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Venom as a source of useful biologically active molecules

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Abstract

In the specialty area of venomology, emergency physicians traditionally have been most interested in the description of a variety of envenomation syndromes and, subsequent to this, the most appropriate investigative and therapeutic strategies to employ when envenomation is present. Taking an alternative viewpoint, in this paper we have reviewed a selection of interesting areas of biomedical research in which venom components are being investigated for their potential as novel therapeutic agents, pesticides and ion-channel probes. In addition, we describe the molecular imaging tools of X-ray crystallography and nuclear magnetic resonance spectroscopy, key techniques in the development of rationally designed therapeutic agents. **See Commentary, page 5.**

Key words: *drug design, ion channel, toxin structure, structural studies, venom.*

Introduction

The world's venomous animals remain responsible for an astonishing number of deaths. In West Africa, it is estimated that snakebite alone is responsible for approximately 23 000 deaths per year, with fewer than 10% of patients being treated in modern medical facilities.[1] It is from this vantage point that the majority of medical interest in the area of venom research has been in the areas of recognition and treatment of envenomation syndromes.

Within Australia, venomous animals loom large in the population's psyche,[2] and with good reason. The line-up of venomous animals includes the following:

- Snakes: Twenty-one of the world's 25 most-deadly snakes by mouse lethality assay reside in Australia, including all of the top 10.[3]
- Jellyfish: The deadly box jellyfish *Chironex fleckeri*, widely recognized as the planet's most deadly venomous animal and its elusive cousin the Irukandji, *Carukia barnesi*.
- Spiders: The potentially lethal spiders *Hadronyche versuta* (the Blue Mountain funnel web) and the redback spider *Latrodectus hasseltii*.
- Cone shells: Carnivorous marine snails that immobilize their prey with a potent cocktail of toxins known as conotoxins.

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Rather than being the subject of generalized fear and loathing and perhaps because, at least in most developed countries, death from envenomation is uncommon, it has been realized that these venomous animals represent a vast and largely untapped source of potent biologically active molecules.

Venoms consist of a complex mixture of toxic components that include protein and peptide toxins, enzymes and other active agents. These molecules serve the dual purposes of prey capture and digestion and/or defence against predators. While the precise mechanisms of action of many venom constituents remain unknown, what is known is that many exert their effects via interactions with specific ion channels, enzymes and membrane components. These highly potent and specific interactions make venom constituents attractive candidates for the development of novel therapeutics, pesticides and as molecular probes of target molecules.

Scope of this review

In this review, we will examine the following areas of interest:

- Sea anemone toxins: several of these have been found to modulate sodium-channel function and are being investigated as drug leads for the development of novel cardioactive agents.
- Conotoxins: potent ion channel modulators found in the venom of a range of marine molluscs known as cone shells. The conotoxins have yielded a novel analgesic agent, known as ziconotide, that is used in the treatment of a variety of pain syndromes.
- Insect-specific toxins from venoms: these peptides, which block insect but not mammalian voltage-gated calcium channels, have tremendous potential in the area of bioinsecticidal control of agricultural pests.
- Antibacterial components of snake and spider venoms: the increasing prevalence of multi-drug-resistant bacteria has led to the screening of a wide number of natural molecules as potential new antibiotic agents.
- The angiotensin converting enzyme inhibitors (ACE inhibitors): for many years, captopril and the ACE inhibitors have been the shining light of practical application of both venom research and rational drug design. As we shall see, captopril was rationally synthesized after isolation and study of an ACE-inhibiting protein from the venom of the snake *Bothrops jararaca*.

The study of these toxin components has encompassed both the mode of action of the molecules and the rigorous analyses of their 3-D structure. Such structure–function studies are at the cutting edge of modern medical science. It is believed that through an understanding of these potent molecules at a structural level, they can be adapted for use as optimized 'rationally designed' agents. Thus the final section in this review outlines the techniques currently employed for such studies of molecular structure.

