PART C

Drug discovery, design and development

Drug discovery, design and development: the past

Before the twentieth century, medicines consisted mainly of herbs and potions and it was not until the mid-nineteenth century that the first serious efforts were made to isolate and purify the **active principles** of these remedies (i.e. the pure chemicals responsible for the medicinal properties). The success of these efforts led to the birth of many of the pharmaceutical companies we know today. Since then, many naturally occurring drugs have been obtained and their structures determined (e.g. morphine from opium, cocaine from coca leaves, quinine from the bark of the cinchona tree).

These natural products sparked off a major synthetic effort where chemists made literally thousands of analogues in an attempt to improve on what nature had provided. Much of this work was carried out on a trial and error basis, but the results obtained revealed several general principles behind drug design. Many of these principles are described in Chapters 13 and 14.

An overall pattern for drug discovery and drug development also evolved, but there was still a high element of trial and error involved in the process.The mechanism by which a drug worked at the molecular level was rarely understood, and drug research focused very much on what is known as the **lead**[1] **compound**—an active principle isolated from a natural source or a synthetic compound prepared in the laboratory.

Drug discovery, design and development: the present

In recent years, medicinal chemistry has undergone a revolutionary change. Rapid advances in the biological sciences have resulted in a much better understanding of how the body functions at the cellular and the molecular level. As a result, most research projects in the pharmaceutical industry or university sector now begin by identifying a suitable target in the body and designing a drug to interact with that target. An understanding of the structure and function of the target, as well as the mechanism by which it interacts with potential drugs is crucial to this approach.

[1] Pronounced '*leed*'.

Generally, we can identify the following stages in drug discovery, design and development.

Drug discovery: finding a lead (Chapter 12)

- Choose a disease!
- Choose a drug target.
- Identify a bioassay.
- Find a 'lead compound'.
- Isolate and purify the lead compound if necessary.
- Determine the structure of the lead compound if necessary.

Drug design (Chapters 13 and 14)

- Identify structure-activity relationships (SARs).
- Identify the pharmacophore.
- Improve target interactions (pharmacodynamics).
- Improve pharmacokinetic properties.

Drug development (Chapter 15)

- Patent the drug.
- Carry out preclinical trials (drug metabolism, toxicology, formulation and stability tests, pharmacology studies etc).
- Design a manufacturing process (chemical and process development).
- Carry out clinical trials.
- Register and market the drug.
- Make money!

Many of these stages run concurrently and are dependent on each other. For example, preclinical trials are usually carried out in parallel with the development of a manufacturing process. Even so, the discovery, design and development of a new drug can take 15 years or more, involve the synthesis of over 10 000 compounds, and cost in the region of $800 million or £450 million.

There are three case studies in this section covering the discovery and design of clinically important agents. Case study 2 covers the design of ACE inhibitors, which are important cardiovascular drugs that act as antihypertensives. Case Study 3 describes the discovery of the antimalarial agent artemisinin, and the design of analogues based on an understanding of its mechanism of action. Case Study 4 is an example of how traditional drug design strategies were used in the design of important drugs that are used against the tropical disease of bilharzia.

12 Drug discovery: finding a lead

In this chapter, we shall look at what happens when a pharmaceutical company or university research group initiates a new medicinal chemistry project through to the identification of a lead compound.

12.1 Choosing a disease

How does a pharmaceutical company decide which disease to target when designing a new drug? Clearly, it would make sense to concentrate on diseases where there is a need for new drugs. However, pharmaceutical companies have to consider economic factors as well as medical ones. A huge investment has to be made towards the research and development of a new drug. Therefore, companies must ensure that they get a good financial return for their investment. As a result, research projects tend to focus on diseases that are important in the developed world, because this is the market best able to afford new drugs. A great deal of research is carried out on ailments such as migraine, depression, ulcers, obesity, flu, cancer, and cardiovascular disease. Less is carried out on the tropical diseases of the developing world. Only when such diseases start to make an impact on western society do the pharmaceutical companies sit up and take notice. For example, there has been a noticeable increase in antimalarial research as a result of the increase in tourism to more exotic countries, and the spread of malaria into the southern states of the USA (see Case Study 3).

Choosing which disease to tackle is usually a matter for a company's market strategists. The science becomes important at the next stage.

12.2 Choosing a drug target

12.2.1 Drug targets

Once a therapeutic area has been identified, the next stage is to identify a suitable drug target (e.g. receptor, enzyme, or nucleic acid). An understanding of which biomacromolecules are involved in a particular disease state is clearly important (Box 12.1). This allows the medicinal research team to identify whether agonists or antagonists should be designed for a particular receptor, or whether inhibitors should be designed for a particular enzyme. For example, agonists of serotonin receptors are useful for the treatment of migraine and antagonists of dopamine receptors are useful as antidepressants. Sometimes it is not known for certain whether a particular target will be suitable or not. For example, **tricyclic antidepressants** such as **desipramine** (Fig. 12.1) are known to inhibit the uptake of the neurotransmitter **noradrenaline** from nerve synapses by inhibiting the carrier protein for noradrenaline (section 23.12.4). However, these drugs also inhibit uptake of a separate neurotransmitter called **serotonin,** and the possibility arose that inhibiting serotonin uptake might also be beneficial. A search for selective serotonin uptake inhibitors was initiated, which led to the discovery of the best-selling antidepressant drug **fluoxetine** (**Prozac**) (Fig. 12.1), but when this project was initiated it was not known for certain whether serotonin uptake inhibitors would be effective or not.

12.2.2 Discovering drug targets

If a drug or a poison produces a biological effect, there must be a molecular target for that agent in the body. In the past, the discovery of drug targets depended on finding the drug first. Many early drugs such as the analgesic **morphine** are natural products derived from plants, and just happen to interact with a molecular target in the human body. As this involves coincidence more than design, the detection of drug targets was very much a hit and miss affair. Later, the body's own chemical messengers started to be discovered and pointed the finger at further targets. For example, since the 1970s a variety of peptides and proteins have been discovered which act as the body's own analgesics (enkephalins

BOX 12.1 Recently discovered target: the caspases

The **caspases** are examples of recently discovered enzymes that may prove useful as drug targets. They are a family of protease enzymes, which catalyse the hydrolysis of important cellular proteins, and which have been found to play a role in inflammation and cell death. Cell death is a natural occurrence in the body, and cells are regularly recycled. Therefore, caspases should not necessarily be seen as 'bad' or 'undesirable' enzymes. Without them, cells could be more prone to unregulated growth resulting in diseases such as cancer.

The caspases catalyse the hydrolysis of particular target proteins such as those involved in DNA repair, and the regulation of cell cycles. By understanding how these enzymes operate, there is the possibility of producing new therapies for a variety of diseases. For example, agents which promote the activity of caspases and lead to more rapid cell death might be useful in the treatment of diseases such as cancer, autoimmune disease, and viral infections. Alternatively, agents that inhibit caspases and reduce the prevalence of cell death could provide novel treatments for trauma, neurodegenerative disease, and strokes. It is already known that the active site of caspases contains two amino acids that are crucial to the mechanism of hydrolysis—cysteine, which acts as a nucleophile, and histidine, which acts as an acid–base catalyst. The mechanism is similar to that used by acetylcholinesterase (section 22.14.3.2).

Caspases recognize aspartate groups within protein substrates and cleave the peptide link next to the aspartate group. Selective inhibitors have been developed which include aspartate or a mimic of it, but it remains to be seen whether such inhibitors have a clinical role.

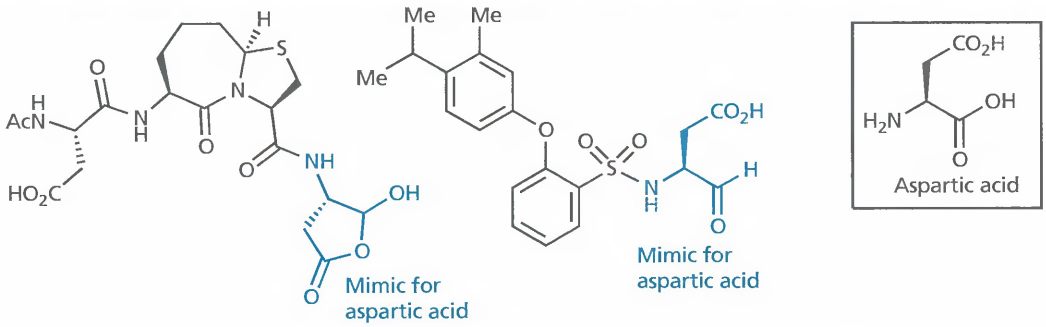


FIGURE 1 Selective caspase inhibitors.

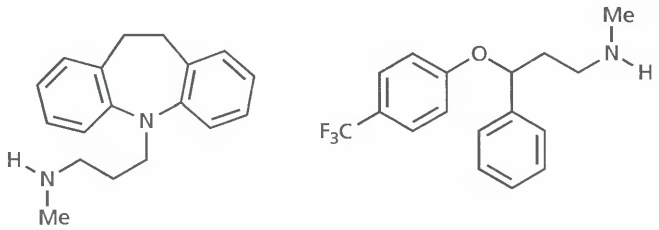


FIGURE 12.1 Antidepressant drugs.

and endorphins). Despite this, relatively few of the body's messengers were identified, either because they were present in such small quantity or because they were too short lived to be isolated. Indeed, many chemical messengers still remain undiscovered today. This in turn means that many of the body's potential drug targets remain hidden. Or at least it did! The advances in genomics and proteomics have changed all that. The various genome projects which have mapped the DNA of humans and other life forms, along with the newer field of proteomics (section 2.6), are revealing an ever increasing number of new proteins which are potential

drug targets for the future. These targets have managed to stay hidden for so long that their natural chemical messengers are also unknown and, for the first time, medicinal chemistry is faced with new targets, but with no lead compounds to interact with them. Such targets have been defined as **orphan receptors**. The challenge is now to find a chemical which will interact with each of these targets in order to find out what their function is and whether they will be suitable as drug targets. This has been one of the main driving forces behind the rapidly expanding area of **combinatorial synthesis** (chapter 16).

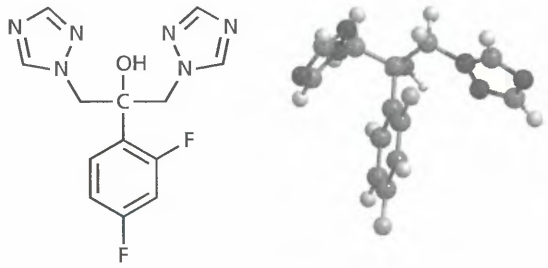


FIGURE 12.2 Fluconazole.

12.2.3 Target specificity and selectivity between species

Target specificity and selectivity is a crucial factor in modern medicinal chemistry research. The more selective a drug is for its target, the less chance there is that it will interact with different targets and have undesirable side effects.

In the field of antimicrobial agents, the best targets to choose are those that are unique to the microbe and are not present in humans. For example, **penicillin** targets an enzyme involved in bacterial cell wall biosynthesis. Mammalian cells do not have a cell wall, so this enzyme is absent in human cells and penicillin has few side effects (section 19.5). In a similar vein, sulfonamides inhibit a bacterial enzyme not present in human cells (section 19.4.1.5), and several agents used to treat AIDS inhibit an enzyme called retroviral reverse transcriptase which is unique to the infectious agent HIV (section 20.7.3).

Other cellular features that are unique to microorganisms could also be targeted. For example, the microorganisms which cause sleeping sickness in Africa are propelled by means of a tail-like structure called a **flagellum.** This feature is not present in mammalian cells, so designing drugs that bind to the proteins making up the flagellum and prevent it from working could be potentially useful in treating that disease.

Having said all that, it is still possible to design drugs against targets which are present both in humans and microbes, as long as the drugs show selectivity against the microbial target. Fortunately, this is perfectly feasible. An enzyme which catalyses a reaction in a bacterial cell differs significantly from the equivalent enzyme in a human cell. The enzymes may have been derived from an ancient common ancestor, but several million years of evolution have resulted in significant structural differences. For example, the antifungal agent **fluconazole** (Fig. 12.2) inhibits a fungal demethylase enzyme involved in steroid biosynthesis. This enzyme is also present in humans, but the structural differences between the two enzymes are significant enough that the antifungal agent is highly selective for the fungal enzyme. Other examples of bacterial or viral enzymes which are sufficiently different from their human equivalents are dihydrofolate reductase (section 19.4.2) and viral DNA polymerase (section 20.6.1).

