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Medicinal Chemistry of ATP Synthase: A Potential Drug Target of Dietary Polyphenols and Amphibian Antimicrobial Peptides

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Abstract

In this review we discuss the inhibitory effects of dietary polyphenols and amphibian antimicrobial/antitumor peptides on ATP synthase. In the beginning general structural features highlighting catalytic and motor functions of ATP synthase will be described. Some details on the presence of ATP synthase on the surface of several animal cell types, where it is associated with multiple cellular processes making it an interesting drug target with respect to dietary polyphenols and amphibian antimicrobial peptides will also be reviewed. ATP synthase is known to have distinct polyphenol and peptide binding sites at the interface of α/β subunits. Molecular interaction of polyphenols and peptides with ATP synthase at their respective binding sites will be discussed. Binding and inhibition of other proteins or enzymes will also be covered so as to understand the therapeutic roles of both types of molecules. Lastly, the effects of polyphenols and peptides on the inhibition of *Escherichia coli* cell growth through their action on ATP synthase will also be presented.

Keywords

F_1F_o ATP synthase; ATPase; *E. coli* ATP synthase; dietary polyphenols; amphibian antimicrobial peptides; enzyme inhibitors

INTRODUCTION

ATP synthase is the fundamental means of cellular energy production in animals, plants, and almost all microorganisms. ATP synthase is also the final enzyme in the oxidative phosphorylation pathway and is responsible for ATP synthesis by oxidative or photophosphorylation in the membranes of bacteria, mitochondria, and chloroplasts. It is the smallest known biological nanomotor, found from bacteria to man. In order to synthesize ATP, the cell's energy currency, a mechanical rotation mechanism is used in which subunits rotate at approximately 100 times per second in order to convert food into energy by oxidation. A typical 70 kg human with a relatively sedentary lifestyle will generate around 2.0 million kg of ATP from ADP and Pi (inorganic phosphate) in a 75-year lifespan [1]. ATP synthase functions in the same way in both prokaryotes and eukaryotes [2]. For

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different organisms estimates of the number of protons required to synthesize one ATP molecule have ranged from three to four, with the possibility that cells can vary this ratio to suit different conditions [3–5].

STRUCTURE FUNCTION RELATIONSHIP OF ATP SYNTHASE

F_1F_o-ATP synthase is structurally and functionally similar whatever the source. In its simplest form, as shown in Fig. (1), *Escherichia coli* ATP synthase contains eight different subunits, namely $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10}$. The total molecular mass is ~530 kDa. F_1 corresponds to $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_o to ab_2c_{10}. In chloroplasts, the structure is the same except that there are two isoforms. In mitochondria, there are 7–9 additional subunits, depending on the source, but *in toto* they contribute only a small fraction of additional mass and may have regulatory functions [6–8]. ATP hydrolysis and synthesis occur on three catalytic sites in the F_1 sector, whereas proton transport occurs through the membrane embedded F_o sector. The γ-subunit forms a coiled coil of α-helices that go right up into the central space of the $\alpha_3\beta_3$ hexagon. Proton gradient-driven clockwise rotation of γ (as viewed from the outer membrane) leads to ATP synthesis and anticlockwise rotation of γ results in ATP hydrolysis. In recent nomenclature, the rotor consists of $\gamma\varepsilon c_n$, and the stator consists of $\alpha_3\beta_3\delta ab_2$ [9–11]. The function of the stator is to prevent co-rotation of catalytic sites with the rotor. Current understanding of the F_1F_o structure and mechanism has been thoroughly reviewed by Senior's group and others [1, 11–22].

The three catalytic sites located on the F_1 sector of ATP synthase are designated βTP, βDP, and βE by x-ray crystallographers, based on the binding of ATP, ADP, and Pi respectively [23, 24]. βE is the empty site into which Pi (inorganic phosphate) must initially bind for ATP synthesis. It has been proposed that the synthesis reaction in the three catalytic sites does not occur independently but occurs sequentially. In this mechanism, the three catalytic sites have different affinities for nucleotides at any given moment. Each catalytic site undergoes conformational transitions that lead to the following sequence: substrate (ADP +Pi) binding → ATP synthesis → ATP release. Experimental observations of rotation verified the predication made by Boyer [2, 25, 26] that catalysis requires the sequential involvement of three catalytic sites, each of which changes its binding affinity for substrates and products as it proceeds through the cyclical mechanism, hence the term "binding change mechanism." Proton motive force is converted in F_o to a mechanical rotation of the rotor shaft, which drives conformational changes of the catalytic domains in F_1 to synthesize ATP. Conversely, hydrolysis of ATP induces reverse conformational changes of F_o sector and consequently reverses rotation of the shaft. Conformational changes in the catalytic sites are connected to the γ-subunit rotation. γ-Subunit rotation in isolated $\alpha_3\beta_3\gamma$ subcomplex has been observed directly by Yoshida and Kinosita with colleagues in Japan, and subsequently by several other labs [4, 27–31]. The reaction mechanism of ATP hydrolysis and synthesis in F_1F_o and their relationship to the γ-subunit mechanical rotation is not the focus of this review, but is a fundamental question that remains to be elucidated. This question also applies to many other ATPases and GTPases, with a relevance to nanotechnology and nanomedicine [32, 33]. Therefore, it is important to summarize the catalytic function of ATP synthase in some detail before describing inhibition chemistry of ATP synthase by polyphenols or peptides [34, 35].

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CATALYTIC AND MOTOR FUNCTION OF ATP SYNTHASE

Previous studies were focused on determining the Pi binding residues in the catalytic site so as to better understand the reaction mechanism of ATP synthesis, hydrolysis and their relationship to mechanical rotation. Identification of Pi binding residues and residues surrounding the Pi binding subdomain in the catalytic site is imperative for answering the following two important questions. (I) How does the enzyme bind ADP and Pi rather than ATP at catalytic sites? This is an often overlooked but crucial question in the mechanism of ATP synthesis. In active cells, the cytoplasmic concentrations of ATP and Pi are approximately in the 2–5 mM range, whereas that of ADP is at least 10–50-fold lower. Equilibrium binding assays have established that both ADP and ATP bind to catalytic sites of purified F_1 and detergent solubilized F_1F_o with relatively similar binding affinities [36–39]. Obviously, a specific mechanism must have evolved for selectively binding ADP into catalytic sites while contemporaneously discouraging access to ATP during proton driven rotation and ATP synthesis. One hypothesis is that during ATP synthesis, proton gradient driven rotation of subunits drives an empty catalytic site to bind Pi tightly, thus stereo-chemically precluding ATP binding and therefore selectively favoring ADP binding [12]. (II) How does subunit rotation affect Pi binding [25, 40, 41]? It was shown that Pi binding appears to be “energy linked”, implying that it is linked directly to subunit rotation [6, 42, 43]. Therefore, for formulating a mechanism for ATP synthesis it is of paramount importance to understand the features that determine Pi binding. Moreover, in the near future it may be possible to use molecular features of Pi binding, derived from mutational and biochemical studies, in the development of potent and novel molecular inhibitors of ATP synthase.

X-ray crystallographic structure of the catalytic sites of ATP synthase shows the following residues αPhe-291, αSer-347, αGly-351, αArg-376, βLys-155, βArg-182, βAsn-243, and βArg-246 in close proximity to bound phosphate analogs AlF_3 or SO_4^{2-} suggesting that these residues are involved in Pi binding [24, 44]. [*E. coli* residue numbers are used throughout]. Earlier attempts to measure Pi binding in purified *E. coli* F_1 using [^{32}P] Pi [40] or by competition with ATP or AMP-PNP in fluorescence assays of nucleotide binding [37, 45] failed to detect appreciable Pi binding at physiological Pi concentration. An assay devised by Perez *et al.* [46] in which the protection afforded by Pi against inhibition of ATPase activity induced by covalent reaction with 7-chloro-4-nitrobenzo-2-oxa-1, 3,-diazole (NBD-Cl) provided a measure of Pi binding. Earlier Orris *et al.* [47] showed by X-ray crystallography that the covalent adduct formed by NBD-Cl is specifically in the βE catalytic site, thus protection afforded by Pi indicates that binding of Pi occurs at the βE catalytic site. By modifying the above assay for use with *E. coli* purified F_1 or membrane bound F_1F_o, we have thus far investigated the relationship between Pi binding and catalysis for eight residues, namely αPhe-291, αSer-347, αGly-351, αArg-376, βLys-155, βArg-182, βAsn-243, and βArg-246. We found that the following five residues; αSer-347, ocArg-376, βLys-155, βArg-182, and βArg-246 are grouped in a triangular fashion, and are involved in Pi binding. The other three residues; αPhe-291, αGly-351, and βAsn-243 are not [17, 33, 48–52]. Presence of Pi binding residues in the catalytic site explains the preferential binding of ADP over ATP. The story doesn’t end here as there are many other residues in close

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proximity to Pi binding subdomain in the catalytic sites which may have a direct or indirect role in Pi binding and thus require further characterization.