Sea anemone toxins as modulators of sodium channel function

Sea anemones contain potent venom for the purpose of prey capture. The venom is fired into the victim via a barbed tubule that emerges from venom-containing cysts (nematocysts) on their tentacles. This both paralyses the prey and allows it to be drawn towards the digestive apparatus of the anemone.

The paralytic action of the venom is due to a mixture of protein and peptide toxins, several of which are potent inhibitors of sodium channel inactivation.[4–6] One of the best characterized of these is anthopleurin-A (AP-A) (Fig. 1)

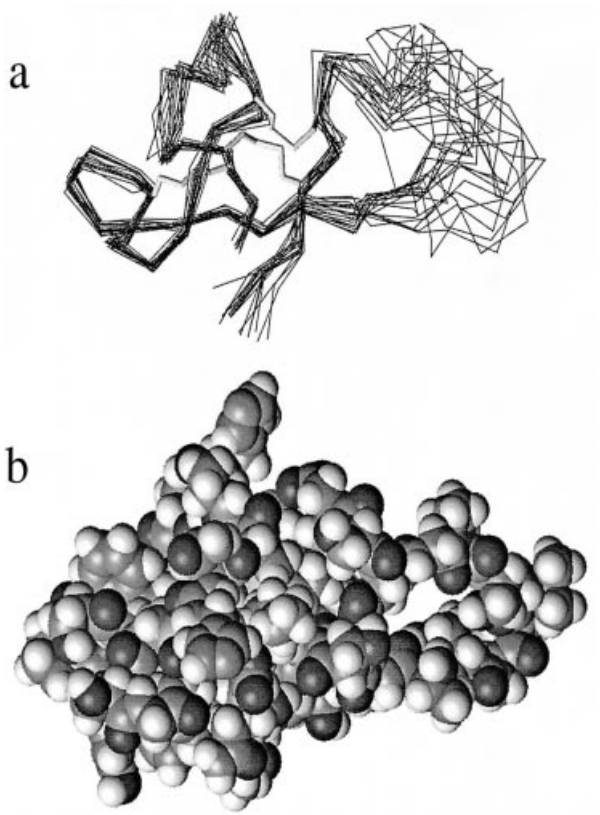

Figure 1. The sodium channel modulator anthopleurin-A from the venom of the giant green sea anemone *Xanthopleura xanthogrammica*: a. the backbone fold of the 20 nuclear magnetic resonance (NMR)-derived structures (black) and the three disulphide bonds which stabilize the structure (grey); b. the space-filling model of one of the NMR-derived structures (pdb code: 1AHL).

which was found to be active as a cardiac stimulant at nanomolar concentrations *in vivo* and not associated with any significant effects on heart rate or blood pressure in mammals.[7,8] This was of great interest, especially since it was found to be approximately 200-fold more potent than digoxin and to possess fewer undesirable side-effects. It was thus a potentially valuable lead in the development of a novel drug for the treatment of chronic heart failure.

Since AP-A was discovered, a whole family of sea anemone sodium channel modulators have been isolated from various species of anemone. Many are polypeptides of about 49 amino-acid residues in length and contain three disulfide bonds which help to stabilize their 3-D structure. Despite their potency, however, the use of these 'natural products' as drugs is not ideal because of their low stability following oral administration and their antigenicity.[9] The research effort has thus been to use the 3-D structural information and knowledge of the mode of action of these peptides in order to design smaller, non-peptide molecules with the same activity.

The toxins are known to bind to specific sites on the myocardial voltage-gated sodium channel,[10] known as 'site 3'. Other polypeptides that also bind to site 3 include several 'short' anemone polypeptides[6,11] that are neurotoxic to crustacea, a class of anemone peptides with antihypertensive and antiviral activity[12,13] and α-scorpion toxins.[14] By the comparison of the structures of these different classes of peptides, the important structural features of the molecules can be identified. This, along with knowledge of their relative binding affinities, underpins the effort to define the cardioactive pharmacaphore.

