1. Quantitative structure-activity relationship (QSAR) studies on bioactive cyclopeptides

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Abstract. Cyclopeptides show a variety of structures, from cyclodipeptides through cyclohepta- and octapeptides to cyclotides with 30 amino acids, which can be found in bacteria, insects, higher plants, fungi, animals, and humans. Peptides and in particular cyclopeptides are therapeutic targets which play an important role in medicinal chemistry, including drug design and quantitative structure-activity relationships (QSARs). However, few QSAR studies have been carried out on cyclopeptides in comparison with small organic molecules. Some structural features, substructures, and functional groups contribute to enhance the bioactivity. Configurational and conformational studies of cyclopeptides from plant and fungal origin were analysed, and related to antitumour activity and toxicity. Some physico-chemical properties, \textit{e.g.}, partition coefficient ($\log P$) and several molecular parameters have been found to be relevant for activity. QSAR models, including a variety of descriptors, have been applied to synthetic and natural

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cyclopeptides as well as to their analogues. The balance between antimicrobial and haemolytic properties for the design of antimicrobial cyclic peptides is discussed. Considerations about anticancer and cytotoxic activity of cyclopeptides are also included. This kind of studies also help the design of selective drugs.

**Abbreviations**

QSARs, quantitative structure-activity relationships; SARs, structure-activity relationships; NMR, Nuclear Magnetic Resonance; MS, mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; LPS, lipopolysaccharide; LA, lipid A; MIC, minimum inhibitory concentration; IC50, inhibition constant for 50% inhibition; 2D-NMR, bidimensional NMR; 3D-QSAR, three-dimensional QSAR; Abu, L-α-aminobutyric acid; Ala, alanine; Arg, arginine; Asn, asparagine; Cys, cysteine; β-Phe, β-phenylalanine; Phe, phenylalanine; Gly, glycine; Leu, leucine; Lys, lysine; Pro(Cl2), β,γ-dichlorinated proline; HOPro, hydroxyproline; D-Pro, D-proline; Pro, proline; Ser, serine; alloThr, allothreonine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine. A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, hystidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

**Introduction**

Proteins and peptides play an important role in biological systems and affect most physiological processes of plants, animals and microorganisms, in particular in humans, usually acting as agonists and/or antagonists at specific receptors. Bioactive cyclopeptides or cyclic peptides have been found in animals, plants, fungi, bacteria, insects and humans. Cyclopeptides have shown cytotoxic, cytostatic, antifungal, antiviral, antibacterial, plant-stimulating, insecticidal, antimalarial, estrogenic, sedative, nematicidal, immunosuppressive, and enzyme-inhibitory activities. Recently, the occurrence, structures and bioactivity of cyclopeptides in higher plants and higher fungi have been reported [1].

In fact, many biologically active proteins and peptides, including cyclopeptides, are involved in disease processes, and the abnormal expression of these peptides has also been associated with human disease [2]. The experimental demonstration of the occurrence of antimicrobial peptides in several unexplained human inflammatory disorders can provide novel therapeutic approaches to the treatment of disease. Therefore, these compounds can be therapeutic targets, which can be used in the development of future drugs [3].
A variety of antimicrobial peptides is naturally produced by different organisms through either ribosomal (defensins and small bacteriocins) or non-ribosomal synthesis (peptaibols, cyclopeptides and pseudopeptides). Some of these natural antimicrobial peptides are used for the design of new synthetic analogues, and have been also expressed in transgenic plants to confer disease protection. These compounds are also secreted by microorganisms, being active ingredients of commercial biopesticides [4,5].

However, most native peptides have a limited applicability as drug candidates due to: (a) rapid metabolism by proteolysis, (b) poor absorption by the gastrointestinal tract, and poor transport over the blood brain barrier, (c) rapid excretion by the liver and kidneys, and (d) lack of receptor specificity due to the conformational flexibility [6]. Therefore, the structure and key pharmacophore groups of native peptides are usually converted into new nonpeptidic drug candidates [7], the so-called peptidomimetics.

The development of peptidomimetic protease inhibitors has been feasible in recent years by the three-dimensional (3D) structural information on proteases from X-ray diffraction and NMR spectroscopy [8, 9]. Until the 3D structures of the important group of the seven transmembrane G-protein-coupled receptors (GPCRs) have been resolved [10], it was necessary to rely on homology modelling of the receptors based on the structure of bovine rhodopsin and site-specific mutagenesis to provide important clues in the development of new drugs for these receptors [11].

**Structural features of the peptides**

Peptides have a large degree of conformational freedom due to their free rotating bonds, thus giving rise to a large number of conformations. However, in solution and in the absence of receptors or enzymes the biologically active conformation may be poorly populated. Conformationally constrained analogues can significantly contribute to the identification of these conformations. Hence, peptidomimetics provide valuable information on SARs of both peptides and complex proteins [12].

Once the primary structure of the biologically active peptide has been identified, the first design step is to remove the amino acids from the amino and carboxy termini, one at a time, in order to obtain the smallest biologically active peptide fragment. Subsequently, side chain requirements, e.g., pharmacophore groups, can be determined by systematically replacing each residue in the peptide with a specific amino acid and evaluating the biological activity. The most often used amino acid is Ala, but occasionally Gly is used [13].
Constrained structures mimicking the bioactive conformation will be less exposed to proteolytic cleavage, may give more selective ligands, providing an entropy advantage in receptor binding compared to the more flexible linear peptides [12,14].

After the SAR of each amino acid in the peptide has been determined the next step is to try to establish the bioactive conformation(s). The conformational freedom of the highly flexible peptide can be reduced by the introduction of local and/or global constraints. Methods of obtaining local constraints include incorporation of modified amino acids, e.g., D-amino acids and N-methyl amino acids, introduction of modified amide bonds or short-range cyclizations forming a link between two backbone termini, between one of the termini and one side chain, between two side chains, or between backbone atoms other than the termini. Secondary structure mimetics can also be used as constraints. When incorporated into a peptide, a secondary structure mimic enforces a particular conformation. Incorporation of such a moiety provides additional information about requirements for receptor binding and/or activation [14].

SARs of the constrained analogues together with information obtained from NMR spectroscopy and molecular modelling can, in an iterative process, give a 3D pharmacophore model of the bioactive conformation. The last step is to introduce the pharmacophore groups onto a nonpeptidic scaffold in the correct spatial arrangement, in agreement with the obtained model of the bioactive conformation [12-14].

The receptor-based screening of natural and synthetic compound collections has proved to be a useful method for identifying peptidomimetics [11]. Furthermore, design, combinatorial chemistry and classical medicinal chemistry play important roles.

Hirschmann et al. [15] reported an example in which pharmacophore modelling was used to obtain a peptidomimetic agonist of the cyclopeptide hormone somatostatin. Furthermore, a systematic exploration of the conformational space for a series of analogues of FC131, a cyclopentapeptide CXCR4 antagonist, has been recently performed, thus leading to a minimalistic 3D pharmacophore model for binding of these cyclopentapeptides [16]. The chemokine receptor CXCR4 is involved in HIV entry, and therefore, is an attractive target for antiretroviral drugs.

The most essential conformational components of peptides and proteins are the secondary structure elements: α-helices, β-sheets, reverse turns and loops. These elements are often located on the protein surface and seem to act as molecular recognition sites in biological processes. Even small peptide fragments can fold into turn conformations in which the amino acid side chains are displayed on the surface of a compact backbone core [17]. There is
a lot of evidence suggesting that the side chain groups in the peptide are the most important recognition elements in peptide-receptor interaction [11]. The reversed turns can be divided into β-turns and γ-turns.

