An Introduction to Key Concepts in Medicinal Chemistry

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Contents

Introduction

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This volume in *Chemistry Learning Trends* introduces readers to a key chapter from the 4th edition of Camille Wermuth's *Practice of Medicinal Chemistry* and highlights the interdisciplinary nature of medicinal chemistry. The succeeding articles, from the ScienceDirect Reference Module in Chemistry, Molecular Sciences and Chemical Engineering, will introduce readers to important themes and valuable methods raised in this chapter.

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CHAPTER

3

Drug Targets, Target Identification, Validation, and Screening

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OUTLINE

It doesn't matter how beautiful your theory is, it doesn't matter how smart you are or what your name is, if it doesn't agree with experiment, it's wrong. ***Richard P. Feynman (American theoretical physicist 1918–1988)***

I. INTRODUCTION

For ages, humans have been using medicinal substances without tools like DNA microarrays to identify them. Instead, they were guided by theories like the concept of the four humors in Greco-Roman medicine or by spiritual systems like animism. The chances are high that modern medicinal chemists would fully reject these rationales. Today we believe that the essential first step in the discovery of a new cure for a disease is the identification of the protein that is at the basis of that disease. The chances are high that medicinal chemists would fully agree with this rationale, but maybe they shouldn't. In this chapter, we will see why.

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First, we examine why the definition of a drug target is already a bit misleading. Then we explore whether the mantra "first a target, then a drug" is a good guideline. We compare the three most used strategies for drug discovery today and assess the role of target identification in these strategies. The next question is what kind of targets we should try to identify. Is the search for the cause of a disease a fruitful road to find new cures? Can we find cures altogether? Finally, after having established the difference between the two meanings of target identification, we describe briefly the current and most frequently used methods to identify and validate drug targets.

II. WHAT IS A DRUG TARGET?

In 1891, Paul Ehrlich was experimenting with dyes to stain bacteria. He had already made outstanding contributions in treating infected patients with antitoxins. Together with vaccines, these account for the successful immunotherapy. Ehrlich saw this immunotherapy as chemical reactions between very complex structures. At that time, the concepts of cells and microorganisms were very new and nobody understood the composition of cells. Maybe a cell was one big molecule, (i.e., a cell-molecule). Ehrlich believed that cell-molecules had side-chains to receive nutrients from outside, and he called these side-chains receptors. He thought that bacteria also had receptors and that the staining of bacteria was a chemical reaction between the dye molecule and the receptors. What if this reaction could not only stain the bacteria but also kill them? What if this dye could do the same in an infected patient? Ehrlich showed that methylene blue was taken up by the malaria parasite and had modest effects in two patients. He was extremely excited by this and coined the term "chemotherapy." The difference with immunotherapy was that now the antitoxins—which were very complex and difficult to produce and standardize—could be replaced by well-identified chemicals (small molecules) that were easier to produce and handle.

We owe the concept that a drug acts by binding to a target molecule to Paul Ehrlich. In his own words:,"Corpora non agunt nisi fixata" or "substances don't act unless they are bound." Today this concept is still valid. The *Oxford Dictionary of Biochemistry* defines a drug target as "a biological entity (usually a protein or gene) that interacts with, and whose activity is modulated by, a particular compound." Peter Imming [1] uses the following working definition: a molecular structure (chemically definable by at least a molecular mass) that will undergo a specific interaction with chemicals we call drugs because they are administered to treat or diagnose a disease. The interaction has a connection with the clinical effect(s).

These definitions could give the misleading impression that a drug−target interaction is a one-to-one relation [2], as if every drug acted by binding to one and only one single specific target. This impression is further strengthened by the ambition of every medicinal chemist, starting with Paul Erhlich himself, to synthesize a "magic bullet," an ultra-specific compound that would bind only to the target and to nothing else. However, evidence is growing that many drugs are successful just because they act on multiple different—not co-located—targets, potentially even hitting several pathways together [3]. Of the 1366 drugs reported in DrugBank 2.0, about 960 have more than one therapeutic target [4], a phenomenon called polypharmacology. As a consequence, searching for a super-selective drug may not always lead to the most active compound. In this perspective, target-based drug screening is not well suited to discover these so called "dirty drugs."

The one-to-one relation also doesn't fit with drugs that act by binding to a complex of proteins or even a complex between proteins and nucleic acids. Many proteins form dimers, trimers, or even more complex constellations. In these cases, the drug binding pocket could contain parts of two or more proteins. But the target discovery tools are less well suited to find such targets.

Yet another—very obvious—violation of the one-to-one relation is that the same pocket can accommodate many different small molecules. A substantial part of all new drugs is based on this promiscuous behavior of many pockets. The production of close analogues—or, more pejoratively, "me too drugs" —is often seen as a risk averse and profit driven strategy. Nevertheless, these drugs often result in an important incremental progress in activity, side effect profile, or administration facility [5].

A less obvious violation of the one-to-one relation is the fact that a protein can contain multiple pockets. Usually these pockets are all different and could partially overlap, be indirectly connected by allosteric regulation, or be completely separated. The binding to these different pockets could result in different effects. For example, the binding with nucleoside drugs to the active site of a viral polymerase makes it more difficult for the virus to build resistance than with nonnucleoside drugs that have their binding site outside the active site of the enzyme.

These comments make the picture of a drug target more complex. We could define a drug target as the minimal constellation of molecules that elicit a medically desired effect when bound by a drug.

III. THE PURPOSE OF TARGET IDENTIFICATION

Before exploring the plethora of methods to identify drug targets, we should discuss the role and the value of target identification in the drug discovery process. We will describe the role of target identification in the following three drug discovery strategies for small molecules:

- Target-Based Screening Strategy
- Phenotypic Screening Strategy
- Fast Follower Strategy

A. Target-Based Screening

Target identification is the cornerstone of target-based screening. The concept underlying this strategy is that at the most fundamental level, most drugs work by binding to a specific target. Therefore, if you want to make a truly new drug, the first thing you have to do is to find a new target. The next step is to find small molecules that bind to this target, preferably as specific as possible. This procedure looks so overwhelmingly self-evident, innovative, and scientific that the complete pharmaceutical research community has been dreaming for decades about realizing this strategy. With tremendous efforts, some even succeeded in making drugs this way (e.g., mercaptopurine and cimetidine), but in general the tools were inadequate.

Beginning in the 1980s with the breakthrough in gene technologies, along with the invention of the extremely versatile polymerase chain reaction in 1983 and the publication of the human genome by HUGO and Craig Venter in 2001, pharmaceutical scientists finally received the tools they needed to turn the blind old-fashioned dull drug screening into a highly rational, hypothesis-driven, reductionistic and efficient drug discovery engine. Target-based screening was now possible, and the entire industry embraced it, largely replacing the phenotypic screens [6,7]. Even today, in many presentations on drug discovery for the general public and in many textbooks [8] and publications [9,10], the mantra "first a target, then a drug" is still presented as the main road for drug discovery. The technological advancements are indeed enormous. Today we can sequence the genome of entire organisms in days and measure gene activity in single cells. We can trace individual molecules as they move around in a cell. We can screen millions of compounds in miniaturized and robotized high-throughput assays. Crystallographers can observe protein targets at atomic scale. Faster than ever before, chemists can synthesize very complex molecules, and these can be quantified and identified in very small amounts. Bio-informatics can mine big databases and simulate biological pathways and systems. These technological advancements are certainly as profound and extensive as those in the electronics industry. Many people in the field, particularly molecular biologists and young managers, expected to see an explosion of new drugs against diseases formerly untreatable. But today, most diseases are still here, and the only thing that really exploded was the cost to discover and develop new drugs. In a recent article [11], the authors plotted the number of drugs that could be developed with 1 billion dollar over the years, beginning as early as 1950. The investments were corrected for inflation. It's remarkable that the exponential decrease in output is almost constant over the entire time-span. There is no such thing as a dramatic revolution in increased output. This constant exponential decrease in itself is not scientific proof that there is something wrong with the target-based screening strategy. There could be—and there certainly are—other reasons that could explain the steady increase in R&D cost per drug. But the least thing it proves is that target-based screening and all the new technologies have not brought the expected quantum leap in R&D efficacy. A more specific investigation [12] tracked down the research strategies for all 259 drugs that were approved by the FDA between 1999 and 2008. For the so called first-in-class small molecules (molecules with a new target, not the me-too ones) 38 percent came out of target-based screening. The other 62 percent came out of phenotypic screening. And 62 percent is even an underestimation of the success-rate of phenotypic screening, because this strategy was used less by industry. (Figure 3.1).

Target-based screening is now more and more brought into question [6,12–17]. Although this strategy has certainly led to many successes, it has failed more than expected. Often the targets thrown up by this reductionistic, bottom-up approach were wonderful *in vitro* but disastrous in the clinic due to lack of efficacy or unexpected toxicity [18].

B. Phenotypic Screening

The under-performance of the above described target-based screening leads us to the question whether it's possible to develop drugs without knowing the target in the first place. The answer is, of course, a big "yes." Aspirin

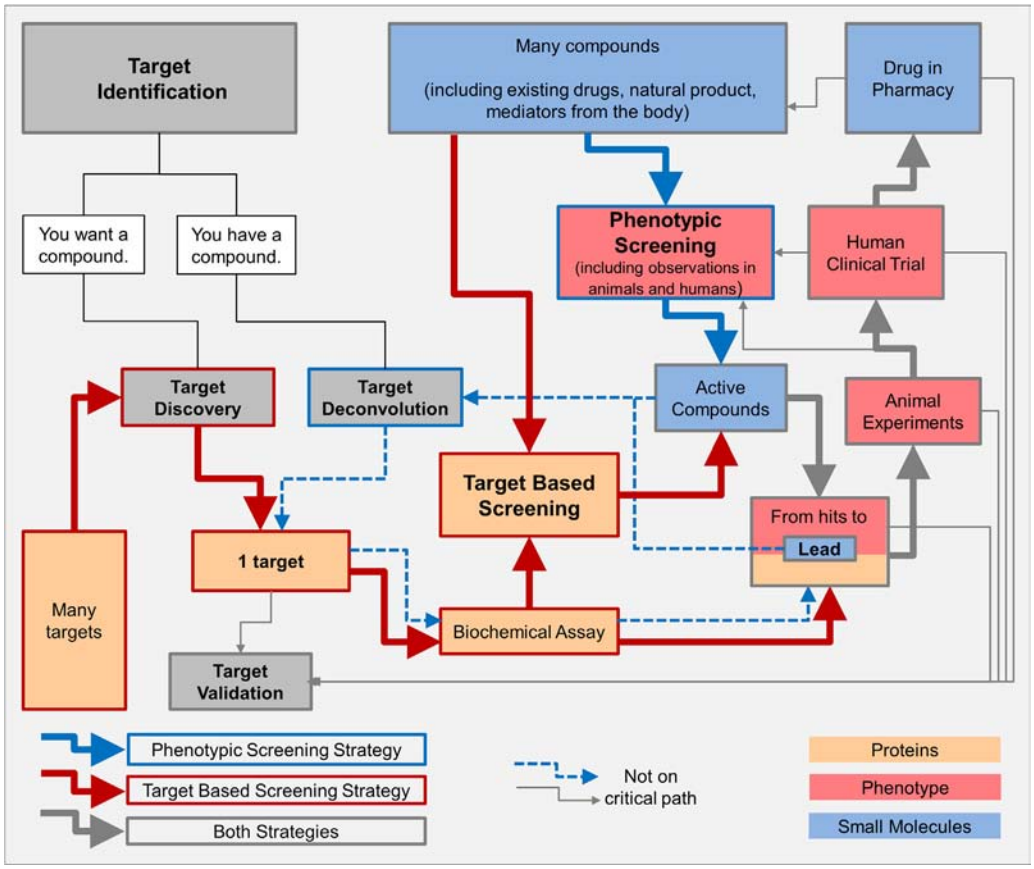

FIGURE 3.1 Target identification can be split into target deconvolution and target discovery. The former is used to identify the target of an active compound, usually obtained by phenotypic screening (blue arrows). The latter is used to discover a new target whose modulation would be of medical use. This target is the starting point for a target-based drug screening project (brown arrows) resulting in an active compound. Target-based screening is a longer process because you first have to screen for a target before you can screen for a compound. In phenotypic screening, the target identification can be done in parallel with the further lead optimisation or even be omitted, and therefore is not on the critical path (dotted arrows).

was synthesized in 1897, but its mechanism of action only discovered by Vane [19,20] in 1971 and its target in 1976. Morphine was used for ages, but its main target, the μ-opioid receptor, was identified by Pert and Snyder [21] in 1973, while other targets are still under investigation. The targets of the general anesthetics are only gradually emerging within the last decade, with the $GABA_A$ receptor as the most prominent one [22]. We could tell similar stories for the benzodiazepines, corticosteroids [9], cyclosporine and FK506 [12,23,24], sulfonylureas, antipsychotic drugs, fibrates, vinca alkaloids, and many antidepressants [8]. And although the targets for most antidepressants have been determined by now, their mechanism of action is still a mystery [25–27]. It may also be that the actual declared targets for some drugs are in fact only off-target effects and that the real targets will be discovered in the future. The reader will appreciate that we can't give an example of this last group today.

It is safe to say that most "first in class drugs" developed before the 1980s were discovered by phenotypic screening. First, you discover an active compound, then you try to determine the target. This is exactly the opposite of target-based screening. Today, phenotypic screening is often seen as cellular screening. But cells are only the smallest living organisms that can build up a phenotype out of their genome. All the experiments in which we examine the effect of at least one compound on the phenotypic level are phenotypic screens. A Neanderthal observing papaver somniferum reducing his tooth pain was performing a kind of phenotypic screen. There is an enormous variety in types of cells, cell combinations, tissues, and animals that can be studied [17]. One could rank these experiments on a scale going from the more reductionistic ones on the left to the more holistic ones on the right. The most holistic and closest to real-life situations are experiments in humans, better known as clinical trials. All other experiments more to the left—however sophisticated and ingenious they might be—are always only an approximation of the real thing. Figuring out a mutation in a gene for leptin in a family with extreme obesity is

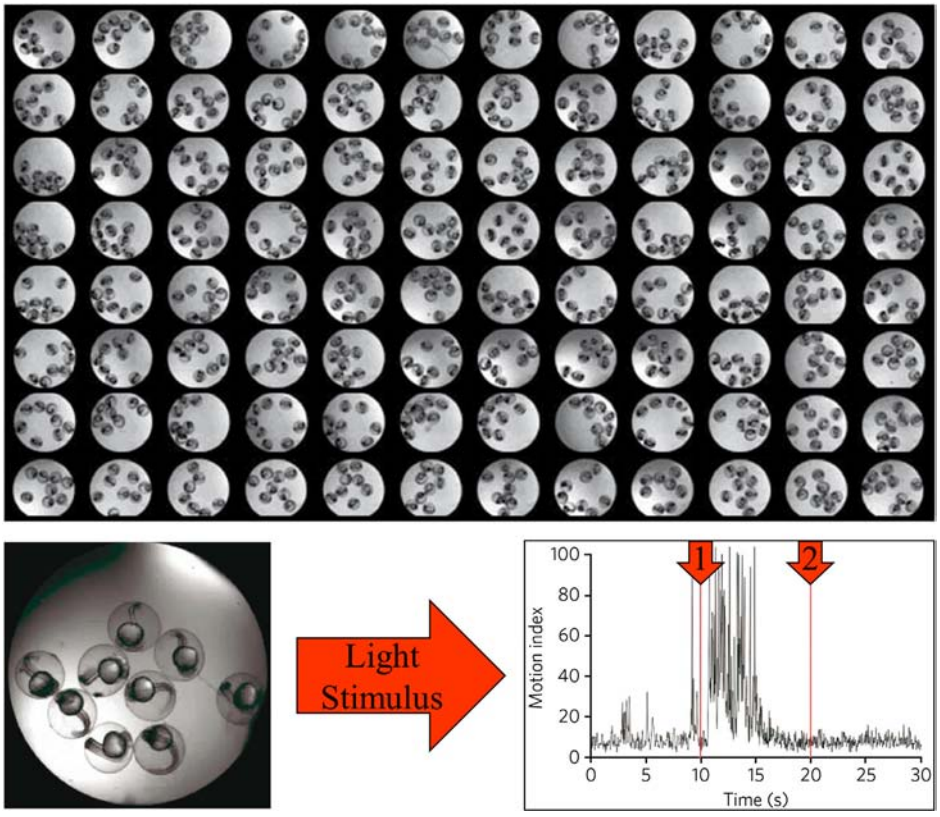

FIGURE 3.2 Eight to ten zebrafish embryos are put in every single well of a 96-well plate. The aggregate motor activity per well is recorded over a 30-second time span, during which two light stimuli are given at 10 and 20 seconds (red arrows). The reactions to the light flashes are translated to distinct behavioral patterns that can be used to evaluate potential neuroactive drugs. Using an automated platform, 5,000 embryos could be screened per microscope-day. *Source: Adapted from reference [28].*

only an approximation of the possible genes causing obesity in the whole population. Indeed, the developed leptin analog was extremely effective, but only in the very small group of people carrying the mutation [13].

Case studies and observations of side-effects in clinical trials (or via drug surveillance) represent an extremely valuable source of information. They form an unplanned phenotypic screen of the highest level. Although a clinical trial is a planned phenotypic experiment, the unexpected side-effect that could eventually result in a drug repositioning exercise was not planned. Drug repositioning and obtaining lead compounds based on unplanned observations are not extremely rare, but they can hardly be conceived as a planned research strategy.

Phenotypic screenings of limited but well-chosen sets of compounds in animals have been a very productive way to find new drugs in the period stretching from the end of World War II until the 1980s. Today, animals are not used anymore to screen compounds for having interesting (unexpected) effects. They are used to confirm expected effects (and to study the pharmacokinetic properties and safety profile of compounds). But the zebrafish is changing this again. For those not familiar with this fish—which is never served in restaurants—it is a very small (4 cm) fish with transparent embryos that for several reasons has become a favorite vertebrate animal model in research. One can easily put 10 embryos in one well of a 96-well microtiter plate. The behavior of these embryos upon treatment with a compound can be monitored automatically. For example, the team of Randall Peterson [28] measured the motor activity upon stimulation with intensive light and could link the so called photomotor response profiles to different classes of neuroactive drugs. In an automated platform, 5,000 embryos could be observed per microscope per day. In total they screened 14,000 different compounds, and the behavioral information of more than a quarter million embryos was collected. (Figure 3.2).

Most phenotypic screens today are still performed in cells. Again, we have to be aware that cells are only an approximation of tissues and organs. And cells in laboratories—often immortalized cancer cell-lines, possibly manipulated to mimic a disease state—could behave totally differently than normal cells. Nevertheless, these cellular phenotypic screens have many advantages over biochemical screens with purified targets. A major advantage is that in a cell, the target is in its normal biological context: it is present in the real compartment of the cell, in the real constellation with other proteins and membranes, at the real concentration, in the real phosphorylation-ubiquitination-glycosylation-acylation and whatever state, and embedded in its full metabolic and regulatory pathways.

A second advantage is that cells contain more than one target. Throw a potential bactericidal compound on a culture of *M. tuberculosis* and you are testing its activity on 614 vital proteins, about 3,400 other proteins, and an unknown set of nonprotein targets [29]. In contrast, in a biochemical screen you only test one known target at a time and it can take years to set up an assay for a second protein. Then there is a good chance that this protein is in an artificial, biologically irrelevant state. The only advantage is that the high-throughput capacity is usually higher, but critics would say that this only helps to produce an even bigger pile of irrelevant hits.

A third advantage is that phenotypic screens do not fail to identify prodrugs, which need to be converted to active drug by a host cell or a bacterial cell. Prodrugs are not active in biochemical assays.

For the fourth advantage of phenotypic screening, it is instructive to look at the search for antivirals against the hepatitis C virus (HCV). Compared with a human or even with a single cell, a virus is a simple organism. You sequence its little genome and try to figure out the function of the handful of viral proteins. You can bet that all of them must be vital and hence must represent good drug targets, especially the enzymes. Target-based screening makes much sense here. You synthesize the enzyme, make a biochemical screen, test thousands of compounds, and—with some luck—you obtain active inhibitors. If, after further optimization, these inhibitors have an acceptable oral bioavailability, if they have a good half-life, if they can penetrate the liver cells, and if they have no safety issues, you have a drug. This turned out to be the case for compounds targeting the HCV protease enzyme, while compounds targeting the HCV polymerase enzyme [30] are in late stage clinical development. However, the class of replicase complex inhibitors—formerly known as NS5A inhibitors—could only be found by phenotypic screening. These compounds have picomolar *in vitro* activities and about eight of them are now in phase 2–3 studies [30]. The exact role of the nonstructural viral NS5A protein and the way the compounds bind to it is still a matter of debate. This enigmatic and intractable protein would never have been selected as a target in the target-based screening strategy. This example shows that phenotypic screening can be used to access less obvious targets. Mark Fishman, president of the Novartis Institutes for BioMedical Research (NIBR), who initiated a phenotypic screening effort when he moved to Novartis in 2002, puts it this way: "For me it's a discovery tool. The single biggest impediment to drug discovery is the small number of new, validated targets that we have. Phenotypic screening is one way of moving beyond well-defined targets from the literature to discover new therapeutic targets and new disease biology." [6] Another example is the discovery of bedaquiline, the first anti-tuberculosis drug with a new mechanism of action in 40 years [31]. Bedaquiline [32], synthesized by J. Guillemont, was discovered by a phenotypic screen and its target, the bacterial ATP synthase, was identified afterwards. Again, this target would probably never have been chosen as starting point in a target-based screening campaign and can actually not even be tested in isolation because of its complexity.

What's the role of target identification in the phenotypic screening approach? There are two cases.

- In cases where you use phenotypic screening to find active compounds, it is still very useful to identify the target. First, building a SAR based on activities measured with a biochemical assay gives you a higher resolution because you exclude all the variations caused by cell penetration, off-target binding, cell compartmentalization, and so on. But one has to keep the cellular activity under close surveillance. Working years in the chemistry labs to build a compound that is super-active in the biochemical assay but doesn't penetrate the cell anymore is a waste of time. Second, when 3D-stuctures of the target are available, molecular modeling could assist the SAR. Third, knowing the target could also help in exploring the toxicity and the role of the pathways in human and animal models. Fourth, in general it is substantially more challenging to get a compound without a target through the consecutive approval committees within and outside the company. However, knowledge of the target is not always a regulatory requirement for drug approval. Between 1999 and 2008 the FDA has approved nine compounds with unknown targets [12,19].
- In cases where you use phenotypic screening as a way to find new targets, the role of target identification is self-evident. This approach to finding new targets becomes more and more a valid option for three reasons. First, there is the increased capability to read out ever more complex phenotypes, there is the increased capacity to handle more animals by using robots, and there is the renewed interest in phenotypic screening in general. Second, the methods to determine a target—as will be described further in this chapter—are becoming increasingly efficient. And third, targets found via phenotypic screening are more likely to become valid targets.

C. Fast Follower Strategy

The fast follower strategy consists in synthesizing analogs of an existing drug in the hope of obtaining a compound with at better profile than the starting drug. This strategy is often regarded as not innovative, while there

are many examples where the second or third so called me-too drug offered real clinical improvements [33]. This strategy has many advantages. There is no need to invest in the discovery of a target, and the target is already validated in the best possible way: in humans. Besides the known therapeutic efficacy and safety profile, even the financial risks and benefits can be estimated with more certainty. The probability that at least some of the new analogs will have similar or even better activities is usually high. The new compounds should, of course, fall outside the patent of the original drug. Given the virtually infinite chemical space and the creativity of medicinal chemists, this requirement is an easily attainable goal. It becomes trickier to stay outside the competitor's patents applications on the same target during the 18 months before they are published. Given these advantages, it should not come as a surprise that the number of fast follower drugs is rather high. Of the 259 drugs approved by the FDA between 1999 and 2008, 164 (63 percent) were follower drugs. In the subset of small molecules, the percentage was even higher at 74 percent [12].

The role of target identification in the fast follower strategy is mainly limited to the confirmation that the new compounds still work on the same target. Target knowledge can, of course, be used to drive the SAR.

Chemists have to be aware that the fast follower approach is less attractive for biologists than for chemists. Biologists can be very creative and use their full intellectual potential in proposing new hypotheses on working mechanisms, in the endeavor to find new targets and in the design of new assays. But in the fast follower approach, most of this early work is already done and described by the originators. Biologists merely have to reproduce parts of this work. For the medicinal chemists, however, the fast follower approach stretches their chemical knowledge and creativity to the limit. They don't have to reproduce but instead come with something better in a new way.

IV. TARGET OPTIONS AND TREATMENT OPTIONS

In this section, we deal with questions like "What are good targets?"; "Which targets should we try to discover?"; "What can we achieve with targets?"; and "What are the therapeutic targets and treatment options?"

Today, most drugs are discovered and developed by commercial organizations. Even if their shareholders would not be interested in making money, companies at least have to make sufficient profit to cover the cost of new research and development. Therefore, the selection of a target is driven by scientific as well as commercial considerations [5]. In an ideal world, the commercial value of a drug should be in parallel. (Figure 3.3).

with its therapeutic value. The therapeutic value can be determined by calculating the DALYs (Disability Adjusted Life Years) that a disease or condition causes. Severe but rare diseases result in fewer DALYs compared to less severe but very common chronic diseases. In the real world, one should add the difference in purchasing power of patients to the equation. Even then, predicting the return of a drug has proven to be very hard. For example, several companies were not interested in licensing atorvastatin (Lipitor) [5]. Here we will focus on the scientific considerations. Given a selected disease, what are the chances of finding a small molecule against it? What is a good target if we want to cure the disease? Or is this already a wrong starting point?

In some languages, the word "drug" or "medicine"—not to mention the word "cure" —is translated by a word that literally means "agent that cures." That sounds fair and familiar, but most of us don't realize how wrong this really is. The majority of drugs synthesised and sold today don't cure at all. A cure should result in the permanent end to the specific instance of the disease, without further need for medication. A cure also implies that a relapse should not be the consequence of an inadequate treatment. Proton pump inhibitors or H2 antagonists can cure a patient with a duodenal ulcer within several weeks. More precisely, the temporary suppression of the normal gastric acid production permits the body to repair the gastric mucosa. But they do not remove the major causative agent, the bacterium Helicobacter pylori, and within a year the patient can relapse. Therefore, antibiotics are added to complete the treatment. Cases where a patient relapses after antibiotic treatment could be due to inadequate cure (i.e., not all bacteria were killed) or to reinfection from outside.

A quick look at the top 200 pharmaceutical products by 2009 worldwide sales [34] gives us plenty of examples of drugs that don't cure: cholesterol lowering drugs, anticoagulants, anti-asthmatics, anti-psychotics, anti-rheumatics, insulin, anti-diabetics, anti-Alzheimer products, blood pressure lowering drugs, painkillers, erythropoietin, anti-epileptics, erectile dysfunction products, immunosuppressive agents, nasal vasoconstrictors, hypnotics, sedatives, vaccines, urinary incontinence products, anti-Parkinson products, and narcotic analgesics. The pharmacological classes that do cure—at least sometimes—form a much shorter list; anti-infective drugs and anti-cancer drugs are the most prominent ones. Anti-ulcerants, as explained above, are a bit of a special case. The acute use of anticoagulants to dissolve a blood clot in the coronary arteries just after a heart attack could also be regarded as curative.

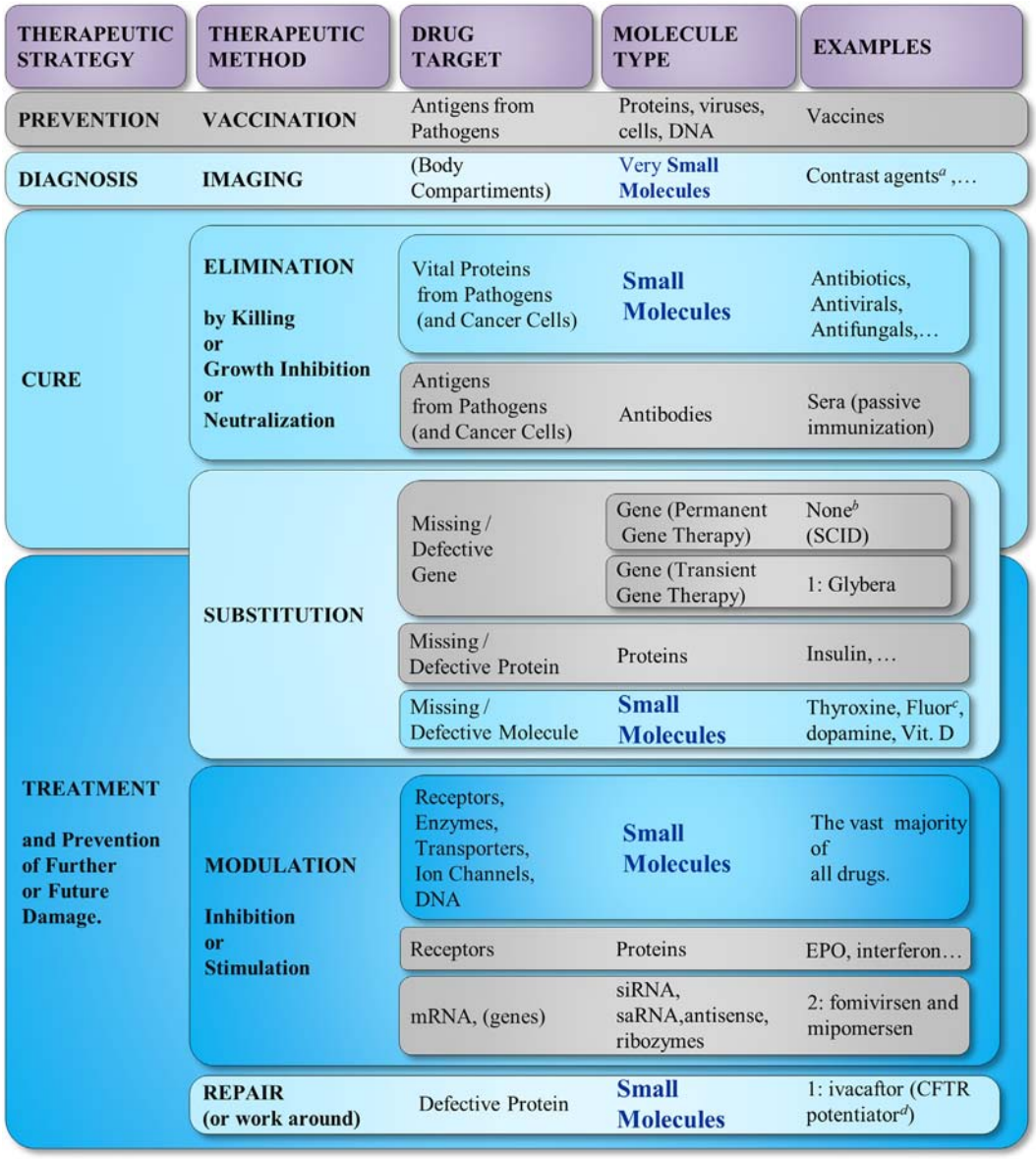

FIGURE 3.3 A simplified overview of the drug landscape. The areas in blue are covered by small molecules. The darker the blue, the more predominant the small molecules are. Biologicals are in grey. In general, it's very hard to cure a disease with small molecules, except for infections and cancer. Therefore, the targets for small molecules can best be found in the pathways that could modify the disease. The proteins that are on the causative path may be of indirect use but are rarely direct targets for small molecules, except again for infections and cancer. Some remarks to the figure: a. By definition, only molecules that are introduced into the body can form part of the diagnostic drugs and they are mainly used for imaging. b. Although there have been some curative gene therapy interventions in the past (like for SCID), at the moment the only approved gene therapy is Glybera, but that works only transiently and is therefore grouped under treatment. c. Nutrient supplements are grouped under the treatment of a shortage with preventive effects. d. Ivacaftor could also be seen as an allosteric agonist.

The observation that most drugs don't cure is not mind-blowing, but surprisingly this observation is often ignored by scientists looking for a new target. They persist in looking for the cause of a disease. They are looking for the gene mutations that cause cystic fibrosis, schizophrenia, Alzheimer disease, diabetes, obesity, and others. They are looking for the medical insults in early life that may cause epilepsy or autism or depression. But when they finally determine the deletion of three nucleotides that result in the loss of amino acid phenylalanine in position 508 of the CFTR protein, causing this protein to fold abnormally and finally being responsible for two-thirds of the cystic fibrosis cases, what then? Can they undo the deletion with a small molecule? No. Can they replace the CFTR gene with a small molecule? No. Can they replace the cystic fibrosis transmembrane conductance regulator by a small molecule? No. What about when scientists finally discover that 5 percent of schizophrenia is

caused by being born in winter or by hypoxia during fetal development? Can they change the birthday of the patient with a small molecule? Can they influence fetal conditions decades after birth with a small molecule? We could go on with other examples. The bottom line is that in general it is not possible to remove the cause of a disease with small molecules. Only when the cause is a cancer cell or a pathogen, things that don't have to be repaired or replaced but simply have to be killed, only then can small molecules cure.

There are of course exceptions. The small molecule ivacaftor is the first approved molecule that repairs a defective protein. The defective protein is the CFTR protein with the less frequent G551D mutation that accounts for 4–5 percent cases of cystic fibrosis. The word "repair" is perhaps not the best term, because ivacaftor most probably facilitates the impaired channel gating via an allosteric potentiating effect [35]—a kind of patch, so to speak. Ivacaftor has to stay in place to keep the protein channel open. It works only for a small subset of the cystic fibrosis patients. Again, ivacaftor is an exception, and although we can't exclude that science will change this tomorrow, the lesson today is that proteins or genes that cause disease could be very interesting but are rarely good drug targets for small molecules.

What are then better targets? Beta2-adrenoceptor agonists are effective in the acute treatment of asthma, although asthma is not caused by a defective sympathetic nervous system stimulation. The underlying cause of essential hypertension is unknown but can be treated—not cured—with alfa1-adrenoreceptor antagonists (among others). These examples show that it is not mandatory to explore the full pathogenesis of a disease to invent drugs that relieve symptoms, that counteract potential lethal situations, and that prevent further damage or future damage. It also points to where we should look for targets. In order to mitigate or counteract the effects of a disease, we should look for powerful master switches: proteins that are specialized in modulating cell functions. These proteins have a name: receptors. So it makes complete sense that 60 percent of all targets are located on the cell surface [36] and that 44 percent of the human targets are receptors [37]. Of these receptors, 82 (42 percent) are G protein-coupled receptors targeted by 357 unique drugs [37]. Of the 1,062 drugs that act on human targets, 563 (53 percent) of them bind to receptors. In fact, except for the 88 cancer targets, almost all other human targets are involved in disease modifying therapy. We should also not forget that more and more drugs have nothing to do with disease. Examples include women who want to become temporally infertile and the elderly who want to smooth out their wrinkles or extend their normal life-span.

In most articles [1,2,36–38] that estimate the number of actual and potential targets, the authors are rather prudent and conservative. Despite our 20,000 genes, the number of targets is often estimated to be only a few thousands. We are not going to refute this guesstimate but will make three remarks:

1. The famous visionary and science fiction writer Arthur C. Clarke once said that "If an elderly but distinguished scientist says that something is possible, he is almost certainly right; but if he says that it is impossible, he is very probably wrong." [39]
2. In the years just before the Wright brothers took off with their motorized airplane, elderly distinguished scientists declared that it was impossible to fly with machines heavier-than-air. These scientists apparently never observed a bird.
3. In the years just after the first antisense drugs were approved by the FDA, imagine how many more drug targets we would have if we were able to deliver easily [40] antisense and siRNA constructs into human cells. Imaging we would be able to activate genes with saRNA or be able to repair genes with rather small molecules [41].

V. TARGET DECONVOLUTION AND TARGET DISCOVERY

Figure 3.1 illustrates the two different meanings and applications of target identification. To avoid confusion, we will call the first target deconvolution and the second target discovery. In literature, target identification is often used for both, although target deconvolution is used more and more.

1. Target deconvolution.

   With target deconvolution, we mean that we have an active compound for which we want to find out, elucidate, assess, determine, and identify the target on which it is working. Target deconvolution [42] is the more recent appellation. Most often the active compound comes out of a phenotypic screening project. Therefore, target deconvolution is seen as an important and almost indispensable component of the phenotypic screening strategy. It is, however, not on the critical path in the sense that a project could advance to lead optimisation and in principle even to clinical trials and market approval without target knowledge.

The target identification can be executed in parralel with the further process. As stated before, the FDA approved 9 drugs [12,19] without known targets between 1999 and 2008.

2. Target discovery.

With target discovery, we mean that we want to find a potential target that can be used in a target-based screening project to obtain active compounds. The starting point is different, as we have no compound. We have a disease for which we want to find, screen, and explore possible targets. Target discovery is an absolute indispensable starting point for every target-based screening campaign. Target discovery can also be a part of academic research into the pathogenesis of a disease. One valid way to discover new targets is to start a phenotypic screening followed by target deconvolution of the lead compound. Most of the methods used for target discovery can also be used for target deconvolution.

VI. METHODS FOR TARGET IDENTIFICATION AND VALIDATION

There are dozens of methods to identify drug targets and the field is heavily based on extremely rapidly evolving technologies such as molecular biology, miniaturization, microscopy, automation, and informatics. Although we selected the most frequently used methods, the list is certainly not complete and risks becoming outdated very rapidly. No single method or guideline can be applied to every target, and the methods are complementary. There is also no common way to classify the different methods [9,23,42–50]. Therefore, we have gathered them in an overview (Table 3.1) so that the reader can classify them using multiple criteria.

A. Affinity Chromatography

Concept. An immobilized small molecule is used as a bait to fish the best binding protein(s) out of a mixture [42,44–46,51].

Input. An active small molecule and a mixture of possible targets (proteins).

Output. A fraction of the mixture that is enriched in targets that have a high binding affinity to the small molecule.

Group. Affinity based method. Direct method. Compound-centric chemical proteomics method. Target deconvolution. Bottom-up. One of the most widely used methods, especially for targets that are only present in mammalian cells or whole organisms [44].

Description. Affinity chromatography is a method to separate or purify mixtures based on different affinities toward a solid phase. Here the mixture is a cell or tissue lysate that contains the target(s) for the small molecule of interest. The small molecule is chemically attached to the solid phase. This solid phase (also referred to as stationary phase, column packing, matrix, beads, or resin) often consists of agarose or sepharose. The cell lysate acts as the mobile phase and is incubated with the solid phase. The components that have the highest affinity for the small molecule will bind preferentially to the solid phase. The unbound components of the cell lysate are then washed away or eluted from the column using an appropriate buffer. The next step is the elution of the bound target. This can be done with a buffer that disrupts the interaction between the target and the immobilized small molecule. Alternatively, the target can be eluted using an excess of free small molecules. The purified target now remains to be identified using advanced mass spectrometry or immunodetection on a SDS-PAGE gel.

Requirements. This method can only be applied when one has already obtained a (small) molecule by phenotypic screening or other sources. The second requirement is that it must be possible to chemically attach the small molecule to the solid phase in such a way (or under the assumption) that it doesn't interfere with the binding toward the target protein. It can be helpful to dispose of a SAR to identify parts of the molecule that can be used to attach the linker.

Advantages. This method has been very successful, and a large number of modifications are available to overcome several drawbacks. The most important advantage is that although the binding happens *in vitro*, the targets in the cell lysate are still in a very natural state [42,51]. The cells can be primary cells, even from human biopsies. These cells contain the entire proteome for that cell-type and disease-state. This is an unbiased probe compared to individually recombinant synthesized proteins in artificial prokaryotic cells, missing post-translational modifications. In the lysed cells, the proteins were embedded in their full metabolic and regulatory pathways, possibly in some relevant activation and labeling state.

TABLE 3.1 Some Methods for the Deconvolution, Discovery and Validation of Targets

Method	Objective	Group	Small molecule state	Binding occurs in	Target state	Target production	Detection of binding	Target identification
Affinity Chromatography (A)	T. Deconvolution	affinity methods	linked to solid phase	vitro	in lysate	normal + lysate	affinity separation	mass spectrometry
Fractionation (A)	T. Deconvolution	affinity methods	labeled but free	vitro	in lysate	normal + lysate	fraction is labeled	mass spectrometry
Phage Display (A)	T. Deconvolution	affinity methods	linked to solid phase	vitro	linked to phage	expression-cloning	affinity separation	sequencing
mRNA Display (A)	T. Deconvolution	affinity methods	linked to solid phase	vitro	linked to mRNA construct	expression-cloning	affinity separation	sequencing
Haploinsufficiency Profiling (HIP) (C)	T. Deconvolution	genetic methods	totally free	cells	normal	modulated expression	change in phenotype	sequencing
siRNA (C)	T. Deconvolution	genetic methods	totally free	cells	normal	modulated expression	change in phenotype	sequencing
Resistant mutants (D)	T. Deconvolution	genetic methods	totally free	cells	normal vs mutated	normal	change in phenotype	sequencing
siRNA (E)	T. Discovery T. Validation	genetic methods	na	na	normal	modulated expression	na	change in phenotype
Yeast three-hybrid (F)	T. Deconvolution	genetic methods	free but with linker	cells	linked to activating domain	expression-cloning	change in phenotype	sequencing
DNA microarrays (G)	T. Discovery	genetic methods	na	na	normal	normal vs altered	na	spot location + database
Protein microarrays (G)	T. Deconvolution	microarrays	labeled but free	vitro	linked to solid phase	not defined	affinity separation	spot location + database
Gene expression Profiling (H)	T. Deconvolution	Profiling	totally free	cells	normal	normal	change in transcriptome	profile comparison
Analysis of patho-physiology (I)	T. Discovery	top-down approach	useful	cells	normal	normal	not defined	not defined
Study of "old" drugs (J)	T. Discovery	top-down approach	not defined	not defined	not defined	not defined	not defined	not defined
Systems biology (K)	T. Discovery	in silico	in silico	in silico	in silico	in silico	in silico	in silico
Simulation of a human patient (L)	complete knowledge	in silico	in silico	in silico	in silico	in silico	in silico	in silico

The letters in brackets refer to the section in this chapter where the method is described. T.: Target. Na: not applicable. Normal means that the target is in its normal physiological state and location. The grouping of methods is rather arbitrary. Some authors group phage display under genetic methods, while there are reasons to group protein microarrays under affinity methods.

Disadvantages. First, the immobilization of the small molecules is not a routine operation and can abrogate the biological interaction with the protein. It will not be possible to find a good linker for every compound. Second, the assumption that the protein with the highest affinity to the small molecule is likely to be the biological target can be wrong. Also, when the small molecule has to bind to more than one protein in order to exert its biological activity, only the most abundant or strongest binding protein will be recovered. Third, the method works best for high-affinity bindings with dissociation constants ranging from 10^{-7} to 10^{-15}M. The lower the binding affinity, the more target proteins will be lost [42]. Fourth, this method is best suited to soluble proteins. Membrane bound proteins or nonproteins will be hard to identify with the standard set up [46]. Fifth, the conditions have to be adapted for every small molecule. This is not a high-throughput assay. Further disadvantages are nonspecific protein binding, the use of complex instrumentation, and the trial and error aspects in the fine-tuning [44].

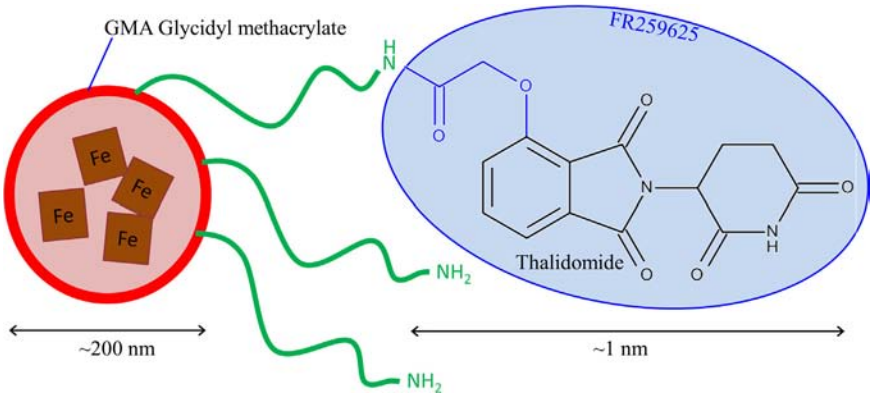

FIGURE 3.4 The FR529625 carboxy derivative (in blue ellipse) of thalidomide (in black) is bound to the amino-groups of the linkers (in green) of the FG beads (in red). The ferrite-glycidyl methacrylate (FG) beads allow for magnetic separation of target molecules that bind to thalidomide from the cell extract. The coating with GMA (Glycidyl mathecrylate) has a very low nonspecific protein adsorption. *Redrawn based on reference [54].*

Examples. Because affinity chromatography is one of the most used methods for target deconvolution, the number of examples is huge. Some examples are: tubulexin A binding to CSE1L and tubulin, Methyl gerfelin binding to glyoxalase 1, piperlongumine binding to GSTP1, Imatinib binding to kinases, and resveratrol binding to eIF4A

Case study. A dramatic example is the identification of a primary target of thalidomide teratogenicity in 2010 by the group of Ito [52]. Thalidomide was marketed during the late 1950s as a safe sedative because no lethal dose could be established in animals. Therefore, it was prescribed to thousands of pregnant women, especially for morning sickness. Soon thereafter, an epidemic of typical birth defects broke out. Thalidomide could be identified as the cause and was removed from the market, but for more than 10,000 children it was too late. Thalidomide was the biggest disaster in pharmaceutical history and was the start of a much more stringent FDA. But, amazingly, the drug is back. After the serendipitous discovery in 1965 by Dr. Sheskin of its miraculous effect on erythema nodosum leprosum [53], a very painful leprosy complication, the drug is now used under strict control for the treatment of leprosy and multiple myeloma. Because the effects of the drug are that unique, several hundreds of clinical trials are now undertaken, mainly in the field of cancer. Unfortunately, in South America children are again born with limb and other birth defects due to noncompliant use of thalidomide. If the mechanism of action of thalidomide's teratogenicity could be found, safe analogs could perhaps be conceived. Many mechanisms were proposed, but the direct target was not known. The group of Ito performed a classic affinity purification using magnetic FG beads. To bind the compound to the beads, a carboxylic derivate (FR259625) was made [54] and bound covalently to the amino-group of the Glycidyl methacrylate. The loaded beads were then incubated with HeLa cell extracts (a cancer cell once taken without permission from the cervix of Henrietta Lacks). The beads were washed very stringently and bound proteins finally eluted using free thalidomide [55]. The eluate was put on a SDS gel electrophoresis and silver stained. Only two protein bands could be found and identified with mass spectrometry: cereblon (CRBN) and DNA binding protein 1 (DDB1). The latter in fact did not bind directly to thalidomide but was piggy-backing on cereblon. After identification of the target, several target validation experiments were performed, as will be described at the end of this chapter. (Figure 3.4 and Figure 3.5)

Variations.