ROLE OF ATP SYNTHASE IN HUMAN HEALTH AND DISEASES

ATP synthase is critical to human health. Malfunction of this complex has been implicated in a wide variety of diseases including cancer, tuberculosis, neuropathy, Alzheimer's, Parkinson's, and a class of severely debilitating diseases known collectively as mitochondrial myopathies. This enzyme is also a likely therapeutic target in the treatment of diseases such as, cancer, heart disease, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, tuberculosis, Parkinson's, and Alzheimer's ([53] and reference therein). One of the forms of Leigh syndrome, a neurodegenerative disease, is the result of mutation in the *a*-subunit of ATP synthase [54]. The c-subunit of ATP synthase is involved in both the lysosomal storage diseases, Kufs' and Battens'. Low expression of β-subunit and accumulation of α-subunit in the cytosol is associated with Alzhimer's disease. The neuropathy, ataxia, is also caused by dysfunction of ATP synthase. Presence of circulating subunit F6 has been associated with hypertension. Recent studies have also suggested that the presence of ATP synthase on the surface of several animal cell types is correlated with multiple cellular processes including lipid metabolism, angiogenesis, intracellular pH regulation, and programmed cell death [55–59]. Angiostatin is a known inhibitor of angiogenesis, which was shown to bind to ATP synthase on the surface of human endothelial cells. It was shown that angiostatin's antiproliferative effect on endothelial cells was dependent on its interaction with the α-subunit of ATP synthase. The mechanism behind this is the transport of H across the plasma membrane by mitochondrial ATP synthase causing cytolysis of tumor cells [60].

In addition to the above mentioned conditions, ATP synthase is also a target enzyme for antimicrobial agents. *Streptococcus mutans* is a primary microbial agent in the pathogenesis of dental caries through biofilm formation and acid production. Inhibition of ATP synthase of *S. mutans* inhibits biofilm formation and acid production [61, 62]. Also in *Mycobacterium* ATP synthase, two mutations in the c-subunit (D32V and A63P) confer resistance to the new tuberculosis drug diarylquinoline [63, 64]. Thus a better understanding of ATP synthase inhibition may aid in the treatment of these diseases.

ATP SYNTHASE INHIBITION

A wide range of natural and synthetic products are known to bind and inhibit both bovine mitochondrial and *E. coli* ATP synthase [53, 65, 66]. The inhibitory effects and the extent of inhibition on molar scale are variable among different inhibitors. Also, some of the inhibitors are known to inhibit only ATP synthesis and not hydrolysis or vice versa while others inhibit both synthesis and hydrolysis equally. Among the potent inhibitors of ATP synthase are: 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl), sodium azide (NaN_3), aluminum fluoride (AlFx), scandium fluoride (ScFx), beryllium fluoride (BeFx), dicyclohexylcarbodiimide (DCCD), several naturally occurring antibiotics such as oligomycin, efrapeptins, aurovertins, leucinostatins, a number of polyphenols like resveratrol, piceatannol, quercetin, morin, epicatechin, and quite a few peptides such as

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melittin, melittin related peptide (MRP), ascaphin, aurein, caerin, dermaseptin, and maganain II [17, 33–35, 65, 67–75].

The importance of ATP synthase as a promising target for drug development is evident from the fact that many antibiotics such as efrapeptins, aurovertins, and oligomycins inhibit its function. Antibiotics efrapeptins and aurovertins inhibit both synthesis and hydrolysis of ATP by ATP synthase [73, 76]. The efrapeptins bind to ATP synthase at a site extending from the rotor, across the central cavity of the enzyme, into the specific βE-subunit catalytic site. This binding prevents the closure of the βE subunit during the rotary cycle [73, 77]. Aurovertins are known to bind and inhibit mitochondrial ATPase, thereby uncoupling oxidative phosphorylation. Two molecules of aurovertin bind simultaneously to the cleft between nucleotide binding and C-terminal domain of two β subunit domains [76]. Aurovertin was shown to bind non-covalently to wild-type *E. coli* F_1 with K_d of 1μM [78, 79]. Weber and Senior [65] using 10 μM aurovertin found that it inhibited ATPase activity of β-Trp-331 F_1 by 87%. Another interesting observation was that aurovertin is an uncompetitive inhibitor of *E. coli* and mitochondrial F_1-ATPase and does not achieve total inhibition of hydrolysis [80].

Oligomycins are macrolides generated by *Streptomyces* and can be poisonous to other organisms. The macrolides are a group of antibiotic drugs whose activity depends on the presence of a macrolide ring. Macrolides are secondary metabolites from bacteria, fungi, plants, and animals belonging to the polyketide class of natural products [81]. Common macrolide antibiotics in use are Azithromycin, Clarithromycin, Dirithromycin, and Erythromycin. Antibiotic macrolides are used to treat infections such as respiratory tract and soft tissue infections. The antimicrobial spectrum of macrolides is slightly wider than that of penicillin, and therefore macrolides are a common substitute for patients with a penicillin allergy. Beta-hemolytic *Streptococci, Pneumococci, Staphylococci* and *Enterococci* are usually susceptible to macrolides. Unlike penicillin, macrolides have been shown to be effective against mycoplasma, mycobacteria, some rickettsia, and chlamydia.

In addition, oligomycin is a potent inhibitor of ATP synthase by binding in the F_o sector and blocking proton conduction. Oligomycin, was shown to induce an apoptotic suicide response in cultured human lymphoblastoid and other mammalian cells within 12–18 hrs, but not in ρo cells that are depleted of a functional mitochondrial respiratory chain [82]. A similar study suggested that interaction with components of mitochondrial pathways by oligomycin may lead to apoptosis of select cells, *via* CD14 [83]. Thus, it is quite possible that some degree of inhibition, or other interactions between mitochondrial ATP synthase and polyphenols could occur and play a considerable role in apoptosis *via* mitochondrial pathways [34, 84, 85].

INHIBITORY EFFECTS OF POLYPHENOLS

Polyphenols are naturally occurring plant based phyhtochemicals which possess antioxidant, chemopreventive, and chemotherapeutic properties [84, 86, 87]. Apples, berries, cantaloupe, cherries, grapes, pears, plums, broccoli, cabbages, and onions are rich in polyphenols [88]. A variety of flavonoids or polyphenolic compounds exert a broad range of pharmacological

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effects, including protection of cells or tissues and invoking multiple responses, including cell death, through their actions on a multitude of targets. A large body of experimental data is available on the effects of dietary polyphenolic compounds and their derivates on human health. Some polyphenols are known to block the action of enzymes and other substances that promote the growth of cancer cells [66, 89–93]. Physiological relevance of dietary polyphenols can be ascribed to their interaction with the mitochondria of eukaryotic cells, while degenerative diseases such as cancer, aging, and neurological disorders are attributed to mitochondrial dysfunction [94, 95]. It was shown that known cardiovascular benefits of dietary polyphenols may derive in part from their inactivation of plasminogen activator inhibitor type 1(PAI-1). PAI-1 has been implicated in variety of pathological processes, such as angiogenesis and tumor metastasis [96, 97].

In vitro studies using rat hepatic mitochondria suggested that flavonoid extracts from Algerian plants have some protective effects against oxidative stress by protecting the mitochondria [98]. Resveratrol (trans-3,4',5,-trihydroxy-stilbene), a phytoalexin, is a toxic antimicrobial compound produced by plants under stress conditions, or in response to pathogen infection or parasitic attack. Phytoalexins are broad spectrum inhibitors that are chemically different among plant species. Phytoalexins are toxic to the pathogens and they may puncture cell walls, delay maturation, disrupt metabolism or prevent reproduction. Inhibition of phytoalexin biosynthesis has been found to result in increased susceptibility of plant tissues to infections [99].

