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ATP Synthase: A Molecular Therapeutic Drug Target for Antimicrobial and Antitumor Peptides

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Abstract

In this review we discuss the role of ATP synthase as a molecular drug target for natural and synthetic antimi-crobial/antitumor peptides. We start with an introduction of the universal nature of the ATP synthase enzyme and its role as a biological nanomotor. Significant structural features required for catalytic activity and motor functions of ATP synthase are described. Relevant details regarding the presence of ATP synthase on the surface of several animal cell types, where it is associated with multiple cellular processes making it a potential drug target with respect to antimicrobial peptides and other inhibitors such as dietary polyphenols, is also reviewed. ATP synthase is known to have about twelve discrete inhibitor binding sites including peptides and other inhibitors located at the interface of α/β subunits on the F_1 sector of the enzyme. Molecular interaction of peptides at the β DEELSEED site on ATP synthase is discussed with specific examples. An inhibitory effect of other natural/synthetic inhibitors on ATP is highlighted to explore the therapeutic roles played by peptides and other inhibitors. Lastly, the effect of peptides on the inhibition of the *Escherichia coli* model system through their action on ATP synthase is presented.

Keywords

F_1F_o ATP synthase; ATPase; *E. coli* ATP synthase; antimicrobial peptides; antitumor peptides; enzyme inhibitors

1. INTRODUCTION

ATP synthase is the primary means of cellular energy production in all animals, plants, and almost all microorganisms. ATP, the universal energy currency, is generated by ATP synthase by oxidative or photophosphorylation in the membranes of bacteria, mitochondria, and chloroplasts. The overall reaction sequence is: ATP synthase + ADP + Pi ↔ ATP Synthase + ATP. ATP generation requires a mechanical rotation mechanism in which ATP

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CONFLICT OF INTEREST

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synthase subunits rotate at approximately 100 times per second in order to convert food into energy by oxidation. ATP synthase works like a motor and is indeed one of the smallest biological nanomotors found in all livings systems. An average human leading a normal life is expected to generate approximately 2.0 million kg of ATP from ADP and Pi (inorganic phosphate) in a 75-year lifetime. [1–3]. The structural and functional activity of ATP synthase enzymes are essentially the same in all prokaryotes and eukaryotes [4–8]. The total number of protons required to synthesize one ATP molecule among different organisms ranges from three to four, with the possibility that cells can vary this ratio to suit their physiological and environmental conditions [9–11].

2. ATP SYNTHASE ENZYMES

All the metabolic and physiological processes performed by living organisms require energy. This source of energy is adenosine triphosphate (ATP). Consequently, ATP is the universal energy currency used by all cells from bacteria to human. The third phosphate bond of ATP is extremely unstable and its hydrolysis releases a significant amount of free energy (~7kcal/mol). The continuous use of ATP in a multitude of functions means every cell must generate ATP on a constant basis.

ATP synthase is one of the oldest and most highly conserved enzymes. Consequently, ATP synthases, from the inner membrane of mitochondria and chloroplast thylakoid membranes, show identical structural and functional properties to their counterparts from the plasma membrane of bacteria. ATP synthase (EC 3.6.3.14) is a general term for an enzyme that can synthesize adenosine triphosphate (ATP) from adenosine diphosphate and inorganic phosphate. ATP synthase molecules are membrane-bound transporters that couple ion movement through a membrane with the synthesis or hydrolysis of an ATP nucleotide. A variety of membrane- bound ATP synthases evolved to fulfill the explicit needs of different cell types. Based on the particular function, these enzymes are categorized as F-, V-, A-, P-, or E-type ATP synthase [12–15]. Synthesis and hydrolysis of ATP is the sole function of all these forms of ATP synthases.

Before discussing the detailed structure of F_1F_o ATP synthase (see Fig. 1A), it would be appropriate to briefly describe other types of ATPases. The F-type ATP synthase (for ‘phosphorylation Factor’, and also known as H^+-transporting ATPases or F_1F_o-ATPases) are extraordinarily conserved among organisms and are the principal enzymes performing ATP synthesis in living systems. They are located in the plasma membranes of bacteria, in the thylakoid membranes of chloroplasts, and in the inner membranes of mitochondria. In certain bacteria, Na^+-transporting F-ATP synthase is also present. The V-type ATP synthase (for ‘Vacuole’) is found in the eukaryotic endomembrane systems, e.g. in vacuoles, the Golgi apparatus, endosomes, lysosomes, and in the plasma membrane of prokaryotes and certain specialized eukaryotic cells. V-ATPases hydrolyze ATP to drive a proton pump, but cannot work in reverse to synthesize ATP [16, 17]. The A-type ATP synthases (A-ATPases, for ‘Archaea’) are found solely in Archaea and have a similar function to F-ATPases (reversible ATPases). A-type ATPases may have arisen as an adaptation to different cellular needs and the more extreme environmental conditions faced by Archaeal species. The P-type ATP synthases (P-ATPases, also known as E1-E2 ATPases) are found in bacteria and

in a number of eukaryotic plasma membranes and organelles. P-ATPases function to transport a variety of different compounds, including ions and phospholipids, across a membrane using ATP hydrolysis for energy. There are many different classes of P-ATPases, each of which transport a specific type of ion: H^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Ag^+ and Ag^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Cu^+ and Cu^{2+}. The E-type ATP synthases (E-ATPases, for 'Extracellular') are membrane-bound cell surface enzymes that have broad substrate specificity, hydrolyzing other NTPs besides ATP, as well as NDPs – although their most likely substrates are ATP, ADP, and UTP, as well as extracellular ATP [18–22].

The F_1F_o ATP synthase has a long research history and is the prime focus of this review. The F_1 particle was first isolated by Ephraim Racker in 1961 (for factor 1). The name F_o comes from the oligomycin inhibition of the membrane-embedded portion of ATP synthase. Fundamentally, F_1F_o-ATP synthase is structurally and functionally similar whatever the source. In its simplest form, as shown in Fig. (1A), *Escherichia coli* ATP synthase contains eight different subunits, namely $\alpha_3\beta_3\gamma\varepsilon ab_2c_{10}$. The total molecular mass is ~530 kDa. F_1 corresponds to $\alpha_3\beta_3\gamma\delta\varepsilon$ and F_o to ab_2c. The yeast ATP synthase is one of the most complex known enzymes with ~20 different subunit types [7, 8, 23]. In plants ATP synthase is also present in chloroplasts (CF_oF_1-ATP synthase). The enzyme is integrated into the thylakoid membrane where; the CF_1-part inserts into the stroma, and is integral to the dark reactions of photosynthesis (Calvin cycle) and ATP synthesis. 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 [24–26]. 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 extends 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 $\alpha_3\beta_3\delta ab_2$ [27–29]. 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 [1–3, 29–42].