1. Reduction of sample complexity. When the drug binds many proteins in an unspecific way, one can try first to eliminate the proteins that are not of interest [56].
2. Compound analogs. A way to validate the target is to synthesize compounds with weaker and stronger affinities for the protein and then to examine the correlation between the phenotypic effect and the affinity [44]. The extreme is to compare with a nonactive analog [42].
3. Biotinylation. When biotin can be attached as linker, the biotin can then be bound with high affinity to streptavidin.
4. Fractionation. In this method, the small molecule is not immobilized but free. The molecule is radioactively or fluorescently labeled and incubated with the cell lysate. This mixture is then fractionated and the radioactivity or fluorescence is measured to determine which fraction contains the bound target protein. Radioactive labeling can be a solution when it's impossible to attach a linker to the molecule, but this is an expensive approach. The fractionation or separation can be done using affinity chromatography, 2D gel electrophoresis, or any other technique [44].

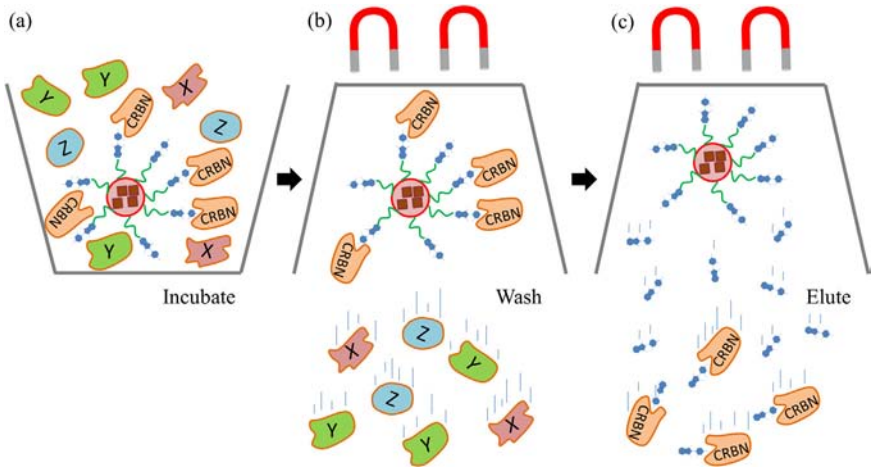

FIGURE 3.5 (a) The magnetic FG beads are incubated with a cell extract. The protein CRBN (cereblon) binds to thalidomide (blue molecule) while other proteins (X, Y, Z...) don't. (b) Using the magnets, it's easy to wash the beads and remove the nonbinding proteins. (c) In a last step the CRBN protein is eluted using free thalidomide. *Based on reference [55].*

5. Magnetic beads. Ferrite-glycidyl methacrylate (FG) beads can be used to allow magnetic separation of the solid phase from the cell extract [54].
6. Quantitative mass spectrometry. Different methods using isotopes (iTRAQ, ICAT, SILAC) [51] can help to make mass spectrometry more quantitative.
7. Phage display. The different target proteins are displayed by phages on their surface. The phage population is separated by affinity chromatography with the small molecule and the enriched elution is amplified in bacteria. With this target enriched phage collection, another affinity round is then performed. This way, low abundant binding proteins are amplified [42].
8. mRNA display. Similar to the principle above. The target proteins are linked to their encoding mRNA's instead of a phage. After affinity enrichment, the mRNA can be amplified with PCR and translated *in vitro* to obtain more of the target protein. With this target enriched collection, a second cycle is started [42].

B. Genetic Methods

Description. Today the molecular biology tools to play with genes are extremely versatile and powerful, yet at the same time often easy to use at a gradually decreasing cost. As a consequence, more and more methods for target identification and validation are directly or indirectly based on them.
A first approach is modulate gene expression for target deconvolution. The principle is simple. Imagine a (small) drug molecule that changes the phenotype of a cell by blocking an unknown target protein. What will happen when we change the concentration of this protein by modulating the expression of its gene? Decreasing the target concentration will make it easier to block the target with the same amount of drug. The cells become more sensitive to the drug. Decreasing the target concentration to zero by deleting the gene or suppressing the expression completely will (in an ideal situation) mimic the phenotype induced by the drug. An alternative to deleting the gene is to mutate the gene in such a way that the protein loses its function, also resulting in mimicking the drug phenotype. Increasing the target protein concentration by overexpressing its gene will make the cells less sensitive to the drug. How can we use all these modulations to identify the drug target when we don't know the gene to modulate? Well, we "simply" modulate all possible genes one-by-one, and each time we look for a change in drug induced phenotype. When there is a change, we have found the target gene and hence the target protein, or to put it more accurately, we found at least one target.

Down-regulating expression can be done in many ways:

- deletion of the gene (knock down [57], used in the Haploinsufficiency profiling in yeast method);
- mutating the gene (randomly chemical induced mutations);
- binding to the DNA with so called zinc finger proteins [58];
- binding to the mRNA with antisense RNA; or
- silencing the gene with siRNA.

Up-regulating expression can be done, for example, by transfecting the cells with cDNA or plasmids or viral vectors. A new tool to activate expression is the use of saRNA, but this is still under debate [59,60].
A further extension to the above approach is not to modulate existing genes but to introduce new ones. For example, transfect yeast with all human genes one-by-one, like in the yeast three hybrid method, or make viruses that express human proteins on their surface like in the phage display method.
The modulation methods for target deconvolution are known under the name "chemical genomic methods."
A second approach is to modulate gene expression for target discovery. Again we modulate the genes one-by-one, and we look for changes in phenotype induced by this modulation, usually without giving any drug. This way we can infer the function of a gene. We can also look for specific phenotypic changes. When, for example, cancer cells don't grow anymore after a certain gene is down-regulated, than the gene-product is possibly an interesting drug target.
A third approach is to modulate a specific gene to validate that a given protein is indeed a good target. Modulation, usually down-regulation, mimics the effect of a drug without yet having a drug for the target. This down-regulation can even be done in animals to further add confidence to the target. When one has a drug, an extra confirmation consists in mutating the target gene in such a way that the protein keeps its function but can't be blocked anymore by the drug, rendering the cell insensitive to the drug. This would prove that the drug doesn't work via other mechanisms.
A fourth approach is to identify existing modulations in gene expression (including mutations) associated with diseases or drug resistance, using DNA micro-arrays or sequencing. (Figure 3.6).

C. Haploinsufficiency Profiling in Yeast

Concept. Lowering the expression of a target by deleting one of its two genes in the diploid yeast makes the strain more sensitive to a drug that acts on that target and often results in decreased growth [33,44,46,58,61].
Input. At least one compound of interest and a collection of strains, each with a deleted gene.
Output. Genes that code for proteins that directly or indirectly interact with the compound.
Group. Direct Methods. Genetic deconvolution methods. Chemical genomics.
Description. Imagine the picture of a drug that inhibits the growth of yeast by inhibiting a certain enzyme. Increasing the abundance of this enzyme will render the compound less effective. Decreasing the abundance of this enzyme will make the yeast more sensitive to the drug. One way to decrease the abundance of the enzyme is to shut down one copy of every two genes in the diploid yeast that codes for the enzyme. This method is based on the natural phenomenon of haploinsufficiency: in diploid organisms, deletions or mutations in one copy of the diploid set of a gene may result in an abnormal phenotype, especially when it is a vital gene. A deletion of a gene that doesn't code for the enzyme will not change the sensitivity to the drug, unless the gene

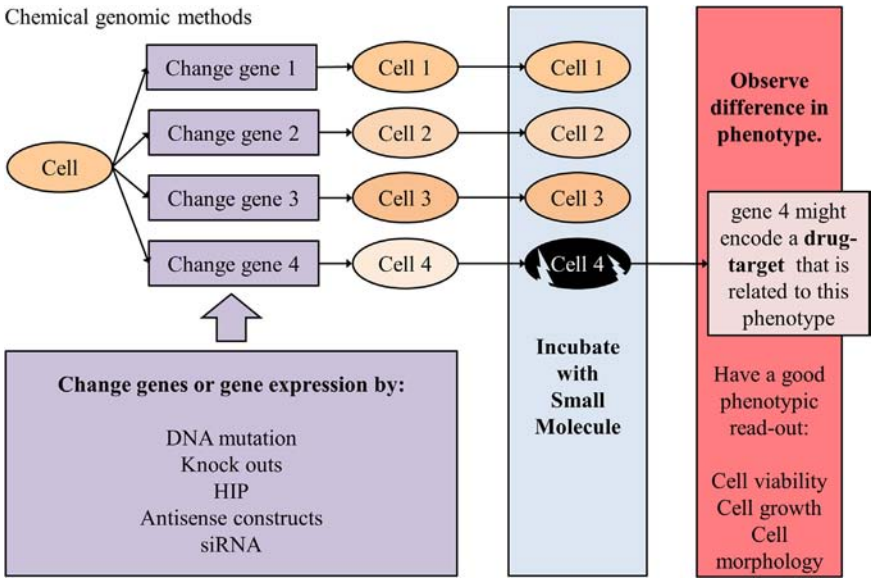

FIGURE 3.6 A collection of cells is made in which all possible genes are modulated one-by-one, and each time we look for a difference in drug induced phenotype. When a target is, for example, less expressed, then the cell could become more sensitive to the drug. Here crippled cell 4 indicates that gene 4 could code for a protein interacting with the drug.

has something to do with the pathway that comprises the enzyme or—more indirectly—results in the inhibition of an efflux pump, thereby increasing the concentration of the small molecule. Hence, observing which deletions affect the growth in the presence or absence of the compound can pinpoint possible targets of that compound or at least components that are part of the working or not working of the drug or even its toxicity. The Yeast KnockOut (YKO), a pooled collection of S. cerevisiae strains where each one of the more than 6,000 genes are completely deleted and replaced by a unique 20 base pair sequence—a "barcode" —is now available [33]. When the pooled collection grows in contact with the small molecule, some strains will grow less. This can be monitored by extracting the genomic DNA and quantifying the "barcodes" with PCR. This way the strain that contains the known deletion that interacts with the small molecule is identified.

Requirements. This method can only be applied when one already has a (small) molecule obtained by phenotypic screening or other sources.

Advantages.

1. The small molecule can interact with the proteins in their normal *in vivo* constitution (except for an artificially decreased abundance in one specific protein) [46].
2. The small molecule does not have to be linked to a bead or a hybrid protein. This not only makes life easier but also guarantees that the binding capacity is not hindered or influenced.
3. Only 0.1−1.0 mg compound per assay is required.
4. Because all genes are tested in the pooled collection, the identification of multi-target interactions is possible. Also, proteins that do not bind at all with the compound but have an effect because they are part of the pathway could be identified. Given the fact that good drugs are often "dirty drugs" that hit multiple targets, this method is well placed to study not only the target but also the whole mechanism of action of a drug.
5. There is a good chance that the cellular localization of the yeast protein mimics that of the human homolog. This further increases the real-life path that the drug has to follow to reach its targets.

Disadvantages.

1. In the yeast three hybrid, one can test (in principle) any human protein. But the protein is not in its normal state and habitat. In the haploinsufficiency model, the proteins are in their normal state and habitat, but this only approximates the human proteins and conditions. Human proteins that lack a yeast homolog will not be picked up.
2. A second limitation is that only interactions between small molecules and target proteins that impair cell growth (a specific phenotypic condition) will be picked up [33].

Examples.

Brefeldin A binding on Sec7p, doxorubicin binding on SIZ1, cisplatin binding on FCY2, NMD2, NOT3, SKY1, methotrexate binding to DFR1, FOL1, FOL2., 5-fluorouracil binding to CDC21, RRP6, RRP41, RRP44, RRP46, NOP4, MAK21, SSF1, YPR143W and tunicamycin binding to ALG7p, HAC1, GFA1.

Variations.

The key of the HIP method is that the target of interest is decreased in concentration. Deleting one copy of the diploid set is one option to achieve this, but one could also use RNA interference to reduce the target protein concentration. Other variations just do the opposite: increase the target concentration using plasmid overexpression. A nice experiment is to combine the under- and overexpression. When a reduced protein makes the cells more sensitive to the drug and at the same time makes the cell more resistant to the drug when overexpressed, then the probability that this protein interacts with the small molecule is high [46].

D. Analysis of Resistant Mutants

Concept. When a bacterium is treated with an antibiotic, resistant mutants can be selected and their genome sequenced. The mutated gene often reveals the target of the antibiotic [62].

Input. A drug and a resistant mutant.

Output. The mutated gene.

Group. Genetic methods. Target deconvolution.

Description. This method is very useful in determining the target of molecules obtained from a phenotypic screen with bacteria or viruses. As viruses and bacteria replicate quickly with a high frequency of spontaneous mutations, they easily acquire mutations resulting in resistance to a given drug. Selecting resistant mutants and sequencing their complete genomes to identify the mutation resulting in resistance is nowadays a very feasible exercise.

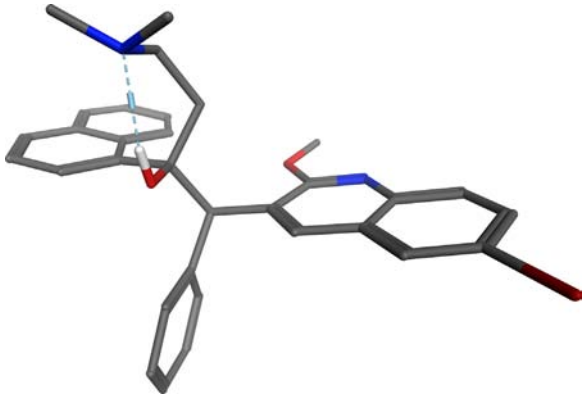

FIGURE 3.7 Structure of bedaquiline rendered in MOETM based on crystallographic data in reference [32]. The target of bedaquiline, the ATP synthase of *M. tuberculosis*, was identified by sequencing resistant mutants of the bacterium.

Case study. Using a phenotypic screen, the diarylquinoline bedaquiline (R207910) was found to be active against *M. tuberculosis* (TB) with a minimum inhibitory concentration (MIC) of 0.030 μg/mL. The activity was specific for mycobacteria and resistant strains were still sensitive to several other TB drugs, suggesting a new mechanism of action. The group of Koen Andries compared sequences of resistant and sensitive strains and identified two point mutations in the atpE gene, coding for a protein of the mycobacterial ATP synthase [31,63]. The ATP synthase enzyme is essential for the generation of energy in mycobacteria, and had previously not been described as a target for a TB drug or in fact any antibiotic. In December 2012, the FDA granted bedaquiline accelerated approval, based on phase II clinical trial data, as part of combination therapy to treat adults with multi-drug resistant pulmonary tuberculosis when other alternatives are not available [39]. (Figure 3.7)

E. siRNA for Target Validation

Concept. Temporary suppression of a gene-product with siRNA mimics the effect of an antagonistic drug. The value of the protein as a potential drug-target can be tested without having a drug. More recently discovered saRNA seems to up-regulate genes and could perhaps mimic some agonists [64].
Input. A potential valuable drug target.
Output. The phenotypic effect of the decreased production of the target protein.
Group. Target validation. Genetic methods. This method can, however, also be used for target discovery and target deconvolution.
Description. To add confidence that a protein would be a valuable drug target to treat a disease or at least to confirm that the protein is implicated in the disease, one could remove the protein by simply deleting the gene for it. But this is rather drastic and in reality far from simple, especially in animals. The gene could be vital during embryonic development and the production of knock-out animals is time consuming and demanding. It would be much more convenient if we could turn off the gene during a limited period (e.g., during the experiment). By preference, this would be done just with an injection, almost like a drug.
This is possible with siRNA. siRNA stands for small interference RNA and consists of 21−25 nucleotides long, double-stranded RNA molecules, complementary to a sequence in the gene one would like to knockdown. The detailed mechanism of the knockdown is rather complicated, involving several enzymes, and is still not fully understood. The bottom line, however, is that the mRNA is cleaved and hence the translation is blocked. The nice thing is that in principle any mRNA can be deleted this way. One only needs to have a unique sequence of 21 nucleotides belonging to the mRNA. In practice, not every sequence works equally well, and other sequences turn out not to be selective enough. Delivery of the siRNAs into the cell is another challenge. High pressure injection of naked siRNAs into the tail vein of mice resulted in effective uptake into the liver, kidney, lung, and muscle. Delivery of siRNA via expression out of a viral vector is a useful option. Even more sophisticated is the creation of a cell (or transgenic animal) that expresses the siRNA under control of a promotor that can be activated with an existing small molecule.

Advantages.

1. The biggest advantage is that you can study the effect of inhibiting a target without having a drug that does the job.
2. Down-regulating gene expression with siRNA can mimic drug effects in a much better way than knocking out a gene by deletion or mutation. With siRNA, the cell or animal can develop and behave normally as long as no siRNA is given. The siRNA also has only a temporary effect, almost like a drug.
3. In principle one only needs to have a unique "antisense" sequence of 21−25 nucleotides to block a protein. No knowledge of the structure of the protein is required. In theory, all antagonistic drugs could be replaced by siRNA molecules that could be designed in silico in—let's exaggerate—a few hours. The fact that the last ten years only two such drugs made it to the market is due to the famous delivery problem, and to some other issues like off-target effects and lack of efficacy.
4. siRNA is a relatively fast and inexpensive method.

Disadvantages.

1. Down-regulating a gene is not the same as blocking a specific part of the gene-product. A drug could, for example, completely block one function of a receptor, but the receptor is still present and can have other functions that are not affected. Knockingdown the gene will decrease all functions of that protein.
2. Down-regulating a gene can have more effect than just a decrease in concentration of the one gene-product.
3. The delivery of siRNA remains a challenge.
4. The activity and selectivity of the siRNA sequences are hard to predict.
5. The down-regulation is not 100 percent; there might still be some protein synthesis ongoing.

Variations.

Here we described the use of siRNA to block one specific gene in order to validate its gene-product as a drug target. siRNA can, however, also be used for target discovery by blocking genes one-by-one in order to correlate genes with phenotypic effects. And in combination with a (small) molecule, siRNA can be used like HIP to determine the target of the drug

Additional comments.

A small disadvantage is that only antagonists and enzyme inhibitors can be mimicked with siRNA. But this could change since the recent discovery [59] that short double stranded RNA complementary with the promotors of a gene can strongly activate the transcription. These so called saRNA (for small activating RNA) can mimic agonistic drug effects. Whether they do this via direct activation of the promotor or by silencing an upstream repressor is still under debate [60].

F. Yeast Three-Hybrid System

Concept. A cell (often yeast) is genetically engineered to give a signal when a small molecule (the bait) binds with protein X (the prey). Every cell is transfected to express another protein X. Transfecting plasmids are made from a library of cDNA [42,44,65].

Input. A small molecule and a set of cells, each expressing one member out of a set of possible target proteins.

Output. A read-out that permits the identification and selection of the cell (or colony) in which the small molecule binds to the expressed protein. This protein is localized in the cell nucleus.

Group. Expression cloning approaches.

Description. This system also is based on the affinity between the compound and the target protein, but it is used to circumvent the problem of low protein abundance by expressing the protein in genetically engineered cells. To understand this method, one has to dive into some molecular biology. Because that is not the purpose of this book, we will not dive very deep. The method is derived from the well-known yeast two-hybrid system. The yeast two-hybrid system or Y2H was developed by Stanley Fields and Ok-Kyu Song [66] in 1989 in order to detect protein−protein interactions. The key underlying the Y2H assay is that most eukaryotic transcription factors can be split into two parts and still activate transcription on condition that the two parts are sufficiently close together. A direct physical contact between the two parts is not necessary. The two parts are the DNA binding domain (DBD) and the activation domain (AD). To study the interaction between known protein 1 and unknown protein X, Fields and Song made two hybrids: they fused protein 1 with the DNA binding domain (hybrid 1, called the bait) and protein X with the activation domain (hybrid 2, called the prey). These hybrids were expressed in the yeast cells by transfecting them with vectors (plasmids) encoding the two hybrid proteins. In fact, for every protein X a

different vector is made. When the bait (protein 1) "captures" the prey (protein X), they bring their attached domains to each other, thereby bringing the activation domain close enough to the transcription start site to activate the transcription of the reporter gene. In the original assay, the reporter gene was the lacZ gene of E. coli. This gene encodes for β-galactosidase, an enzyme that cleaves lactose into glucose and galactose. Lactose can now be replaced by artificial derivatives that produce a useful read-out when cleaved. For example, cleaving the derivative 5-bromo-4-chloro-3-indolyl-β-D-galactoside turns the cells blue. This indicates that protein 1 binds with protein X. In the yeast three-hybrid system, a third hybrid is made: a fusion between a small molecule 1 of interest and a ligand molecule like methotrexate. The protein 1 is replaced by a ligand binding protein like dihydrofolate reductase that will bind strongly to methotrexate. The net result is an assay in which the colonies will turn blue when the bait—now ending with small molecule 1—captures the prey, protein X. The blue cells are then selected, and protein X is identified based on its DNA (Figure 3.8).

Requirements. This method can only be applied when one already has a (small) molecule obtained by phenotypic screening or other sources. The second requirement is that it must be possible to chemically attach the small molecule to a ligand like methotrexate using a linker like polyethylene glycol and in such a way (or under the assumption) that it doesn't interfere with the binding toward the target protein. It can be helpful to dispose of a SAR to identify parts of the molecule that can be used to attach the linker. A third requirement is that one needs to make use of a cDNA library containing the proteins to study. The compilation of this library (genomic DNA, normalized cDNA, full-length cDNA, etc.) can affect the protein expression. Finally, the cell type should be appropriate for your proteins.

Advantages.

1. As the target proteins are expressed artificially, they can be in higher concentrations than in a cell lysate.
2. The whole mammalian proteome can be screened in intact cells.
3. Once you have the complete set of transfected yeast cells, the technology is rather easy and can be automated.

Disadvantages.

1. Yeast is not a mammalian cell. However, mammalian versions (like MASPIT [42]) are now available
2. When molecules can activate the reporter gene via other ways, this will result in false positives.
3. Many false negatives due to steric hindrance of the protein in its fusion complex.
4. The small molecule has to be linked to methotrexate.
5. Proteins have to be fused with the activation domain and are possibly not in their natural state.
6. The binding takes place in the nucleus of the cell, hence membrane penetration is part of the equation.

Examples.

Atorvastatin binding to PDE6D, kinase inhibitors like purvalanol B to bind with known targets (like CDK1, CDK5, CDK6) and unknown targets (CLK3, PCTK1, PCTK2, PAK4, RSK3, FYN, YES, EPHB2, and FLT4) [42], Sulfosalazine binding to SPR.

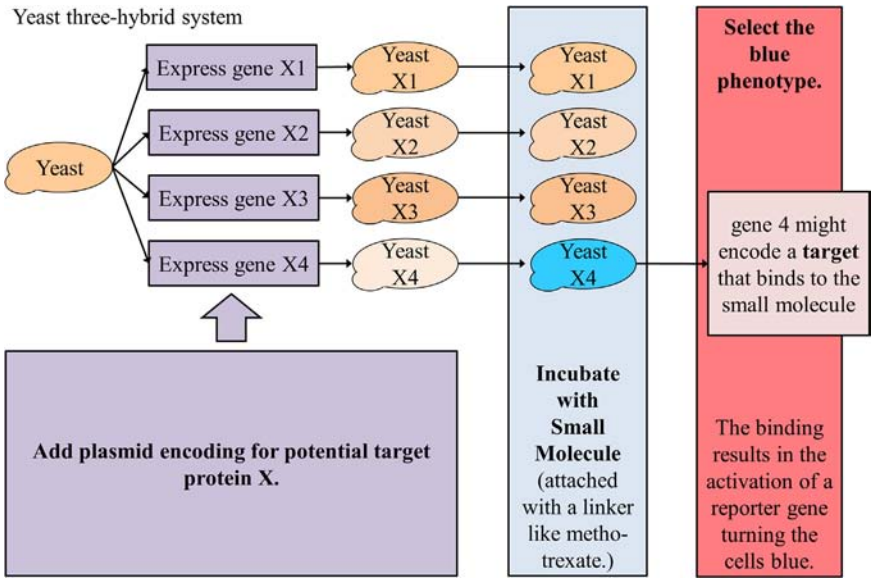

FIGURE 3.8 A collection of yeast cells is built in which all target proteins of interest are introduced one-by-one. This is done by introducing a gene construct that codes for the protein linked to a reporter gene activating domain. When a drug binds to the target protein, the reporter gene is activated. In case the reporter gene codes for β-galactosidase the yeast can be made to turn blue.

Variations.
Since its creation in 1989 the Y2H technique has seen a bewildering number of variations and improvements, very well described by Stynen et al. [65] We only mention the MASPIT variant. MASPIT stands for mammalian small molecule protein interaction trap. The protein hybrids are replaced by a JAK-STAT hybrid system inducing a reporter gene. The advantage, besides being in a mammalian cell, is that the binding occurs in the cytoplasm rather than in the nucleus.

G. DNA Microarrays

Concept. A DNA microarray is a surface with an array of microscopic spots of DNA. Each spot contains short single strand DNA molecules, all with the same unique sequence. Each gene expressed in a sample will hybridize with its corresponding spot, which can be identified using fluorescence readout [67–70].
Input. cDNA converted from mRNA out of a cell lysate.
Output. A list of all of up- and down-regulated genes.
Group. Target discovery. No small molecule needed. Target validation. Target deconvolution: see comparative profiling.
Description. Most disease phenotypes must be ultimately based on changes in gene expression in some cells. Hence, a list of all genes whose expression is up- or down-regulated in a disease can lead to potential drug targets. A gene that is mutated and causes a disease is probably less useful than a healthy gene whose altered expression or gene products are involved in the disease phenotype.
Today, microarrays are the technology of choice to discover these genes. The principle is simple, but the practice is a combination of art and science [69]. A DNA microarray is a surface of a few square centimeters that contains thousands or even millions of spots of DNA. Every single spot contains a unique probe consisting of single-strand DNA molecules with a unique sequence representing a specific gene. It is of the utmost importance that one knows which spot belongs to which gene. With a spot size of 16 μm, one can put 40,000 spots on a square centimeter, sufficient to probe the whole human transcriptome. Every spot contains 10 million identic single strand DNA molecules with a length of 25 to over 100 nucleotides long. These microarrays (or "gene chips") are manufactured industrially using several different techniques like robotic contact spotting, inkjet deposition, or on-chip synthesis using photolithography (Affymetrix) [69].
To identify which genes are expressed in a specific tissue (e.g., a liver cancer), one proceeds as follows. The mRNA is extracted and converted to cDNA because RNA is less stable. The cDNA can be concentrated or amplified when needed. The cDNA is labeled with a fluorochrome and incubated on the microarray chip for hybridization during several hours. Nonbound material is washed away, the chip is laser-scanned by a robot, and the data are processed, analyzed, and visualized [69–72]. To interpret the results, one should compare the gene expression from the liver cancer cells with the corresponding expression in normal liver cells. The method of choice to do this is to repeat the exercise with the normal cells on a second chip. Until very recently, a convenient alternative was to process the normal cells in parallel with the cancer cells from the beginning and use a different dye, usually green for control cells and red for the cells of interest. The red and green labeled cDNAs are then put together in equal amounts onto the chip. After hybridization and washing, the red spots indicate genes that are more expressed in the cancer cells than in the normal cells, and green spots indicate genes that are less expressed in the cancer cells than in normal cells. Equally expressed spots will be yellow. The problem with this pairwise analysis is that it is less suited for later comparisons due to standardization issues. For this reason, the pairwise analysis is increasingly abandoned.
Advantages.
1. Microarrays allow us to test the whole human transcriptome in one experiment.
2. The technology is now a standard laboratory tool and many services are available.
3. The technology is very versatile and can be used in a broader way than just for comparing the transcriptome. Other applications are chromosome aberrations studies, methylation analysis, single-nucleotide polymorphism detection, toxicogenomics, and diagnostic use. Today 45 million sequences can be probed with one chip.
4. The technology is becoming cheaper, comparable to PCR (Polymerase Chain Reaction).
5. With the "maskless" photolithographic production, probes can be made on-demand without having to interrupt the production process.
6. Limited sample need. For the transcriptome analysis, you need about 20,000 cells. But with a preparation step, one could start from a single cell [73].

Disadvantages.

1. When millions and millions of molecules interact and hybridize with each other you may expect some variation and unexpected things to happen, resulting in false positives and false negatives. For example, the cDNA can form tertiary structures preventing some parts from hybridizing with the probes.
2. Not all mRNAs are equally well extracted, and some mRNA are very unstable.
3. Important pharmacological targets such as GPCRs, ion channels, and transporters have mRNA in low concentrations and need enhanced sensitivity of the microarray to be picked up.
4. The transcriptome doesn't equal the proteome. mRNA can be regulated before translation, and many post-translational regulations and modifications to proteins can make them inactive and hence virtually absent.
5. Some closely related genes can easily hybridize with the probes of their close family members (called cross-hybridization).
6. The need for skilled technical personnel.

Examples.

Potential targets were identified related to asthma, SARS, arthritis, and systemic lupus erythematosus. In the cancer field, clinicians are using DNA microarrays to distinguish between cancers and to predict which treatments will have the most effect [67]. Using protein microarrays, SMIR4, a small-molecule inhibitors of rapamycin, has been found [74].

Variations.

1. The list of variations based on probes, array size, production methods, and applications is endless. Smaller spots allow the production of microarrays with millions of spots to interrogate the single-nucleotide polymorphisms (SNPs). Microarrays for yeast, viruses and bacteria, lab animals, and per chromosome are all available or can be produced.
2. Microarrays loaded with proteins (directly or via antibodies) permit the study of protein−protein interaction, protein−DNA/RNA interactions, and protein−small molecule interactions.
3. Microarrays with living cells attached to the surface by antibodies or proteins are used mainly in cancer research.

H. Comparative Profiling

Concept. A compound is added to a cell. This alters the gene-expression profile, called the "signature." This signature is then compared with a database of signatures obtained from treating cells with known compounds. The underlying idea is that compounds that produce about the same signature have about the same target(s) [45]. Gene-expression is a convenient profile, but the concept of comparative profiling is also applicable to protein expression, toxicity pattern, metabolome expression, morphological aspects, and other aspects.

Input. The gene-expression signature of an uncharacterized compound.

Output. Matching drug−target pairs based on profile similarity, to be further validated.

Group. Indirect. Deconvolution. Profiling. In silico. Genetic.

Description. As an example, we describe gene expression profiling using the Connectivity Map (also known as cmap) developed at the Broad Institute [75−77]. The concept is based on the pioneering work of Hughes in yeast [78]. The first step is to incubate relevant cells or tissues with the compound to be characterized. Cells from treated patients or animals could also be used. Then the complete gene-expression pattern is obtained using micro-arrays that cover the whole genome. The second step is to feed this gene-expression pattern into the cmap website [79] and click query. A pattern-matching algorithm based on the Kolmogorov-Smirnov statistic now compares the profile with all the profiles in the database. At the moment of writing, the so-called build02 database contains over 7,000 transcriptional expression profiles from the treatment of a very limited set of cultured human cell lines with 1,309 small molecules at a limited set of concentrations. As a comparison, build01 contained only 164 compounds. Pattern-matching algorithms can be fine-tuned in myriads of ways, but the cmap website keeps that very limited so that scientists at the bench don't need to be experts in bio-informatics or statistics. The main output of the algorithm is—to put it simply—a list of compounds ranked by how well their induced up- and down-regulated gene-expression mimics that of the test compound. It is important to note that the cells used to obtain the pattern to be searched (the "query signature") should not be from the same cell line and not even from the same species as the ones used to build the database. Of course, only genes that are present and expressed in both cell types can be compared. This is less a shortcoming than one would suspect, because many genes are up- or down-regulated in clusters, and missing one or two genes still permits finding the overall matching patterns. On the other hand, a compound like estrogen will not change the expression profile when the estrogen receptor is absent in the cell (Figure 3.9).

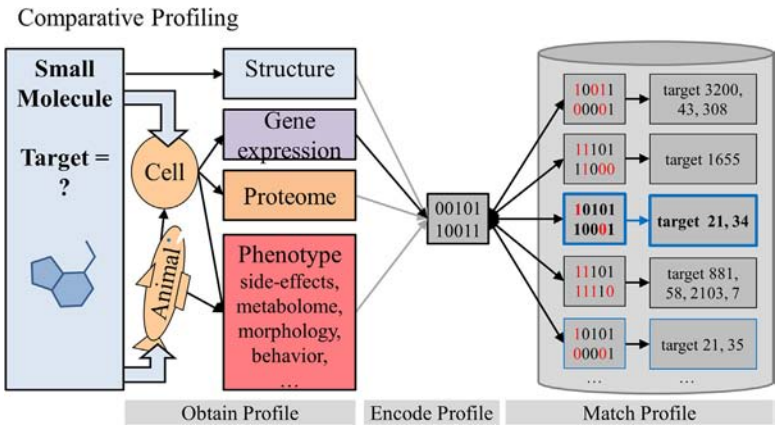

FIGURE 3.9 A method to infer the target of a compound is to first get a profile of a compound. A profile can be anything, such as the structure of the compound, the behavioral impact in an animal experiment, or the gene expression change in a cell. It is important that one uses a database containing the profiles of many other compounds together with their target(s). The profiles are encoded in such a way that matching algorithms can find the profiles in the database that most resembles the profile of the drug. The underlying hypothesis is that compounds with the same profile would also have the same target.

Requirements. Comparative profiling is only possible when there is something with which to compare. One has to use of a database that contains the signatures of many known compounds and a pattern-matching algorithm.
Advantages.
1. The availability of freely accessible profiling databases growing over time.
2. Web-based, easy-to-use interface.
3. The test compound is used in its normal state in a normal cell.
4. Multiple effects are measured, not just the binding with one single protein.

Disadvantages.
1. The output is not a guaranteed target but rather a proposal of possible targets or pathways. And when an expression match is found with a compound with unknown targets, you still have no target clue.
2. The cells used to build the database are special human cell lines, living on plastic, instead of primary cells living in the body.
3. Targets or pathways that were never hit before will not result in a match.

Examples.
Tools: the COMPARE NCI60 analysis [80], the JFCR39 database [81], the Connectivity Map database developed at the Broad Institute [75,76]. Drug-targets identified [45]: gedunin-HSP90, droxinostat-HDAC, bisebromoamide-actin, glucopiericidin A-glucotransporter, BNS22-topoisomearse II, Theonellamide F-3b-Hydroxysterols.
Case study.
The group of Wei and Armstrong [82] was confronted with the resistance to glucocorticoid treatment of childhood acute lymphoblastic leukemia (ALL), resulting in a poor prognosis. Comparing gene-expression profiles obtained from sensitive and resistant patients was not really helpful, but querying the Connectivity Map database pointed to the connection between glucocorticoid sensitivity and rapamycin. Rapamycin, a mTOR inhibitor, is an FDA-approved immunosuppressant, and it would be very promising if this drug could restore the sensitivity of the cancer to dexamethasone. Wei could prove this concept to work in cells. Another group showed that mTOR inhibitors are synergistic with methotrexate in mice [83]. Promising *in vitro* results with temsirolimus, another mTOR inhibitor, in prostate cancer didn't translate into a positive clinical outcome [84].
Variations.
1. Other databases, other algorithms, limited gene sets, other techniques to measure the gene expression. Example: the Luminex 1000 Profiling Approach [85].
2. Comparative profiling of protein-expression patterns.
3. Comparative profiling of side effects.
4. Comparative profiling of the metabolome.
5. Comparative profiling of the morphology of the cell.
6. Comparative profiling of the structure of the compound.

I. Analysis of the Pathophysiology

Concept. The detailed analysis of the pathophysiology could reveal new interesting targets.
Input. A disease of interest.
Output. Potential targets, pathways, or active compounds. Insight.

Group. Target discovery. Conventional. Top down.
Description. It sounds logical and scientific that the way to find a drug-target is the detailed study of a disease, unraveling and exploring the consecutive layers, starting from the macroscopic manifestations, drilling down to changes in tissues and cells, and ending with characterizing the molecular culprits. However, most of the time there is no need to travel down the entire way, nor to understand every element on the path to find a drug. When George Hitching and Gertrude Elion first started to synthesize analogs of purine to block DNA synthesis, the role of DNA was still uncertain. They knew that the purine metabolite was needed by bacteria to produce DNA, and so false purines could perhaps block the enzymes and function as "antimetabolites." In general, studying the functions and interactions of endogenous small molecules—metabolites, hormones, neurotransmitters, cytokines—is very rewarding. Formulating hypotheses, even when they turn out decades later to be wrong, is another useful approach. Finding targets that could be used to modify or counteract the consequences of a disease—instead of finding the cause of a disease—is often more fruitful, because removing the cause remains even today an almost unattainable goal. Infectious diseases are the biggest exceptions to this rule. Cancer is already less an exception. The reason is that in the case of bacteria, the complete organism can be put in a test tube. When a compound is able to kill the bacterium in a test tube, there is some chance that it will maintain that capacity in our body. Of course, the drug has to reach the bacterium in our body. For cancer, the situation is more complex. A single cancer cell is not the same thing as a cancer tissue with all its different cell types. Blood circulation is different in cancer tissues. Oxygen levels and acidity could be different. Analysis of the pathophysiology is a top-down, classic strategy that still works well today and certainly has a bright future with all the new molecular and imaging research tools available.
Advantages.
1. New targets and pathway can be found.
2. No need to start from an active molecule, although starting from endogenous molecules is often key.
3. Proven track record.
Disadvantages.
Takes a long time without guaranteed success. The war on cancer started decades ago.
Examples. Some targets identified by analysis of the pathophysiology are many viral enzymes like reverse transcriptase, dihydrofolate reductase, 5HT transporter, COX-2, cysteinyl leukotriene receptor, angiotensin-converting enzyme, estrogen receptor, and dopamine receptor.

J. The Study of Existing Drugs

Concept. The thorough study of "old" and even more recent drugs with the newest technologies and insights can reveal new potential targets [44].
Input. An existing drug.
Output. The drug-target or additional knowledge on the mechanism of action and affected pathways, including new subtypes of receptors or potential new targets.
Group. Target discovery. Target deconvolution. Conventional. Top down.
Description. Because in the past most drugs were discovered via phenotypic screening, a lot of drugs were on the to-do list for target deconvolution. Consequently many new targets were discovered this way. Since the decline in phenotypic screening in favor of target-based screening, the to-do list has shrunk. The recent revival of phenotypic screening and the screening of exotic (natural) molecules will further sustain the need for target deconvolution. The study of toxic effects of drugs can also lead to new targets or to deeper knowledge of existing target effects. Recently [86] a group revealed that COX-2 inhibition in the cardiomyocytes in mice resulted in enhanced susceptibility to induced arrhythmogenesis. The study of COX-2 also led to the discovery of the COX-3 enzyme, a splice variant which was subsequently found to be nonactive in humans.
Examples. $GABA_A$ receptor, COX enzymes, tubulin, L-type calcium channels, KATP channels, dopamine D2 receptor, PPARa, monoamine transporters.

K. Systems Biology

Concept. The study of the dynamic interactions between all components of a biological system.
Input. All kinds of so-called -omics data sets [10]. Modelling software.
Output. A better understanding of the role and value of specific targets in their biological context.

Group. In silico methods.
Description. To understand and predict the behavior of an organism, one should study not only all its individual parts in a reductionistic way but also their dynamic interactions in an integrated holistic perspective. The concept of systems biology is certainly not new. Simulating the dynamic interaction between two components of an axon (a sodium and a potassium channel) to explain how a nerve signal emerged was published in 1952 [87]. Today the number of components in the actual datasets is huge: 29,000 transcriptomic elements, 20–30 million epigenetic elements, 22,000–39,000 proteins (each present in a multitude of different states, like phosphorylation states) and around 40,000 small cellular biomolecules [10]. The next step is to collect the interactions between these components. The BioGRID database contains over 139,000 nonredundant interactions between over 18,000 human proteins. Then comes the software and the mathematical models to make predictions. As the models are fitted with the actual data, they are usually good in predicting what we know already. Most of the targets proposed so far using systems biology were not easily druggable and provoked a renewed interest in phenotypic screening. Systems biology is, however, an ideal working instrument to increase our understanding of the mechanism of actions of drugs in the body.
Example. Pyrvinium [10].

L. In Silico Simulation of the Human Patient

Concept. The ultimate goal of systems biology is the understanding of functioning of a whole organism [10]. Understanding in this field is achieved by modeling or simulation. We are, however, so far from the complete simulation of a patient that I think it is appropriate to address this topic with a sense of humor.
Input. A demand for a safe, effective, inexpensive oral drug against disease x.
Output. After pushing the button, the chemical formula of a perfect drug rolls out of the computer.
Group. Futuristic methods.
Requirements. An enormous dose of luck and a pile of quantumcomputers.
Additional comments. Trying to simulate a human patient in silico would redirect funding for drug research to the informatics industry. But one should be realistic. A human body contains about 10 trillion interacting cells and every cell contains about 23 trillion molecules (of which about 8 billion are proteins) that interact with one another. Today it's very hard to predict the behavior of one single small molecule in a very confined part of a protein. We can't predict consistently the structure of a protein out of the gene sequence. We can't consistently predict the function of a protein. Today we can't even predict consistently the interaction between one small molecule and a bunch of water molecules—called dissolution. One could say that we don't have to simulate all the billions of molecules to predict the overall behavior. Yet, a change in one molecule in one single cell can lead to the death of a person within a year. (Figure 3.10).

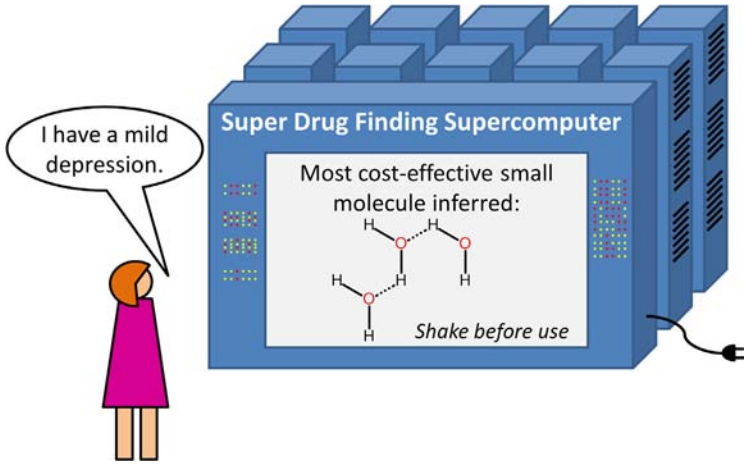

FIGURE 3.10 The complete simulation of a human patient at work.

VII. TARGET VALIDATION

It can't be stressed enough that the only validation that really matters is the proof in the clinic. It adds, of course, confidence that a target seems to work in dozens of different cell-types and in several animal models, but at the end this is all ersatz compared to the ultimate test: does a compound that effectively acts on the target really work in humans?

Further, target validation is not different from the usual scientific process. When an interesting target is described in a publication, the first step is to reproduce the experiments. Although not well studied, this reproducibility turns out to be a bit problematic. Florian Prinz examined 67 projects—mostly in oncology—and found that in only 20–25 percent of them the in-house findings were completely in line with the relevant published data [15,88].

The next step is to make variations in one of the three elements in the ligand–target–environment equation. Modulating the affinity of the small molecule for the target protein should correlate with a modulated activity of the molecule. Mutations in the binding domain of the protein should result in modulation or loss of activity of the ligand. Changing the cell-type should or should not change the effect of the molecule. As already stated, the use of siRNA is an invaluable tool for target validation. It can be used in cell experiments and animal models to study the effect of inhibiting or blocking a target without the need to have a small molecule.

As an example we continue with the thalidomide story. In 2010, Ito [52] identified cereblon as a target protein for thalidomide's teratogenicity using affinity chromatography. How did he validate the target? One way was to go *in vivo*. Thalidomide is not teratogenic in mice and rats. But what about in zebrafish? Thalidomide given to zebrafish embryos resulted in disturbed pectoral fins. Zebrafish have a cereblon orthologous gene, zcrbn, and its protein could also be affinity-extracted with thalidomide. Then Ito's team blocked the gene with antisense constructs, resulting in the same teratogenic effects as thalidomide. Injection of correct mRNA for the gene rescued the defects. They then made a mutant zCrbn protein that could not bind to thalidomide but was still functioning. Overexpression of this mutant made the embryos insensitive to thalidomide.

VIII. CONCLUSION

In the last decades, many successful new small molecules and biologicals have enriched our therapeutic capabilities. Yet despite all the new fantastic technologies, we have not seen an explosion in R&D efficacy. The small molecules that came out of the target-based screening strategy often missed efficacy or showed unexpected toxicity in clinical trials. Today, the follower strategy, based on targets validated in humans, is by far the most successful strategy measured by the number of drugs it produces. The second most productive strategy is phenotypic screening, which is probably even more successful than has been acknowledged. The renewed interest in phenotypic screening and the increased capabilities to handle thousands of animals like zebrafish in an automated way will result in an increased demand for target deconvolution. The methods for target deconvolution will remain a rapidly evolving and highly entangled landscape. The ultimate target validation method, however, will still be the human clinical trial. Chemists should think critically about targets that were identified based solely on highly reductionistic methods. Otherwise, they might blindly chase for years the perfect compound for the wrong target.

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Medicinal and Pharmaceutical Chemistry

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The Pre-Medicinal Chemistry Era

As a scientific discipline medicinal chemistry could only emerge after it became generally accepted that the properties of the active ingredients of medicinal products determine the pharmacological and consequently the therapeutic effects of the active principle. Until synthetic organic chemistry developed, coincidently almost during the same period in which pharmacology became an experimental science, medicinal products were mainly obtained from natural materials, from plants especially; to a certain extent mineral products were also used. It was the shape, the colour of (parts of) plants which were considered to be indicative for the biological activities.

Not long after the first paper[3] on synthetic organic chemistry appeared (synthetic dyes, Perkin, 1856)[9] it was a series of milestone publications by Crum Brown and Fraser (1868)[10] which initiated the development of synthetic medicinal agents. In the years thereafter several scientists believed that from then on new medicines could be developed on basis of knowledge (!) of relationships between chemical structure and biological activities. 'Soon pharmacopeia will be composed on basis of structure–activity relationships' (Richardson, 1877),[14] and 'Soon doctors will have series of medicines in order of potency to influence practically any physiological action' (Brunton, 1889)[11] were too optimistic opinions. They were followed by more realistic views. Hopkins (1901)[12] concluded that 'things had developed in a very disappointing way.' It was in 1937 that the famous pharmacologist Clark[15] felt that structure–activity relationships had been 'investigated so intensively that one had a fairly clear idea of the extent of our ignorance.'

Indeed, notwithstanding the extreme enthusiasm about the possibilities to develop new medicines at the end of the 19th century not much exiting things happened during the following half a century. The process meant to lead to new therapies remained a matter of trial and error only. There have been several reasons for lack of real progress. The major one was the almost complete absence of any reliable information on physiological and pathological mechanisms. An almost as important cause was the limited insight in the real structure of the molecules synthesized. The central question was – and to some level this is still true –, the mechanism of action involved when a compound exerts a certain effect.

At the very end of the 19th century Langley had suggested that there should be 'receptive substances' present in the body and Ehrlich introduced the term receptor: 'That combining group of the protoplasmic molecule to which a foreign group, when introduced attaches itself.' Neither Langley nor Ehrlich[4] had an idea about the chemical nature of a receptor and to what kind of primary effects the interaction of the introduced molecule and the receptor would lead. Medicinal Chemistry was not born yet; drug development was based on two separated disciplines: synthetic chemistry and pharmacology. There were high and solid walls between the disciplines and even in the years 2000 these walls are still existing, but have to be turned down for making drug design a reality. Whitesides and Deutch[1] phrased it very adequately: 'Chemistry should cluster its teaching and research around the exciting and uncertain future rather than the historical ossified past'; such is especially true for the field of medicinal chemistry.