Resveratrol has the potential for multiple uses, with multiple benefits in humans, including but not limited to increased life span, anticancer/antitumor effects, and antimicrobial activities [100]. Administration of resveratrol was shown to increase life span of yeast, *Caenorhabditis elegans, Drosophila melanogaster*, and mice [101–104]. Resveratrol was also shown to induce apoptosis *via* mitochondrial pathways [90, 105]. Aziz *et al.* [93] demonstrated that resveratrol has chemopreventive properties against prostate cancer. They found that treatment with resveratrol concentrations of up to 50 μmol/L/day resulted in stimulation of apoptosis in androgen-responsive human prostate carcinoma cells (LNCaP). At similar concentrations resveratrol had no effect on the rate of cell death in normal human prostate cells.

Fig. (2) illustrates some known polyphenol inhibitors of ATP synthase. These polyphenolic compounds have varying degree of inhibitory effects on ATPase activity as well as on *E. coli* cell growth. One of the best sources of natural polyphenols is tea. Tea has been associated with multiple health benefits that are attributed to the presence of polyphenolic compounds. Catechins are polyphenolic antioxidant plant metabolites that constitute ~25% of the dry weight of fresh tea leaves [106]. The term catechin is also commonly used to refer to the related family of flavonoids. Actual catechin content varies depending on the climate where tea is being grown. Catechins are present in practically all teas made from *Camellia sinensis*, including white tea, green tea, black tea and Oolong tea. Catechins are also present in chocolate [107], fruits, vegetables and in many other plant species [108, 109]. Catechin gallates are gallic acid esters of the catechins. Epigallocatechin gallate (EGCG) is the most abundant catechin in tea. Catechin and epicatechin are epimers, with (–)-epicatechin and (+)-catechin being the most common optical isomers found in nature. Epigallocatechin and

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gallocatechin contain an additional phenolic hydroxyl group when compared to epicatechin and catechin, respectively. The health benefits of catechins include reduction in atherosclerotic plaques and reduced carcinogenesis *in vitro* and in animal models [110, 111]. Green tea polyphenols such as EGCG and epicatechin gallate (ECG) were shown to modulate insulin secretion by inhibiting glutamate dehydrogenase activity [112]. EGCG also causes apoptosis of breast cancer cells by blocking *in vitro* Fatty acid synthase (FASN) activity that is over expressed in human breast carcinomas [113].

Mice fed catechins showed decreased levels of aging, lowering of oxidative stress in mitochondria, and an increase in mRNA transcription of mitochondria related proteins [114]. Previous studies indicated concentration dependent effects of epicatechin on biomarkers of oxidative stress in hypertensive and normal patients. The mode of action involves reduction of malondi-aldehyde (MDA) and protein carbonyl content with an increase in glutathione (GSH) and membrane sulfhydryl (–SH) content [115]. Recently we also found that epicatechin is a potent inhibitor of *E. coli* ATP synthase. Both cell growth and ATPase activity were abrogated (IC_{50} ~4.0 mM) [85].

Polyphenolic compounds also have promising antimicrobial activities. For example, quercetin (3,3',4',5,7-pentahy-droxyflavone) and apigenin (4',5,7-trihydroxyflavone) both show antibacterial activity against D-Ala:D-Ala ligase (Ddl) and *E. coli* ATP synthase [34, 116] and [85]. They function as reversible inhibitors of Ddl and compete with its ATP substrate but are non-competitive inhibitors with substrate DAla. Both quercetin and apigenin are reversible inhibitors of *E. coli* ATP synthase and bind non-covalently at the polyphenol binding site. Quercetin was also found to be a more potent inhibitor on molar scale, having higher affinity than apigenin against *Helicobacter pylori* Ddl, *E. coli* Ddl, and *E. coli* ATP synthase [34, 116] and [85].This can be attributed to the two additional hydroxyl groups on the flavone skeleton of the quercetin, thus facilitating its inhibitory and binding activity to Ddl and ATP synthase (see Fig. 2).

It was shown that polyphenols can also inhibit biofilm formation and acid production by *S. mutans*. One of the pathways through which polyphenols are active against *S. mutans* is the inhibition of proton–translocating F_1-ATPase activity [61, 62]. The role of mycobacterial ATP synthase is also of interest as tuberculosis (TB) still claims about 2 million lives worldwide yearly. It is interesting that two mutations, D32A and A63P, in the c-subunit of mycobacterium ATP synthase confer resistance to diarylquinoline, a tuberculosis drug [63, 64]. A wide range of polyphenols have been shown to bind at the distinct polyphenol binding site and inhibit ATP synthase partially or maximally. Fig. (3) shows the polyphenol binding pocket of ATP synthase both in unbound and polyphenol bound form [34].

Among the many polyphenols studied so far, piceatannol is one of the most portent inhibitors of ATP synthase. The polyphenol binding pocket lies at the interface of the α, β, and γ-subunits of F_1 sector. In the polyphenol bound ATP synthase crystal structure the polyphenols piceatannol, resveratrol, or quercetin were found to bind in a slightly distorted planar conformation through H-bonds and hydrophobic interactions [84]. As can be seen in Fig. (3B), piceatannol can form hydrophobic interactions with γGln274 (γLys-260), γThr-277 (γIle-263), βAla-264 (βAla-278), or βVal-265 (βVal-279), and an additional non-

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polar interaction with residues γAla-270 (γAla-256), γThr-273 (γThr-259), γGlu-278 (γGlu-264), αGly-282 (αGly-290), or αGlu-284 (αGlu-292) which are within 4Å of the bound compounds [*E. coli* residue numbers are used throughout. Bovine residue numbers are shown in parentheses]. Overall, the polyphenol binding pocket residues are highly conserved among different species including human, bovine, rat, and *E. coli* [117, 118]. The Polyphenol binding pocket residues of *E. coli* ATP synthase are identical to the bovine polyphenol binding pocket residues, except for two changes, namely γQ274K and γT277I where Gln is replaced by Lys and Thr is replaced by Ile in bovine. Distance measurements using Deep View Swiss-Pdb Viewer, Version 4.01 (http://spdbv.vital-it.ch/) suggested that the –OH group of γThr-277 generates an additional H-bond with the –OH group of γSer-281 [34] and can form additional H-bond with the oxygen or –OH groups of the polyphenol compounds.

Earlier in this review several potentially relevant effects of natural dietary polyphenolic compounds as antimicrobial and antitumor agents were discussed. Many polyphenols (see Fig. 2) have been shown to bind and inhibit ATP synthase, suggesting that the dietary benefits of these compounds are in part linked to the inhibition of ATP synthesis in tumor cells, thereby leading to apoptosis [34, 119]. All polyphenols illustrated in Fig. (2) cause partial or complete inhibition of ATP synthase and Fig. (4) shows the inhibitory effects of three well known polyphenols (piceatannol, quercetin, or resveratrol) on ATP synthase. On molar scale piceatannol was found to be the most potent inhibitor of ATPase activity and caused 100% inhibition (IC_{50}~ 14μM). Quercetin causes ~80% inhibition (IC_{50}~ 33μM) while resveratrol exerted only ~40% inhibition (IC_{50}~ 94μM) [34]. Our results [85] suggest that all other polyphenols illustrated in Fig. (2) exert partial or maximal inhibition of *E. coli* ATP synthase. The inhibitory effects of polyphenols have been found to be reversible in all cases. These polyphenols also inhibit intact *E. coli* cell growth to varied degrees on limiting glucose media [34]. We also found that structural modulation of polyphenols such as resveratrol have the potential to increase inhibitory effects by as much as 100-fold. Our recent unpublished results demonstrate that introduction of an imino group, repositioning of the –OH groups, and or introduction of nitro group enhances inhibitory effects of resveratrol from ~40% to 100% and IC_{50}~ 94μM to IC_{50}~2.25μM.

INHIBITORY EFFECTS OF AMPHIBIAN ANTIMICROBIAL PEPTIDES

Fig. (5) shows the x-ray crystallographic structure of βDELSEED-loop (residue numbers β380–386) of ATP synthase along with two cationic α-helical peptides maganin II and dermaseptin. Indirect experimental evidence on protection against the inhibition of quinacrine mustard by melittin, the α-helical 26-residue cationic peptide from honey bee venom (*Apis mellifera*), suggested a common βDELSEED binding site. Several peptides form basic amphiphilic α-helical structures that have been shown to bind at the βDELSEED-loop of *E. coli* ATP synthase. Peptides such as bacterial/chloroplast ATP synthase ε-subunit, melittin, and the synthetic derivative of cytochrome oxidase SynA2 are known inhibitors of ATP synthase [120–122].