One of the most common structure motifs in proteins is the β-turn [18]. To be considered a β-turn the tetrapeptide sequence should not be part of an α-helical region, and the distance between C-α of the first and C-α of the fourth amino acid residue should be $\leq 7 \text{ Å}$. A number of slightly different turn types has been reported [19]. A hydrogen bond between the carbonyl in the first residue and the NH group in the fourth residue is often present to stabilize the turn in a pseudo-ten-membered ring structure.

A γ-turn, which is a more rare reversed turn, consists of three amino acid residues. It is defined by a hydrogen bond between the carbonyl group in the first residue and the NH-group of the third residue forming a pseudo-seven-membered ring. γ-Turns are divided into two classes, inverse and classic. The second side chain is oriented in an equatorial position in the most common inverse γ-turn, while the rare classic γ-turn has an axial second side chain. γ-Turns are rare in proteins but are frequently found in small peptides, especially in small cyclic peptides [20].

**Cyclotides**

Cyclotides are small plant proteins, e.g., cyclic disulfide-rich plant peptides of about 30 amino acids, found in plants of the Rubiaceae, Violaceae and Cucurbitaceae families [21], and are believed to be part of the host defence system [22] on the basis of their high expression levels in plants, and their toxic and growth retardant activity in feeding trials against *Helicoverpa* spp. insect pests [23].

These compounds have a macrocyclic peptide backbone and a cystine knotted arrangement made up of six conserved cysteine residues (three conserved disulfide bonds), which makes them very stable [24]. This unique structure, together with a variety of biological activities, makes them of great interest as possible leads in drug development or as carriers of grafted peptide sequences [25,26]. A database of cyclic protein sequences and structures, with applications in protein discovery and engineering is known [27] as well as chemical and biomimetic total syntheses of natural and engineered MCoTI cyclotides [28].

Molecular dynamics simulations and MM-PBSA free energy calculations have been carried out to elucidate structure and folding of these disulfide-rich miniproteins [29]. NMR spatial structure of ternary complex Kalata B7/Mn$^{2+}$/DPC micelle has been recently reported [30].
The insecticidal activity of cyclotides and the comparison with structurally similar cystine knot proteins from peas (\textit{Pisum sativum}) and an amaranth crop plant (\textit{Amaranthus hypocondriacus}) have been recently reviewed [23].

In addition to their insecticidal effect, cyclotides have also shown to be cytotoxic, anti-HIV [31], anthelmintic [32,33], molluscicidal [34], antimicrobial and haemolytic agents (see Anticancer and cytotoxic activities section).

**Configurational and conformational studies on bioactive cyclopeptides**

Conformational studies of the natural cyclopeptides and their derivatives were related to the stereochemical requirements for a bioactive compound.

Antitumour cyclic pentapeptides, astins A - H, have been isolated from \textit{Aster tataricus} (Asteraceae), which is known as a Chinese medicine and as an ornamental higher plant [35]. Astins contain a 16-membered ring system with a unique mono- or dichlorinated proline and/or allothreonine residues. The main active principle, astin B, contains a $\beta\gamma$-dichlorinated Pro, an alloThr, a Ser, a $\beta$-Phe and an $\alpha$-aminobutyric acid, and was identified as cyclo(Pro(Cl$_2$)-alloThr-Ser-$\beta$-Phe-Abu). Astin C was identified as cyclo(Pro(Cl$_2$)-Abu-Ser-$\beta$-Phe-Abu) [36].

Thus, astins A-C (Fig. 1) showed to be similar to cyclochlorotine, cyclo(Pro(Cl$_2$)-Abu-Ser-$\beta$-Phe-Ser), which has been isolated from \textit{Penicillium islandicum} Sopp. (Fig. 2).

In order to understand the mechanisms involved in the action of cyclic peptides, it was necessary to assess their conformational characteristics. The conformational analysis of this antitumour astin B was performed by 2D NMR techniques [9], temperature effects on NH protons, rate of hydrogen-deuterium exchange, vicinal NH-C$\alpha$H coupling constants, and NOE experiments [37].

![Figure 1](image-url)

**Figure 1.** Structures of Astin A, B and C from the higher plant \textit{Aster tataricus} (Asteraceae).
The combination of 2D NMR analysis with molecular dynamics and mechanics calculations allowed to determine the energetically favourable conformation of astin B in solution. The methods of molecular mechanics and restrained molecular dynamics calculations were applied to understand the energetic preferences of various conformations of astin B. A conformational difference was observed between astin B, showing a cis configuration in a Pro amide bond, and cyclochlorotine from *Penicillium islandicum*, showing all trans amide configurations [38].

A detailed knowledge of the conformation of astin B in a polar solvent such as DMSO-d$_6$ was considered the basis for SARs, allowing the design of new analogues with higher activity [39].

The assignments of $^1$H and $^{13}$C NMR signals of astin B were made by combination of $^1$H-$^1$H COSY, HMQC and HMBC spectra [35,36]. The HMBC, which provided $^1$H-$^{13}$C long-range couplings, proved to be extremely valuable for the assignments. The conformational determination of astin B in solution was made on the basis of the results of the following experiments: Hydrogen bonding, vicinal NH-CαH coupling, NOE enhancements, and quenched molecular dynamics [36,38].

Computational procedures using NMR data were applied to the elucidation of the solution conformation of astin B and further to the disclosure of the difference between the conformations in the solid and solution states. Molecular dynamics techniques were applied to astins. Three distance constraints involved in the hydrogen bondings, as also found in the crystal state, and four distance constraints derived from the NOE experiments were used to show that this solution structure of astin B was consistent with experimental data [38].

Conformations, dipole moments and toxicity

Conformation of the cyclopeptides isolated from the higher fungus *Amanita phalloides* (Vaill. ex Fr.) Secr. has been related to its toxicity. Hence, the electronic structures and conformations of the cyclopeptides,
$O$-methyl-α-amanitin, phalloidin, and antamanide, were obtained from molecular parameters on the basis of semiempiric and *ab initio* methods [40].

The electronic structures and conformational analysis of the toxic cyclopeptides, α-amanitin, $O$-methyl-α-amanitin, S-deoxo-α-amanitin, α-amanitin-(S)-sulfoxide and α-amanitin-sulfone (Fig. 3) were obtained from molecular parameters on the basis of AM1 and *ab initio* methods [41].

Accordingly, the planar indole moiety of α-amanitin showed to be ahead from the rest of the bean-shaped bicyclic structure (Fig. 4). Therefore, the upper and lower sides of the $\pi$-heterocycle were available for interacting with any $\pi$-compounds, forming stable $\pi$-complexes. This region was also so lipophilic as required for transport through membranes to enter cells [40,41].

Total and point-charge dipole moments and $sp$ hybrid were calculated for the five cyclic peptides of Fig. 3. The negative charge was then located on the sulphur atom of α-amanitin, $O$-methyl-α-amanitin, S-deoxo-α-amanitin, α-amanitin-(S)-sulfoxide and α-amanitin-sulfone towards the inner cavity. Then, this cavity was negative, nucleophilic, and thus adequate for scavenging cations in order to form complexes with probably a high stability constant. Inclusion of molecules was also possible depending on the inner cavity’s size of each compound, ranging from nearly 10 Å to 6-8 Å [41].

Smaller dipole moment values accounted for a major toxicity of the molecule examined. The lowest point-charge dipole moment was that of α-amanitin-sulfone, similar to that of the thioether S-deoxo-α-amanitin, while the highest point-charge dipole moment was achieved for α-amanitin-(S)-sulfoxide, followed by the (R)-isomer, α-amanitin, due to the distinct...