Notwithstanding the absence of understanding which mechanisms are involved in the biological activity of organic compounds, many effective new medicines have been identified and introduced during the first sixty years of the 20th century. A major drawback has been the slow development in pharmacological techniques though. Pharmacological investigations were almost exclusively involving in vivo experiments. The outcome of in vivo experiments is obviously determined by more factors than the ultimate interaction of the active principle with its target. Absorption, distribution, metabolism, excretion, interaction with other sites contribute all to the activity as measured finally. The relationships between structure and activity remained therefore very much unreliable. Especially Rudolf Magnus (1873–1927) saw the advantages of using isolated organs at an early stage. His work with isolated organs, especially isolated intestine, has contributed much to make structure–activity relationship studies more reliable.

The question on the nature of receptors remained, however, unanswered still. In the foreword to the famous book Molecular Pharmacology by Ariëns.[2] The receptor is compared with a attractive lady to whom you may send letters, who sometimes answers the letter, but who never shows up. And Ariëns who by applying a wide variety of isolated organs, introducing simple mathematics of compound-receptors interactions, admitted in 1967 at a symposium of the NY Academy of sciences: 'Yes, I know when I am

talking about receptors I am talking about, something I do know nothing about.'[16] It has been Nauta,[13] who has been most likely the first who suggested that a receptor was a helix-shaped protein (1968), but any solid evidence was not available yet.

The Birth of the New Discipline

In 1909 the American Chemical Society founded its 'Division of Pharmaceutical Chemistry'; the division was later renamed as 'Division of Chemistry of Medicinal Products.' In 1948 the term medicinal chemistry emerged, 'Division of Medicinal Chemistry.' A new discipline was born, but it took quite a time before it was accepted as such. Medicinal chemistry meanwhile found a place within the pharmaceutical industry and at universities. Some universities gave the field a place in departments of chemistry, but many thought it was a pharmaceutical field and should be part of their Faculty of Pharmacy. But almost always the two major actors, synthetic chemistry and pharmacology, remained separated. The one group synthesized new compounds; the other tested ('screened') them. Burger[3] gave an excellent account on his matter.

In the following years societies of medicinal chemistry were started in many countries; some were independent societies, other parts (division) of chemical and again other of pharmaceutical societies. In 1970 a Section Medicinal Chemistry was started by the Organic Chemistry Division of the IUPAC, followed by the founding of the European Federation of Medicinal Chemistry in 1972. These developments, the differences in the framework and the organizational structures have not helped to arrive at an internationally accepted definition of the new science. It proved even to be difficult to decide on the name of the field: medicinal chemistry (English), Chimie therapeutique (French), Wikstofforschung (German), Chimia Farmaceutica (Italian), and Farmacochemie (Dutch). The use of the term 'Pharmaceutical Chemistry' when medicinal chemistry is meant is especially confusing as pharmaceutical chemistry is the established term for chemistry related to finished medicinal products. In textbooks dating from the early 1970s several definitions could be found, reason why the IUPAC published in 1973 as a comprehensive definition: '*Medicinal chemistry concerns the discovery, the development, the identification and the interpretation of the mode of action of biologically active compounds at the molecular level. Emphasis is put on the discovery and development of drug, as indicated by the adjective 'medicinal.' However, as the definition indicates, the interests of Medicinal Chemistry are not restricted to drugs but also imply bio-active compounds in general.*' And pharmaceutical chemistry was left as the established term for finished medicinal products.

'*Medicinal Chemistry is also concerned with the study, identification and synthesis of metabolic products of drugs and related compounds.*' The IUPAC recommended the sole use of the term 'medicinal chemistry,' but in several countries such was not followed up. More importantly, it seems that the interdisciplinary working medicinal chemists of the first hours did not foresee the rapid developments taking place in the years to follow. Although the mile-stone papers of Hansch appeared during the sixties of last century, terms as structure–activity relationships, or drug design are absent in the 1973 IUPAC definition!

In 1959 the American Chem. Soc. started to publish the interdisciplinary Journal of Medicinal Chem. originally published as J. of Medicinal and Pharmaceutical Chem. in the United Kingdom. The current name was installed in 1963. In 1966 the publication of Annual Reports in Medicinal Chemistry, started, also by the ACS. Both publications are leading in the field.

Medicinal Chemistry in the 20th Century; Some Dreams Come True

Until the 1960s medicinal chemistry, could be described as a field in which scientists from different disciplines cooperated, but which was yet far from an integrated discipline. Through the activities of the several national societies, their – often international – symposia, the publishing of interdisciplinary journals, but especially by the developments in neighbouring field's things changed rapidly. The introduction of the use of computers in science had a great impact. In the 1960s the multivariate, quantitative structure–activity studies (QSAR) had become possible by the use of computers. Researchers became over-enthusiastic; within the pharmaceutical industry management was even concerned about these, as it was thought that the possibility to predict properties of not yet synthesized compounds would mean the end of the patentability of any potentially new medicine.

The transfer of pharmacology into a molecular science made a proper identification of receptors and subsequently of subtypes of several receptors and thereafter the selection of – for those receptors – selective ligands feasible. The adrenergic α- and β-receptors and ligands, muscarinic receptor subtypes, histamine H_1 and H_2, a variety of serotonin receptors. The introduction of ligand displacement (radiolabeled ligands) techniques leading to the determination of reliable affinity parameters became very important. This period has been called a golden age of the pharmaceutical industry. The fruit was relatively low hanging; when most of the fruit had been picked, the expectations became less and less optimistic, however.

Breakthroughs in biological sciences opened new vistas though. Receptor machineries were unravelled. The structure of receptors being proteins in α-helix shape was confirmed; ion-channels became chemically identified, together with their function; intracellular receptors got their place. Receptors proteins were identified for which no physiological functions were available: orphan receptors; Lefkowitz defined a 'reversed pharmacology': receptors without known functions. Later such receptors could be deorphanized. The developments had great impact on the new possibilities of medicinal chemistry. Moreover, the one intriguing result obtained by molecular was followed by the other. Spontaneously active receptors were found, leading to the discrimination of receptor blocking agents as (neutral) antagonists and inverse agonists. Mutated receptors expressed in isolated cell systems became important tools. Point mutations of receptors were found to be the cause of known diseases.[8]

The impact of the successes of molecular biology has not been restricted to the design of new active compounds. The impact has been as important for the design of less toxic compounds. And also for the so-called ADME fields: absorption, distribution, metabolism excretion, i.e. the kinetic profile of medicinal agents. Where medicinal chemists profited much of the results of their colleagues in biology research, the molecular biologists were much dependent on the enormous progress in analytical chemistry. Chromatography and mass spectroscopy made the elucidation of the primary structure of receptor proteins possible; computational approaches followed to study the molecular mechanisms involved in receptor binding and receptor activation. The same analytical technologies led to concept of 'systems biology,' with which the complexity of physiological systems became understood and transparent: the old idea of multimarket medicines got a new meaning.[6]

Dreams became true when receptors – especially the G-protein coupled receptors, the GPCR's – were isolated and crystallized finally. Now medicinal chemists could really start to think of designing new molecules from scratch. The impact of the combined developments remained not unnoticed: in 2012 the Nobel Award for Chemistry (not for 'Physiology or Medicine'!) went to the molecular pharmacologists Lefkowitz and Kobilka for their work on isolating and crystallizing GPCR proteins.

Current Medicinal Chemistry; An Integrated Interdisciplinary Branch of Chemistry

Besides its scientific relevance, the main objective of medicinal chemistry has always been and still is to identify new compounds which can be used as the active principle of effective and safe medicines.[5] In the early days a synthetic chemist synthesized compounds and a pharmacologist 'screened' them, in the beginning in vivo only. Sometimes interesting compounds were found, some reached clinical applications when not a mere idea about the mechanisms involved were available; the diazepams constituted a matter in case.

Things have changed! The main objective of the field did not though, but the pattern of the process did very much. Currently the process starts with a decision on the target which could be used to arrive at new interesting compounds. Biochemistry, molecular biology, genetics, the study of systems biology all became very important contributors to medicinal chemistry as the sources for new targets, pharmacology remaining irreplaceable, however. When a target has been identified and a pharmacological testing system selected it should be decided to investigate which compounds have to be made.

At-random synthesis has proved to be not productive anymore. Some 15–20 years ago combinatorial chemistry had been considered as a promising approach, but this typical example of modern, more or less random, synthesis did not live up to the high hopes. Currently the fragment-based route of selecting new interesting structures seems to offer better chances; this approach is followed by many modern drug hunters. It is very promising that more and more crystal structures of target molecules are obtained. The search after new molecules from natural sources, especially from plants used in traditional medicines seems to remain an attractive alternative, but the positive results remain limited though.

New chances are likely coming from detailed information on the structure of target molecules. Designing molecules from scratch, based on the structure of the selected target will offer possibilities of which medicinal chemists could so far dream only. Until recently the design of new molecules was based especially on the structure of known ligands: ligand based design. With target-based design rational drug design seems to become reality finally.

Rational drug design, however, seems to be a misnomer. Medicinal chemists are first of all the makers of compounds with a wanted activity, compounds which interfere with a target. But an active molecules is not a drug, a medicine yet, in this sense the term 'medicinal chemistry' is debatable. For being suitable as an active ingredient of a medicine, a compound should fulfil more requirements. The active principle of a medicine should be able to reach the target for which it is meant and if possible be kept away from any other site which it potentially could influence. The pharmacokinetic profile of the compound constitutes an important aspect of the usefulness of the given compound. The pharmacokinetic behaviour of a compounds includes phenomena like absorption from the site of application, distribution through the total organism, metabolism (especially by the liver after oral application), and excretion (especially but not exclusively) via the urine or faeces.

But there is still more. The way by which a compound is presented to the body, as a tablet, by intra-muscular injection, via the skin, by inhalation and so on, is at least as important for the final efficacy of the medication. The speed of dissolution of a tablet or capsule determines e.g. to a large extent the speed of absorption; this part of the discipline concerns 'pharmaceutical chemistry.' It should be clear that the distinction what is included in medicinal chemistry and what in pharmaceutical chemistry is not always unambiguous; it may differ from country to country, being partly determined by local 'tradition.'

When finally a compound with – at least – both an interesting pharmacological and pharmacokinetic profile has been obtained a medicine becomes within reach, but for one matter, relative safety and as much as possible an absence of side effects are required as well. During quite a long time, investigations into these issues only started after the profiling for activity had been completed; toxicology was considered as a black box. Here too times have changed; currently studies in unwanted effects start early, molecular toxicology was introduced as a new discipline. The aim of this discipline is to design structures of compounds without affinities for targets which could lead to toxicity, often referred to as anti-targets. As toxicity is often caused by especially metabolites of the primary agent, studying 'drug metabolism' constitutes an important aspect of the field of molecular toxicity.[7]

A special group of medicines is formed by chemotherapeutic agents, antibiotics included. For these medicaments toxicity is the wanted property. The purpose of chemotherapy is to kill cells, microbes (including virus particles) and tumor cells. The main property, cell toxicity is a relatively easy requirement, but the real issue is in this case is obviously selectivity, no harm for the host cells or the non-cancer cells. By making use of differences in the biochemistry or the constitution between microbial and mammal

cells, relatively safe antimicrobials have been not difficult to be identified (e.g. sulfonamides, penicillines), but it took long before effective and relatively safe and viral compounds could be introduced. Currently viricidal derivatives not killing non-infected cells are effective viral killing compounds. The intrinsic problem for anti-cancer medicines continuous to be problematic; cancel cells are still showing properties of the healthy cells of the patient; toxicity of anti cancer agents continues to be a very serious problem.

An additional problem both for antimicrobials and anti cancer medicines is the occurrence of resistance; this matter is for great concern for antimicrobials especially; microbes insensitive for any of the available chemotherapeutics are a real threat.

Comprehensive Medicinal Chemistry

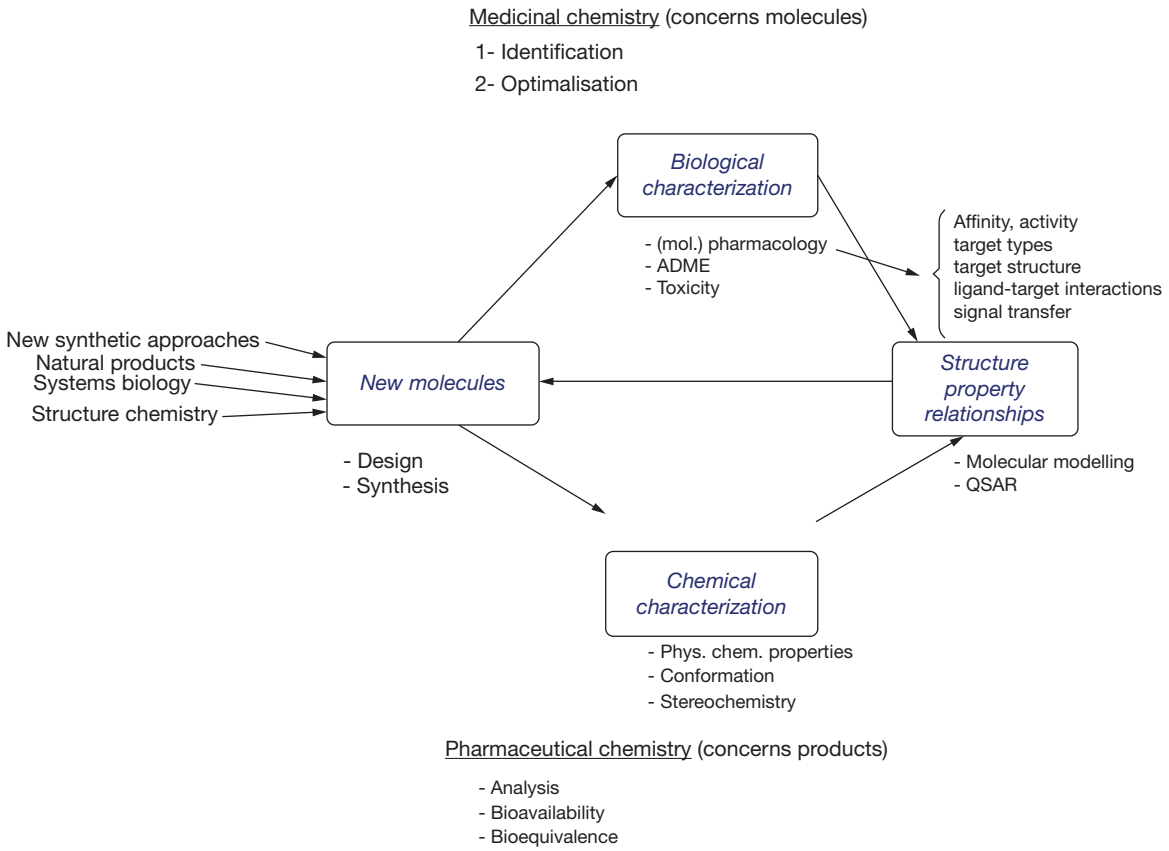
In the **Scheme 1** '*Comprehensive Medicinal Chemistry*' the process of identifying new compounds as potentially interesting for use as active principle of a medicinal product is summarized and separated from pharmaceutical chemistry.

This process is presented an interactive circular sequence of contributions from different disciplines a medicinal chemistry programme can start at any of the mentioned parts of the total process. This scheme shows the very specific character of the discipline as, as described by Burger[3] as being interdependent and yet independent. It is not a special type of synthetic chemistry to which a bit of pharmacology has been added etc. but a really interdisciplinary chemical discipline. Another eye-catching feature is the continuous evolution of the discipline. New approaches, often from neighbouring fields (biology, medical sciences) are offering new vistas for the medicinal chemists. In recent years the development of systems biology has opened new routes to interesting targets. For these developments the enormous progress the field of analytical chemistry (chromatography, NMR, MS) had and will have great impact for medicinal chemistry.

In the several *Chapters Medicinal and Pharmaceutical Chemistry* of the comprehensive work *Chemistry, Molecular Sciences and Engineering* the field will be presented in details, according to **Scheme 1**, as an interdisciplinary circular process and related to Pharmaceutical chemistry.

Subareas will be presented in separate sections; they involve e.g.

Classes of medicinal agents (such as cardiovasculars, antihistamines);
Characterizing of Compounds, both in a chemical and a biological sense;
In Silico Compound Design; ligand based and target based;



Scheme 1 *Comprehensive Medicinal Chemistry treated* as an interactive circular process.

Synthetic approaches;
Society and Politics; patents, prices, neglected areas; tropical diseases, drug resistant microbes and cells;
Personal Essays of; selected scientists;
Case Studies of medicines, discovery and impact;
Pharmaceutical Chemistry; finished medicines; analysis.

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Perspectives in Drug Discovery☆

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Three case histories are cited to provide significant lessons to all medicinal chemists. The first case is a classical one of bioisosteres and understanding a carcinogenicity: if the tumors are caused by a chemical toxicity of the drug substance, it is not registrable, whereas if tumor incidence is a pharmacologic result from ultra high doses over the lifetime of rodents, it may be registrable. The second case illustrates licensing a late stage drug candidate in order to market a first-of-type drug and then improve its profile for a best-of-class drug before the competition does. The last case illustrates the need to investigate preliminary pharmacokinetics and drug disposition early in lead optimization so that different structure–activity relationships for different properties can be converged before clinical trials.

Introduction

The role of a medicinal chemist in drug discovery is the design, synthesis, and registration of the best compound for treating a particular disease condition. The job is not finished when the most potent compound for a given target (receptor, ion channel, enzyme) is identified. The best compound of the series for bioavailability to the target must also be found, the active compound with least metabolism or most predictable pharmacokinetics, and certainly the compound with fewest other effects or greatest selectivity for the desired effect must be identified. The convergence of these properties is the best compound for the disease until a more relevant mechanism or target is found that provides a more effective compound.

My perspectives on drug discovery have developed over the 50+ years that I have worked in the field, but many of the views were clear early in this time before combinatorial chemistry, high-throughput screening, and cloned and expressed receptor subtypes. The case histories I describe here focus on whether to discontinue or redirect projects; only the time required to complete these projects is shortened by new technologies, but the strategic lessons are still valid today.

Case 1

In the early 1960s, I joined a company effort to explore beta-adrenergic agents because of clinical use of epinephrine for cardiac rescue and isoproterenol for bronchodilation in asthma. Pharmacologic identification of the first beta-blocker, sotalol, concurrent with ICI's pronethalol which soon yielded to the more potent propranolol, hit a development wall – what is the disease condition and clinical measurement? The marketing director clearly asked, 'What kind of patient needs their betas blocked?' In 1964, the British physician Brian Pritchard reported significant lowering of blood pressure in hypertensives by propranolol, opposite to what was predicted because of its predominant effect on cardiac function rather than peripheral vasculature. By following rather than leading with clinical trials, sotalol was registered first in Europe but its sales were dwarfed by propranolol. In later years, probing clinical research established a greater β1-receptor selectivity and a significant role for sotalol as an antiarrhythmic, a use not anticipated by early animal pharmacology.

More lessons emerged from the adrenergic studies. Our major thesis for structurally modifying the catecholamines was to replace phenolic –OH groups with the methanesulfonamide group in hopes of achieving some organ or functional selectivity. With sotalol (4-$MeSO_2NH$–) we achieved good beta-blocking potency, some $beta_1$/$beta_2$-selectivity, and good oral bioavailability. The more interesting experiment was replacing one phenolic –OH at a time with $MeSO_2NH$ in the catecholamines to retain beta agonist potency (Figure 1). The 4-OH, 3-$NHSO_2Me$ analog was equipotent to isoproterenol with much longer $t_{1/2}$ and more $beta_2$-selective, whereas the 3-OH, 4-$NHSO_2Me$ was over 10 000 times less active. The more acidic proton of $MeSO_2NH$ had to be *meta* to the phenethanolamine for beta-adrenergic potency, and the group is more ionized at blood pH than the catechol, whereas the ionized

☆*Change History:* August 2013. One major change is the elimination of the first case on understanding clinical endpoints before screening biological targets; the other three cases were retained and the last two were slightly modified. Case 2 now highlights a significant movement of drug discovery from large pharmas to small biotech and even startup companies for innovative targets and molecules. The third case updates the information and issues from preclinical development to highlight new requirements of chemistry before commencing clinical trials. Reference 3 was added to provide background for case 3. My biographical sketch was changed to reflect forward chronology and add some recent activities.

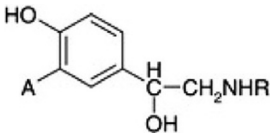

Figure 1 A = HO, R = *i*Pr, isoproterenol; A = $MeSO_2NH$, R = *i*Pr soterenol; A = $HOCH_2$, R = *t*Bu, albuterol.

group *para* to the side chain does not function as an agonist. Furthermore, the *meta* $MeSO_2NH$ group is not a good substrate for catechol-*o*-methyltransferase (COMT) methylation, which explains why these bioisosteres were not rapidly metabolized and have a longer $t_{1/2}$. Three compounds from this series (different amine substituents) were clinically evaluated and found to be very safe, effective, and selective agonists.[1,2] Further studies showed the 3-$MeSO_2NH$ group to be a useful bioisostere in phenylalanines, but just as it was not a good substrate for COMT it did not permit decarboxylation with L-DOPA decarboxylase. This meant the methanesulfonamide bioisostere of L-DOPA would not provide an alternative Parkinson's drug, nor did this methanesulfonamide bioisostere provide an effective long-lasting alternative to estradiol, and its usefulness remained primarily in the catecholamines.

A major lesson came belatedly from the clinical trials and toxicological studies of the beta agonists. Soterenol, the bioisostere of isoproterenol, had almost completed phase III clinical trials as a bronchodilator for asthmatics, and mesuprine was in phase III for peripheral vascular disease and premature labor as a uterine relaxant when preliminary results from chronic toxicity and carcinogenicity studies became available. At necropsy 20 of 50 rats in the high dose group had benign tumors, mesovarial leiomyomas, which appeared like a third ovary. No tumors were found after 18 months, only after 24 months. With a heightened concern for tumors and carcinogenicity at the Food and Drug Administration (FDA) in the 1960s, all clinical trials were stopped and all patients who had received drug were to be followed for their lifetime. The FDA even assumed the $MeSO_2NH$ group was responsible for the leiomyomas, although the rats were dosed with 10 000 times the amount of compound that would have killed them with epinephrine. The concern at that time was not benefit/risk ratio or separation between active and toxic dose, but an absolute concern for tumors. All development projects with sulfonamido beta-adrenergic agonists were stopped and no products were marketed.

About 7 years later, Glaxo developed a similar compound salbutamol/albuterol (see **Figure 1**), which differed from isoproterenol and soterenol only by having the *meta* OH or $MeSO_2NH$ isostere replaced by CH_2OH and *t*-butylamine in place of *i*-propylamine. The amine substituent was a trivial change because all three series of catecholamines had *t*-butyl > *i*-Pr > Me in potency with no appreciable difference in pharmacologic or kinetic/disposition properties. The pK_a of the catechol or 'mixed catechol' group was $MeSO_2NH > OH > CH_2OH$, but all somewhat acidic due to the *para* OH, and only the OH was readily metabolized by COMT to an inactive metabolite. At that time, the Mead Johnson (Bristol-Myers) management agreed to repeat the carcinogenicity studies to see if the leiomyomas were truly drug related, even though it cost nearly 3 years and > $2 million at that time. The new study included four arms with a small group necropsied at 18 months: (1) were the results reproducible? (2) were they reproducible with a different strain of rats? (3) were they reproducible if drug was administered orally by gavage or in feed? (4) were they reproducible if a similar dose of albuterol was used instead of soterenol? The answer to all questions was yes with nearly identical results. Albuterol had been licensed to Schering Plough for the US market, and the carcinogenicity study was conducted by Schering Plough, but no mesovarial leiomyomas were found. We called the head of R&D at Schering Plough who asked their pathologist to re-examine the preserved rat ovaries from their study. Although surprised, they found a similar incidence of leiomyomas when they knew what to look for. At that time, our tissue pharmacologists measured the effect of soterenol on isolated mesovarial tissue and found the density of beta-adrenergic receptors to be much greater than the density in rat uterus, the classical tissue for measuring beta-adrenergic potency. We were convinced that the unusual benign tumors were an extension of the compounds' pharmacology at very high doses and lifetime dosing rather than a specific chemical toxicity. Subsequent to these findings, Glaxo repeated the carcinogenicity studies with albuterol and found a similar incidence of tumors at their laboratories, but their convincing finding came from a parallel treatment arm with enough propranolol added to block the adrenergic effects of albuterol – no tumors!

I believe this was one of the first examples clearly showing that animal tumors were the result of high dose pharmacology rather than chemical toxicity. The FDA's conclusion was to accept these findings as establishing the dose-limiting toxicity, and the likely dosage or even overdose consumed by people did not indicate significant risk, so albuterol was approved and Bristol-Myers was allowed to resume clinical trials with soterenol and mesuprine. After patients were followed for over 11 years, no major adverse effects or toxicities were reported. Although the sulfonamide adrenergic drugs were first-of-type to be developed by many years, they would be second to the marketplace, and Bristol-Myers' management did not see a marketing advantage at that time, so they were never commercialized. By contrast, Glaxo marketed both oral and inhaled formulations of albuterol (Ventolin) as it quickly became the leading drug for asthma and obstructive lung diseases. It has remained champion of its class without significant competition for over 25 years.

Several perspectives or morals are to be learned from this example – primarily, do not accept an unusual effect or toxicity in a nonhuman species as a basis for killing a clinical project – first, understand it, especially if it is an extension of the desired pharmacology at very high doses. Design the toxicology protocols so you can understand what is responsible for unexpected effects.

Another perspective I learned as a medicinal chemist from this work is that you cannot deviate very far from the structure of neurotransmitters or hormones if you want to develop receptor agonists. whereas there can be considerable variation for antagonists. This holds up well for adrenergics, serotonin, acetylcholine, glutamate, glycine, and estrogen. The $MeSO_2NH$ mimic for OH was most effective as part of a catechol group for agonists. Even the $-OCH_2-$ linker for propranolol compared to pronethalol retains a similar conformation for the side chain and improves affinity for antagonists but not agonists. Simple replacement of functional groups with bioisosteric groups that do not change stereochemistry, pK_a, hydrophilicity, or bioavailability are about all that is permitted for a true transmitter or hormone mimetic.

Case 2

A major new perspective was learned in the 1970s and 1980s when therapeutic area teams of chemists, biologists, and clinicians were assigned to intervene in major disease conditions. Our understanding of mechanism, relevant animal models, and preferred points for disease intervention was very limited at that time. We made structural assumptions for prototypical compounds with known activity, synthesized a few grams from multistep pathways, tested this compound in several in vitro assays for potency and selectivity, and assumed their mechanism of action would translate to clinical activity. Research groups from other companies were following the same process and would occasionally announce exciting new structural leads at scientific meetings. We often made the mistake that we were competing within structural classes or mechanism (usually unknown) rather than disease class. Bristol-Myers took an early lead at that time by licensing lead compounds from other companies or academic groups and probing disease targets with clinical trials; the mechanism or target was learned only after clinical efficacy was established. Trazodone (Desyrel) blazed the trail for other serotonergics in depression, and was replaced by the nonsedating nefazodone from internal discovery. Treatment of cancer became an achievable goal and a significant business with cyclophosphamide, bleomycin, mitomycin, cisplatin, and Taxol – structurally diverse, different mechanisms but clinically effective. Smart variations of a known active compound were easier and faster if you owned the lead molecule. Each of these first-of-type products was licensed with minimal clinical results, but spawned improved drugs from in-house research; the structural lead came from licensing and was still novel. During the past decade most big pharmas have significantly reduced their drug discovery efforts and license first-of-type clinical stage products from smaller biopharmas, so a greater understanding of SAR for ADME and pk and for system biology of the disease target are now required by discovery chemists.

Case 3

The final perspective I would like to pass to younger medicinal chemists is the need to optimize structure for treating the disease, not just the molecular target. The typical drug discovery process today is to screen more than 100 000 compounds from small molecule libraries against a cell-based or cell-free target in high-throughput manner. The iterative process between synthesis and screening is so fast now because many analogs are prepared at the same time by parallel synthesis in tubes or 96-well plates, then screened against molecular targets in high-throughput mode. There is a tendency in many organizations to assign enough chemists to optimize the 'hit' structure for potency, then explore the most active compounds by in vivo assays. It is my opinion that optimal compounds in a series for clinical development can be identified more quickly if most compounds synthesized are evaluated for aqueous solubility and partition coefficient, cytotoxicity, induction of P450 liver enzymes, and a conscious animal model of the disease being investigated.

Seldom does the structure–activity relationship (SAR) for the primary molecular target parallel the SAR for oral bioavailability (indicated by in vivo potency and water solubility) or for safety (indicated by cytotoxicity and P450 enzyme induction). Convergence of SAR for in vivo potency, safety, bioavailability, and drug disposition ($t_{1/2}$) is essential to select an optimal compound for clinical development. Obviously this perspective pertains to systemic diseases more than to invading microbes (bacteria, viruses, fungi, etc.). With high-throughput synthesis capabilities, the assays are rate-limiting rather than the preparation of compounds, so the convergence of different SAR can be achieved by getting multiple data points on each compound as it is synthesized.

The disparity of SAR has never been greater in my experience than for the project in which I am currently involved, inhibiting brain levels of $A\beta_{42}$ for Alzheimer's disease. We use a demanding human-cell-based assay and look for nanomolar potency in the primary screen, then we use mixed brain culture to confirm activity and potency for decreasing $A\beta_{42}$. Early in our efforts to optimize structure, we learned that removing a methyl group from a heteroaromatic ring decreased potency slightly but increased brain levels of drug and induced P450 enzyme activity. Then when we varied the substituent pattern on an aromatic ring, we found a most potent series that lacked aqueous solubility and oral bioavailability. Even slight structural changes that improved potency worsened several other properties which made the newer analogs less desirable. We recognized the need for convergence of SAR for all of these properties before we pursued multiple dose pharmacology or other receptors/ion channels/enzymes in search of specificity. To achieve optimal clinical efficacy for this series, we wanted a compound to have good oral bioavailability and brain levels (brain/plasma ratio >1), no cytotoxicity or liver enzyme induction, significant plasma $t_{1/2}$ and area under the curve (AUC), in order to safely dose patients orally and once daily in clinical trials for several months.[3] The simultaneous study of potency, bioavailability, and preliminary pharmacokinetics shortened the time to select an optimal clinical candidate. This perspective is certainly aided by

the high-throughput screening of many compounds against these drug properties. In preclinical development and clinical candidate selection, we dropped the concept that water solubility is essential for oral absorption and focused on good brain/plasma ratios. We found acceptable oral absorption and brain distribution with ultra fine particle size (nanoparticles) and dosing with food, which reduced high blood levels and liver exposure. These concepts have revolutionized clinical formulations for orally dosed compounds that are desired for brain distribution.

In concluding my perspectives on the foremost considerations for medicinal chemists, probably top of the list is patentability of the lead compounds. In this day of court decisions on infringement and 'first-to-file' applications, issued patents on drug targets or mechanism of action and diagnostic tools enable the patent holder to use them and possibly license them but not prevent infringement if used by others for drug development. This makes the composition of matter patents, plus synthetic process and formulation patents, king of intellectual property and sole protector of a product in the market place. For this reason, the medicinal chemist needs to synthesize compounds beyond the optimal clinical candidate which might be anticipated as second generation products, then secure broad patent coverage to prevent 'me-too' developments. Do not try to patent too broadly and include inactive compounds because that will weaken the patent for unanticipated active analogs. Do not file too early because that will shorten the patent life of products. Good patent strategy drives much of the synthetic effort for a first-of-type series.

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LIQUID CHROMATOGRAPHY | Affinity Chromatography☆

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Introduction

Affinity chromatography can be defined as a liquid chromatographic method in which a biological agent or biomimetic ligand is used for the selective retention of complementary compounds. This form of liquid chromatography was originally employed by Starkenstein in 1910 for the purification of amylase through the use of starch as a solid support and stationary phase. This method continued to slowly develop over the next 50 years. However, it was not until the 1960s that suitable supports like beaded agarose, as developed by Hjerten, became available, as well as relatively simple immobilization techniques for these supports. An important advancement in this latter area was a paper in 1967 by Axen, Porath, and Ernback, in which the cyanogen bromide method for protein and peptide immobilization was first reported. This immobilization approach was then utilized in 1968 by Cuatrecasas, Anfinsen, and Wilchek to purify enzymes through the use of immobilized enzyme inhibitors. It was also at this time that the term 'affinity chromatography' was proposed to describe this separation technique.

Affinity chromatography is relatively simple to perform and is a powerful tool for the separation of biological macromolecules. The high selectively of this approach often allows single-step purification strategies to be developed, even when working with dilute and highly complex mixtures. This simplicity and the variety of ligands that can be used with this approach have made affinity chromatography an important tool in process-scale separations. However, modern affinity chromatography also plays an significant role in the analysis and study of biological systems. For instance, most forms of chiral liquid chromatography, such as those using immobilized cyclodextrins or serum proteins, can be considered to belong to a subcategory of affinity chromatography.

Basic Principles of Affinity Chromatography

The Process

Affinity chromatography relies on the specific recognition that occurs between many biological molecules, such as the binding of an antibody with an antibody or the interaction of a hormone with its receptor. These interactions are used in affinity chromatography by permanently bonding (or 'immobilizing') onto a solid support an appropriate binding agent. This immobilized agent is known as the affinity ligand and represents the stationary phase for this method. Once the affinity ligand and its support have been placed within a column, this column can be used to retain any substance that will form a strong but reversible complex with the ligand. The optimum association constant (K_a) for such a system in purification work is generally 10^4–10^8 l mol^{-1}. However, stronger and weaker interactions can be used in analytical applications of affinity columns.

The most common format employed in affinity chromatography is given in **Figure 1**. In this format, a solution containing the target of interest is passed through a column containing an immobilized ligand capable of binding to the target. This step is performed in the presence of an application buffer that allows such binding to occur. Because the resulting interaction is usually selective in nature, the ligand will recognize and retain the target while allowing other compounds in the sample to pass through the column in the nonretained peak. However, due to the strong binding that is often present between the target and ligand, the target is held within the column until the mobile phase or chromatographic conditions are varied. This variation in conditions is often accomplished by passing an elution buffer through the column. As the target elutes from the column, the target is captured for

☆*Change History:* March 2013. DS Hage updated the main body of the text and the suggested articles for further reading.

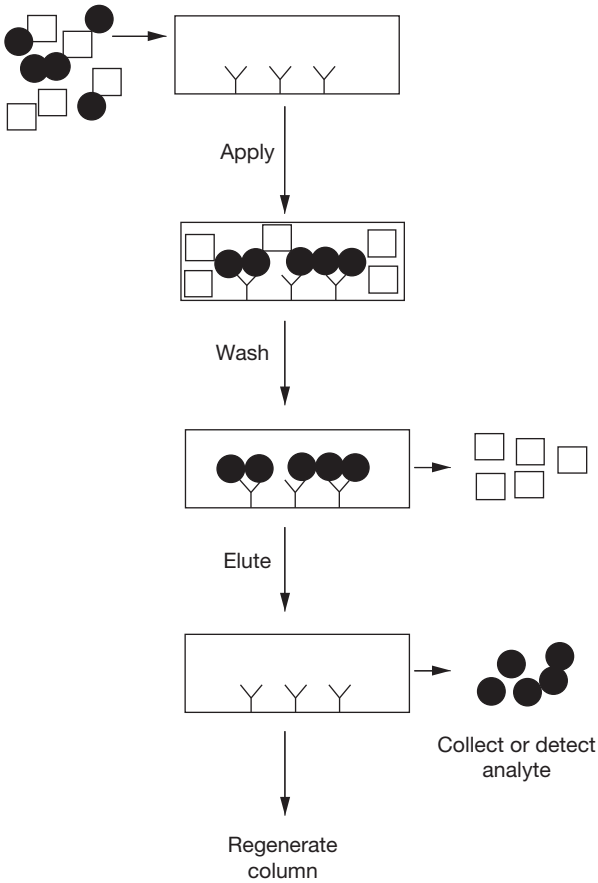

Figure 1 Typical separation scheme for affinity chromatography, showing the steps for sample application, column washing, analyte/target elution, and column regeneration.

Table 1 Affinity ligands and their target compounds

Type of affinity ligand	*Typical targets*
Biological agents	
Lectins	Glycoproteins, cells
Carbohydrates	Lectins and other carbohydrate-binding proteins
Nucleic acids	Exonucleases, endonucleases, and polymerases
Cofactors, substrates, and inhibitors	Enzymes
Protein A and protein G	Antibodies
Hormones and drugs	Receptors
Antibodies	Antigens
Nonbiological ligands	
Metal-ion chelates	Proteins and peptides that bind metal ions
Synthetic dyes	Enzymes and various nucleotide-binding proteins

further use or monitored by an online detector. The column is then cleaned, allowed to regenerate in the application buffer, and used for application of the next sample. As this scheme suggests, there are several factors to consider in the design of an affinity separation. These factors include the ligand, support material, immobilization method, and application or elution conditions.

The Affinity Ligand

Examples of ligands that are used in affinity chromatography are given in Table 1. The ligand can be a naturally occurring biomolecule, an engineered macromolecule, or a synthetic substance. Examples of naturally occurring ligands that are employed in this technique include enzyme inhibitors, hormones, lectins, antibodies, and nucleic acids, which are used to bind enzymes,

receptors, polysaccharides, antigens, and nucleic acid-binding proteins, respectively. Nonbiological ligands that have been used in affinity columns include immobilized chelates of metal ions (e.g., Ni^{2+}), reactive dyes, and molecularly imprinted polymers.

As shown in Table 1, many ligands are biological macromolecules. These ligands can take part in several types of interactions as they bind to their targets, including electrostatic forces, hydrogen bonding, dipole–dipole interactions, and van der Waals forces. The specific recognition between the ligand and target is due to a combination of these interactions as well as the fit between these species. This is the source of the high affinity that is seen for many biological ligands in affinity columns.

All affinity ligands can be grouped into one of two categories: group-specific ligands and high-specificity ligands. High-specificity ligands are generally based on biological agents. A common example is the binding of an antibody with the foreign substance (or antigen) to which the antibody was initially raised. These ligands tend to have relatively large association constants and generally require a step gradient for elution in affinity chromatography. Group-specific (or general) ligands are agents that bind to a class of related molecules. These ligands recognize a common structural feature on their targets and can be either biological or nonbiological in origin. Examples include protein A, protein G, lectins, boronates, dyes, and immobilized metal-ion chelates. Depending on their affinity, group-specific ligands may require step elution (e.g., as often used for lectins, protein A, and protein G) or may allow the use of isocratic elution (e.g., as occurs in some applications of boronates and immobilized metal-ion chelates).

There are several terms used to classify affinity methods based on the ligands they employ. For instance, bioaffinity chromatography (or biospecific adsorption) is a term used to describe any type of affinity chromatography that has a biological molecule as the ligand. This category can be further subdivided into other techniques. As an example, the use of antibodies (or immunoglobulins) as affinity ligands is often referred to as immunoaffinity chromatography, and the use of lectins as ligands is commonly known as lectin affinity chromatography. Other classifications based on the type of ligand include (1) dye-ligand or biomimetic affinity chromatography, which generally uses an immobilized synthetic dye; (2) immobilized metal-ion affinity chromatography, in which the ligand is a metal ion complexed with an immobilized chelating agent; and (3) boronate affinity chromatography, in which boronic acid or one of its derivatives is utilized as the ligand.

The Support

The support is the material or matrix that holds the ligand within the affinity column. Table 2 shows various materials that have been employed for this purpose. Ideally, the support should have low nonspecific binding for sample components but should be easy to modify for ligand attachment. This material should also be stable under the flow rate, pressure, and solvent conditions to be employed in the analysis or purification of samples. In addition, the support should be readily available and simple to use in method development.

Depending on what type of support material is being used, affinity chromatography can be characterized as either a low- or high-performance technique. In low-performance (or column) affinity chromatography, the support is usually a large diameter, nonrigid gel. Many of the carbohydrate-based supports and synthetic organic materials listed in Table 2 fall within this category. The low back-pressures and reasonable costs of these supports make them useful for large-scale processing and small-scale preparative work. However, these supports also tend to have slow mass transfer and limited stability at high flow rates and pressures. These factors limit the usefulness of these supports in analytical applications, where both rapid and efficient separations are often desired. In high-performance affinity chromatography (HPAC, also known as high-performance affinity liquid chromatography), the supports generally consist of small, rigid particles capable of withstanding the flow rates and/or pressures characteristic of high-performance liquid chromatography (HPLC). Examples of supports suitable for such work include modified silica or glass, hydroxylated polystyrene media and some types of monolithic supports. The mechanical stability and efficiency of

Table 2 Materials used as supports in affinity chromatography

Support material	*Approximate usable pH range*
Carbohydrate-based supports	
Agarose	2–14
Cellulose	1–14
Dextran	2–14
Synthetic organic polymers	
N-Acryloyl-2-amino-2-hydroxymethyl-1,3-propane diol	1–11
Hydroxyethylmethacrylate polymer	2–12
Oxirane-acrylic polymer	0–12
Polyacrylamide	3–10
Polytetrafluoroethylene	0–14
Poly(vinyl alcohol)	1–14
Styrene–divinylbenzene polymer	1–13
Inorganic supports	
Glass	2–8
Silica	2–8

these supports allows them to be used with standard HPLC equipment. This results in a separation with good speed and precision, making it useful in analytical applications.

One desirable characteristic for the support is that it should allow easy access of the target to the ligand. For a porous material, this requires that the support have pores that are at least several-fold larger than the target. However, the use of a support with large pores will also produce a low surface area per unit volume, which will limit the number of ligands that can be attached to the surface. In process-scale work, another requirement for the support is that it should be possible to routinely sanitize this material without causing damage. This requirement generally means that the support should be resistant to reagents such as concentrated sodium hydroxide or 8 mol l^{-1} urea.

Immobilization Methods

The third item to consider in the development of an affinity technique is the way in which the ligand is attached to the solid support (i.e., the immobilization method). There are many ways immobilization of the ligand can be accomplished. These approaches include simple adsorption to a solid support, bioselective adsorption to a secondary ligand (e.g., the noncovalent binding of antibodies to immobilized protein A), entrapment, imprinting, and covalent attachment.

There are several criteria that should ideally be met for the immobilization method. First, this approach must allow the affinity ligand to be coupled to the support without significantly affecting the ligand's binding properties. Second, this method must allow the immobilized ligand to still be accessible to its target for binding to occur. In addition, the immobilization method should not introduce any groups to the support that can give rise to nonspecific interactions. Fourth, the amount of ligand coupled to the support should be optimal for binding to the target and the ligand should be immobilized in a manner that prevents the loss of ligand over time.

Covalent attachment is the most common approach used for ligand immobilization. Some techniques available for this approach are listed in Table 3. For a protein or peptide, this process generally involves coupling the ligand through amine, carboxylic acid, or sulfhydryl residues that are present in its structure. Immobilization of a ligand through other functional sites (e.g., aldehyde groups produced by carbohydrate oxidation) is also possible.

Application and Elution Conditions

The final set of items to consider in the use of affinity chromatography is the selection of application and elution conditions. Most application buffers in affinity chromatography are solvents that mimic the pH, ionic strength, and polarity experienced by the target and ligand in their natural environment. These conditions generally allow for optimum binding between the target and ligand. Any

Table 3 Examples of covalent immobilization methods

Techniques for ligands with amine groups
Cyanogen bromide (CNBr) method
Divinylsulfone method
Epoxy (bisoxirane) method
Ethyl dimethylaminopropyl carbodiimide (EDC) method
N-Hydroxysuccinimide ester (NHS) method
Schiff base (reductive amination) method
Tresyl chloride/tosyl chloride method
Techniques for ligands with free sulfhydryl groups
Divinylsulfone method
Epoxy (bisoxirane) method
Iodoacetyl/bromoacetyl methods
Maleimide method
Pyridyl disulfide method
TNB-thiol method
Tresyl chloride/tosyl chloride method
Techniques for ligands with carboxylate groups
Ethyldimethylaminopropyl carbodiimide (EDC) method
Techniques for ligands with hydroxyl groups
Cyanuric chloride method
Divinylsulfone method
Epoxy (bisoxirane) method
Techniques for ligands with aldehyde groups
Hydrazide method
Schiff base (reductive amination) method

cofactors or metal ions required for solute–ligand binding should also be present in this solvent. In addition, surfactants and blocking agents may be added to the buffer to prevent nonspecific retention on the support or affinity ligand.

The elution buffer can be either a solvent that produces weak binding or one that decreases the extent of this binding by using a competing agent that displaces the target from the column. These two approaches are known as nonspecific elution and biospecific elution, respectively. Biospecific elution is the gentler of these two methods because it is carried out under essentially the same solvent conditions as used for sample application. This feature makes this approach attractive for purification work, where a high recovery of active target is desired. In nonspecific elution, a change in column conditions is used to weaken the interactions between retained compounds and the ligand. This result can be accomplished by changing the pH, ionic strength, or polarity of the mobile phase. The addition of denaturing or chaotropic agents to the mobile phase can also be employed. These changes can alter interactions of the target with the ligand, leading to lower retention. Nonspecific elution tends to be much faster than biospecific elution in removing compounds from affinity columns, resulting in sharper peaks and lower limits of detection for use in analytical applications of affinity chromatography. This method can also be used in purifying solutes, but there is a greater risk of target denaturation with this approach than there is with biospecific elution. Furthermore, in nonspecific elution care must be taken to avoid using conditions that may harm the support or result in an irreversible loss of ligand activity.

Applications of Affinity Chromatography

Preparative Applications

The most popular use of affinity chromatography is in the purification of proteins and other biological agents. The use of this method in enzyme purification is particularly important, with hundreds to thousands of applications having been reported in this field alone. Ligands used for this purpose include enzyme inhibitors, coenzymes, substrates, and cofactors. For instance, nucleotide mono-, di-, and triphosphates can be used for the purification of various kinases, NAD has been used to isolate dehydrogenases, and RNA or DNA has been used for the preparation of polymerases and nucleases.

Antibodies have also been popular ligands for the purification of biological compounds. There are now thousands of examples of immunoaffinity methods that have been developed for the isolation of hormones, peptides, enzymes, recombinant proteins, receptors, viruses, and subcellular components. In addition, immobilized antigens are frequently used to isolate specific types of antibodies. A more general purification scheme for antibodies can be obtained by using antibody-binding proteins like protein A and protein G. These latter ligands have the ability to bind to the constant region of many types of immunoglobulins. Both protein A and protein G have their strongest binding to immunoglobulins at or near neutral pH but readily dissociate from these when placed into a lower pH buffer.

Dye–ligand affinity chromatography is often used in large-scale protein and enzyme purification, with hundreds of such compounds having been isolated by this technique. In this method, an immobilized synthetic dye is used that binds to the active site of a target by mimicking the structure of its substrate or co-factor. The most common dye used for this purpose is Cibacron Blue 3G-A (see **Figure 2**). Other dyes that have been used in this approach include Procion Blue MX-3G or MX-R, Procion Red HE-3B, Thymol Blue, and Phenol Red. Although these ligands were originally discovered on a trial and error basis, recent work in the area of biomimetic affinity chromatography has used computer modeling and 3D protein structures to develop dyes that compliment the binding pockets of specific target proteins.