3. FORMATION OF ATP THE ENERGY CURRENCY

A total of six nucleotide binding sites exist on the F_1 sector of ATP synthase. The three that are catalytic are mainly contributed by the β-subunit, and the three that are non-catalytic are mainly contributed by α-subunits. The three catalytic sites are designated as βTP, PDP, and βE by x-ray crystallographers based on the binding of ATP, ADP, and Pi respectively [43, 44]. Pi initially binds to the βE (the empty site) for ATP synthesis. The synthesis reaction in the three catalytic sites is interdependent and occurs successively. The three catalytic sites are known to have different affinities for nucleotides at any given moment. Each catalytic site undergoes a conformational change that results in the following sequence: substrate (ADP+Pi) binding → ATP synthesis → ATP release. Boyer [4, 45, 46] predicted that catalysis requires the sequential involvement of three catalytic sites, each of which changes its binding affinity for substrate and product as it proceeds through the cyclical mechanism. Boyer named this sequence the "binding change mechanism." In F_o, a proton motive force is

converted to a mechanical rotation of the rotor shaft, which drives conformational changes of the catalytic domains in F_1 to synthesize ATP. The reverse reaction hydrolysis of ATP induces reverse conformational changes of the F_o sector and consequently reverses rotation of the rotor shaft. These conformational changes in the catalytic sites are linked to rotation of the γ-subunit. Yoshida and Kinosita, with colleagues in Japan, and subsequently by several other labs [10, 47–51], have observed the γ-subunit rotation in an isolated $\alpha_3\beta_3\gamma$ subcomplex. The catalytic function of ATP synthase with respect to ATP hydrolysis or synthesis in F_1F_o and its relationship to the mechanical rotation of γ-subunit is not the focus of this review. However, a better understanding of the structure and function of F_1F_o would illuminate the possible pathways to developing ATP synthase as a molecular drug target and its use in nanotechnology and nanomedicine [52–55]. Hence, understanding the structure and catalytic function of ATP synthase, particularly Pi binding leading to ATP formation, is of paramount importance to embarking on the details of its inhibition by peptides [2, 3, 56–59].

4. SIGNIFICANCE OF INORGANIC PHOSPHATE (Pi) BINDING

Understanding Pi binding can reveal a wealth of information on the reaction mechanism of ATP synthesis, hydrolysis, and the γ-subunit rotation induced conformational changes of αβ-subunits. These relationships are appreciated from the following two central questions. (I) What causes the ATP synthase to bind ADP and Pi rather than ATP at catalytic sites? The interesting fact is that in active cells, the cytoplasmic concentrations of ATP and Pi are approximately in the 2–5 mM range, whereas the ADP concentration is at least 10–50-fold lower. However, it has been established from the equilibrium binding assays that both ADP and ATP bind to catalytic sites of purified F_1 and detergent solubilized F_1F_o with nearly comparable binding affinities [60–63]. Apparently, a specific mechanism favors the selective binding of ADP into catalytic sites while simultaneously obstructing 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 stereochemically preventing ATP binding and resulting in ADP binding [30]. (II) How does subunit rotation affect the Pi binding [45, 64, 65]? Theoretical and experimental evidence suggests that Pi binding appears to be “energy linked”, implying that it is linked directly to subunit rotation [24, 66, 67]. Thus, the details of Pi binding are not only necessary for understanding the mechanism of ATP synthesis, but as suggested earlier, molecular features of Pi binding derived from mutational and biochemical studies may in the near future, assist in the development of potent and novel molecular drug inhibitors of ATP synthase [2, 59, 68, 69].

Selective Pi binding, carried out by the catalytic site Pi binding subdomain residues, is the key for ATP formation. Residues αPhe-291, αSer-347, αGly-351, αArg-376, βLys-155, βArg-182, βAsn-243, βArg-246, and other highly conserved αVISIT-DG sequence residues are found in close proximity to bound phosphate analogs AlF_3 or SO_4^{2-} in the X-ray crystallographic structure of ATP synthase catalytic sites, which suggests their involvement in preferential Pi binding [44, 70]. [*E. coli* residue numbers are used throughout]. Moreover, Orris *et al*. [71] showed by X-ray crystallography that the covalent adduct formed by NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1, 3,-diazole) is specifically in the βE catalytic site, thus the

protection afforded by Pi against the NBD-Cl inhibition of ATP synthase indicates that Pi binding occurs at the βE catalytic site.

For mitochondrial ATP synthase Perez *et al*. [72] showed that Pi protects against the NBD-Cl inhibition of ATPase activity, providing a means to measure Pi binding. Alteration of the Pi protection against the NBD-Cl assay for *E. coli* purified F_1 or membrane bound F_1F_o resulted in defining the relationship between Pi binding and catalysis for eight residues, namely aPhe-291, aSer-347, aGly-351, aArg-376, βLys-155, βArg-182, βAsn-243, and βArg-246. The following five residues; αSer-347, αArg-376, βLys-155, βArg-182, and βArg-246 grouped in a triangular fashion, were found to be involved in Pi binding. Three other residues; αPhe-291, αGly-351, and βAsn-243, though important for function and overall structural maintenance, are not directly involved in Pi binding [35, 53, 73–78]. The presence of Pi binding residues in the catalytic site causes the preferential binding of ADP over ATP. As mentioned elsewhere [3] there are other residues, such as rest of the αVISIT-DG sequence residues, in close proximity to Pi binding subdomain in the catalytic sites that appear to be potential candidates for direct or indirect Pi binding and require further characterization.

5. ATP SYNTHASE AND DISEASE STATES

It is well known that failure of the ATP synthase complex can result in a wide variety of diseases and that this enzyme may also be used as a therapeutic drug target in the treatment of many diseases such as cancer, tuberculosis, obesity, neuropathies, Alzheimer's, microbial infections, mitochondrial diseases, immune deficiency, cystic fibrosis, diabetes, ulcers, and Parkinson's [2, 56, 79, 80]. For example, one of the forms of Leigh syndrome, a neurodegenerative disease, is the result of mutation in the *a*-subunit of ATP synthase [81]. The c-subunit of ATP synthase is involved in both lysosomal storage diseases and Batten disease. Alzheimer's disease patients show accumulation of α-subunit and low expression of β-subunit in the cytosol. The presence of circulating subunit F6 has been associated with hypertension [82, 83]. Furthermore, ATP synthase is a possible molecular target for antiobesity drugs. The inhibition of non-mitochondrial ATP synthase resulted in the inhibition of cytosolic lipid droplet accumulation [84]. The presence of ATP synthase on the surface of multiple animal cell types is also correlated with several other cellular processes including angiogenesis, intracellular pH regulation, and programmed cell death [85–90]. Angiostatin, a known inhibitor of angiogenesis, was shown to bind to ATP synthase on the surface of human endothelial cells. The transport of H^+ across the plasma membrane by mitochondrial ATP synthase was associated with cytolysis of tumor cells and is the basis for angiostatin's antiproliferative effect on endothelial cells because of its interaction with the α-subunit of ATP synthase [91].

