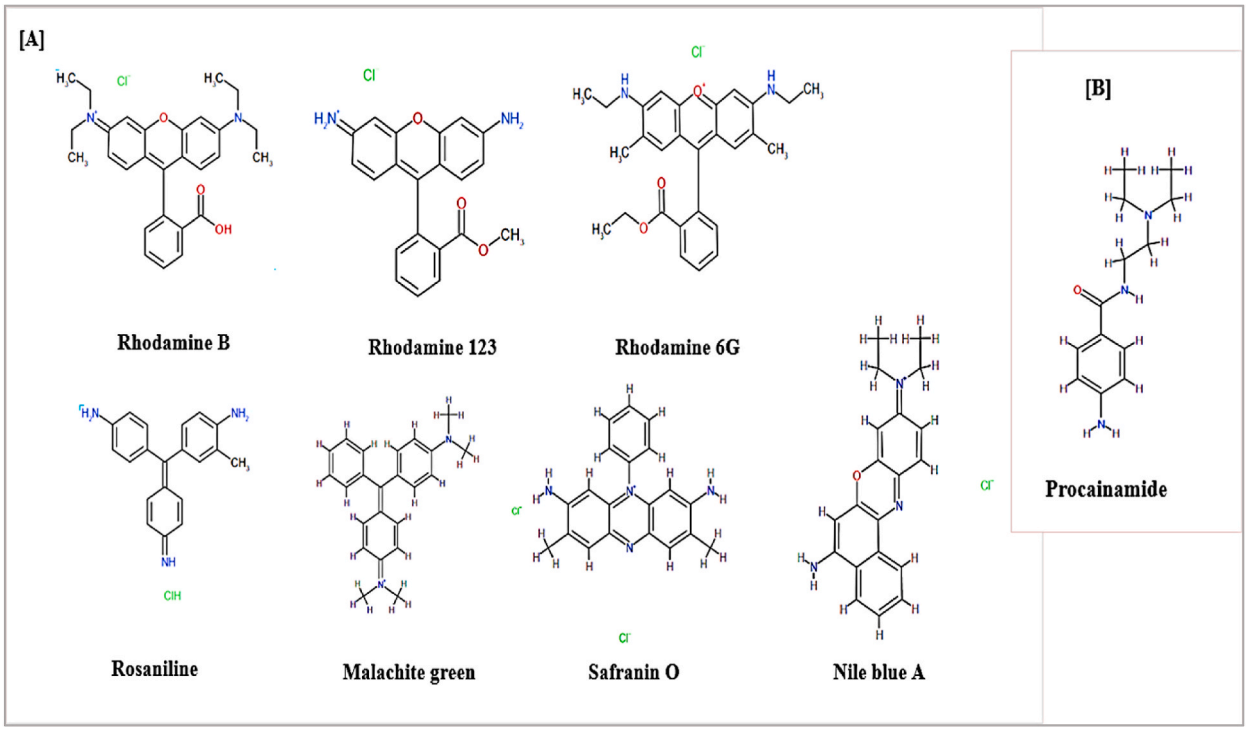

Fig. 7. Structures of cationic inhibitors. (A) Amphiphilic cationic dyes. (B) Procainamide (TALAs and related compounds). The figures were drawn with the program Chem4Word.

creates a picosecond spike in temperature, which may explain the temperature difference observed by Chrétien and his team [88].

Researchers have used a temperature-sensitive fluorescent probe to measure the temperature inside mitochondria under different conditions [90]. The family of rhodamine dyes possesses advantageous photophysical properties, one of which is the fluorescence intensity's insensitivity to changes in pH [88]. RH-123 is less lipophilic than other cation dyes, allowing it to diffuse through the mitochondrial membrane in response to potential and concentration gradients. However, its slower *trans*-bilayer diffusion means it cannot keep up with changes in Δψmit. RH-123 slightly affects the membrane surface potential and has similar kinetic constants for influx and efflux from the mitochondrial matrix [91]. Although it cannot measure the kinetics of Δψmit formation directly, RH-123 is a good candidate for measuring the actual membrane potential because its uptake is proportional to Δψmit. Therefore, the rate of fluorescence quenching is also dependent on Δψmit and the steady-state level of fluorescence decrease [91].