The sodium channel site 3 binding face of the toxins has now been identified[9] and both charged and hydrophobic residues are thought to be important for binding affinity. It now remains for the synthesis and testing of mimics of this pharmacaphore to be carried out. This may be achieved by designing a linear peptide mimetic, or it may be necessary to develop a molecule of a completely different architecture which maintains the spatial arrangement and chemical properties of the toxin binding face. Such 'rational drug design' is at the cutting edge of medical research today.

Cone shell venom: The conotoxins

Conotoxins have been the subject of intensive research efforts in recent times and much of this work has been based in Australia. Animals of the genus *Conus* are predatory molluscs, found in tropical waters such as the Great Barrier Reef area of Queensland, that also immobilize their prey by injecting them with venom via a harpoon-like apparatus. The conotoxins of each species of cone shells are targeted to specific receptors in the cone shells' prey.[15] There are over 500 species currently identified, with both inter- and intraspecies variation of venom components. In fact, within the same animal there can be significant variation in the composition of venom over time. Seemingly minor variations to the basic protein structure of these conotoxins can result in widely varying affinities for molecular targets.

Conotoxins consist of a variety of peptidic components, notable for their relatively small size (9–35 amino acids[15]), complex post-translational modifications, distinctive disulphide bonds and potent inhibitory effect on a variety of neuronal ion channels. Thus far, around 60 unique conotoxins have been identified. This is, however, only the tip of the iceberg. Analysis of whole cone shell venoms suggests that this represents less than 0.1% of the total peptide load contained within these venoms.[16]

The majority of conotoxins are potent neurotoxins, being highly selective for neuronal ion channel subtypes. They are divided into six major classes, based upon their structure and channel-blocking activity. Conotoxins have been shown to have activity, usually antagonistic on nicotinic acetylcholine receptors, sodium channels, calcium channels and *N*-methyl-D-aspartate (NMDA) glutamate receptors.[16] They were initially used as ion-channel probes and have assisted in both the characterization of specific ion-channel subtypes and the elucidation of the 3-D structure of the ion-channel binding surface.

Conotoxins have been recognized for their potential as therapeutic agents owing to their relatively small size and the excellent stability conferred by the disulphide bonds. The most spectacular current therapeutic application of the conotoxins is, however, that of the omega conotoxin MVIIA, a 25-amino-acid disulfide-bridged polypeptide derived from the venom of *Conus magnus*. This conotoxin, which has been chemically synthesized and will be marketed under the generic name of ziconotide, is an antagonist of N-type neuronal calcium channels[16] (Fig. 2), which play an important role in the spinal transmission of pain.

Ziconotide has been shown to be effective in the treatment of both nociceptive and neuropathic pain syndromes in both animal[17] and human models of pain. Atanassoff *et al.*[18] recently reported a randomized, double-blinded clinical trial of postoperative intrathecal ziconotide infusions in the treatment of patients undergoing elective total abdominal hysterectomy, radical prostatectomy or total hip replacement. Both outcome measures of mean daily morphine patient-controlled analgesia consumption and visual-analogue pain-intensity scores reached statistical