12.2.4 Target specificity and selectivity within the body

Selectivity is also important for drugs acting on targets within the body. Enzyme inhibitors should only inhibit the target enzyme and not some other enzyme. Receptor agonists/antagonists should ideally interact with a specific kind of receptor (e.g. the adrenergic receptor) rather than a variety of different receptors. However nowadays, medicinal chemists aim for even higher standards of target selectivity. Ideally, enzyme inhibitors should show selectivity between the various isozymes of an enzyme (isozymes are the structural variants of an enzyme that result from different amino acid sequences or quaternary structure). Receptor agonists and antagonists should not only show selectivity for a particular receptor (e.g. an adrenergic receptor) or even a particular receptor type (e.g. the β-adrenergic receptor), but also for a particular receptor subtype (e.g. the β_2-adrenergic receptor).

One of the current areas of research is to find antipsychotic agents with fewer side effects. Traditional antipsychotic agents act as antagonists of dopamine receptors. However, it has been found that there are five dopamine receptor subtypes and that traditional antipsychotic agents antagonise two of these (D_3 and D_2). There is good evidence that the D_2 receptor is responsible for the undesirable Parkinsonian type side effects of current drugs, and so research is now underway to find a selective D_3 antagonist.

12.2.5 Targeting drugs to specific organs and tissues

Targeting drugs against specific receptor subtypes often allows drugs to be targeted against specific organs or against specific areas of the brain. This is because the various receptor subtypes are not uniformly distributed around the body, but are often concentrated in particular tissues. For example, the β-adrenergic receptors in the heart are predominantly β_1 whereas those in the lungs are β_2. This makes it feasible to design drugs that will work on the lungs with a minimal side effect on the heart, and *vice versa*.

Attaining subtype selectivity is particularly important for drugs that are intended to mimic neurotransmitters. Neurotransmitters are released close to their target receptors and once they have passed on their message, they are quickly deactivated and do not have the opportunity to 'switch on' more distant receptors. Therefore, only those receptors which are fed by 'live' nerves are switched on.

In many diseases, there is a 'transmission fault' to a particular tissue or in a particular region of the brain. For example, in Parkinson's disease, **dopamine** transmission is deficient in certain regions of the brain although it is functioning normally elsewhere. A drug could be given to mimic dopamine in the brain. However, such a drug acts like a hormone rather than as a neurotransmitter because it has to travel round the body in order to reach its target. This means that the drug could potentially 'switch on' all the dopamine receptors around the body and not just the ones that are suffering the dopamine deficit. Such drugs would have a large number of side effects, so it is important to make the drug as selective as possible for the particular type or subtype of dopamine receptor affected in the brain. This would target the drug more effectively to the affected area and reduce side effects elsewhere in the body.

12.2.6 Pitfalls

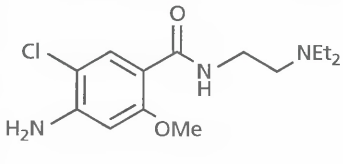
A word of caution! It is possible to identify whether a particular enzyme or receptor plays a role in a particular ailment. However, the body is a highly complex system. For any given function, there are usually several messengers, receptors, and enzymes involved in the process. For example, there is no one simple cause for hypertension (high blood pressure). This is illustrated by the variety of receptors and enzymes which can be targeted in its treatment. These include β_1-adrenoceptors, calcium ion channels, angiotensin-converting enzyme (ACE), and potassium ion channels.

As a result, more than one target may need to be addressed for a particular ailment (Box 12.2). For example, most of the current therapies for asthma involve a combination of a bronchodilator (β_2-agonist) and an anti-inflammatory agent such as a corticosteroid.

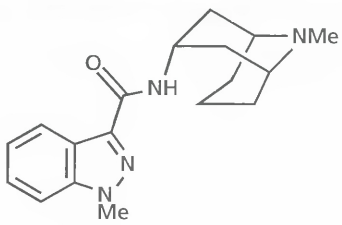
Sometimes, drugs designed against a specific target become less effective over time. Because cells have a highly

BOX 12.2 Pitfalls in choosing particular targets

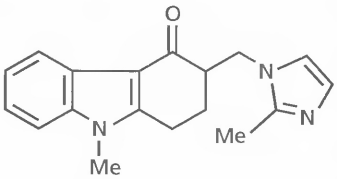
Drugs are designed to interact with a particular target because it is believed that target is important to a particular disease process. Occasionally though, a particular target may not be so important to a disease as was first thought. For example, the dopamine D_2 receptor was thought to be involved in causing nausea. Therefore, the D_2 receptor antagonist **metoclopramide** was developed as an antiemetic agent to prevent nausea. However, it was found that more potent D_2 antagonists were less effective, implying that a different receptor might be more important in producing nausea. Metoclopramide also antagonizes the 5-hydroxytryptamine ($5HT_3$) receptor, so antagonists for this receptor were studied, which led to the development of the antiemetic drugs **granisetron** and **ondansetron**.



Metoclopramide



Granisetron



Ondansetron

Antiemetic agents

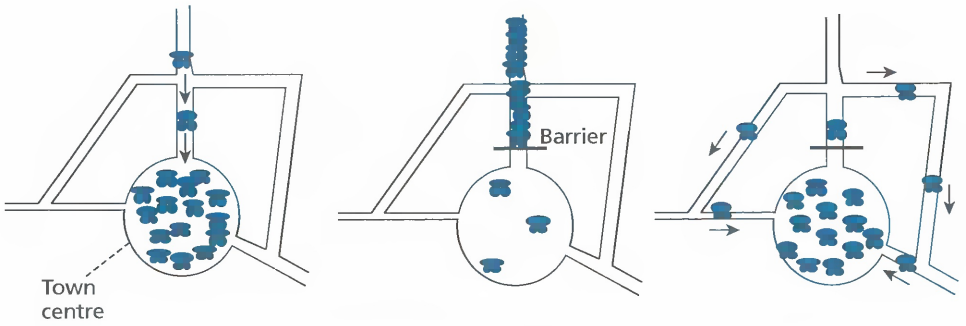


FIGURE 12.3 Avoiding the jam.

complex system of signalling mechanisms, it is possible that the blockade of one part of that system could be bypassed. This could be compared to blocking the main road into town to try and prevent congestion in the town centre. To begin with, the policy works, but in a day or two commuters discover alternative routes and congestion in the centre becomes as bad as ever (Fig. 12.3).

12.2.7 Multi-target drugs

In certain diseases and afflictions, there can be an advantage in 'hitting' a number of different targets selectively, as this can be more beneficial than hitting just one. Combination therapy is normally used to achieve this by administering two or more drugs showing selectivity against the different targets. This is particularly the case in the treatment of cancer (chapter 21) and HIV infection (Box 20.2). However, combination therapies are also used in a variety of other situations (sections 19.4.2.1 and 19.5.4). The disadvantage of combination therapies is the number of different medications and the associated dose regimens. Therefore, there are benefits in designing a single drug that can act selectively at different targets. Many research projects now set out to discover new drugs with a defined profile of activity against a range of specific targets. For example, a research team may set out to find a drug that has agonist activity for one receptor subtype and antagonist activity at another. A further requirement may be that the drug does not inhibit metabolic enzymes (section 11.4) or does not act on targets that can lead to toxicity (Box 12.3). A current area of research is in designing dual-action drugs to treat depression (section 23.12.4 and Case Study 7). Another

BOX 12.3 Early tests for potential toxicity

In vivo and *in vitro tests* are often carried out at an early stage to find out whether lead compounds or candidate drugs are likely to have certain types of toxicity. One such test is to see whether compounds inhibit HERG potassium ion channels in the heart. HERG stands for the gene that codes for this protein, the so called Human Ether-a-go-go Related Gene! Who makes up these names? Several promising drugs have had to be withdrawn at a very late stage in their development because they were found to inhibit the HERG potassium ion channels. Inhibition can result in disruption of the normal rhythm of the heart, leading to fibrillation, heart failure and death. The gastric agent **cisapride** (Fig. 1) and the antihistamine **terfenadine** (section 11.4.6) both had to be withdrawn from the market because of this problem. A large variety of other structures have been found to have this unwanted effect, and so tests to detect this property are best done as early as possible in order to remove this property as part of the drug optimization process.

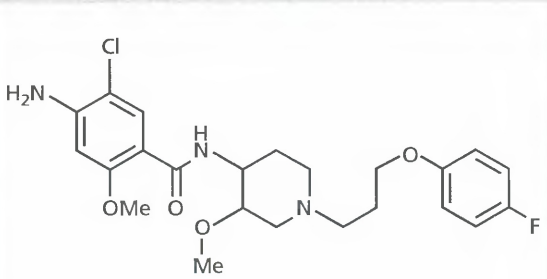


FIGURE 1 Cisapride.

(Continued)

BOX 12.3 Early tests for potential toxicity *(Continued)*

The **Ames test** is another early test that is worth carrying out in order to detect potential mutagenicity or carcinogenicity in new compounds. It involves the use of a mutated bacterial strain of *Salmonella typhimurium* that lacks the ability to synthesize the amino acid histidine, and will grow only in a medium containing histidine. The test involves growing the mutant strain in a medium that contains a small amount of histidine, as well as the test compound. Since there is only a small amount of histidine present, the mutant bacteria will soon stop growing and dividing. However, some of the mutant bacteria will 'back mutate' to the original wild type strain. These cells are now able to synthesize their own histidine and will keep growing. The bacterial colonies that are present on the plate are subcultured onto plates lacking histidine to detect the presence of the wild-type strains, allowing a measure of the mutation rate. Any mutagenic or carcinogenic drug that is present in the original medium will increase the mutation rate, relative to a reference culture containing no drug.

Many research groups now concentrate on 'taming' AMES and HERG liabilities at an early stage of drug development. For example, structure I (Fig. 2) is an antagonist for the **melanin-concentrating hormone receptor** (MCH-R)—a receptor that has been identified as an important target for novel anti-obesity drugs. Unfortunately, structure I blocks HERG ion channels and has Ames liability (i.e. it has mutagenic properties). A library of analogues was prepared by parallel synthesis (chapter 16), which identified structure II as a potent antagonist having no Ames liability. Further work led to structure III, which lacked the Ames liability and had a greatly reduced capacity to block HERG ion channels.

Another example where studies were carried out to remove unfavourable interactions with the HERG ion channels is in the development of the antiviral agent maraviroc (section 20.7.5).

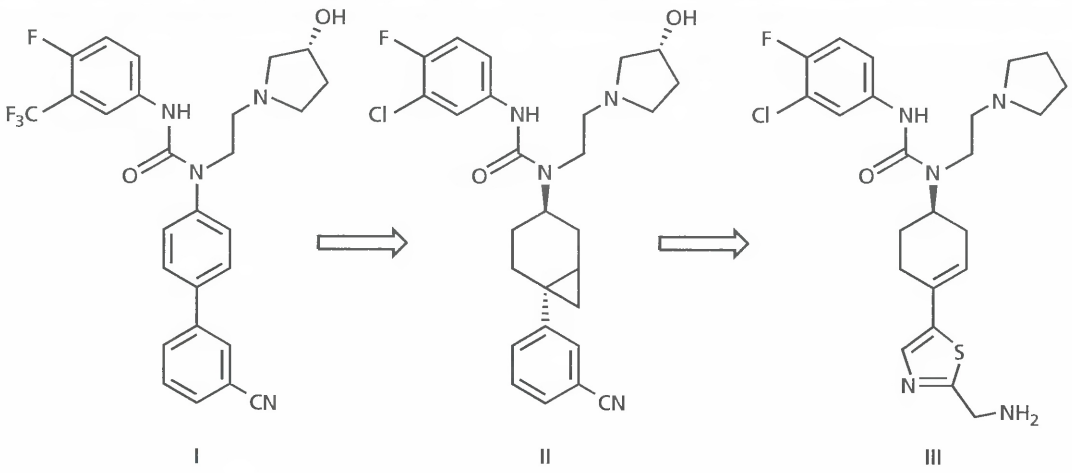


FIGURE 2 The development of agents to remove undesirable properties.

example is **olanzapine** (Fig. 12.4). This drug binds to more than a dozen receptors for serotonin, dopamine, muscarine, noradrenaline and histamine. This kind of profile would normally be unacceptable, but olanzapine has been highly effective in the treatment of schizophrenia, probably because it blocks both serotonin and dopamine receptors. Drugs which interact with a range of targets are called **promiscuous ligands** or **dirty drugs**.

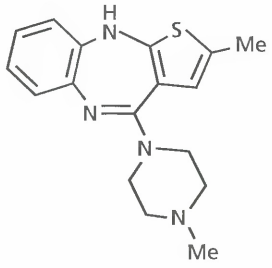


FIGURE 12.4 Olanzapine.