Tertiary amine local anesthetics (TALAs) comprise an aromatic portion, an intermediate chain, and a terminal amine group. The intermediate chain can either be an ester (found in tetracaine and procaine) or an amide (found in dibucaine and lidocaine) group [92]. TALAs group can bind to ATP synthases of mitochondria and some bacteria and inhibit ATP hydrolysis activity. TALAs can block both membrane-bound and soluble MF1. The inhibition of MF1 is reversible, and the concentration required for inhibition is like that needed for blocking nerve conduction [93]. Procainamide (Fig. 7 (B)), among other TALAs, can activate F1's ATPase activity at low concentrations but inhibits it at high concentrations. This phenomenon is not observed with other TALAs. The mechanism by which TALAs inhibit MF1 is still debated. One theory suggests that TALAs induce the structural dissociation of F1's multi-subunit structure, while another indicates that TALAs interact with F1's catalytic sites [94].

3.7. Substrates and substrate analogs

3.7.1. Phosphate analogs

Arsenate (Fig. 8) can negatively impact energy production by blocking ATP synthesis at the active site of ATP synthase through competition with phosphate. In addition, it can also block the exchange of P_i and H_2O, as well as ATP and P_i, which is catalyzed by ATP synthase. Arsenate is more effective in inhibiting ATPase when low phosphate levels [95]. On the other hand, azide (Fig. 8) inhibits the ATPase activity of F1 in mitochondria, bacteria, and chloroplasts. However, it does not have any effect on ATP synthesis. The inhibition mechanism of azide is non-competitive, and it only occurs in the presence of ADP and ATP [96]. Azide binding in the catalytic site is very tight and requires the previous binding of both ADP and Mg^{2+}. The inhibition depends on the ATP concentration and can be reversed by adding phosphate to compete for the azide binding site [96].

3.8. Other inhibitors

F1-ATPase is activated by divalent metal ions, but they can also act as inhibitors at high concentrations and in their free form. Free Mg^{2+}, Mn^{2+}, and Ca^{2+} ions can competitively inhibit F1-ATPase [60]. Additionally, excess-free ATP can also act as an inhibitor of ATP synthase. In ox heart mitochondria, inhibiting F1-ATPase activity by free ATP can be second-order/parabolic. Although ADP is a substrate for F1-ATPase, preincubation of F1-ATPase with ADP and Mg^{2+} causes hysteretic inhibition [97]. Furthermore, this inhibition occurs when the bound Pi is absent, Mg^{2+} is related to F1, and ADP is attached to a single catalytic site. The inhibition induced by Mg^{2+} ADP is slow and partial and can be reversed by adding ATP in the absence of Mg^{2+} [97].

3.9. Physical inhibitory factors

Exposure to high hydrostatic pressure levels (above 60–80 MPa) can lead to reversible inhibition of membrane-bound and isolated FoF1 and irreversible inactivation of soluble F1 from mitochondria [98]. However, hydrostatic pressure levels below the values mentioned above can have a stimulating effect on ATPase activity. The extent of inactivation due to high pressure depends on the protein concentration [98,99]. The inhibition caused by high hydrostatic pressure is linked to the dissociation that interferes with the required contacts for conveying conformational information between subunits, thereby disrupting rotational catalysis [99].

Another factor that can cause inhibition of membrane-bound and soluble F1 of mitochondrial ATP synthase is exposure to far-UV

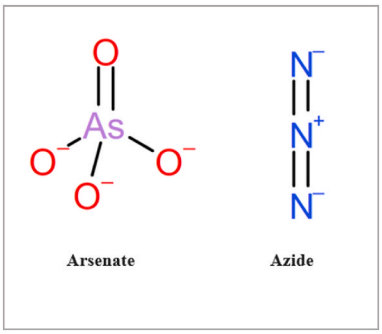

Fig. 8. Structure of Arsenate and Azide. The figures were drawn with the program Chem4Word.

irradiation, which occurs in a time-dependent manner. This causes F1 to release tightly bound adenine nucleotides. Furthermore, UV irradiation alters the basic-Tyr residue at F1's active site, influencing subsequent structural alterations in F1 [100].