![Figure 3. Structures of α-amanitin, O-methyl-α-amanitin, S-deoxo-α-amanitin α-amanitin-(S)-sulfoxide and α-amanitin-sulfone.](image-url)
Figure 4. Spatial structure of α-amanitin.

The orientation of the oxygen atom in relation to the inner cavity, thus being able to modify its negative charge [41]. The α-amanitin-(R)-sulfoxide decreased the inner negative charge, while the (S)-compound increased it. The important difference was the direction of the dipole moment vector of both cyclic compounds.

Dipole moment's direction was nearly alike for α-amanitin, O-methyl-α-amanitin, S-deoxo-α-amanitin, and α-amanitin-sulfone, only α-amanitin-(S)-sulfoxide showed quite a distinct orientation. In the case of α-amanitin-(S)-sulfoxide, HOPro (amino acid 2), Asn (amino acid 1) and Cys (amino acid 8) were situated on the axis of the dipole moment. The dipole moment pointed away from the sulphur atom towards this positively charged portion of the molecule. Therefore, in the (S)-sulfoxide, the distribution of charges was disturbed owing to the occurrence of a marked polar character of one side, which usually should take part in the binding to macromolecules. Furthermore, this feature made difficult its passing through membranes. Hence, on the basis of dipole moment calculations it was possible to explain the decrease in toxicity and binding of this molecule, and the results were in agreement with the inhibitory constants Ki of RNA-polymerase II and lethal doses in mice [1,41].

Hydrophobicity of cyclopeptides

The physical properties of peptides have not been studied so much as those of small organic molecules. In fact, only few QSAR studies on peptides and proteins have been carried out [42].

Physico-chemical descriptors, such as the partition coefficient, are useful for selecting compounds for screening and development of predictive QSAR models. In fact, experimental partition coefficients are main descriptors of
lipophilicity or hydrophobicity, and many other ADMET (absorption, distribution, metabolism, excretion and toxicity) properties [43-47]. Hydrophobicity governs a variety of biological processes, such as transport, distribution and metabolism of biological molecules, molecular recognition, and protein folding. Therefore, such a parameter is essential to predict the transport and activity of drugs and potential pharmaceuticals.

As it is known, the partition coefficient ($P$) is the ratio between the molar concentration of a chemical compound in an organic nonpolar layer, e.g., $n$-hexane, and that in an aqueous layer, e.g., water. This partition coefficient is expressed as $\log P$. Elution times from RP-HPLC have been used as a measure of relative hydrophobicity of peptides and peptide analogues [48]. Unfortunately, the availability of measured $\log P$ values for peptides is limited [42].

During the past three decades, many methods for the prediction of $\log P$ have been reported [49-51]. Various physico-chemical parameters were used in these models, including structural effects, $\beta$-turn formation corrections, $N$- and $C$-terminal effects, etc. Akamatsu’s results were incorporated into the PLogP program [52]. At present, the most widely used method is an additive approach, where a molecule is divided into fragments, and the $\log P$ value is obtained by summing the contributions of each fragment. Addition of the $\log P$ values of each atom within the compound is also used, e.g., XLogP program [53]. Other approaches are based upon the use of topological indices and quantum mechanics.

Thompson et al. [42] have recently reported on the accuracy of available programs for the prediction of $\log P$ values for peptides as effective measures of hydrophobicity for use in peptide QSAR studies [54]. Eight $\log P$ prediction programs were tested, of which seven programs were fragment-based methods. Owing to the different input requirements of each program, various representations of the structures were used: amino acid sequences for use with PlogP [52]; SMILES strings [55] for ALogP, LogKow and Interactive Analysis’s LogP (IALogP); 2D SYBYL ‘mol2’ files for XlogP [53]; 3D structures from Corina [56] for MLogP and one whole-molecule approach (QikProp).

The dataset consisted of 340 peptides, varying from 2 to 16 amino acids in length, and included 141 blocked peptides, 158 unblocked peptides, and 41 cyclic peptides [42]. The predictive accuracy of the programs was assessed using $r^2$ values, with ALogP being the most effective program, and MLogP the least one. Blocked, unblocked, and cyclic peptide structures were studied. All programs gave better predictions for blocked peptides, while, in general, $\log P$ values for cyclic peptides were under-predicted and those of unblocked peptides were over-predicted. The performance of the programs (from best to
worse) for cyclopeptides was as follows: LogKow, ALogP, XLogP, MLogP, QikProp, ACDLogP, IALogP [42].

Hattotuwagama and Flower [57] developed a new approach to the prediction of log \( P \) values for both blocked and unblocked peptides based on an empirical relationship between global molecular properties and measured physical properties. The final model consisted of five physico-chemical descriptors: molecular weight, number of single bonds, bidimensional van der Waals (2D-VDW) volume, bidimensional hydrophobic and polar van der Waals surface area (2D-VSA). The approach was peptide specific and its predictive accuracy was high, but was not applied to cyclopeptides.

It is worth to mention that many authors has suggested that measuring the partition into other organic phases, such as phospholipids bilayers or micelles, might be more adequate than \( n \)-hexane for seeking biologically-relevant measures of peptide hydrophobicity.

**Antimicrobial activity of peptides**

The extensive clinical use of the classical antibiotics has led to resistant bacteria strains, in particular those responsible for infectious diseases [58,59]. Then, new effective antibiotics are required [60]. Cationic antimicrobial peptides can represent such a class of antibiotics [61,62].

The endotoxin of the Gram-negative bacteria is a lipopolysaccharide (LPS), which is a component of the outer membrane of these bacteria, and the endotoxic membrane anchor moiety is a lipid A (LA) [63]. LPS is spread out during Gram-negative bacterial infection and antimicrobial therapy and/or bacteria lysis, and may result in a lethal endotoxemia [64]. Therefore, the target of any novel class of antimicrobial peptides is the neutralization of LPS and/or LA [65].

Endotoxin-binding host defence proteins showed that an LPS- and LA-binding substructure was formed by amphipathic sequences, \( e.g., \) hydrophilic and hydrophobic moieties into opposite faces of the molecule [66,67], rich in cationic residues with a \( \beta \)-sheet conformation [68,69]. The amphipathicity of antimicrobial peptides was necessary for their mechanism of action, because the positively charged polar face would help the molecules reach the biomembrane through electrostatic interaction with the negatively charged head groups of phospholipids, and then the nonpolar face of the peptides will allow insertion into the membrane through hydrophobic interactions, causing increased permeability and loss of barrier function of target cells [68,70]. Then, amphipathic cationic peptides were proposed as antimicrobials against Gram-negative bacteria by targeted disruption of LPS [71].
Antimicrobial peptides take part in the innate immune response by providing a rapid first-line defence against infection [72]. Examples of antimicrobial peptides are magainins, cecropins, defensins, lactoferricins, tachypleins, protegrins, thanatin, and others [73,74]. Antimicrobial peptide databases have been recently reported [75,76].

These compounds have been classified into three classes on the basis of secondary structures: a) linear peptides with propensity for amphiphilic α-helical structure [77,78], which mainly occur as disordered structures in aqueous media and become amphipathic helices upon interaction with the hydrophobic membranes [79,80], e.g., cecropins, magainins, and melittins; b) peptides with β or αβ structure stabilized by different number of disulfide bridges. The β-sheet class consists of cyclic peptides constrained in this conformation either by intramolecular disulfide bonds, e.g., defensins [81] and protegrins [82], or by an N-terminal to C-terminal covalent bond, e.g., gramicidin S and tyrocidins [83]; and c) peptides with over-representation of certain amino acids or unusual structures. The third group has been recently reviewed [84]; it includes aromatic amino acid-rich peptides, (Pro-Arg)-rich peptides, unusual defensins and defensin-like molecules, unusual antimicrobial peptides from amphibians, bacteriocins with unusual structure and anionic antimicrobial peptides [84].