Previous studies indicated that during conditions of high gradients and low ATP concentrations, the C-terminal α-helical domain of the ε-subunit of F_1-ATPase undergoes

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large conformational changes and interacts with the $\alpha_3\beta_3$ hexagon ring, where it then comes in close proximity to the βDELSEED-loop. Electrostatic interactions between basic residues of the ε-subunit and the acidic residues of βDELSEED-motif cause inhibition of ATPase activity [123–125]. Another natural regulatory peptide known to affect ATP synthase activity is IF_1 of the mitochondria [126, 127]. IF_1 is a natural regulatory peptide of between 56–87 residues in length that inhibits ATPase activity of ATP synthase in a reversible and noncompetitive fashion [128]. Crystal structure of IF_1 with the F_1- subunit shows the N-terminal domain of IF_1 bound at the interface of the α and βF_1 subunits [129].

Melittin is an α-helical basic peptide also known to have inhibitory effects on the ATPase activity of F_1-ATP synthase [35, 120, 121]. The peptide is composed of 26 residues and is the primary component of honey bee venom (*A. mellifera*). Melittin is a potent inhibitor of both *E. coli* and bovine ATP synthase (IC50~5μM) [35, 121]. The inhibitory activity of melittin is suggested to be similar to that of other known α-helical peptide inhibitors, IF_1, Wild–type yeast cytochrome oxidase, and synthetic Syn-A2. All the above inhibitors are known to act in a reversible and noncompetitive fashion [120, 121, 130–133].

Defenses against pathogens include a wide variety of systems in both plants and animals. Among these defenses are various types of oligopeptides and peptides [134]. Antimicrobial peptides (AMPs) are a component of vertebrate innate immunity that has been present in most living organisms for over 2.6 billion years [135]. AMPs were first described in insects as an inducible system of protection against bacterial infection [136–138]. Most recently Laughlin and Ahmad [35] showed that several cationic α-helical amphibian AMPs have reversible inhibitory effects on the ATPase activity of *E. coli* ATP synthase. AMPs are generally cationic and amphipathic molecules that are less than 50 amino acids in length. They have been isolated from all investigated phyla, including microbes, plants, invertebrates and vertebrates. AMPs show potent activity against Gram-positive, Gram-negative bacteria, fungi, parasites, and enveloped viruses [135]. It is noteworthy that several amphibian AMPs are also known to have selective anticancer activity [139]. AMPs are also characterized for having multiple functions with unclear modes of actions. Additionally, AMPs have a neutralizing effect on bacterial endotoxins that are a primary cause of lethality in sepsis [140–142].

Presently there are 1504 entries in the Antimicrobial Database [143], (http://aps.unmc.edu/AP/main.php), of which 1154 (77%) are identified as having antibacterial activity, 438 with antifungal activity, 94 with anticancer activity and 86 with antiviral activity. Amphibian skin is the single largest source of AMPs identified in the database, being 36.4% (N=548) of all listed AMPs. Of all animal AMPs (N=1046) 52.4% are derived from amphibians. The first amphibian AMPs identified were the magainins from skin secretions of the frog *Xenopus laevis* [144]. Currently known amphibian AMPs in the database were derived from the European toad in the family Bufonidae, South American tree frog species of the family Hylidae and species of frogs in the family Ranidae in Europe, North America or South America [140, 145]. Frogs skin secretions contain a variety of AMPs with up to 100 unique amino acid sequences per species[146]. Based on structural similarity and species of origin there are four identified classes of amphibian AMPs: 1) maginins from *Xenopus*, 2) dermaseptins from species in the genus *Phyllomedusa*, 3)

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bombinins and bombinin H from European toads, and 4) temporins, brevinins, esculentins, ranalexins and ranauerins from species in the genus *Rana* [140].

The mean length of all peptides in the AMP database is 29.96 residues, and the mean net charge is +3.81. Identification of secondary structure among database AMPs resulted in 14.89% (N=224) as α-helical, 2.92% (N=44) as β-conformation, and 2.32% (N=35) as α+β. Whereas 24.46% (N=368) were found to have disulfide bonds and 5.85% (N=88) were rich in unusual amino acids [143].

Most of the anuran produced AMPs are cationic, between 10 and 50 residues in length, and frequently include a C-terminal amide group. Mode of action studies indicate that AMPs appear to interact with negatively charged phospholipids and then insert into the bacterial cell membrane, or they may also move across the cell membrane by passive transport and there disrupt a number of cellular processes. AMPs are associated with a number of other antimicrobial processes as well, including cell proliferation and angiogenesis [135]. Several mechanisms have been hypothesized regarding the activity of AMPs, including membrane permeabilization and cell death by either a "barrel-stave" model [147] or a "torodial pore" model proposed for magainins from *Xenopus* skin [148–150]. Dermaseptins appear to cause a non-pore-dependent cytolytic activity that causes membrane bilayer miscellization and disintegration [151]. This leads to the question of whether or not some of the antimicrobial effects of amphibian AMPs could be through their inhibitory actions on ATP synthase.

Following the discovery of AMPs in insects, biochemically active substances in frog skin were identified as bioactive peptides [144, 152, 153]. Frogs and toads secrete AMPs from granular glands of the skin, typically in response to infection or environmental stress [154] and at concentrations as high as mg/g of wet skin [153]. AMPs have become potential sources of compounds with useful pharmacological properties and medical utility in antimicrobial [155–157] and anticancer [158] applications. One probable drawback of the usefulness of these molecules is that they seem to be effective only at very high doses [159]. This may be related to the observed high concentrations *in vivo*, but may possibly be improved for pharmacological purposes by modifications of amino acid sequences or functional groups, based on molecular modeling studies, as has been observed recently with polyphenolic compounds [P. Dadi and Z. Ahmad unpublished results]. Synergistic effects with AMPs among different dermaseptins have also been observed, which may be related to the large number of different isoforms found within different species [160]. This suggests that evaluation of AMPs potential activity as ATP synthase inhibitory molecules may also be enhanced by combinatorial studies.

The amphibian AMPs with potential inhibitory effects on ATP synthase through binding at the βDELSEED-loop are expected to be relatively short cationic peptides of approximately 10–30 amino acid residues, with α-helical secondary structure, and having previously identified anti-bacterial or anti-cancer effects. Table 1 identifies a list of sixty candidate amphibian AMPs selected from the AMP database based on an α-helical secondary structure, a net positive charge, a total length of 10–30 residues, and identified antibacterial or anti-cancer activity. We very recently tested fifteen peptides from this candidate list for ATP synthase inhibitory activity [35] based on the previously identified ATP synthase

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inhibition by the melittin peptide [35, 120, 121]. As shown in Fig. (6) we found that MRP and MRP-amide strongly inhibited the ATPase activity of ATP synthase. However, maginin II, maganin II-amide, or caerin 1.9 only partially inhibited ATPase activity. Other peptides exerting partial inhibition of *E. coli* ATP synthase but not shown in Fig. (6) were ascaphin-8, aurein 2.2, aurein 2.3, citropin 1.1, and maculatin 1.1. Presence of an amide group at the C-terminal end of MRP and magainin II caused an additional ~20–40% inhibition. All the above amphibian AMPs had varying degrees of effect on *E. coli* cell growth. Ascaphin-8, aurein 2.2, aurein 2.3, caerin 1.9, citropin 1.1, dermaseptin, magaininII-NH_2, MRP, or MRP-NH_2 resulted in significant inhibition of cell growth, indicative of complete abrogation of ATP synthesis [35].

It seems probable that there will be variable results in terms of ATP synthase and cell growth inhibition among different dietary polyphenols and amphibian AMPs, depending on the target molecules and organisms. We also expect that various modifications of naturally occurring dietary polyphenols and amphibian AMPs may be used to modulate their effectiveness on a molar scale with regard to both ATP synthase inhibition and cytotoxicity. By virtue of the great variability in the structures and potential functions exhibited by both dietary polyphenols and amphibian AMPs, these relatively simple molecules may constitute a formidable natural resource of new drug compounds that may be targeted at microorganisms and neoplasms through their inhibitory actions on ATP synthase.