The potential use of ATP synthase protection against microbial infections is straight forward because it is an appropriate target enzyme for antimicrobial agents. Protection against dental cavities caused by the microbe *Streptococcus mutans* presents a nice example for this potential. *S. mutans* is an important microbial agent in the pathogenesis of dental cavities through acid production and biofilm formation. Inhibition of *S. mutans* ATP synthase provides a prophylactic effect against *S. mutans* metabolism by arresting biofilm formation

and acid production [92, 93]. Another instructive example is the case of *Mycobacterium tuberculosis* ATP synthase, where two mutations in its c-subunit (D32V and A63P) confer resistance to the tuberculosis drug diarylquinoline [94, 95], providing insight into the causes of drug resistance against tuberculosis. Hence, a better understanding of ATP synthase inhibition and its interaction with known inhibitors may be of value in the treatment of these and other diseases.

The importance of ATP synthase as a promising target for drug development is also evident from the fact that many antibiotics such as efrapeptins, aurovertins, and oligomycins inhibit its function. Efrapeptins and aurovertins inhibit both synthesis and hydrolysis of ATP by ATP synthase [96, 97]. Oligomycin on the other hand is a potent inhibitor of ATP synthase by binding in the F_o sector and blocking proton conduction. One study showed that oligomycin induces 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 [98]. Another similar study suggested that oligomycin interaction with components of mitochondrial pathways may lead to apoptosis of select cells via CD 14 [99]. Thus, it is quite possible that some degree of similar inhibition, or interactions between ATP synthase and other inhibitors, may occur and play a significant role in apoptosis via mitochondrial pathways [68, 69, 79].

6. NON PEPTIDE ATP SYNTHASE INHIBITORS

A wide variety of natural and synthetic products are known to bind and inhibit ATP synthase [2, 3, 56, 59, 68, 69, 100–104]. 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl), sodium azide (NaN3), aluminum fluoride (AlFx), scandium fluoride (ScFx), beryllium fluoride (BeFx) are known inhibitors. (In a biochemical reaction Al, Sc, and Be can be coordinated by different numbers of fluoride ions. The presence of F- species is indicated with x). Several naturally occurring antibiotics such as oligomycin, efrapeptins, aurovertins, leucinostatins, a number of polyphenols like resveratrol, piceatannol, quercetin, morin, epicatechin, and peptides to be discussed later are additional inhibitors of ATP synthase [2, 35, 53, 59, 68, 69, 97, 101, 105–112]. Fig. (3) shows inhibitory effects of some inhibitors.

Recently there has also been a focus on polyphenol induced inhibition of ATP synthase. This is due to their natural occurrence, compatibility with the human system, and ubiquitous availability. Polyphenols are mainly naturally occurring, but are also synthetic or semisynthetic organic chemicals characterized by the presence of single or multiple phenolic structural units. The number and characteristics of the phenolic groups stimulates the unique metabolic, toxic, or therapeutic properties associated with them [113–115].

A variety of dietary flavonoids or polyphenolic compounds exert a broad range of pharmacological effects, such as protection of cells or tissues through 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 [102, 116–120]. 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 [121, 122]. Interestingly enough, experimental results suggest that the mechanism of inhibitory actions of both polyphenols and peptides on ATP synthase may be somewhat similar [59, 68, 69, 119]. X-ray crystallographic structures, in addition to biochemical assays of polyphenol and peptide inhibited ATP synthase, may help in revealing the exact inhibitory mechanism.

7. ANTIMICROBIAL AND ANTITUMOR PEPTIDES - THE POTENTIAL THERAPEUTIC DRUGS INHIBITORS OF ATP SYNTHASE

Insulin, thyroid hormone, and factor VII were among the first peptide drugs [123]. Recent interest in peptides for therapeutic applications has developed in part from the need for new antibiotics because of evolved bacterial resistance to traditional molecules [124]. The potential utility of AMPs for the development of new antibiotics is also related to the observation that there may be no general mechanism for the evolution of bacterial resistance to the activity of different peptides [2, 59, 125, 126]. Additionally, peptides are characterized by a wide spectrum of activity against multiple bacteria types and by low levels of evolved resistance [127]. Bio-active peptides can be grouped into a number of functional classes such as 1) peptide hormones and neurohormones, 2) peptide toxins, 3) antimicrobial peptides (AMPs), and 4) cell penetrating peptides, with these having a number of different effects, such as being neurotoxic, cytolytic, necrotic, hemor-rhagic, anti-inflammatory and analgesic, among others [128].

A number of peptides with antimicrobial activity known as Antimicrobial Peptides (AMPs) or Host Defense Peptides (HDPs) are in various stages of development. Possible clinical applications include anticancer activity, immunomodulation, wound healing, drug carriers, vaccine adjuvants, innate defense regulators, and both pro and anti-inflammatory agents [125]. Potential general applications under development include topical antibiotics and antiseptics, anti-inflammatory activity, nosocomial infections, and respiratory [129]. Present efforts to develop AMPs for specific therapeutic applications include therapies for oral diseases [130], biofilm infections [131], bacterial sepsis [132], antimalarial host-directed adjunctive therapy [133], and methicillin-resistant *Staphylococcus aureus* [134].

Since 2000 about 20 new antibiotics have been developed and 40 compounds are in clinical development. At least 15 peptides or peptide mimics for therapeutic applications are in active development [129]. A predominance of natural product compounds now present in late stage trials suggests the possibility that natural products such as AMPs may have an increased likelihood of success [135]. Potential weaknesses of AMPs as candidates for new antibiotics include weak activity, nonspecific cytotoxicity, susceptibility to proteolysis, high production costs, loss of activity, potential interference with host innate immunity, and interference with normal flora [126, 127, 136, 137].

Natural defenses against pathogens include a wide variety of systems in both plants and animals and include various types of oligopeptides and peptides [138]. AMPs are a component of vertebrate innate immunity that have been present in most living organisms for over 2.6 billion years [139] and were first described in insects as an inducible system of

protection against bacterial infection [140–142]. AMPs are generally cationic and amphipathic molecules of less than 50 amino acids residues. They have been isolated from all investigated phyla, including microbes, plants, invertebrates, and vertebrates. AMPs have been shown to exhibit inhibitory activity against Gram-positive and Gram-negative bacteria, fungi, parasites, and viruses [139]. Plant AMPs also exhibit activity against human pathogens [136]. A large number of AMPs are known to have selective anticancer activity as well [143]. AMPs have a neutralizing effect on bacterial endotoxins that are a primary cause of lethality in sepsis [144–146] and may have multiple additional inhibitory properties with unclear modes of actions [137].