Design of new molecules has been achieved using combinatorial-chemistry procedures coupled to high-throughput screening systems and data processing with design-of-experiments (DOE) methodology to obtain QSAR equation models and optimized compounds. Upon selection of best candidates with low cytotoxicity and moderate stability to protease digestion, anti-infective activity has been also evaluated in plant-pathogen model systems [85].

Large-scale production can be achieved by solution organic or chemoenzymatic procedures in the case of very small peptides, but, in many cases, production can be performed by biotechnological methods using genetically modified microorganisms (fermentation) or transgenic crops (plant biofactories) [85].

A variety of human proteins and peptides has antimicrobial activity and plays important roles in innate immunity. There are three important groups of human antimicrobial peptides, defensins, histatins, and cathelicidins [86]. Defensins are cationic non-glycosylated peptides containing six cysteine residues that form three intramolecular disulfide bridges, resulting in a triple-stranded β-sheet structure, e.g., α-defensins and β-defensins in humans. The second group is the family of histatins, which are small, cationic, histidine-rich peptides present in human saliva. Histatins adopt a random coil
conformation in aqueous solvents and form α-helices in non-aqueous solvents. The third group comprises only one antimicrobial peptide, the cathelicidin LL-37. This peptide is derived proteolytically from the C-terminal end of the human CAP18 protein. Just like the histatins, it adopts a largely random coil conformation in a hydrophilic environment, and forms an α-helical structure in a hydrophobic environment [86]. Cathelicidin and defensin gene families are multifunctional natural antibiotic peptides and signalling molecules that activate host cell processes involved in immune defence and repair [87,88]. In mammals, defensins have evolved to have a central function in the host defence properties of granulocytic leukocytes, mucosal surfaces, skin and other epithelia. Three structural subgroups of mammalian defensins are involved as effectors of antimicrobial innate immunity [89].

Furthermore, over 80 different α-defensin or β-defensin peptides are expressed by the leukocytes and epithelial cells of birds and mammals. Although these compounds may be candidates for therapeutic development due to the broad-spectrum antimicrobial properties, there are technical limitations related to their size (30-45 residues) and complex structure. Therefore, minidefensins have been developed, which are antimicrobial peptides with 16-18 residues, approximately half the number found in α-defensins [90]. The θ-defensins are evolutionarily related to α- and β-defensins, but other minidefensins probably arose independently. Like α- or β-defensins, minidefensin molecules have a net positive charge and a largely β-sheet structure that is stabilized by intramolecular disulfide bonds. Whereas α-defensins are found only in mammals and θ-defensins only in nonhuman primates, the other minidefensins come from widely divergent species, including horseshoe crabs, spiders, and pigs. Several α-defensins and minidefensins are effective inhibitors of HIV-1 infection in vitro, and recent evidence implicates α-defensins in resistance to HIV-1 progression in vivo [90].

Cyclic peptides have been and are under study as potential antimicrobial therapeutic agents. These peptides usually exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, fungi and enveloped viruses [86]. Combinatorial synthesis of cyclic peptides together with antimicrobial screening and other bioactivities can provide new lead identification, and construction of QSARs. Redman et al. [91] reported a new sequencing protocol for rapid identification of the members of a cyclic peptide library based on automated computer analysis of mass spectra, new lead identification, and construction of QSARs. The utility of the new MS-sequencing approach was demonstrated using sonic spray ionization ion trap MS and MS/MS spectrometry on a single compound per bead cyclic peptide
library and validated with individually synthesised pure cyclic $D,L$-$\alpha$-peptides [91].

Also, complex libraries of glycosidated cyclic peptides, which are an important class of drug-like compounds, have been developed by incorporating glycosidated amino acids into linear peptides via solid-phase peptide synthesis followed by thioesterase-mediated peptide cyclization [92].

Then, the two major classes of cationic amphipathic antimicrobial peptides are $\alpha$-helical and $\beta$-sheet peptides [93,94]. In fact, potent cyclic antimicrobial peptides selective for Gram-negative bacteria have been successfully developed on the basis of the $\beta$-stranded framework mimicking the putative LPS-binding sites of the LPS-binding protein family [95].

The disadvantage of antimicrobial peptides for clinical use as antibiotics is their toxicity or ability to lyse eukaryotic cells [61]. Then, it would be necessary to dissociate anti-eukaryotic activity from antimicrobial activity in order to use them as broad-spectrum antibiotics.

SAR studies indicated that changes in the amphipathicity of these antibacterial peptides could be used to dissociate the antimicrobial activity from the haemolytic activity [96,97]. Furthermore, peptide cyclization increased the selectivity for bacteria because of substantially reducing the haemolytic activity [98].

Antimicrobial and haemolytic activities of de novo designed cyclic $\beta$-sheet gramicidin S analogues have been successfully dissociated by systematic alterations in amphipathicity/hydrophobicity through $D$-amino acid substitutions [99,100]. Chen et al. [48,101] also demonstrated that in linear peptides the helix-destabilizing properties of $D$-amino acids offered a systematic approach to the controlled alteration of the hydrophobicity, amphipathicity, and helicity of amphipathic $\alpha$-helical model peptides.

Frecer et al. [102] reported the de novo design of a series of synthetic cyclic amphipathic cationic peptides for which a high affinity of binding to LA was predicted from molecular modelling. These V peptides were composed of two identical symmetric amphipathic LPS- and LA-binding motifs containing cationic residues, such as $HBHPHBH$ and $HBBHBHBH$ (where $B$ is a cationic residue, $H$ is a hydrophobic residue, and $P$ is a polar residue), that formed two strands of a $\beta$-hairpin joined by a $G_9S_{10}G_{11}$ turn on one side and a disulfide bond between C$_1$ and C$_{19}$ bridging the N- and C-terminal residues on the other side (Cys$_1$-Cys$_{19}$ disulfide bridge linking the terminal residues) (Fig. 5). The structure of each peptide was cyclized via a disulfide bridge, and showed a $\beta$-sheet conformation, which would bind to the bisphosphorylated glucosamine disaccharide head group of LA, primarily by ion-pair formation between anionic phosphates of LA and the cationic side chains [68].
The V peptides contained seven alternating H and B or P residues with the general sequence Ac-C-HBHB(P)HBHGSG-HBHB(P)HBH-C-NH₂, where Ac was an acetyl group. The two LPS- and LA-binding sites showed structural similarity to cyclic β-sheet defence peptides, such as protegrin 1, thanatin, and androctonin [73]. Peptides were further characterized by electrospray ionization mass spectrometry (ESI-MS) and amino acid analysis.

MD simulations showed that the backbone conformations of free V peptides evolved from the initial β-hairpin with defined patterns of secondary structure into flexible random conformations. The patterns of the molecular shape fluctuations and torsional flexibility indicated high degrees of flexibility of the free V peptides in solution [102].

Antibacterial activity test, haemolytic activity assay, and cytotoxicity test were carried out. The therapeutic index, which is a widely used parameter to represent the specificity of antimicrobial reagents, was calculated by the ratio of minimal haemolytic concentration (MHC) (haemolytic activity) and minimal inhibitory concentration (MIC) (antimicrobial activity); thus, larger values in therapeutic index indicated higher antimicrobial specificity [59,103].

The therapeutic index could be increased either by increasing antimicrobial activity or decreasing haemolytic activity, while maintaining antimicrobial activity.