12.3 Identifying a bioassay

12.3.1 Choice of bioassay

Choosing the right bioassay or test system is crucial to the success of a drug research programme. The test should be simple, quick, and relevant, as there is usually a large number of compounds to be analysed. Human testing

is not possible at such an early stage. so the test has to be done *in vitro* (i.e. on isolated cells, tissues, enzymes or receptors) or *in vivo* (on animals). In general, *in vitro* tests are preferred over *in vivo* tests because they are cheaper, easier to carry out, less controversial and they can be automated. However, *in vivo* tests are often needed to check whether drugs interacting with a specific target have the desired pharmacological activity, and also to monitor their pharmacokinetic properties. In modern medicinal chemistry, a variety of tests is usually carried out both *in vitro* and *in vivo* to determine not only whether the candidate drugs are acting at the desired target, but also whether they have activity at other undesired targets (Box 12.3). The direction taken by projects is then determined by finding drugs that have the best balance of good activity at the desired target and minimal activity at other targets. In this way, there is less likelihood of millions of dollars being wasted developing a drug that will either fail clinical trials or be withdrawn from the market with all the associated litigation that involves – a '**fail fast fail cheap**' strategy.

12.3.2 *In vitro* tests

In vitro tests do not involve live animals. Instead, specific tissues, cells or enzymes are used. Enzyme inhibitors can be tested on the pure enzyme in solution. In the past, it could be a major problem to isolate and purify sufficient enzyme to test, but nowadays genetic engineering can be used to incorporate the gene for a particular enzyme into fast-growing cells such as yeast or bacteria. These then produce the enzyme in larger quantities, making isolation easier. For example, **HIV protease** (section 20.7.4.1) has been cloned and expressed in the bacterium *Escherichia coli*. A variety of experiments can be carried out on this enzyme to determine whether an enzyme inhibitor is competitive or non-competitive, and to determine IC_{50} values (section 7.8.2).

Receptor agonists and antagonists can be tested on isolated tissues or cells which express the target receptor on their surface. Sometimes these tissues can be used to test drugs for physiological effects. For example, bronchodilator activity can be tested by observing how well compounds inhibit contraction of isolated tracheal smooth muscle. Alternatively, the affinity of drugs for receptors (how strongly they bind) can be measured by **radioligand studies** (section 8.9). Many *in vitro* tests have been designed by genetic engineering where the gene coding for a specific receptor is identified, cloned, and expressed in fast-dividing cells such as bacterial, yeast, or tumour cells. For example, **Chinese Hamster Ovarian cells** (CHO cells) are commonly used for this purpose, as they express a large amount of the cloned receptor on their cell surface. *In vitro* studies on whole cells are useful because there are none of the complications of *in vivo* studies where the drug has to cross barriers such as the gut wall, or survive metabolic enzymes. The environment surrounding the cells can be easily controlled and both intracellular and intercellular events can be monitored, allowing a measurement of efficacy and potency (section 8.9). Primary cell cultures (i.e. cells that have not been modified) can be produced from embryonic tissues; transformed cell lines are derived from tumour tissue. Cells grown in this fashion are all identical.

Antibacterial drugs are tested *in vitro* by measuring how effectively they inhibit or kill bacterial cells in culture. It may seem strange to describe this as an *in vitro* test, as bacterial cells are living microorganisms. However, *in vivo* tests are defined as those that are carried out on animals or humans to test whether antibacterial agents combat infection.

In vitro tests are also used to test for the pharmacokinetic properties of compounds. For example, the Caco-2 cell monolayer absorption model is used to assess how well a drug is likely to be absorbed from the gastrointestinal tract. Microsomes and hepatocytes extracted from liver cells contain cytochrome P450 enzymes, and can be used to assess the likely metabolism of drug candidates, as well as identifying possible drug-drug interactions.

12.3.3 *In vivo* tests

In vivo tests on animals often involve inducing a clinical condition in the animal to produce observable symptoms. The animal is then treated to see whether the drug alleviates the problem by eliminating the observable symptoms. For example, the development of non-steroidal inflammatory drugs was carried out by inducing inflammation on test animals then testing drugs to see whether they relieved the inflammation.

Transgenic animals are often used in *in vivo* testing. These are animals whose genetic code has been altered. For example, it is possible to replace some mouse genes with human genes. The mouse produces the human receptor or enzyme and this allows *in vivo* testing against that target. Alternatively, the mouse's genes could be altered such that the animal becomes susceptible to a particular disease (e.g. breast cancer). Drugs can then be tested to see how well they prevent that disease.

There are several problems associated with *in vivo* testing. It is slow and it also causes animal suffering. There are the many problems of pharmacokinetics (chapter 11), and so the results obtained may be misleading and difficult to rationalize if *in vivo* tests are carried out in isolation. For example, how can one tell whether a negative result is due to the drug failing to bind to its target or not reaching the target in the first place? Thus, *in vitro* tests are usually carried out first to determine whether a drug interacts with its target, and *in vivo* tests are then carried out to test pharmacokinetic properties.

Certain *in vivo* tests might turn out to be invalid. It is possible that the observed symptoms might be caused by a different physiological mechanism than the one intended. For example, many promising antiulcer drugs which proved effective in animal testing were ineffective in clinical trials. Finally, different results may be obtained in different animal species. For example, **penicillin methyl ester prodrugs** (Box 19.7) are hydrolysed in mice or rats to produce active penicillins, but are not hydrolysed in rabbit, dogs or humans. Another example involves **thalidomide** which is teratogenic in rabbits and humans but has no such effect in mice.

Despite these issues, *in vivo* testing is still crucial in identifying the particular problems which might be associated with using a drug *in vivo* and which cannot be picked up by *in vitro* tests.

12.3.4 Test validity

Sometimes the validity of testing procedures is easy and clear-cut. For example, an antibacterial agent can be tested *in vitro* by measuring how effectively it kills bacterial cells. A local anaesthetic can be tested *in vitro* on how well it blocks action potentials in isolated nerve tissue. In other cases, the testing procedure is more difficult. For example, how do you test a new antipsychotic drug? There is no animal model for this condition and so a simple *in vivo* test is not possible. One way round this problem is to propose which receptor or receptors might be involved in a medical condition and to carry out *in vitro* tests against these in the expectation that the drug will have the desired activity when it comes to clinical trials. One problem with this approach is that it is not always clear-cut whether a specific receptor or enzyme is as important as one might think to the targeted disease (Box 12.2).

12.3.5 High-throughput screening

Robotics and the miniaturization of *in vitro* tests on genetically modified cells has led to a process called high-throughput screening (HTS), which is particularly effective in identifying potential new lead compounds. This involves the automated testing of large numbers of compounds versus a large number of targets; typically, several thousand compounds can be tested at once in 30–50 biochemical tests. It is important that the test should produce an easily measurable effect which can be detected and measured automatically. This effect could be cell growth, an enzyme-catalysed reaction which produces a colour change, or displacement of radioactively labelled ligands from receptors.

Receptor antagonists can be studied using modified cells which contain the target receptor in their cell membrane. Detection is possible by observing how effectively the test compounds inhibit the binding of a radiolabelled ligand. Another approach is to use yeast cells that have been modified such that activation of a target receptor results in the activation of an enzyme which, when supplied with a suitable substrate, catalyses the release of a dye. This produces an easily identifiable colour change.

In general, positive hits are compounds which have an activity in the range of 30 μM–1 nM. Unfortunately, HTS can generate many false-positive hits, and there is a high failure rate between the number of hits and those compounds which are eventually identified as authentic lead compounds. One of the main causes of false hits is what are known as **promiscuous inhibitors.** These are agents which appear to inhibit a range of different target proteins and show very poor selectivity. It is believed that agents working in this manner come together in solution to form molecular aggregates which adsorb target proteins onto their surface, resulting in the inhibition observed. The effect is more pronounced if mixtures of compounds are being tested in solution, such as those prepared by combinatorial syntheses. This kind of inhibition is of no use to drug design, and it is important to eliminate these agents early on as potential lead compounds, such that time is not wasted resynthesizing and investigating them. One way of finding out whether promiscuous inhibition is taking place is to add a detergent to the test solution. This reverses and prevents the phenomenon.

Other false hits include agents which are chemically reactive and carry out a chemical reaction with the target protein, such as the alkylation or acylation of a susceptible nucleophilic group. This results in an irreversible inhibition of the protein since the agent becomes covalently linked to the target. Although there are important drugs which act as irreversible inhibitors, the emphasis in HTS is to find reversible inhibitors which interact with their targets through intermolecular binding interactions. For that reason, known alkylating or acylating agents should not be included in HTS, or if they are, they should not be considered as potential lead compounds. Examples of reactive groups include alkyl halides, acid chlorides, epoxides, aldehydes, α-chloroketones and trifluoromethyl ketones.

12.3.6 Screening by NMR

Nuclear magnetic resonance (NMR) spectroscopy is an analytical tool which has been used for many years to determine the molecular structure of compounds. More recently it has been used to detect whether a compound binds to a protein target. In NMR spectroscopy, a compound is radiated with a short pulse of energy which excites the nuclei of specific atoms such as hydrogen, carbon or nitrogen. Once the pulse of radiation has stopped,

the excited nuclei slowly relax back to the ground state giving off energy as they do so. The time taken by different nuclei to give off this energy is called the **relaxation time** and this varies depending on the environment or position of each atom in the molecule. Therefore, a different signal will be obtained for each atom in the molecule, and a spectrum is obtained which can be used to determine the structure.

The size of the molecule also plays an important role in the length of the relaxation time. Small molecules such as drugs have long relaxation times whereas large molecules such as proteins have short relaxation times. Therefore it is possible to delay the measurement of energy emission such that only small molecules are detected. This is the key to the detection of binding interactions between a protein and a test compound.

First of all, the NMR spectrum of the drug is taken, then the protein is added and the spectrum is re-run, introducing a delay in the measurement such that the protein signals are not detected. If the drug fails to bind to the protein, then its NMR spectrum will still be detected. If the drug binds to the protein, it essentially becomes part of the protein. As a result, its nuclei will have a shorter relaxation time and no NMR spectrum will be detected.

This screening method can also be applied to a mixture of compounds arising from a natural extract or from a combinatorial synthesis. If any of the compounds present bind to the protein, its relaxation time is shortened and so signals due to that compound will disappear from the spectrum. This will show that a component of the mixture is active and determine whether it is worthwhile separating the mixture or not.

There are several advantages in using NMR as a detection system.

- It is possible to screen 1000 small-molecular-weight compounds a day with one machine.
- The method can detect weak binding, which would be missed by conventional screening methods.
- It can identify the binding of small molecules to different regions of the binding site (section 12.4.10).
- It is complimentary to HTS. The latter may give false-positive results, but these can be checked by NMR to ensure that the compounds concerned are binding in the correct binding site (section 12.4.10).
- The identification of small molecules that bind weakly to part of the binding site allows the possibility of using them as building blocks for the construction of larger molecules that bind more strongly (section 12.4.10).
- Screening can be done on a new protein without needing to know its function.

12.3.7 Affinity screening

A nice method of screening mixtures of compounds for active constituents is to take advantage of the binding affinity of compounds for the target. This not only detects the presence of such agents but picks them out from the mixture. For example, the vancomycin family of antibacterial agents has a strong binding affinity for the dipeptide D-Ala-D-Ala (section 19.5.5.2). D-Ala-D-Ala was linked to sepharose resin and the resin was mixed with extracts from various microbes which were known to have antibacterial activity. If an extract lost antibacterial activity as a result of this operation, it indicated that active compounds had bound to the resin. The resin could then be filtered off, and by changing the pH, the compounds could be released from the resin for identification.