There are 2065 entries in the Antimicrobial Peptide Database (APD) [147], (http://aps.unmc.edu/AP/main.php), of which 1664 (80.6%) are identified as having antibacterial activity, 732 (35.4%) have antifungal activity, 141 (6.8%) have anticancer activity, and 125 (6.0%) have antiviral activity. The mean length of all peptides in the APD is 30.63 residues and the mean net charge is +3.11. Identification of secondary structure among database AMPs shows 14.67% (N=303) are α-helical, 4.35% (N=90) are in β-conformation, and 2.76% (N=57) are α+β. 17.96% (N=371) were found to have disulfide bonds and 5.52% (N=114) were rich in unusual amino acids [147]. While the updated APD is sufficient to serve our purpose in this article, interested readers may refer to other databases dedicated to AMPs listed in the APD links. Additional information on helical AMPs from both synthetic and natural sources can be found in YADAMP, which was built based on the information from the APD as well as other literature sources [148].

Of all APD listed animal derived AMPs, 1490 (56.5%) are from amphibians. Following the discovery of AMPs in insects, biochemically active substances in frog skin were identified as bioactive peptides [149–151]. AMPs are gene-encoded and produced by phagocytes and epithelial cells [128]. Frogs and toads secrete AMPs from granular glands of the skin, typically in response to infection or environmental stress [152] and at concentrations as high as mg/g of wet skin [150]. The single largest source of AMPs found in the APD is from amphibian skin with 842 (40.7%) of all listed AMPs from this source. The first amphibian AMPs identified were the magainins from skin secretions of the frog *Xenopus laevis* [149]. Currently known amphibian AMPs 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 and South America [146, 153]. Frog skin produces a variety of AMPs with up to 100 unique amino acid sequences per species [154]. Based on structural similarity and species of origin, there are four identified classes of amphibian AMPs: 1) magainins from Xenopus, 2) dermaseptins from species in the genus Phyllomedusa, 3) bombinins and bombinin H from European toads, and 4) temporins, brevinins, esculentins, ranalexins, and ranauerins from species in the genus Rana [146].

Most 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 [139]. Several mechanisms have been

hypothesized regarding the activity of AMPs, such as membrane permeabilization and cell death by either a “barrel-stave” model [155] or a “torodial pore” model proposed for magainins from *Xenopus* skin [156–158]. Dermaseptins appear to cause a non-pore-dependent cytolytic activity that causes membrane bilayer micellization and disintegration [159]. Laughlin and Ahmad [59] showed that several cationic α-helical amphibian AMPs such as Ascaphin-8, XT-7, Citropin 1.1, Aurein 2.2, Aurein 2.3, Maculatin 1.1, Melittin-related peptide, Carein 1.8, Carein 1.9, Maganin II, Maganin II -amide, and dermaseptin have reversible inhibitory effects on the ATPase activity of *E. coli* ATP synthase. ATP synthase is present of the plasma membrane of bacteria while in eukaryotic cells it is present on the inner membrane of mitochondria. Therefore, inhibitory actions of membrane targeting AMPs may be attributable to their interactions with membrane bound ATP synthase. Similarly in tumor cells the presence of the F_1 sector of ATP synthase on the plasma membrane [56].

AMPs have become potential sources of compounds with useful pharmacological properties and medical utility in antimicrobial [160–162] and anticancer [163] applications. One probable drawback of the usefulness of these molecules is that they seem to be effective only at very high doses [164]. 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 [100] and peptides [Z. Ahmad and T. Laughlin unpublished results]. Synergistic effects with AMPs among different α-helical peptides have also been observed [165] and may be related to the large number of different isoforms found in several species [166]. This suggests that the evaluation of potential ATP synthase inhibitory activity by AMPs may be enhanced by combinatorial studies.

A number of experimental studies described below used α-helical peptide to induce inhibition of ATP synthase. α-helical peptides used to inhibit ATP synthase have been derived from a variety of organisms, including insects, yeasts, and frogs Apparently the sterochemical interactions of α-helical peptides with the β-DELSEED-motif of ATP synthase are more compatible than other secondary structures.

Melittin, the primary component of honey bee venom (*A. mellifera*), is an α-helical basic peptide composed of 26 residues and is known to have inhibitory effects on the ATPase activity of F_1-ATP synthase [2, 57–59]. The peptide is a potent inhibitor of both *E. coli* and bovine ATP synthase (IC50~5μM) [58, 59], and may have an effect similar to that of other known α-helical peptide inhibitors, IF_1, Wild-type yeast cytochrome oxidase, and synthetic Syn-A2 [57, 58, 167–171].

Fig. (1B) shows the x-ray crystallographic structure of anionic βDELSEED-loop (residue numbers β380–386) of ATP synthase, while some select cationic α-helical peptides with potential therapeutic properties are shown in Fig. (2). Indirect experimental evidence on protection against the inhibition of quinacrine mustard by melittin suggested a common βDELSEED binding site for peptides. To date, several peptides that form basic amphiphilic α-helical structures have been shown to bind at the βDELSEED-loop of *E. coli* ATP synthase. [57, 58].

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 large conformational changes and interacts with the $\alpha_3\beta_3$ hexagon ring, where it comes in close proximity to the βDELSEED-loop. Electrostatic interactions between basic residues of the ε-subunit and the acidic residues of βDEL-SEED-motif cause inhibition of ATPase activity [172–174]. ATP synthase activity is also affected by mitochondrial if_1 [175, 176]. if_1 is a natural regulatory peptide of 56–87 residues in length that inhibits the ATPase activity of ATP synthase in a manner that is both reversible and noncompetitive [177]. A crystal structure of if_1 with the F_1- subunit shows the N-terminal domain of IF_1 to be bound at the α and βF_1 interface [178].

We expect AMPs with potential inhibitory effects on ATP synthase through binding at the βDELSEED-loop 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. Candidate AMPs for anti ATP synthase activity and anti-microbial activity (Table I and Table II) were selected from the APD based on an α-helical secondary structure, a net positive charge, a total length of 10–30 residues, and identified antibacterial, antiviral, anticancer, or antifungal activity. Laughlin and Ahmad [59] tested fifteen peptides for ATP synthase inhibitory activity based on the previously identified ATP synthase inhibition by the melittin peptide [57, 58]. It was shown that MRP and MRP-amide strongly inhibited the ATPase activity of ATP synthase. However, magainin II, magainin II-amide, and caerin 1.9 only partially inhibited ATPase activity. Other peptides exerting partial inhibition of *E. coli* ATP synthase, but not shown in Fig. (2), were ascaphin-8, aurein 2.2, aurein 2.3, citropin 1.1, and maculatin 1.1. The 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, magainin II-NH2, MRP, or MRP-NH2 resulted in significant inhibition of cell growth, which was interpreted as a possible result of anti ATP synthase activity [2, 59].