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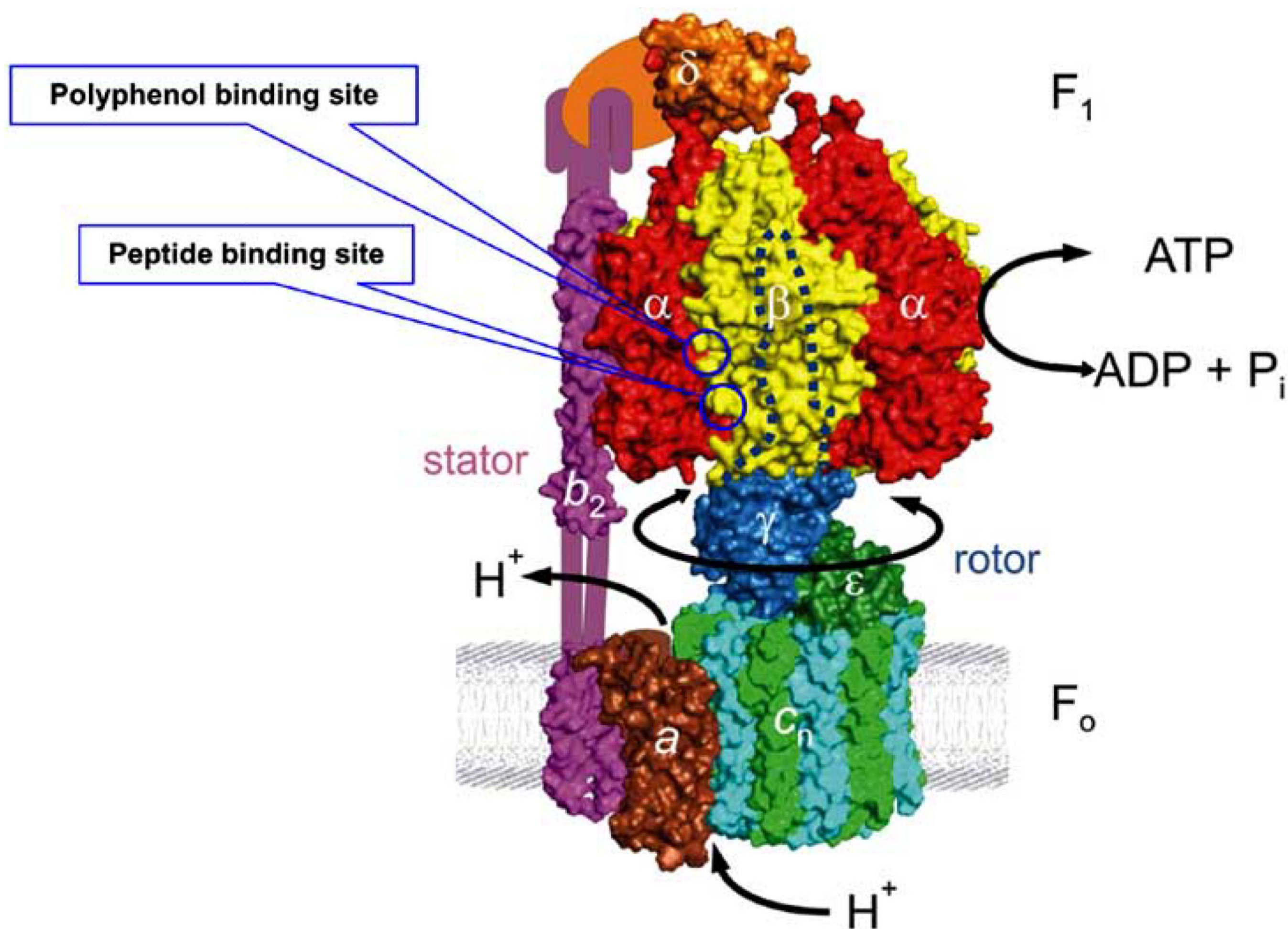

Fig 1. Structure of *Escherichia coli* ATP synthase

In its simplest form in *E. coli* this enzyme is composed of two sectors, water soluble F_1 and membrane bound F_o. Catalytic activity occurs at the interface of α/β subunits of F_1 sector which consists of five subunits ($\alpha_3\beta_3\gamma\delta\varepsilon$) and proton conduction occurs at the F_o sector consisting of three subunits (ab_2c). In mitochondria and chloroplasts additional subunits are present. Polyphenol and peptide binding sites are identified with circles at the interface of α/β subunits. This model of *E. coli* ATP synthase is reproduced from Weber [11] with permission; copyright Elsevier.

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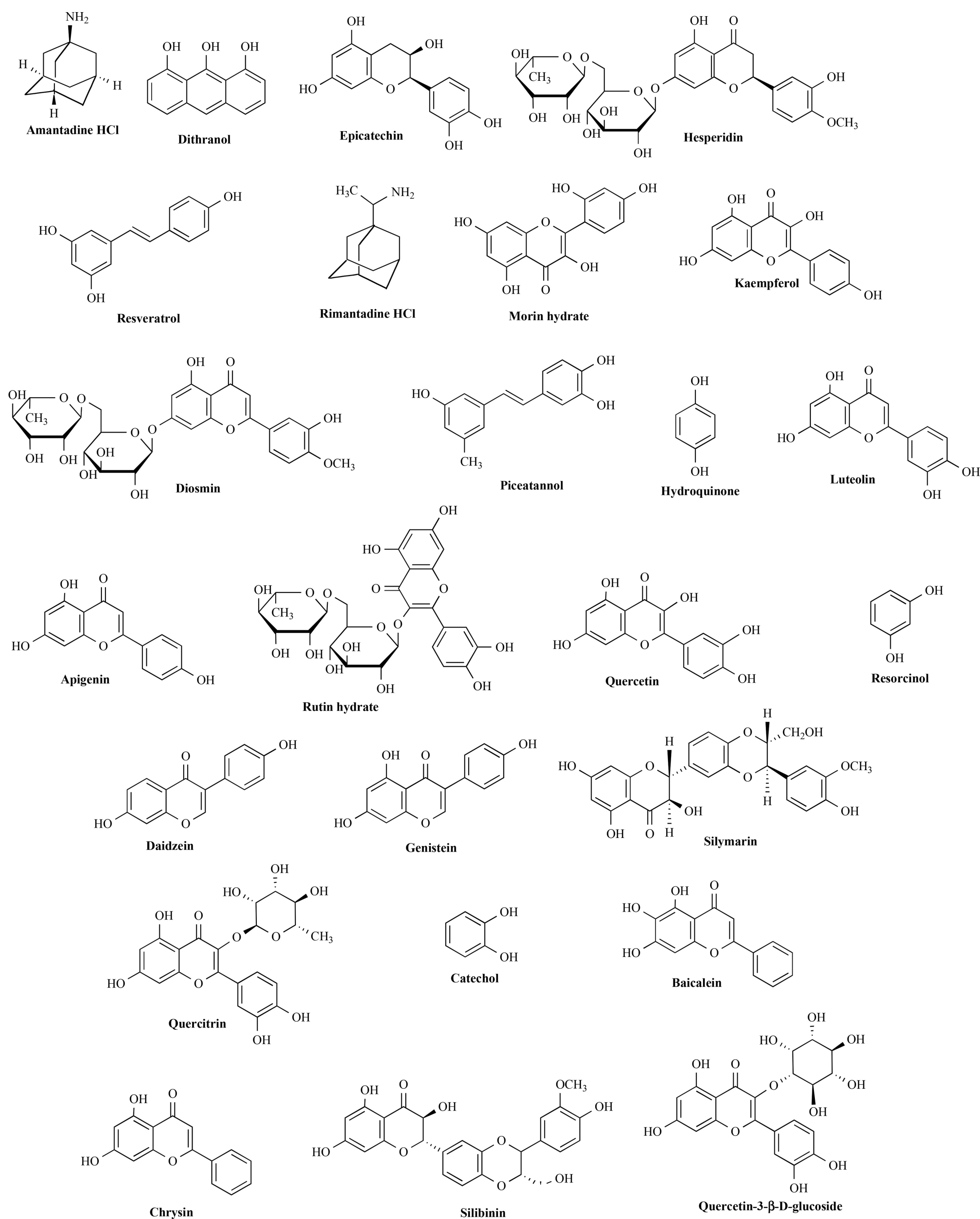

Fig 2. Structure of polyphenolic compounds

All polyphenolic compound indentified here are known to bind and inhibit ATP synthase.