Analytical Applications

Although affinity chromatography was originally created as a preparative method, the past few decades have seen this method also become an important tool in analytical applications. **Table 4** summarizes some strategies that can be employed in these applications and gives some representative examples. The simplest format for using affinity chromatography in analysis involves the traditional step gradient mode, as shown in **Figure 1**. The advantages of using this approach in analytical applications, particularly when performed by HPLC, include its speed, relative simplicity, and good precision.

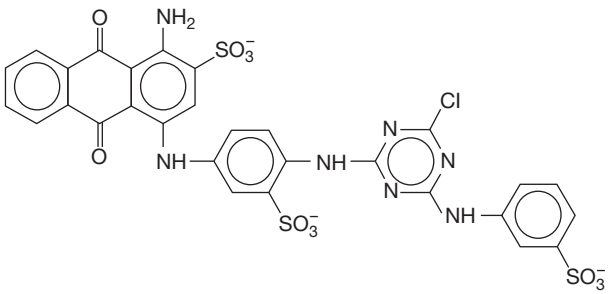

Figure 2 Structure of Cibacron Blue 3G-A, a stationary phase often used in dye–ligand affinity chromatography.

Table 4 Analytical applications of affinity chromatography[a]

General application	*Examples of analytes*
Direct detection	Anti-idiotypic antibodies, antithrombin III, bovine growth hormone, fibrinogen, fungal carbohydrate antigens, glucose tetrasaccharide, glutamine synthetase, granulocyte colony stimulating factor, group A-active oligosaccharides, human serum albumin, immunoglobulin G, immunoglobulin E, interferon, interleukin-2, lymphocyte receptors, β_2-microglobulin, tissue-type plasminogen activator, transferrin
Offline affinity extraction	Aflatoxin, albuterol, benzodiazepines, cytokinins, fumonisin, human chorionic gonadotropin, ivermectin and avermectin, nortestosterone, ochratoxin A, oxytocin, phenylurea herbicides, sendai virus protein, trenbolone, triazine herbicides
Online affinity extraction	Aflatoxin M1, β-agonists, α_1-antitrypsin, atrazine, atrazine metabolites, benzylpenicilloyl-peptides, bovine serum albumin, carbendazim, carbofuran, chloramphenicol, clenbuterol, cortisol, dexamethasone, diethylstilbestrol, digoxin, estrogens, hemoglobin, human epidermal growth factor, human growth hormone variants interferon α-2, LSD, lysozyme variants
Chromatographic immunoassays	Adrenocorticotropic hormone, α-amylase, atrazine/triazines, 2,4-dinitrophenyl lysine, human chorionic gonadotropin, human serum albumin, immunoglobulin G, isoproturon, parathyroid hormone, phenytoin, testosterone, theophylline, thyroid stimulating hormone, thyroxine, transferrin, transferrin, trinitrotoluene

[a]The information in this table is based on data provided in Hage (1998).

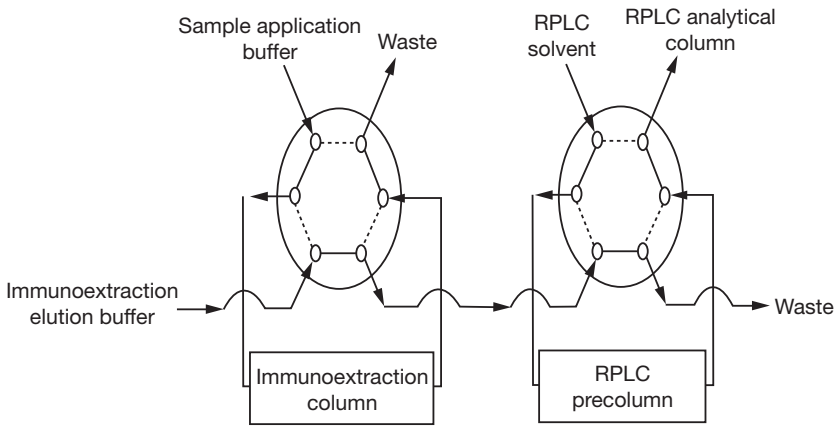

Figure 3 Scheme for interfacing an immunoextraction column with reversed-phase liquid chromatography (RPLC). The two circles represent six-port switching valves, with the solid and dashed lines showing the two positions of these values and the flow of sample and solvents in each of the two positions. The operation of this system is described in the text.

Affinity extraction is another approach that can be used for solute detection. In this method, an affinity column is used for the removal of a specific solute or group of solutes from a sample prior to their determination by a second method. This approach employs the same operating scheme as shown in **Figure 1** but now involves combining the affinity column either offline or online with some other method for the actual quantitation of analytes. This scheme often involves the use of antibodies as ligands, but other binding agents can also be employed.

Offline extraction is the easiest and most common way for combining affinity columns with other analytical techniques. This method typically involves the use of antibodies that are immobilized and packed into a disposable syringe or solid-phase extraction cartridge. After conditioning the affinity column with the necessary application buffer or conditioning solvents, the sample is applied and undesired sample components are washed away. An elution buffer is then passed through the column and the target is collected. If desired, the collected fraction can be analyzed directly or first dried down and reconstituted in a solvent that is more compatible with the method to be used for quantitation.

Online affinity extraction can also be used. A typical scheme for performing online immunoextraction with reversed-phase liquid chromatography (RPLC) is shown in **Figure 3**. This particular scheme involves injecting the sample onto an immunoextraction column, with this column later being switched online with a RPLC column. An elution buffer is then applied to dissociate any retained analyte, which will be captured and reconcentrated at the head of the RPLC column. After all solutes have left the immunoaffinity column, this column is switched back offline and regenerated by passing through the initial application buffer. Meanwhile, the RPLC column is developed with either an isocratic or gradient elution scheme that uses a mobile phase with increased organic modifier content. As the solutes elute through the RPLC column, they are monitored and quantitated through the use of an online detector.

A third way affinity chromatography has been used in analytical applications is through a chromatographic, or flow-injection, immunoassay. This method is used in determining trace analytes that do not directly produce a readily detectable signal. The competitive binding assay is the most common format used in such an assay. This is generally accomplished by mixing the sample

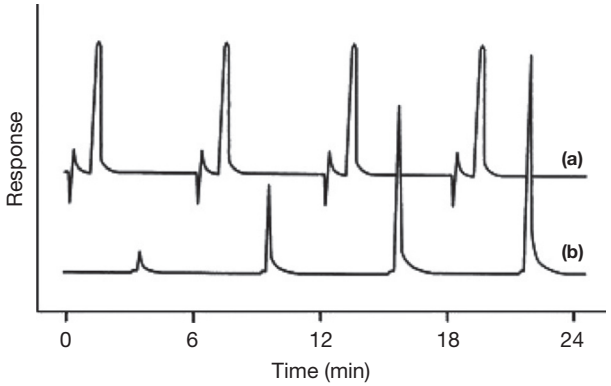

Figure 4 Detection of a parathyrin in human plasma by a chromatographic-based sandwich immunoassay. The results in (a) show the injection spikes and nonretained fractions for four sequential injections of human plasma samples with increasing parathyrin levels. The results in (b) show the response due to the retained parathyrin. This plot was obtained from Hage, D.S.; Kao, P.C., *Anal. Chem.* **1991**, *63*, 586–595.

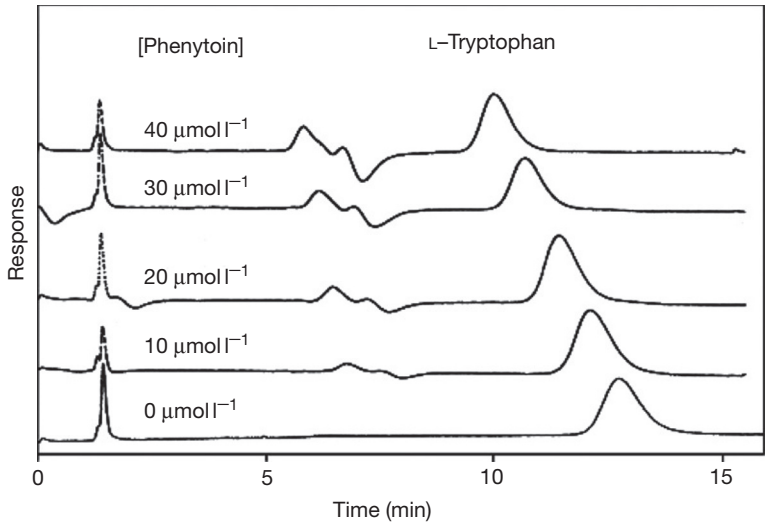

Figure 5 An example of a zonal elution experiment, in which small injections of L-tryptophan are made onto an immobilized human serum albumin column in the presence of increasing amounts of phenytoin in the mobile phase.

with a fixed amount of a labeled analyte analog (i.e., the 'label') and simultaneously or sequentially injecting these onto an immunoaffinity column that contains a limiting amount of antibody. Immunometric assays have also been performed on chromatographic systems. For instance, in a sandwich immunoassay (i.e., a two-site immunometric assay), two different types of antibodies are used (see Figure 4). The first of these two antibodies is attached to a solid-phase support and used to extract the analyte from samples. The second antibody contains an easily measured tag and serves to place a label on the analyte, thus allowing it to be quantitated.

Biophysical Applications

Besides its use in separating and quantitating sample components, affinity chromatography can also be employed as a tool for studying solute–ligand interactions. This approach is called analytical, or quantitative, affinity chromatography. Using this technique, information can be obtained regarding the equilibrium and rate constants for biological interactions, as well as the number and types of sites involved in these interactions.

Information on the equilibrium constants for a solute–ligand system can be acquired by using the methods of zonal elution or frontal analysis. Zonal elution involves the injection of a small amount of solute onto an affinity column in the presence of a mobile phase that contains a known concentration of competing agent. The equilibrium constants for the ligand with the solute (and competing agent) can then be obtained by examining how the solute's retention changes as the competing agent's concentration is varied (see Figure 5). This method has been used to examine a number of biological systems, such as enzyme-inhibitor binding,

protein–protein interactions, and drug–protein binding. Frontal analysis is used in a similar manner but is performed by continuously applying a known concentration of solute to the affinity column. The moles of analyte required to reach the mean point of the resulting breakthrough curve is then measured and used to determine the equilibrium constants and number of binding sites for solute–ligand binding.

Information on the kinetics of solute–ligand interactions can also be obtained using affinity chromatography. A number of methods have been developed for this, including techniques based on band-broadening measurements, peak fitting, the split-peak effect, and peak decay analysis. These methods are generally more difficult to perform than equilibrium constant measurements but represent a powerful means for examining the rates of biological interactions. Systems studied by these techniques have included the binding of lectins with sugars, protein A, or protein G with immunoglobulins, antibodies with antigens, and drugs with serum proteins. The recent creation of commercial sensors for biointeraction studies is one result of such work.

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3.05 Microarrays

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3.05.1 Introduction

For the pharmaceutical industry, undoubtedly the most enticing prospect stemming from the modern genomics revolution is the promise it offers of improved drug target identification. A drug target is a molecule in the body, usually a protein, that is intrinsically associated with a particular disease process and that could be addressed by a drug to produce a desired therapeutic effect. Typical examples for such targets are receptors on the surface of a cell that can be triggered to give a signal upon stimulation with a drug or enzymes whose activity of converting one substance to another is modulated via a drug. The identification, characterization, and validation of a drug target is a long and difficult exercise, demanding, as it does, a profound understanding of a disease's etiology and the biological processes associated with it, coupled with a fair amount of trial and error experimentation. In fact, up to now, drugs have been developed to address only a few hundred known drug targets. On the other hand, the anticipation is that today's research in genomics and proteomics will yield several thousand new drug targets.

Furthermore, it is likely that these drug targets will lead to drugs that are therapeutically more precise and more effective than those available today. Many of today's drugs were identified by happenstance and show a multitude of side effects. While some lack of specificity may be desirable in certain circumstances, it is still preferable to have substances that are as specific toward their respective targets as possible to avoid undesirable adverse events. Using our ever-increasing understanding of how proteins react and interact with each other, it is expected that, for a given disease, the best possible molecular intervention point should be precisely identifiable. In order to get there, there is a need for experimental procedures that will capture the behavior of all involved interaction partners simultaneously. Such procedures are currently emerging in the genomics area with technologies such as deoxyribonucleic acid (DNA) microarrays for gene activity (= mRNA) profiling, and in related areas such as proteomics, metabolomics, and lipidomics.

The advent of DNA microarrays was heralded by the seminal paper of Schena *et al.*,[1] who introduced the concept of immobilizing microscopic amounts of DNA fragments on glass substrates, and the subsequent success story of Golub *et al.*,[2] who demonstrated that such microarrays could be used both to distinguish among clinically similar types of

cancer, acute myeloid leukemia, and acute lymphoblastic leukemia, as well as to generate a list of genes associated with the classification.

The power of DNA microarray technology is that it provides a high throughput instrument for simultaneously screening thousands of genes for differential expression across a series of different conditions. Despite the fact that it may not have sufficient sensitivity to detect subtle expression changes such as those of regulatory genes, a well-designed and properly executed microarray experiment should be able to detect the downstream effects of such changes, which generally tend to be more dramatic. Since these effects are the ones most likely to be associated with druggable targets, DNA microarrays are an important tool for identifying drug targets.

The remainder of this chapter is organized as follows. Section 3.05.2 outlines DNA microarray technology and experiments. Section 3.05.3 describes the various data analysis considerations that are necessary to make sense out of the enormous datasets generated by these experiments. Section 3.05.4 presents a series of case studies from the neuroscience area, although the concepts illustrated therein apply much more globally. Finally, Section 3.05.5 offers some concluding remarks as well as a quick look to the future.

3.05.2 Deoxyribonucleic Acid Microarray Experiments

DNA microarray technology enables scientists to simultaneously assess the relative transcription levels of several (if not all) of the genes within a cell across a series of different conditions: for example, across different pathological conditions, such as healthy versus diseased, different disease states, across time during the progression of a disease or infection, or during therapy and so on. Under the premise that some genes would be differentially expressed as a consequence of a disease process, monitoring expression patterns in this way provides scientists with potentially valuable clues and insights for scrutinizing and understanding the biological and cellular processes underlying that disease.

DNA microarrays are small solid supports (such as glass microscope slides, silicon chips, beads, or nylon membranes) onto which nucleic acid sequences (probes) have been robotically attached in a rectangular array format. Typically, the number of probes arrayed is very large and would correspond to a significant representation of the genome under study, which is in keeping with the concept that this technology is essentially a high throughput screen of the transcriptome. The probes are usually made of small single-stranded DNA sequences (either oligonucleotides or denatured polymerase chain reaction (PCR) products), which are expected to be exactly matching (complementary) to the single-stranded fluorescently labeled target that is finally applied to the microarray. The selection of such short probes is driven by the annotation of the genome of a given organism. The information at the start and the end of a gene plus the untranslated regions of the corresponding mRNA is of high importance for the actual selection of the probe sequence. Owing to the working mechanism of one key enzyme (the reverse transcriptase), the probe sequences tend to be preferentially selected from the 3′-end or the untranslated region of the mRNA.

The labeled target is prepared from mRNA extracted from a biological sample. The mRNA is reverse transcribed using oligo dT as primer, linearly amplified, fluorescently labeled by incorporating Cy5- or Cy3-deoxynucleotides or biotinylated deoxynucleotides to which fluorochromes can attach, solubilized in hybridization buffer, and then dispersed over the surface of the microarray. The microarray slide is then inserted into a sealed hybridization chamber to allow hybridizations between corresponding sequences on the microarray (i.e., the probes) and in the sample (i.e., the labeled target) to occur. Following incubation, the slide is washed using stringent and nonstringent buffers to remove excess sample from the array and to reduce cross-hybridization. The occurrences of any true hybridization for any of the probes are indicated by the remaining fluorescent intensities as measured by a confocal laser microscope or similar instrument. **Figure 1** shows schematically the key steps involved in a typical microarray experiment.

A standard microarray experiment would comprise several samples run through the above process. Comparing the resulting fluorescent intensities across the different samples would indicate which genes are differentially expressed across them.

3.05.2.1 Microarray Technologies

Several different microarray technologies are commercially available from a number of suppliers, such as Affymetrix and GE Healthcare, or they may be produced by an in-house laboratory. A few of the more common microarray technologies are described below.

In a cDNA microarray, the probes are long DNA fragments (a few hundred bases to several kilobases in length) with sequences complementary to the sequences of the genes of interest or representative subsequences thereof.[1] Regions of low complexity are usually excluded as they could diminish hybridization specificity. cDNA probes can be obtained

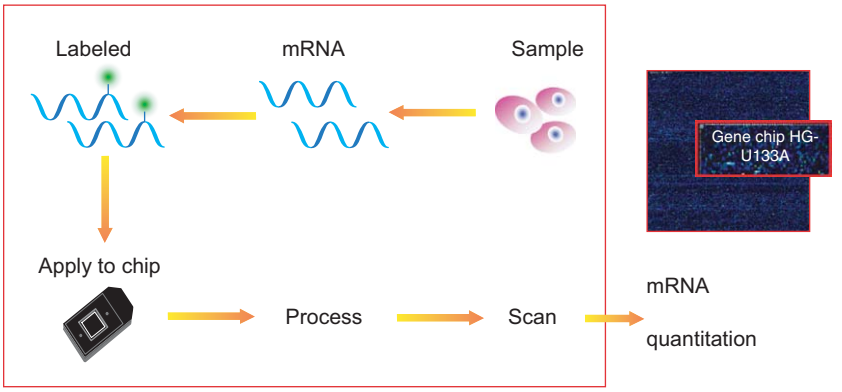


Figure 1 A schematic display of the key steps of a microarray experiment.

from commercial cDNA libraries or, alternatively, PCR can be used to amplify specific genes from genomic DNA to generate cDNA probes. Once obtained, the double-stranded DNA probes are denatured to generate single-stranded probes, which are then robotically spotted onto a glass slide.

Experiments involving cDNA microarrays may be single- or multichannel. Two-channel experiments are the most common. In a two-channel experiment, two samples (e.g., one from control tissue and one from diseased tissue) are prepared for hybridization to the array with different samples labeled with different fluorescent dyes. They are then combined and hybridized to the microarray together. The two samples will hybridize competitively to the probes on the array with the sample containing more transcript for a particular probe prevailing. The two sets of fluorescent intensities are recorded in two scans.

Another type of DNA microarray technology is the oligonucleotide microarray. Affymetrix manufactures a particular type of oligonucleotide microarray[3] in which a gene is represented by a set of 11–20 25-mer oligonucleotide probes called perfect match (PM) probes. The PM probes are carefully selected to have little cross-reactivity with other genes so that nonspecific hybridization is minimized. Nevertheless, some nonspecific hybridization will tend to occur. A second probe called a mismatch (MM) probe, which is identical to the PM probe except for a mismatched base at its center, is placed adjoining to the PM to combat this effect. The idea behind the mismatch probe is to subtract any background hybridization as measured by the MM probe signal from the PM probe signal. Affymetrix synthesizes the probes in situ onto silicon chips using a proprietary photolithographic process.[4] A technological variant of this in situ synthesis process is carried out by Affymetrix' partner NimbleGen. They use a maskless approach based on almost a million tiny mirrors for the photolithographic synthesis of the oligonucleotides. Advantages of this approach are flexibility in the array design (custom chips) and much shorter time frames between the design of a chip and its actual manufacturing as there is no need to generate masks.[5]

Other platforms also use oligonucleotides instead of cDNAs. There is a tendency in these platforms toward using medium-length oligonucleotides as they are thought to provide more of a balance between sensitivity and specificity. Longer oligonucleotides have higher sensitivity as they emit stronger signals and therefore have the ability to detect less abundant transcripts. On the other hand, shorter oligonucleotides have higher specificity as the risk of cross hybridization also increases with length. This makes it challenging to select a set of oligonucleotides to be arrayed such that cross hybridization among known similar sequences is minimized as much as possible and yet sufficiently strong and clear signals are obtained. The most suitable compromise is still an issue for debate. In these platforms, the oligonucleotides are presynthesized as usual rather than synthesizing them in situ. In addition, a substrate other than glass or silicon may be used for immobilizing them.

For instance, in GE Healthcare's Codelink microarrays, presynthesized 30-mer oligonucleotides are inkjet spotted onto a three-dimensional polyacrylamide gel matrix. The polyacrylamide gel can bind considerably more oligonucleotide molecules than a conventional glass surface and the three-dimensional nature of the slide surface enhances the sensitivity of the assay. Applied Biosystems' expression array system uses chemiluminescence hybridization signaling chemistry of 60-mer oligonucleotide probes that are immobilized on a three-dimensional porous nylon substrate. Agilent manufactures two-channel 60-mer oligonucleotide arrays using a proprietary dispensing process similar to the inkjet technology known from current computer printers.

Owing to the diversity of microarray technologies, processes, experimental designs, and even data formats, the Microarray Gene Expression Data (MGED) Society developed the MIAME (minimum information about a microarray

experiment) guidelines as a standardized platform for storing and annotating microarray data with a view toward encouraging the generation, storage, and exhange of microarray data in a controlled manner.[3]

Each microarray platform has its own advantages and disadvantages.[6] The greater length of the probes arrayed on cDNA microarrays ensures that they hybridize to sample sequences with higher stringency and are less likely to be affected by minor mutations, but they exhibit lower hybridization sensitivity as longer sequences have higher likelihood for nonspecific binding. They are less expensive to produce than commercial off-the-shelf arrays when produced in quantity and thereby offer some flexibility along with the ability to be customized if manufactured in-house. Then again, they tend to demand much more time and labor-intensive effort in manufacturing, clone maintenance, PCR work, and quality control. On the other hand, commercial oligonucleotide microarrays tend to yield more reproducible results that often also show higher specificity.[7] The disadvantage of using such platforms is certainly the price, and often there are organizational problems caused by having to rely on central facilities. Owing to the necessity of utilizing comparably expensive specialized equipment, many research institutions have had to set up core teams dedicated to performing the microarray work. Regrettably, this removes part of the control a researcher has over his whole experiment.

Other applications of DNA microarrays in drug target research have recently gained more attention but will not be addressed in this chapter further as they require their own experimental design and analysis techniques. Some of these new applications are comparative genomic hybridization (CGH)[8] for the detection of deletions and/or amplifications of longer DNA stretches in the genome, resequencing of specific DNA sequences in a high throughput fashion, and single nucleotide polymorphism (SNP) analysis[9] for the study of inter-individual differences in single nucleotides within the genome, to name a few.

3.05.2.2 Experimental Design Considerations

It is imperative to design a microarray experiment so that its objectives can be met. The objective of many microarray experiments is to identify which genes are differentially expressed among several sets of samples. In such comparative experiments, it is important to be able to generate statistics about what could be considered a differentially expressed gene. This means that replicate samples must be run in order to generate enough data for a statistical analysis to have adequate power to detect gene expression changes. As microarray experiments tend to be expensive, researchers are sometimes reluctant to do too many replicates. However, both natural biological variability and the series of highly technical steps that constitutes a microarray experiment cause the data to be subject to various sources of variation. Minor differences in extraction, amplification, labeling, hybridization, and even the skill and experience of the experimenter could result in substantial differences among what would otherwise be true replicates.

Only with sufficient replication can this variability be overcome and statistical significance assessed adequately. There are two types of replication. One type is ‘biological replication,’ which involves extracting mRNA from several biological samples and running each sample on a different microarray in order to compensate for intrinsic biological variability in gene expression. The other type is ‘technical replication,’ which involves running the same sample on multiple arrays in order to compensate for the technical variability of the experimental process.

Sometimes an experiment might turn out to be too large or too complex to be completed in a single session. In that case, it is important to allocate, organize, and run the arrays in such a way that the effects of interest are not confounded with extraneous effects, such as operator effects, manufacturing batch effects, and hybridization day effects.

In general, it is crucial to pay careful attention to quality issues in order to ensure validity of the conclusions reached from the study. The mRNA source and the preparation of the samples should be controlled and their quality assessed. The most common techniques used nowadays are classical agarose gel electrophoresis to assess potential mRNA degradation, spectrophotometrical analysis using a very small quantity of total RNA in a Nanodrop, and miniaturized electrophoresis technologies such as the Agilent Bioanalyzer, which can produce data on mRNA degradation as well as mRNA quantity. The whole issue of mRNA quality is largely dependent on the source from which the mRNA is prepared. Cells cultivated in in vitro cultures tend to be much more homogeneous and generally yield much more consistent mRNA quality and quantity as compared to mRNA from tissues. Furthermore, it is much easier to extract mRNA from some tissues than others: While it is fairly easy to extract mRNA from liver, tissues such as heart or spleen require much more experience to obtain the high-quality mRNA necessary for microarray analysis.

In addition, several control probes are often arrayed on the microarrays for various quality control purposes. The most common are negative control probes, which are used to assess background and nonspecific hybridization, and positive control probes, which are used to measure the abundance of nucleic acid sequences spiked in to the labeled target as a way of assessing labeling and hybridization efficiencies.

3.05.3 Data Analysis Considerations

Owing to the volume and complexity of the data generated by DNA microarray experiments, the data analysis aspect of these studies acquires greater importance than other areas of biomedical research. In order to derive substantive biological information from these studies, careful experimental design and proper statistical and bioinformatics analysis are vital. Typically, a data analysis would consist of several stages. The first stage is always a data preprocessing step, which will be described next. The subsequent stages depend on the objective of the experiment.

3.05.3.1 Data Preprocessing

Preprocessing the raw intensity data enhances the sensitivity of the downstream comparisons. Some preprocessing steps, in the sequence in which they would normally be performed, are:

1. Array quality check: While a certain amount of variation in the intensity values across even identical arrays is inevitable and not a cause for concern, it is not uncommon, given the technical complexity of the experimental process, that a few arrays or a few samples in a study turn out to be defective. It is essential, however, to detect and eliminate them so that they do not unduly affect the data analysis. Arrays or groups of arrays that are substantially different from the rest can be identified by calculating Spearman correlation coefficients between all pairs of arrays. The resulting correlation matrix can be displayed on a heatmap by coloring each correlation on the basis of its value. This will highlight any unusual arrays, samples of poor quality, and bad hybridizations, which will generally tend to have low correlations with most, if not all, of the other arrays and will therefore stand out in the plot.
2. Data re-expression: Microarray data are generally very heavily skewed. Furthermore, the distribution of the intensity values could be quite different for different genes, most noticeably between low-expressing genes and high-expressing genes. The within-gene distribution can be symmetrized and the heterogeneity of variances across the genes greatly reduced by transforming the data. More often than not this can be achieved via a simple logarithmic transformation (logarithms of base 2 are often used). However, sometimes, some other transformation, such as a variance stabilizing transformation, a hybrid linear log transformation, or a started log transformation will do better.
3. Normalization: Spot intensity data will often need to be normalized to reduce monotonic nonlinear array effects. Care must be taken when doing so to ensure that gene-specific effects are at most only slightly dampened, otherwise detecting differential expressed genes will become impossible. Quantile normalization is a popular normalization procedure. A reference array, to which all the arrays are normalized, is defined, usually as an average microarray, such as by calculating gene-wise either the mean or the median across all the arrays. The distributions of the transformed spot intensities are coerced to be as similar as possible to that of the transformed spot intensities of the reference array. Quantile normalization is useful for normalizing a series of arrays where it is believed that a small but indeterminate number of genes may be differentially expressed across the arrays, yet it can be assumed that the distribution of spot intensities does not vary substantially from array to array. This assumption is often valid, and when it is not, the normalization would have to be customized to the particular data being analyzed.
4. Final quality checks: Inevitably, the data will contain a few unusual spot intensity values (i.e., outliers). By identifying and either removing or downweighting such aberrant values, the adverse impact that they would otherwise have on subsequent analyses can be reduced. This can be done by comparing replicates, which can be expected to be very similar to one another. In addition, a final array check can be done via a principal component analysis (PCA) or spectral map analysis as described in (*see* Section 3.05.4.2).

Details of these preprocessing steps and others are described in [10].

3.05.3.2 Visual Inspection of the Data

Gene expression data is typically organized into a gene expression matrix in which the columns represent the samples or experiments and the rows represent the expression vectors for the genes being interrogated by the microarray. The matrix elements are either the spot intensities preprocessed as above or fold changes based on the spot intensities. In trying to expose patterns in this data, it is helpful to somehow render this matrix visually.

A commonly used simple approach is to display the gene expression matrix as a heatmap by coloring each matrix element on the basis of its value. It is likely that the heatmap will initially appear to be devoid of any apparent pattern or order. However, by reordering the rows and/or columns on the basis of a clustering of the genes and/or samples, potentially interpretable patterns of gene expression will often emerge, as groups of co-regulated genes should occupy adjacent or nearby rows and similar samples should occupy adjacent or nearby columns in the display.

PCA is another method for visually presenting the data with a view toward revealing structures in the gene expression vectors. PCA is a technique for summarizing a multidimensional dataset in a few dimensions. Noting that the gene expression matrix has as many dimensions as it has genes, some serious dimension reduction is necessary without too much reduction in information in order for this to produce a meaningful representation. Methodologically, PCA finds a new coordinate system such that the first coordinate, a linear combination of the columns of the data matrix, has maximal variance, the second coordinate has maximal variance subject to being orthogonal to the first, and so on. Plotting the first few coordinates often reveals interesting patterns. For instance, samples sharing similar profiles will tend to lie close to each other. A rough visual estimation of the number of clusters represented in the data should also be possible. In addition, PCA may reveal oddities in the data, such as unusual arrays, and it therefore provides a useful supplementary array quality check as stated in (*see* Section 3.05.3.1).

A number of methods similar to PCA have been developed and project different views of the data. Factor analysis, correspondence factor analysis, multidimensional scaling, and projection pursuit are some of the better known ones.

The biplot and spectral map analysis are extensions of these ideas. The PCA described above is PCA applied to the samples (i.e., to the columns of the gene expression matrix). PCA can also be applied to the genes (i.e., to the rows of the gene expression matrix). When a form of PCA, called the singular value decomposition, is applied to both samples and genes, the simultaneous representation of both samples and genes in a single plot is called a biplot. Thus, a biplot will consist of both sample-related points and gene-related points. As in a PCA plot, the distances between the sample-related points are related to the dissimilarities between the samples; the closer a set of sample-related points, the more similar the samples to which they correspond. Most gene-related points will clump together in the center of the biplot; these would correspond to genes that are not differentially expressing across the samples. Any gene-related points that lie substantially away from this central clump in the direction of a cluster of sample-related points would correspond to genes whose expression profiles are associated with that cluster of samples. Thus, a biplot could be used to reveal not only clusters of samples but also the genes associated with them. As a variation, prior to constructing a biplot, the rows and columns of the gene expression matrix can be centered and weighted in specific ways to allow certain features of the biplot to be highlighted. This is spectral map analysis.[11]

3.05.3.3 Identifying and Studying Differentially Expressing Genes

Identifying genes whose changes in expression are most associated with differences between the samples is one of the fundamental questions addressed in the analysis of gene expression data. Thus, a comparative analysis of the gene expression levels from a diseased cell versus those of a normal cell will help in the identification of the genes and perhaps the biological processes and pathways affected by the disease process. Researchers can then use this information to synthesize drugs that influence and intercede in this process. In order to address this question, both the magnitude and the consistency of the expression changes must be assessed and a valid and reliable metric of differential gene expression derived from the preprocessed spot intensity data.

When comparing two sets of samples, the significance of any gene expression change can be assessed by calculating a t statistic for each gene. The t statistic is essentially a signal-to-noise ratio, in which the difference in mean log spot intensities (essentially the mean fold change on a log scale) is the signal and the standard error is the noise. Note that the fold change by itself, although used occasionally, is unreliable because it does not take into account the fact that not all fold changes can be treated equally which itself is because different genes have different levels of variability. Of course, the t test must be used carefully too. For instance, when the sample sizes are small, as is common in many microarray experiments, the value of the t test statistic tends to be overly dependent on the estimate of noise, to combat which modified versions of the t test (e.g., significance analysis of microarray data-t by [12] and Conditional t by [10]) have been proposed.

When comparing gene expression levels across multiple sets of samples, the t test statistic would be replaced by the F test statistic. In the event that the experimental situation is more complex, such as a temporal or dose–response study, appropriate F test statistics associated with linear ANOVA (analysis of variance) models could be used.

Genes that yield statistically significant t (or F) statistics should be examined for corroborating evidence of biological relevance. Thus, a potentially useful supplementary exercise is to superimpose inter-gene functional relationships onto the t test (or F test) results.[13] Accordingly, a list of genes with statistically significant expression changes can be examined for the repeated occurrence of various functional annotations, such as gene ontology classifications, biochemical pathways, or protein–protein interactions. Via a simple statistical analysis, it can be ascertained whether the set of significant genes contains an over-representation of genes in certain functional categories. There is at present one obstacle to doing this well and that is the scarcity and the questionable quality of the annotation data available for genes and their functional interactions. Nevertheless, as annotation databases improve

in coverage, quality, and complexity, their integration into microarray results will be more viable and become an increasingly valuable exercise.

Another potentially useful supplementary exercise is to cluster genes from multiple sets of samples into clusters with similar expression patterns. Hierarchical and nonhierarchical algorithms such as agglomerative nesting, *k*-means, and self-organizing maps have been implemented to cluster similar gene expression patterns. Presuming that genes performing similar functions or belonging to the same biochemical pathway will exhibit a tendency to co-regulate, inferences could be made regarding the unknown genes that share a cluster with genes whose functions or pathways are known. Further inferences can be made by interrogating these clusters as above for over-representation in certain functional categories.[13]

3.05.3.4 Sample Classification

When the samples are heterogeneous, they could be clustered using one of the methods listed above. A cluster analysis will reveal groups of similar samples. If the sample types are known, this exercise can be used as validation that the experiment was successful at separating the samples. In addition, clustering may indicate the existence of new subclasses of existing classes of a phenotype or a disease, thereby leading to a better understanding of the situation at the gene level.

When the samples are known to come from a number of different classes, another primary objective of many DNA microarray experiments is the classification of the samples into the separate classes. This has two objectives. The first is to identify the features (in this case, the genes or certain combinations of genes) that are most associated with the classification. The second is to predict the classification of new samples. Accordingly, classification addresses the question: given a set of samples, how can one assign each sample into the set of known classes with minimum errors? To do this, a set of feature variables is defined and a decision rule (i.e., a classifier) based on these feature variables is derived such that, given the measured values for any sample, the classifier would map it into one of the classes. A number of methods can be used for classification, including linear discriminant analysis and variants, *k* nearest neighbors, random forests, neural networks, and support vector machines. Readers should refer to the References for examples of various microarray classification methods.[10,14]

3.05.4 Case Studies

In an ideal situation the identification of novel drug targets in a given disease is performed using diseased and control tissue. However, biological samples derived from a precisely defined patient population and matched controls are scarce. An exception to this rule is the field of oncology research, where profiling experiments can be performed on tumor tissue and healthy control tissue derived from the same patient. In contrast, at the other end of the spectrum, are the diseases of the central nervous system for which it is much more complicated to obtain affected tissue. As an alternative, post-mortem samples are mostly used in diseases such as bipolar disorder, major depression, and schizophrenia. This complication might be resolved in the future if reports on the possible correlation between expression patterns observed in the brains and lymphoblastoid cells in patients with bipolar disorder, are confirmed.[15]

As an alternative to patient samples, scientists have turned to animal and cellular models of disease to study changes in gene expression related to the etiology and progression of disease states. Both animal and cellular systems can also be used for mode of action studies. In the following case studies, we will address experimental design, microarray experiments, and data analysis in real-life examples.

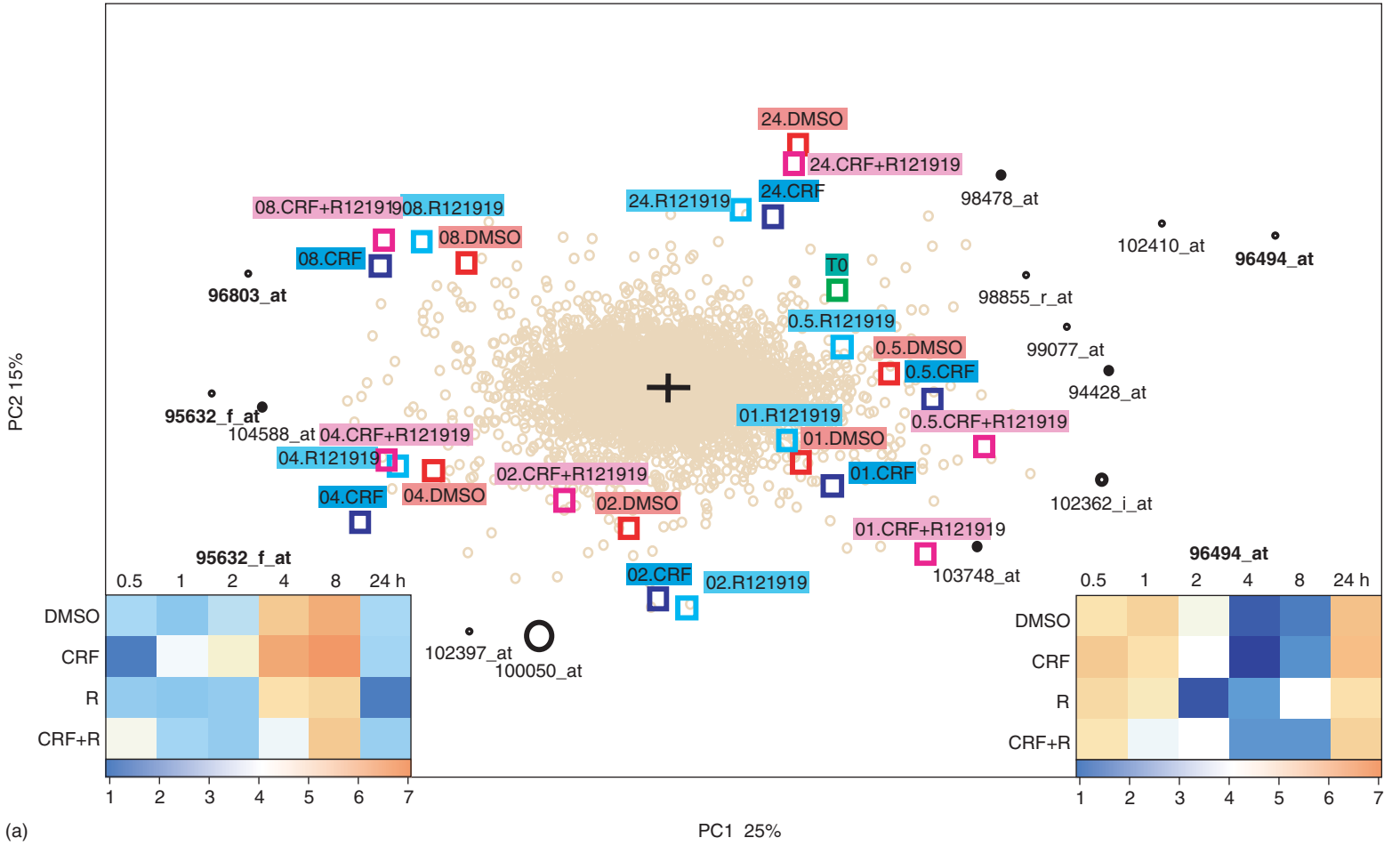
3.05.4.1 Case Study 1

The aim of the first case study was to elucidate pathways downstream of the corticotropin-releasing factor (CRF) receptor CRF_1 in order to identify novel modulators of this receptor-signaling pathway.[16] CRF is a 41-amino-acid polypeptide that plays a central role in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, mediating neuroendocrine, autonomic, and behavioral responses to various stressors.[17] Hypothalamic neurons release CRF in response to stress, stimulating the secretion of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn leads to increased release of glucocorticoids from the adrenal glands. Alterations in the CRF system activity have been linked to a number of psychiatric disorders, including anxiety and depression. Since the pituitary is the main target organ for CRF, the study was performed on a mouse pituitary-derived cell line called AtT-20 that expresses the CRF_1 receptor. Experimenting on freshly isolated pituitary cells probably would be closer to the in vivo situation compared to the artificial AtT-20 cell line. However, the pituitary is a heterogeneous organ, comprising many different cell types, of which

only a subset (the corticotrophs) respond to CRF. Therefore, changes in gene expression profile in this subset of cells might be diluted when studying the whole organ. In that sense, not withstanding the fact that AtT-20 cells are derived from a mouse pituitary cancer, they are considered a good cellular model of corticotrophs. The experimental setup of this case was as follows: AtT-20 cells were exposed either to CRF, CRF in combination with the CRF_1 antagonist R121919,[37] antagonist alone, or vehicle (DMSO). The transcriptional response to these four treatments was followed over time starting from 0 to 24 h after start of the treatment. Thus, in total 25 samples were processed: time 0 and 6 other time points for each of the four treatments. A downside of this design is the lack of replicates of a specific treatment at each time point. After processing the RNA samples on Affymetrix microarrays, data were preprocessed as described above. In this case data were transformed using a logarithmic transformation with base 2. After transformation, data were normalized using quantile normalization,[18] taking into account the fact that Affymetrix arrays have a limited array-to-array variation. After this preprocessing, data were visually inspected using spectral map analysis. As described above (*see* Section 3.05.3.2), this summarizing technique allows similarities between samples to be identified in an unbiased way based on overall gene expression patterns. In addition, relations between samples and genes can be identified on the biplot. In this specific case the spectral map allowed the identification of both an effect of progressing time and CRF exposure on gene expression. As shown in **Figure 2**, in the biplot of the first two principal components, samples are clustered clock-wise according to time exposed to a certain treatment, irrespective of the nature of this treatment. Interestingly, when plotting the first versus the third principal component differences between the treatments are visualized. This biplot was further applied to identify genes that contribute to the differences between the samples. Genes that do not contribute to this difference end up in the middle of the plot around the so-called centroid. Genes that co-cluster with samples at the periphery of the biplot are genes that show a striking upregulation in that sample. In this manner several genes were found to be strongly upregulated by CRF exposure. The alternative approach that was followed consisted of analyzing expression measurements in the different treatments relative to those observed in the corresponding time point in DMSO-treated control samples. Regulated genes were defined as those showing a greater than two fold change in transcript levels at any one time point. Eighty-eight genes showed a difference in expression after treatment with CRF compared to treatment with the antagonist. Based upon the time point where the response was maximal, genes were classified into 'early responders,' 'intermediate responders,' and 'late responders.' Among the responders were known players in the pathways downstream of the CRF_1 such as the transcription factors Nurr1, Nurr77, and Jun-B, indicating that the setup could confirm previous data obtained by other means. Of interest is the observation that 50 of the 88 genes that were identified using this arbitrary fold change criterion were also identified in the unsupervised spectral map analysis demonstrating the power of this graphical visualization tool.

Confirmation of array data is often done using quantitative PCR techniques such as Taqman technology. In this case study 21 of the most regulated genes were confirmed using quantitative PCR on an ABIPrism 7700 cycler with commercially available assays from Applied Biosystems. Data obtained in this way on all of the tested genes confirmed expression profiles previously observed on the array. Overall, the magnitudes of changes observed with microarray technology were lower than those obtained using quantitative PCR. Ultimately, confirmation needs to be done using a non-RNA-dependent method. This could be based on the protein or on protein function. An example of this is the use of RNA interference (RNAi) technology. By knocking down expression of specific genes identified in the array experiments using RNAi, one could immediately test hypotheses generated by array data analysis.

Figure 2 Spectral map analysis of the microarray data of AtT-20 cells. (a) The first two principal components (PC) of the weighted spectral map analysis applied to the normalized microarray data for all time points and all treatments. On the spectral map, squares depict different samples whereas circles depict different genes (the size of the circle corresponds to intensity on an Affymetrix array). The distances between squares are related to the dissimilarities between the samples; the closer a set of squares, the more similar the samples to which they correspond. Most circles will clump together in the center of the biplot around the centroid (depicted by a cross); these correspond to genes that are not differentially expressed across the samples. Any circle that lies substantially away from this central clump in the direction of a cluster of squares corresponds to a gene whose expression profile is associated with that cluster of samples. Those genes contributing significantly (measured by their distance from the centroid) to difference between samples are annotated with their Affymetrix identifier.[36] The first two PCs identified time as the major discriminator between the samples, distributing the samples clockwise over the plot starting at the T_0 time point and ending at the T_{24} time point. Inserted heat-maps (insets) show representative genes (indicated in bold in biplot) that are maximally induced either after 4 and 8 h or at 0.5, 1, and 24 h, corresponding to their position on the biplot. (b) Biplot of first and third PCs. The third PC identifies the specific CRF effect on AtT-20 cells over time. (c) Summary views on the first three dimensions of the spectral map, showing how this technique identified time and CRF effects in the microarray dataset. uvc,unit column-variance scaling; RW, row weight; CW, column weight. Closure = none; center = double; norm. = global; scale = uvc; RW, mean; CW, constant. (Reproduced from Peeters, P. J.; Gohlmann, H. W.; Van, D. W. I.; Swagemakers, S. M.; Bijnens, L.; Kass, S. U.; Steckler, T. *Mol. Pharmacol*. **2004**, *66*, 1083–1092.)