High peptide hydrophobicity and amphipathicity also led to a higher peptide self-association in solution. When the self-association of a peptide in aqueous media was too strong, it would decrease the ability of the peptide to dissociate and penetrate into the biomembrane and to kill target cells. Temperature profiling in RP-HPLC from 5 to 80°C was used to measure self-association of small amphipathic molecules, including cyclic β-sheet peptides [104], accounting for dimerization of the peptides at 5 °C and the monomerization of peptides at 80 °C because of dissociation of the dimers [102].

A higher ability to self-associate in solution was correlated with weaker antimicrobial activity and stronger haemolytic activity of the peptides. In addition, self-associating ability was correlated with the secondary structure of peptides, i.e. disrupting the secondary structure by replacing the L-amino
acid with its $D$-amino acid counterpart decreased the peptide association parameter ($PA$) values [102].

Therefore, the $D$-amino acid substituted peptides possessed an enhanced average antimicrobial activity compared with $L$-diastereomers.

**QSAR models for antimicrobial cyclopeptides**

QSARs were obtained by associating the experimental biological potencies to physico-chemical molecular properties obtained from the peptide sequences [102]. A rational strategy was used to design cationic antimicrobial peptides via repeated sequences of alternating cationic and nonpolar residues.

According to the above mentioned considerations, to achieve a high level of antimicrobial activity and selectivity toward bacteria instead of eukaryotic cells, systematic modifications of molecular properties were made by varying the amino acid residues of the amphipathic LPS- and LA-binding motifs [68] while preserving the size, symmetry, and amphipathic character of the peptides.

In peptides V1 to V7, the molecular charges, amphipathicities, and lipophilicities of the peptides were modulated by varying the cationic (polar) amino acid residues in the center of the binding motifs, where $B(P)$ was Lys or Arg (Ser or Gln), and the hydrophobic residues, where $H$ was Ala, Val, Phe, or Trp, which preserved the symmetries, sizes, and amphipathic characters of the peptides with alternating polar and nonpolar residues. Lysine residues were previously shown to contribute mostly to the high affinity to LA when placed at the flanking basic residue position of the $H_B/B(P)/B_H$ motif with a β-sheet conformation [68,102].

**QSAR analysis** of peptide sequences and their antimicrobial, cytotoxic, and haemolytic activities revealed that site-directed substitutions of residues in the hydrophobic face of the amphipathic peptides with less lipophilic residues selectively decreased the haemolytic effect without significantly affecting the antimicrobial or cytotoxic activity. On the other hand, the antimicrobial effect was enhanced by substitutions in the polar face with more polar residues, which increased the amphipathicity of the peptide [105].

The combination of three molecular properties (charge, amphipathicity, and lipophilicity) was found to correlate with the observed antimicrobial, haemolytic, and cytotoxic activities of the V peptides. Single-variate QSAR correlations of these properties to the biological effects could not be established, suggesting that the membrane disruption involved a concerted process [43,106,107].

The V peptides exhibited strong effects against five Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*), with MICs in the nanomolar range, and low cytotoxic and
QSAR of cyclopeptides

haemolytic activities at concentrations significantly exceeding their MICs. Then, simple properties derived from the peptide sequences, such as the molecular charge ($Q_M$), amphipathicity index ($AI$), and lipophilicity index ($\Pi_{o/w}$), were correlated to the mean antimicrobial effect against Gram-negative bacteria by multivariate linear regression as shown in eq. 1 [102].

For antimicrobial effect:

$$\ln (\text{MIC}) = 9.49 \cdot Q_M + 10.17 \cdot AI - 0.05 \cdot \Pi_{o/w} - 22.16 \quad \text{(equation 1)}$$

The $t$ test of the multivariate correlation equation revealed that the antimicrobial effect on bacteria was mainly determined by the V-peptide charge ($Q_M$) and amphipathicity ($AI$), i.e., by the number of cationic and polar residues forming the polar face of the V peptides and their distribution throughout the two symmetric amphipathic LPS- and LA-binding motifs [102]. In fact, a higher affinity to the outer bacterial membrane seemed to be a favourable prerequisite for the antimicrobial effects, since the V peptides displayed low micromolar $K_d$ values and antimicrobial activities at concentrations in the nanomolar range. $K_d$ accounted for the dissociation constant of the peptide-LA.

The haemolytic activity of the peptides against human erythrocytes was determined as a main measure of peptide toxicity towards higher eukaryotic cells. Since both antimicrobial and haemolytic activities of the cationic peptides involved cell membrane lysis, and depended on the same physico-chemical properties [99,106] a similar correlation equation (eq. 2) was obtained for the haemolytic activities of the V peptides [102].

For haemolysis:

$$\ln (\text{EC}_{50}) = - 5.34 \cdot Q_M - 4.94 \cdot AI - 0.23 \cdot \Pi_{o/w} + 31.87 \quad \text{(equation 2)}$$

In this case the correlation parameters and the $t$ statistics showed that the haemolytic activity against eukaryotic cells was mainly influenced by the molecular lipophilicity, i.e., the sum of the lipophilicities of all residues, with the major contribution coming from the $H$ residues, which formed the nonpolar face of the V peptides, which was predicted to acquire a $\beta$-hairpin-like structure in the peptide-LA complexes [102].

The EC$_{50}$ values of the V peptides for cytotoxicity ranged from 40 M to 5.7 mM, which exceeded their MICs by up to 3 orders of magnitude. For the cytotoxic effects of the V peptides, the correlation (eq. 3) was obtained [102].

For cytotoxicity:

$$\ln (\text{EC}_{50}) = 8.98 \cdot Q_M + 11.74 \cdot AI - 0.04 \cdot \Pi_{o/w} - 8.70 \quad \text{(equation 3)}$$
Therefore, the correlation parameters and t statistics indicated that the cytotoxic activity was determined mainly by the peptide charge and the amphipathicity.

Amphipathicity of the L-amino acid substituted peptides was determined by the calculation of hydrophobic moment [108] using the software package Jemboss [109], modified to include the determined hydrophobicity scale. Hydrophobicity coefficients were determined by RP-HPLC at pH 7 (phosphate buffer) [102].

Since the antimicrobial activities of the V peptides strongly increased with the increasing amphipathicity of the molecules at constant $Q_M$ and $\Pi_{o/w}$, then, aggregates of V peptides rather than individual molecules would behave as strong antimicrobials [102]. The ability of cationic peptides to form aggregates has been related to their antimicrobial potencies, as previously reported for dermaseptin S4 [110,111], protegrin-1 [112], and human defensins [59,113].

The validity of the QSAR model for the antimicrobial potencies of the V peptides against Gram-negative bacteria was verified with the set of cyclic cationic amphipathic peptides designed by Muhle and Tam [95], which were similar to the V peptides, e.g., cyclo(PACRCRAG-PARCRCAG) sequences constrained by two cross-linking disulfide bonds. These peptides displayed potent activities against Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa). The MICs of these peptides were 20 nM for E. coli. Frecer’s correlation equation for the antimicrobial activity (eq. 1) was able to reproduce the qualitative rank order of antimicrobial potencies at low salt concentrations for eight of the nine peptides [95].

Then, on the basis of QSARs, new analogues that had strong antimicrobial effects but that lacked haemolytic activity have been proposed [102].