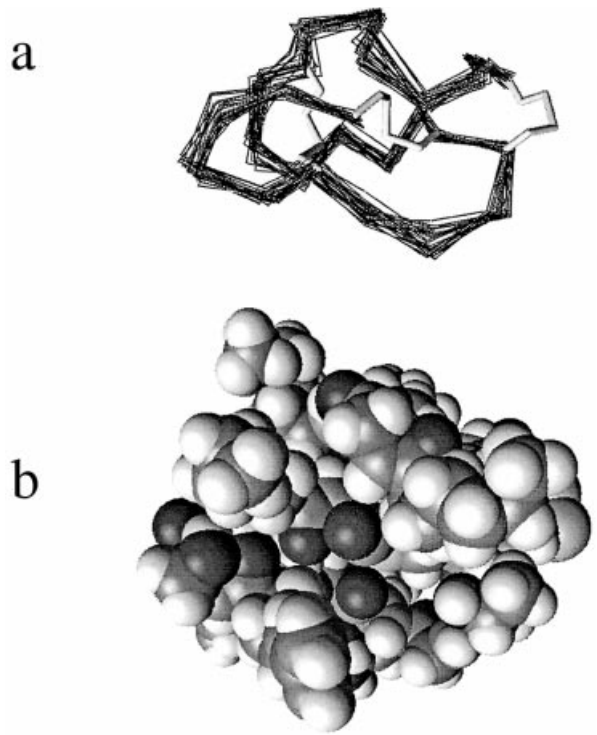

Figure 2. The nuclear magnetic resonance (NMR)-derived structure of omega conotoxin MVIIA (ziconotide), a potent inhibitor of N-type calcium channels: a. the backbone fold of the 32 NMR-derived structures (black) and the three disulphide bonds which stabilize the structure (grey). b. the space-filling model of one of the NMR-derived structures (pdb code: 1DW4).

significance in the ziconotide-treated group, with an acceptable side-effect profile. However, only 26 patients were available for analysis and it is obvious that further large-scale investigation is required.

In addition to its proposed use in patients with nociceptive and neuropathic pain, ziconotide has also proven to be an effective neuroprotective agent in animal models of traumatic brain injury[19] and cerebral and spinal ischaemia.[20] Following acute cerebral ischaemia, there is loss of cytosolic calcium homeostasis, with increased levels of intracellular calcium, which in turn leads to mitochondrial dysfunction. Use of N-type calcium-channel blocking agents partially reverses this rise of intracellular calcium, which is seen particularly in the setting of reperfusion injury.

The search continues for other therapeutic agents based on the conotoxins. Potential therapeutic applications include the setting of severe head injury, epilepsy, cerebrovascular disease and spinal cord injury.

Insecticidal components of animal venoms

A very exciting and currently active area of venom research involves the search for insecticidal venom components that can be used as an environmentally friendly form of agricultural pest control. The work in this area has been focused in two areas: (i) the search for an appropriate insecticidal peptide toxin that is toxic to the target insects but not other plants and animals in the food chain; and (ii) the appropriate vector for delivering the insecticide to its site of action.

Agricultural pests remain both difficult to control and economically devastating throughout the developed world. Current control techniques focus on the use of noxious chemical insecticides such as organophosphates and organochlorines that possess major environmental problems in their routine use. In addition, resistance to these agents develops in target insects over time. The production of a viable bioinsecticide will provide another avenue of pest control that is both more specific to the target pest and less harmful to the wider ecosystem in which they are deployed.

Fletcher and colleagues have been active in examining the venom of the Australian funnel web spider for active insecticidal components, with particular reference to the larva of the cotton bollworm *Helicoverpa armigera*.[21] The omega-atracotoxin (ACTX) HV1 (Fig. 3) from this spider is a 37-residue peptide toxin that contains three intramolecular disulphide bonds. It is lethal to the larvae of *H. armigera*, while being harmless to newborn mice at doses up to 2.5 mg/kg.[21]

The 3-D structure of omega ACTX-HV1 has been solved and surprisingly it is structurally similar to many of the conotoxins, but functionally dissimilar. The toxin has been shown to block insect, but not mammalian, voltage-gated calcium channels,[21] and is thus a good candidate as a bioinsecticide. Insecticidal venom fractions have also been isolated from scorpion venom,[22] spiders other than the funnel web[23] and centipedes.[24] It may be that a cocktail of such insect-specific toxins will ultimately be most effective.