24.DMSO
24.CRF+R121919
24.CRF
24.R121919
98478_at
08.CRF+R121919
08.R121919
08.DMSO
08.CRF
96803_at
T0
102410_at
96494_at
98855_r_at
0.5.R121919
99077_at
0.5.DMSO
0.5.CRF
94428_at
95632_f_at
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04.CRF+R121919
01.R121919
0.5.CRF+R121919
01.DMSO
04.R121919
02.CRF+R121919
01.CRF
102362_i_at
04.CRF
04.DMSO
02.DMSO
01.CRF+R121919
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02.CRF
02.R121919
102397_at
100050_at
95632_f_at
0.5 1 2 4 8 24 h
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CRF
R
CRF+R
1 2 3 4 5 6 7
96494_at
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DMSO
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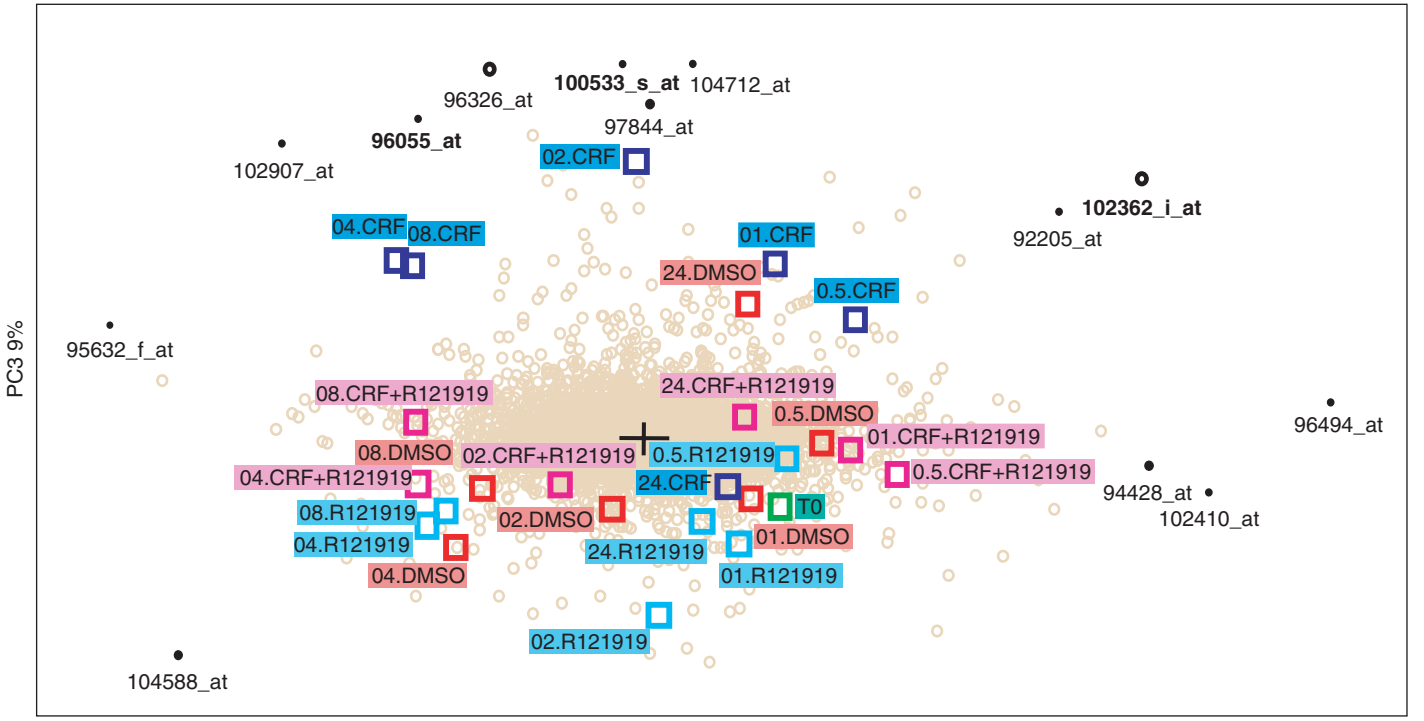
CRF+R
1 2 3 4 5 6 7
PC2 15%
PC1 25%
(a)



(b)

Figure 2 Continued

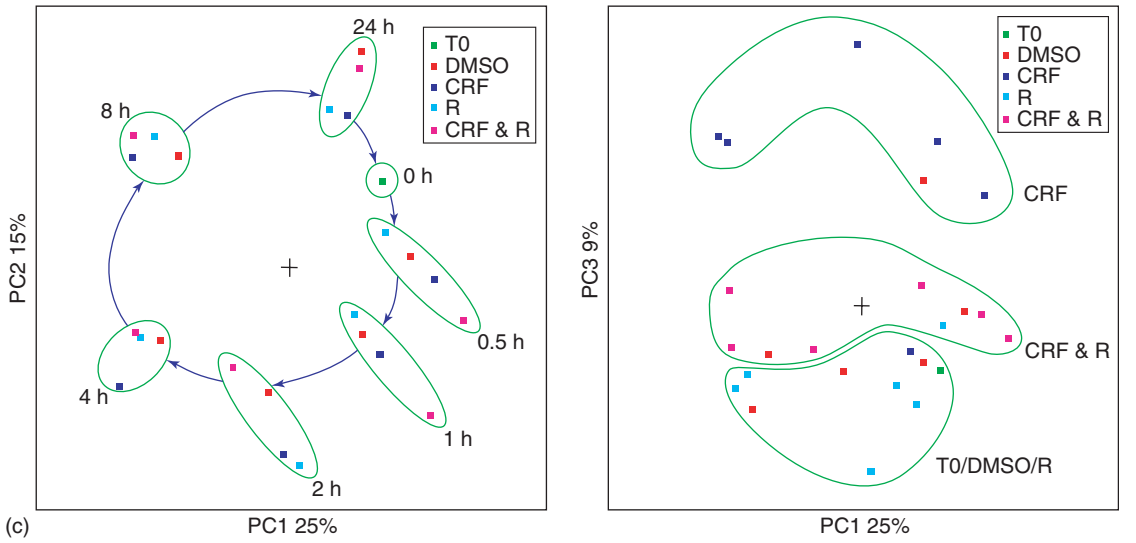


Figure 2 Continued

3.05.4.2 Case Study 2

In a second case study profiling experiments were done on transgenic animals rather than cell cultures. The aim of this study was to elucidate molecular signatures present in different brain regions of transgenic mice overexpressing CRF (CRF-OE).[19] These signatures are hypothesized to underlie homeostatic mechanisms induced by lifelong elevated CRF levels in the brain. In line with a role of brain CRF in the mediation of endocrine, autonomic, and behavioral responses to stress, transgenic mice overexpressing CRF (CRF-OE) have been reported to show increased anxiety-related behavior, cognitive impairments, and an increased HPA axis activity in response to stress.[20,21] These animals therefore are considered to constitute a good animal model for chronic stress and as an extension thereof, depression. At least part of the behavioral and cognitive changes observed in CRF-OE mice can be attenuated by central administration of a CRF antagonist.

The experimental design of this study aimed at elucidating differences between CRF-OE animals and their wild-type littermates in addition to the effects on gene expression profiles of administrating 10 mg kg^{-1} of R121919, a CRF_1 receptor antagonist. To this end six experimental groups were defined. Half of the groups consisted of CRF-OE animals, the other half of wild-type littermates. Animals were either untreated, vehicle treated, or compound treated twice a day for five consecutive days. In experiments using transgenic animals the number of animals is often the limiting factor, which is certainly the case with CRF-OE mice, since these mice are notoriously difficult to breed. As a result each experimental group only consisted of three to four age-matched male animals.

Rather than analyzing whole brain samples, dissection of brain was performed in order to get regional expression data. In total, expression patterns in six regions were studied, including cerebellum, hippocampus, frontal cortex, temporal area, nucleus accumbens, and pituitary. The importance of performing these dissections in a standardized way is exemplified in **Figure 3**. As shown in the spectral map biplot each mouse brain region is characterized by a specific gene expression pattern. Deviations in removing certain brain areas resulting in more or less contribution of surrounding tissue will result in an overall increase of the noise. Therefore consistency is required in removing the tissue (removal of tissue was performed by one operator).

RNA isolated from different animals was processed separately per individual animal and per brain region on Affymetrix murine U74 arrays. As in the previous case study, genes that were called absent (nonreliable detection) in all samples according to Affymetrix's MAS 5.0 software were removed from further analysis. Raw fluorescence intensities from each array were $\log_2$ transformed and data were quantile normalized. Following the group-wise quantile normalization per treatment and genotype, a second quantile normalization was carried out across the data of all samples of a given brain area.[18]

Visual inspection of overall changes in gene expression profile among different treatment groups was done using spectral map analysis per brain region. This analysis revealed overt differences in gene expression pattern predominantly at the level of the pituitary and less so at the level of the other brain areas. Prolonged elevated levels of CRF as present in CRF-OE are expected to induce major changes in the expression profiles in this organ since it is the

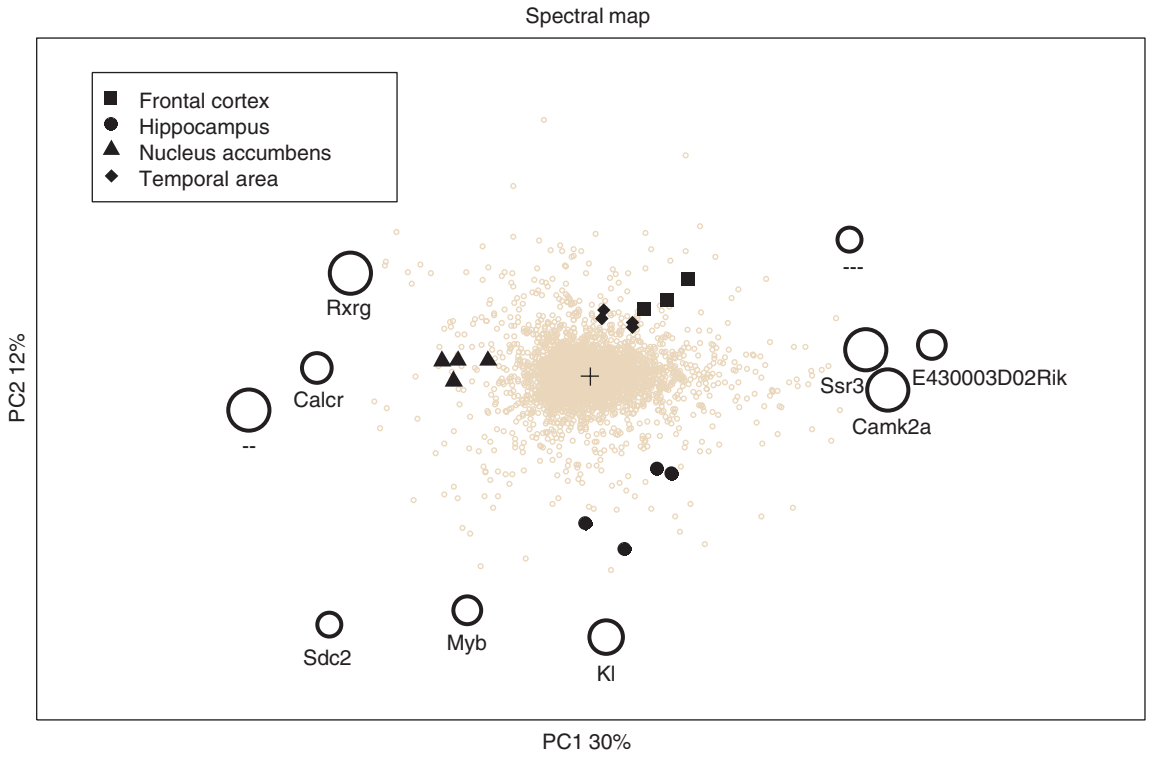


Figure 3 Spectral map analysis of brain regions from mice. The first two components of this spectral map show clustering of samples according to the brain region they are derived from. Genes contributing most are depicted by their gene symbol if applicable. The gene for the calcitonin receptor (Calcr) is, for example, more expressed in the nucleus accumbens than in other brain regions. In contrast, the gene encoding the calcium/calmodulin-dependent protein kinase II-alpha (Camk2a) is more abundant in the other brain regions than in the nucleus accumbens. uvc, unit column-variance scaling; RW, row weight; CW, column weight. Closure = none; Center = double; Norm. = global; Scale = uvc; RW = mean; CW = mean.

primary target of CRF. On the pituitary data spectral map the CRF-OE and wild-type samples (depicted by squares in Figure 4) cluster at opposite sites in the biplot. Genes at the periphery of the biplot (contributing significantly to the differences in expression profiles between samples) included kallikreins (Klk9, Klk13, Klk16, and Klk26), a family of serine proteases involved in the processing of biologically active peptides. Upregulation of these enzymes might be an indication for an overactive pituitary. Treatment with the CRF_1 antagonist R121919 or treatment with vehicle did not induce gross changes in expression profile and did not normalize expression patterns in CRF-OE relative to those observed in wild-type animals, as illustrated by the spectral map. An underpowered study as a result of the limited number of animals in each group might account for this negative result. Another more likely explanation is that whereas changes in behavior of CRF-OE mice are the immediate consequence of CRF_1 antagonism, the changes in gene expression are the consequence of prolonged HPA axis activation that are not easily overcome by treatment for 5 days with the CRF_1 antagonist R121919 as in the above experimental setup. A prolonged treatment and/or a larger group of animals might overcome these issues, once again stressing the importance of good experimental design.

Since spectral map analysis only revealed differences between transgenic animals and their wild-type littermates, post hoc analysis was performed on untreated CRF-OE and their corresponding wild-type littermates. Given the low number of animals per group the significance analysis of microarray data algorithm[12] was chosen as an adapted *t* test for multiple observations. Significance analysis of microarray data assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, significance analysis of microarray data uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, i.e., the false discovery rate (FDR).[22] Thus, the FDR is the expected proportion of false positives among the tests found to be significant. An extension of this FDR is the so-called *q*-value.[16] This *q*-value is similar to the well-known *p*-value. It gives each hypothesis test a measure of significance in terms of a certain error rate. The *p*-value of a test measures the minimum false-positive rate that is incurred when calling that test significant. Likewise, the *q*-value of a test measures the minimum false discovery rate that is incurred

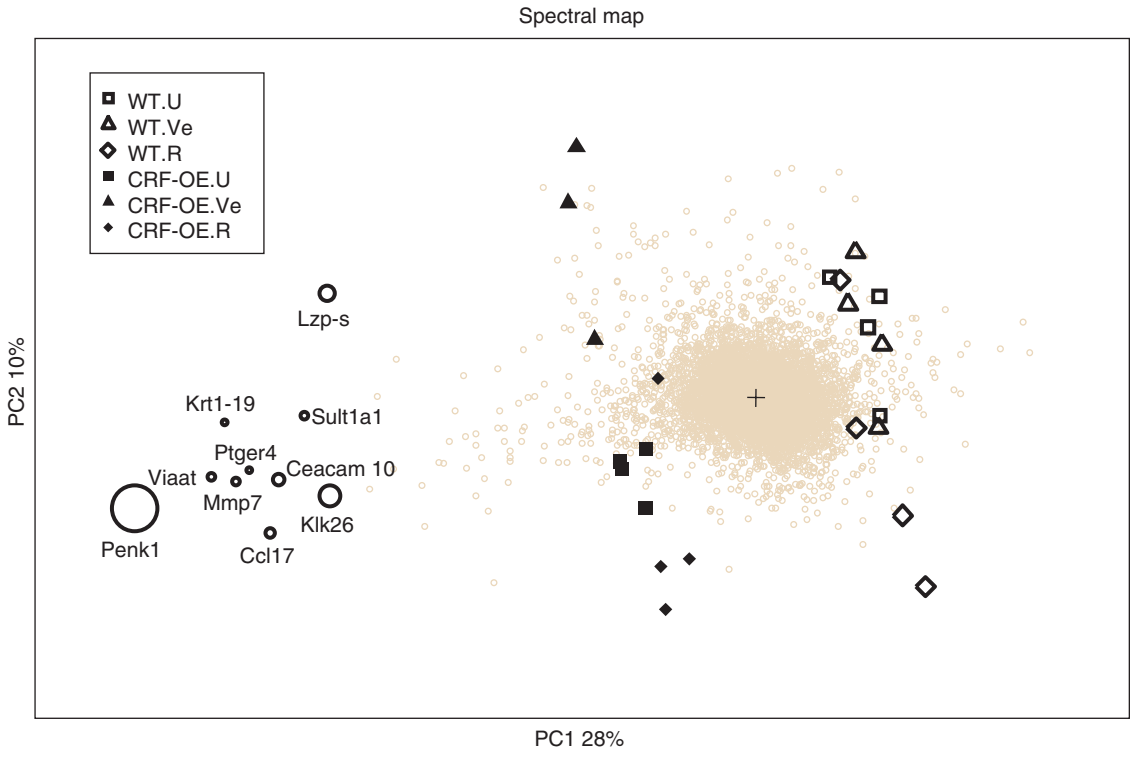


Figure 4 Spectral map analysis of pituitary-derived gene expression profiles of CRF overexpressing mice (CRF-OE) compared to wild-type (WT) mice. Spectral map analysis of microarray data obtained in the pituitary showing the projection of both genes and samples in two dimensions. Positioning of the samples derived from CRF-OE on the opposite side of the centroid compared to the WT samples along the x-axis (PC1) indicates that 28% of the variation in gene expression levels is explained by the genotype of the animals. Genes that contribute largely to the difference between WT and CRF-OE (indicated by their positioning at the extremities of the graph) are highlighted and depicted by their gene symbol. uvc, unit column-variance scaling; RW, row weight; CW, column weight. Closure = none; Center = double; Norm. = global; Scale = uvc; RW = mean; CW = mean.

when calling that test significant. Whereas the *p*-value is commonly used for performing a single significance test, the *q*-value is useful for assigning a measure of significance to each of many tests performed simultaneously, as in microarray experiments. A 10% threshold is accepted practice for array data analysis (as applied for pituitary data). However, deviation of this rule of thumb can be made when the number of significantly changed genes is limited. Therefore, a *q*-value below 20% was used in all other datasets.

In agreement with the spectral map, only a limited number of genes were significantly altered in most brain areas with only 10 genes being downregulated in the hippocampus. In the nucleus accumbens 50 genes were downregulated whereas 11 genes were upregulated. Recurring changes in expression patterns in several brain areas could be clustered into a few pathways such as the glucocorticoid signaling pathway (exemplified by downregulation of 11β-hydroxysteroid dehydrogenase type 1 and upregulation of the immunophilin Fkbp5). Adaptations in the glucocorticoid pathway fit nicely with the CRF changes in CRF signaling, since continued production of CRF in the CRF-OE animals will lead to an excess of glucocorticoids. This excess of glucocorticoids is counteracted by changing the levels of important players in that pathway such as 11β-hydroxysteroid dehydrogenase type 1 and Fkbp5. These changes in individual gene expression profiles in different brain areas could be confirmed by quantitative PCR.

In agreement with results of the spectral map analysis, at the level of the pituitary many more genes (114 genes) differed significantly in their expression between untreated wild-type and CRF-OE animals and were more than two times up- or downregulated. When comparing wild-type to CRF-OE animals, 102 genes had a *q*-value below 10% and were more than 1.5-fold downregulated in CRF-OE. Similarly, 180 genes had a *q*-value below 10% and were more than 1.5-fold upregulated in CRF-OE. In agreement with the spectral map, significance analysis of microarray data identified kallikrein genes *Klk9*, *Klk13*, *Klk16*, and *Klk26*, but in addition also identified *Klk5* and *Klk8* to be significantly upregulated in CRF-OE.

A recurring theme revealed by microarray analysis was the downregulation of neurotensin receptor 2 (*Ntsr2*) mRNA levels in several brain regions including the hippocampus (− 54%), the nucleus accumbens (− 38%), and the frontal cortex (− 59%), but not in the pituitary. Although levels of *Ntsr1* mRNA were below the detection limit in microarray analysis, the observation of *Ntsr2* mRNA downregulation prompted investigators to study the expression levels of all members of the neurotensin (NT) receptor family by quantitative PCR. *Ntsr3* mRNA did not show any change in expression in any area tested. *Ntsr1* mRNA was significantly downregulated in CRF-OE, with the most pronounced effect in the hippocampus. Quantitative PCR also demonstrated a 67% downregulation of *Ntsr2* mRNA in the hippocampus, confirming the microarray data. In order to evaluate the changes in Ntsr1 and Ntsr2 mRNA at the protein level, the presence of receptor (Ntsr1 to Ntsr3) was assessed by autoradiography of [^{125}I]NT binding on brain sections. In agreement with array and quantitative PCR results, autoradiography data demonstrated an overall (Ntsr1 to Ntsr3), genetically determined downregulation of the [^{125}I]NT binding capacity in CRF-OE versus wild-type animals. Blocking of the Ntsr2 receptors by a saturating concentration of levocabastine, unmasked prominent differences in expression of Ntsr1. A pronounced downregulation of the [^{125}I]NT binding in the CRF-OE animals was observed in specific brain regions such as the stratum radiatum ($-63.8 \pm 6.5\%$, $p < 0.001$), the retroslenial granular cortex ($-49.4 \pm 7.5\%$), and the temporal cortex ($-34.8 \pm 3.5\%$). Also, the autoradiography data demonstrated no effect of subchronic administration of R121919 as seen in the gene expression profiling data. These data clearly demonstrate the utility of non-RNA-based technologies such as radioligand binding studies to further validate and investigate microarray data. In addition to the confirmation of quantitative information provided by microarray and PCR this radioligand binding technique supplies further spatial information on where in the brain changes have occurred.

3.05.4.3 Case Study 3

This final case study deals with the complexity of an animal model for irritable bowel syndrome (IBS) in combination with the fact that changes in gene expression are believed only to occur in a small subset of cells. The aim of this study was to investigate long-term changes in gene expression in viscera-specific neurons of both nodose ganglia (NG) and dorsal root ganglia (DRG) in a postinfectious mouse model of IBS.[23] The pathogenesis of IBS is heterogeneous, but at least in a subpopulation of patients emotional stress and enteric infection have been implicated.[24] Therefore, the mouse model of IBS that was chosen for this study consisted of a transient inflammation induced by the nematode *Nippostrongylus brasiliensis* (Nb) combined with exposure to stress. Gene expression profiles were measured both in NG and DRG visceral sensory neurons because the gastrointestinal tract receives dual extrinsic sensory innervation. Vagal afferents have their cell bodies in the NG and project centrally to make synaptic connections in the brainstem, mainly at the level of the nucleus tractus solitarius while spinal afferents arise from the DRG and project into the dorsal horn of the spinal cord (**Figure 5**).[25] Currently, there is a common view that vagal and spinal afferents have different functional roles: spinal afferents play a major role in nociception, while vagal afferents mediate physiological responses and behavioral regulation, particularly in relation to food intake, satiety, anorexia, and emesis. However, there is some overlap, and vagal and spinal afferents share a number of features in common. Both NG and DRG neurons have been shown to become sensitized following inflammatory insult, demonstrating plasticity in the mechanisms that regulate neuronal excitability, which has implications for pain processing.

In this way, extrinsic afferent neurons supplying the gut are prime targets for new treatments of chronic visceral pain disorders such as IBS. However, fiber-tracing experiments measuring the extent to which abdominal viscera-projecting neurons[23] contribute to the total pool of sensory neurons in the DRG and NG, showed that only 3% are visceral sensory neurons. This gives an estimate of the extent to which changes in gene expression occurring in this subpopulation is likely to be diluted when whole ganglia expression is assessed. To circumvent these issues, viscera-specific sensory neurons were labeled using retrograde labeling with fluorescently tagged cholera toxin beta subunit. Labeled neurons in DRG (T10 to T13) and NG were isolated using laser-capture microdissection. This laser-captured material was linearly amplified using a three-round amplification and labeling protocol. To check for linearity of amplification, laser-captured material was spiked with four poly-adenylated prokaryotic genes at a fixed ratio. For these genes probes are present on Affymetrix's microarrays, allowing the efficiency and linearity of the amplification procedure to be monitored.

As mentioned above, the mouse model of IBS consisted of exposing animals to a combination of stress and infection with the nematode *N. brasiliensis*, leading to a transient jejunitis.[26] To assess inflammation at different time points after Nb infection IgE serum levels and jejunal mast cell counts as well as histological analysis were determined. At 3 weeks postinfection, the acute phase of inflammation was finished. Cortisol levels were significantly increased in stressed animals, regardless of infection, at 21 days postinfection. Based on these results, the assessment of mRNA expression levels in viscera-specific neurons was done at 21 days postinfection. At this time-point both NG and DRG visceral

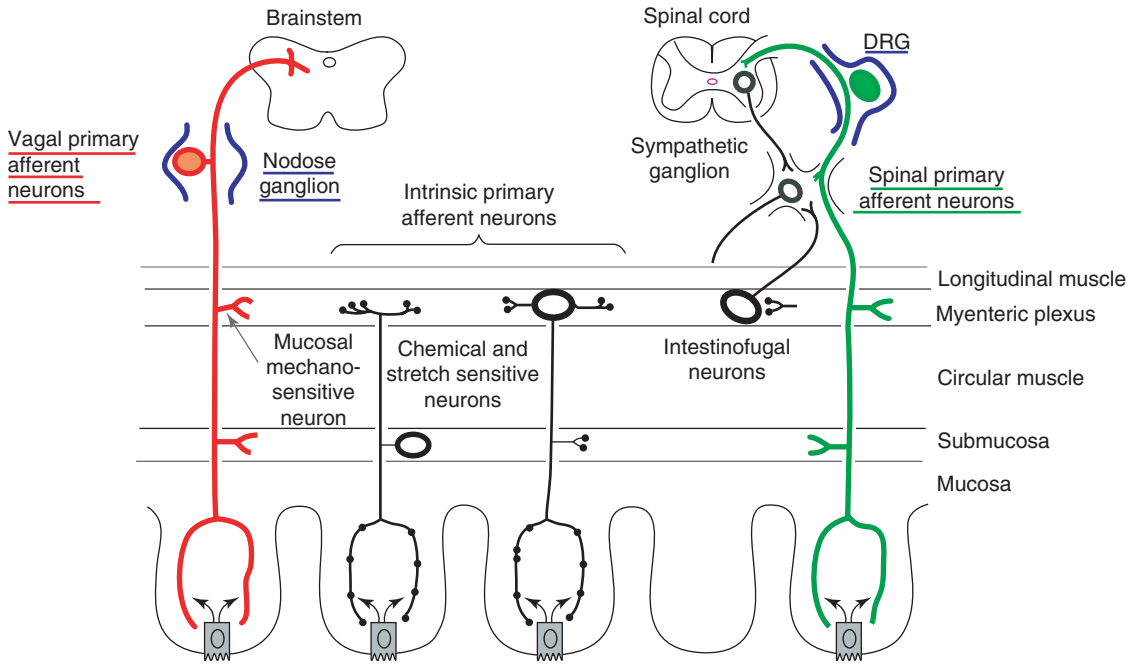


Figure 5 Vagal and spinal afferents in the gastrointestinal system. This simplified diagram of the enteric nervous system indicates that visceral sensory information is relayed from the gut wall to the brain via two main routes. Vagal afferents have their cell bodies in the nodose ganglia and spinal afferents have their cell bodies in the dorsal root ganglia. (Reproduced from Furness, J. B.; Kunze, W. A.; Clerc, N. *Am. J. Physiol.* **1999**, *277*, G922–G928, with permission from the American Physiological Society.)

neurons derived from infected animals showed hyperexcitability in patch clamp electrophysiology experiments. Rather than firing one action potential (as observed in neurons derived from noninfected animals), infected neurons fired a train of action potentials when a current was injected.

In order to evaluate both the effects of infection and stress, four experimental groups were defined each containing 10–12 animals. The first group was exposed to a sham infection and was housed in no stress conditions (SH-NS). The second group was exposed to Nb infection and housed in no stress conditions (INF-NS). A third group was housed in stress conditions but was not exposed to infection (SH-ST). The last group was housed in the same stress conditions but was also infected (INF-ST).

Spectral map analysis of the gene expression in viscera-specific spinal neurons (DRG) revealed no differences among the four experimental groups. Significance analysis of microarray data analysis found no significant differentially expressed genes between the extreme groups (SH-NS versus INF-ST). Upon spectral map analysis of viscera-specific vagal neurons (NG), the animals of groups SH-NS and INF-ST were separated by the first two principal components (Figure 6). Animals of groups SH-ST and INF-NS were plotted at the center in the middle, suggesting that their contribution to the variation in the dataset is relatively small compared to the two extreme groups.

A gene-specific two-way ANOVA model was used to analyze the microarray data in more detail:

$$\log_2(Y_{ijk}) = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

where α is the infection effect ($i = 1, 2$), β the stress effect ($j = 1, 2$), and $\alpha\beta$ the interaction.

An overall F test was performed to identify significant genes with any effect resulting in a list of 284 genes. A subsequent test was done to look for significant genes with interaction, infection, and stress effect at the same time. False discovery rate was controlled at 10% at each stage using the algorithm of Benjamini and Hochberg.[27] In this way genes could be classified as affected by stress (59 genes) or infection (13 genes), affected by both (139 genes) and in addition genes were identified that were affected by interaction between stress and infection (73 genes). Of interest in this study is the identification of genes such as the serotonin receptor subtype $5HT_{3A}$, for which antagonists are already on the market for treatment of IBS (Figure 7).[28] It is this type of corroborating clinical evidence that further strengthens the data obtained in an animal model. Furthermore, it allows the search for genes showing a similar

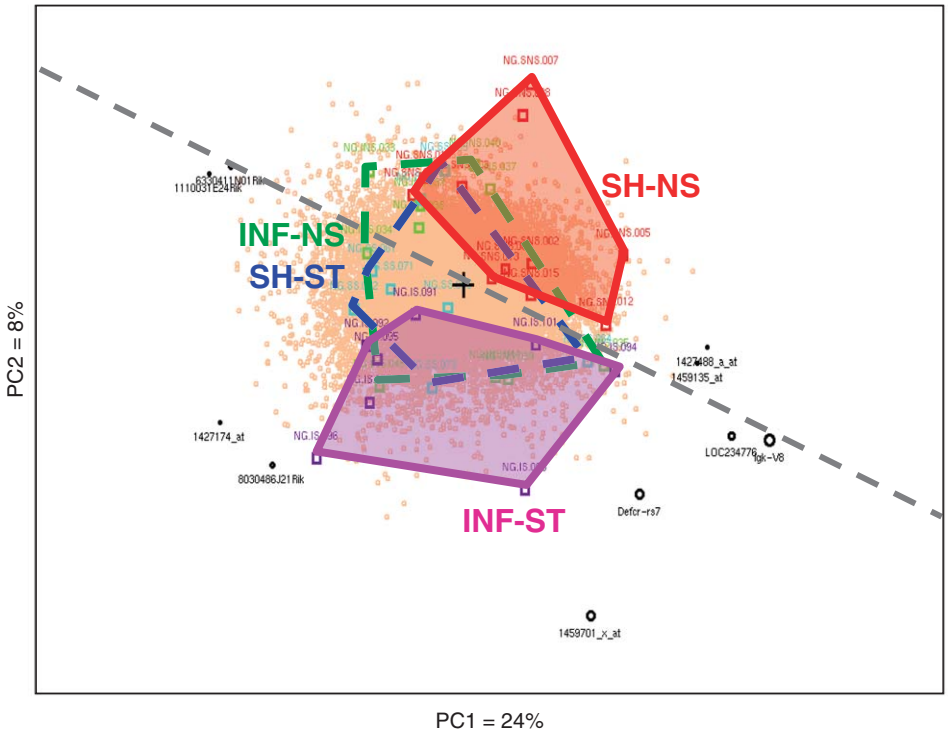


Figure 6 Spectral map analysis of specific gene expression profiles of visceral sensory neurons in nodose ganglia obtained in a mouse model for irritable bowel syndrome (IBS). The spectral map indicates that the first two principal components allow discrimination of the sham/nonstressed animals (SH-NS) from the infected/stressed animals (INF-ST). Samples derived from infected/nonstressed (INF-NS) and sham/stressed (SH-ST) animals end up more in the middle of the biplot in between the two other groups indicating that expression profiles differ the most in the INF-ST versus the SH-NS animals.

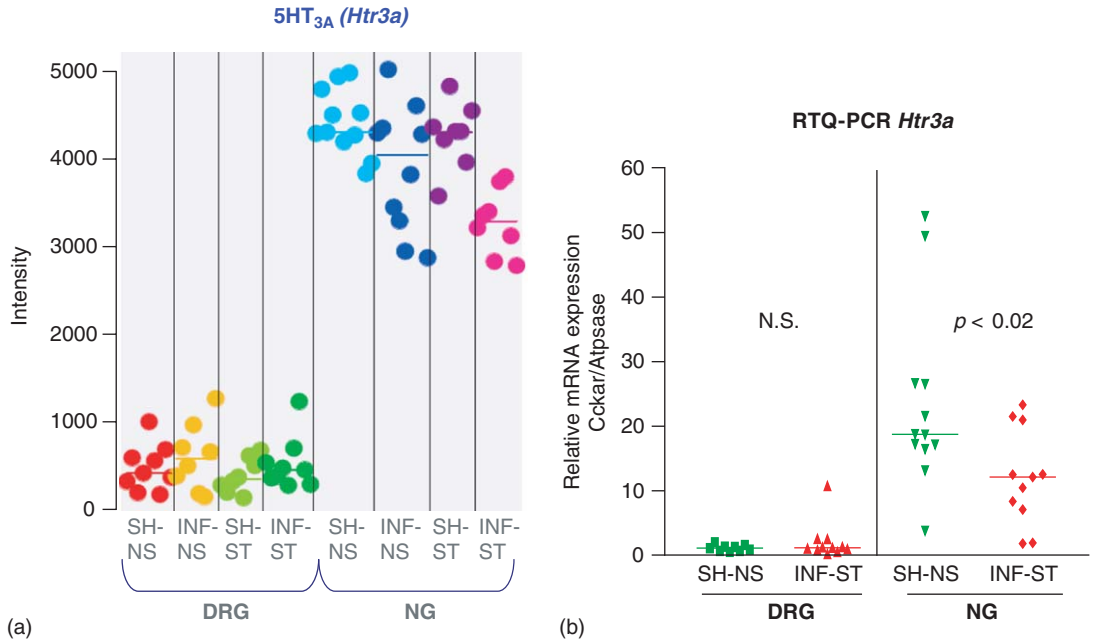


Figure 7 Expression of the $5HT_{3A}$ receptor in a mouse model for IBS. Expression levels of the $5HT_{3A}$ receptor mRNA (*Htr3a*) as assessed both by microarray (a) and quantitative PCR (b) in vagal sensory afferents in mice exposed to stress and *Nippostrongylus brasiliensis* infection compared to control animals.

expression pattern. It is clear that the identification of genes for which pharmacological tools are readily available allows for a straightforward hypothesis testing in animal models. In this way the loop can be closed in the sense that the animal model is first used for target identification followed by validation of that novel target in the same model. Further on, when compounds modulating the target have been identified, the model can again be used for lead generation and optimization.

3.05.4.4 Conclusions from the Case Studies

Although the above-mentioned examples relate mainly to neuroscience research it is clear that the setup of the different studies is generally applicable. Over the past few years a plethora of studies have been performed in several disease areas with the aim of finding novel drug targets.[29–32] In addition to hypothesis generation, microarrays are increasingly used for hypothesis testing. Moreover, with more data becoming available, comparisons between different animal models for the same disease will provide a more powerful approach to the understanding of disease etiology and progression.

3.05.5 Discussion and a Look to the Future

The prevalence of DNA microarray technology testifies to how invaluable a tool it has become in the armamentarium of the research molecular biologist, whether in an academic laboratory or in the pharmaceutical industry. With a well-designed experiment and rigorous standard operating procedures, microarrays will provide valid assessments of the relative transcription levels for thousands of genes simultaneously.

As microarray technology has matured over the past few years and microarray-specific data analysis techniques have been developed, it has become increasingly apparent that microarrays are very comprehensive in characterizing a complex biological sample. Even though the technology itself can surely be further optimized (e.g., with regards to sensitivity and reproducibility), the data generated today is already of high quality. This is reflected in the articles on microarray studies published most recently.[6,33] Use of technical replicates has decreased while use of biological replicates has increased. The main reason can be found in comparably higher variability due to biological variance between individuals rather than technological sources of variance.

A logical consequence emerging from this finding is the need to emphasize a proper design of experiment. Owing to the complex and thereby comprehensive characterization of the samples on the mRNA level, flaws in the experimental design and/or the execution of the biological experiment can often be detected. Combined with the still substantial costs surrounding this genomic technology it becomes apparent that successful microarray studies require not only the input of a statistician determining the right tools for the data analysis but also some level of guidance from a scientist experienced in conducting microarray and/or mRNA experiments. While the former will avoid wasting time after the data is generated (the design of the experiment will define the analysis technique used in the end), the latter will avoid wasting money on microarrays and wasting time on having to repeat experiments. Some of the common avoidable problems include:

- The wealth of data generated by microarrays tends to persuade people to look at answers to biological questions that were not incorporated in the experimental design. Especially when the primary question does not lead to conclusive answers, a feeling of ‘but can we not get something out of the data’ creeps in. This rarely results in conclusive answers to such secondary questions and tends to be quite time consuming as the data analyst has to apply analysis techniques for which the experiment was not set up. In turn, this results in nonconclusive answers and can spark even more attempts by the scientist interested in the biological interpretation of the results to look even further for information in the data for which the experiment was not designed.
- Trying to incorporate too many different groups into one single experiment usually makes the analysis more difficult and also tends to sacrifice sufficient number of biological replicates per treatment group. In the extreme scenario people will have done a big experiment but might not be able to draw any conclusions because of lack of statistical power. Then the experiment turns out to be a very expensive pilot study. This is even more of an issue if it turns out that due to a large experimental setup that was supposed to cover many different aspects of a certain biological question or phenomenon, the person conducting the biological experiment introduces more technical variance due to time constraints in conducting the experiment.
- There is a lack of experience in working with RNA. Some people are tempted to base their experimental setup on findings, e.g., from behavioral assays or protein work. They then ignore the difference in timing that takes place. While a certain effect may take 4 h to become apparent in a Western blot or a behavioral assay, the effect on the mRNA might have taken place after just 20 min.

What is the future outlook for microarrays in molecular biology research? As the data analysis and the biological interpretation of gene expression data is regarded as the most time-consuming part of a typical microarray, the prospect of future DNA microarrays that would increase even further the amount of data retrieved per sample stresses the importance to continue investing heavily in the development of suitable statistical algorithms and bioinformatics tools to avoid drowning in data. Suppliers such as Affymetrix have already started shipping tiling arrays that cover whole genomes with probes in equal distances rather than with probes for individual genes to discover transcriptional activity outside the current definition of where genes are located. Another approach that is being evaluated and that will also substantially increase the amount of data is the splice variants array, where probes are designed to cover all known gene splice variants.

Recent advances in RNA research have unveiled the presence of so-called microRNAs (miRNAs), small noncoding RNA molecules present in the genome of all metazoa, which function as regulators of gene expression.[34] The few hundred miRNAs that have been discovered so far have been suggested to play an important role in animal development and physiology. Moreover, profiling of expression of some 200 miRNAs in cancer patients proved to be more predictive for classification than mRNA profiling.[35] As insight into the function of these miRNAs continues to grow and, as is likely, their number increases, profiling of noncoding RNAs might become more important in the near future.

Finally, we note that, besides drug target identification, DNA microarrays and associated technologies can be used to identify biomarkers of various kinds to aid in drug development. Efficacy biomarkers would provide gene-level evidence of the efficacy of a drug. Subgroup biomarkers would identify subgroups of patients who would benefit most from a given therapy, giving rise to the optimism of 'personalized medicine.' Toxicity biomarkers would predict adverse events and would be used for compound screening. And so on. Continuing innovations in genomics and analytical technologies are, by the day, offering exciting new strategies for researchers as they search for safe and efficacious drugs.

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Biographies

Dhammika Amaratunga is Senior Research Fellow in Nonclinical Biostatistics at Johnson & Johnson Pharmaceutical Research & Development LLC, where he has been since 1989, working with researchers in drug discovery. In recent years, his primary focus has been on gene expression data analysis. He and his collaborators have written a book (in fact, the first fully authored book on statistical analysis of DNA microarray data) and numerous publications, taught courses and given over 50 (national and international) invited presentations. Over the years, he has been actively involved in a number of committees, including currently PhRMA's Statistics Expert Team on Pharmacogenomics. He has a BSc in Mathematics and Statistics from the University of Colombo, Sri Lanka, and a PhD in Statistics from Princeton University, USA, which he received working under the supervision of Prof John W Tukey.

Hinrnich Göhlmann was born in 1970 in Osnabrück, Germany. He studied Biology at the Technische Hochschule Darmstadt, Germany, and received his Diploma in 1995. His thesis was based on glycolysis research with the yeast *Saccharomyces cerevisiae* in the group of Prof F K Zimmermann. In 1995 he joined the group of Prof R Herrmann at the Zentrum für Molekulare Biologie of the University of Heidelberg, Germany. He received his doctoral degree on his work regarding a whole genome expression analysis of *Mycoplasma pneumoniae* in 1999. Following this in 1999, he joined the department of Functional Genomics of Johnson & Johnson Pharmaceutical Research and Development in Beerse,

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Pieter J Peeters was born in 1972 in Turnhout, Belgium. In 1995, he obtained the degree of bioengineer with a specialization in cellular and genetic biotechnology at the Catholic University of Leuven, Belgium. His first work in the genomics field was in the yeast *Saccharomyces cerevisiae* on the functional analysis of open reading frames, in collaboration with Prof P Philippsen from the *Biozentrum* in Basel, Switzerland. He obtained his PhD in the lab of Prof P Marynen at the Center for Human Genetics at the Catholic University of Leuven in 2000. During his PhD he studied the role of the ETS-variant gene 6 (ETV6) in different mechanisms for leukemogenesis. In 1999, he joined the Johnson & Johnson Pharmaceutical Research and Development organization in Beerse, Belgium as a Research Scientist. There he has been involved in target identification and validation in different disease areas, including affective spectrum disorders such as depression, Alzheimer's disease, and visceral pain, using small model organisms such as *C. elegans*, DNA microarray, and RNA interference technology. He is the author of several peer-reviewed articles.

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4.09 Systems Biology

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Symbols

$\mathbf{B}$	regression matrix for regressing $\mathbf{y}$ on $\mathbf{T}$	J	number of metabolites of a particular platform
c	component number	n	number of samples
$E_{x0–x9}$	residuals of the model for $\mathbf{X_9}$–$\mathbf{X_0}$	p	number of variables
$\mathbf{E}_x$	residuals of the model for $\underline{\mathbf{X}}$	T	number of time points
$\mathbf{e}_y$	residuals of the model for $\mathbf{y}$	$\mathbf{t}_c$	scores for component c
$\mathbf{G}$	core array	$\mathbf{V}$	matrix of weighing coefficients that can be written in terms of $\mathbf{w}$
I	number of subjects		

$\mathbf{w}_c^M$, $\mathbf{w}_c^K$, $\mathbf{w}_c^J$	vector of weights	$\underline{\mathbf{X}}$	Multiway matrix with peak areas or metabolite concentrations
$\mathbf{W}^M$, $\mathbf{W}^K$, $\mathbf{W}^J$	Matrices with weights	$\mathbf{X}_0$	metabolic responses on day 0
		$\mathbf{X}_9$	metabolic responses on day 9
$\mathbf{X}$	Matrix with peak areas or metabolite concentrations	$\mathbf{y}$	treatment group membership

4.09.1 Introduction

4.09.1.1 Introduction to Systems Biology

The development of high-throughput DNA-sequencing technologies during the late 1980s facilitated enormous boost in the emergence of novel methodologies aiming at unraveling the molecular complexity of biological systems. The availability of rapidly increasing numbers of complete genome sequences, including that of humans, created a demand for techniques and analysis methods that are as comprehensive as the sequences themselves. The advances in high-throughput genome-wide ('-omics') technologies, such as transcriptomics, proteomics, and metabolomics, enabled rapid and accurate quantification of most or all components (i.e., genes, proteins, or metabolites) of the experimental system in a single experiment. This opened new avenues in molecular biosciences, facilitating a shift of the paradigm from studying one molecule or process at the time to the more comprehensive, holistic concept characterizing the systems biology approaches.[1–3]

The scaling-up of fundamental experimentation in molecular biology to genomics proportions in the 1990s resulted in the generation of large volumes of data. Consequently, novel computational approaches were necessary to facilitate comprehension of the great amount of information produced, leading to a boost in the bioinformatics field. The bioinformatics and computational biology methods, initially focused on object classification approaches and statistical models, are continuously evolving to accommodate the need for understanding the function of biological systems as a whole. The major challenge in such efforts resides in integration of complex, abundant, and diverse data into a unique conceptual framework that is quantitative and predictive.[4] As a result of recent developments in this field, two distinct systems biology approaches have arisen: the 'top-down' systems biology, aiming at identifying molecular interaction networks based on observations derived from various '-omics' experimental data sets, and the 'bottom-up' systems biology, which uses computational modeling to predict system behavior based on the functional properties of a subsystem characterized to a high level of mechanistic detail by 'classical' molecular methods.[5]

To achieve the ambitious task of unraveling the complexity of biological systems in a holistic manner and to apply the discovered fundamental principles in areas of interest such as human health, systems biology requires bridging the gap between various nonbiological disciplines such as information technology, mathematics, physics, and chemometrics. The chemometrics methods include powerful multivariate statistical tools that can handle large numbers of highly correlated variables – as is the case in '-omics' data sets, especially when they are combined. Therefore, the potential of chemometrics in extracting the relevant systematic information from large-scale -omics data sets is high and it has been proven in a number of applications.[6] Nevertheless, this field is still in its infancy and significant effort is required before robust statistical modeling systems are established for more comprehensive characterization of biological systems and their interactions.

A typical workflow used for systems biology experiments is shown in **Figure 1**.

4.09.1.2 Systems Biology and Metabolomics in Nutrigenomics Research

The primary goal of nutrition research is to optimize health through dietary intervention so that the onset of disease can be prevented or delayed. As our diets represent complex mixtures of many bioactive

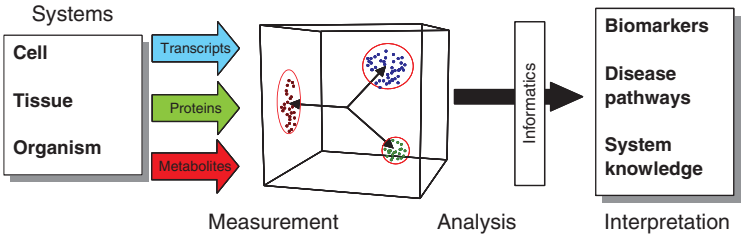

Figure 1 Systems biology: parallel analyses of proteins, metabolites, and mRNA from complex biological samples and integration of data using chemometrics to study complex interaction in biological systems.

compounds that are administered over a long period of time and in various concentrations, they are expected to have multifactorial molecular effects, which include subtle perturbations in gene and protein expression as well as enzyme and metabolite concentrations. Therefore, the recent development of various -omics and systems biology approaches greatly benefits the understanding of the mechanistics of the effects of diet by monitoring the entire complexity of multiple molecular changes with high sensitivity.[7–9] Following this trend, a number of nutrigenomics approaches have emerged.[10] By definition, nutrigenomics is the study of molecular relationships between nutrition and the complexity of molecular processes measured with '-omics' technologies, with the aim to extrapolate how such subtle changes can affect human health.[7,11–13] The focus of nutrigenomics is the discovery of genes, metabolites, and proteins that have a (regulatory) role in the mechanisms that lead to the development of nutrition-related disorders.

Within the field of nutrigenomics, a major emphasis of systems biology approach is the discovery of biomarkers to monitor health–nutrition relationships as well as the discovery of bioactive component patterns and possible synergetic effects.

Although the analytical platforms used to provide a profile of biological compound classes are similar, the challenges in terms of diversity, matrix type, and concentration are quite different for systems biology applications in nutritional sciences compared to, for example, pharmaceutical research.

Within nutrition research, the systems biology approach taken is often based on comparative analytics in which a control group is compared to one or more groups subjected to different food regimes or divided in different health states. The latter is an important aspect of biomarker discovery in food sciences as compared to pharmaceutical research. In nutritional studies, the aim is to use food, functional food, or nutraceuticals either to prevent the development of disease or to assist the biological system to regulate back into homeostasis from a very preliminary state of perturbation (unbalance or trigger for disease development). In practice, it means that subtle differences should be detected as compared to pharmaceutical applications where often comparison of control versus disease is done at a clinically very well-defined and developed disease status. From the analytical point of view, the effects are more pronounced and biomarkers of disease as well as biomarkers for drug intervention monitoring are more readily detected than in the nutrition area. Another important finding that has evolved over the last decade is the recognition that a single biomarker cannot describe a complex disease because most diseases are not monogenetic but have multiple pathways/networks involved. The concept of biomarker fingerprints is becoming widely accepted.[14] In nutrition, the concept of multifactorial approaches is the basis given the complexity of our food products, and from that perspective studies are of the most complex nature in life sciences: perturbing complex biological organisms with complex mixtures.