12.3.8 Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical method of detecting when a ligand binds to its target. The procedure is patented by Pharmacia Biosensor as **BIAcore** and makes use of a dextran-coated, gold-surfaced glass chip (Fig. 12.5). A ligand that is known to bind to the target is immobilized by linking it covalently to the dextran matrix, which is in a flow of buffer solution. Monochromatic, plane-polarized light is shone at an angle of incidence (α) from below the glass plate and is reflected back

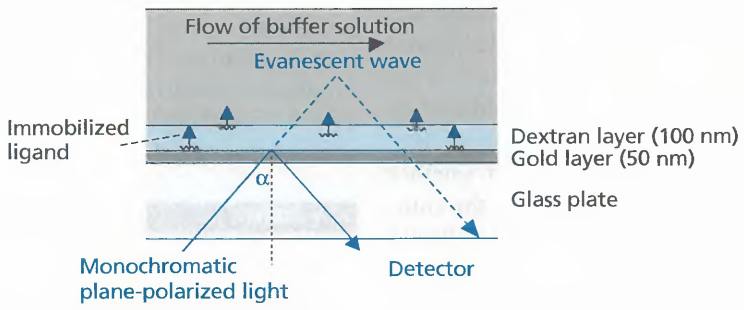


FIGURE 12.5 Surface plasmon resonance. The word evanescent means 'passing out of sight'.

at the interface between the dense gold-coated glass and the less dense buffer solution. However, a component of the light called the evanescent wave penetrates a distance of about one wavelength into the buffer/dextran matrix. Normally, all of the light including the evanescent wave is reflected back, but if the gold film is very thin (a fraction of the evanescent wavelength), and the angle of incidence is exactly right, the evanescent wave interacts with free oscillating electrons called **plasmons** in the metal film. This is the surface plasmon resonance. Energy from the incident light is then lost to the gold film. As a result, there is a decrease in the reflected light intensity, which can be measured.

The angle of incidence when SPR occurs depends crucially on the refractive index of the buffer solution close to the metal film surface. This means that if the refractive index of the buffer changes, the angle of incidence at which SPR takes place changes as well.

If the macromolecular target for the immobilized ligand is now introduced into the buffer flow, some of it will be bound by the immobilized ligand. This leads to a change of refractive index in the buffer solution close to the metal-coated surface which can be detected by measuring the change in the angle of incidence required to get SPR. The technique allows the detection of ligand-target binding and can also be used to measure rate and equilibrium binding constants.

Suppose now we want to test whether a novel compound is binding to the target. This can be tested by introducing the novel compound into the buffer flow along with the target. If the test compound *does* bind to the target, less target will be available to bind to the immobilized ligands, so there will be a different change in refractive index and the change in the angle of incidence will also be different.

12.3.9 Scintillation proximity assay

Scintillation proximity assay (SPA) is a visual method of detecting whether a ligand binds to a target. It involves the immobilization of the target by linking it covalently to beads which are coated with a scintillant. A solution of a known ligand labelled with iodine-125 is then added to the beads. When the labelled ligand binds to the immobilized target, the ^{125}I acts as an energy donor and the scintillant-coated bead acts as an energy acceptor, resulting in an emission of light which can be detected. In order to find out whether a novel compound interacts with the target, the compound is added to the solution of the labelled ligand and the mixture is added to the beads. Successful binding by the novel compound will mean that less of the labelled ligand will bind, resulting in a reduction in the emission of light.

12.3.10 Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) is a technique that is used to determine the thermodynamic properties of binding between a drug and its protein target, in particular the binding affinity and enthalpy change. Two identical glass cells are used which are filled with buffer solution. One of the cells acts as the reference cell, while the other acts as the sample cell and contains the protein target in solution. The reference cell is heated slightly to a constant temperature. The sample cell is heated to the same temperature through an automatic feedback system, whereby any temperature difference between the two cells is detected and power is applied to the sample cell to equalise the temperature. Once the apparatus has stabilized, a constant level of power is being used to maintain the two cells at the same constant temperature.

The drug is now added to the sample cell and binds to the protein target. If the binding interaction is exothermic, heat energy is generated within the sample cell and so less external power is needed to maintain the cell temperature. If the interaction is endothermic, the opposite holds true and more external power has to be applied to maintain the temperature. The external power required to maintain the temperature of the sample cell is measured with respect to time, with power 'spikes' occurring every time the drug is injected into the cell. Measurement of these spikes allows the determination of the thermodynamic properties of binding.

12.3.11 Virtual screening

Virtual screening involves the use of computer programs to assess whether known compounds are likely to be lead compounds for a particular target. There is no guarantee that 'positive hits' from a virtual screening will in fact be active, and the compounds still have to be screened experimentally, but the results from a virtual screening can be used to make experimental screening methods more efficient. In other words, if there are several thousand compounds which are available for testing, virtual screening can be used to identify those compounds which are most likely to be active and these would take priority for actual screening. Virtual screening can involve a search for pharmacophores known to be required for activity or by virtual docking of the compounds into target binding sites (sections 17.11–13).

KEY POINTS

- Pharmaceutical companies tend to concentrate on developing drugs for diseases that are prevalent in developed countries, and aim to produce compounds with better properties than existing drugs.

- A molecular target is chosen which is believed to influence a particular disease when affected by a drug. The greater the selectivity that can be achieved, the less chance of side effects.
- A suitable bioassay must be devised which will demonstrate whether a drug has activity against a particular target. Bioassays can be carried out *in vitro* or *in vivo*, and usually a combination of tests is used.
- HTS involves the miniaturization and automation of *in vitro* tests such that a large number of tests can be carried out in a short period of time.
- Compounds can be tested for their affinity to a macromolecular target by NMR spectroscopy. The relaxation times of ligands bound to a macromolecule are shorter than when they are unbound.
- SPR, SPA, and ITC are two visual methods of detecting whether ligands bind to macromolecular targets.
- Virtual screening can be used to identify compounds most likely to be active in experimental screening.

12.4 Finding a lead compound

Once a target and a testing system have been chosen, the next stage is to find a lead compound—a compound which shows the desired pharmacological activity. The level of activity may not be very great and there may be undesirable side effects, but the lead compound provides a start for the drug design and development process. There are various ways in which a lead compound might be discovered as described in the following sections.

12.4.1 Screening of natural products

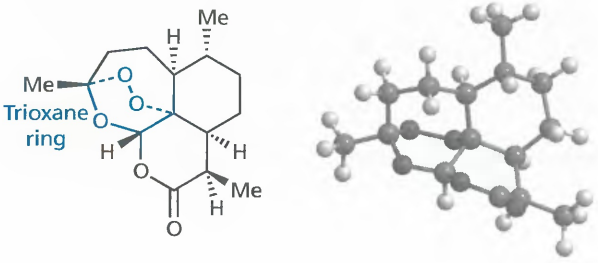
Natural products are a rich source of biologically active compounds. Many of today's medicines are either obtained directly from a natural source or were developed from a lead compound originally obtained from a natural source. Usually, the natural source has some form of biological activity, and the compound responsible for that activity is known as the **active principle.** Such a structure can act as a lead compound. Most biologically active natural products are **secondary metabolites** with quite complex structures. This has an advantage in that they are extremely novel compounds. Unfortunately, this complexity also makes their synthesis difficult and the compound usually has to be extracted from its natural source—a slow, expensive, and inefficient process. As a result, there is usually an advantage in designing simpler analogues (section 13.3.8).

Many natural products have radically new chemical structures which no chemist would dream of synthesizing. For example, the antimalarial drug **artemisinin** (Fig. 12.6) is a natural product with an extremely unstable looking trioxane ring—one of the most unlikely structures to have appeared in recent years (Case Study 3).

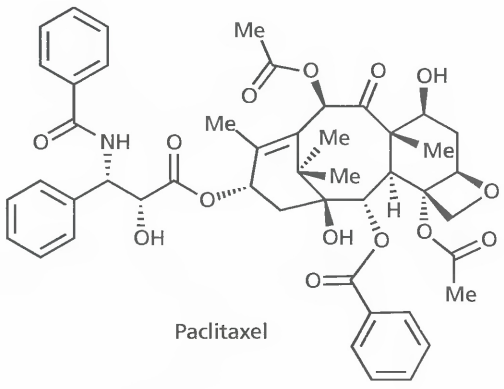
12.4.1.1 Plant kingdom

Plants have always been a rich source of lead compounds (e.g. **morphine, cocaine, digitalis, quinine, tubocurarine, nicotine,** and **muscarine**). Many of these lead compounds are useful drugs in themselves (e.g. morphine and quinine), and others have been the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). Plants still remain a promising source of new drugs and will continue to be so. Clinically useful drugs which have recently been isolated from plants include the anticancer agent **paclitaxel (Taxol)** from the yew tree, and the antimalarial agent artemisinin from a Chinese plant (Fig. 12.6).

Plants provide a bank of rich, complex, and highly varied structures which are unlikely to be synthesized in laboratories. Furthermore, evolution has already carried out a screening process whereby plants are more likely to survive if they contain potent compounds which deter animals or insects from eating them. Considering the debt medicinal chemistry owes to the natural world, it



Artemisinin



Paclitaxel

FIGURE 12.6 Plant natural products as drugs.

is sobering to think that very few plants have been fully studied and the vast majority have not been studied at all. The rainforests of the world are particularly rich in plant species which have still to be discovered, let alone studied. Who knows how many exciting new lead compounds await discovery for the fight against cancer, AIDS or any of the other myriad of human afflictions? This is one reason why the destruction of rain forests and other ecosystems is so tragic; once these ecosystems are destroyed, unique plant species are lost to medicine for ever. For example, **silphion**—a plant cultivated near Cyrene in North Africa and famed as a contraceptive agent in ancient Greece—is now extinct. It is certain that many more useful plants have become extinct without medicine ever being aware of them.

12.4.1.2 Microorganisms

Microorganisms such as bacteria and fungi have also provided rich pickings for drugs and lead compounds. These organisms produce a large variety of antimicrobial agents which have evolved to give their hosts an advantage over their competitors in the microbiological world. The screening of microorganisms became highly popular after the discovery of **penicillin.** Soil and water samples were collected from all round the world in order to study new fungal or bacterial strains, leading to an impressive arsenal of antibacterial agents such as the **cephalosporins, tetracyclines, aminoglycosides, rifamycins, chloramphenicol** and **vancomycin** (Chapter 19). Although most of the drugs derived from microorganisms are used in antibacterial therapy, some microbial metabolites have provided lead compounds in other fields of medicine. For example, **asperlicin**—isolated from *Aspergillus alliaceus*—is a novel antagonist of a peptide hormone called **cholecystokinin** (CCK) which is involved in the control of appetite. CCK also acts as a neurotransmitter in the brain and is thought to be involved in panic attacks. Analogues of asperlicin may therefore have potential in treating anxiety (see also Box 13.2).

Other examples include the fungal metabolite **lovastatin,** which was the first of the clinically useful statins found to lower cholesterol levels (Case Study 1), and another fungal metabolite called **ciclosporin** (Fig. 12.7) which is used to suppress the immune response after transplantation operations. **Lipstatin** (Fig. 12.7) is a natural product which was isolated from *Streptomyces toxytricini* and inhibits pancreatic lipase. It is the lead compound for the anti-obesity compound orlistat (Box 7.2)

12.4.1.3 Marine sources

In recent years, there has been great interest in finding lead compounds from marine sources. Coral, sponges, fish, and marine microorganisms have a wealth of biologically potent chemicals with interesting inflammatory, antiviral, and anticancer activity. For example, **curacin A** (Fig. 12.8) is obtained from a marine cyanobacterium, and shows potent antitumour activity. Other antitumour agents derived from marine sources include **eleutherobin, discodermolide, bryostatins, dolostatins,** and **cephalostatins** (sections 21.5.2 and 21.8.2).

12.4.1.4 Animal sources

Animals can sometimes be a source of new lead compounds. For example, a series of antibiotic polypeptides known as the **magainins** were extracted from the skin of the African clawed frog *Xenopus laevis*. These agents protect the frog from infection and may provide clues to the

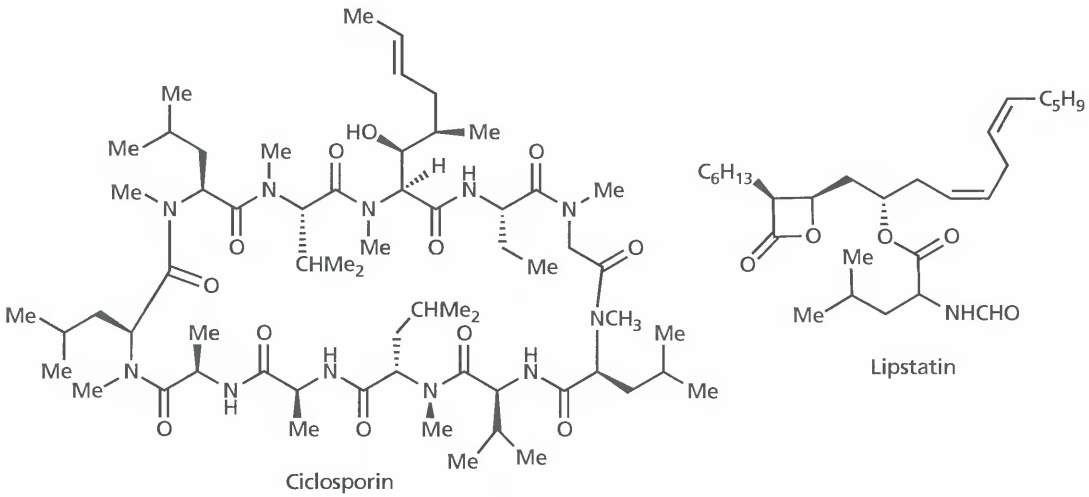


FIGURE 12.7 Lead compounds from microbiological sources.