Baculoviruses expressing insect-selective neurotoxins are currently thought to be the most efficient mechanism for delivering the toxin to its site of action. This virus specifically infects *H. armigera*, but without the addition of a venom-derived neurotoxin they are very slow to cause larval mortality. This enables the infected larvae to continue their course of crop destruction prior to dying. The addition of one or more insect-selective neurotoxins provides a method for rapid attack of insect hosts, with greatly reduced time to host death.[25]

Antibacterial components of snake and spider venoms as potential therapeutic agents

The continuing emergence of multiple-antibiotic-resistant bacteria, together with the increase in both viral and bacterial illness associated with immunosuppressive conditions, has driven the search for new antibiotics. Antibacterial toxins and molecules have been isolated in a wide variety of species including bacteria, insects, spiders, scorpions, amphibians and mammals.[26]

In the case of snake venom, it is known that in a number of different snake species, infection following snake bite without necrosis is rare. Whole snake venom from a variety of South African snakes has been demonstrated to have antibacterial properties against aerobic and anaerobic bacteria and both Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Clostridium perfringens*.[27]

The venom of the neotropical wandering spider, *Cupiennius salei*, has also been examined in detail. Fractionation of the venom revealed five peptides with molecular masses of approximately 4 kDa, all with unique antibacterial activities, that appear to exert their effects by cell lysis by formation of channels or pores within the bacterial cell wall.[26]

Subsequent to the identification of the amino acid sequence and structural configuration of these peptides, 'analogue and hybrid peptides'[26] can be developed that will possess even more powerful antibacterial activity. This is an area of venom research that will become increasingly important over the next few years as the true potential of these molecules is realized.

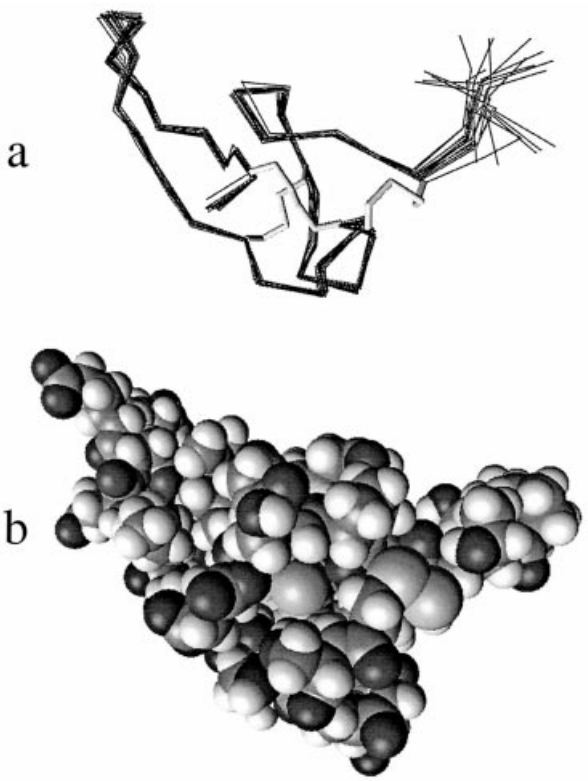

Figure 3. The nuclear magnetic resonance (NMR)-derived structure of the insecticidal atracotoxin HV1 from *Hadronyche versuta* (the Blue Mountain funnel web spider): a. the backbone fold of the 20 NMR-derived structures (black) and the three disulphide bonds which stabilize the structure (grey); b. the space-filling model of one of the NMR-derived structures (pdb code: 1AXH).

Captopril: A snake-venom-derived metallopeptidase inhibitor

The discovery of captopril provides an excellent example of a snake-venom-derived metallopeptidase inhibitor already in widespread clinical use. The critical link in the development of this novel class of antihypertensive agents was made in 1967, when it was realized that the enzyme responsible for the conversion of angiotensin-I to angiotensin-II (a potent vasoconstrictor) was also responsible for the inactivation of bradykinin.[28] Bradykinin is an endogenous nonapeptide that results in increased vascular permeability, dilation of blood vessels and contraction of non-vascular smooth muscle.

