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REVIEW

NMR and protein structure in drug design: application to cyclotides and conotoxins

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Abstract Nuclear magnetic resonance spectroscopy (NMR) is a powerful technique for determining the structures, dynamics and interactions of molecules, and the derived information can be useful in drug design applications. This article gives a brief overview of the role of NMR in drug design and illustrates this role with examples studied in our laboratory in recent years on disulfide-rich peptides, including cyclotides and conotoxins. Cyclotides are head-to-tail cyclized proteins from plants that are exceptionally stable and hence make useful templates for the stabilization of bioactive peptide epitopes as well as potential leads for anti-HIV drugs. Natural cyclotides target cell membranes, so understanding cyclotide-membrane interactions is useful in applying cyclotides in drug design applications. NMR studies of these interactions are described in this article. Conotoxins are disulfide-rich peptides, from the venoms of marine cone snails, which are of pharmaceutical interest because they potently interact with a range of ion channels, transporters and other receptor sites implicated in disease states. Chemically re-engineering conotoxins to give them a cyclic backbone has been used to engender them with improved biopharmaceutical properties, such as are observed in cyclotides.

Membrane-active peptides: 455th WE-Heraeus-Seminar and AMP 2010 Workshop.

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Introduction

This manuscript follows the content of a lecture given by David Craik at the 1st Australian-Croatian conference on membrane-acting peptides held in Split in August 2010. The purpose of the lecture was two-fold: first, to give an overview of general applications of NMR spectroscopy in drug design and development, and second, to illustrate the principles of NMR and drug design with particular applications to two classes of molecules that have been extensively studied in our laboratory, namely cyclotides (Craik et al. 1999; Göransson et al. 2004; Craik et al. 2006a, b) and conotoxins (Daly and Craik 2009; Clark et al. 2010). The overriding aim was to illustrate how a diverse range of NMR techniques can assist in the drug design and development process, with particular reference to peptides, since peptides were the major theme topic of the conference.

Figure 1 gives a schematic overview of the general process of drug design. It starts with the premise that the action of a drug, which might be a small molecule, peptide or protein, involves interaction with a receptor site, which might be a protein, nucleic acid or lipid/glycolipid as part of a membrane. Binding of the drug to the receptor is an essential step for subsequent signal transduction and pharmacological action. For the purpose of this talk the focus is on the drug being a peptide and the receptor being either a lipid as part of a membrane, in the case of the cyclotides, or a membrane-bound receptor in the case of the conotoxins. The peptides that we focus on are typically smaller than 50 amino acids in size and therefore are ideally suited to structural studies using NMR spectroscopy.

By contrast, most receptors are of higher molecular weight, typically greater than 25 kDa for proteins, and are therefore better suited to structure determination by X-ray crystallography.

The arrow in the lower section of Fig. 1 illustrates the traditional drug screening process whereby small molecules are tested directly for their pharmacological activity; this information is then used in the optimization of initial hits. Such screening does not necessarily require isolation and purification of the receptor targets and, indeed, in many early studies the screening was done directly in an organ or organism, rather than at the receptor level. NMR does not necessarily play a major role in this type of screening, except as a routine structural tool to characterize compounds synthesized in the hit-to-lead optimization process. By contrast with this traditional screening approach, over the last decade NMR has made major inroads into new generation screening processes involving fragment-based screening methods. In these approaches, the *binding* process is the detection endpoint rather than the pharmacological *activity*. The arrow in Fig. 1 that illustrates this process is strategically located to emphasize that here both the drug and the purified receptor are involved in the assay procedure and the endpoint is binding. The underlying assumption is that binding necessarily leads to modulation of activity, and that binding is easier to measure in a robust biophysical screen using NMR than is pharmacological activity in an in vitro or in vivo screen. The modulation of activity might include activation (agonists), or blocking (antagonists), or either (for allosteric modulators).

Fragment-based methods started with the SAR by NMR (Structure–Activity Relationships by NMR) method developed at Abbott Laboratories in the mid 1990s (Shuker et al. 1996) and are now widely used in the pharmaceutical industry, with a number of variations having been developed. These methods have not yet led to a marketed drug, but several candidates are in clinical trials, including ABT-263 (Tse et al. 2008), and with the widespread use of fragment-based screening methods it is a relatively safe prediction that success stories will soon emerge.

The remainder of this article focuses on another way besides screening in which NMR can contribute to the drug design process, i.e., via studies of specific interactions between ligands and macromolecular targets, known generically as structure-based design. As indicated in Fig. 1, there are two general approaches for structure-based design: (i) ligand-based design and (ii) receptor-based design. In ligand-based design, particularly where the ligands are small bioactive peptides, NMR is an ideal technique for determining the three-dimensional structure of the ligand, which may then be used to guide the design process. As part of the lecture course at the 1st Australian-Croatian conference on membrane-acting peptides, a workshop on determining structures of peptides by NMR was given but will not be discussed further in this article. Several texts and review articles are available to describe the general principles of determining the structures of peptides and proteins by NMR (Wüthrich 1986; Craik and Daly 2007; Guntert 2009). We focus here on applications of two classes of peptides that we have examined in ligand-based molecular design approaches, i.e., cyclotides and conotoxins. Although the focus is on structure, it is important to recognize that NMR can also provide a great deal of information about molecular dynamics and interactions, and about other parameters relevant to drug design, as summarized in Table 1 (Craik et al. 2010). We will touch on dynamics aspects later in this article in relation to the translational diffusion of cyclotides when bound to