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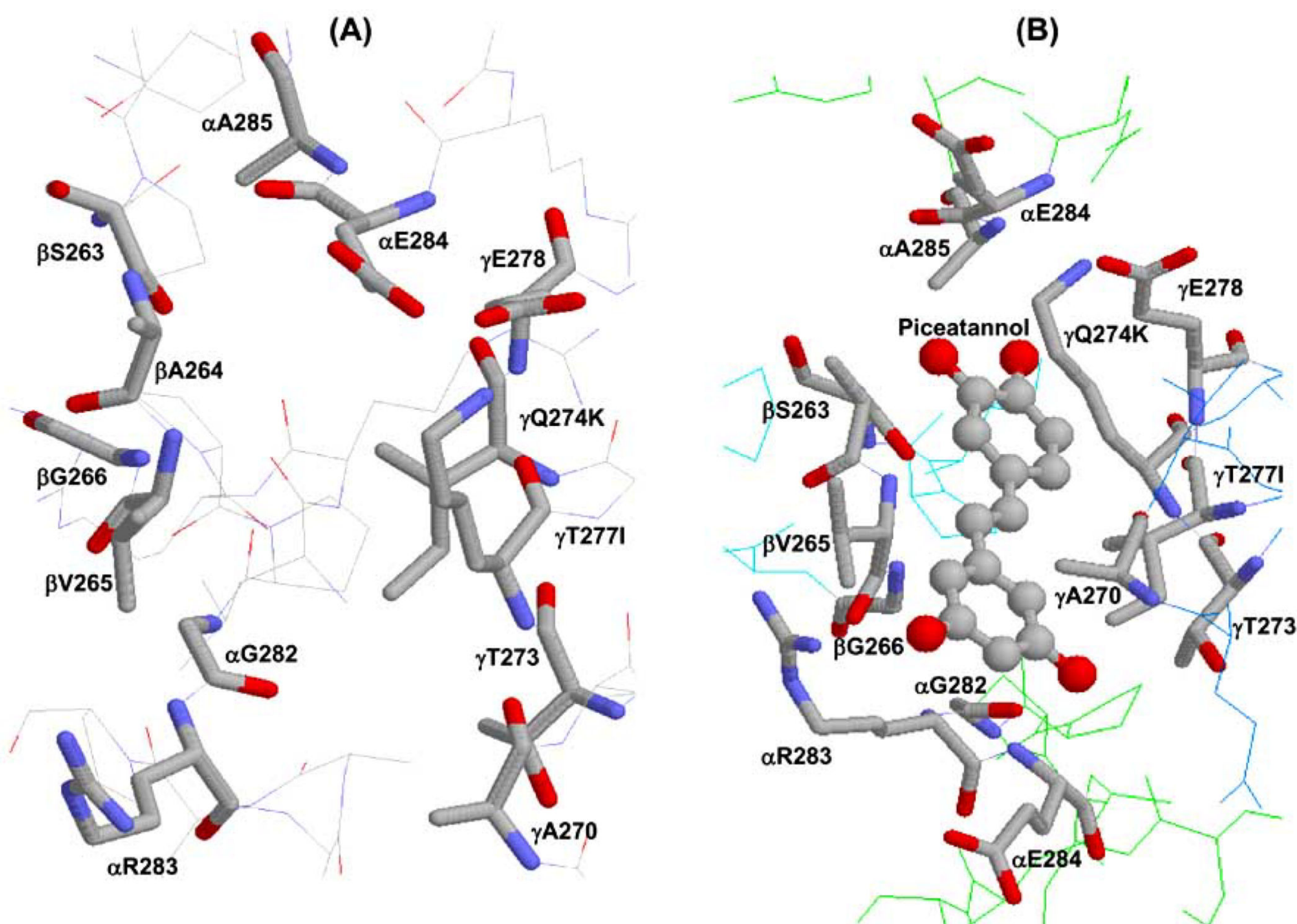

Fig 3. X-ray crystallographic structure of polyphenol binding site of ATP synthase
(**A**) Empty and (**B**) piceatannol bound polyphenol binding pocket. Residues from α, β, and γ subunits involved in interaction with polyphenols are identified. In bovine two variants, Q274K and T277I, occur in the γ subunit and are identified in the figure. PDB file 2jj 1 [84] with RasMol [161] was used to generate this figure.

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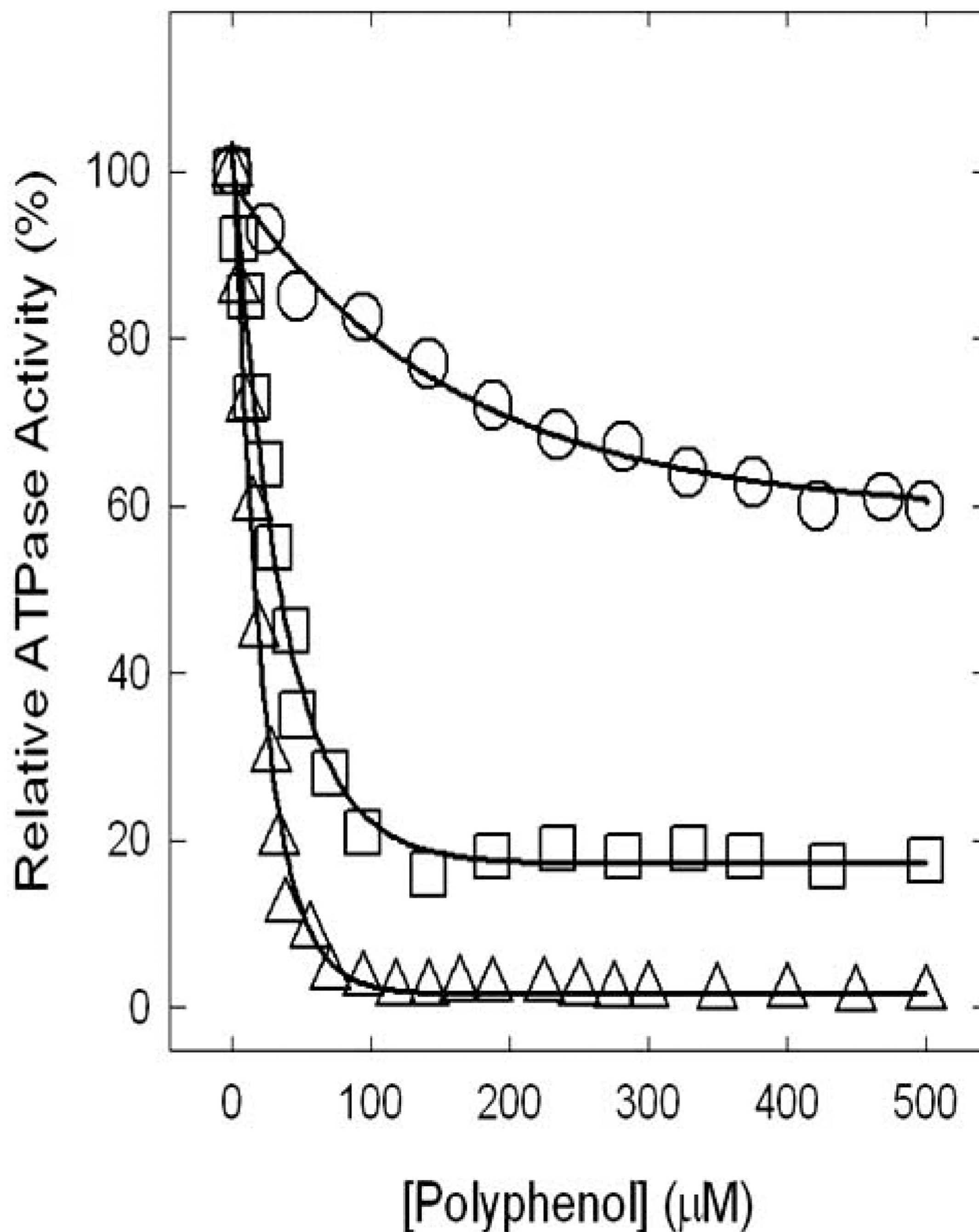

Fig 4. Inhibitory effects of polyphenols on ATP synthase

Inhibition profiles induced by resveratrol (o), quercetin (□), and piceatannol ( ) resulting in partial or complete inhibition of ATP synthase are shown. For experimental details see Dadi *et al.* [34].