The catalytic component of the mitochondrial ATP synthase (F1) is a cold-labile portion, and its activity decreases quickly when incubated at low temperatures [101]. This inactivation is unaffected by ATP, ADP, or $Mg2+$ and is restored when the enzyme solution is warmed under appropriate circumstances. The inactivation triggered by cold temperatures dissociates the enzyme complex into subunits [102].

4. Toxic effect of ATP synthase inhibitors

Various ways can trigger the production of ATP in mitochondria, one of which is using ATP synthase inhibitor drugs. These drugs are often used as antibiotics, fungicides, and herbicides because they impede the energy metabolism of living organisms and plants. However, they can also have toxic effects on animals and humans.

Oligomycin is a well-known ATP synthase inhibitor commonly used in research to study the activity of mitochondria. It has been shown to induce cardiac hypertrophy and heart failure in animal models [28,29], and it has also been found to significantly affect the kidneys in a toxic manner [30]. Azithromycin, another antibiotic, and ATP synthase inhibitor has also shown a highly neurotoxic effect in animal models [31]. Herbicides like paraquat, which have similar activity as ATP synthase inhibitors, can lead to Parkinson's disease and other immune disorders in humans [32].

Aside from their toxic effects, ATP synthase inhibitors can also play a role in developing drug resistance in bacteria, as seen in oligomycin [103]. Given the potential risks of these inhibitors to human health, it is crucial to understand their mechanisms and toxic side effects better. In summary, ATP synthase inhibitors harm several organs, such as the nervous system, heart, and kidneys, in humans and animals. Their usage must be regulated and minimized to decrease their harmful side effects.

5. Discussion and conclusions

Previously, ATP synthase was thought to exist only in the inner membrane of mitochondria, the plasma membrane of bacteria, and the thylakoid membrane of chloroplasts. It was believed to only play a role in ATP synthesis and proton gradient generation. However, recent evidence suggests that ATP synthase is also present on the surfaces of several types of animal cells and acts as a receptor for different ligands, contributing to various cellular processes like lipid metabolism, angiogenesis, intercellular pH regulation, and the cytolytic pathway of tumor cells [27,104–108]. Due to its multiple functions, ATP synthase is now considered a promising molecular target for treating various diseases.

A remarkable feature of mitochondrial ATP synthases is their ability to form dimers that create long rows on the inner membrane. Subunits e, g, k, and b-subunits were found to be associated with dimers; of these subunits, e, and g are essential for dimer formation and important for cristae morphology but not for ATP synthase activity [3].

The discovery that the PTP forms from the F-ATP synthase has redirected research toward the mechanisms that switch this key enzyme from an energy-conserving to an energy-dissipating device. This can improve our understanding of the pathophysiological events that trigger heart disease transitions and set a logical framework for therapeutic strategies.

F1FO-ATPase is a complex molecular machinery with interconnecting subunits that interact. Even minor disruptions to its structure or conformation can hinder ATP synthesis and/or hydrolysis. Genetic mutations that affect F1FO-ATPase biogenesis, underdeveloped cristae, insufficient F1FO-ATPase populations, and poor prognosis are common causes of mitochondrial diseases [109,110].

Targeting ATP synthase through various small molecules (modulators/inhibitors) has several advantages. Firstly, it plays a crucial role in energy metabolism. If selectively targeting, it may help to eradicate certain types of cancer. For instance, an immunomodulatory drug, Benzodiazepine Bz-423, triggers the mitochondrial pathway of apoptosis selectively in pathogenic lymphocytes by binding to the OSCP of Fo mitochondrial F1Fo-ATPase [20,21]. Additionally, resveratrol and piceatannol, possible anti-angiogenesis agents, have been shown to block tumor development by binding to the β subunit of F1 [24].

Further, its complex subunit composition makes it an ideal target for managing various other diseases (e.g., neurodegenerative disorders like Alzheimer's and Parkinson's and metabolic diseases like diabetes) [75]. Certain conditions have similar mechanisms of cell death as aging. As a result, some severe illnesses are closely linked to increased lifespan and age. Since mPTP formation is one of these age-related mechanisms, it's clear that the pore shutters have multiple therapeutic roles and anti-aging properties. Although pore modulators that target the F1FO-ATP synthase may be effective in treating neurodegenerative disorders and other mPTP-related diseases, alternative therapeutic methods based on F1FO-ATP synthase modulation are being considered. Furthermore, a synthetic compound, J147, has emerged as a promising therapeutic molecule because it can improve severe cognitive deficits in aged transgenic mice. J147 targets the mitochondrial alpha-F1-ATP synthase, causing a slight enzyme inhibition that activates AMP-activated protein kinase [111].