It seems probable that there will be variable results from the testing of AMPs for inhibition of ATP synthase or of cell growth for therapeutic applications, depending on the particular target molecules and organisms. We also found that various modifications of naturally occurring AMPs may be used to modulate their effectiveness on a molar scale with regard to both ATP synthase inhibition and cytotoxicity (Z. Ahmad and T.F Laughlin unpublished results). By virtue of the great variability in the structures and potential functions of AMPs, these relatively simple molecules may constitute a rich natural resource of new drug compounds that may be targeted at microorganisms and neoplasms through inhibitory effects on ATP synthase.

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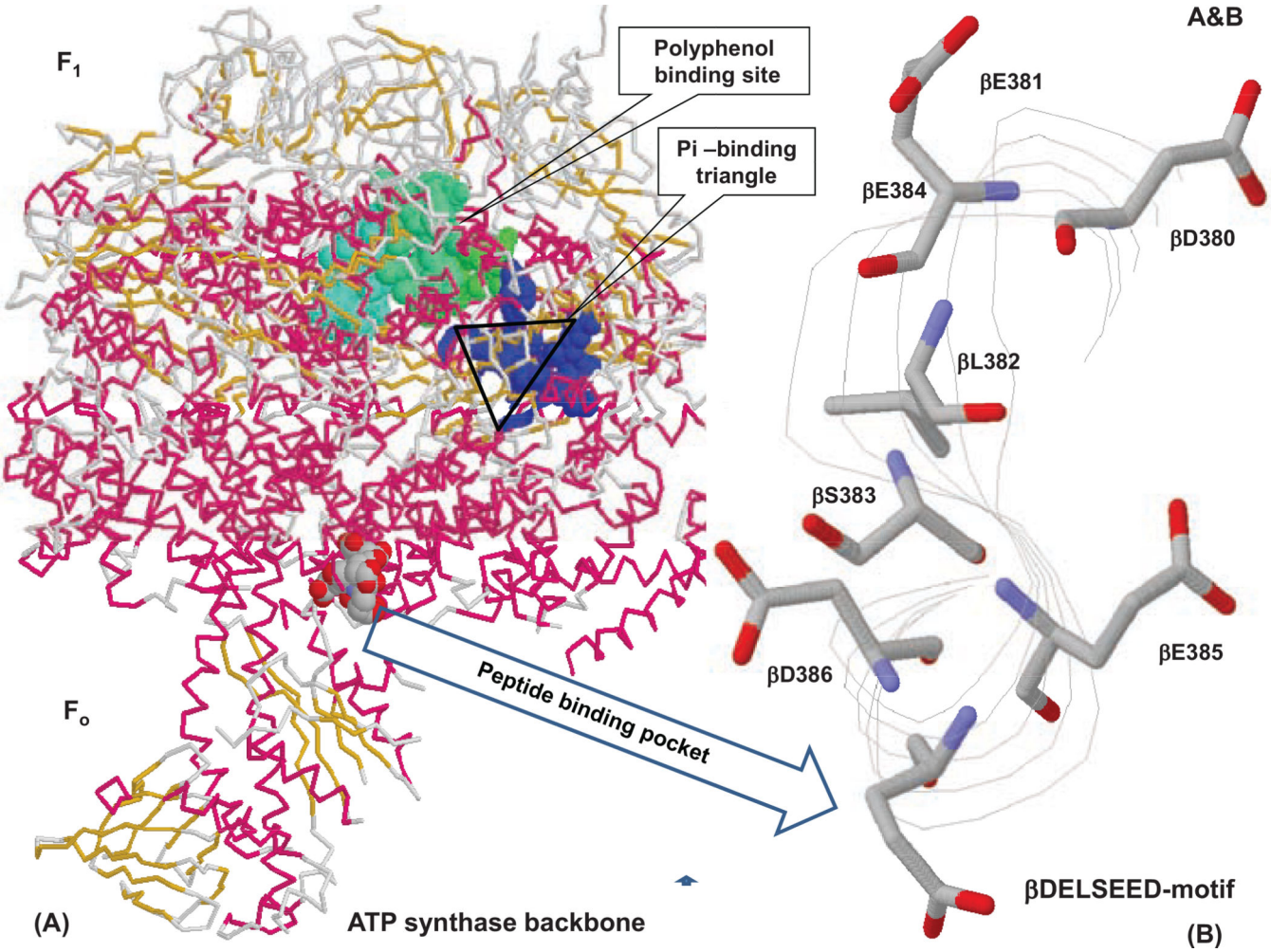


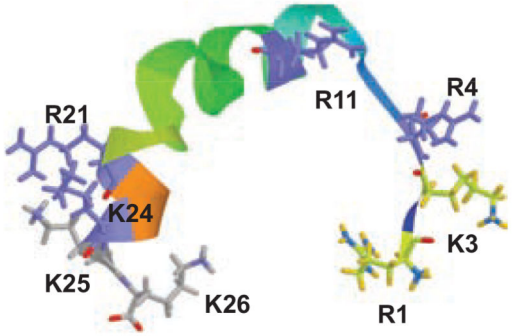
Fig. (1). Structure of Escherichia coli ATP synthase and peptide binding siteβDELSEED

(**A**) Water soluble F_1 form of *E. coli* enzyme in backbone form showing catalytic Pi binding subdomain triangle, polyphenol, and peptide binding sites in space fill form. Membrane bound F_o sector is also show in backbone form. Polyphenol and peptide binding sites are identified with circles at the interface of α/β subunits. (**B**) Enlarged peptide binding pocket in wireframe form identifies the involved residues. Figure was generated by PDB file 1H8E [44] using Rasmol [179].