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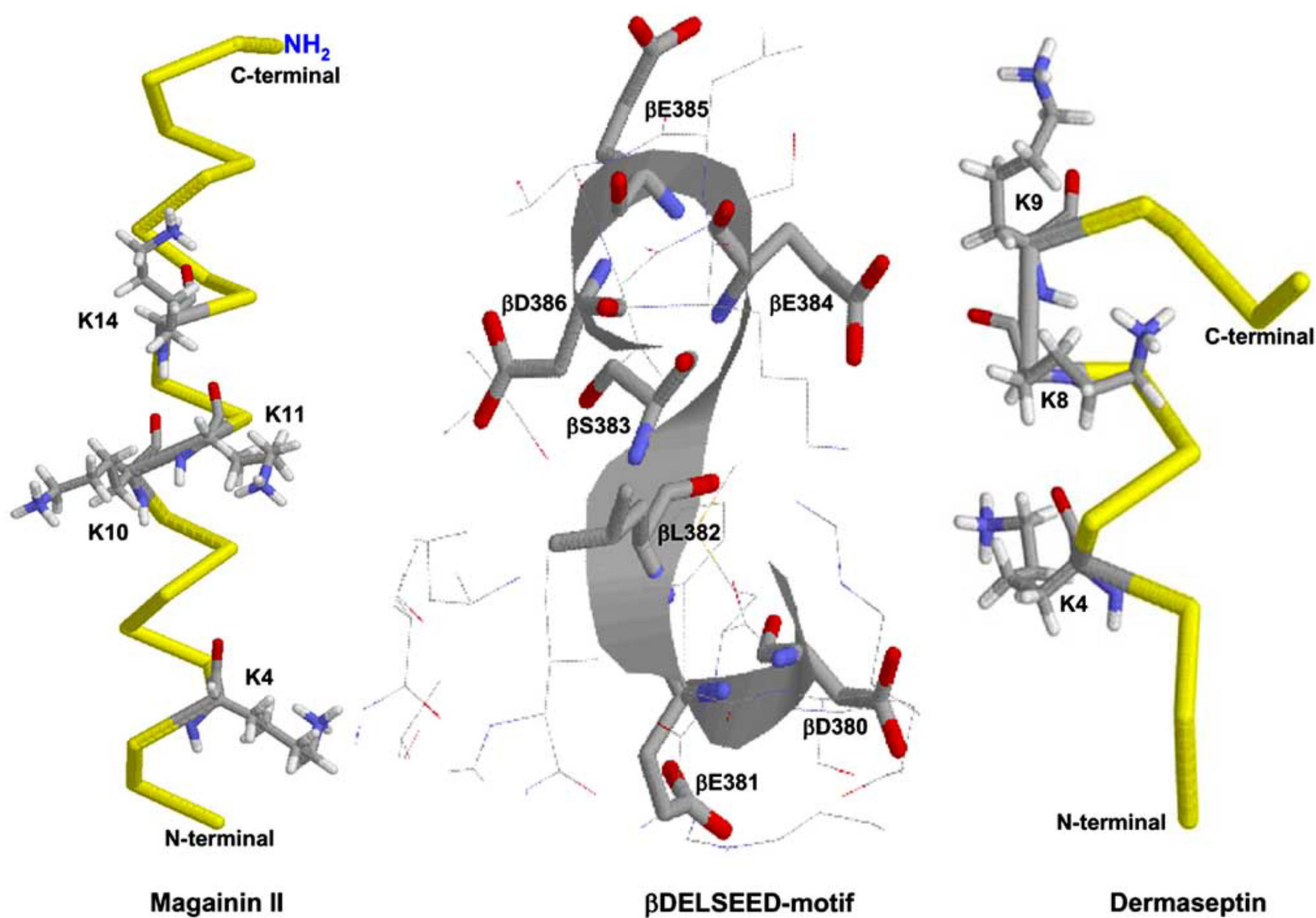

Fig 5. X-ray crystallographic structure of proposed peptide binding βDELSEED-loop of ATP synthase with amphibian AMPs magainin II and dermaseptin

Important residue side chains involved in the electrostatic interaction between βDELSEED-loop and peptides are illustrated. PDB files used were 2MAG [162], 2DCX [163], and 1H8E [24]. RasMol [161] was used to generate this figure.

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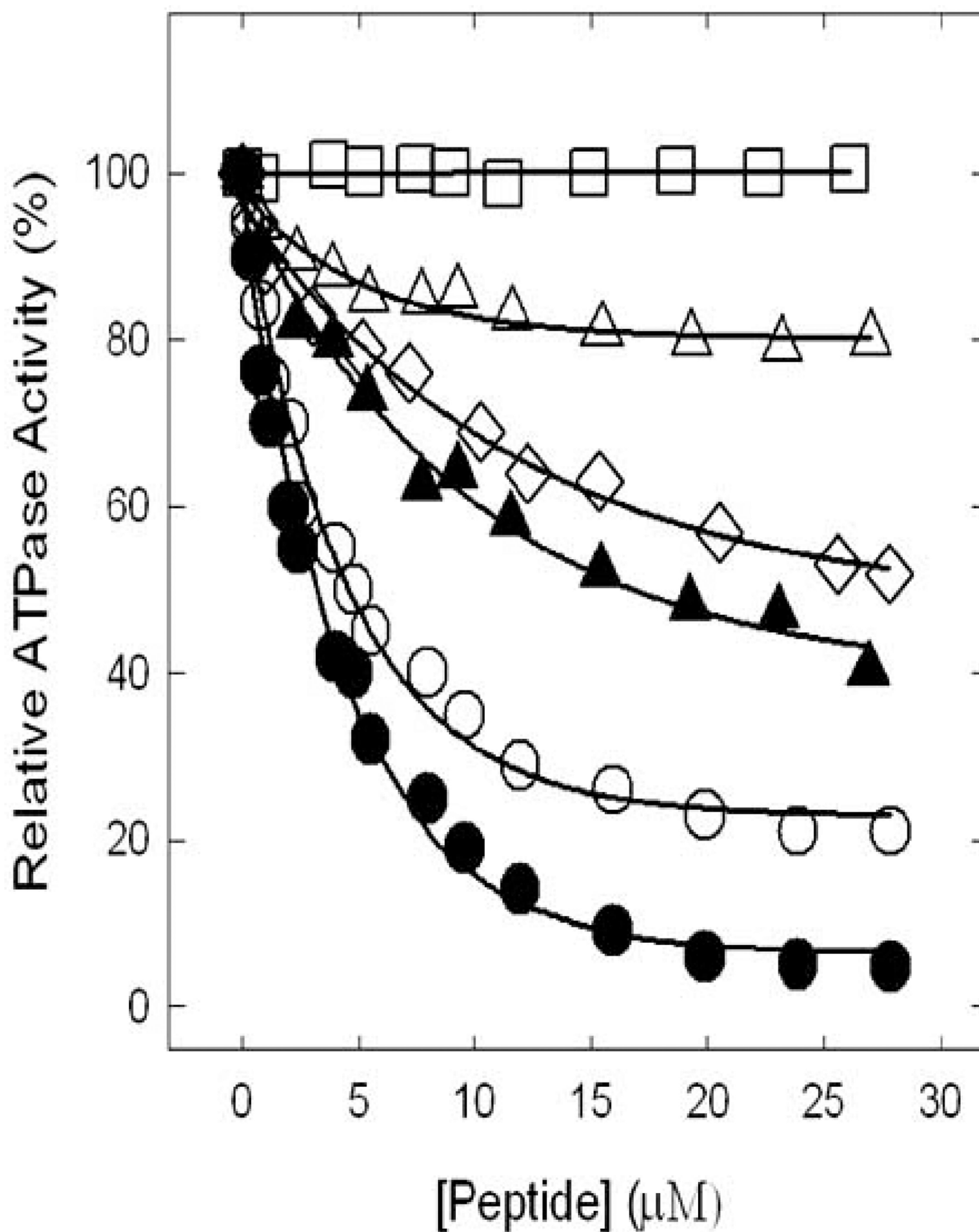

Fig. 6. Inhibitory effects of amphibian AMPs on ATP synthase

Inhibition profiles induced by MRP (o), MRP-NH_2 (●), maginin II ( ), maginin II-NH_2 (▲), caerin 1.8 (□), and caerin 1.9 ( ) resulting in partial or complete inhibition of ATP synthase are shown. For experimental details see Laughlin and Ahmad [35].