QSAR eq. 1 predicted rapid increases in the antimicrobial activity with an increase in the molecular charge $Q_M$ over 4 (in units of electron charge) when amphipathicity and hydrophobicity were kept constant at the levels of the most promising peptide, V4. Model analogues of V4 that shared the polar HKHQHKH motif and that differed only in the $H$ residues (which retained the amphipathicity index of V4) were predicted to possess decreasing haemolytic activities with decreasing lipophilicities, while their predicted antimicrobial and cytotoxic activities remained unchanged. In other analogues of V4, the replacement of the two central Gln residues by more polar Asn residues was predicted to lead to significantly increased antimicrobial potencies due to the increased amphipathicity independent of the $H$ residues (predicted MICs were lower than that of V4) [102].
Thus, variations in the $H$ residues forming the hydrophobic face of the analogues of V4 mainly affected the haemolytic activity, which was shown to depend strongly on $\Pi_{o/w}$, but did not affect the predicted antimicrobial activity of the analogues. Therefore, replacement of the $H$ residues with less hydrophobic residues in the nonpolar face of the amphipathic analogues was appropriate for decreasing the haemolytic activities of the V peptides.

On the other hand, directed substitutions of the $B$ and $P$ residues in the polar faces of the V peptides with more polar residues, which increased the amphipathic character (more negative $AI$ values) of the peptide while keeping the net charge, the symmetry of the binding motifs, and the composition of the hydrophobic face, were predicted to bring about a significant increase in antimicrobial potencies [59,102].

The positively charged antimicrobial peptide cyclo[VKLdKVdYPLKVKL dYP] (GS14dK4), which is a diastereomeric lysine ring-size analogue of the naturally occurring antimicrobial peptide gramicidin S (Fig. 6), exhibited enhanced antimicrobial and markedly reduced haemolytic activities compared with gramicidin S itself [114].

The binding of GS14dK4 to various lipid bilayer model membranes has been recently studied using isothermal titration calorimetry [114]. Dynamic light scattering results indicated the absence of any peptide-induced major alteration in vesicle size or vesicle fusion under the experimental conditions. The binding of GS14dK4 was significantly influenced by the surface charge density of the phospholipid bilayer and by the presence of cholesterol. The presence of cholesterol markedly reduced the affinity of a peptide for phospholipid bilayers. The binding isotherms could be described quantitatively by a one-site binding model. The measured endothermic binding enthalpy ($\Delta H$) varied strongly (+6.3 to +26.5 kcal/mol) and appeared to be inversely related to the order of the phospholipid bilayer system. However, the negative free energy ($\Delta G$) of binding remained relatively constant (-8.5 to -11.5 kcal/mol) for all lipid membranes examined. The

![Figure 6. Structure of gramicidin S.](image)
relatively small variation of negative free energy of peptide binding together with a pronounced variation of positive enthalpy produced an equally strong variation of $T\Delta S$ (+16.2 to +35.0 kcal/mol), indicating that GS14dK4 binding to phospholipids bilayers was primarily entropy driven [114].

The properties and SAR studies of a macrocyclic analogue of porcine protegrin-1 have been recently reported [115]. Protegrin-1 (PG-1) is an 18-residue β-hairpin peptide containing two disulfide bridges (Cys$_{6-15}$ and Cys$_{8-13}$) that belongs to the cathelicidin class of antimicrobial peptides (Fig. 7). These disulfides constrained the peptide backbone into a β-hairpin conformation, with a β-turn formed by residues 9–12, as detected by NMR.

SAR studies [116] led to the discovery of analogue IB367 with Cys$_{5-14}$ and Cys$_{7-12}$ disulfide bridges, which has been clinically tested to treat ulcerative oral mucositis, ventilator associated pneumonia, and respiratory infections associated with cystic fibrosis. An approach to PG-1 peptidomimetics has been earlier reported [117,118] based on the use of β-hairpin-stabilizing organic templates. The template D-Pro-Pro was chosen for its ability to promote a β-hairpin loop structure. This design was used to prepare β-hairpin peptidomimetics [119-122].

The lead compound, containing the sequence cyclo(Leu-Arg-Leu-Lys-Lys-Arg-Arg-Trp-Lys-Tyr-Arg-Val-D-Pro-Pro), showed antimicrobial activity against Gram-positive and Gram-negative bacteria, but a much lower haemolytic activity than PG-1.

SAR studies were carried out on over 100 single site substituted synthetic analogues, and the biological profiles were assessed. Some analogues showed slightly improved antimicrobial activities (2–4-fold lowering of MICs), whereas other substitutions caused large increases in haemolytic activity [115].

Frecer [123] quantitatively analysed antimicrobial and haemolytic activities of protegrin-1 mimetics-cyclic cationic peptides with β-hairpin fold synthesised by Robinson et al. [115] (Fig. 7).

![Figure 7. Structure of protegrin-1 (PG-1) and cyclic β-hairpin peptides-analogues of PG-1.](image-url)
The polar face of the cyclic lead peptide R1 [115] was formed by the side chains of cationic residues 2, 4, 6, 7, 9, 11 and D-Pro13 and Pro14, while the nonpolar face consisted of residues 1, 3, 5, 8, 10 and 12 with predominant lipophilic/aromatic character.

For the QSAR models, the selected properties, which characterized peptide's charge, lipophilicity, amphipathicity, size, shape and flexibility were used, including the following descriptors: charge ($Q$), overall lipophilicity ($L$), lipophilicity of polar and nonpolar faces ($P$ and $N$), surface areas of polar and nonpolar faces ($S_P$ and $S_N$), molecular mass of the polar and nonpolar faces ($M_{WP}$ and $M_{WN}$), count of small lipophilic, highly lipophilic and aromatic residues forming the nonpolar face ($C_{SL}$, $C_{HL}$ and $C_{AR}$), total number of hydrogen bond donor and acceptor centres ($HB_{don}$ and $HB_{acc}$), total number of rotatable bonds (RotBon) and various amphipathicity descriptors ($P/L$, $P/N$, $L/N$, $Q/L$, $Q/N$, $S_P/S_N$, $M_{WP}/M_{WN}$, $Q/C_{SL}$, $Q/C_{HL}$ and $Q/C_{AR}$) [123]. These simple additive molecular descriptors were easily derived from peptide sequences and tabulated amino acid properties [124].

Freer [123] assumed that the analogues adopted an amphipathic β-hairpin secondary structure, which was in agreement with the fact that protegrins and synthetic analogues with a constrained β-hairpin conformation displayed higher antimicrobial potencies than linear or nonconstrained counterparts [69,125].

The best models obtained by application of genetic function approximation algorithm correlated antimicrobial potencies (log $MIC_a$) to peptide's charge and amphipathicity index, while haemolytic effect (log %Hem) correlated well with the lipophilicity of residues forming the nonpolar face of the β-hairpin [123].

The lipophilicity of the nonpolar face $N$ and the amphipathicity parameter $Q/N$ showed some relation to the antimicrobial activity, while $N$ and the intercorrelated count of highly lipophilic residues in the nonpolar face ($C_{HL}$) appeared to be related to the haemolytic activity. This finding was consistent with the above-mentioned QSAR studies (eqs. 1-3), which suggested that charge and amphipathicity correlated with MIC of cyclic cationic peptides and overall lipophilicity was very important for the haemolytic activity [102].

A large set of QSAR models combining up to five descriptors in each correlation equation was prepared by the genetic function approximation (GFA) algorithm [126] of the Cerius² package. The fitness of each generated model was evaluated by using the lack-of-fit score [127]. The best performing QSAR model of the antimicrobial effect of R1 analogues accounted for two descriptors, molecular charge $Q$ and amphipathicity parameter $Q/N$, which are
determined by the cationic residues forming mainly the polar face and the lipophilicity of the nonpolar face, $N$ (eq. 4) [123].

$$\log \text{MIC}_a = 1.291 - 0.180 \cdot Q + 1.438 \cdot (Q/N)$$  \hspace{1cm} \text{(equation 4)}

Antimicrobial effect of cationic peptides related to molecular charge and amphipathicity has been previously reported [102,128].