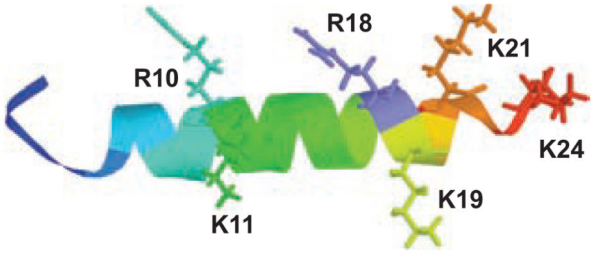
A

Alyteserin-1c (2L5R)

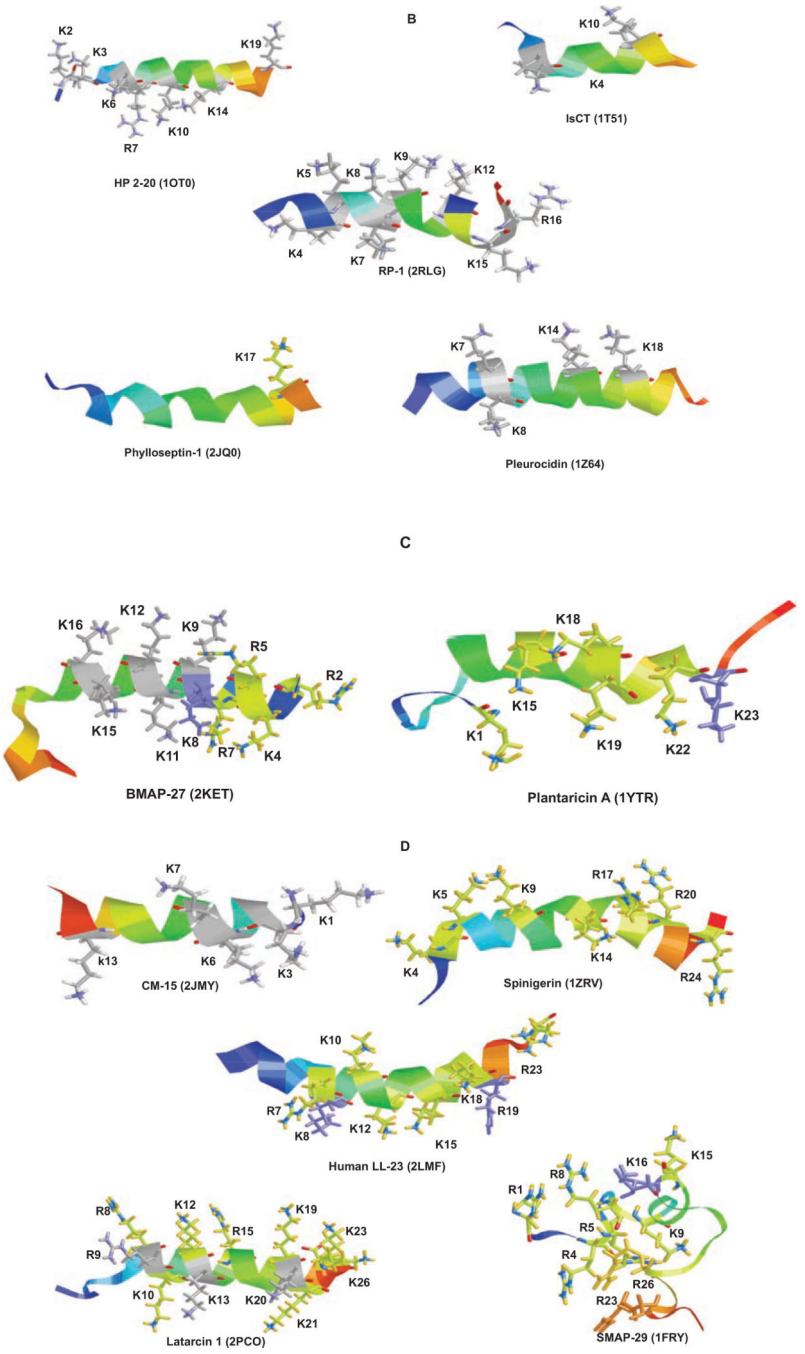
Bombinin H2 (2AP7)



Chicken CATH-1 (2AMN)



Distinctin chain D (1XKM)



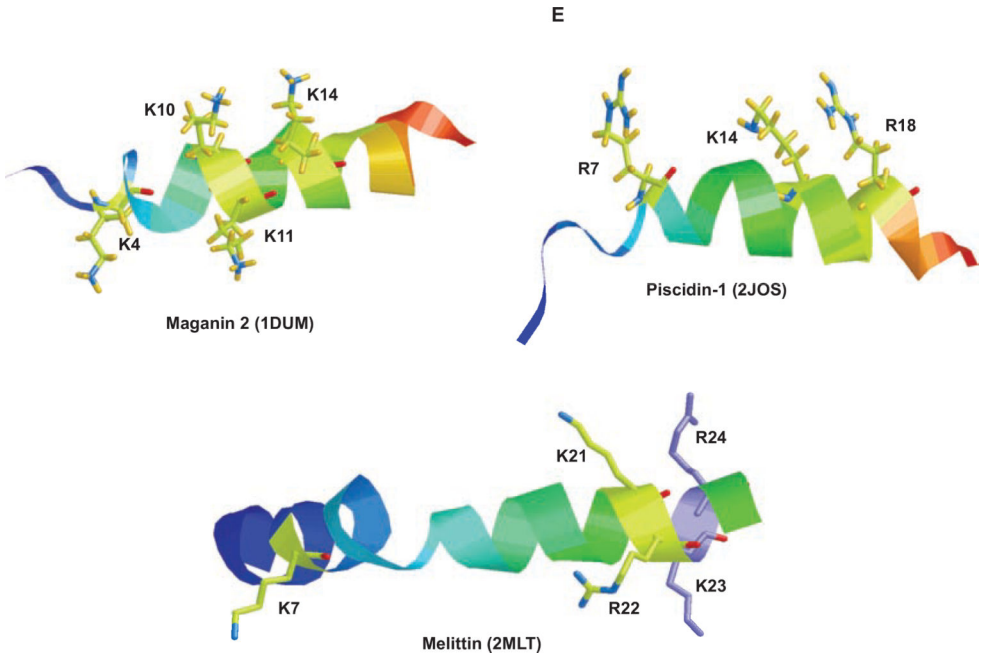


Fig. (2). X-ray crystallographic structures of select peptides

(**A** & **B**) found to have only antimicrobial properties, (**C**) with antibacterial and anticancer properties, (**D**) with antibacterial and anti fungal properties, and (**E**) with antibacterial, anti cancer, and anti-fungal properties. Associated positively charged residues are identified for each peptide. RasMol [179] was used to generate these figure using PDB files 2LRR [180], 2AP7 *Zangger, K., Jilek, A., Khatai, L. "Solution structure and orientation of bombinin H2 and H4 in a membrane-mimetic environment*",2AMN [181], 1XKM [182], 1OT0 *Lee, K.H., Lee, D.G., Park, Y., Hahm, K.-S., Kim, Y. "Structure of Antimicrobial Peptide, HP (2–20) and its Analogues Derived from Helicobacter pylori, as Determined by 1H NMR Spectroscopy*", 1T51 [183], 2RLG [184], 2JQ0 [185], 1Z64 [186], 2KET *Yang, S., Jung, H., Kim, J. "solution structure of BMAP-27*", 1YTR [187], 2JMY [188], 1ZRV [189], 2LMF [190], 2PCO [191], 1FRY [192], 1DUM [193], 2JOS [194], 2MLT [168].