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Table 1

Amphibian Cationic, Alpha-Helical, Antimicrobial/Anticancer Peptides

Amphibian peptides	Sequence	Length	Charge	Species of origin
Ascaphin-8 [a], [164]	GFKDLLKGAAKALVKTVLF	19	3	*Ascaphus truei*
Aurein 2.2 [a], [165]	GLFDIVKKVVGALGSL-NH_2	16	1	*Littoria aurea, L. raniformis*
Aurein 2.3 [a], [165]	GLFDIVKKVVGAIGSL-NH_2	16	1	*Littoria aurea*
Bombinin H2[b], [166]	IIGPVLGLVGSALGGLLKKI	20	2	*Bombina variegata*
Bombinin H4[b], [166]	LIGPVLGLVGSALGGLLKKI	21	2	*Bombina. variegata*
Bombinin-like pep3[b], [167]	GIGAAILSAGKSALKGLAKGLAEHF	25	3	*Bombina orientalis*
Bombinin-like pep4[b], [167]	GIGAAILSAGKSIIKGLANGLAEHF	25	2	*Bombina orientalis*
Buforin II [a], [168]	TRSSRAGLQFPVGRVHRLLRK	31	7	*Bufo bufo gargarizans*
Caerin 1.1 [a], [169]	GLLSVLGSVAKHVLPHVVPVIAEHL	25	3	*Littoria splendida*
Caerin 1.2 [b], [170]	GLLGVLGSVAKHVLPHVVPVIAEHL	25	3	*Litoria caerula*
Caerin 1.3 [a], [170]	GLLSVLGSVAQHVLPHVVPVIAEHL	25	2	*Littoria caerula*
Caerin 1.4 [b], [170]	GLLSSLSSVAKHVLPHVVPVIAEHL	25	3	*Litoria caerula*
Caerin 1.7 [a], [171]	GLFKVLGSVAKHLLPHVAPVIAEK	24	4	*Littoria chloris*
Caerin 1.8 [a], [171]	GLFKVLGSVAKHLLPHVVPVIAEK	24	4	*Littoria chloris*
Caerin 1.9 [b], [171]	GLFGVLGSIAKHVLPHVVPVIAEK	24	3	*Littoria chloris*
Citropin 1.1[a], [172]	GLFDVIKKVASVIGGL	16	1	*Littoria citropa*
D-1CDYa [b], [173]	IIPLPLGYFAKKT	13	2	*Rana chensinensis*
Dermaseptin-B4[b], [174]	ALWKDILKNVGKAAGKAVLNTVTDMVNQ	28	2	*Phyllomedusa bicolor*
Dermaseptin-B5[b], [174]	GLWNKIKEAASKAAGKAALGFVNEMV	26	2	*Phyllomedusa bicolor*
Dermaseptin-B9[b], [174]	ALWKTIIKGAGKMIGSLAKNLLGSQAQPES	30	3	*Phyllomedusa bicolor*
Dermaseptin S1[a], [175]	ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ	34	4	*Phyllomedusa sauvagei*
Dermaseptin-S3[b], [175]	ALWKNMLKGIGKLAGKAALGAVKKLVGAES	30	5	*Phyllomedusa sauvagei*
Dermaseptin-S4[b], [175]	ALWMTLLKKVLKAAAKALNAVLVGANA	27	4	*Phyllomedusa sauvagei*
Dermaseptin-S5[b], [175]	GLWSKIKTAGKSVAKAAAKAAVKAVTNAV	29	6	*Phyllomedusa sauvagei*
Dermaseptin-S9[b], [176]	GLRSKIWLWVLLMIWQESNKFKKM	24	4	*Phyllomedusa sauvagei*
Distinctin[b], [177]	NLVSGLIEARKYLEQLHRKLKNCKV	25	5	*Phyllomedusa disticta*
Fallaxidin 4.1[b], [178]	GLLSFLPKVIGVIGHLIHPPS	21	3	*Litoria fallax*
Frenatin 3[b], [179]	GLMSVLGHAVGNVLGGLFKS	20	2	*Litoria infrafrenata*
Hylaseptin P1[b], [180]	GILDAIKAIAKAAG	14	1	*Hyla punctata*
Hylin a1[b], [181]	IFGAILPLALGALKNLIK-NH_2	18	2	*Hypsiboas albopunctatus*
Japonicin-CDYa[b], [173]	FFPLALLCKVFKKC	14	3	*Rana chensinensis*

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Amphibian peptides	Sequence	Length	Charge	Species of origin
Kassinatuerin-1[b], [182]	GFMKYIGPLIPHAVKAISDLI- NH_2	21	2	*Kassina senegalensi*
Maculatin 1.1 [a], [183]	GLFVGVLAKVAAHVVPAIAEHF- NH_2	22	2	*Litorria genimaculate*
Magainin II [a], [144]	GIGKFLHSAKKFGKAFVGEIMNS	23	4	*Xenopus laevis*
Magainin II amide [a], [144]	GIGKFLHSAKKFGKAFVGEIMNS-NH_2	23	4	*Xenopus laevis*
MRP [b], [184]	AIGSILGALAKGLPTLISWIKNR	23	3	*Rana tagoi*
MRP amide [b], [184]	AIGSILGALAKGLPTLISWIKNR-NH_2	23	3	*Rana tagoi*
Nigrocin 2[b], [185]	GLLSKVLGVGKKVLCGVSGLC	21	3	*Rana nigromaculata*
Ocellatin-F1[b], [186]	GVVDILKGAAKDIAGHLASKVMNKL	25	3	*Leptodactylus fallax*
Odorranain-B1[b], [187]	AALKGCWTKSIPPKPCFGKR	20	5	*Odorrana grahami*
Odorranain-F1[b], [187]	GFMDTAKNVAKNVAVTLIDNLKCKITKAC	29	3	*Odorrana grahami*
Odorranain-G1[b], [187]	FMPILSCSRFKRC	13	2	*Odorrana grahami*
Odorranain-H1[b], [187]	GIFGKILGVGKKVLCGLSGWC	21	3	*Odorrana grahami*
Odorranain-T1[b], [187]	TSRCYIGYRRKVVCS	15	4	*Odorrana grahami*
PGLa[b], [188]	GMASKAGAIAGKIAKVALKAL	21	4	*Xenopus laevis*
Phylloseptin-H1[b], [189]	FLSLIPHAINAVSAIAKHN	19	3	*Phyllomedusa hypochondrialis*
Phylloseptin-H2[b], [189]	FLSLIPHAINAVSTLVHHF- NH_2	19	3	*Phyllomedusa hypochondrialis*
Phylloseptin-H3[b], [189]	FLSLIPHAINAVSALANHG- NH_2	19	2	*Phyllomedusa hypochondrialis*
Phylloxin[b], [190]	GWMSKIASGIGTFLSGMQQ	19	1	*Phyllomedusa bicolor*
Pseudin 1[b], [191]	GLNTLKKVFQGLHEAIKLINNHVQ	24	4	*Pseudis paradoxa*
Pseudin 3[b], [191]	GINTLKKVIQGLHEVIKLVSNHE	23	3	*Pseudis paradoxa*
Pseudin 4[b], [191]	GINTLKKVIQGLHEVIKLVSNHA	23	4	*Pseudis paradoxa*
Ranalexin[b], [192]	FLGGLIKIVPAMICAVTKKC	20	3	*Rana catesbeiana*
Temporin A[b], [193]	FLPLIGRVLSGIL	13	1	*Rana temporaria*
Temporin B[b], [193]	LLPIVGNLLKSLL-NH_2	13	1	*Rana temporaria*
Temporin L[b], [193]	FVQWFSKFLGRIL	13	2	*Rana temporaria*
Temporin-SHa[b], [194]	FLSGIVGMLGKLF	13	1	*Pelophylax saharica*
Temporin-SHc[b], [194]	FLSHIAGFLSNLF	13	1	*Pelophylax saharica*
Uperin 3.6[b], [195]	GVIDAAKKVVNVLKNLF-NH_2	17	2	*Uperoleia mjobergii*
XT-7 [a], [196]	GLLGPLLKIAAKVGSNLL	18	2	*Xenopus tropicalis*

[a]Peptides known for both antibacterial and anticancer activity.

[b]Peptides known for antibacterial activity only.