The focus of treating amyotrophic lateral sclerosis is to preserve the function of mitochondria. This often shows a strong link between the formation of mPTP and the activity of F1FO-ATP synthase. A rise in ion conductance due to mPTP formation, detected in the c-subunit of F1FO-ATP synthase, can be prevented with dexpramipexole. This neuroprotective medication causes changes in the F1FO complex's structure [112].

ATPase inhibitory factor 1 (IF1) interacts with ATP synthase and prevents it from breaking down ATP. In experiments with diabetic animals, giving them recombinant IF1 improved their ability to tolerate glucose and lowered their blood sugar levels. IF1 also stops β-F1-ATPase from breaking down ATP in the cell membrane, which increases the amount of ATP outside the cell and activates the protein kinase B (Akt) pathway. This ultimately helps with glucose uptake. Scientists believe that IF1 is a new type of "myokine" and

may help people with diabetes. These findings suggest that both AMPK and Akt contribute to the positive effects of IF1 on glucose tolerance. Overall, IF1 shows promise as a potential treatment for diabetes [113].

Lastly, ATP synthase has been identified as a promising drug target for developing antimicrobials. The approval of Bedaquiline in 2012 for treating tuberculosis is a testament to its potential efficacy. Bedaquiline is a fantastic medication that has unique anti-mycobacterial properties. It targets the proton pump of mycobacterial adenosine triphosphate (ATP) synthase, vital for the survival of both prokaryotic and eukaryotic cells. By binding to the subunit c of mycobacterial ATP synthase, Bedaquiline stops the production of ATP, resulting in the eradication of bacteria [23]. This medication works exceptionally well against mycobacterium and has minimal impact on host cells because it has a much stronger affinity for mycobacterial ATP synthase than human mitochondrial ATP synthase [114]. In contrast, aurovertin is an antibiotic that attaches to the β subunit in the F1 portion and inhibits ATP synthesis in preference to ATP hydrolysis [63].

The ATP synthases found in mitochondrial and plasma membranes have subunits not present in bacterial or chloroplast ATP synthases, for the subunit contains additional subunits such as e, f, g, i/j, k, and A6L, the specific functions of which are still unknown. Recent evidence suggests that the "extra" subunits of mitochondria have additional functions, although they were initially thought to play only a role in ATP synthesis [6]. For example, subunit F6 has been shown to regulate blood pressure [115]. Subunit e has been reported to regulate gene expression for subunit g of the ATP synthase and is highly susceptible to various physiological modifications and stresses [116]. Further research is necessary to investigate the regulatory roles of these additional subunits, which could lead to the ATP synthase's use as a drug target.

While ATP synthase inhibitors have shown potential therapeutic benefits, some of these compounds have been found to have toxic effects on various organs, including the nervous system (such as azithromycin) [31], heart (such as oligomycin) [28,29], and kidneys (such as aurovertin) [30], in both humans and animals. Additionally, they can impact drug response and resistance, making it necessary to regulate and minimize their usage to reduce harmful side effects.

Many synthetic and natural ATP synthase inhibitors provide a promising foundation for developing new therapeutics for human diseases. It's essential to consider the selectivity of an ATP synthase inhibitor as a therapeutic agent, especially when targeting microbial and mammalian ATP synthase enzymes. With careful consideration and application, these inhibitors can potentially revolutionize the treatment of various illnesses.

The discovery of ATP synthase's role in the life or death of cells has opened new possibilities for drug development. Thanks to a combination of computational and experimental approaches, scientists have identified and created modulators and inhibitors for the enzyme. Bioinformatics has also provided crucial insights into creating drugs that target Fo subunits [117]. And with ongoing research exploring the potential of modifying c-subunits [118], the future of drug inhibition looks brighter than ever.

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CRediT authorship contribution statement

Arwa R. Althaher: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Mohammad Alwahsh:** Writing – review & editing, Writing – original draft, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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