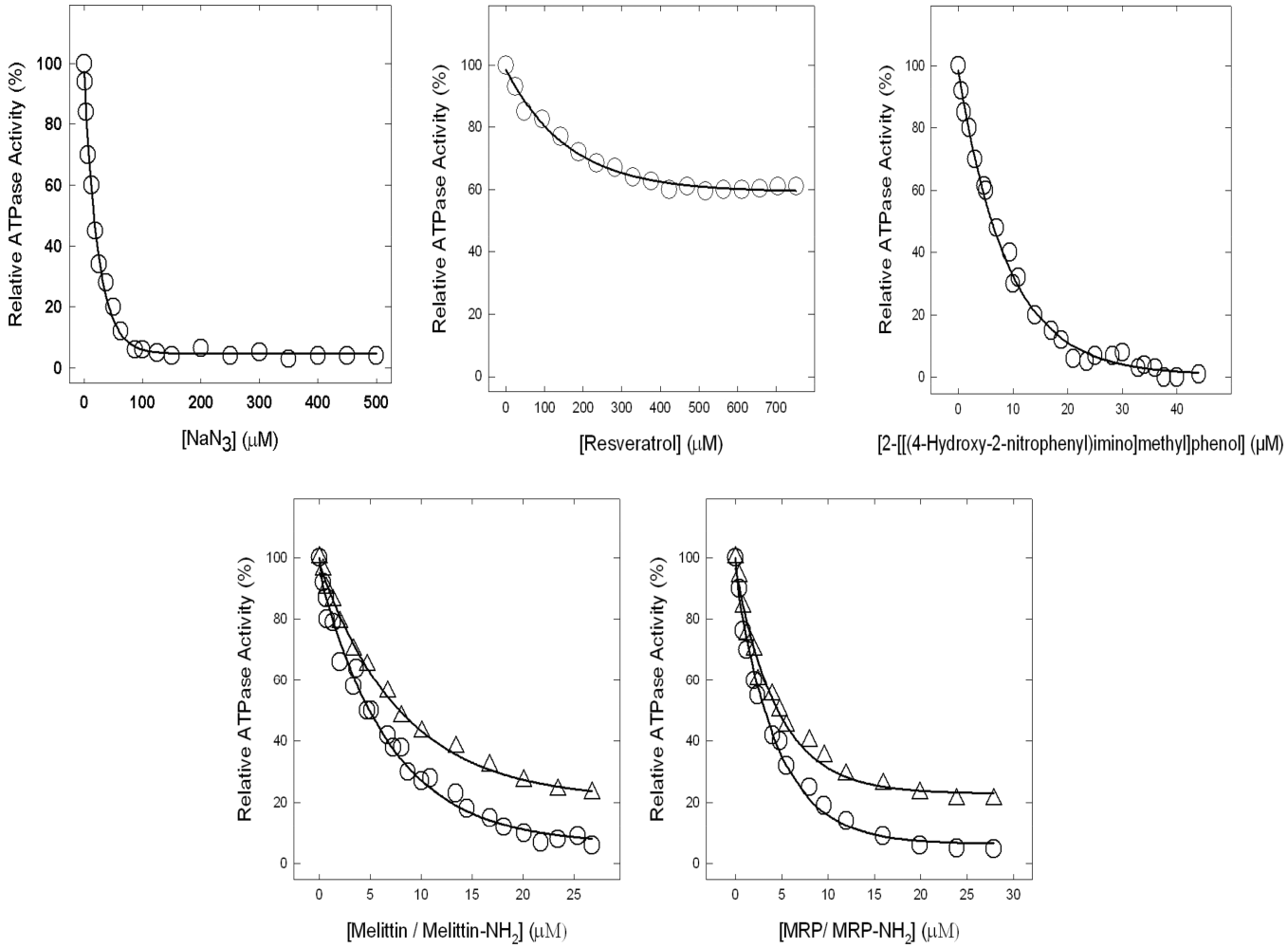


Fig. (3). Inhibition of ATP synthase

Inhibition profiles induced by NaN_3 taken from [74], resveratrol taken from [68], modified resveratrol 2-[[(4-hydroxy-2-nitrophenyl)imino]methyl] phenol taken from [100], melittin/ melittin-NH_2, MRP/MRP-NH_2 taken from [59]. For experimental details see the associated references.

Table 1

Cationic α-helical Peptides Having Antibacterial, Anticancer, Antifungal, and Antiviral Properties. + Equals Activity Against. Peptide Data Taken From Antimicrobial Peptide Database [147]

Name	Length	Charge	Bacteria	Cancer Cells	Fungi	Viruses
Alyteserin-1c	23	3	+			
Ascaphin-8	19	4	+	+	+	+
Aurein 2.2	16	1	+	+	+	+
Bactrocerin-1	20	6	+		+	
BMAP-27	27	10	+	+	+	+
Bombinin H2	20	3	+			
Bombinin-like peptide 1	27	3	+			
Brevinin-1BYa	24	4	+	+	+	
Buforin II	21	6	+	+	+	
Caerin 1.6	24	2	+	+		
Cathelicidin-BF	30	11	+		+	
Ceratotoxin A	29	6	+			
Chicken CATH-1	26	8	+			
Chrysophsin-1	25	6	+	+		
Ci-MAM-A24	24	7	+		+	
Citropin 1.1	16	2	+	+	+	
Clavanin A	23	1	+			
Clavaspirin	23	1	+			
CM15	15	5	+		+	
CM-3	18	6	+		+	
Cryptonin	24	8	+		+	
Decoralin	11	2	+		+	
Dermaseptin-S3	30	5	+		+	
Dicynthaurin	30	4	+			
Distinctin	25	4	+			
Eumenitin	15	3	+			

Name	Length	Charge	Bacteria	Cancer Cells	Fungi	Viruses
Stylin A	19	5	+			
Substance P	11	3	+		+	
Temporin A	13	1	+			+
The K4 peptide	14	4	+			
Uperin 3.6	17	2	+			+
WLBU2	24	13	+			
XT-7	18	3	+	+	+	

Table 2

Sequence and Origin of Table I Peptides. Peptide Data Taken From Antimicrobial Peptide Database [147]