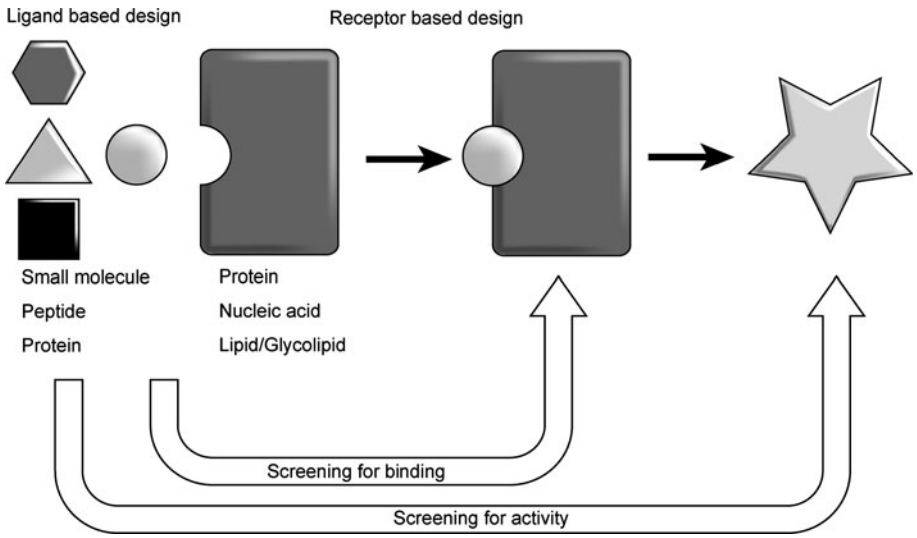

Fig. 1 Schematic overview of drug action and the drug development process. Ligand (*drug*) recognition by the receptor depends on complementary shape matches as well as the chemical microenvironment such as hydrophobicity and charge, with binding to the receptor a pre-requisite to a biological response. The *broad arrows* at the bottom of the figure highlight the traditional screening process, where the readout is biological activity (indicated by the *big star*), and NMR-based screening approaches where the readout is binding

Table 1 NMR parameters and their applications in drug design/discovery

Parameter	Information relevant to drug design
Chemical shift	Reflects local chemical environment; provides a fingerprint marker of structure and interactions (particularly in HSQC spectra)
Coupling constants	Conformational analysis, establishing molecular connectivity
Nuclear overhauser effect	Determining interproton distances, three-dimensional structures
Relaxation times	Molecular dynamics
Line-shape	Detecting and quantifying chemical exchange processes
Peak intensities	Reflect relative number of nuclei in a given peak, molecular symmetry. Also reflect binding interactions in saturation transfer difference spectra
Amide exchange rates	Reflect hydrogen bonding or solvent exposure of amide protons
Amide temperature coefficients	Reflect hydrogen bonding and/or molecular flexibility

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membrane-mimicking micelles, as well as approaches to detect intermolecular interactions, which are central to drug-receptor interactions.

Peptides as drug leads

It is widely recognized that peptides make excellent drug leads (Vlieghe et al. 2010) but in general this potential has not been translated into drugs themselves. The reason that peptides make great leads is that they have evolved to be highly specific and potent for their receptors and typically have low toxicity. However, combined with these advantages are some disadvantages, including poor stability in vivo, poor oral bioavailability, high cost, as well as high clearance from the body and, occasionally, solubility challenges. Work in our laboratory has focused on attempts to overcome these disadvantages and, in particular, to develop cyclization as a tool to stabilize peptide-based drug leads. The impetus for this approach came from discoveries of naturally-occurring cyclic peptides over the last decade. Figure 2 illustrates some examples of these naturally-occurring peptides and shows that they occur in all kingdoms of life, including bacteria, plants and animals (Trabi and Craik 2002; Craik et al. 2003; Craik 2006).

The cyclic peptides we have focused on most are the cyclotides, a large family of disulfide-rich head-to-tail cyclized proteins from plants (Craik et al. 1999). The original work in this field was initiated by Gran, a Norwegian doctor, who discovered that women in Africa used a medicinal tea from the plant *Oldenlandia affinis* to accelerate child birth (Gran 1970, 1973a, b). In 1995 we reported the NMR-determined structure (Saether et al. 1995) of the bioactive uterotonic ingredient, kalata B1, from this plant and showed that it was very unusual, comprising a head-to-tail cyclized backbone and knotted arrangement of three disulfide bonds referred to as a cyclic cystine knot (CCK) motif (Craik et al. 1999). This unique structural motif explains the exceptional stability of kalata B1, which is resistant to enzymes, heat and acid treatment (Colgrave and Craik 2004), consistent with its bioavailability in indigenous medicinal uses after being boiled to make a tea.