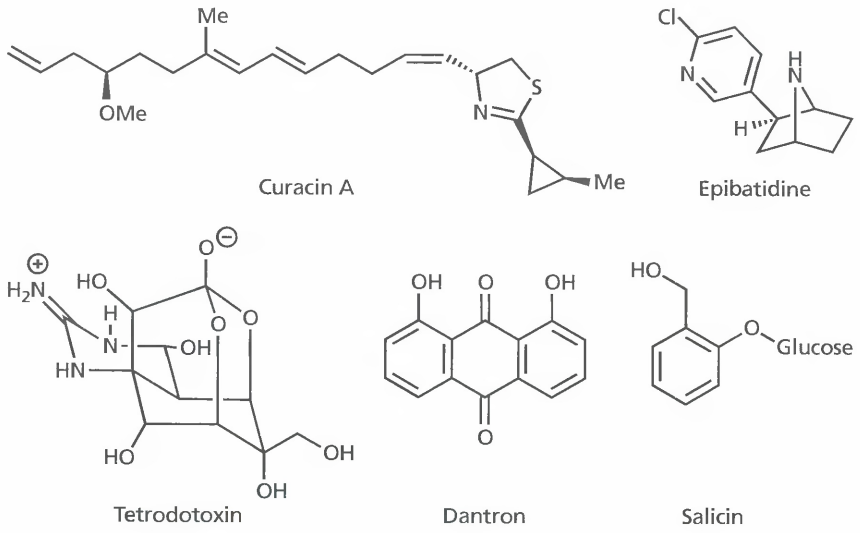


FIGURE 12.8 Natural products as drugs.

development of novel antibacterial and antifungal agents in human medicine. Another example is a potent analgesic compound called **epibatidine** (Fig. 12.8), obtained from the skin extracts of the Ecuadorian poison frog.

12.4.1.5 Venoms and toxins

Venoms and toxins from animals, plants, snakes, spiders, scorpions, insects, and microorganisms are extremely potent because they often have very specific interactions with a macromolecular target in the body. As a result, they have proved important tools in studying receptors, ion channels, and enzymes. Many of these toxins are polypeptides (e.g. α-**bungarotoxin** from cobras). However, non-peptide toxins such as **tetrodotoxin** from the puffer fish (Fig. 12.8) are also extremely potent.

Venoms and toxins have been used as lead compounds in the development of novel drugs. For example, **teprotide,** a peptide isolated from the venom of the Brazilian viper, was a lead compound for the development of the antihypertensive agents **cilazapril** and **captopril** (Case Study 2).

The neurotoxins from *Clostridium botulinum* are responsible for serious food poisoning **(botulism)**, but they have a clinical use as well. They can be injected into specific muscles (such as those controlling the eyelid) to prevent muscle spasm. These toxins prevent cholinergic transmission (chapter 22) and could well prove a lead for the development of novel anticholinergic drugs.

Finally, **conotoxin** is a peptide toxin derived from the marine cone snail, and has very powerful analgesic properties in humans. A synthetic form of conotoxin called **ziconotide** was approved in 2004 for the treatment of chronic pain.

12.4.2 Medical folklore

In the past, ancient civilizations depended greatly on local flora and fauna for their survival. They would experiment with various berries, leaves and roots to find out what effects they had. As a result, many brews were claimed by the local healer or shaman to have some medicinal use. More often than not, these concoctions were useless or downright dangerous, and if they worked at all, it was because the patient willed them to work—a **placebo effect**. However, some of these extracts may indeed have a real and beneficial effect, and a study of medical folklore can give clues as to which plants might be worth studying in more detail. **Rhubarb** root has been used as a purgative for many centuries. In China, it was called 'The General' because of its 'galloping charge'! The most significant chemicals in rhubarb root are anthraquinones, which were used as the lead compounds in the design of the laxative—**dantron** (Fig. 12.8).

The ancient records of Chinese medicine also provided the clue to the novel antimalarial drug **artemisinin** mentioned in section 12.4.1 above (Case Study 3). The therapeutic properties of the opium poppy (active principle **morphine**) were known in Ancient Egypt, as were those of the *Solanaceae* plants in ancient Greece (active principles **atropine** and **hyoscine**; section 22.11.2). The snakeroot plant was well regarded in India (active principle **reserpine**; Fig. 12.9), and herbalists in medieval England used extracts from the willow tree (active principle **salicin**; Fig. 12.8) and foxglove (active principle **digitalis**—a mixture of compounds such as digitoxin, digitonin, and digitalin). The Aztec and Mayan cultures of South America used extracts from a variety of bushes and trees including the ipecacuanha root (active principle

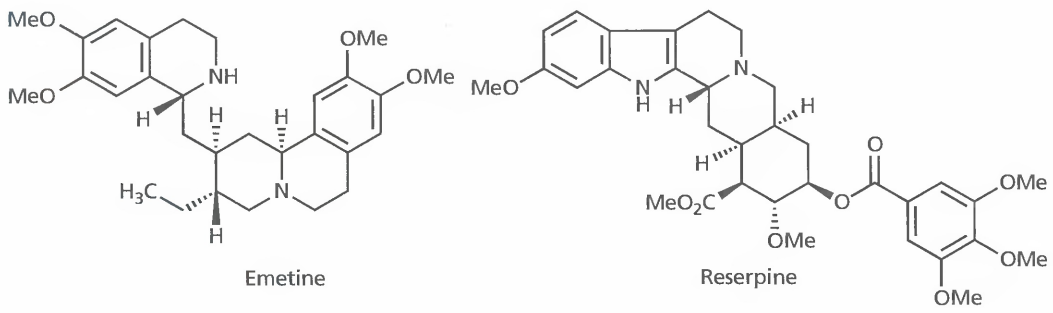


FIGURE 12.9 Active compounds resulting from studies of herbs and potions.

emetine; Fig. 12.9), coca bush (active principle **cocaine**), and cinchona bark (active principle **quinine**).

12.4.3 Screening synthetic compound 'libraries'

The thousands of compounds which have been synthesized by the pharmaceutical companies over the years are another source of lead compounds. The vast majority of these compounds have never made the market place, but they have been stored in compound 'libraries' and are still available for testing. Pharmaceutical companies often screen their library of compounds whenever they study a new target. However, it has to be said that the vast majority of these compounds are merely variations on a theme; for example 1000 or so different penicillin structures. This reduces the chances of finding a novel lead compound.

Pharmaceutical companies often try to diversify their range of structures by purchasing novel compounds prepared by research groups elsewhere—a useful source of revenue for hard-pressed university departments! These compounds may never have been synthesized with medicinal chemistry in mind and may be intermediates in a purely synthetic research project, but there is always the chance that they may have useful biological activity.

It can also be worth testing synthetic intermediates. For example, a series of thiosemicarbazones was synthesized and tested as antitubercular agents in the 1950s. This included isonicotinaldehyde thiosemicarbazone, the synthesis of which involved the hydrazide structure **isoniazid** (Fig. 12.10) as a synthetic intermediate. It was subsequently found that isoniazid had greater activity than the target structure. Similarly, a series of **quinoline-3-carboxamide** intermediates (Fig. 12.10) were found to have antiviral activity.

12.4.4 Existing drugs

12.4.4.1 'Me too' and 'me better' drugs

Many companies use established drugs from their competitors as lead compounds in order to design a drug that gives them a foothold in the same market area. The aim is to modify the structure sufficiently such that it avoids patent restrictions, retains activity, and ideally has improved therapeutic properties. For example, the antihypertensive drug captopril was used as a lead compound by various

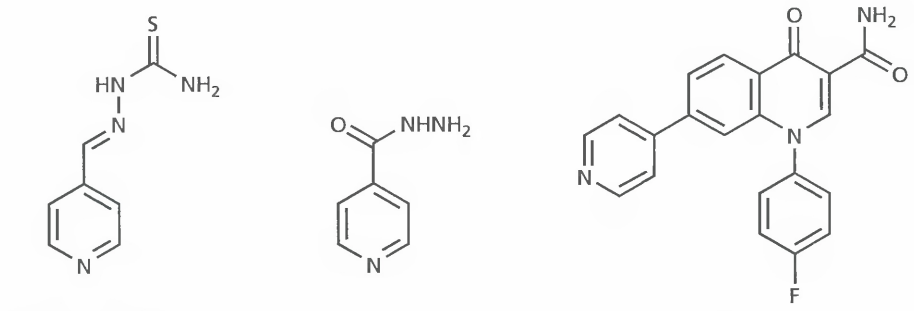


FIGURE 12.10 Pharmaceutically active compounds discovered from synthetic intermediates.

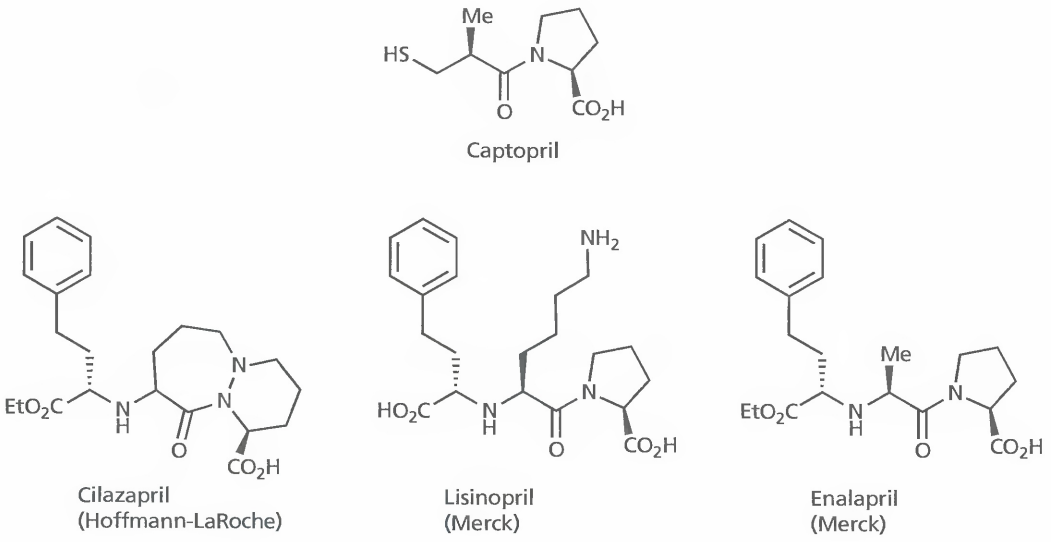


FIGURE 12.11 Captopril and 'me too' drugs.

companies to produce their own antihypertensive agents (Fig. 12.11, and Case Study 2).

Although often disparaged as 'me too' drugs, they can often offer improvements over the original drug ('me better' drugs). For example, modern penicillins are more selective, more potent, and more stable than the original penicillins. Newer statins that lower cholesterol levels also have improved properties over older ones (Case Study 1).

12.4.4.2 Enhancing a side effect

An existing drug usually has a minor property or an undesirable side effect which could be of use in another area of medicine. As such, the drug could act as a lead compound on the basis of its side effects. The aim would then be to enhance the desired side effect and to eliminate the major biological activity. This has been described as the SOSA approach—**selective optimization of side activities**. Choosing a known drug as the lead compound for a side effect has the advantage that the compound is already 'drug-like' and it should be more feasible to develop a clinically useful drug with the required pharmacodynamic and pharmacokinetic properties. Many of the 'hits' obtained from HTS do not have a 'drug-like' structure and it may require far more effort to optimize them. Indeed, it has been argued that modifications of known drug structures should provide lead compounds in several areas of medicinal chemistry. Many research groups are now screening compounds that are either in clinical use or reached late-stage clinical trials to see whether they have side activities that would make them suitable lead compounds. The Johns Hopkins Clinical Compound Library is one such source of these compounds.