Name	Sequence	Source
Alyteserin-1c	GLKEIFKAGLGSLVKGIAAHVAS	Amphibian
Ascaphin-8	GFKDLLKGAAKALVKTVLF	Amphibian
Aurein 2.2	GLFDIVKKVVGALGSL	Amphibian
Bactrocerin-1	VGKTWIKVIRGIGKSKIKWQ	Insect
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHLG	Mammal
Bombinin H2	IIGPVLGLVGSALGGLLKKI	Amphibian
Bombinin-like peptide 1	GIGASILSAGKSALKGLAKGLAEHFAN	Amphibian
Brevinin-1BYa	FLPILASLAAKFGPKLFCLVTKKC	Amphibian
Buforin II	TRSSRAGLQFPVGRVHRLLRK	Amphibian
Caerin 1.6	GLFSVLGAVAKHVLPHVVPVIAEK	Amphibian
Cathelicidin-BF	KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	Reptile
Ceratotoxin A	SIGSALKKALPVAKKIGKIALPIAKAALP	Insect
Chicken CATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	Bird
Chrysophsin-1	FFGWLIKGAIHAGKAIHGLIHRRRH	Fish
Ci-MAM-A24	WRSLGRTLLRLSHALKPLARRSGW	Urochordate
Citropin 1.1	GLFDVIKKVASVIGGL	Amphibian
Clavanin A	VFQFLGKIIHHVGNFVHGFSHVF	Urochordate
Clavaspirin	FLRFIGSVIHGIGHLVHHIGVAL	Urochordate
CM15	KWKLFKKIGAVLKVL	Synthetic
CM-3	ALKAALLAILKIVRVIKK	Synthetic
Cryptonin	GLLNGLALRLGKRALKKIIKRLCR	Insect
Decoralin	SLLSLIRKLIT	Insect
Dermaseptin-S3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES	Amphibian
Dicynthaurin	ILQKAVLDCLKAAGSSLSKAAITAIYNKIT	Urochordate
Distinctin	NLVSGLIEARKYLEQLHRKLKNCKV	Amphibian
Eumenitin	LNLKGIFKKVASLLT	Insect
Fallaxidin 4.1	GLLSFLPKVIGVIGHLIHPPS	Amphibian
FK-13	FKRIVQRIKDFLR	Synthetic
Frenatin 3	GLMSVLGHAVGNVLGGLFKS	Synthetic
Halictine 1	GMWSKILGHLIR	Insect
Halocyntin	FWGHIWNAVKRVGANALHGAVTGALS	Urochordate
Hedistin	LGAWLAGKVAGTVATYAWNRYV	Annelid
HFIAP-3	GWFKKAWRKVKNAGRRVLKGVGIHYGVGLI	Hagfish
HP 2–20	AKKVFKRLEKLFSKIQNDK	Synthetic
Human Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	Human
Human KR-20	KRIVQRIKDFLRNLVPRTES	Human
Human KS-30	KSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human

Name	Sequence	Source
Human LL-23	LLGDFFRKSKEKIGKEFKRIVQR	Human
Hylaseptin P1	GILDAIKAIAKAAG	Amphibian
Hylin a1	IFGAILPLALGALKNLIK	Amphibian
IsCT	ILGKIWEGIKSLF	Arachnid
Japonicin-1CDYa	FFPLALLCKVFKKC	Amphibian
Japonicin-1Npa	FLLFPLMCKIQGKC	Amphibian
Kassinatuerin-1	GFMKYIGPLIPHAVKAISDLI	Amphibian
L5K5W	KKLLKWLKKLL	Snythetic/amphibian
Lasiocepsin	GLPRKILCAIAKKKGKCKGPLKLVCKC	Insect
Lasioglossin LL-I	ILGKLLSTAAGLLSNL	Insect
Latarcin 1	SMWSGMWRRKLKKLRNALKKKLKGEK	Arachnid
Lycotoxin I	IWLTALKFLGKHAAKHLAKQQLSKL	Arachnid
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	Amphibian
Mastoparan B	LKLKSIVSWAKKVL	Insect
Maximin 4	GIGGVLLSAGKAALKGLAKVLAEKYAN	Amphibian
MB-21	FASLLGKALKALAKQ	Synthetic
Melectin	GFLSILKKVLPKVMAHMK	Insect
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ	Insect
Meucin-13	IFGAIAGLLKNIF	Arachnid
Misgurin	RQRVEELSKFSKKGAAARRRK	Fish
Moronecidin	FFHHIFRGIVHVGKTIHKLVTG	Fish
MUC7 20-Mer	LAHQKPFIRKSYKCLHKRCR	Human
Nigrocin-2	GLLSKVLGVGKKVLCGVSGLC	Amphibian
Ocellatin-F1	GVVDILKGAAKDIAGHLASKVMNKL	Amphibian
Odorranain-B1	AALKGCWTKSIPPKPCFGKR	Amphibian
Oxt 4a	GIRCPKSWKCKAFKQRVLKRLLAMLRQHAF	Arachnid
P-18	KWKLFKKIPKFLHLAKKF	Synthetic
Pandinin 2	FWGALAKGALKLIPSLFSSFSKKD	Arachnid
Parasin I	KGRGKQGGKVRAKAKTRSS	Fish
Parkerin	GWANTLKNVAGGLCKITGAA	Amphibian
Pd_mastoparan PDD-A	INWKKIFEKVKNLV	Insect
Pep27	MRKEFHNVLSSGQLLADKRPARDYNRK	Bacteria
PGLa	GMASKAGAIAGKIAKVALKAL	Amphibian
Phylloseptin-H1	FLSLIPHAINAVSAIAKHN	Amphibian
Phylloxin-B1	GWMSKIASGIGTFLSGMQQ	Amphibian
Piscidin 1	FFHHIFRGIVHVGKTIHRLVTG	Fish
Plantaricin A	KSSAYSLQMGATAIKQVKKLFKKWGW	Bacteria
Pleurocidin	GWGSFFKKAAHVGKHVGKAALTHYL	Fish
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	Mammal

Name	Sequence	Source
Polybia-MP-I	IDWKKLLDAAKQIL	Insect
Ponericin G1	GWKDWAKKAGGWLKKKGPGMAKAALKAAMQ	Insect
PP13	GAARKSIRLHRLYTWKATIYTR	Insect
Pseudin-1	GLNTLKKVFQGLHEAIKLINNHVQ	Amphibian
Ranalexin	FLGGLIKIVPAMICAVTKKC	Amphibian
RP-1	ALYKKFKKKLLKSLKRL	Synthetic
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	Mammal
Spinigerin	HVDKKVADKVLLLKQLRIMRLLTRL	Insect
Styelin A	GFGKAFHSVSNFAKKHKTA	Urochordate
Substance P	RPKPQQFFGLM	Human
Temporin A	FLPLIGRVLSGIL	Amphibian
The K4 peptide	KKKKPLFGLFFGLF	Synthetic
Uperin 3.6	GVIDAAKKVVNVLKNLF	Amphibian
WLBU2	RRWVRRVRRWVRRVVRVVRRWVRR	Synthetic
XT-7	GLLGPLLKIAAKVGSNLL	Amphibian