After determination of this structure, and seeing reports of other related macrocyclic peptides containing six Cys residues (Schöpke et al. 1993; Gustafson et al. 1994; Witherup et al. 1994) we became convinced that kalata B1 might be just one member of a much larger family of proteins, so we started a systematic discovery effort to find other examples. This search led to numerous such examples of peptides (Göransson et al. 1999; Craik et al. 1999), to which we gave the systematic name cyclotides (cyclo peptides) (Craik et al. 1999). As it happens, the cyclotides are just one class of head-to-tail cyclic disulfide-rich peptides that have since been discovered and are now being

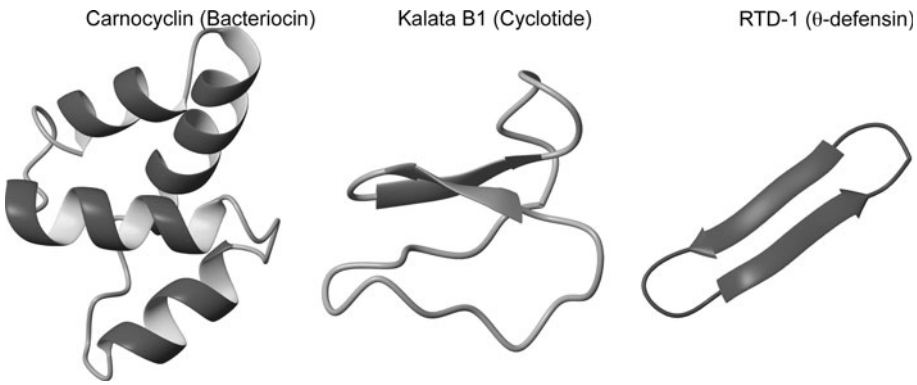

Fig. 2 Three-dimensional structures of selected naturally-occurring macrocyclic peptides. The structures shown are carnocyclin (bacteriocin) (PDB ID code 2KJF), kalata B1 (cyclotide) (PDB ID code 1NB1) and RTD-1 (defensin) (PDB ID code 1HVZ). The helices are shown with *thickened ribbons* and the *β*-strands are *arrows*. The diagram was made using MOLMOL (Koradi et al. 1996)

exploited as useful frameworks in drug design. Others being studied in our lab include θ-defensins (Tang et al. 1999; Daly et al. 2007) and sunflower trypsin inhibitors (Luckett et al. 1999; Korsinczky et al. 2001).

Cyclotides

Cyclotides are small disulfide-rich peptides from plants in the size range 28–37 amino acids that contain a cyclic cystine knot motif. Kalata B1 (Fig. 3) is the prototypic member, but we now believe that cyclotides are an exceptionally large family probably comprising around 50,000 members (Simonsen et al. 2005; Gruber et al. 2008). This large number of peptides is generated by variations in the peptide sequences in the backbone loops between successive Cys residues, leading to the idea that cyclotides can be regarded as a natural combinatorial template (Craik et al. 2006a, b). Associated with this combinatorial diversity, cyclotides have a diverse range of biological activities, including insecticidal, anti-HIV, antimicrobial, antifouling, hemolytic, and other activities (Daly et al. 2009), but our main interest in them is that they are ultra-stable molecules. As already noted, this stability derives from their cyclic cystine knot motif, illustrated in Fig. 3, in which two disulfide bonds and their connecting backbone segments form a ring that is threaded by the third disulfide bond. Cyclotides fall into three subfamilies: Möbius, bracelet and trypsin inhibitor cyclotides. Prototypical members of the first two of these families include kalata B1 and cycloviolacin O1 (Rosengren et al. 2003), whereas the trypsin inhibitor subfamily is exemplified by the structure of MCoTI-II (Felizmenio-Quimio et al. 2001; Heitz et al. 2001).

NMR has played a major role in determination of the structure of cyclotides (Craik and Daly 2007). Figure 4 illustrates selected examples of recently determined structures showing that they all conform to the same basic CCK scaffold, but that in addition to differences in the sequence there are differences in the lengths and flexibilities of the various loops. Onto the basic scaffold are superimposed the amino acid sidechains and it is the surface-exposed residues that are responsible for the interesting properties of cyclotides. In particular, cyclotides typically have a surface-exposed patch of hydrophobic residues. We assume that the patch arises because of the inaccessibility of the inner core of the protein to these residues since it is occupied by the cystine knot motif. Whatever the reason for the existence of the hydrophobic patch, it certainly affects the biophysical properties of cyclotides and, in particular, makes them late eluting on HPLC (Daly et al. 1999) and also appears to be responsible for their membrane binding (Kamimori et al. 2005; Shenkarev et al. 2006).

Biological activities of cyclotides

Table 2 summarizes some of the major reported activities of cyclotides, including uterotonic (Gran 1973a, b), anti-HIV (Gustafson et al. 1994), antimicrobial (Tam et al. 1999a), hemolytic (Daly et al. 1999), anti-tumor (Lindholm et al. 2002), anti-fouling (Göransson et al. 2004), nematocidal (Colgrave et al. 2008a, b), and insecticidal activities (Jennings et al. 2001). Whereas the pharmaceutically directed activities, such as anti-HIV activity (Gustafson et al. 2004), are serendipitous activities that are unrelated to the natural function of cyclotides, many of the other activities have the common theme of toxic properties that are associated with host defense functions of cyclotides. So far, the most extensively studied host defense activity is the insecticidal activity of cyclotides which dramatically affect the growth and development of Helicoverpa larvae (Jennings et al. 2001, 2005). These caterpillars are major pests on cotton and corn crops; the mode of action of cyclotides against them was reported recently to be disruption of the insect midgut membranes (Barbeta et al. 2008). Thus, we now describe some recent studies in which NMR and other biophysical techniques have played an important role in defining the nature of cyclotide-membrane interactions. The idea that cyclotides interact