For example, most sulfonamides have been used as antibacterial agents. However, some sulfonamides with antibacterial activity could not be used clinically because they had convulsive side effects brought on by **hypoglycaemia** (lowered glucose levels in the blood). Clearly, this is an undesirable side effect for an antibacterial agent, but the ability to lower blood glucose levels would be useful in the treatment of diabetes. Therefore, structural alterations were made to the sulfonamides concerned in order to eliminate the antibacterial activity and to enhance the hypoglycaemic activity. This led to the antidiabetic agent **tolbutamide** (Fig. 12.12). Another example was the discovery that the anticoagulant **warfarin** is also a weak inhibitor of a viral enzyme that is important in the life cycle of HIV. Warfarin was used as the lead comound in the development of an anti-HIV drug called **tipranavir** (section 20.7.4.10).

In some cases, the side effect may be strong enough that the drug can be used without modification. For example, the anti-impotence drug **sildenafil** (Viagra) (Fig. 12.12) was originally designed as a vasodilator to treat angina and hypertension. During clinical trials, it was found that it acted as a vasodilator more effectively in the penis than in the heart, resulting in increased erectile function. The drug is now used to treat erectile dysfunction and sexual impotence. Another example is the antidepressant drug **bupropion**. Patients taking this drug reported that it helped them give up smoking and so the drug is now marketed as an antismoking aid (Zyban). **Astemizole**

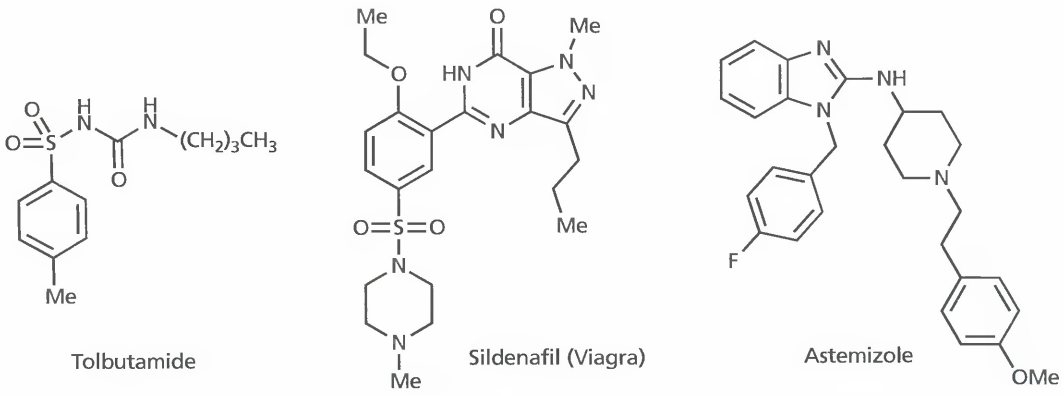


FIGURE 12.12 Tolbutamide and sildenafil (Viagra).

(Fig. 12.12) is a medication used in the treatment of allergy, but has been found to be a potent antimalarial agent.

The moral of the story is that a drug used in one field of medicinal chemistry could be the lead compound in another field (Box 12.4). Furthermore, one can fall into the trap of thinking that a structural group of compounds all have the same type of biological activity. The sulfonamides are generally thought of as antibacterial agents, but we have seen that they can have other properties as well.

12.4.5 Starting from the natural ligand or modulator

12.4.5.1 Natural ligands for receptors

The natural ligand of a target receptor has sometimes been used as the lead compound. The natural neurotransmitters **adrenaline** and **noradrenaline** were the starting points for the development of adrenergic β-agonists such as **salbutamol**, **dobutamine** and **xamoterol** (section 23.10), and 5-hydroxytryptamine (5-HT) was the

BOX 12.4 Selective optimization of side activities (SOSA)

Several drugs have been developed by enhancing the side effect of another drug. **Chlorpromazine** is used as a neuroleptic agent in psychiatry, but was developed from the antihistamine agent **promethazine**. This might appear an odd thing to do, but it is known that promethazine has sedative side effects and so medicinal chemists modified the structure to enhance the sedative effects at the expense of antihistamine activity. Similarly, the development of sulfonamide diuretics such as **chlorothiazide** arose from the observation that **sulfanilamide** has a diuretic effect in large doses (due to its action on an enzyme called **carbonic anhydrase**).

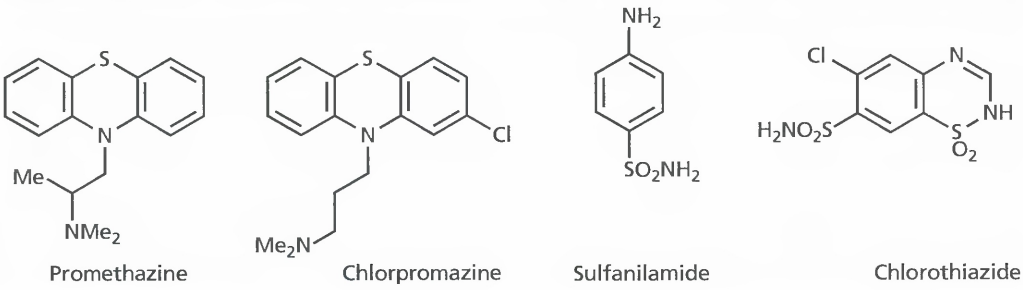


FIGURE 1 Drugs developed by enhancing a side effect.

BOX 12.4 Selective optimization of side activities (SOSA) *(Continued)*

Sometimes slight changes to a structure can result in significant changes in pharmacological activity. For example, **minaprine** (Fig. 2) is an antidepressant agent that acts as a serotonin agonist. Adding a phenolic substituent resulted in **4-hydroxyminaprine,** which is a potent dopamine agonist, whereas adding a cyano substituent gave **bazinaprine,** which is a potent inhibitor of the enzyme monoamine oxidase-A. Minaprine also binds weakly to muscarine receptors (K_i = 17 μM) and modifications were successfully carried out to give structure I with nanomolar activity (K_i = 3 nM) for the muscarinic receptor and negligible activity for dopamine and serotonin receptors. Minaprine also has weak affinity for the cholinesterase enzyme (IC_{50} = 600 μM). Modifications led to structure II, which has strong affinity (IC_{50} = 10 nM) for this target.

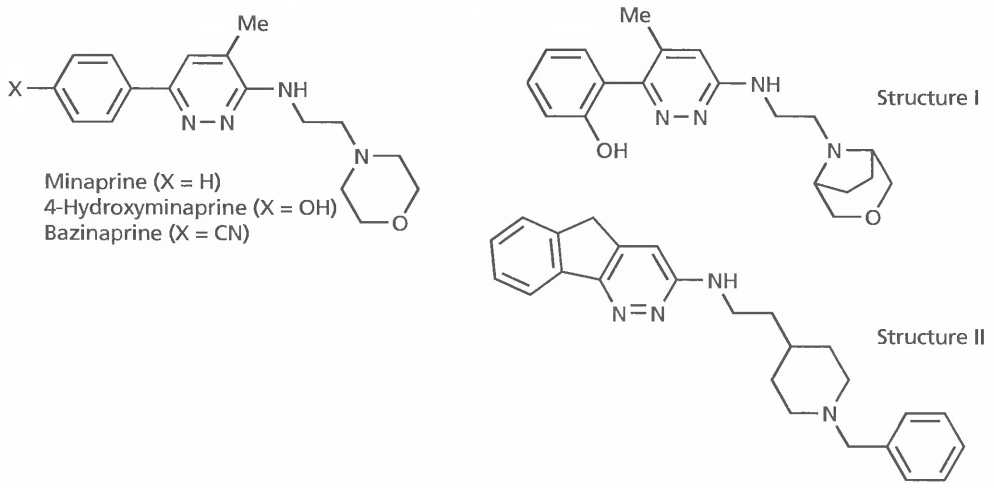


FIGURE 2 Structures with different pharmacological properties derived from the lead compound minaprine.

starting point for the development of the 5-HT_1 agonist **sumatriptan** (Fig. 12.13).

The natural ligand of a receptor can also be used as the lead compound in the design of an antagonist. For example, **histamine** was used as the original lead compound in the development of the H_2 histamine antagonist **cimetidine** (section 25.2). Turning an agonist into an antagonist is frequently achieved by adding extra binding groups to the lead structure. Other examples include the development of the adrenergic antagonist **pronethalol** (section 23.11.3.1), the H_2 antagonist **burimamide** (section 25.2), and the 5-HT_3 antagonists **ondansetron** and **granisetron** (Box 12.2).

Sometimes the natural ligand for a receptor is not known (an **orphan receptor**) and the search for it can be a major project in itself. If the search is successful, however, it opens up a brand-new area of drug design (Box 12.5). For example, the identification of the opioid receptors for **morphine** led to a search for endogenous opioids (natural body painkillers) which eventually led to the discovery of **endorphins** and **enkephalins** as the natural ligands, and their use as lead compounds (section 24.8).

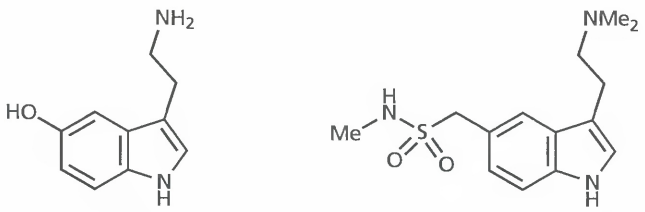
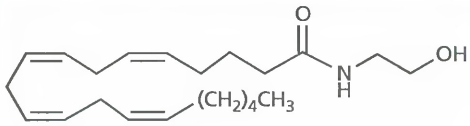


FIGURE 12.13 5-Hydroxytryptamine and sumatriptan.

BOX 12.5 Natural ligands as lead compounds

The discovery of **cannabinoid** receptors in the early 1990s led to the discovery of two endogenous cannabinoid messengers—**arachidonylethanolamine (anandamide)** and **2-arachidonyl glycerol.** These have now been used as lead compounds for developing agents that will interact with cannabinoid receptors. Such agents may prove useful in suppressing nausea during chemotherapy, or in stimulating appetite in patients with AIDS.



Anandamide.

12.4.5.2 Natural substrates for enzymes

The natural substrate for an enzyme can be used as the lead compound in the design of an enzyme inhibitor. For example, **enkephalins** have been used as lead compounds for the design of enkephalinase inhibitors. **Enkephalinases** are enzymes which metabolize enkephalins, and their inhibition should prolong the activity of enkephalins (section 24.8.3).

The natural substrate for HIV-protease was used as the lead compound for the development of the first protease inhibitor used to treat HIV (section 20.7.4). Other examples of substrates being used as lead compounds for inhibitors include the substrates for farnesyl transferase (section 21.6.1) and matrix metalloproteinase (section 21.7.1).

12.4.5.3 Enzyme products as lead compounds

It should be remembered that enzymes catalyse a reaction in both directions and so the product of an enzyme-catalysed reaction can also be used as a lead compound for an enzyme inhibitor. For example, the design of the carboxypeptidase inhibitor **L-benzylsuccinic acid** was based on the products arising from the carboxypeptidase-catalysed hydrolysis of peptides (Case Study 2).

12.4.5.4 Natural modulators as lead compounds

Many receptors and enzymes are under allosteric control (sections 3.6 and 8.3.2). The natural or endogenous chemicals that exert this control (modulators) could also serve as lead compounds.

In some cases, a modulator for an enzyme or receptor is suspected but has not yet been found. For example, the **benzodiazepines** are synthetic compounds that modulate the receptor for **γ-aminobutyric acid** (GABA) by binding to an allosteric binding site. The natural modulators for this allosteric site were not known at the time benzodiazepines were synthesized, but endogenous peptides called **endozepines** have since been discovered which bind to the same allosteric binding site and which may serve as lead compounds for novel drugs having the same activity as the benzodiazepines.

12.4.6 Combinatorial and parallel synthesis

The growing number of potentially new drug targets arising from genomic and proteomic projects has meant that there is an urgent need to find new lead compounds to interact with them. Unfortunately, the traditional sources of lead compounds have not managed to keep pace and in the last decade or so, research groups have invested greatly in combinatorial and parallel synthesis in order to tackle this problem. Combinatorial synthesis is an automated solid-phase procedure aimed at producing as many different structures as possible in as short a time as possible. The reactions are carried out on very small scale, often in a way that will produce mixtures of compounds in each reaction vial. In a sense, combinatorial synthesis aims to mimic what plants do, i.e. produce a pool of chemicals, one of which may prove to be a useful lead compound. Combinatorial synthesis has developed so swiftly that it is almost a branch of chemistry in itself and a separate chapter is devoted to it (chapter 16). Parallel synthesis involves the small scale synthesis of large numbers of compounds at the same time using specialist miniaturised equipment. The synthesis can be carried out in solution or solid phase, and each reaction vial contains a distinct product (chapter 16).