Based on this QSAR model, the best variants of R1 lead should have the polar faces formed only by charged residues and the nonpolar faces by highly lipophilic residues in order to display strong antimicrobial activity. Thus, any analogue with the same charge as the lead peptide R1 ($Q = 7 \hat{e}$) should show more potent antimicrobial activity than R1 when the lipophilicity of its nonpolar face $N \geq 6.84$ (value of $N$ for R1) [123]. MIC$_a$ values were validated for the 97 peptides of Robinson et al. [115] for their $Q$ and $N$ descriptors [123].

The best QSAR model of the haemolytic effect for the R1 analogues related the lysis of human erythrocytes to the lipophilicity of the nonpolar face, $N$ (eq. 5) [123].

$$\log \%\text{Hem} = -2.551 + 0.431 \cdot N$$  \hspace{1cm} \text{(equation 5)}

Therefore, the haemolytic potency of R1 analogues depended mainly on the lipophilicity of the nonpolar face, thus being almost independent on the charge and composition of the polar face. Based on this QSAR model, any analogue with $N \leq 6.84$ should show lower levels of haemolytic activity than the lead peptide R1 [123]. The %Hem values of the 97 peptides of Robinson et al. [115] fitted this model.

The combination of the QSAR models with the cyclic backbone of the protegrin analogues (constant turns, residues 6, 7 and 13, 14), sequence amphipathicity (regular alternation of cationic and nonpolar residues) and peptide symmetry provided a sufficient strategy for peptide design. The secondary structure of the backbone of the peptides NR7–NR9 was stabilized by a Cys$_3$-Cys$_{10}$ disulfide bridge in the form of a cyclic $\beta$-hairpin [123]. Tam et al. [128] showed by circular dichroism experiments that cyclic protegrins containing one to three cysteine bonds displayed some degree of $\beta$-strand structure in solution. The occurrence of the $\beta$-hairpin fold was essential for membrane permeation/disruption by PG-1 analogues as previously reported [128-130].

Recently, Bhonsle et al. [131] used 3D-QSAR for identification of descriptors defining bioactivity of antimicrobial peptides. The resulting 3D-physico-chemical properties were controlled by the placement of amino acids with well-defined properties (hydrophobicity, charge density, electrostatic
potential, and those mentioned above) at specific locations along the peptide backbone. These peptides exhibited different *in vitro* activity against *Staphylococcus aureus* and *Mycobacterium ranae*. The differences in the biological activity seem to be due to different physico-chemical interactions that occur between the peptides and the cell membranes of the bacteria. 3D-QSAR analyses showed that specific physico-chemical properties were responsible for antibacterial activity and selectivity. There were five physico-chemical properties specific to the *S. aureus* QSAR model, while five properties were specific to the *M. ranae* QSAR model. Accordingly, for any particular antimicrobial peptide, organism selectivity and potency are controlled by the chemical composition of the target cell membrane [131].

As described above, cationic peptide antibiotics possess amphiphilic structure, thereby displaying lytic activity against bacterial cell membranes. Naturally occurring antimicrobial peptides contain a large number of amino acid residues, which limit their clinical applicability. Recent studies indicated that it is possible to decrease the chain-length of these peptides without loss of activity, and suggested that a minimum of two positive ionizable (hydrophilic) and two bulky groups (hydrophobic) are required for antimicrobial activity. By employing the HipHop module of the software package CATALYST, these experimental findings have been translated into 3D pharmacophore models by finding common features among active peptides [132]. Positively ionizable and hydrophobic features were the important characteristics of compounds used for pharmacophore model development. Based on the highest score and the presence of amphiphilic structure, two separate hypotheses, Ec-2 and Sa-6 for *Escherichia coli* and *Staphylococcus aureus*, respectively, were selected for mapping analysis of active and inactive peptides against these organisms. The resulting models not only provided information on the minimum requirement of positively ionizable and hydrophobic features but also indicated the importance of their relative arrangement in space. The minimum requirement for positively ionizable features was two in both cases, but the number of hydrophobic features required in the case of *E. coli* was four, while for *S. aureus* it was found to be three [132]. Hypotheses were further validated using cationic steroid antibiotics, a different class of facial amphiphiles with the same mechanism of antimicrobial action as that of cationic peptide antibiotics. The results showed that cationic steroid antibiotics also require similar minimum features to be active against both *E. coli* and *S. aureus*. 
Interaction of antimicrobial peptides in biomembranes

Cytoplasmic membrane is the main target of some antimicrobial peptides [133]. In fact, all cationic amphipathic peptides interact with membranes [134,135]. Cationic peptides first bind to the negatively charged LPS or LA of Gram-negative bacteria [98,136], then permeate the membrane by different mechanisms, finally leading to bacteria death. The development of resistance to membrane active peptides whose target is the cytoplasmic membrane is not expected because this would imply severe changes in the lipid composition of cell membranes of microorganisms.

The mechanism of bacterial membrane disruption by cationic amphipathic peptides should involve several molecular properties of the peptides: a net positive charge (attachment to anionic outer membrane constituents), amphipathicity (aggregation on the membrane surface), and lipophilicity (permeation into the membrane), as has been discussed above [137].

Many models have been proposed on the interaction of cationic amphipathic antimicrobials with the cytoplasmic membrane [61,136-139] because lethal action could be either from membrane disruption or from translocation through the membrane to target receptors inside the cell.

Two main proposed mechanisms are: (i) The “barrel-stave” mechanism: the peptide may form transmembrane channels/pores, as their hydrophobic surfaces interact with the lipid core of the membrane and the hydrophilic surfaces point inwards, producing an aqueous pore [140]; (ii) The “carpet” mechanism: peptides lie at the interface parallel with the membrane allowing their hydrophobic surface to interact with the hydrophobic component of the lipid, and the positive charge residues can still interact with the negatively charged head groups of the phospholipid [141].

An NMR study of the amphipathic cyclic β-sheet antimicrobial peptide of gramicidin S [142] supported the interface model. However, neither of these mechanisms alone could fully explain the reported data.

The mechanism of action depends upon the difference in membrane composition between prokaryotic and eukaryotic cells [143]. If the peptides formed pores/channels in the hydrophobic core of the eukaryotic bilayer, they would cause the hemolysis of human erythrocytes. On the contrary, for prokaryotic cells the peptides lysed cells in a detergent-like mechanism as described in the carpet mechanism.

In fact, the extent of interaction between peptide and biomembrane depends on the composition of the lipid bilayer. Liu et al. [144,145] used a polyleucine-based α-helical transmembrane peptide to demonstrate that the peptide reduced the phase transition temperature to a higher extent in
phosphatidylethanolamine (PE) bilayers than in phosphatidylcholine (PC) or phosphatidylglycerol bilayers, indicating a higher disruption of PE organization. The zwitterionic PE is the main lipid component in prokaryotic cell membranes, and PC is the main lipid component in eukaryotic cell membranes [146].

According to the results, the carpet mechanism is essential for strong antimicrobial activity, and if there were a preference by the peptide for penetration into the hydrophobic core of the bilayer, the antimicrobial activity would actually decrease [143].