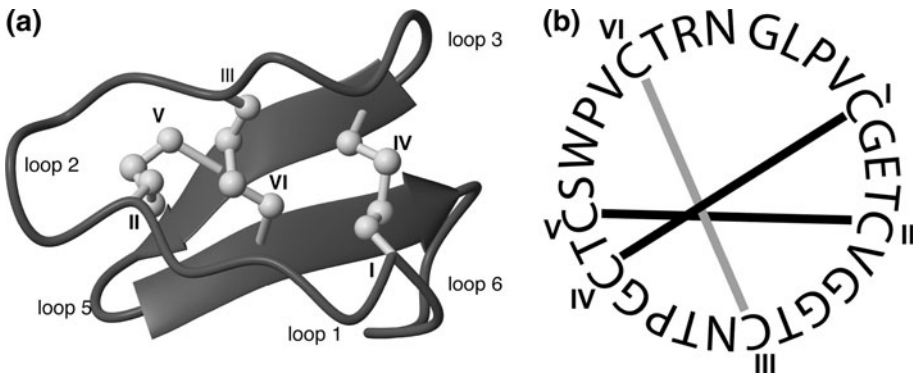

Fig. 3 Cyclic cystine knot motif of the cyclotides. **a** The structure of kalata B1 (PDB: 1nb1) highlighting the knotted conformation of the three disulfide bonds. **b** The sequence of kalata B1 with the three disulfide bonds shown. The penetrating disulfide bond is shown in *grey*

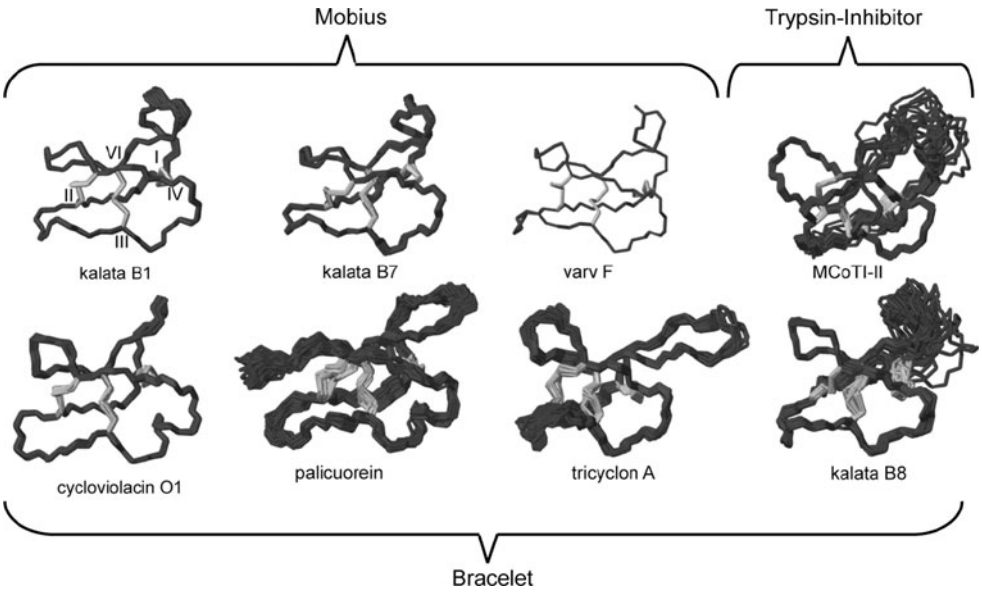

Fig. 4 Selected structures of cyclotides. The structures shown are kalata B1 (PDB ID code 1NB1), kalata B7 (PDB ID code 2JWM), the crystal structure of varv F (PDB code 3E4H), MCoTI-II (PDB ID code 1IB9), cycloviolacin O1 (PDB ID code 1NBJ), palicourein (PDB ID code 1R1F), tricyclon A (PDB ID code 1YP8), and kalata B8 (PDB ID code 3E4H). Selected Möbius and trypsin inhibitor cyclotides are represented at the *top* of the diagram and bracelet cyclotides at the *bottom*

with membranes was discussed in detail in a talk by Sónia Henriques at the Australia-Croatia conference, and aspects of that talk are included herein.

To help define the structure–activity relationships of cyclotides we have undertaken a number of biophysical studies over recent years, including alanine (Simonsen et al. 2008) and lysine scanning mutagenesis of kalata B1 (Huang et al. 2010), as well as surface plasmon resonance studies (Kamimori et al. 2005). These studies have been underpinned by synthetic methods that have been developed to produce both native and modified cyclotides (Daly et al. 1999; Tam et al. 1999b; Camarero et al. 2007; Thongyoo et al. 2008) and to correctly fold them (Daly et al. 1999; Göransson and Craik 2003; Leta Aboye et al. 2008; Gunasekera et al. 2009).

Alanine scanning is a technique commonly used to derive structure–activity relationships in drug design studies, whereby each residue in a peptide or protein is replaced one at a time by an alanine residue and then tested for activity. A reduction in activity is taken to signify that the original residue replaced by the alanine played some role in the bioactivity. Of course, to draw this conclusion one needs to be sure that the alanine mutation did not change the three-dimensional shape of the molecule; this is where NMR is extremely useful. Figure 5 shows plots of the αH NMR chemical shifts for a series of point alanine mutations of the prototypical cyclotide, kalata B1 (Simonsen et al. 2008). αH chemical shifts are excellent markers of structure and the fact that the shifts are essentially unchanged across the suite of mutants provides strong support for the fact that mutagenesis does not lead to structural perturbations. We did structures of selected alanine mutants to verify the predictions made by the alanine scanning mutations (Simonsen et al. 2008).