In order to achieve its aim, analytical platforms underlying nutrigenomics research should be able to provide a comprehensive profiling of all biological levels coupled with excellent quantification, as it is mandatory for the nonlinear analysis. This is still a challenging task. At the transcriptomic level, the coverage aspect is well achieved but quantification is relatively poor. At the protein level, the challenge to cover the proteome, for instance in plasma, is open for many improvements and the challenge is clear if one considers the 10 orders of magnitude in concentration range that need to be covered. In nutritional studies, measurement at the metabolite level is very attractive, as metabolomics – which involves the study of the repertoire of small molecules or metabolites present in a cell, tissue, organ, or biological fluid – is capable of measuring both exogenous (such as drugs, xenobiotics, and food) and

endogenous small molecules (metabolites involved in or resulting from primary and intermediary metabolism) in one run. Therefore, metabolomics is the ultimate technique to understand the effect of exogenous compounds (nutrition) on metabolic regulation in humans. It may identify those small molecules that make the difference between the effects of different diets and it can deepen our knowledge of human health and the interacting and regulatory roles of nutrition.[15] However, the application of metabolomics to nutritional research meets unique challenges. The chemical diversity of the metabolites is enormous in addition to a large dynamic concentration range. A wide variety of methods have been used to separate and quantify the components of the metabolome, and no single analytical platform can capture all metabolites in one sample. For that reason, only a technology platform consisting of several analytical approaches based on different techniques offers a solution today.

4.09.1.3 Chemometric Challenges in Metabolomics Data Analysis

Within metabolomics research, often rather expensive experiments are performed such as clinical studies with animals or humans to determine the effects that are due to illness or treatment thereof. Samples taken from the study subjects are analyzed analytically and after data preprocessing the analytical profile of these samples is available for further statistical processing by means of univariate or multivariate statistics. Mostly, multivariate statistics is the method of choice thanks to the strong correlation structure within the obtained data sets. Processing metabolomics data by means of multivariate statistics is challenging because of the nature of the data, and special caution needs to be taken before blindly applying the available techniques offered within software tools. A summary and explanation of specific problems within statistics for metabolomics are given here.

The data obtained within metabolomics studies are megavariate in nature, meaning that the number of variables (p) largely exceeds the number of samples (n), which is also known as the 'large p, small n' or '$n << p$' problem. Because of the megavariate nature of the data, it is futile to apply standard methods, for example, power analysis or setup of an experimental design. Power analysis is normally used to estimate the minimum number of experiments to be performed to optimize the possibility that an experiment will result in models that can discriminate between, for example, healthy and diseased or treated and untreated. Given a certain expected magnitude of difference between groups and known variations due to biological and analytical sources, the experimental design used for the experiment, and the number of metabolites measured within the samples, it should be possible to estimate the minimum number of subjects to be included within the experiment. However, whereas power analysis is a common method in traditional clinical studies where univariate statistics is commonly used, no standard methodology is available as of today for estimating the number of experiments required for studies where multivariate statistics is used. Power calculations are well developed for univariate analysis. The first approach to estimating sample size in 2D gel electrophoresis was recently introduced but not for multivariate effects.[16] For multivariate measurements (e.g., in epidemiology), limited results are available that require nonsingular covariance matrices.[17] However, in the megavariate metabolomics data, covariance matrices are usually not nonsingular. In the megavariate gene expression analysis, the usual approach is to use false discovery rate (FDR)[18,19] or other multiple testing approaches.[20] Such methods disregard the highly colinear character of metabolomics data. The use of the colinear structure within the metabolomics data should allow for a relatively small number of experiments than would be the case when univariate statistics is applied, but so far only very little work has been done on this subject.

A similar reasoning can be followed for the experimental design when the standard experimental design theory cannot handle megavariate data and is suitable for only a limited number of variables. For determining an optimal experimental design, the focus should be on the reduction of unwanted effects such as analytical variation and biological variation, for example, subject-to-subject differences. The effects measured are often small in comparison to the analytical and biological variation, which is especially the case where nutritional effects are to be picked up by metabolomics. The differences in experimental results are due to individual differences between study subjects (animals/humans) and due to the challenge given to the subjects by means of treatment by food or food additives. The effects are small in comparison to the

biological variation, which makes the chance of finding a metabolite that can serve as a marker for health even more difficult.

Analytical data obtained within metabolomics studies are often not quantitative and at the most semiquantitative, which means that measured intensities in different samples are more or less comparable to each other. When hundreds of metabolites are measured per sample, it becomes impossible to set up calibration lines for each metabolite even if the identities of all metabolites are known. Although quantification in the normal definition of the word is impossible, the goal should be to standardize the measured intensities such that multiple individual experiments can be combined later on and semiquantitative data can be transferred into quantitative data once a calibration line is determined for a certain metabolite. Quantification is of utmost importance to enable biological interpretation of assessed results by means of known reaction mechanisms. A related challenge is the identification of all metabolites as measured with the numerous analytical methods available for analytical scientists in the field of metabolomics. Unfortunately, the biological expert is more than often confronted with markers of disease with the not so informative name of 'unknown number' *x*. A workaround for quantification as well as identification is to report only those metabolites that have a known identity and for which quantitative data can be obtained. This hampers however the possibility of finding new hypotheses and a rather large amount of data is discarded from the measured analytical profiles.

From an analytical point of view, the data have become of a much higher quality than years ago thanks to the effort put in by instrumental vendors to obtain more reproducible data with higher resolution both in retention time and in spectral sense. The availability of mass spectral instruments with higher mass resolution however also poses a new challenge when untargeted approaches are used. Normally, alignment of the retention time axis is required; however, now alignment of mass spectral information is also needed. The development of chemometrics tools for processing analytical data in metabolomics studies is lagging behind the developments in analytical chemistry, and experimentalists are often forced to use targeted approaches thereby discarding large numbers of metabolites. Some challenges in data extraction from raw analytical profiles are, among others, drift correction and alignment; both are the subject of other chapters.

The challenges in obtaining predictive models from both analytical data and biological information are multiple as well. Earlier it was mentioned that the effects due to nutrition or medicines can be subtle but it becomes even more difficult when the effects are different between individuals because of their different physiological status at the time of treatment. It is known that many medicines work only for a certain percentage of patients and the individual differences largely hamper the possibility of obtaining a generic model from the assessed data. Another subject of concern is that different measurements are performed on a single sample to obtain a comprehensive chemical profile of the sample, which means that information is obtained for as many chemical components as possible. In order to model the data obtained with different analytical platforms, the data need to be concatenated or rather fused to a single data matrix, which can then be treated with standard chemometrics tools and tricks. However, the different analytical platforms have different characteristics and moreover there does not exist a single preprocessing tool that can be used to process data from all analytical platforms. The differing characteristics of the platforms result in different noise structures within the blocks of data obtained from each instrument. This is comparable to the problem of heteroscedasticity, which is normally solved by means of log transformation depending on the relation between noise and magnitude of the data. Besides the issue of differences between instrument characteristics, partial overlap of the analytical profiles is an issue that has to be dealt with. Some chemical compounds are simultaneously measured on different analytical platforms, and without correction this compound will end up in the data matrix multiple times leading to more concentration toward this compound in the model thanks to the high colinearity between these variables. If the identity of all variables is known, the problem would be easy to solve but the comprehensive approach of metabolomics is far from having reached that stage of maturity.

The main approach in modeling metabolomics data has been the classification between groups but now the focus is more on generation of hypotheses for mechanisms. This should provide a clear indication of which mechanisms are responsible for health and disease and also an understanding of biological mechanisms. In order to allow the generation of hypotheses, new tools are required that (1) allow the inclusion of prior knowledge, (2) make it possible to find cause and effect relationships, and (3) simplify the models by discarding uninformative parts of the data sets (variable selection). These challenges require collaboration between different

fields of expertise because all three fields involved in metabolomics (analytics, biology, and statistics) are equally important but none can solve the puzzle on its own.

A final challenge is the validation of results and this can be approached from different angles depending on the aim of validation. For model validation, methods such as Jack-knifing, bootstrapping, leave-one-out cross-validation, and double cross-validation have become common techniques, reducing the chances of reporting artifacts as biomarkers. From an analytical point of view, the validation is found by tracing back the interesting metabolites within the raw data to ensure that no mistakes were made in the complex process of data handling or that internal standards (IS) are mistakenly reported as biomarkers, and with having only a few metabolites as potential biomarkers the effort required to perform identification of unknowns has become less. The proof of the pudding however is the biological validation because once a modeled mechanism can be understood and is proven by an independent repeated experiment, the chances that the model is valid are high.

A great help in model validation and model interpretation is the use of visualization tools. Whereas experts in the field of chemometrics are used to interpret the models, the users of the tools are not used to that. A simple representation of the models with easy access to raw data and other sources of information will greatly help in the acceptance of chemometrics as a general tool for metabolomics research. Bioinformatics in combination with data warehousing tools can bring together data and prior knowledge and make it available to the metabolomics researcher.

4.09.1.4 Human Example Study

A typical metabolomics workflow integrated in a nutritional system biology study is depicted in **Figure 2**. Each section of this chapter will follow step-by-step this working process and start with an overview of the state of

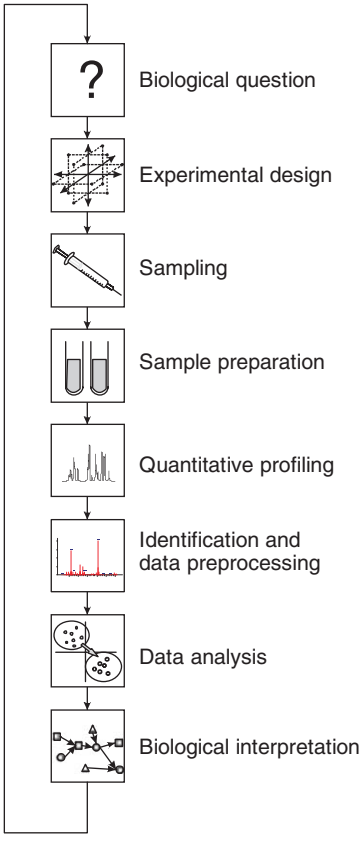

Figure 2 Schematic representation of a metabolomics workflow.

the art of the different steps involved in the nutritional metabolomics and systems biology workflow. This will be focused on the application of chemometrics in this research field. Each section will end with the demonstration of how the different topics were applied in a human intervention study as published by Wopereis and coworkers[21] and Rubingh and coworkers.[22] The main focus of the example study was to demonstrate and quantify the consequence of an antiinflammatory intervention by the antiinflammatory compound diclofenac on metabolism in mildly overweight subjects with low-grade state of inflammation. Different analytical methods were used for the detection of high- and low-abundant plasma metabolites to obtain a comprehensive picture of metabolic changes induced by this mild antiinflammatory drug intervention.

4.09.2 Study Setup

4.09.2.1 Study Design

Basically, two main types of design are being applied in clinical trials, namely a parallel design and a crossover design (see **Figure 3**). In a parallel design, at least two groups of people are followed up in time, where each group gets a treatment. The intervention effect is defined as the difference in the parameter of interest between start and end of the intervention (treatment A in **Figure 3**). This difference occurring in the treatment period is compared to the difference observed between start and end in the control treatment (treatment B in **Figure 3**).

In a crossover design, each person will undergo all treatments by switching treatment in each experimental period including a control treatment period. The intervention effect is defined as the difference in the parameter of interest at the end of the intervention (treatment A in **Figure 3**) as compared to the same parameter at the end of the control period (treatment B in **Figure 3**).

Each of the two designs has its own advantages and disadvantages. The parallel design has the advantage that many treatments can be included in the study and the subjects will be followed up during the same time period, so that specific changes over time, such as seasonal variations, will affect all experimental groups similarly. The disadvantage of this design is that the start condition of all experimental groups needs to be the same for those parameters that are being evaluated; in other words, randomization of the individuals to the various treatments needs to be well controlled. This may be difficult if not all randomization factors are known or if there are many randomization factors to be considered. Other disadvantage is that interindividual variation is an important source of variation, which means that the statistical power is lower and that more individuals are needed in this type of study.

The advantage of the crossover trial is that randomization will not be essential as it is in the parallel design, because all individuals will receive all treatments. Consequently, each individual will function as his or her own control. Other advantage of this design is that the statistical power is higher in crossover trials as only intraindividual variation is to be considered. Therefore, less people need to be included in crossover trials.

The disadvantage of crossover trials is their longer duration, which may limit their feasibility. Potentially order effects (a previous treatment affecting the following one) may play a role in crossover studies. However,

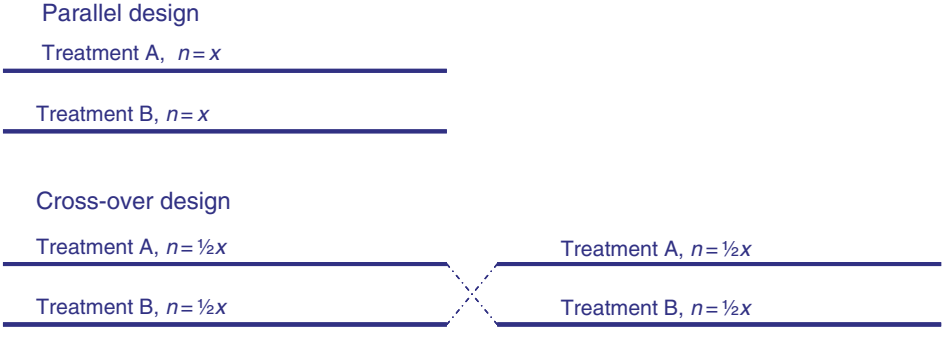

Figure 3 Two main types of study designs in clinical trials.

in case the treatment orders are randomized, for example, by using a Latin square, such problems are reduced to a minimum.

Variations on these two main intervention types exist, but are not discussed in this chapter.

Choosing the right control may be difficult in a trial, specifically when the components have clear immediate effects on behavior or when taste or other product characteristics are difficult to disguise. Furthermore, control products may differ from the experimental condition only in the ingredients of interest.

Confounding of study outcomes may occur. Some of the potentially confounding factors include blinding, bad compliance, unwanted side effects, and insufficient power.

Clinical studies are preferentially blinded, which means that not only the participants do not know which treatment they receive (single blinded) but also those conducting the trial (double blinded).

Bad compliance may be a determining factor in observing no effect and therefore compliance marker may be evaluated or other means of maximizing compliance (more control, motivation, extra return products) need to be considered.

One of the unwanted side effects occurs in diet-controlled studies. In these studies, caloric intake may be underestimated, because part of the daily intake is simply forgotten. Also, physical activity needs to be estimated and needs to be compensated for. If not, applying a controlled diet may decrease caloric intake, reduce body weight, and lead to false conclusions because the observed effects are not mediated by the intervention but by weight loss.

Including too low numbers of volunteers may result in type 1 error (a positive effect of the intervention is picked up) – the effect is due to chance – or type 2 error (no effect of the intervention is observed) – the absence of an effect is due to limited statistical power. These errors can be quantified as their probabilities and need to be used to estimate the number of participants needed in a study preventing type 1 and 2 errors.

In conclusion, many aspects of trial design need to be taken into account before one gets the most out of it, important among which are the type of design, power, and potentially confounding factors.

As stated earlier, nutritional metabolomics studies (and nutrigenomics studies in general) often have to deal with large interindividual diversity (confounders). Furthermore, an organism under mild stress, for example a nutritional intervention, attempts to maintain homeostasis and metabolic control, which makes it difficult to identify alterations in biofluids such as plasma. Furthermore, plasma and urine are biofluids that represent whole body metabolism, which means that the concentration of a metabolite is often the sum of different organs and thus the representation of several biological processes. These complexity issues might be addressed by designing an experiment that makes use of a 'challenge test'. This experimental design uses one or more stresses in such a way that an individual's homeostasis is reversibly and safely perturbated. This is based on the concept that the resilience of the system can be assessed after challenging or perturbing a homeostatic situation. Application of a metabolic perturbation in concert with metabolomics may identify a set of metabolites that are predictive of differences in response to the challenge. Although this concept is new in nutrigenomics research, nutrient challenges are quite common in medical sciences. The oral glucose tolerance test (OGTT) is an example of a challenge test used for the diagnosis of type 2 diabetes.[23–25]

4.09.2.2 Sampling/Sample Preparation

Both sampling and sample preparation can have a large influence on the variation within the final data used for modeling. In metabolomics-driven studies, the aim is to obtain a quantitative view on the status of the system at the time of sampling. The samples that are drawn from biological systems such as a microbiological fermentation or from body fluids such as blood or urine are highly susceptible to changes owing to biological reactions taking place especially when the environment of the sample changes. It is therefore essential that changes in metabolites are minimized during sampling and sample preparation. In the metabolomics society, it is recognized that there is a great need for standardization of sample collection and preparation protocols for clinical studies. These standardized protocols should incorporate the optimal conditions from a metabolomics point of view as well as from a clinical point of view. Although it is

impossible to prevent any changes in the samples during sampling, it is of utmost importance that sampling is carried out in a similar way for all samples in a study using standard protocols and that all steps are recorded during the procedure.

Sampling can be both invasive (blood, cerebrospinal fluid (CSF), serum, tissues, intracellular metabolites in plants and microorganisms) or noninvasive (urine). Although the strategies of sampling may vary, the general idea of sampling is to minimize the formation or degradation of metabolites due to remaining enzymatic activity or oxidation processes. The time and method of sampling can have great influence on the reproducibility of the analytical sample. The storage of samples is also important as the continued freeze/thawing of samples can have a great influence on the stability and composition of samples.[26–28]

The sampling procedure depends strongly on the type of sample collected. Collection of plasma usually requires the addition of an anticlogging agent, such as heparin, followed by centrifugation and subsequent storage at −80 °C. For urine, the general procedure is to store the samples at low temperature (−20/−80 °C) in order to inhibit metabolic reactions. Preservatives are in general not used for urine samples. Tissue samples are mostly freeze-dried and stored at −80 °C. Special precautions must be taken when reactive metabolites are under study, like adding antioxidants to prevent oxidation of neurotransmitters.

Further sample preparation prior to analysis is mainly determined by the analytical method used. For global profiling methods, such as nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography-mass spectrometry (LC-MS), or gas chromatography-mass spectrometry (GC-MS), sample preparation is minimized in order to retain as many metabolites as possible in the sample. In many applications, no further isolation of metabolites from the sample matrix is performed and samples are diluted and analyzed directly or after chemical derivatization. For example, the analysis of biofluids by NMR requires no or only very limited sample preparation.[26,29,30] The same is true for the analysis of urine by LC-MS.[28,31–34] The analysis of plasma or tissue by MS-based methods usually requires protein precipitation and/or extraction of (certain classes of) metabolites. GC-MS analysis used in metabolomics includes chemical derivatization by oximation and silylation.[35,36] In certain cases, especially for low-abundant metabolites, further isolation of metabolites is necessary using solid-phase extraction (SPE) or liquid–liquid extraction (LLE). As said in the beginning of this section, sample preparation can have a large influence on the variation within the final data. It is therefore necessary that every step in the sample preparation is validated as much as possible using, for example, stable isotope–labeled standards or quality control (QC) samples.

4.09.2.3 Analytical Measurements

The ultimate goal of metabolomics is the quantitative analysis of all metabolites in a sample. This is dramatically more complex than analyzing the genome or the proteome. This is due to the number of metabolites that constitute the metabolome as well as their structural and compositional diversity. Moreover, the concentration range to be covered in metabolomics is also extremely large. Comprehensive analysis of the metabolome thus requires analytical techniques and instruments that offer high resolving power, sensitivity, and dynamic range. This is a major challenge and therefore a lot of research is done to improve analytical measurements in metabolomics, which is reflected in the numerous reviews on this subject in the recent literature.[29,34,36–47]

Numerous analytical platforms have been used for metabolomics studies, such as NMR, Fourier transform infrared (FT-IR) spectroscopy, and MS using flow injection or coupled to separation techniques. The advantages of NMR are the minimal requirement for sample preparation, the nondiscriminating and nondestructive nature of the technique, and the potential for high-throughput analysis.

The major disadvantage is the fact that only medium- to high-abundant metabolites are detected. Moreover, the identification of individual metabolites based on chemical shift signals is very difficult. MS-based metabolomics offers high selective and sensitive analysis and the potential to identify metabolites. However, MS-based techniques usually require sample extraction, which can cause metabolite losses and discrimination of metabolite classes. Also, very low-abundant metabolites can not be analyzed by MS-based profiling methods.

The chemical diversity of metabolites exceeds the span of any analytical method. Application of a single analytical method in metabolomics studies will therefore give only limited information. It is mandatory to combine different analytical methods into an overall metabolomics platform to improve the metabolite

coverage.[48–52] GC-MS, LC-MS, and NMR-based metabolomics platforms are mostly used for the global profiling and quantification of biochemical changes.[53,54] At the broad level, metabolomics platforms that are developed in the context of a specific compound class may be used. Mostly, LC-MS-based techniques are used for this goal. Lipidomics platforms, for example, are specifically developed for the analysis of lipid biochemical pathways.[55] In addition, approaches have been described for the analysis of oxylipids,[56] acylcarnitines,[57] etc. Finally, at the focus level, very specific analytical platforms that often detect signaling molecules available in the lowest concentration ranges have been developed. The (trace)-element status for example can be monitored using induced coupled plasma (ICP)-MS[58] and neutral eicosanoid profiles can be generated via LC-MS/MS analysis.[59]

Figure 4 illustrates an example of such a metabolomics platform including global profiling methods based on NMR, GC-MS, and LC-MS for relatively medium- to high-abundant metabolites, a collection of class-targeted profiling LC-MS methods for some specific classes of metabolites and metabolites with lower abundance, and target LC-MS/MS methods for specific low-abundant metabolites. These methods cover a wide range of different classes of metabolites, with the total number of distinct metabolites being in the order of 60–800.[48] Often not all methods are used but a selection is made based on prior knowledge, sample volume, time schedule, and budget constraints.

The analysis of the metabolome in body fluids has been approached via other techniques as well as GC in combination with flame ionization detection (FID) for the profiling of the lipid domain, which is a very quantitative detection strategy.[60] Other examples are LC coupled to electrochemical detection (ECD), allowing the detection of metabolites involved in neurotransmitter pathways and pathways involved in oxidative stress, and LC using diode-array and fluorescence detection.[61,62] Most or all of these technical platforms are already familiar in the field of pharmacology and toxicology. The difference in metabolomics used in different research fields lies not in the platforms, but rather in the specific way in which these analytical tools are applied, the samples used, and the chemometric methods applied to the data.

Reports of the plasma metabolome composition are widely variable but range from 10 to 30 thousand small molecules. The coverage today is still limited, with NMR typically covering the mg ml^{-1}, µg ml^{-1}, and at best the high ng ml^{-1} range measuring in the order of 300 – maximum 500 – components for a 600 MHz to mass spectrometric applications covering also the ng ml^{-1} and pg ml^{-1} range when applied on focused metabolite classes. The strength of NMR is typically its power of quantification for several high-concentration compound classes, but nonpolar metabolites such as lipids and low-abundant metabolites such as neurotransmitters, which are very important in many diseases and in nutritional studies, are not measurable. Currently, MS-based methods are mostly used in metabolomics studies, often in combination with separation methods, that is, LC-MS and GC-MS. Resolution of these methods improves dramatically by using high-resolution mass spectrometers, for example, time-of-flight (ToF) and Fourier transform-ion cyclotron resonance (FT-ICR). The coupling of separation and MS increases the resolving power, dynamic range, and sensitivity. Sensitivity is very

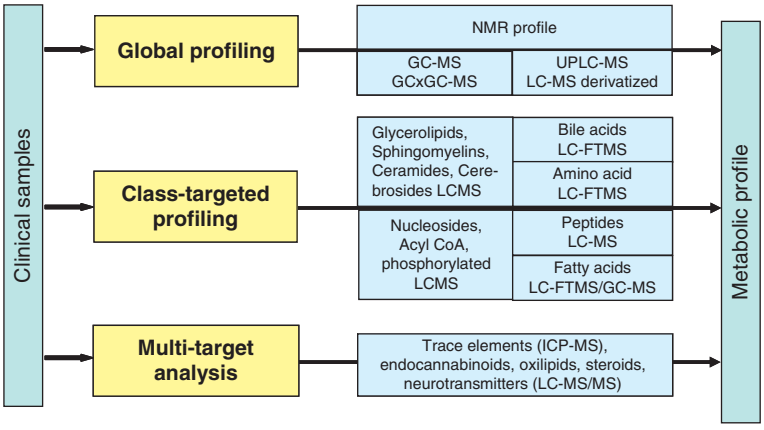

Figure 4 Example of a comprehensive metabolomics platform combining global and targeted analytical methods.

important in metabolomics for including low-abundant metabolites and for working with limited sample volumes. The major challenges for MS-based methods are quantification and identification, which will be described later.

Besides good coverage and sensitivity, it is of utmost importance that the analytical methods used in metabolomics studies show good analytical performance. The statistical analyses that are performed on the final data are often complicated especially when longitudinal sampling and different subgroups are included. As a result, the order in which samples are measured within an analytical series of measurements is critical, while variations within the instrument and/or method can easily induce differences or trends that can be mistaken for biological effects.

4.09.2.4 Quality Control

Variation in sample preparation and analysis can easily induce differences or trends that can be mistaken for biological effects. The magnitude of this problem is proportional to the scale of the study. Small-scale studies, which contain only a small number of samples in a single batch of experiments, may only suffer from instrumental drift for example due to ion source contamination. Owing to the use of IS, much of the instrumental drift and offset in between series can be corrected for but the IS are not available for each individual metabolite, and hence the remaining drift has to be taken into account. Large-scale studies, such as nutritional metabolomics studies, are even more challenging while these studies result in large time intervals between the analyses of different batches of samples from the same study sample set.[55] Sometimes, parallel instruments of the same type are used in the same laboratory or even in different laboratories. All these aspects will induce variation for which corrections should be made. Relevant biological information can be obtained from these studies only if the reliability, repeatability, and reproducibility of the different steps in the workflow are excellent.

The only way to achieve this is to develop validated protocols for sample preparation and analysis.[32,48,63] All samples should be treated in a similar way in order to be able to compare samples within a study. Validation of the different steps in sample preparation can be carried out using standard addition experiments and stable isotope–labeled standards, and corrections, for example recovery, can be made after analysis. Analytical methods should be used only if they are extensively validated.[31,33,35] Furthermore, standardized analysis designs should be applied using QC samples[26,31–33,63] or preferably biological calibration samples,[48] calibration standards for quantification of specific metabolites and randomized study samples. Which randomization is the proper method depends on the study design and the way the data analysis is performed, which calls for an early choice of the data analytical method to be used. QC samples are used to rigorously monitor the performance of the method.[32] These samples are preferably pooled study samples that are analyzed at the beginning, end, and randomly through the run. In practice, these QC samples are used only qualitatively to determine for example whether a batch of experiments failed based on preset acceptance criteria. Recently, Van Der Greef *et al.*[48] described an alternative approach using biological calibration samples and this can be used to quantitatively correct for intrabatch temporal trends and remove systematic batch-to-batch differences. Although with this approach no real quantitative data are obtained, that is, absolute concentration, it is at least possible to compare responses of metabolites between different samples analyzed in different batches. In this way, analytical variation is removed almost completely and will thus not hamper statistical analysis of the final data.

Standardization at an international level is needed in order to be able to improve the universal significance of data generated from different studies at different sites and this will improve the overall understanding of complex biological systems.

4.09.2.5 Human Example Study: Design and Measurements

4.09.2.5.1 Design and samples

The study was designed as a double blind, randomized, parallel trial, in which subjects were treated with diclofenac ($n = 10$) or placebo ($n = 10$). Randomization of subjects to treatment groups was restricted by the levels of high-sensitivity C-reactive protein (hsCRP), body mass index (BMI), fasting glucose, and age. Subjects consumed one capsule (placebo or 50 mg diclofenac) ~1 h before breakfast, lunch, and dinner for 9 days.

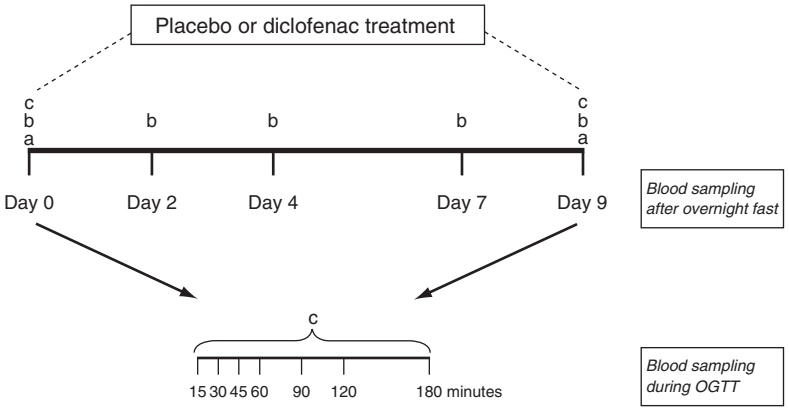

Figure 5 Overview of the study design of diclofenac study and time points at which the metabolome was measured.

Subjects were instructed to keep their habitual diet during the study. Nineteen men completed the study. One person dropped out on the first day of the study for reasons unrelated to the study. The metabolomics approach was applied not only in fasting (homeostatic) conditions, but also on multiple time points during an oral glucose challenge test (OGTT). This is based on the concept that the resilience of the system can be assessed after challenging or perturbing a homeostatic situation. Application of a metabolic perturbation in concert with metabolomics may identify a set of metabolites that are predictive of differences in response to the challenge, which is measured as a change in time to return to baseline concentrations. Blood samples were taken after an overnight fast on days 0, 2, 4, 7, and 9. Subjects underwent an OGTT on days 0 and 9. Blood samples were taken just before (0 min) and 15, 30, 45, 60, 90, 120, and 180 min after the administration of the glucose solution (70 g). **Figure 5** shows the study design and time points at which metabolome measurements were made.

4.09.2.5.2 Metabolomics measurements

LC-MS and GC-MS techniques were applied on the plasma samples. LC-MS was used to measure the compound classes lipids (LC-MS lipids), free fatty acids (LC-MS FFA), and polar compounds (LC-MS polar).[55,64] Plasma GC-MS was applied to screen for metabolites on the global level.[35] In total, 19 plasma samples per person were measured on all 4 metabolite platforms, which is a total of 361 plasma samples. Furthermore, a QC sample was prepared by pooling plasma from all subjects. The pool was divided into 10 µl aliquots and these were extracted the same way as the study samples. The QC samples were placed at regular intervals in the analysis sequence (one QC after every 10 samples). The QC samples serve two purposes. The first is a regular QC sample to monitor the LC-MS response in time. After the response has been characterized, the QC samples are used as standards of unknown composition to calibrate the data.[48] The LC-MS lipids platform detected a total of 61 different lipids, the free fatty acid platform a total of 14 fatty acids, and the LC-MS polar a total of 130 polar compounds. The GC-MS platform detected a total of 137 metabolites. Thus, in total, about 340 different plasma metabolites were analyzed per subject.

4.09.3 Data Preprocessing

4.09.3.1 Signal Alignment

The information acquired with instrumental measurements such as GC-MS, LC-MS, and NMR is sometimes used as is, without extraction of individual peaks based on the total ion current (TIC) of the GC-MS and LC-MS data files or the individual spectra of the NMR measurements.[65] Owing to variations within the measurements such as pH differences or instrumental changes (shortening of the analytical column in GC measurements, variations in eluent composition for LC instruments, etc.), the position of the individual peaks with the same identity within the different data files will vary and correction is required even when the total signal (profile) is used for data analysis. In that case, alignment methods such as correlation optimized warping (COW) are applied (see Chapter 2.06). For

instance, in GC analysis, a small part of the GC column is removed after a certain number of measurements to prevent unwanted effects on the analysis results owing to fouling of the column. Shortening of the column will result in a distinct shorter retention for all the metabolites and a simple additive correction procedure will solve this problem. For NMR, alignment is mostly performed after peak extraction where each peak can have a different shift behavior owing to variations in the pH of the sample. It should be remembered that with NMR the whole sample is measured at once without prior separation of the individual compounds. It would be possible to perform LC-NMR measurements in which prior to the NMR measurements a separation by means of LC is performed, but this methodology is not common practice yet in metabolomics research (For more information, please see Chapter 3.04).

4.09.3.2 Peak Extraction

Metabolomics measurements can be performed on different instruments (or platforms, in the metabolomics jargon). Mainstream methods are NMR, GC-MS, and LC-MS. All three methods have their own characteristics and require preprocessing to clean up the data. For NMR, this comes down to correcting for peak shifts. For GC-MS and LC-MS, the characteristics of the clean data change depending on the type of preprocessing. Essentially, there are three ways to preprocess GC-MS and LC-MS data: target analysis, peak-picking, and deconvolution. In target analysis, a specific m/z channel is sought in the MS domain that is specific for a metabolite. Then the associated chromatographic peak can be integrated and used as pseudo-concentration. Each metabolite is represented by one variable in the final data table.

In peak-picking, first the m/z traces containing meaningful information are selected (e.g., with CODA[66] or Impress[67]) and then the peaks in the selected ion traces are integrated to obtain single-intensity measures for complete peak profiles. This results in a data table in which one metabolite is represented by different variables (peaks representing different ions as present in the mass spectrum).

In deconvolution, the coeluting peaks in the chromatograms are mathematically resolved where analytical resolution of the peaks remained incomplete. Multiple overlapping peaks representing different metabolites are deconvoluted. After that, the resulting pure chromatographic peaks are integrated. This results in a table of areas per chromatographic peak (= metabolite), representing pseudo-concentrations. A second table is produced as well containing the mass spectral information, which can be used for metabolite identification (For more information, please see Chapter 4.13).

From the above descriptions, it is clear that different data structures arise for GC-MS and LC-MS depending on the type of preprocessing. In the target and deconvolution case, a single variable in the data table also represents a single metabolite. For the peak-picked data (and also for the NMR data), multiple variables can describe one metabolite. The latter means that there are two types of correlation in a data table: the 'analytical' correlation (more variables for the same metabolites) and 'biological correlation', the correlation between metabolites induced by the underlying biology. Peak-picked data sets are usually megavariate, but do not necessarily contain more metabolites than a smaller targeted or deconvoluted data set.

4.09.3.3 Peak Alignment

Given an optimal instrument and standardized samples (same pH for instance in the case of NMR measurements), it should be possible to obtain intensities of the same chemical entities within different samples at the same retention time (chromatography) or the same chemical shift (NMR). However, the situation is not optimal and some differences between samples exist and as a result after peak extraction from the raw data, equal chemical entities are reported at different retention times or chemical shifts. To correct this, multiple methods have been developed. Chapter 2.06 of this book deals in great detail with the subject of alignment.

4.09.3.4 Quantification

Metabolomics comprises the quantitative analysis of metabolites. However, to obtain the actual concentrations of metabolites in samples, analyses using stable isotope–labeled IS and calibration standards are necessary for every metabolite. This is possible for target analysis, but quite a challenge for global profiling methods, if not

impossible. For known metabolites, in global profiling methods it is possible to use stable isotope–labeled IS in order to obtain quantitative data. However, generally, these methods are able to measure hundreds of metabolites, many of them being unknowns. Practically, class-targeted and global profiling analysis is nothing more than recording the response of substances in samples in which the response is only a measure of concentration. In the ideal case, the peak areas for the same metabolite in different samples should be directly comparable with each other on a relative scale. However, as mentioned earlier, the response is determined not only by the actual concentration, but also by various other factors. These factors should be prevented or corrected for in order to be able to compare metabolite responses. Peak extraction of metabolomics data generally leads to raw peak areas of metabolites. These peak areas should then be corrected for the biological (e.g., biomass, creatinine for urine) and analytical variation (intrabatch drifting and batch-to-batch differences) using biological calibration samples[48] to obtain relative peak areas that can be compared between samples in different batches. Using calibration standards, known metabolites can then be at least semiquantified. Quantitative aspects are even more challenging in large-scale human studies, such as nutritional metabolomics studies, because these studies result in large time intervals between the analyses of different batches of samples from the same study sample set. In metabolomics, it is often sufficient to know whether there are metabolites that increase or decrease in concentration as a result of a specific treatment or illness and the identity of these metabolites. However, if these metabolites are present and identified, the next question is often what the exact concentration is in order to compare the results with other results from clinical studies.

4.09.3.5 Human Example Study

The raw analytical data with the diclofenac study were processed with homemade comprehensive peak-picking software Impress.[68,69] This software is able to extract peaks from multiple data files and perform alignment of the peaks. Still, manual setting of parameters is required and to circumvent subjectivity of the preprocessing the produced peak list was used only to extract all possible features from the total set of data files. From the extracted features, all isotopes were removed, resulting in the removal of multiple entries of the same metabolite and a data set was obtained containing only one entry per metabolite. These features were then entered into the XCalibur software version 1.4 (Thermo Electron Corp., San Jose, CA, USA). Within this software, retention time windows and mass windows are entered for each metabolite, and for each data file the matching peak intensity is reported.

Data of each subject were corrected for the recovery of the IS for injection. Batch-to-batch differences in data were removed by synchronizing the medians of QC samples per batch. QC samples are produced by pooling true samples from the study forming a large quantity of one composite sample that contains all metabolites that are also present in the individual sample. The theory is that repeated measurements should result in the same analytical result and that deviations from that assumption are due to analytical effects. For each metabolite within an individual sample, a matching metabolite is available within the pooled QC sample. After each 6th or 10th study sample, a QC measurement is performed, depending on the analytical method. The measured intensities in the first batch are taken as reference batch and the QC measurements in each consecutive batch are matched toward those QC measurements. Different ways of correction are possible depending on the type of deviation within the measurements of which a simple offset is the simplest one.

For all platforms, duplicate measurements were combined into a single measurement. When both analytical duplicates had a zero value or when both had a nonzero value, measurements were averaged, whereas the single value was taken when only one of the duplicates was above zero.[55] To avoid trivial results, data were additionally cleaned up by removing glucose-related metabolites and IS isotopes in the LC-MS polar data and glucose metabolites in the GC-MS global data set.

4.09.4 Data Analysis

Data from metabolomics studies have some specific characteristics that are different from other types of data. Many of the metabolomics-specific issues have already been mentioned in Section 4.09.1.3. Additional issues to consider are data fusion, scaling, and centering.

Metabolomics measurements are often performed on the same samples using different platforms in order to obtain an as comprehensive view on the sample composition as possible. Owing to the different characteristics of the instruments and different data preprocessing strategies used, combination of data sets is not straightforward and calls for data fusion strategies.[70] Data sets obtained from different instruments have unequal noise characteristics, which can be due to, for instance, chemical reactions within the ion spray in LC. Another aspect to take into account when combining data sets from different instruments is whether the relation between measured intensity and true concentration is comparable between instruments. This is especially of concern when combining data sets from instruments with different working mechanisms such as NMR and MS. The final concern is the method used to convert raw data into peak tables. One method will result in peak tables in which each metabolite is represented by one variable (target analysis and deconvolution), whereas another method will result in a peak table in which metabolites are represented by multiple variables (e.g., peak-picking). The process of combining or fusing the data is completely context dependent and will not be discussed in detail here. Needless to say, fusing data of different platforms only aggravates the problem of low sample-to-variable ratios.

Another special issue is scaling and centering within metabolomics data analysis especially where different groups and dose and time effects are included within one study. In two-way analysis scaling and centering are quite straightforward, whereas in multiway analysis they are more complicated, because centering across or scaling within a certain mode might disturb prior centering and/or scaling steps.[71–73]

4.09.4.1 Metabolomics-Specific Modeling

Many if not all chemometrical modeling techniques have been used to process data obtained from metabolomics experiments. While metabolomics studies often suffer from a large number of variables (p) in comparison to the number of objects or samples (n), certain methods such as multiple linear regression (MLR) will not fulfill the purpose of metabolomics modeling. Regularization methods such as ridge regression (RR), support vector machine (SVM), and least absolute shrinkage and selection operator (LASSO) are suitable for solving the problem of (near) colinearity, which results in unstable models with highly variable regression coefficients (For more information, please see Chapters 4.08, 4.12, 3.01 and 3.05). However, the general method used for a first view of metabolomics data sets is still the principal component analysis (PCA), enabling a general view of the structure of the data at hand (For more information, please see Chapter 2.13). Gross errors, outliers, and a first idea of the amount of clustering are quickly discovered looking at the PCA score plots. Likewise, partial least squares (PLS) is used to correlate the sample composition to a kind of phenotype such as the yield of a fermentation process (For more information, please see Chapter 3.01). PCA and PLS are well suited for two-way data sets, whereas structured data need to be processed with methods such as variance (ANOVA)-simultaneous component analysis (ASCA) and n-PLS, which can handle the underlying structure in the data sets. For example, when at the same time healthy versus diseased and a development in time of the metabolic changes are to be traced, the structure of the study design needs to be included to ensure that the different sources of variation are not mixed up. Inclusion of the experimental design into model assessment also helps in obtaining results that can be interpreted more easily (For more information, please see Chapters 4.05, 4.12, 1.02, 2.23, and 3.03).

In many metabolomics studies, the general question is to find metabolites that are able to discriminate between healthy and diseased or between treated and untreated patients. Once the metabolites are found that are discriminating between the groups, biological interpretation using genetic information or pathway information can be performed. Examples of suitable methods for classification are PLS-discriminant analysis (PLS-DA) in the case of a two-way data structure and n-PLS-DA for multiway data structures.

A new direction that is being developed within metabolomics research is to build models using prior knowledge. Prior knowledge can be the nearness of metabolites within a metabolic pathway and thereby their simultaneous reaction to disturbances within the pathway. Among others, methods that can be used to incorporate prior knowledge are Grey component analysis (GCA)[74] and Bayesian statistics (For more information, please see Chapters 2.23, 3.23, and 2.03).[75]

4.09.4.2 Model Validation

Although it is impossible in many cases to expand the number of experiments of metabolomics studies owing to costs or for ethical reasons in the case of animal or human studies, statistical resampling techniques need to be

applied to perform model validation. In applications where the availability of samples is less problematic, data sets can be subdivided into training sets and validation sets. This is not the case in metabolomics and techniques such as double cross-validation (2CV) can be applied;[76,77] it is currently the best method to mimic validation with a true test set. Using 2CV, cross-validation can still be used to find a good estimate of the prediction error without having to define a dedicated data set for model building, model validation, and estimation of prediction errors. Determining the tunable parameter (e.g., number of latent variables (LVs) in PLS) with cross-validation is part of the procedure to build a model. The entire modeling procedure has to be cross-validated in order to obtain the prediction error. The double cross-validation consists of two nested cross-validation loops. The modeling procedure, including the cross-validation that determines the tunable parameter, forms the inner loop. The cross-validation for the error estimation takes place in the outer loop.

Biological and analytical validations of the model results are as important as the statistical validation. Variables that appear to be of importance, as deduced from statistical analysis, should be traced back in the analytical data to ensure that these variables are not just artifacts for instance due to extraction procedures. Also, biological validation as far as information is available about the variables should reveal whether certain effects as explained within the statistical model can be explained. Examination of the feasibility of the statistical models can be based upon metabolic pathways or upon thermodynamic information.[78]

4.09.4.3 Human Example Study

PLS-DA[79] was used to identify metabolites that differed in their change between days 0 and 9 in fasted conditions between treatment groups (**Figure 5**, analysis a). As the interest was in intraindividual differences between days 0 and 9, the X-block was defined for each metabolite platform by subtracting the day 0 values from the day 9 values, which removed interindividual differences.[80]

For the identification of metabolites that differed in their change caused by the treatments between days 0, 2, 4, 7, and 9 time course and days 0 and 9 OGTT time course, the multiway generalization of PLS-DA was used (n-PLS-DA).[81–83] In this way, metabolites can be identified that differ in changes over time between the treatment groups, by creating a so-called three-way matrix of size $19 \times \mathcal{J} \times T$, where 19 is the number of subjects, $\mathcal{J}$ is equal to the number of metabolites of a particular platform, and T is equal to the number of time points, which were either days 0, 2, 4, 7, and 9 (**Figure 5**, analysis b) or the time points after glucose administration on days 0 and 9 (**Figure 5**, analysis c). For the statistical analysis of the OGTT time-course data, the day 0 data were subtracted from the day 9 data, removing interindividual differences. The GC-MS global and LC-MS polar data sets were centered across subjects and followed by scaling within the metabolite mode $\mathcal{J}$, whereas the LC-MS lipids and fatty acids data sets were centered only across subjects. The centering step was performed to remove constants between the subjects, whereas scaling within the metabolite mode resulted in standardized metabolites. By performing the scaling step after the centering step, the prior centering remained unaffected.[72,73,83]

In (n-)PLS-DA, a Y variable containing class membership information is correlated to the data block X. In the present study, the metabolic response ($\mathbf{X}$) is related to treatment groups; hence $\mathbf{y}$ is not a continuous parameter as in regular regression, but a dichotomous vector containing the treatment group membership. The subjects who received the placebo treatment were assigned to class '0' and the subjects who received diclofenac were assigned to class '1'.

The following model was used in the analysis of the 0, 2, 4, 7, and 9 days time course:

$$\begin{aligned}
&\mathbf{T} = \mathbf{XV} \\
&\mathbf{X} = \mathbf{TG}\left(\mathbf{W}^{M} \otimes \mathbf{W}^{K} \otimes \mathbf{W}^{\mathcal{J}}\right)' + \mathbf{E}_x \\
&\mathbf{y} = \mathbf{TB} + \mathbf{e}_y \\
&\max \operatorname{cov}\left(\mathbf{t}_c, \mathbf{y}^{(c-1)}\right); c = 1, \ldots, C \\
&\mathbf{w}_c^{M}, \mathbf{w}_c^{K}, \mathbf{w}_c^{\mathcal{J}}
\end{aligned} \tag{1}$$

where $\mathbf{V}$ is a matrix of weighing coefficients which can be written in terms of $\mathbf{W}$, $\mathbf{G}$ is the core array, $\mathbf{B}$ is the regression matrix for regressing $\mathbf{y}$ on $\mathbf{T}$, and $\mathbf{E}_x$ and $\mathbf{e}_y$ are the residuals of the model for $\mathbf{X}$ and $\mathbf{y}$, respectively.[71]

For the analysis of the OGTT time course, the multilevel variant of multiway PLS-DA was used. As the interest is in intraindividual differences specifically, the interindividual variation can be removed by subtracting the day 0 data from the day 9 data. The multilevel multiway model was created, which regresses parameter **y** containing the treatment group membership to the changes in metabolic response between days 0 and 9, $\mathbf{X}_9 - \mathbf{X}_0$ (size $I \times \mathcal{J} \times K$). The model used was adapted as follows:

$$\begin{aligned}
&\mathbf{T} = (\mathbf{X}_9 - \mathbf{X}_0)\mathbf{V} \\
&(\mathbf{X}_9 - \mathbf{X}_0) = \mathbf{TG}\left(\mathbf{W}^M \otimes \mathbf{W}^K \otimes \mathbf{W}^{\mathcal{J}}\right)' + \mathbf{E}_{\mathbf{X}_0 - \mathbf{X}_9} \\
&\mathbf{y} = \mathbf{TB} + \mathbf{e}_y \\
&\max \operatorname{cov}\left(\mathbf{t}_c, \mathbf{y}^{(c-1)}\right); c = 1, \ldots, C \\
&\mathbf{w}_c^M, \mathbf{w}_c^K, \mathbf{w}_c^{\mathcal{J}}
\end{aligned} \tag{2}$$

The creation of the **X**-block that was used for n-PLS-DA modeling is illustrated in **Figure 6**. First of all, a three-way matrix $\mathbf{X}_0$ of size $19 \times \mathcal{J} \times 8$ was created. This matrix contained the metabolic data of day 0, determined at eight different time points for each subject. A matrix $\mathbf{X}_9$ of the same size was also created, containing similar information for the day 9 measurements. Finally, the $\mathbf{X}_0$ matrix was subtracted from the $\mathbf{X}_9$ matrix and this **X**-block was used for data analysis.