This link was to prove very important because, shortly afterwards, it was found that the venom of the Brazilian snake *Bothrops jararaca* contained an inhibitor of the enzyme responsible for the breakdown of bradykinin, which was subsequently realized to be identical to ACE.[29]

Following this, the active component of the snake venom was purified and the nonapeptide, teprotide, was identified and then synthesized. Teprotide was shown to be an excellent antihypertensive in both animal and human models via ACE inhibition but had the drawback of requiring intravenous administration. With knowledge of the clinical efficacy of teprotide in humans, a hypothetical model of the active site of ACE was developed. Increased understanding of the chemical and enzymatic properties of ACE, including the mechanism of inhibition by snake venom peptides, made it possible to begin to design simple non-peptide molecules that would interact with great affinity at the active site of this enzyme.[30]

From this hypothetical working model of the active site of ACE came the rationally designed captopril, a

novel and highly effective antihypertensive drug,[30] and the prototype for a new class of therapeutic agents, the ACE inhibitors (Fig. 4).

Studies of molecular structure for 'rational drug design'

Rational drug design, as opposed to serendipitous drug discovery, depends upon knowledge of either the molecular structure of the target molecule or the ligands which bind it. Ideally the full 3-D structure of the ligand : target complex to atomic resolution should be known, but this is often not the case. Some proteins, particularly large multidomain proteins or those that are membrane bound, are difficult to structurally determine and thus not available to assist in the process. The chemical features of the binding site may alternatively be inferred from the structure of the natural ligand, often a small, more soluble compound which is more easily structurally analysed. When many ligands to the target molecule are structurally characterized, their common chemical features can provide a very good picture of the active pharmacophore. This then, can be used as a template for the design of a drug molecule with potent binding characteristics that is suitable for commercial production and use.

Determining the 3-D structure of any biological macromolecule requires the use of X-ray crystallographic, nuclear magnetic resonance (NMR) spectroscopic or electron diffraction techniques. None of these methods are routine in their application and some are better suited to particular structural problems than others. This final section aims to provide a brief overview of the capabilities and limitations of these technologies.

X-ray crystallography is a technique which directly images the molecule of interest. X-rays (with a wavelength of approximately 1 Å) rather than light (with a minimum wavelength of approximately 4000 Å) must be used because their shorter wavelength is sensitive to the distances of interest (a typical covalent bond is 1.5 Å). The molecule is exposed to a beam of monochromatic X-rays, which are diffracted by the electrons surrounding and connecting each atom in the molecule. The diffraction pattern is recorded onto an X-ray sensitive recording plate and is then mathematically re-focused in order to visualize the 'electron density' of the molecule. This is analogous to the need for reflected light rays to be re-focused by the lenses in our eyes before we can interpret visual information.

In order to be able to measure the X-ray diffraction signal, the molecules must be prepared in crystalline form. When the individual molecules are in such an ordered arrangement, their diffraction pattern is effectively amplified and can be recorded (Fig. 5). The requirement for a good diffracting crystal, however, is not always readily achieved. There is no guaranteed method for growing good quality diffracting crystals and macromolecules, particularly those which occur naturally in a lipid environment of the cell, are notoriously difficult to

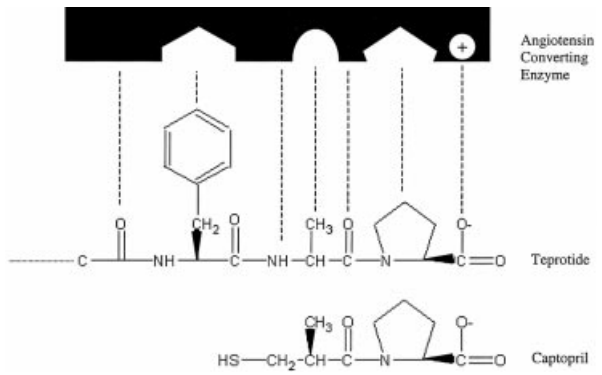

Figure 4. Development of captopril. The amino acid sequence of the snake-venom-derived peptide, teprotide, was used to develop a model of the active site of the enzyme angiotensin converting enzyme (ACE). The synthetic drug captopril was designed to mimic the C-terminus of teprotide.