As a result of the alanine scanning mutagenesis, we identified a region of kalata B1 that we refer to as the "bioactive face". Replacement by Ala of any of six residues located in a single contiguous patch on the surface (Fig. 6) causes a drop in insecticidal activity. One residue whose replacement also causes a loss of activity, Gly12, is separated from this patch. We believe this is due to a small structural perturbation caused by replacement of the glycine residue by alanine, as is apparent from the perturbations in αH chemical shifts (Fig. 6). Glycine is a small amino acid known for its conformational adaptability and, clearly in this case, occupies a position of strain in the molecule, which when replaced by alanine causes a structural perturbation. Putting this result aside, the basic result from the alanine scan is that there is a patch of "bioactive" residues all located on one face of the molecule. Interestingly, this face is different from the hydrophobic patch of cyclotides, which is also illustrated in Fig. 6.

The finding that the bioactive region of the molecule, as detected by Ala scanning mutagenesis, is different from the hydrophobic face raised the question of which part of the molecule interacts with membranes? We undertook surface plasmon resonance studies to unequivocally determine that cyclotides do bind to membranes (Kamimori et al. 2005). Subsequent NMR studies confirmed membrane binding and helped define the residues involved in the interaction (Shenkarev et al. 2006). In order to do solution-type NMR measurements, Shenkarev et al. used DPC micelles as membrane surrogates and undertook a series of NMR experiments to determine the nature of cyclotide-micelle interactions. These included chemical shift titrations as a function of micelle/kalata B1 molar ratio as well as translational diffusion measurements that detected changes

Table 2 Reported bioactivities of cyclotides

Activity	Compound	Reference[a]
Antifouling	cycloviolacin O2	Göransson et al. (2004)
Anti-HIV	circulin A and B	Gustafson et al. (1994)
	circulin C–F	Gustafson et al. (2000)
	cycloviolacin A–D	Hallock et al. (2000)
	cycloviolacin O13, O14 and O24	Ireland et al. (2008)
	cycloviolacin Y4 and Y5	Wang et al. (2008)
	kalata B1	Daly et al. (2004)
	kalata B8	Daly et al. (2006)
	palicourin	Bokesch et al. (2001)
	varv E	Wang et al. (2008)
	vhl-1	Chen et al. (2005)
Antimicrobial	circulin A and B	Tam et al. (1999a)
	cyclopsychotride A	Tam et al. (1999a)
	cycloviolacin O2	Pranting et al. (2010)
	kalata B1	Tam et al. (1999a)
	kalata B7	Gran et al. (2008)
Antitumor	cycloviolacin O2	Lindholm et al. (2002)
	varv A and F	Lindholm et al. (2002)
	varv E	Svangard et al. (2004)
	vibi E–H	Herrmann et al. (2008)
	vitri A	Svangard et al. (2004)
Cell-penetrating	MCoTI-II	Greenwood et al. (2007)
Hemolytic	circulin A and B	Tam et al. (1999a)
	cyclopsychotride A	Tam et al. (1999a)
	cycloviolacin O2, O13, O14, O15 and O24	Ireland et al. (2006)
	kalata B1	Daly et al. (1999)
	varv A	Ireland et al. (2006)
	violapeptide 1	Schöpke et al. (1993)
Insecticidal	kalata B1 and B2	Jennings et al. (2001)
Molluscidal	cycloviolacin O1	Plan et al. (2008)
	kalata B1, B2	Plan et al. (2008)
Nematocidal	kalata B1, B2	Colgrave et al. (2008b)
	cycloviolacin O2, O3, O8, O13, O14, O15 and O16	Colgrave et al. (2008b)
Neurotensin antagonist	Cyclopsychotride A	Witherup et al. (1994)
Trypsin inhibitor	MCoTI-I	Hernandez et al. (2000)
	MCoTI-II	Hernandez et al. (2000)
Uterotonic	kalata B1, B2	Gran (1973b)

[a] Reference in which the activity was reported/suggested for the first time

in the dynamics of cyclotides associated with their micelle binding. Both sets of measurements showed a clear binding interaction, as shown in Fig. 6, and the thermodynamic parameters derived from fitting of Langmuir binding curves suggested a binding energy of approximately 5.4 kcal/mol, in good agreement with measurements from surface plasmon resonance results (Kamimori et al. 2005). Studies in which paramagnetic spin labels were embedded at two locations, deep and shallow, in the micelle caused broadening of NMR signals from specific cyclotide residues. These studies were used to define the orientation of kalata B1 (Shenkarev et al. 2006) and related cyclotides to the membrane (Shenkarev et al. 2008), and showed that the hydrophobic patch was the important binding region.

The combination of these surface plasmon resonance and NMR measurements has led to a model for binding interactions of cyclotides with membranes. In this model, cyclotides associate with membranes via their hydrophobic