Cross-validation was used to validate the (n-)PLS-DA models, using a 'leave-one-out' cross-validation scheme.[84] Data of one subject were left out in the first cross-validation step, a (n-)PLS-DA model was built, and the treatment class membership of the subject who was left out was predicted. This was repeated until all 19 subjects were left out once. The error rate of the model was determined by comparing the original class membership and the predicted one. The optimal number of LVs was determined based on the minimum value of the error rate. The final fit of the model was made using the number of optimal LVs. (n-)PLS-DA models for which an error rate was found below 35% were optimized by performing metabolite selection based on a jack-knife approach.[84] Data of one subject were left out and a (n-)PLS-DA model was made using the same number of LVs as those used for the final model. This was repeated until all 19 subjects were left out once. This resulted in 19 sets of regression coefficients, the standard deviation of which was used to determine the relative standard deviations (RSDs) of each regression coefficient. Only those metabolites that had an RSD of less than 50% were included in the new data set, which was used to build a second (n-)PLS-DA model. Metabolites that contributed to treatment differences were identified based on absolute regression coefficients of this second model.

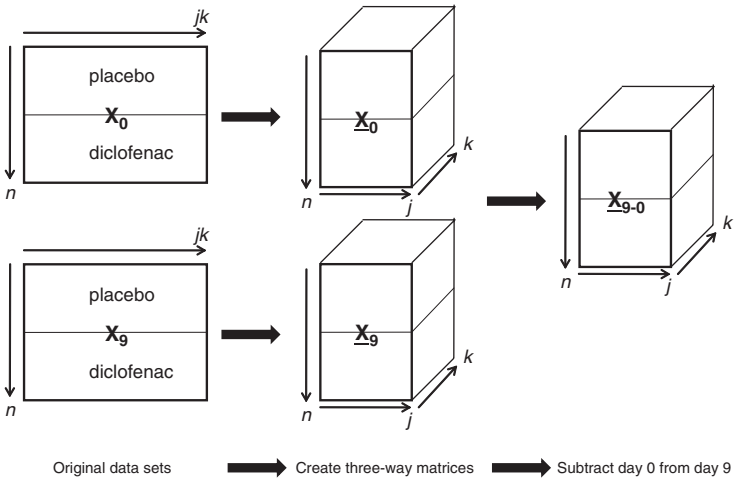

Figure 6 The creation of the **X**-block that was used for multilevel n-PLS-DA modeling.

A permutation test was performed to test whether the treatment differences were indeed true differences. One thousand dichotomous **y** vectors were randomly created using the same proportion of zeros and ones as the vector that was used for modeling. For each random vector, a multilevel n-PLS-DA model was made using the same 'leave-one-subject-out' cross-validation approach and the error rate was calculated. A distribution was made of all thousand error rates and the error rate for the original model was compared to this distribution, which resulted in a *p*-value for the significance of treatment differences. All analyses were performed using Matlab Version 7.0.4 R14 (The Mathworks, Inc.) and the n-way toolbox version 2.11.[85]

4.09.4.3.1 Results

Table 1 shows the results of these multivariate analyses of the different data sets derived from the four metabolomics platforms.

Comparison of the fasting state metabolomes between subjects treated with placebo and diclofenac on day 9 compared to day 0 resulted in PLS-DA models (Figure 5, analysis a) with high error rates, indicating no significant difference in fasted plasma samples.

Extension of the n-PLS-DA models with the metabolomics data of the plasma samples taken in fasted conditions on several intermediate days (Figure 5, analysis b) during the intervention for the various metabolomics platforms also resulted in high error rates. This confirms that no significant metabolomic changes could be detected between subjects treated with placebo and diclofenac at the fasted (homeostatic) condition.

Metabolic perturbation by the OGTT considerably improved the metabolomics-based differentiation between the treatments. The n-PLS-DA models on plasma samples taken in an eight-point time course after glucose administration of subjects on day 9 versus day 0 (Figure 5, analysis c) resulted in improved error rates for all metabolomics platforms compared to PLS-DA and n-PLS-DA models based on plasma in fasted conditions, between the control and antiinflammatory treatment. The n-PLS-DA models for metabolome data from GC-MS global, LC-MS polar, and LC-MS lipids were optimized by using a jack-knife approach. If a subject is left out and the regression coefficient changes a lot, this will result in a relatively high RSD for that particular variable. A variable with a high RSD was considered to be unstable and hence unreliable for explaining the differences in response between the placebo and the diclofenac group. The n-PLS-DA models after jack-knifing for GC-MS global and LC-MS polar metabolome data resulted in models with error rates of 10.5 and 5.0% respectively, indicating that the GC-MS global model and the LC-MS polar model misclassified respectively only 2 and 1 persons out of 19. The model of the LC-MS lipids data set resulted in a model with an error rate of 16%, indicating that 3 out of 19 subjects were misclassified. For the LC-MS polar model, for example, 10 of the variables that appeared in the top of the model based on the original 120 variables

Table 1 Results of multivariate data analysis, expressed as error rates, of various metabolomics data sets

Method	*GC-MS global (137[a])*	*LC-MS polar (130[a])*	*LC-MS lipids (61[a])*	*LC-MS FFA (14[a])*
PLS-DA Day 9 vs. day 0	42.0%	53.0%	37.0%	37.0%
n-PLS-DA Days 0, 2, 4, 7, and 9	57.0%	42.0%	52.5%	47.0%
n-PLS-DA Day 9 vs. day 0; 0–15–30–45–60–90–120, and 180 min	31.5%	31.5%	21.0%	31.5%
n-PLS-DA after metabolite selection Day 9 vs. day 0; 0–15–30–45–60–90–120, and 180 min	$\mathcal{F}=77$ 10.5%	$\mathcal{F}=31$ 5.0%	$\mathcal{F}=25$ 16.0%	NA

[a] Number of metabolites.

Note: Metabolite selection was applied only if the error rate of the original model of the complete data set was below 35% and the data set contained more than 50 metabolites. NA, not analyzed.

overlapped with the model using 31 variables. So, essentially the same information could be described using fewer variables, illustrating the fact that many variables were unimportant for the model. Overall, it was concluded that significant metabolomic changes due to the treatment could be detected only in the metabolomics data of the OGTT time course.

4.09.4.3.2 Statistical interpretation

The optimized LC-MS polar model will be used for the statistical interpretation of the results from the multilevel multiway models. The multiway regression model resulted in a regression matrix of size $\mathcal{J}^* \times K$, in which $\mathcal{J}^*$ is the number of variables after variable selection. To determine the variables that contributed most to treatment differences, the regression coefficients were sorted by their absolute value in descending order per time point K. For each time point, the first 10 variables were selected and used for interpretation. The selected variables are presented in **Table 2**.

The contribution of each variable to the treatment effect can be followed over time by investigating its appearance in the list of parameters that contribute most to the differences between treatments. Some metabolites were important over the whole range of time, whereas others were contributing only for a certain period of time. The variables that appeared in the top 10 for only one time point were considered to be coincidently related to the treatment. Variables V01 and V02 will be used to illustrate further interpretation.

V01 is an example of a metabolite that contributes to the response differences between treatments at each measurement point, as illustrated by the light-gray shade in **Table 2**. This means that the response of this metabolite between days 0 and 9 differed during the whole time course in subjects treated with diclofenac compared to the placebo group. This effect is illustrated in **Figure 7**, in which the mean difference between days 9 and 0 response for V01 is plotted per treatment group. The placebo group had at fasting state ($t0$) a mean change of about zero between days 9 and 0, whereas at the same time point the diclofenac group had a mean decrease of 2.5 units. The difference between treatment groups fluctuates between 1 and 2.5 units, depending on the time point, but it remains quite stable over time. In **Figure 8**, the regression coefficient of this variable is plotted against the time. The same conclusion can be drawn toward this metabolite from this figure.

Variable V02 was only seen in the top 10 of contributing variables at 90 min and later of the OGTT, as illustrated by the dark-gray shade in **Table 2**. This metabolite was ranked 13 at $t90$ and therefore not included in **Table 2** for this time point. The ranking at $t0$, $t15$, $t30$, and $t45$ was 21, 18, 29, and 26, respectively. So, after 1 h, this metabolite differed in response to the challenge test between days 9 and 0 in subjects treated with diclofenac compared to the placebo group. This effect is illustrated in **Figure 9**: The differences in response are more or less the same up to 45 min and around zero, whereas they deviate from $t60$ and later. In **Figure 10**, the regression coefficient of this variable is plotted against the time. There is no significant contribution to treatment differences over the first 60 min of the curve. Only after an hour, this variable becomes more important.

For the interpretation of the results of this type of modeling, it must be kept in mind that the regression coefficients, which were used to rank the metabolites, are based on a model in which other metabolites were also included. So, each coefficient reflects the relation between the treatment group and that

Table 2 Overview of variables selected and used for interpretation

	Ranking									
Time point (minutes)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
0	V01	V12	V51	V13	V48	V42	V53	V17	V27	V86
15	V31	V51	V44	V17	V12	V01	V15	V54	V59	V38
30	V01	V12	V51	V13	V17	V48	V42	V53	V86	V08
45	V01	V12	V17	V51	V13	V31	V08	V42	V48	V44
60	V01	V12	V13	V51	V17	V16	V08	V77	V48	V02
90	V54	V17	V77	V88	V76	V59	V53	V86	V16	V19
120	V48	V01	V08	V02	V13	V17	V36	V12	V16	V27
180	V01	V12	V17	V51	V13	V16	V08	V48	V02	V31

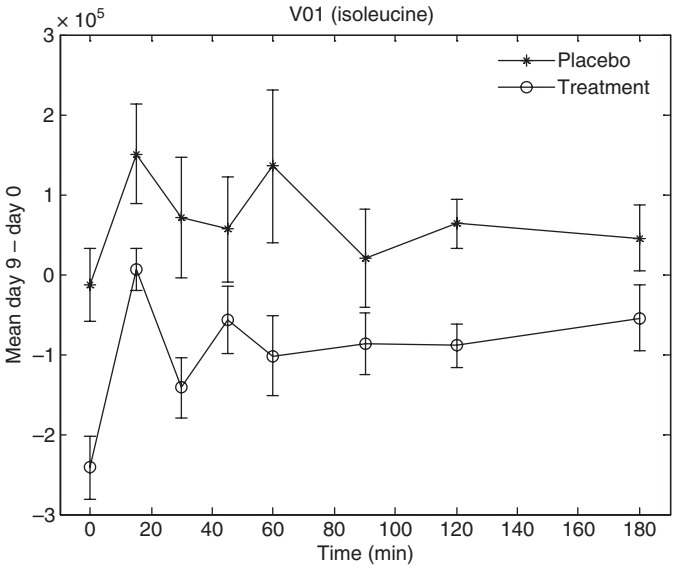

Figure 7 Mean change (±se) in metabolic response to the challenge test between days 9 and 0 for subjects on placebo and diclofenac treatment, for variable V01 – identified as isoleucine – that contributes to treatment differences over the whole time course.

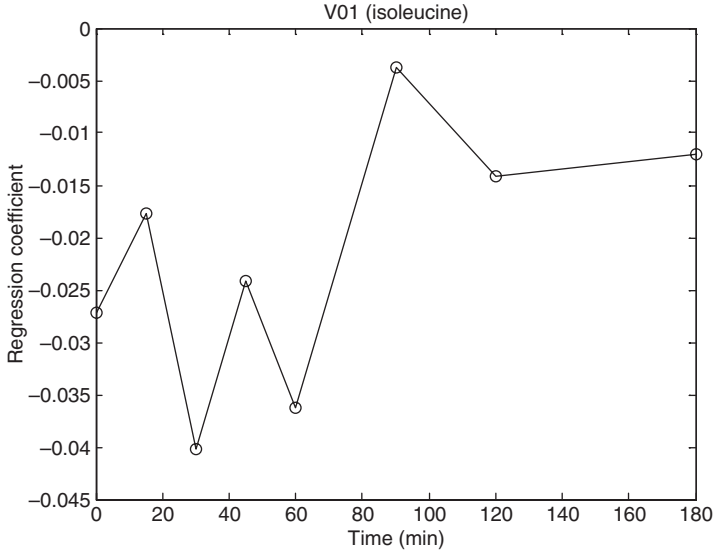

Figure 8 Regression coefficients over time of a multilevel n-PLS-DA model for variable V01 – identified as isoleucine – that contributes to treatment differences at each time point of the time course.

particular metabolite, given the presence of the other metabolites that were used in that particular model. In **Figures 8** and **10**, the other metabolites are not taken into account, and hence these are univariate illustrations of multivariate results.

In **Figure 11**, the results of the permutation test are shown. The asterisk represents the error rate of the n-PLS-DA model that was made, whereas the histogram represents the distribution of error rates based on permuted classes. In **Figure 11**(a), the results of the overall multilevel n-PLS-DA model are given, and in **Figure 11**(b), the results of the optimized multilevel n-PLS-DA are given. The results for the overall model are very moderate ($p = 0.472\,5$), but the treatment differences become more clear after optimization of the model ($p = 0.005\,9$).

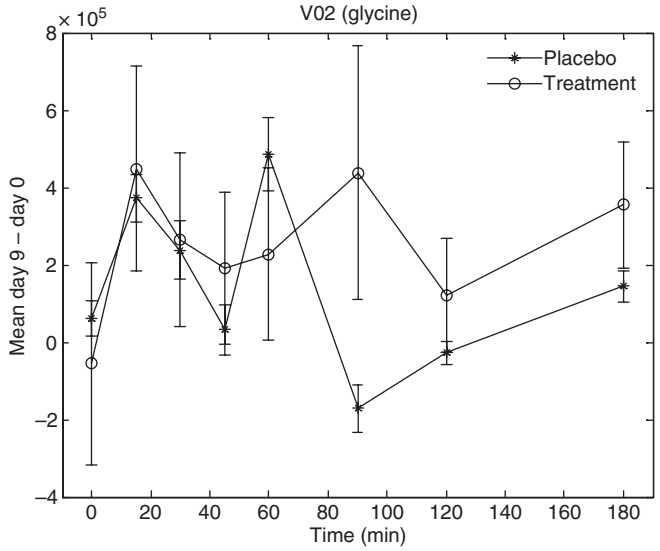

Figure 9 Mean change (±se) in metabolic response to the challenge test between days 9 and 0 for subjects on placebo and diclofenac treatment, for variable V02 – identified as glycine – that contributes to treatment differences in the second part of the time course.

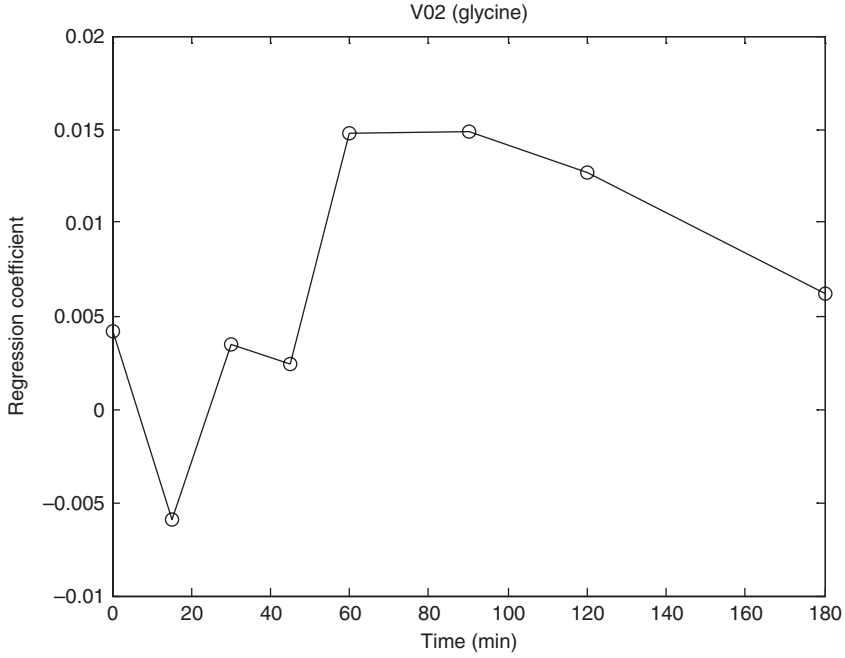

Figure 10 Regression coefficients over time of a multilevel n-PLS-DA model for variable V02 – identified as glycine – that contributes to treatment differences only in the second part of the time course.

4.09.5 Metabolite Identification

4.09.5.1 Identification

The identity of statistically relevant metabolites in metabolomics studies is a prerequisite for biological interpretation. The Human Metabolome Database (HMDB, www.hmdb.ca) is the most complete and comprehensive database of human metabolites, containing records of more than 2000 endogenous metabolites.[86,87]

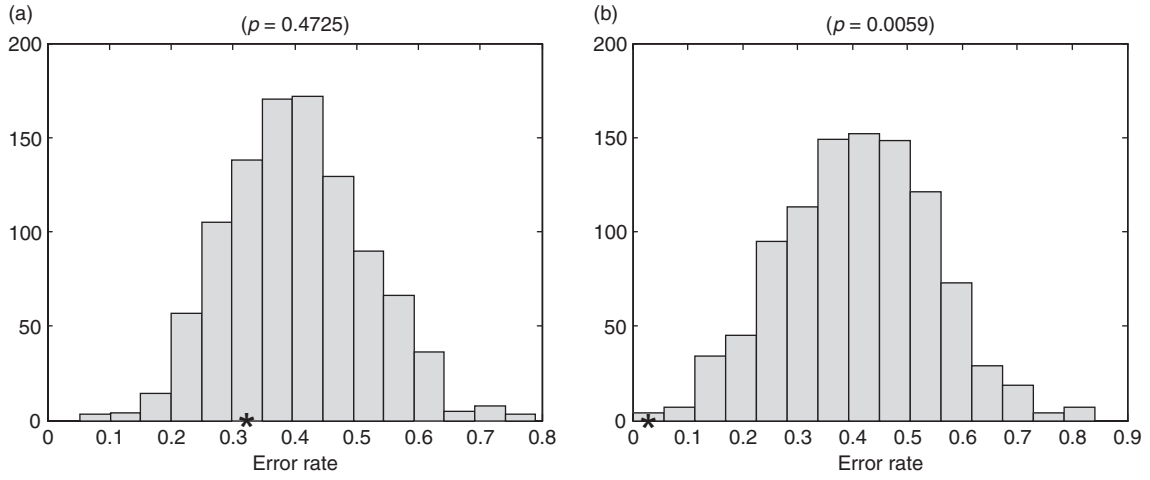

Figure 11 Permutation test results for the original multilevel n-PLS-DA model (a) and the optimized multilevel n-PLS-DA model (b).

Besides literature-derived data, the database also contains experimental metabolite concentration data compiled from mass spectra and NMR analyses performed on urine, blood, and CSF. Furthermore, thousands of NMR and MS spectra of purified, reference metabolites are collected. Besides knowing what metabolites are present in biofluids, it is important to know what metabolites can be analyzed with a specific analytical method. As explained earlier, analytical methods with different comprehensiveness can be applied for metabolomics studies. For target methods and target biomarker assays, it is exactly known which metabolites can be measured and identification is not an issue. For class-targeted profiling methods, it is known which class of compounds can be analyzed and databases for these methods can be made based on reference compounds and the HMDB. If unknown metabolites are detected in these methods, one already knows what type of metabolite it is. In most cases, further identification using high-resolution MS and MS/MS is necessary, as will be explained further on. In global profiling methods, such as NMR and GC-MS, metabolites with different chemical structures can be analyzed within a single method. An unknown peak in such a method is by all means a real unknown. For these methods, it is essential that databases are built based on reference compounds. If methods are standardized and widely accepted, it is possible to build open-access databases, such as the Golm Metabolome Database (www.csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) for GC-MS.[88]

Chromatograms of biofluids analyzed by global profiling methods will always contain unknown peaks. For identification of unknown peaks detected by LC-MS, the ideal strategy would be the isolation of the peak by fractionation of the LC eluent and subsequent structure elucidation using NMR and MS/MS, which can be directly coupled.[89] However, as metabolites to be identified are often only present in small amounts in very complex mixtures, fractionation and subsequent NMR analysis are not straightforward. In this case, a combination of LC-MS/MS, MS^n, and high-resolution MS detection can be applied. Valuable information can be obtained by FT-ICR-MS because of its ultra-high resolution and mass accuracy, which allows the derivation of the elemental composition. However, identification based on accurate mass and the resulting elemental composition only is certainly not routine and further experiments, that is, high-resolution MS/MS, are necessary in most cases.[90,91] MS/MS data obtained from unknown peaks can be compared to spectra of authentic compounds compiled in spectral libraries, for example, METLIN database (http://metlin.scripps.edu/), MassBank (www.massbank.jp), HMDB, MoTo database (http://appliedbioinformatics.wur.nl/).

For the identification of unknown compounds detected by GC-MS, fractionation of peaks is not a straightforward option, although preparative GC is possible. Identification can be achieved using chemical ionization to determine the molecular weight and high-resolution MS detection for the determination of the elemental composition of the molecular ion and characteristic fragments. MS/MS experiments can then be used for further structure elucidation. An important current development for identification of unknown peaks detected by GC-MS is algorithm-based substructure recognition from electron impact spectra.[36]

It is important to mention that a metabolite is identified only when it is confirmed by analyzing authentic standards or complete structure elucidation by NMR. One should make a clear distinction between identified metabolites and tentatively assigned metabolites. In practice, the real identification of an unknown metabolite is a time-consuming and expensive job with a low rate of success.

4.09.5.2 Human Example Study

Metabolites were annotated using an in-house metabolite database containing retention time information, electron impact MS spectra (GC-MS data), ion trap MS/MS spectra (LC-MS), and accurate mass data (LC-MS) of reference substances. The confidence of identification is 100% unless indicated otherwise. Accurate mass MS and MS/MS data of reference substances and metabolites in the study samples were acquired using LTQ-FT and LTQ-Orbitrap instruments.

4.09.5.2.1 Results

The metabolites with the 10 highest absolute regression coefficients per time point (see also **Table 2**) were selected from the LC-MS polar and GC-MS global n-PLS-DA models as being most discriminative between subjects treated with diclofenac and placebo. This resulted in a total of 15 metabolites from the GC-MS global data set and a total of 24 metabolites from the LC-MS polar data set for metabolite identification. Ultimately, 69% of the selected metabolites could be identified (14 out of 15 in the GC-MS global data set; 13 out of 24 in the LC-MS polar data set). **Table 3** lists these most discriminating metabolites that could be identified.

Table 3 Overview of most discriminating metabolites, ranked according to their importance to the model, and their metabolite response in the OGTT time course

	Metabolite	*Response type*
GC-MS global	Uric acid	A
	1,2-Diglyceride (C36:2)	A
	Proline	A
	Isoleucine	A
	1-Aminocyclopentanecarboxylic acid	A
	Threonine	A
	4-Hydroxyproline	A
	2,3,4-Trihydroxybutanoic acid	A
	Aminoadipic acid	A
	Arabitol, ribitol, or xylitol	A
	Ornithine	A
	Mannose or galactose	A
	Palmitoleic acid (C16:1)	A
	Palmitic acid (C16:0)	A
LC-MS polar	Isoleucine	A
	Glycine	B
	2-Amino-2-methyl butanoic acid	A
	5-Oxoproline	B
	1-Aminocyclopentanecarboxylic acid	A
	4-Hydroxyproline	A
	Isoleucine and leucine (not resolved)	A
	Hippuric acid	A
	5-Oxoproline (acetonitrile adduct)	B
	Aspartic acid	B
	Glutamic acid	B
	Citric acid	A

A, contributed to treatment differences at each time point of the time course (**Figure 7**);
B, contributed to treatment differences in the second part of the time course (**Figure 9**).

4.09.6 Interpretation and Visualization

The transformation from statistical results derived from metabolomics data into biological knowledge is still in its infancy. Many bioinformatics strategies dealing with transcriptomics data have been reported in the literature. However, tools for the biological interpretation of metabolomics data are scarce at the moment. Pathway tools such as Pathvisio (open source, see www.pathvisio.org) and biological network tools such as Metacore (GeneGo Inc., St. Joseph, MI, USA) and IPA (Ingenuity Systems, Redwood City, CA, USA) provide a first attempt to connect these components but are not (yet) up to the task. Several challenges have to be faced when translating metabolomics results into biology. First of all, metabolomics data in human nutritional studies are often generated in plasma and urine matrices. The existing pathway and biological network tools deal with intracellular pathways and processes, but this cannot be directly translated into biology of the biofluids that represents whole body metabolism. The 'intrinsic' biological function of glucose, for example, is as an energy source, whereas increased glucose concentrations in urine are an indicator for diabetes and increased concentrations in plasma might be related to the postprandial state and/or insulin (in)sensitivity. Many other metabolites have less known or more complex functions. Intraorgan concentrations have different relevance, and even intracellular compartmentalization plays a role. The HMDB database (http://www.hmdb.ca/) provides a treasure of information on metabolite properties. NuGOwiki (http://www.nugowiki.org/) potentially adds to this knowledge base.

Furthermore, it is not possible to simply compare metabolic organ changes with plasma changes. The body strives to maintain homeostasis, and organs need to work hard to keep plasma concentrations between acceptable boundaries, thereby showing much more fluctuation than for example in the liver. Inverse correlations may even be observed: Oxidative stress produces a sharp drop of glutathione (GSH) in the liver but owing to liver damage, leaking of GSH may increase its concentration in plasma. Urine functions as an accumulating waste basket of exogenous compounds and their metabolites, whereas plasma levels of these compounds need to be as low as possible. So, translation is not straightforward. Statistical relationships between various compartments should therefore be treated with care.

Another important concern is the interactions between gut flora and host metabolism. The large-bowel microflora produces metabolic signals that might overwhelm the true metabolic signals of nutrients in human biofluids.[92,93] Furthermore, food will be metabolized in macronutrients that are partly equal to endogenous metabolites. Nutritional researchers like to understand the effects of nutrition on endogenous metabolic regulation and their impact on health. How to separate the exogenous from endogenous metabolomes? Metabolomics reveals both, which complicates the interpretation. It is already shown for instance that consumption of different diets leads to different metabolic profiles in different populations; increases of specific metabolites could be correlated to a high-meat, vegetarian, Atkins, or high-fish diet.[94] It is also shown that the metabolic variability remains under the consumption of a standard diet. A reduction in interindividual variation was observed only in urine after a short-time consumption of standard diet and not in plasma or saliva.[95] Moreover, it will not be simple to understand the separate effects of age, gender, physical activity, stress, drugs, region, etc. Clearly, significant effort is required before results from chemometrics can straightforwardly be translated into biological knowledge. Nevertheless, to make nutritional metabolomics or systems biology a real success, biological understanding of the changes detected is crucial.

4.09.6.1 Human Example Study

Only identified metabolites were used for further interpretation of the statistical analysis. Detailed pathway and biological network analysis was performed in Metacore version 4.3 (GeneGo Inc., St. Joseph, MI, USA). Only curated interactions were used for biological network analysis. The following metabolites were not available in Metacore and therefore not used for pathway and network analysis: 1,2-diglyceride (C36:2), 2,3,4-trihydroxybutanoic acid, and 2-amino-2-methyl butanoic acid, 1-aminocyclopentanecarboxylic acid. Pathway maps were edited in Mapeditor (GeneGo Inc., St. Joseph, MI, USA) version 2.1.0.

4.09.6.1.1 General interpretation

Most of the selected metabolites (81%, **Table 3**) showed a difference in offset that is constant during the OGTT time course (**Figure 7**, response type A). In other words, these metabolites are discriminating between the treated and untreated subjects independent of time during the OGTT time course. This indicates that only minor differences exist between the treatment groups, which can be identified only by repeated measurements. Indeed, time-independent PLS-DA analysis yielded a similar error rate (11%). Several amino acids ($n = 6$), organic acids ($n = 7$), carbohydrates ($n = 2$), and fatty acids and lipids ($n = 3$) were categorized with a response type A. The majority of the response type A metabolites showed a decreased concentration in plasma in response to diclofenac treatment compared to subjects treated with placebo (results not shown).

Some of the selected metabolites (19%) showed contribution to treatment differences only in the second part of the OGTT time course (**Figure 9**, response type B). This indicates that these four metabolites differed between treatments only when challenging the metabolic situation, leading to alterations in dynamic response to the perturbation. Indeed, time-independent PLS-DA analysis increased the error rate of the LC-MS polar model (26%). The amino acids glycine, aspartic acid, and glutamic acid and the organic acid 5-oxoproline were categorized with a response type B. All response type B metabolites showed higher concentrations in the diclofenac-treated group.

Some metabolites were identified as being discriminating between the treatments in data from both analytical platforms (isoleucine, 1-aminocyclopentanecarboxylic acid, and 4-hydroxyproline), validating their contribution to the differences between the treatment groups.

4.09.6.1.2 Detailed pathway and biological network analysis

Detailed pathway and network analysis identified the modes of action of diclofenac (**Figure 12(a)**). Diclofenac is known to inhibit and activate several enzymes and transporters.[96–104] The inhibition of the enzyme aminopeptidase N (CD13) by diclofenac might explain the lower plasma concentrations of the neutral amino acids L-isoleucine, L-threonine, and L-leucine. CD13 is a broad-specificity aminopeptidase that cleaves specifically the N-terminal bound neutral amino acids from oligopeptides. Also most of the other neutral amino acids measured showed a lower plasma concentration in diclofenac-treated subjects (L-valine, L-serine, L-cysteine, L-phenylalanine, L-tyrosine, and L-tryptophan, but not L-alanine and L-methionine), whereas most of the basic or acidic plasma amino acids (L-aspartic acid, L-glutamic acid, L-asparagine, and L-histidine) did not show this concentration difference (exception is L-lysine). This further confirms that inhibition of CD13 might explain the differences found in plasma amino-acid concentrations between the treatment groups. Besides its role in the digestion of peptides, CD13 is also known to significantly suppress inflammatory immune responses.[105,106]

Furthermore, diclofenac is known to increase the concentration of kynurenic acid, an endogenous antagonist of the *N*-methyl-D-aspartate receptor (NMDA receptor), having therefore analgesic properties.[107,108] The NMDA receptor is a glutamate receptor, which requires binding of glutamate, aspartate, and the coagonist glycine derived from blood for its efficient opening.[105] The NMDA receptor is activated by insulin.[109] This might explain the concentration differences of the amino acids L-glutamate, L-aspartate, and L-glycine that develop at 90–120 min after glucose intake (metabolites with response type B). Insulin activates the NMDA receptor of placebo-treated subjects in response to glucose intake, whereas the NMDA receptor of diclofenac-treated subjects will be insensitive to insulin because of the increased levels of the antagonist kynurenic acid (**Figure 12(b)**).

Besides the identification of metabolite changes likely caused by the modes of action of diclofenac, metabolite changes could be identified that were associated with metabolic effects caused by modulation of the inflammatory status. Elevated serum uric acid levels are associated with metabolic syndrome, insulin resistance, and type 2 diabetes.[110,111] **Figure 12(c)** presents an overview of the modes of action of uric acid and its relation to glucose metabolism and inflammation. Uric acid inhibits endothelial nitric oxide bioavailability, which is required for insulin to stimulate glucose uptake in cells. Besides its role in the development of insulin resistance, elevated levels of uric acid are associated with inflammation, as serum uric acid has been suggested as a sensitive marker for vascular inflammation.[110–112] The diclofenac treatment resulted in lower levels of uric acid, suggesting that vascular inflammation might be lowered, contributing to increased insulin sensitivity in these subjects.

Finally, L-5-oxoproline (pyroglutamic acid) is an intermediate of GSH synthesis via the intracellular γ-glutamylcysteine cycle. **Figure 12(d)** presents an overview of the GSH synthesis pathway and its relation to glucose and insulin. Higher clearance of plasma L-5-oxoproline represents a lower GSH synthesis.[113] GSH synthesis is predominantly regulated by the activity of γ-glutamylcysteine synthetase (γ-GCS). Interestingly, insulin increases and glucose decreases the regulation of GSH synthesis by GCS.[114–116] In the current study, the control group showed a clear decrease of 5-oxoproline concentration at 120 min after glucose intake, whereas 5-oxoproline concentration in the diclofenac-treated group remained at similar levels compared to previous time points (response type B metabolite). GSH, measured with the LC-MS polar platform, showed a similar dynamic response to glucose as 5-oxoproline. This indicates that diclofenac treatment resulted in a higher GSH synthesis response after the glucose bolus compared to placebo, suggesting improved insulin sensitivity in diclofenac-treated subjects.

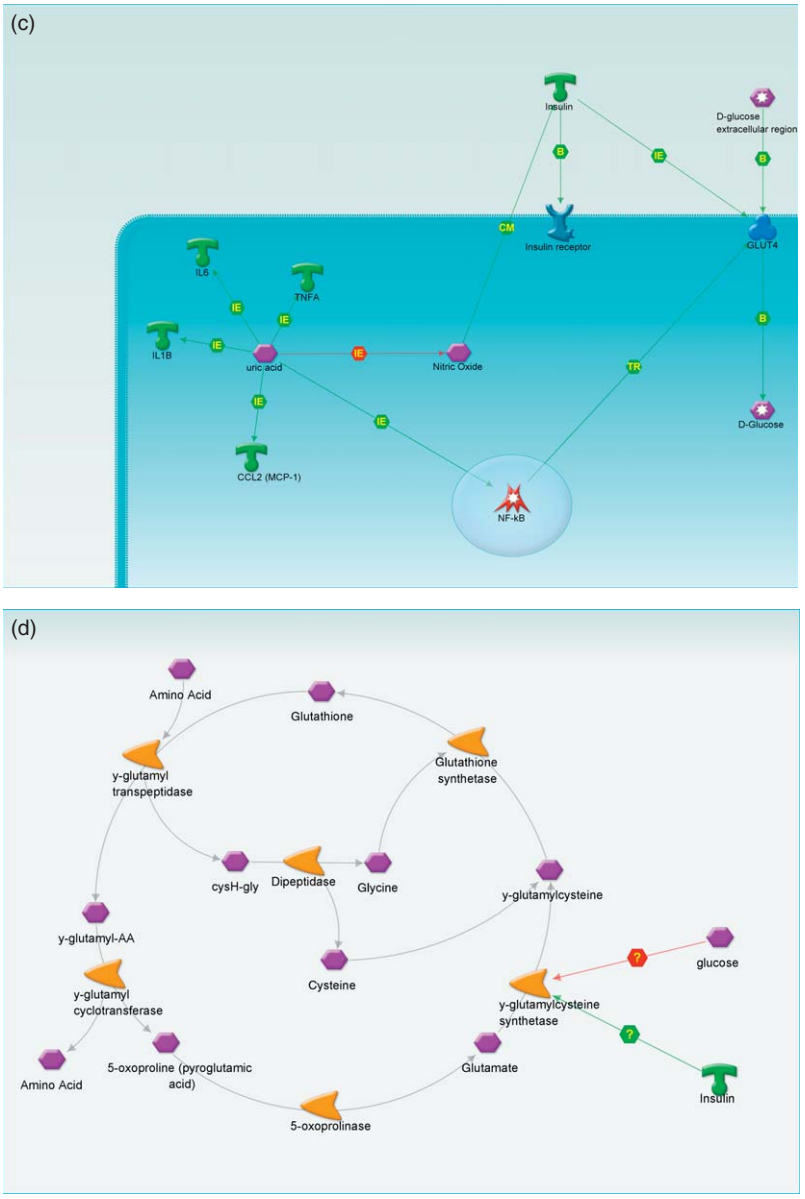

Figure 12 (a) Modes of action of the nonsteroidal antiinflammatory compound diclofenac. (b) Diclofenac and the NMDA receptor. Insulin activates the action of the NMDA receptor, which in turn needs glycine, glutamic acid, and/or aspartic acid for its activation. Diclofenac increases concentrations of the endogenous metabolite kynurenic acid, which blocks the NMDA receptor. (c) Uric acid and its connection to glucose metabolism and inflammation. High levels of uric acid result in lowering of concentrations of nitric oxide (Nitric oxide is needed for the activation of insulin), are associated with insulin resistance, and result in higher expression of the proteins TNFA, Il6, Il1B, and MCP-1 and the transcription factor NF-κB. (d) GSH synthesis pathway and its connection to glucose and insulin. High levels of glucose inhibit and high levels of insulin activate GSH synthesis via the enzyme γ-GCS (γ-glutamylcysteine synthetase). Connection arrows that are red represent inhibition and those that are green represent activation through binding (B), covalent modifications (CM), competition (CN), influence on expression (IE), transcription regulation (TR), unspecified (?), and cleavage (C). Purple hexagons represent metabolites that have been measured in the metabolomics platforms (GC-MS global and LC-MS polar); purple hexagons with white star represent metabolites that are found to be most discriminative between subjects treated with diclofenac and placebo; purple hexagons with white 'no' sign were not measured in one of the metabolomics platforms. Blue arrows downwards indicate that lower plasma concentration levels were found in the diclofenac-treated group and red arrows upwards indicate that higher plasma concentration levels were found in the diclofenac-treated group. ACCN 3, amiloride-sensitive cation channel 3; CCL2/MCP-1, monocyte chemoattractant protein-1; CD13, aminopeptidase N; COX, prostaglandin G/H synthase; Cyp3A4, cytochrome P450 3A4; DPP4, dipeptidyl peptidase 4; GLUT4, glucose transporter member 4; Il1b, interleukin-1 beta; Il6, interleukin-6; potassium voltage-gated channel subfamily; MCT1, monocarboxylate transporter; SUIS, sucrase-isomaltase, intestinal; TNFA, tumor necrosis factor alpha; UGT, UDP-glucuronosyltransferases 1–7. These figures were created by using MapEditor version 2.1.0 (GeneGo Inc., St. Joseph, MI, USA).

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Biographical Sketches

Leon Coulier holds an M.Sc. degree in Chemistry and Chemical Technology and received his Ph.D. in heterogeneous catalysis/surface science from the Eindhoven University of Technology, the Netherlands (2001). In 2002, he started his career at TNO Quality of Life, Zeist, the Netherlands, as a project manager/scientist at the Analytical Sciences Department. At TNO, he was dealing with different topics in analytical chemistry, for example, polymers, packaging, food, and proteins. Currently, he is responsible for new developments in the field of metabolite analysis, mostly MS-based analytical methods, and the management of metabolomics studies.

Suzan Wopereis received her Ph.D. in the Medical Sciences from the Radboud University (Nijmegen), the Netherlands (2006). During her Ph.D. program, she worked in the field of inborn errors of metabolism where she had her first experiences with several -omics techniques such as proteomics, metabolomics, and genome-wide screening techniques. After that, she started as a scientist at TNO Quality of Life. She is working in a bioinformatics research group active in the field of nutrigenomics and nutritional systems biology, aiming at approaching the biological effects of nutrition on health and disease prevention with -omics techniques. Suzan is responsible for the biological analysis of metabolomics data, in which she closely collaborates with biostatisticians, analytical chemists, and biologists.

Upon her graduation in human nutrition and health at Wageningen University in 1999, Carina Rubingh (1976) started working at TNO Quality of Life, Zeist, The Netherlands. She received her M.Sc. degree in biostatistics in 2004 from the University of Hasselt, Belgium. In 2005, while still working at TNO, she started her Ph.D. at the University of Amsterdam, the Netherlands, on the subject of analysis and validation of metabolomics data. Currently, she is working as a biostatistician in the product group 'Analytical Information Sciences', performing both univariate and multivariate statistics. Her main focus is on data analysis of metabolomics studies, experimental design, and statistical consultancy.

Henk Hendriks (1957) studied Biology at State University Utrecht and received his Ph.D. from the State University Leiden in 1991. In 1986, he started working at TNO (IVEG, Rijswijk) in the Department of Dementia and Cell Physiology. At present, he is working at TNO Quality of Life in Zeist as a senior project leader – Human Volunteer Studies (Department Biosciences). Henk Hendriks' special fields of interest are biomedical aspects of moderate alcohol consumption in man, nutrition and coronary heart disease risk factors, functional food claim support, and novel food safety evaluation.

Marijana Radonjić was born on the 24th of October 1976 in Belgrade (Yugoslavia, Serbia). Upon graduation at The First Belgrade Gymnasium in 1995, she studied Molecular Biology and Physiology at the University of Belgrade and obtained B.Sc./M.Sc. degree in Applied Biochemistry in 2001. From 2001 to 2006, she studied genomics approaches in the field of eukaryotic transcription regulation in the Department of Physiological Chemistry, Utrecht University, the Netherlands. For this research, she received a Ph.D. degree in September 2006. Since 2006, Marijana is employed by the Dutch National Institute for Applied Sciences (TNO), Business Unit Biosciences, Department of Physiological Genomics, as a research scientist responsible for analysis and integration of -omics data in nutritional and toxicological studies.

Renger Jellema, Ph.D., TNO Quality of Life, has a track record of more than 10 years working in the field of chemometrics. His roots are in analytical chemistry for which he obtained a bachelor degree in 1992. Renger studied chemistry at the University of Nijmegen (Radboud University), which he finished in 1995. Subsequently, he did his Ph.D. at the University of Amsterdam in collaboration with the steel company Corus. After a short appointment at the Central Bureau of Statistics (CBS), he obtained his current position at TNO Quality of Life, Zeist where he is currently working in the field of chemometrics as Product Manager of the product group 'Analytical Information Sciences'. The main topics of interest in chemometrics are experimental design, application and development of methods and tools for data preprocessing, multivariate statistics, and visualization.

Comparative Modeling of Drug Target Proteins☆

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☆*Change History:* August 2014. B Webb updated the reference section and brought the entire text up to date.
H Fan updated section 'Comparative Models versus Experimental Structures in Virtual Screening' with new studies, and removed the old Figure 5.
N Khuri added new studies to section 'Use of Comparative Models to Obtain Novel Drug Leads.'
U Pieper updated section 'Automation and Availability of Resources for Comparative Modeling and Ligand Docking,' Table 1, and Figure 5.
GQ Dong added a new section 'Final Assessment of the Fold (Model selection).'

Introduction

Structure-Based Drug Discovery

Structure-based or rational drug discovery has already resulted in a number of drugs on the market and many more in the development pipeline.[1–4] Structure-based methods are now routinely used in almost all stages of drug development, from target identification to lead optimization.[5–8] Central to all structure-based discovery approaches is the knowledge of the three-dimensional (3D) structure of the target protein or complex because the structure and dynamics of the target determine which ligands it binds. The 3D structures of the target proteins are best determined by experimental methods that yield solutions at atomic resolution, such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy.[9] While developments in the techniques of experimental structure determination have enhanced the applicability, accuracy, and speed of these structural studies,[10,11] structural characterization of sequences remains an expensive and time-consuming task.

The Sequence–Structure Gap

The publicly available Protein Data Bank (PDB)[12] currently contains ~92 000 structures and grows at a rate of approximately 40% every 2 years. On the other hand, the various genome-sequencing projects have resulted in over 40 million sequences, including the complete genetic blueprints of humans and hundreds of other organisms.[13,14] This achievement has resulted in a vast collection of sequence information about possible target proteins with little or no structural information. Current statistics show that the structures available in the PDB account for less than 1% of the sequences in the UniProt database.[13] Moreover, the rate of growth of the sequence information is more than twice that of the structures, and is expected to accelerate even more with the advent of readily available next-generation sequencing technologies. Due to this wide sequence–structure gap, reliance on experimentally determined structures limits the number of proteins that can be targeted by structure-based drug discovery.

Structure Prediction Addresses the Sequence–Structure Gap

Fortunately, domains in protein sequences are gradually evolving entities that can be clustered into a relatively small number of families with similar sequences and structures.[15,16] For instance, 75–80% of the sequences in the UniProt database have been grouped into fewer than 15 000 domain families.[17,18] Similarly, all the structures in the PDB have been classified into about 1000 distinct folds.[19,20] Computational protein structure prediction methods, such as threading[21] and comparative protein structure modeling,[22,23] strive to bridge the sequence–structure gap by utilizing these evolutionary relationships. The speed, low cost, and relative accuracy of these computational methods have led to the use of predicted 3D structures in the drug discovery process.[24,25] The other class of prediction methods, *de novo* or *ab initio* methods, attempts to predict the structure from sequence alone, without reliance on evolutionary relationships. However, despite progress in these methods,[26–28] especially for small proteins with fewer than 100 amino acid residues, comparative modeling remains the most reliable method of predicting the 3D structure of a protein, with an accuracy that can be comparable to a low-resolution, experimentally determined structure.[9]

The Basis of Comparative Modeling

The primary requirement for reliable comparative modeling is a detectable similarity between the sequence of interest (target sequence) and a known structure (template). As early as 1986, Chothia and Lesk[29] showed that there is a strong correlation between sequence and structural similarities. This correlation provides the basis of comparative modeling, allows a coarse assessment of model errors, and also highlights one of its major challenges: modeling the structural differences between the template and target structures[30] (**Figure 1**).

Comparative Modeling Benefits from Structural Genomics

Comparative modeling stands to benefit greatly from the structural genomics initiative.[31] Structural genomics aims to achieve significant structural coverage of the sequence space with an efficient combination of experimental and prediction methods.[32] This goal is pursued by careful selection of target proteins for structure determination by X-ray crystallography and NMR spectroscopy, such that most other sequences are within 'modeling distance' (e.g., >30% sequence identity) of a known structure.[15,16,31,33] The expectation is that the determination of these structures combined with comparative modeling will yield useful structural information for the largest possible fraction of sequences in the shortest possible timeframe. The impact of structural genomics is illustrated by comparative modeling based on the structures determined by the New York Structural Genomics Research Consortium. For each new structure without a close homolog in the PDB, on average, 3500 protein sequences without any prior structural characterization could be modeled at least at the level of the fold.[34] Thus, the structures of most proteins will eventually be predicted by computation, not determined by experiment.

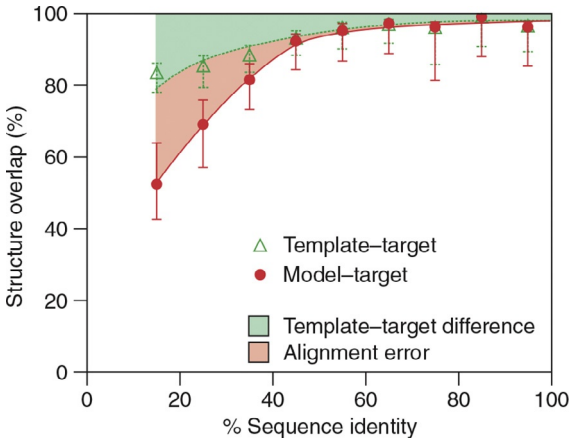

Figure 1 Average model accuracy as a function of sequence identity.[30] As the sequence identity between the target sequence and the template structure decreases, the average structural similarity between the template and the target also decreases (dashed line, triangles).[29] Structural overlap is defined as the fraction of equivalent C^{α} atoms. For the comparison of the model with the actual structure (filled circles), two C^{α} atoms were considered equivalent if they belonged to the same residue and were within 3.5 Å of each other after least-squares superposition. For comparisons between the template structure and the actual target structure (triangles), two C^{α} atoms were considered equivalent if they were within 3.5 Å of each other after alignment and rigid-body superposition. The difference between the model and the actual target structure is a combination of the target–template differences (green area) and the alignment errors (red area). The figure was constructed by calculating 3993 comparative models based on a single template of varying similarity to the targets. All targets had known (experimentally determined) structures.[30]

Outline

In this review, we begin by describing the various steps involved in comparative modeling. Next, we emphasize two aspects of model refinement, loop modeling and side-chain modeling, due to their relevance in ligand docking and rational drug discovery. We then discuss the errors in comparative models. Finally, we describe the role of comparative modeling in drug discovery, focusing on ligand docking against comparative models. We compare successes of docking against models and X-ray structures, and illustrate the computational docking against models with a number of examples. We conclude with a summary of topics that will impact on the future utility of comparative modeling in drug discovery, including an automation and integration of resources required for comparative modeling and ligand docking.