12.4.7 Computer-aided design of lead compounds

A detailed knowledge of a target binding site significantly aids in the design of novel lead compounds intended to bind with that target. In cases where enzymes or receptors can be crystallized, it is possible to determine the structure of the protein and its binding site by **X-ray crystallography.** Molecular modelling software programs can then be used to study the binding site, and to

design molecules which will fit and bind to the site—*de novo* drug design (section 17.15).

In some cases, the enzyme or receptor cannot be crystallized and so X-ray crystallography cannot be carried out. However, if the structure of an analogous protein has been determined, this can be used as the basis for generating a computer model of the protein. This is covered in more detail in section 17.14. NMR spectroscopy has also been effective in determining the structure of proteins and can be applied to proteins that cannot be studied by X-ray crystallography.

12.4.8 Serendipity and the prepared mind

Frequently, lead compounds are found as a result of serendipity (i.e. chance). However, it still needs someone with an inquisitive nature or a prepared mind to recognize the significance of chance discoveries and to take advantage of these events. The discovery of **cisplatin** (section 9.3.4) and **penicillin** (section 19.5.1.1) are two such examples, but there are many more (Box 12.6).

BOX 12.6 Examples of serendipity

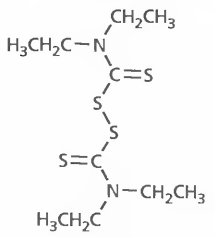
During the Second World War, an American ship carrying **mustard gas** exploded in an Italian harbour. It was observed that many of the survivors who had inhaled the gas lost their natural defences against microbes. Further study showed that their white blood cells had been destroyed. It is perhaps hard to see how a drug that weakens the immune system could be useful. However, there is one disease where this is the case—leukemia. Leukemia is a form of cancer which results in the excess proliferation of white blood cells, so a drug that kills these cells is potentially useful. As a result, a series of mustard-like drugs were developed based on the structure of the original mustard gas (sections 9.3.1 and 21.2.3.1).

Another example involved the explosives industry, where it was quite common for workers to suffer severe headaches. These headaches resulted from dilatation of blood vessels in the brain, caused by handling trinitrotoluene (**TNT**). Once again, it is hard to see how such drugs could be useful. Certainly, the dilatation of blood vessels in the brain may not be particularly beneficial, but dilating the blood vessels in the heart could be useful in cardiovascular medicine. As a result, drugs were developed which dilated coronary blood vessels and alleviated the pain of angina.

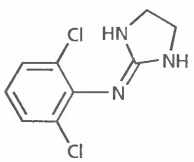
Workers in the rubber industry found that they often acquired a distaste for **alcohol!** This was caused by an antioxidant used in the rubber manufacturing process which found its way into workers' bodies and prevented the normal oxidation of alcohol in the liver. As a result, there was a build up of **acetaldehyde,** which was so unpleasant that workers preferred not to drink. The antioxidant became the lead compound for the development of **disulfiram (Antabuse)**—used for the treatment of chronic alcoholism.

The following are further examples of lead compounds arising as a result of serendipity:

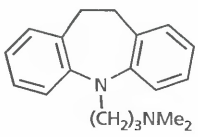
- **Clonidine** was originally designed to be a nasal vasoconstrictor to be used in nasal drops and shaving soaps. Clinical trials revealed that it caused a marked fall in blood pressure, and so it became an important antihypertensive instead.
- **Imipramine** was synthesized as an analogue of chlorpromazine (Box 12.4), and was initially to be used as an antipsychotic. However, it was found to alleviate depression and this led to the development of a series of compounds classified as the **tricyclic antidepressants** (section 23.12.4).



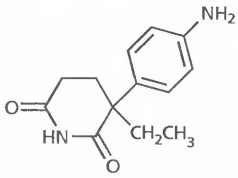
Disulfiram (Antabuse)



Clonidine



Imipramine



Aminoglutethimide

Drugs discovered by serendipity.

(Continued)

BOX 12.6 Examples of serendipity *(Continued)*

- **Aminoglutethimide** was prepared as a potential anti-epileptic drug, but is now used as an anticancer agent (section 21.4.5).
- The anti-impotence drug **sildenafil (Viagra)** (Fig. 12.11) was discovered by chance from a project aimed at developing a new heart drug.
- **Isoniazid** (Fig. 12.9) was originally developed as an antituberculosis agent. Patients taking it proved remarkably cheerful and this led to the drug becoming the lead compound for a series of antidepressant drugs known as the **monoamine oxidase inhibitors** (MAOIs) (section 23.12.5).
- **Chlorpromazine** (Box 12.4) was synthesized as an antihistamine for possible use in preventing surgical shock, and was found to make patients relaxed and unconcerned. This led to the drug being tested in people with manic depression where it was found to have tranquillizing effects, resulting in it being the first of the neuroleptic drugs (major tranquillizers) used for schizophrenia.
- **Ciclosporin A** (Fig. 12.6) suppresses the immune system and is used during organ and bone marrow transplants to prevent the immune response rejecting the donor organs. The compound was isolated from a soil sample as part of a study aimed at finding new antibiotics. Fortunately, the compounds were more generally screened and the immunosuppressant properties of ciclosporin A were identified.
- In a similar vein, the anticancer alkaloids **vincristine** and **vinblastine** (section 10.2.2) were discovered by chance when searching for compounds that could lower blood sugar levels. Vincristine is used in the treatment of Hodgkin's disease.

Sometimes, the research carried out to improve a drug can have unexpected and beneficial spin offs. For example, **propranolol** and its analogues are effective β-blocking drugs (antagonists of β-adrenergic receptors) (section 23.11.3). However, they are also lipophilic, which means that they can enter the central nervous system and cause side effects. In an attempt to cut down entry into the central nervous system, it was decided to add a hydrophilic amide group to the molecule and so inhibit passage through the blood–brain barrier. One of the compounds made was **practolol**. As expected, this compound had fewer side effects acting on the central nervous system, but more importantly, it was found to be a selective antagonist for the β-receptors of the heart over β-receptors in other organs—a result that was highly desirable, but not the one that was being looked for at the time.

Frequently, new lead compounds have arisen from research projects carried out in a totally different field of medicinal chemistry. This emphasizes the importance of keeping an open mind, especially when testing for biological activity. For example, we have already described the development of the antidiabetic drug **tolbutamide** (section 12.4.4.2), based on the observation that some antibacterial sulfonamides could lower blood glucose levels.

12.4.9 Computerized searching of structural databases

New lead compounds can be found by carrying out computerized searches of structural databases. In order to carry out such a search, it is necessary to know the desired **pharmacophore** (section 13.2). Alternatively, docking experiments can be carried out if the structure of the target binding site is known. This type of database searching is also known as **database mining** and is described in sections 17.11–17.13.

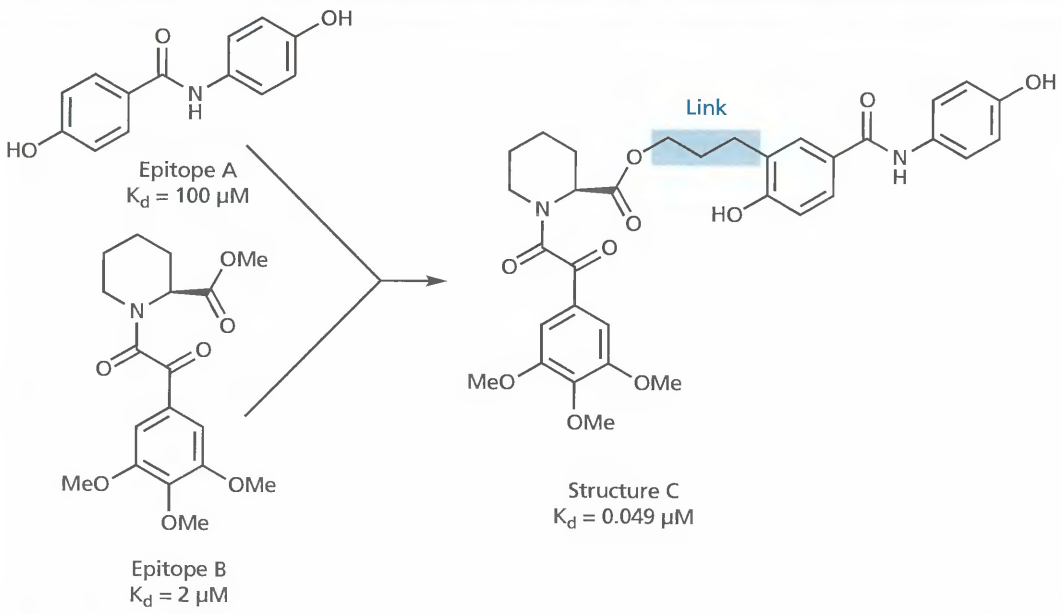
12.4.10 Fragment-based lead discovery

So far we have described methods by which a lead compound can be discovered from a natural or synthetic source, but all these methods rely on an active compound being present. Unfortunately, there is no guarantee that this will be the case. Recently, NMR spectroscopy has been used to *design* a lead compound rather than to discover one (Box 12.7). In essence, the method sets out to find small molecules (**epitopes**) which will bind to specific but different regions of a protein's binding site. These molecules will have no activity in themselves since they only bind to one part of the binding site, but if a larger molecule is designed which links these epitopes together, then a lead compound may be created which *is* active and which binds to the whole of the binding site (Fig. 12.14).

Lead discovery by NMR is also known as SAR by NMR (SAR = structure–activity relationships) and can be applied to proteins of known structure which are labelled with ^{15}N or ^{13}C such that each amide bond in the protein has an identifiable peak.

A range of low-molecular-weight compounds is screened to see whether any of them bind to a specific region of the binding site. Binding can be detected by observing a shift in any of the amide signals, which will not only show that binding is taking place, but will also reveal which part of the binding site is occupied. Once a

BOX 12.7 Use of NMR spectroscopy in finding lead compounds



Design of a ligand for the FK506 binding protein.

NMR spectroscopy was used in the design of high-affinity ligands for the FK506 binding protein – a protein involved in the suppression of the immune response. Two optimized epitopes (A and B) were discovered, which bound to different regions of the binding site. Structure C was then synthesized, where the two epitopes were linked by a propyl link. This compound had higher affinity than either of the individual epitopes and represents a lead compound for further development.

compound (or ligand) has been found that binds to one region of the binding site, the process can be repeated to find another ligand that will bind to a different region. This is usually done in the presence of the first ligand to ensure that the second ligand does in fact bind to a distinct region.

Once two ligands (or epitopes) have been identified, the structure of each can be optimized to find the best ligand for each of the binding regions, then a molecule can be designed where the two ligands are linked together.

There are several advantages to this approach. Since the individual ligands are optimized for each region of the binding site, a lot of synthetic effort is spared. It is much easier to synthesize a series of small molecular weight compounds to optimize the interaction with specific

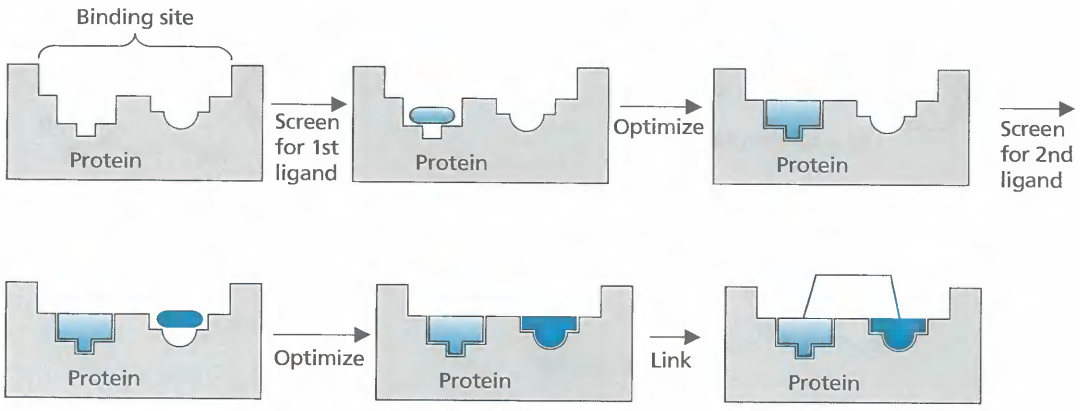


FIGURE 12.14 Epitope mapping.

parts of the binding site, than it is to synthesize a range of larger molecules to fit the overall binding site. A high level of diversity is also possible, as various combinations of fragments could be used. A further advantage is that it is more likely to find epitopes that will bind to a particular region of a binding site than to find a lead compound that will bind to the overall binding site. Moreover, fragments are more likely to be efficient binders, having a high binding energy per unit molecular mass. Finally, some studies have demonstrated a 'super-additivity' effect where the binding affinity of the two linked fragments is much greater than one might have expected from the binding affinities of the two independent fragments.