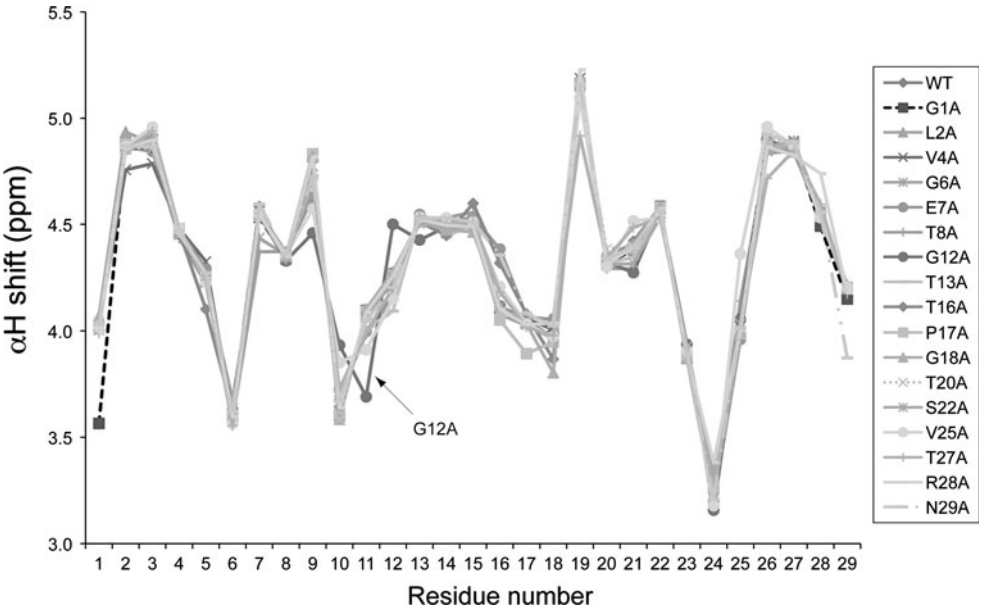

Fig. 5 Chemical shift analysis of kalata B1 mutants in which indicated residues have been substituted with Ala. The αH shifts of 16 alanine mutants are compared with the wild type peptide. Only minor variations are observed, indicating that the global fold is maintained despite the mutations, apart from the Gly12 mutant, which is associated with a small structural perturbation (Simonsen et al. 2008)

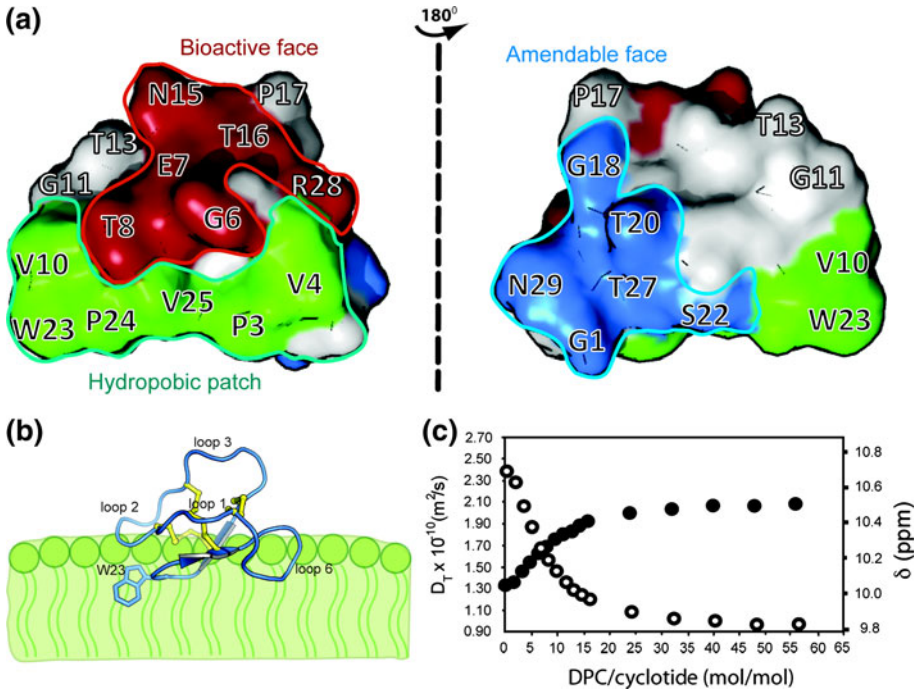

Fig. 6 **a** Surface representation of kalata B1 shown in two views illustrating the hydrophobic patch (*green*), the bioactive face (*red*) and the amendable face (*blue*). **b** kalata B1 membrane-binding orientation based on NMR studies. The hydrophobic patch inserts in the membrane. The Trp-23 side chain is shown. **c** Titration of kalata B1 with DPC in H_2O. The chemical shift, δ of the Hε1 proton of Trp-23, (*filled circles*) and the translational diffusion coefficient, D_T, (*open circles*) are shown as a function of the DPC/kalata B1 mol ratio, as markers of binding

patch as illustrated in Fig. 6b. The hydrophobic patch involves, in many cases, a tryptophan residue (which provides a useful NMR chemical shift titration marker as illustrated in Fig. 6c). In this binding orientation the "bioactive" residues appear not to directly interact with membranes, and we hypothesized that they might be involved in self-association interactions (Simonsen et al. 2008). The proposed model for membrane binding thus involves cyclotides interacting with the membrane surface, self-associating and then ultimately forming pores. Electrophysiology measurements have established that these pores are of large size (>40 Å) and are responsible for the membrane leakage seen when cyclotides are incubated with dye-filled vesicles (Huang et al. 2009).

These studies have been extremely useful in defining cyclotide binding, but a number of questions remain; what is the nature of the self association, what is the geometry of the cyclotide complex in membranes, and is there one mode of action common to all cyclotides? The latter is particularly relevant because a single plant can produce more than 100 different cyclotides; the reasons for this are currently not clear.