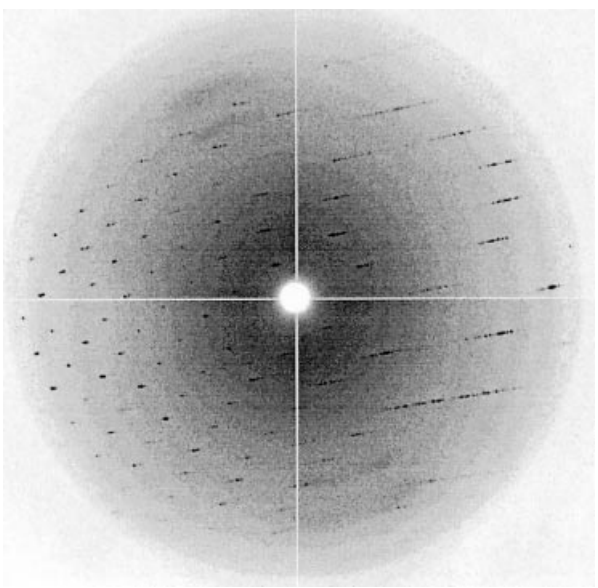

Figure 5. Typical protein crystal X-ray diffraction pattern from which the position and intensity of each diffraction spot can be measured. A set of such images contains the experimental information used to create an electron density map which defines the structure of the macromolecule.

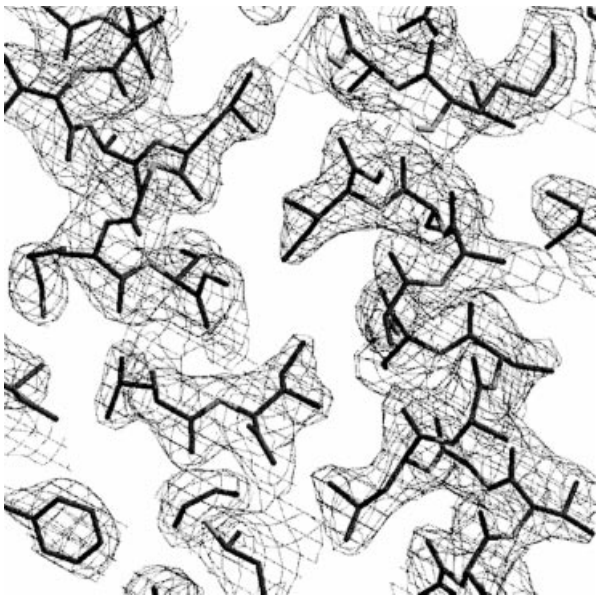

Figure 6. An example of 2.5 Å resolution electron density, shown as a 3-D contour map (grey), into which the atoms and bonds of the macromolecule can be built. The accuracy of the molecular model depends upon the resolution of the map.