The method described above involves the linking of fragments. Another strategy is to 'grow' a lead compound from a single fragment—a process called **fragment evolution**. This involves the identification of a single fragment that binds to part of the binding site, then finding larger and larger molecules that contain that fragment, but which bind to other parts of the binding site as well.

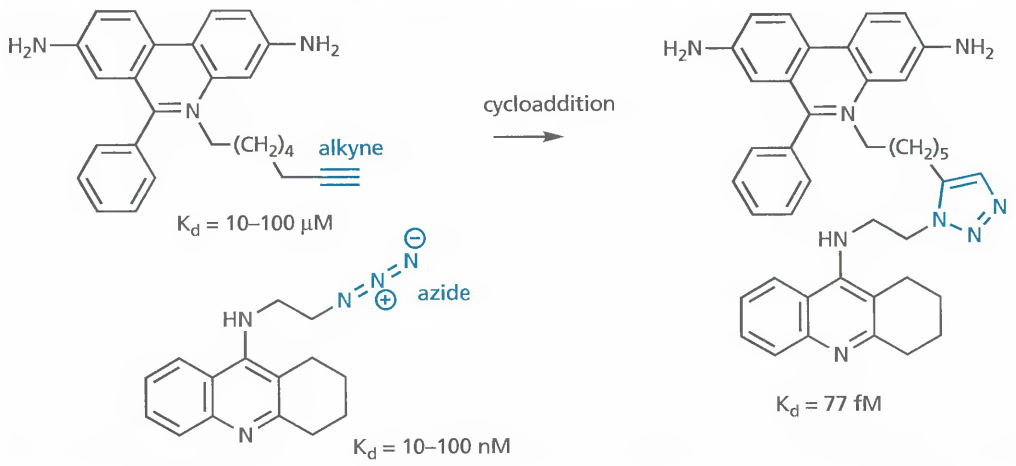
A third strategy is known as **fragment self-assembly** and is a form of dynamic combinatorial chemistry (section 16.6). Fragments are chosen that can not only bind to different regions of the binding site, but can also react with each other to form a linked molecule *in situ*. This could be a reversible reaction as described in section 16.6. Alternatively, the two fragments can be designed to undergo an irreversible linking reaction when they bind to the binding site. This has been called '**click chemistry *in situ***' (Box 12.8).

NMR spectroscopy is not the only method of carrying out fragment-based lead discovery. It is also possible to identify fragments that bind to target proteins using the techniques of X-ray crystallography, *in vitro* bioassays and mass spectrometry. X-ray crystallography, like NMR, provides information about how the fragment binds to the binding site, and does so in far greater detail. However, it can be quite difficult obtaining crystals of protein–fragment complexes because of the low affinity of the fragments. Recently, a screening method called **CrystalLEAD** has been developed which can quickly screen large numbers of compounds, and detect ligands by monitoring changes in the electron density map of protein–fragment complexes relative to the unbound protein.

Finally, it is possible to use fragment-based strategies as a method of optimizing lead compounds that may have been obtained by other means. The strategy is to identify distinct fragments within the lead compound and then to optimize these fragments by the procedures already described. Once the ideal fragments have been identified, the full structure is synthesized incorporating the optimized fragments. This can be a much quicker method of optimization than synthesizing analogues of the larger lead compound.

BOX 12.8 Click chemistry *in situ*

A femtomolar inhibitor for the acetylcholinesterase enzyme was obtained by fragment self-assembly within the active site of the enzyme. One of the molecular fragments contained an azide group while the other contained an alkyne group. In the presence of the enzyme, both fragments were bound to the active site, and were positioned close enough to each other for an irreversible 1,3 dipolar cycloaddition to take place, forming the inhibitor *in situ*. This type of reaction has been called 'click chemistry *in situ*'.



'Click' chemistry by means of a cycloaddition reaction.

12.4.11 Properties of lead compounds

Some of the lead compounds that have been isolated from natural sources have sufficient activity to be used directly in medicine without serious side effects; for example morphine, quinine and paclitaxel. However, most lead compounds have low activity and/or unacceptable side effects which means that a significant amount of structural modification is required (chapters 13 and 14). If the aim of the research is to develop an orally active compound, certain properties of the lead compound should be taken into account. Most orally active drugs obey the rules laid down in Lipinski's Rule of Five or Veber's parameters (section 11.2). A study of known orally active drugs and the lead compounds from which they were derived demonstrates that the equivalent rules for a lead compound should be more stringent. This is because the structure of the lead compound almost certainly has to be modified and increased, both in terms of size and hydrophobicity. The suggested properties for a lead compound are that it should have a molecular weight of 100–350 amu, and a cLog$P$ value of 1–3. (cLog$P$ is a measure of how hydrophobic a compound is; section 14.1). In general, there is an average increase in molecular weight of 80 amu, and an increase of 1 in cLog$P$ when going from a lead compound to the final drug. Studies also show that a lead compound generally has fewer aromatic rings and hydrogen bond acceptors compared to the final drug. Such considerations can be taken into account when deciding which lead compound to use for a research project if several such structures are available. Another approach in making this decision is to calculate the binding or ligand 'efficiency' of each potential lead compound. This can be done by dividing the free energy of binding for each molecule by the number of non-hydrogen atoms present in the structure. The better the ligand efficiency, the lower the molecular weight of the final optimized structure is likely to be.

For fragment-based lead discovery (section 12.4.10), a rule of three has been suggested for the fragments used:

- a molecular weight less than 300
- no more than 3 hydrogen bond donors
- no more than 3 hydrogen bond acceptors
- cLog$P$ = 3
- no more than 3 rotatable bonds
- a polar surface area = 60 Å^2

12.5 Isolation and purification

If the lead compound (or **active principle**) is present in a mixture of other compounds, obtained from a natural source or from a combinatorial synthesis (chapter 16), it has to be isolated and purified. The ease with which the active principle can be isolated and purified depends very much on the structure, stability, and quantity of the compound. For example, Fleming recognized the antibiotic qualities of **penicillin** and its remarkable non-toxic nature to humans, but he disregarded it as a clinically useful drug because he was unable to purify it. He could isolate it in aqueous solution, but whenever he tried to remove the water, the drug was destroyed. It was not until the development of new experimental procedures such as freeze-drying and chromatography that the successful isolation and purification of penicillin and other natural products became feasible. A detailed description of the experimental techniques involved in the isolation and purification of compounds is outwith the scope of this textbook, and can be obtained from textbooks covering the practical aspects of chemistry.

12.6 Structure determination

It is sometimes hard for present-day chemists to appreciate how difficult structure determinations were before the days of NMR and IR spectroscopy. A novel structure which may now take a week's work to determine would have provided two or three decades of work in the past. For example, the microanalysis of **cholesterol** was carried out in 1888 to get its molecular formula, but its chemical structure was not fully established until an X-ray crystallographic study was carried out in 1932.

In the past, structures had to be degraded to simpler compounds, which were further degraded to recognizable fragments. From these scraps of evidence, a possible structure was proposed, but the only sure way of proving the proposal was to synthesize the structure and to compare its chemical and physical properties with those of the natural compound.

Today, structure determination is a relatively straightforward process and it is only when the natural product is obtained in minute quantities that a full synthesis is required to establish its structure. The most useful analytical techniques are **X-ray crystallography** and **NMR spectroscopy**. The former technique comes closest to giving a 'snapshot' of the molecule, but requires a suitable crystal of the sample. The latter technique is used more commonly as it can be carried out on any sample, whether it be a solid, oil, or liquid. There is a large variety of different NMR experiments that can be used to establish the structure of quite complex molecules. These include various two-dimensional NMR experiments which involve a comparison of signals from different types of nuclei in the molecule (e.g. carbon and hydrogen). Such experiments allow the chemist to build up a picture of the molecule atom by atom, and bond by bond.

In cases where there is not enough sample for an NMR analysis, mass spectrometry can be helpful. The

fragmentation pattern can give useful clues about the structure, but it does not prove the structure. A full synthesis is still required as final proof.

12.7 Herbal medicine

We have described how useful drugs and lead compounds can be isolated from natural sources, so where does this place herbal medicine? Are there any advantages or disadvantages in using herbal medicines instead of the drugs developed from their active principles? There are no simple answers to this. Herbal medicines contain a large variety of different compounds, several of which may have biological activity, so there is a significant risk of side effects and even toxicity. The active principle is also present in small quantity, so the herbal medicine may be expected to be less active than the pure compound. Herbal medicines such as St. John's wort can also interact with prescribed medicines (section 11.4.6), and in general there is a lack of regulation or control over their use.

Having said that, these same issues may actually be advantageous. If the herbal extract contains the active principle in small quantities, there is an inbuilt safety limit to the dose levels received. Different compounds within the extract may also have roles to play in the medicinal properties of the plant and enhance the effect of the active principle—a phenomenon known as **synergy.** Alternatively, some plant extracts have a wide variety of different active principles which act together to produce a beneficial effect. The **aloe plant** (the 'wand of heaven') is an example of this. It is a cactus-like plant found in the deserts of Africa and Arizona and has long been revered for its curative properties. Supporters of herbal medicine have proposed the use of aloe preparations to treat burns, irritable bowel syndrome, rheumatoid arthritis, asthma, chronic leg ulcers, itching, eczema, psoriasis, and acne, thus avoiding the undesirable side effects of long-term steroid use. The preparations are claimed to contain analgesic, anti-inflammatory, antimicrobial and many other agents which all contribute to the overall effect, and trying to isolate each active principle would detract from this. On the other hand, critics have stated that many of the beneficial effects claimed for aloe preparations have not been proven and that although the effects may be useful in some ailments, they are not very effective.

KEY POINTS

- A lead compound is a structure that shows a useful pharmacological activity and can act as the starting point for drug design.
- Natural products are a rich source of lead compounds. The agent responsible for the biological activity of a natural extract is known as the active principle.
- Lead compounds have been isolated from plants, trees, microorganisms, animals, venoms, and toxins. A study of medical folklore indicates plants and herbs that may contain novel lead compounds.
- Lead compounds can be found by screening synthetic compounds obtained from combinatorial syntheses and other sources.
- Existing drugs can be used as lead compounds for the design of novel structures in the same therapeutic area. Alternatively, the side effects of an existing drug can be enhanced to design novel drugs in a different therapeutic area.
- The natural ligand, substrate, product or modulator for a particular target can act as a lead compound.
- The ability to crystallize a molecular target allows the use of X-ray crystallography and molecular modelling to design lead compounds which will fit the relevant binding site.
- Serendipity has played a role in the discovery of new lead compounds.
- A knowledge of an existing drug's pharmacophore allows the computerized searching of structural databases to identify possible new lead compounds which share that pharmacophore. Docking experiments are also used to identify potential lead compounds.
- NMR spectroscopy can be used to identify whether small molecules (epitopes) bind to specific regions of a binding site. Epitopes can be optimized then linked together to give a lead compound.
- If a lead compound is present in a natural extract or a combinatorial synthetic mixture, it has to be isolated and purified such that its structure can be determined. X-ray crystallography and NMR spectroscopy are particularly important in structure determination.
- Herbal medicines contain different active principles that may combine to produce a beneficial effect. However, toxic side effects are also possible.

QUESTIONS

1. What is meant by target specificity and selectivity? Why is it important?
2. What are the advantages and disadvantages of natural products as lead compounds?

3. Fungi have been a richer source of antibacterial agents than bacteria. Suggest why this might be so.

4. Scuba divers and snorkellers are advised not to touch coral. Why do you think this might be? Why might it be of interest to medicinal chemists?

5. You are employed as a medicinal chemist and have been asked to initiate a research programme aimed at finding a drug that will prevent a novel tyrosine kinase receptor from functioning. There are no known lead compounds that have this property. What approaches can you make to establish a lead compound? (Consult section 4.8 to find out more about protein kinase receptors.)

6. A study was set up to look for agents that would inhibit the kinase active site of the epidermal growth factor receptor (section 4.8). Three assay methods were used: an assay carried out on a genetically engineered form of the protein that was water soluble and contained the kinase active site; a cell assay that measured total tyrosine phosphorylation in the presence of epidermal growth factor; and an *in vivo* study on mice that had tumours grafted onto their backs. How do you think these assays were carried out to measure the effect of an inhibitor? Why do you think three assays were necessary? What sort of information did they provide?

FURTHER READING

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Titles for general further reading are listed on p. 725.