Recent lysine scanning studies have provided additional insights into the mechanism of action of cyclotides. Unlike the alanine scan, there is a much larger group of residues that cause loss of activity when single amino acids are replaced with lysine (Huang et al. 2010). The results are consistent with the previously presented model in that lysine substitutions in the hydrophobic patch disrupt membrane binding and result in a loss of activity. The Ala scan is a "null" substitution in the hydrophobic patch and this is not informative in this region. The lysine scanning experiments also led to the interesting result that some lysine mutants led to increased activity. This allowed us to identify a third face on the cyclotide molecule, which we refer to as the "amendable" face (the literal meaning of the word "amendable" means changeable for the better) whose location is shown in Fig. 6. This information is being used in current projects in our laboratory to optimize the activity of cyclotides for pharmaceutical and agricultural applications.

Structure, dynamics and interactions

The experiments described so far can be summarized by noting that NMR first provided valuable information on the structures of cyclotides. The structures are conserved, and basically show that cyclotides have a rigid core built around the CCK motif. Onto this CCK structural core are surface-exposed residues in defined orientations that lead to the formation of a hydrophobic patch, a bioactive patch, and an amendable face. The NMR data and other experiments that have defined these patches have been very useful in guiding design studies to understand and optimize cyclotide activities. Dynamic measurements have helped define both the diffusion of cyclotides in solution and their internal molecular dynamics (Puttamadappa et al. 2010). Finally, a range of NMR studies have led to an understanding of interactions between cyclotides and membranes (Shenkarev et al. 2006; Wang et al. 2009).

This is where we will leave the cyclotide story for now and move onto a second example of drug design in which NMR has made significant contributions for a disulfide-rich peptide. In this case the target site is not a membrane, but a membrane-bound receptor and the class of peptides involved is the conotoxins.

Conotoxins

Cone snails are marine snails from the *Conus* genus that are found in tropical and sub-tropical waters, with approximately 500 species worldwide. Each species produces a venom that is used to capture their prey, which consists of worms, other snails or fish. Clearly, for snails to catch fish they need a very potent venom that causes instant paralysis, and the strategy they adopt is to use a cocktail of venom components referred to as conopeptides or conotoxins (Adams et al. 1999; Terlau and Olivera 2004). These conotoxins are typically small peptides of 10–40 amino acids in length, containing up to five disulfide bonds, although two or three disulfide bonds are most common. They target a range of ion channels, transporters and receptors, which, as noted above, in their natural setting are used to immobilize the prey. However, because of their exceptional potency and specificity for a range of receptor subtypes, conotoxins have attracted the attention of pharmaceutical chemists as leads in drug design (McIntosh et al. 1999; Craik and Adams 2007; Bingham et al. 2010). Indeed, one conotoxin, MVIIA, also known as ziconotide or Prialt®, is approved in the USA and EU for the treatment of intractable neuropathic pain (Miljanich 2004), and several others are in clinical or preclinical development (Bingham et al. 2010). Like cyclotides, conotoxins have been described as comprising a core scaffold onto which bioactive amino acid side chains decorate the surface and define their receptor specificity (Hu et al. 1997). In our studies, we aimed to make synthetic conotoxins even more cyclotide-like by re-engineering them with a cyclic backbone to make them more stable than their natural conotoxin counterparts. Here we describe recent results based on cyclizing peptides from the α-conotoxin family (McIntosh et al. 2000; Dutton and Craik 2001).

α-Conotoxins are the smallest of the conotoxins, typically 12–19 amino acids in size, with a conserved four cysteine structural scaffold and helical structure, as illustrated in Fig. 7 (Millard et al. 2004). Typically, they are C-terminally amidated and are potent antagonists of nicotinic acetylcholine receptors (Janes 2005). The example we chose to initiate this work was conotoxin MII (Fig. 7), a small α-conotoxin that is specific for $\alpha 3\beta 2$ subtypes of the nicotinic acetylcholine receptor (Cartier et al. 1996), but it was basically used as a model system for establishing proof-of-concept that disulfide-rich conotoxins could be cyclized to improve their stability. Initial computer-based design studies suggested that linkers of five or more residues would be sufficient to join the ends of MII, and we synthesized a range of cyclic MII analogs with linkers comprising mixtures of glycine and alanine residues. The choice of mixed linkers, rather than a simple poly-Gly linker, was to avoid complexities in the NMR spectra of the synthetic products. It turned out that five residues was too short and that the analogs with six or seven residue linkers maintained full biological activity of the native toxin yet had improved stability (Clark et al. 2005). Consistent with these results, NMR studies showed the peptides with six- or seven-residue linkers were similar to the native structure,

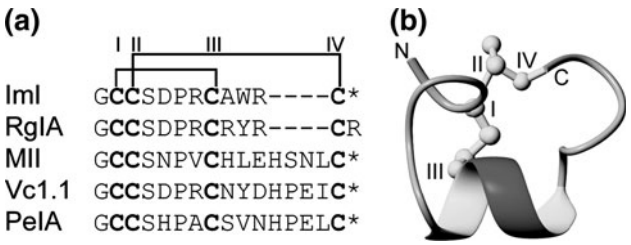

Fig. 7 The α-conotoxin structural framework. **a** Selected sequences of α-conotoxins, highlighting the native disulfide connectivity (CysI-CysIII, CysII-CysIV); **b** the three dimensional structure of MII (PDB ID code 1MII) with the helical region shown with a *thickened ribbon* and the two disulfide bonds shown in *ball-and-stick* format

but the bioactive helix in the five-residue linked conotoxin was perturbed. These structural studies used NMR-derived distance and angle restraints (from NOEs and coupling constants, respectively) as input parameters in simulated annealing calculations to determine the 3D structures (Clark et al. 2005).