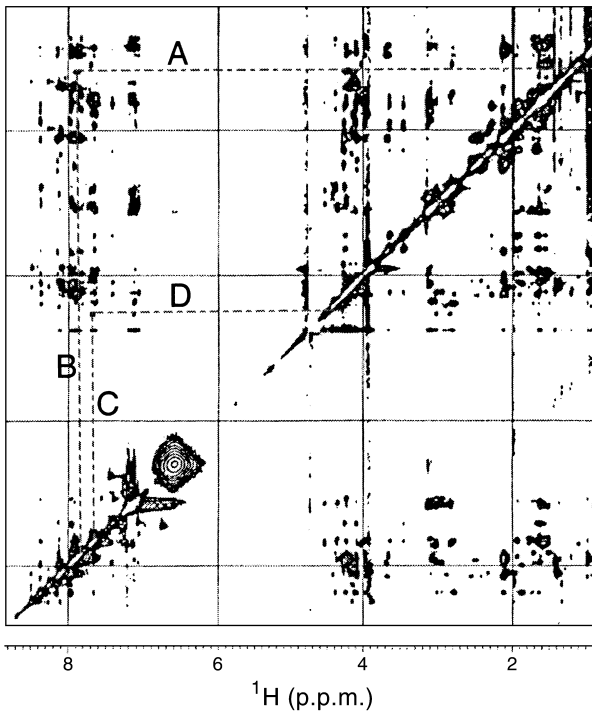

Figure 7. A 2-D ^{1}H nuclear magnetic resonance spectrum of a peptide molecule (recorded at 500 MHz) in which ^{1}H nuclei are distinguished by their resonance frequencies (in p.p.m.). The cross peaks arise from ^{1}H nuclei (e.g. labelled A–D) which exist close to each other in space within the macromolecule. These can be used to define the fold of the molecule in solution.

crystallize. In these cases it is sometimes possible to use one of the other methods of structure determination.

The electron density image that the X-ray experiment gives rise to can then be used to build in the atomic coordinates of the molecule (Fig. 6). A macromolecular structure determined by this method may be highly accurate (to almost 1 resolution), or it might be less well defined (to 4 resolution or greater) depending on the quality of the crystals and the diffraction images collected.

Nuclear magnetic resonance spectroscopy is an alternative method for macromolecular structure determination. It is an indirect method which uses radiofrequency signals emitted from the nuclei of the molecule being investigated to ascertain their spatial arrangement. The molecule is studied while in solution, a fundamental difference from the X-ray crystallographic method. The sample is place in a strong magnetic field in which the magnetically susceptible nuclei (which include ^{1}H nuclei and the less naturally abundant ^{13}C and ^{15}N nuclei) respond like tiny bar magnets and tend to align with the magnetic field. In the NMR experiment, radiofrequency pulses are applied to the sample, which perturb the magnetization of the nuclei from their aligned positions. After the radiofrequency 'excitation' pulse is turned off, the magnetization of the nuclei returns to equilibrium and in doing so each nucleus emits its own characteristic radiofrequency signal. Depending on the radiofrequency pulses that are applied, the emitted radiofrequencies measured can contain information about which signal is coming from which nucleus and, importantly, which nucleus is close in space to which other nucleus (Fig. 7). This provides the information required to determine the 3-D structure of the molecule.

For small macromolecules (< 5 kDa), only two-dimensional (2-D) ^{1}H experiments are required in order to interpret the NMR data. In order to determine larger structures, more sophisticated 3-D and 4-D experiments in which ^{15}N and/or ^{13}C nuclei are detected must be employed. This requires that the macromolecule sample is enriched with ^{15}N and/or ^{13}C isotopes and thus limits the technique to molecules that can be prepared with isotopic enrichment. Nuclear magnetic resonance is also limited to molecules that remain soluble at high concentration (preferably mM concentrations) and that are not greater than approximately 40 kDa (although advances in the field may bring this limit closer to 100 kDa). The final structures tend not to be as well defined as those determined by

X-ray crystallography but may more accurately depict motion which occurs in the molecule in solution.

Finally, electron diffraction is becoming an increasingly useful technique for visualizing macromolecules. Electron diffraction is analagous to X-ray diffraction except that lower wavelength electron frequency waves interact with nuclei as well as the electrons and are used to obtain somewhat lower resolution structural information. Electron diffraction can be conducted in transmission mode through thin films of aligned molecules (or 2-D crystals), which is sometimes the only way that membrane-bound molecules may be structurally analysed. The information that is gained tends to be low resolution (4 Å or greater), but it is anticipated that this technique will also assist in drug design in the future.

The resulting picture of the molecule by these techniques provides information about the arrangement of all of the functional groups of the molecule. Both the chemical nature of the groups and their surface shape form the basis of their binding interaction with their target molecule and therefore provide the information required for rational drug design.

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