Recently, membrane interactions of designed cationic antimicrobial peptides were reported [147]. Novel cationic antimicrobial peptides typified by sequences such as KKKKKAX-AAXAAXA-NH₂, where X = Phe/Trp-displayed high antibacterial activity, but exhibited little or no haemolytic activity towards human red blood cells even at high doses. To clarify the mechanism of their selectivity for bacterial vs. mammalian membranes and to increase the understanding of the relationships between primary sequence and bioactivity, a library of derivatives was prepared by increasing segmental hydrophobicity, in which systematic substitutions of Ala for two, three, or four Leu residues were made. Conformationally constrained dimeric and cyclic derivatives were also synthesised. The peptides were examined for activity against pathogenic bacteria (*Pseudomonas aeruginosa*), haemolytic activity on human red blood cells, and insertion into models of natural bacterial membranes (containing anionic lipids) and mammalian membranes (containing zwitterionic lipids + cholesterol). Results were compared with the corresponding properties of the natural cationic antimicrobial peptides magainin and cecropin. Using circular dichroism and fluorescence spectroscopy, Gluckhov et al. [147] found that peptide conformation and membrane insertion were sequence dependent, both upon the number of Leu residues, and upon their positions along the hydrophobic core. Membrane disruption was likely enhanced by the fact that the peptides contained potent dimerization-promoting sequence motifs, as assessed by SDS-PAGE gel analysis. The overall results led to identify distinctions in the mechanism of actions of these cationic antimicrobial peptides for disruption of bacterial vs. mammalian membranes, the latter dependent on surpassing a "second hydrophobicity threshold" for insertion into zwitterionic membranes.

**Anticancer and cytotoxic activities**

There is a need for novel drugs for the treatment of infectious diseases, autoimmunity and cancer. Cyclic peptides constitute a class of compounds
that have made important contributions to the treatment of certain diseases. Penicillin, vancomycin, cyclosporin, the echinocandins and bleomycin are well-known cyclic peptides [148].

Cyclic peptides, compared to linear peptides, have been considered to have greater potential as therapeutic agents due to their increased chemical and enzymatic stability, receptor selectivity, and improved pharmacodynamic properties. They have been used as synthetic immunogens, transmembrane ion channels, antigens for Herpes Simplex Virus, potential immunotherapeutic vaccines for diabetes and Experimental Autoimmune Encephalomyelitis - an animal model of Multiple Sclerosis, as inhibitors against α-amylase and as protein stabilizers. Cyclic peptides as therapeutic agents in disease have been recently reviewed [148].

Isolation and anti-cancer effects of cyclotides obtained from Violaceae plants have been also reviewed [149]. A fractionation protocol was developed, leading to varv cyclotides from *Viola arvensis* (Violaceae). Separation methods included adsorption, ion exchange chromatography and solvent-solvent partitioning. Structures were determined on the basis of MS for cyclotide sequencing and mapping of disulfide bonds. Finally, to assess SARs, regarding their anti-cancer and cytotoxic effects, the three dimensional structures of cyclotides were characterized by homology modelling techniques [149].

Cytotoxic cyclotides were obtained from *Viola tricolor* [150]. Bioguided fractionation was carried out by RP-HPLC and a fluorometric cytotoxicity assay. Cyclotides were assayed against two human cancer cell lines, U-937 GTB (lymphoma) and RPMI-8226/s (myeloma). The most potent compounds isolated, which showed the lowest IC$_{50}$ values, were: vitri A (IC$_{50}$ = 0.6 μM and IC$_{50}$ = 1 μM, respectively), varv A (IC$_{50}$ = 6 μM and IC$_{50}$ = 3 μM, respectively), and varv E (IC$_{50}$ = 4 μM in both cell lines). Their sequences, determined by automated Edman degradation, quantitative amino acid analysis, and MS, were *cyclo*-GESCVWIPCITSAGCSCSCKVCRYNIGPC (vibri A), *cyclo*-GETCVGTCNTPGCSWCSPVCTNGLPC (varv A), and *cyclo*-GETCVGTCNTPGCSWCSPVCTNGLPI (varv E) [150].

Cycloviolacin H4, a hydrophobic cyclotide, was isolated from the Australian native violet *Viola hederaceae*. Its sequence, *cyclo*-CAESCVEIPCTVTALLGCSCSNVNCYNgP, was determined by nanospray MS/MS and quantitative amino acid analysis. This cyclotide was classified into the bracelet subfamily of cyclotides due to the absence of a cis-Pro peptide bond in the circular peptide backbone. Cycloviolacin H4 exhibited the most potent haemolytic activity in cyclotides, and this activity correlated with the size of a surface-exposed hydrophobic patch. These findings provided insight into the factors that modulate the cytotoxic properties of cyclotides [151].
Recently, the sequences of 11 cyclotides, vibi A-K, isolated from the alpine violet *Viola biflora*, were determined by MS/MS sequencing of proteins and screening of a cDNA library of *V. biflora* in parallel [152]. To correlate amino acid sequence to cytotoxic potency, vibi D, E, G and H were analysed by a fluorometric microculture cytotoxicity assay using a lymphoma cell line. The IC$_{50}$-values of the bracelet cyclotides vibi E, G and H ranged between 0.96 and 5.0 μM while the Möbius cyclotide vibi D was not cytotoxic at 30 μM [152].

Rational design, structure, and biological evaluation of cyclic peptides mimicking the vascular endothelial growth factor (VEGF) have been recently reported [153]. Angiogenesis is the development of a novel vascular network from a pre-existing structure. Blocking angiogenesis is an attractive strategy to inhibit tumor growth and metastasis formation. Based on structural and mutagenesis data, novel cyclic peptides were developed, which mimic, simultaneously, two regions of the VEGF crucial for the interaction with the VEGF receptors. The peptides, displaying the best affinity for VEGF receptor 1 on a competition assay, inhibited endothelial cell transduction pathway, migration, and capillary-like tubes formation. The specificity of these peptides for VEGF receptors was demonstrated by microscopy using a fluorescent peptide derivative. The resolution of the structure of some cyclic peptides by NMR and molecular modelling allowed the identification of various factors accounting for their inhibitory activity [153].

The interest in the application of the QSAR paradigm has steadily increased in recent decades and it may be useful in the design and development of DNA-binding molecules as new anticancer agents. Due to the great potential of DNA as a receptor, many classes of synthetic and naturally occurring molecules exert their anticancer activities through DNA-binding [154].

In the field of antitumour DNA-binding agents, a number of acridine and anthracycline derivatives are in the market as chemotherapeutic agents. However, the clinical application of such compounds has shown multi-drug resistance and secondary and/or collateral effects. Therefore, there has been increasing interest in discovering and developing small molecules that are capable of DNA-binding.

Recently [154], the DNA-binding properties of different compound series have been discussed using 27 QSAR models. The most important determinants for the activity in these models were Hammett electronic (σ and σ+), hydrophobic, molar refractivity, and Sterimol width parameters.

P-glycoprotein is implicated in multiple drug resistance exhibited by several types of cancer against a multitude of anticancer chemotherapeutic agents. Therefore, several research groups searched for effective
P-glycoprotein inhibitors. Cyclosporine A, aureobasidin A and related analogues were reported to possess potent inhibitory actions against P-glycoprotein. Recently, receptor surface analysis was used to construct two satisfactory receptor surface models for cyclosporine- and aureobasidin-based P-glycoprotein inhibitors [155]. These pseudoreceptors were combined to achieve satisfactory 3D-QSAR for 68 different cyclosporine and aureobasidin derivatives. Upon validation against an external set of 16 randomly selected P-glycoprotein inhibitors, the optimal 3D-QSAR was found to be self-consistent and predictive ($r^2_{\text{LOO}} = 0.673$, $r^2_{\text{PRESS}} = 0.600$). The resulting 3D-QSAR was employed to probe the structural factors that control the inhibitory activities of cyclosporine and aureobasidin analogues against P-glycoprotein [155].

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