It is interesting to note that most α-conotoxins have Cys residues near their termini (see Fig. 7), so the introduction of linkers of multiple non-Cys amino acids that extend the sequence beyond the disulfide-rich framework is generally an "out of character" modification. Nevertheless, there are some α-conotoxins that have short peptide tails outside their core region, including GID (Nicke et al. 2003), so the idea that extensions can be made outside the disulfide framework is not without precedent. Such a finding supports the idea that cyclization might be applicable to a broad range of conotoxins.

The initial work on MII led to studies of other α-conotoxins, and in particular the cyclization of α-conotoxin Vc1.1 (Clark et al. 2010), which earlier had been identified as having potent activity in a rat model of neuropathic pain (Sandall et al. 2003). Neuropathic pain is a chronic condition that results from injury to nerves, either in the peripheral or central nervous system. It can cause extreme physical, psychological and social distress. Although there are drugs on the market to treat the condition, they are ineffective in a large proportion of patients and there is a great need for new treatments. More than 35 million people worldwide suffer from neuropathic pain.

α-Conotoxin Vc1.1 was originally found to target the nicotinic acetylcholine receptor but recent studies have shown that it more potently targets the $GABA_B$ receptor (Callaghan et al. 2008), a membrane-bound receptor. It was however, not orally active, thus limiting its potential as a drug. We synthesized cyclized analogues of Vc1.1 and used NMR studies to show that the global fold of the analogs was unchanged by the cyclization process. Importantly, we found that as well as improving stability we also improved the selectivity for the $GABA_B$ receptor over the nicotinic acetylcholine receptor, for one of the cyclic analogs, as illustrated in Fig. 8 (Clark et al. 2010). This illustrates that one of the other advantages of cyclization in that one can achieve increases in potency at

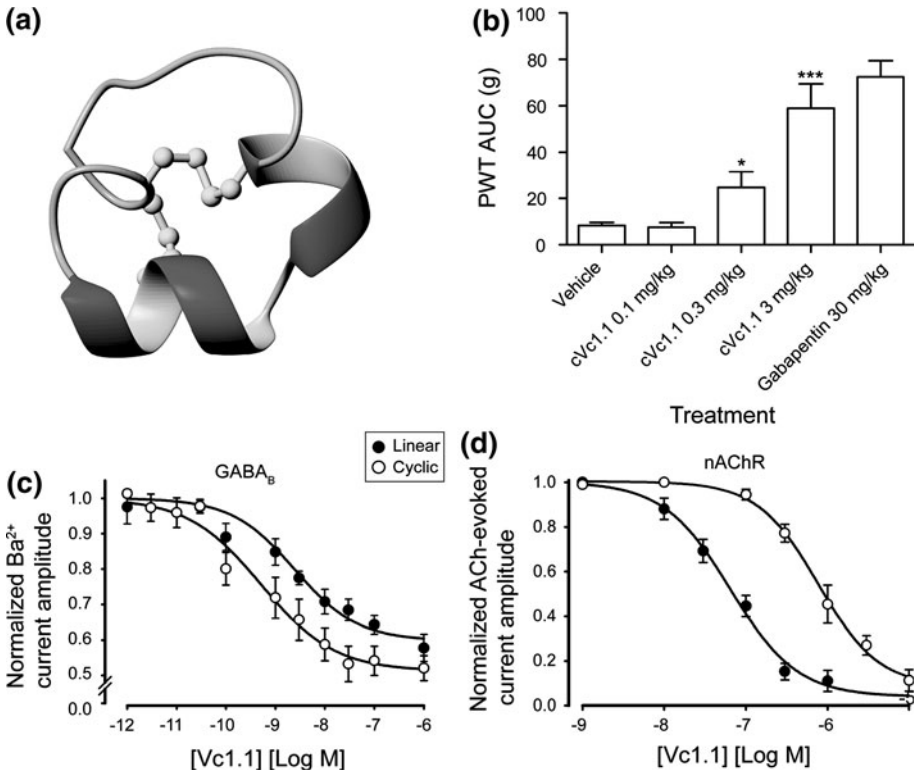

Fig. 8 Structure and activity of cyclic Vc1.1. **a** Three-dimensional structure of cyclic Vc1.1; **b** oral dosing of cyclic Vc1.1 in a rat model of pain produces significant pain relief, as measured by the paw withdrawal threshold, PWT; **c** linear and cyclic Vc1.1 activity at the $GABA_B$ and **d** α9α10 nAChR (Clark et al. 2010)

desired receptors as well as stability increases. Figure 8 shows a comparison of the pain-relieving effects of cyclized Vc1.1 versus the industry gold standard, gabapentin. From this graph it can be clearly seen that cyclic Vc1.1 has similar affects to gabapentin but at much lower dose and therefore, is potentially an exciting new lead molecule for the treatment of neuropathic pain (Clark et al. 2010).

Summary and outlook

The two examples we have described in this review of cyclotide and conotoxin peptides illustrate applications of NMR in drug design for cases where the receptor is a membrane, and a protein-based target, respectively. In both cases, our focus has been on using NMR to derive information about the structures of peptidic ligands and synthetic analogs designed to have improved activities or biopharmaceutical properties. The NMR measurements described are relatively straightforward and yet very valuable to help guide the drug design process. With increasing recognition by the pharmaceutical industry that peptides might represent "the drugs of the future" we anticipate that NMR studies of bioactive peptides will become increasingly valuable.

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