Steps in Comparative Modeling

Comparative modeling consists of four main steps[23] (**Figure 2(a)**): (1) fold assignment that identifies similarity between the target sequence of interest and at least one known protein structure (the template); (2) alignment of the target sequence and the template(s); (3) building a model based on the alignment with the chosen template(s); and (4) predicting model errors.

Fold Assignment and Sequence–Structure Alignment

Although fold assignment and sequence–structure alignment are logically two distinct steps in the process of comparative modeling, in practice almost all fold assignment methods also provide sequence–structure alignments. In the past, fold assignment methods were optimized for better sensitivity in detecting remotely related homologs, often at the cost of alignment accuracy. However, recent methods simultaneously optimize both the sensitivity and alignment accuracy. Therefore, in the following discussion, we will treat fold assignment and sequence–structure alignment as a single protocol, explaining the differences as needed.

Fold assignment

As mentioned earlier, the primary requirement for comparative modeling is the identification of one or more known template structures with detectable similarity to the target sequence. The identification of suitable templates is achieved by scanning structure databases, such as PDB,[12] SCOP,[19] DALI,[36] and CATH,[20] with the target sequence as the query. The detected similarity is usually quantified in terms of sequence identity or statistical measures, such as *E*-value or *z*-score, depending on the method used.

Three levels of similarity

Sequence–structure relationships are coarsely classified into three different regimes in the sequence similarity spectrum: (1) the easily detected relationships characterized by >30% sequence identity; (2) the 'twilight zone,'[37] corresponding to relationships

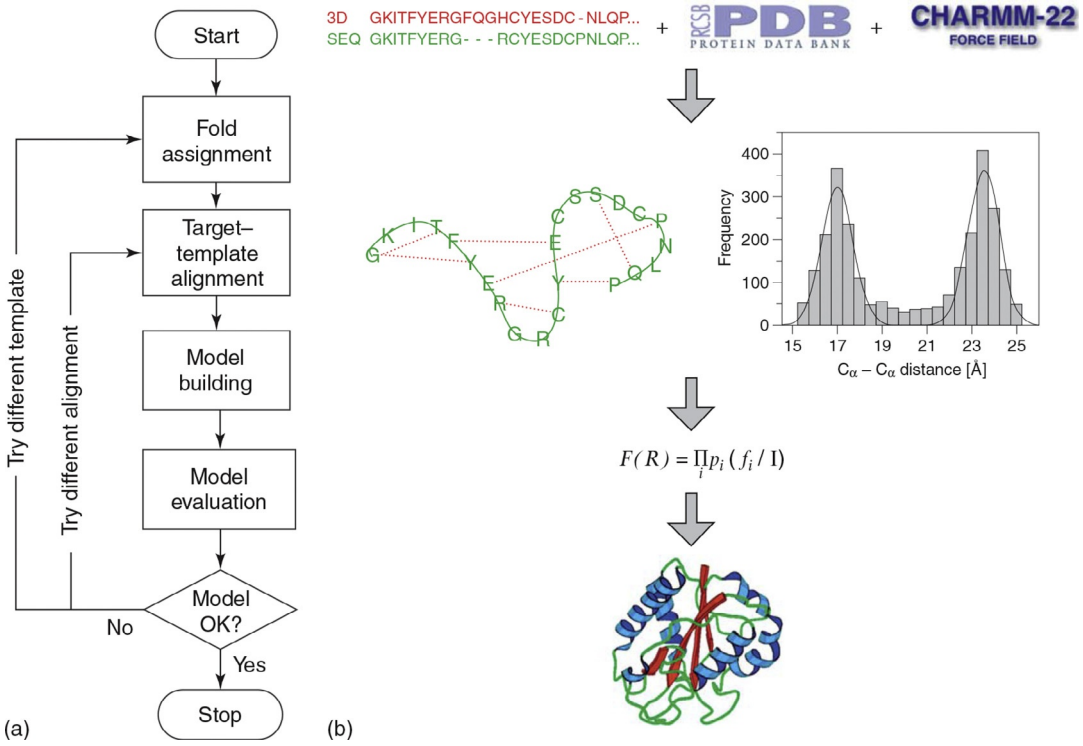

Figure 2 Comparative protein structure modeling. (a) A flowchart illustrating the steps in the construction of a comparative model.[23] (b) Description of comparative modeling by extraction of spatial restraints as implemented in Modeller.[35] By default, spatial restraints in Modeller include: (1) homology-derived restraints from the aligned template structures; (2) statistical restraints derived from all known protein structures; and (3) stereochemical restraints from the CHARMM-22 molecular mechanics force field. These restraints are combined into an objective function that is then optimized to calculate the final 3D model of the target sequence.

with statistically significant sequence similarity in the 10–30% range; and (3) the 'midnight zone,'[37] corresponding to statistically insignificant sequence similarity.

Sequence–sequence methods

For closely related protein sequences with identities higher than 30–40%, the alignments produced by all methods are almost always largely correct. The quickest way to search for suitable templates in this regime is to use simple pairwise sequence alignment methods such as SSEARCH,[38] BLAST,[39] and FASTA.[38] Brenner et al. showed that these methods detect only ~18% of the homologous pairs at less than 40% sequence identity, while they identify more than 90% of the relationships when sequence identity is between 30% and 40%.[40] Another benchmark, based on 200 reference structural alignments with 0–40% sequence identity, indicated that BLAST is able to correctly align only 26% of the residue positions.[41]

Sequence–profile methods

The sensitivity of the search and accuracy of the alignment become progressively difficult as the relationships move into the twilight zone.[37,42] A significant improvement in this area was the introduction of profile methods by Gribskov and co-workers.[43] The profile of a sequence is derived from a multiple sequence alignment and specifies residue-type occurrences for each alignment position. The information in a multiple sequence alignment is most often encoded as either a position-specific scoring matrix (PSSM)[39,44,45] or as a hidden Markov model (HMM).[46,47] To identify suitable templates for comparative modeling, the profile of the target sequence is used to search against a database of template sequences. The profile–sequence methods are more sensitive in detecting related structures in the twilight zone than the pairwise sequence-based methods; they detect approximately twice the number of homologs under 40% sequence identity.[41,48,49] The resulting profile–sequence alignments correctly align approximately 43–48% of residues in the 0–40% sequence identity range;[41,50] this number is almost twice as large as that of the pairwise sequence methods. Frequently used programs for profile–sequence alignment are PSI-BLAST,[39] SAM,[51] HMMER,[46] HHsearch,[52] HHBlits,[53] and BUILD_PROFILE.[54]

Profile–profile methods

As a natural extension, the profile–sequence alignment methods have led to profile–profile alignment methods that search for suitable template structures by scanning the profile of the target sequence against a database of template profiles, as opposed to a database of template sequences. These methods have proven to include the most sensitive and accurate fold assignment and

alignment protocols to date.[50,55–57] Profile–profile methods detect ~28% more relationships at the superfamily level and improve the alignment accuracy by 15–20% compared to profile–sequence methods.[50,58] There are a number of variants of profile–profile alignment methods that differ in the scoring functions they use.[50,55,58–64] However, several analyses have shown that the overall performances of these methods are comparable.[50,55–57] Some of the programs that can be used to detect suitable templates are FFAS,[65] SP3,[58] SALIGN,[50] HHBlits,[53] HHsearch,[52] and PPSCAN.[54]

Sequence–structure threading methods

As the sequence identity drops below the threshold of the twilight zone, there is usually insufficient signal in the sequences or their profiles for the sequence-based methods discussed above to detect true relationships.[48] Sequence–structure threading methods are most useful in this regime as they can sometimes recognize common folds, even in the absence of any statistically significant sequence similarity.[21] These methods achieve higher sensitivity by using structural information derived from the templates. The accuracy of a sequence–structure match is assessed by the score of a corresponding coarse model and not by sequence similarity, as in sequence comparison methods.[21] The scoring scheme used to evaluate the accuracy is either based on residue substitution tables dependent on structural features such as solvent exposure, secondary structure type, and hydrogen bonding properties,[58,66–68] or on statistical potentials for residue interactions implied by the alignment.[69–73] The use of structural data does not have to be restricted to the structure side of the aligned sequence–structure pair. For example, SAM-T08 makes use of the predicted local structure for the target sequence to enhance homolog detection and alignment accuracy.[74] Commonly used threading programs are GenTHREADER,[66,75] 3D-PSSM,[76] FUGUE,[68] SP3,[58] SAM-T08 multitrack HMM,[67,74,77] and MUSTER.[78]

Iterative sequence–structure alignment

Yet another strategy is to optimize the alignment by iterating over the process of calculating alignments, building models, and evaluating models. Such a protocol can sample alignments that are not statistically significant and identify the alignment that yields the best model. Although this procedure can be time-consuming, it can significantly improve the accuracy of the resulting comparative models in difficult cases.[79]

Most Alignment Errors are Unrecoverable

Regardless of the method used, searching in the twilight and midnight zones of the sequence–structure relationship often results in false negatives, false positives, or alignments that contain an increasingly large number of gaps and alignment errors. Improving the performance and accuracy of methods in this regime remains one of the main tasks of comparative modeling today.[80] It is imperative to calculate an accurate alignment between the target–template pair. Although some progress has been made recently,[81] comparative modeling can rarely recover from an alignment error.[82]

Template Selection

After a list of all related protein structures and their alignments with the target sequence have been obtained, template structures are prioritized depending on the purpose of the comparative model. Template structures may be chosen purely based on the target–template sequence identity or a combination of several other criteria, such as experimental accuracy of the structures (resolution of X-ray structures, number of restraints per residue for NMR structures), conservation of active-site residues, holo-structures that have bound ligands of interest, and prior biological information that pertains to the solvent, *p*H, and quaternary contacts. It is not necessary to select only one template. In fact, the use of several templates approximately equidistant from the target sequence generally increases the model accuracy.[83,84]

Model Building

Three Approaches to Comparative Model Building

Once an initial target–template alignment is built, a variety of methods can be used to construct a 3D model for the target protein.[23,82,85–88] The original and still widely used method is modeling by rigid-body assembly.[86,87,89] This method constructs the model from a few core regions, and from loops and side chains that are obtained by dissecting related structures. Commonly used programs that implement this method are COMPOSER,[90–93] 3D-JIGSAW,[94] RosettaCM,[81] and SWISS-MODEL.[95] Another family of methods, modeling by segment matching, relies on the approximate positions of conserved atoms from the templates to calculate the coordinates of other atoms.[96–100] An instance of this approach is implemented in SegMod.[99] The third group of methods, modeling by satisfaction of spatial restraints, uses either distance geometry or optimization techniques to satisfy spatial restraints obtained from the alignment of the target sequences with the template structures.[35,101–104] Specifically, Modeller,[35,105,106] our own program for comparative modeling, belongs to this group of methods.

Modeller: Comparative Modeling by Satisfaction of Spatial Restraints

Modeller implements comparative protein structure modeling by the satisfaction of spatial restraints that include: (1) homology-derived restraints on the distances and dihedral angles in the target sequence, extracted from its alignment with the template structures;[35] (2) stereochemical restraints such as bond length and bond angle preferences, obtained from the CHARMM-22 molecular mechanics force field;[107] (3) statistical preferences for dihedral angles and nonbonded interatomic distances, obtained from a representative set of known protein structures;[108] and (4) optional manually curated restraints, such as those from NMR spectroscopy, rules of secondary structure packing, cross-linking experiments, fluorescence spectroscopy, image reconstruction from electron microscopy, site-directed mutagenesis, and intuition (**Figure 2(b)**). The spatial restraints, expressed as probability density functions, are combined into an objective function that is optimized by a combination of conjugate gradients and molecular dynamics with simulated annealing. This model-building procedure is similar to structure determination by NMR spectroscopy.

Relative Accuracy, Flexibility, and Automation

Accuracies of the various model-building methods are relatively similar when used optimally.[109,110] Other factors, such as template selection and alignment accuracy, usually have a larger impact on the model accuracy, especially for models based on less than 30% sequence identity to the templates. However, it is important that a modeling method allows a degree of flexibility and automation to obtain better models more easily and rapidly. For example, a method should allow for an easy recalculation of a model when a change is made in the alignment; it should be straightforward to calculate models based on several templates; and the method should provide tools for incorporation of prior knowledge about the target (e.g., cross-linking restraints and predicted secondary structure).

Refinement of Comparative Models

Protein sequences evolve through a series of amino acid residue substitutions, insertions, and deletions. While substitutions can occur throughout the length of the sequence, insertions and deletions mostly occur on the surface of proteins in segments that connect regular secondary structure segments (i.e., loops). While the template structures are helpful in the modeling of the aligned target backbone segments, they are generally less valuable for the modeling of side chains and irrelevant for the modeling of insertions such as loops. The loops and side chains of comparative models are especially important for ligand docking; thus, we discuss them in the following two sections.

Loop Modeling

Definition of the problem

Loop modeling is an especially important aspect of comparative modeling in the range from 30% to 50% sequence identity. In this range of overall similarity, loops among the homologs vary while the core regions are still relatively conserved and aligned accurately. Loops often play an important role in defining the functional specificity of a given protein, forming the active and binding sites. Loop modeling can be seen as a mini protein folding problem because the correct conformation of a given segment of a polypeptide chain has to be calculated mainly from the sequence of the segment itself. However, loops are generally too short to provide sufficient information about their local fold. Even identical decapeptides in different proteins do not always have the same conformation.[111,112] Some additional restraints are provided by the core anchor regions that span the loop and by the structure of the rest of the protein that cradles the loop. Although many loop-modeling methods have been described, it is still challenging to correctly and confidently model loops longer than approximately 10–12 residues.[105,113,114]

Two classes of methods

There are two main classes of loop-modeling methods: (1) database search approaches that scan a database of all known protein structures to find segments fitting the anchor core regions[98,115]; and (2) conformational search approaches that rely on optimizing a scoring function.[116–118] There are also methods that combine these two approaches.[119,120]

Database-based loop modeling

The database search approach to loop modeling is accurate and efficient when a database of specific loops is created to address the modeling of the same class of loops, such as β-hairpins,[121] or loops on a specific fold, such as the hypervariable regions in the immunoglobulin fold.[115,122] There are attempts to classify loop conformations into more general categories, thus extending the applicability of the database search approach.[123–125] However, the database methods are limited because the number of possible conformations increases exponentially with the length of a loop, and until the late 1990s only loops up to 7 residues long could be modeled using the database of known protein structures.[126,127] However, the growth of the PDB in recent years has largely eliminated this problem.[128]

Optimization-based methods

There are many optimization-based methods, exploiting different protein representations, objective functions, and optimization or enumeration algorithms. The search algorithms include the minimum perturbation method,[129] dihedral angle search through a rotamer library,[114,130] molecular dynamics simulations,[119,131] genetic algorithms,[132] Monte Carlo and simulated annealing,[133–135] multiple-copy simultaneous search,[136] self-consistent field optimization,[137] robotics-inspired kinematic closure[138] and enumeration based on graph theory.[139] The accuracy of loop predictions can be further improved by clustering the sampled loop conformations and partially accounting for the entropic contribution to the free energy.[140] Another way of improving the accuracy of loop predictions is to consider the solvent effects. Improvements in implicit solvation models, such as the Generalized Born solvation model, motivated their use in loop modeling. The solvent contribution to the free energy can be added to the scoring function for optimization, or it can be used to rank the sampled loop conformations after they are generated with a scoring function that does not include the solvent terms.[105,141–143]

Side-Chain Modeling

Fixed backbone

Two simplifications are frequently applied in the modeling of side-chain conformations.[144] First, amino acid residue replacements often leave the backbone structure almost unchanged,[145] allowing us to fix the backbone during the search for the best side-chain conformations. Second, most side chains in high-resolution crystallographic structures can be represented by a limited number of conformers that comply with stereochemical and energetic constraints.[146] This observation motivated Ponder and Richards[147] to develop the first library of side-chain rotamers for the 17 types of residues with dihedral angle degrees of freedom in their side chains, based on 10 high-resolution protein structures determined by X-ray crystallography. Subsequently, a number of additional libraries have been derived.[148–155]

Rotamers

Rotamers on a fixed backbone are often used when all the side chains need to be modeled on a given backbone. This approach reduces the combinatorial explosion associated with a full conformational search of all the side chains, and is applied by some comparative modeling[86] and protein design approaches.[156] However, $\sim$15% of the side chains cannot be represented well by these libraries.[157] In addition, it has been shown that the accuracy of side-chain modeling on a fixed backbone decreases rapidly when the backbone errors are larger than 0.5 Å.[158]

Methods

Earlier methods for side-chain modeling often put less emphasis on the energy or scoring function. The function was usually greatly simplified, and consisted of the empirical rotamer preferences and simple repulsion terms for nonbonded contacts.[151] Nevertheless, these approaches have been justified by their performance. For example, a method based on a rotamer library compared favorably with that based on a molecular mechanics force field,[159] and new methods continue to be based on the rotamer library approach.[160–162] The various optimization approaches include a Monte Carlo simulation,[163] simulated annealing,[164] a combination of Monte Carlo and simulated annealing,[165] the dead-end elimination theorem,[166,167] genetic algorithms,[155] neural network with simulated annealing,[168] mean field optimization,[169] and combinatorial searches.[151,170,171] Several studies focused on the testing of more sophisticated potential functions for conformational search[171,172] and development of new scoring functions for side-chain modeling,[173] reporting higher accuracy than earlier studies.

Errors in Comparative Models

The major sources of error in comparative modeling are discussed in the relevant sections above. The following is a summary of these errors, dividing them into five categories (**Figure 3**).

Selection of Incorrect Templates

This error is a potential problem when distantly related proteins are used as templates (i.e., less than 30% sequence identity). Distinguishing between a model based on an incorrect template and a model based on an incorrect alignment with a correct template is difficult. In both cases, the evaluation methods (below) will predict an unreliable model. The conservation of the key functional or structural residues in the target sequence increases the confidence in a given fold assignment.

Errors due to Misalignments

The single source of errors with the largest impact on comparative modeling is misalignments, especially when the target–template sequence identity decreases below 30%. Alignment errors can be minimized in two ways. Using the profile-based methods

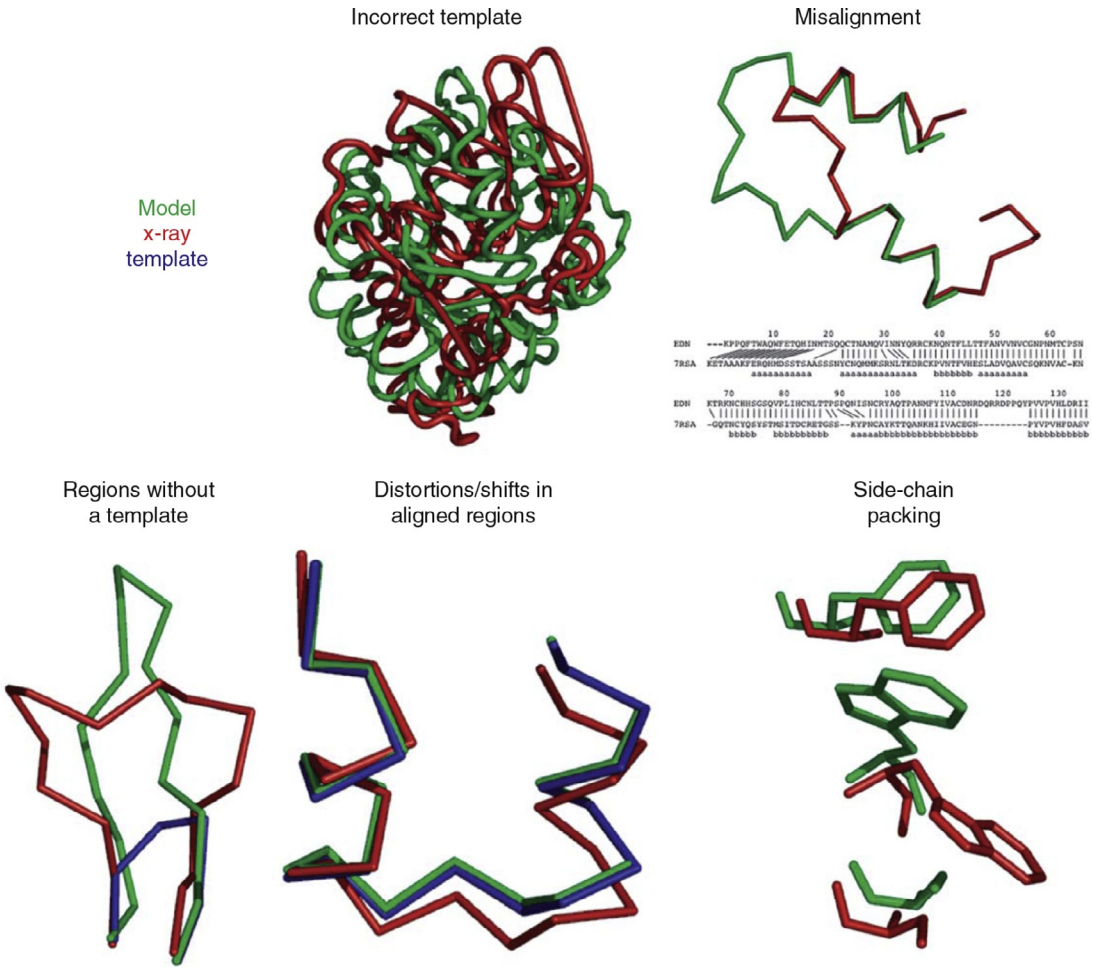

Figure 3 Typical errors in comparative modeling.[23] Shown are the typical sources of errors encountered in comparative models. Two of the major sources of errors in comparative modeling are due to incorrect templates or incorrect alignments with the correct templates. The modeling procedure can rarely recover from such errors. The next significant source of errors arises from regions in the target with no corresponding region in the template, i.e., insertions or loops. Other sources of errors, which occur even with an accurate alignment, are due to rigid-body shifts, distortions in the backbone, and errors in the packing of side chains.

discussed above usually results in more accurate alignments than those from pairwise sequence alignment methods. Another way of improving the alignment is to modify those regions in the alignment that correspond to predicted errors in the model.[83]

Errors in Regions without a Template

Segments of the target sequence that have no equivalent region in the template structure (i.e., insertions or loops) are one of the most difficult regions to model. Again, when the target and template are distantly related, errors in the alignment can lead to incorrect positions of the insertions. Using alignment methods that incorporate structural information can often correct such errors. Once a reliable alignment is obtained, various modeling protocols can predict the loop conformation, for insertions of fewer than 8–10 residues.[105,113,119,174]

Distortions and Shifts in Correctly Aligned Regions

As a consequence of sequence divergence, the main-chain conformation changes, even if the overall fold remains the same. Therefore, it is possible that in some correctly aligned segments of a model, the template is locally different (<3 Å) from the target, resulting in errors in that region. The structural differences are sometimes not due to differences in sequence, but are a consequence of artifacts in structure determination or structure determination in different environments (e.g., packing of subunits in a crystal). The simultaneous use of several templates can minimize this kind of error.[83,84]

Errors in Side-Chain Packing

As the sequences diverge, the packing of the atoms in the protein core changes. Sometimes even the conformation of identical side chains is not conserved – a pitfall for many comparative modeling methods. Side-chain errors are critical if they occur in regions that are involved in protein function, such as active sites and ligand-binding sites.

Prediction of Model Errors

The accuracy of the predicted model determines the information that can be extracted from it. Thus, estimating the accuracy of a model in the absence of the known structure is essential for interpreting it.

Initial Assessment of the Fold

As discussed earlier, a model calculated using a template structure that shares more than 30% sequence identity is indicative of an overall accurate structure. However, when the sequence identity is lower, the first aspect of model evaluation is to confirm whether or not a correct template was used for modeling. It is often the case, when operating in this regime, that the fold assignment step produces only false positives. A further complication is that at such low similarities the alignment generally contains many errors, making it difficult to distinguish between an incorrect template on one hand and an incorrect alignment with a correct template on the other hand. There are several methods that use 3D profiles and statistical potentials,[70,175,176] which assess the compatibility between the sequence and modeled structure by evaluating the environment of each residue in a model with respect to the expected environment, as found in native high-resolution experimental structures. These methods can be used to assess whether or not the correct template was used for the modeling. They include VERIFY3D,[175] Prosa2003,[177,178] HARMONY,[179] ANOLEA,[180] DFIRE,[181] DOPE,[182] QMEAN local,[183] ProQ2,[184] and TSVMod.[185]

Even when the model is based on alignments that have >30% sequence identity, other factors, including the environment, can strongly influence the accuracy of a model. For instance, some calcium-binding proteins undergo large conformational changes when bound to calcium. If a calcium-free template is used to model the calcium-bound state of the target, it is likely that the model will be incorrect, irrespective of the target–template similarity or accuracy of the template structure.[186]

Self-Consistency

The model should also be subjected to evaluations of self-consistency to ensure that it satisfies the restraints used to calculate it. Additionally, the stereochemistry of the model (e.g., bond lengths, bond angles, backbone torsion angles, and nonbonded contacts) may be evaluated using programs such as PROCHECK[187] and WHATCHECK.[188] Although errors in stereochemistry are rare and less informative than errors detected by statistical potentials, a cluster of stereochemical errors may indicate that there are larger errors (e.g., alignment errors) in that region.

Final Assessment of the Fold (Model)

When multiple models are calculated for the target based on a single template or when multiple loops are built for a single or multiple models, it is practical to select a subset of models or loops that are judged to be most suitable for subsequent docking calculations. If some known ligands or other information for the desired model is available, model selection should be guided by this known information.[189] If this extra information is not available, model selection should aim to select the most accurate model. While models or loops can be selected by the energy function used for guiding the building of comparative models or the sampling of loop configurations, using a separate statistical potential for selecting the most accurate models or loops is often more successful.[181,182,190,191]

Evaluation of Comparative Modeling Methods

It is crucial for method developers and users alike to assess the accuracy of their methods. An attempt to address this problem has been made by the Critical Assessment of Techniques for Proteins Structure Prediction (CASP)[192] and in the past by the Critical Assessment of Fully Automated Structure Prediction (CAFASP) experiments,[193] which is now integrated into CASP. However, CASP assesses methods only over a limited number of target protein sequences, and is conducted only every 2 years.[109,194] To overcome this limitation, the new CAMEO web server continuously evaluates the accuracy and reliability of a number of comparative protein structure prediction servers, in a fully automated manner.[195] Every week, CAMEO provides each tested server with the prerelease sequences of structures that are to be shortly released by the PDB. Each server then has 4 days to build and return a 3D model of these sequences. When PDB releases the structures, CAMEO compares the models against the experimentally determined structures, and presents the results on its web site. This enables developers, non-expert users, and reviewers to determine the performance of

the tested prediction servers. CAMEO is similar in concept to two prior such continuous testing servers, LiveBench[194] and EVA.[196,197]

Applications of Comparative Models

There is a wide range of applications of protein structure models (Figure 4).[1,198–204] For example, high- and medium-accuracy comparative models are frequently helpful in refining functional predictions that have been based on a sequence match alone because ligand binding is more directly determined by the structure of the binding site than by its sequence. It is often possible to predict correctly features of the target protein that do not occur in the template structure.[205,206] For example, the size of a ligand may be predicted from the volume of the binding site cleft and the location of a binding site for a charged ligand can be predicted from a cluster of charged residues on the protein. Fortunately, errors in the functionally important regions in comparative models are many times relatively low because the functional regions, such as active sites, tend to be more conserved in evolution than the rest of the fold. Even low-accuracy comparative models may be useful, for example, for assigning the fold of a protein. Fold

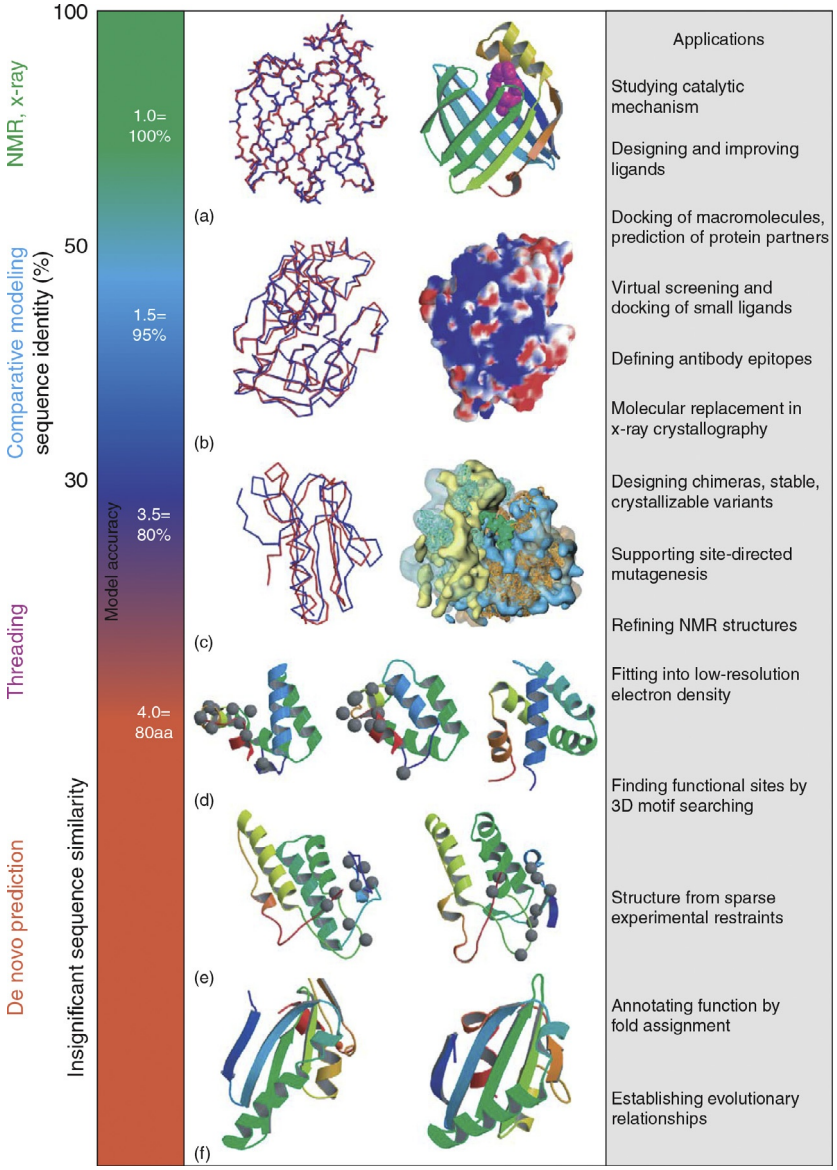

Figure 4 Accuracy and applications of protein structure models.[9] Shown are the different ranges of applicability of comparative protein structure modeling, threading, and *de novo* structure prediction, their corresponding accuracies, and their sample applications.

assignment can be very helpful in drug discovery, because it can shortcut the search for leads by pointing to compounds that have been previously developed for other members of the same family.[207,208]

Comparative Models versus Experimental Structures in Virtual Screening

The remainder of this review focuses on the use of comparative models for ligand docking.[209–211] Comparative protein structure modeling extends the applicability of virtual screening beyond the atomic structures determined by X-ray crystallography or NMR spectroscopy. In fact, comparative models have been used in virtual screening to detect novel ligands for many protein targets,[201] including the G-protein coupled receptors (GPCR),[210,212–223] protein kinases,[224–227] nuclear hormone receptors, and many different enzymes.[228–241] Nevertheless, the relative utility of comparative models versus experimentally determined structures has only been relatively sparsely assessed.[212,224,225,242–244] The utility of comparative models for molecular docking screens in ligand discovery has been documented[245] with the aid of 38 protein targets selected from the 'directory of useful decoys' (DUD).[246] For each target sequence, templates for comparative modeling were obtained from the PDB, including at least one holo (ligand bound) and one apo (ligand free) template structure for each of the eight 10% sequence identity ranges from 20% to 100%. In total, 222 models were generated based on 222 templates for the 38 test proteins using Modeller 9v2.[35] DUD ligands and decoys (98 266 molecules) were screened against the holo X-ray structure, the apo X-ray structure, and the comparative models of each target using DOCK 3.5.54.[247] The accuracy of virtual screening was evaluated by the overall ligand enrichment that was calculated by integrating the area under the enrichment plot (logAUC). A key result was that, for 63% and 79% of the targets, at least one comparative model yielded ligand enrichment better or comparable to that of the corresponding holo and apo X-ray structure.[245] This result indicates that comparative models can be useful docking targets when multiple templates are available. However, it was not possible to predict which model, out of all those used, led to the highest enrichment. Therefore, a 'consensus' enrichment score was computed by ranking each library compound by its best docking score against all comparative models and/or templates. For 47% and 70% of the targets, the consensus enrichment for multiple models was better or comparable to that of the holo and apo X-ray structures, respectively, suggesting that multiple comparative models can be useful for virtual screening.

Use of Comparative Models to Obtain Novel Drug Leads

Despite problems with comparative modeling and ligand docking, comparative models have been successfully used in practice in conjunction with virtual screening to identify novel inhibitors. We briefly review a few of these success stories to highlight the potential of the combined comparative modeling and ligand-docking approach to drug discovery.

Comparative models have been employed to aid rational drug design against parasites for more than 20 years.[132,231,232,240] As early as 1993, Ring et al.[132] used comparative models for computational docking studies that identified low micromolar nonpeptidic inhibitors of proteases in malarial and schistosome parasite lifecycles. Li et al.[231] subsequently used similar methods to develop nanomolar inhibitors of falcipain that are active against chloroquine-resistant strains of malaria. In a study by Selzer et al.[232] comparative models were used to predict new nonpeptide inhibitors of cathepsin L-like cysteine proteases in *Leishmania major*. Sixty-nine compounds were selected by DOCK 3.5 as strong binders to a comparative model of protein cpB, and of these, 21 had experimental IC_{50} values below 100 mmol l^{-1}. Finally, in a study by Que et al.,[240] comparative models were used to rationalize ligand-binding affinities of cysteine proteases in *Entamoeba histolytica*. Specifically, this work provided an explanation for why proteases ACP1 and ACP2 had substrate specificity similar to that of cathepsin B, although their overall structure is more similar to that of cathepsin D.

Enyedy et al.[248] discovered 15 new inhibitors of matriptase by docking against its comparative model. The comparative model employed thrombin as the template, sharing only 34% sequence identity with the target sequence. Moreover, some residues in the binding site are significantly different; a trio of charged Asp residues in matriptase correspond to 1 Tyr and 2 Trp residues in thrombin. Thrombin was chosen as the template, in part because it prefers substrates with positively charged residues at the P1 position, as does matriptase. The National Cancer Institute database was used for virtual screening that targeted the S1 site with the DOCK program. The 2000 best-scoring compounds were manually inspected to identify positively charged ligands (the S1 site is negatively charged), and 69 compounds were experimentally screened for inhibition, identifying the 15 inhibitors. One of them, hexamidine, was used as a lead to identify additional compounds selective for matriptase relative to thrombin. The Wang group has also used similar methods to discover seven new, low-micromolar inhibitors of Bcl-2, using a comparative model based on the NMR solution structure of Bcl-X_L.[233]

Schapira et al.[249] discovered a novel inhibitor of a retinoic acid receptor by virtual screening using a comparative model. In this case, the target (RAR-α) and template (RAR-γ) are very closely related; only three residues in the binding site are not conserved. The ICM program was used for virtual screening of ligands from the Available Chemicals Directory (ACD). The 5364 high-scoring compounds identified in the first round were subsequently docked into a full atom representation of the receptor with flexible side chains to obtain a final set of 300 good-scoring hits. These compounds were then manually inspected to choose the final 30 for testing. Two novel agonists were identified, with 50-nanomolar activity.

Zuccotto et al.[250] identified novel inhibitors of dihydrofolate reductase (DHFR) in *Trypanosoma cruzi* (the parasite that causes Chagas disease) by docking into a comparative model based on ~50% sequence identity to DHFR in *L. major*, a related parasite. The virtual screening procedure used DOCK for rigid docking of over 50 000 selected compounds from the Cambridge Structural

Database (CSD). Visual inspection of the top 100 hits was used to select 36 compounds for experimental testing. This work identified several novel scaffolds with micromolar IC_{50} values. The authors report attempting to use virtual screening results to identify compounds with greater affinity for *T. cruzi* DHFR than human DHFR, but it is not clear how successful they were.

Following the outbreak of the severe acute respiratory syndrome (SARS) in 2003, Anand et al.[251] used the experimentally determined structures of the main protease from human coronavirus (M^{PRO}) and an inhibitor complex of porcine coronavirus (transmissible gastroenteritis virus, TGEV) M^{PRO} to calculate a comparative model of the SARS coronavirus M^{PRO}. This model then provided a basis for the design of anti-SARS drugs. In particular, a comparison of the active site residues in these and other related structures suggested that the AG7088 inhibitor of the human rhinovirus type 2 3C protease is a good starting point for design of anticoronaviral drugs.[252]

Comparative models of protein kinases combined with virtual screening have also been intensely used for drug discovery.[224,225,253–255] The >500 kinases in the human genome, the relatively small number of experimental structures available, and the high level of conservation around the important adenosine triphosphate-binding site make comparative modeling an attractive approach toward structure-based drug discovery.

G protein-coupled receptors are another interesting class of proteins that in principle allow drug discovery through comparative modeling.[212,256–259] Approximately 40% of current drug targets belong to this class of proteins. However, these proteins have been extremely difficult to crystallize and most comparative modeling has been based on the atomic resolution structure of the bovine rhodopsin.[260] Despite this limitation, a rather extensive test of docking methods with rhodopsin-based comparative models shows encouraging results.

The applicability of structure-based modeling and virtual screening has recently been expanded to membrane proteins that transport solutes, such as ions, metabolites, peptides, and drugs. In humans, these transporters contribute to the absorption, distribution, metabolism, and excretion of drugs, and often, mediate drug-drug interactions. Additionally, several transporters can be targeted directly by small molecules. For instance, methylphenidate (Ritalin) inhibiting the norepinephrine transporter (NET) and, consequently, inhibiting the reuptake of norepinephrine, is used in the treatment of attention-deficit hyperactivity disorder (ADHD).[261] Schlessinger et al.[262] predicted 18 putative ligands of human NET by docking 6436 drugs from the Kyoto Encyclopedia of Genes (KEGG DRUG) into a comparative model based on ~25% sequence identity to leucine transporter (LeuT) from *Aquifex aeolicus*. Of these 18 predicted ligands, ten were validated by *cis*-inhibition experiments; five of them were chemically novel. Close examination of the predicted primary binding site helped rationalize interactions of NET with its primary substrate, norepinephrine, as well as positive and negative pharmacological effects of other NET ligands. Subsequently, Schlessinger et al.[263] modeled two different conformations of the human GABA transporter 2 (GAT-2), using the LeuT structures in occluded-outward-facing and outward-facing conformations. Enrichment calculations were used to assess the quality of the models in molecular dynamics simulations and side-chain refinements. The key residue, Glu48, interacting with the substrate was identified during the refinement of the models and validated by site-directed mutagenesis. Docking against two conformations of the transporter enriches for different physicochemical properties of ligands. For example, top-scoring ligands found by docking against the outward-facing model were bulkier and more hydrophobic than those predicted using the occluded-outward-facing model. Among twelve ligands validated in *cis*-inhibition assays, six were chemically novel (e.g., homotaurine). Based on the validation experiments, GAT-2 is likely to be a high-selectivity/low-affinity transporter. Following these two studies, a combination of comparative modeling, ligand docking, and experimental validation was used to rationalize toxicity of an anti-cancer agent, acivicin.[264] The toxic side-effects are thought to be facilitated by the active transport of acivicin through the blood–brain-barrier (BBB) *via* the large-neutral amino acid Transporter 1 (LAT-1). In addition, four small-molecule ligands of LAT-1 were identified by docking against a comparative model based on two templates, the structures of the outward-occluded arginine-bound arginine/agmatine transporter AdiC from *E. coli*[265] and the inward-apo conformation of the amino acid, polyamine, and organo-cation transporter ApcT from *Methanococcus jannaschii*.[266] Two of the four hits, acivicin and fenclonine, were confirmed as substrates by a *trans*-stimulation assay. These studies clearly illustrate the applicability of combined comparative modeling and virtual screening to ligand discovery for transporters.

Future Directions

Although reports of successful virtual screening against comparative models are encouraging, such efforts are not yet a routine part of rational drug design. Even the successful efforts appear to rely strongly on visual inspection of the docking results. Much work remains to be done to improve the accuracy, efficiency, and robustness of docking against comparative models. Despite assessments of relative successes of docking against comparative models and native X-ray structures,[225,244] relatively little has been done to compare the accuracy achievable by different approaches to comparative modeling and to identify the specific structural reasons why comparative models generally produce less accurate virtual screening results than the holo structures. Among the many issues that deserve consideration are the following:

- The inclusion of cofactors and bound water molecules in protein receptors is often critical for success of virtual screening; however, cofactors are not routinely included in comparative models

- Most docking programs currently retain the protein receptor in a rigid conformation. While this approach is appropriate for 'lock-and-key' binding modes, it does not work when the ligand induces conformational changes in the receptor upon binding. A flexible receptor approach is necessary to address such induced-fit cases[267,268]
- The accuracy of comparative models is frequently judged by the C^{α} root mean square error or other similar measures of backbone accuracy. For virtual screening, however, the precise positioning of side chains in the binding site is likely to be critical; measures of accuracy for binding sites are needed to help evaluate the suitability of comparative modeling algorithms for constructing models for docking
- Knowledge of known inhibitors, either for the target protein or the template, should help to evaluate and improve virtual screening against comparative models. For example, comparative models constructed from holo template structures implicitly preserve some information about the ligand-bound receptor conformation
- Improvement in the accuracy of models produced by comparative modeling will require methods that finely sample protein conformational space using a free energy or scoring function that has sufficient accuracy to distinguish the native structure from the nonnative conformations. Despite many years of development of molecular simulation methods, attempts to refine models that are already relatively close to the native structure have met with relatively little success. This failure is likely to be due in part to inaccuracies in the scoring functions used in the simulations, particularly in the treatment of electrostatics and solvation effects. A combination of physics-based energy function with the statistical information extracted from known protein structures may provide a route to the development of improved scoring functions
- Improvements in sampling strategies are also likely to be necessary, for both comparative modeling and flexible docking

Automation and Availability of Resources for Comparative Modeling and Ligand Docking

Given the increasing number of target sequences for which no experimentally determined structures are available, drug discovery stands to gain immensely from comparative modeling and other *in silico* methods. Despite unsolved problems in virtually every step of comparative modeling and ligand docking, it is highly desirable to automate the whole process, starting with the target sequence and ending with a ranked list of its putative ligands. Automation encourages development of better methods, improves their testing, allows application on a large scale, and makes the technology more accessible to both experts and non-specialists alike. Through large-scale application, new questions, such as those about ligand-binding specificity, can in principle be addressed. Enabling a wider community to use the methods provides useful feedback and resources toward the development of the next generation of methods.

There are a number of servers for automated comparative modeling (**Table 1**). However, in spite of automation, the process of calculating a model for a given sequence, refining its structure, as well as visualizing and analyzing its family members in the sequence and structure spaces can involve the use of scripts, local programs, and servers scattered across the internet and not necessarily interconnected. In addition, manual intervention is generally still needed to maximize the accuracy of the models in the difficult cases. The main repository for precomputed comparative models, the Protein Model Portal,[195,198,279] begins to address these deficiencies by serving models from several modeling groups, including the SWISS-MODEL[95] and ModBase[34] databases. It provides access to web-based comparative modeling tools, cross-links to other sequence and structure databases, and annotations of sequences and their models.

A number of databases containing comparative models and web servers for computing comparative models are publicly available. The Protein Model Portal (PMP)[195,198,279] centralizes access to these models created by different methodologies. The PMP is being developed as a module of the Protein Structure Initiative Knowledgebase (PSI KB)[316] and functions as a meta server for comparative models from external databases, including SWISS-MODEL[95] and ModBase,[34] additionally to being a repository for comparative models that are derived from structures determined by the PSI centers. It provides quality estimations of the deposited models, access to web-based comparative modeling tools, cross-links to other sequence and structure databases, annotations of sequences and their models, and detailed tutorials on comparative modeling and the use of their tools. The PMP currently contains 19.5 million comparative models for 4.4 million UniProt sequences (August 2013).

A schematic of our own attempt at integrating several useful tools for comparative modeling is shown in **Figure 5**.[34,291] ModBase is a database that currently contains ~29 million predicted models for domains in approximately 4.7 million unique sequences from UniProt, Ensembl,[269] GenBank,[14] and private sequence datasets. The models were calculated using ModPipe[30,291] and Modeller.[35] The web interface to the database allows flexible querying for fold assignments, sequence–structure alignments, models, and model assessments. An integrated sequence–structure viewer, Chimera,[304] allows inspection and analysis of the query results. Models can also be calculated using ModWeb,[291,309] a web interface to ModPipe, and stored in ModBase, which also makes them accessible through the PMP. Other resources associated with ModBase include a comprehensive database of multiple protein structure alignments (DBALI),[281] structurally defined ligand-binding sites,[319] structurally defined binary domain interfaces (PIBASE),[320,321] predictions of ligand-binding sites, interactions between yeast proteins, and functional consequences of human nsSNPs (LS-SNP).[199,322,323] A number of associated web services handle modeling of loops in protein structures (ModLoop),[324,325] evaluation of models (ModEval), fitting of models against Small Angle X-ray Scattering (SAXS) profiles (FoXS),[326–328] modeling of ligand-induced protein dynamics such as allostery (AllosMod),[329,330] prediction of the ensemble of conformations that best fit a given SAXS profile (AllosMod-FoXS),[331] prediction of cryptic binding sites,[332] scoring protein-ligand complexes based on a

Table 1 Programs, databases and web servers useful in comparative protein structure modeling

Name	*World Wide Web address*
Databases	
Protein Sequence Databases	
Ensembl[269]	http://www.ensembl.org
GENBANK[14]	http://www.ncbi.nlm.nih.gov/Genbank/
Protein Information Resource[270]	http://pir.georgetown.edu/
UniprotKB[13]	http://www.uniprot.org
Domains and Superfamilies	
CATH/Gene3D[20]	http://www.cathdb.info
InterPro[271]	http://www.ebi.ac.uk/interpro/
MEME[272]	http://meme.nbcr.net/meme/
PFAM[17]	http://pfam.sanger.ac.uk/
PRINTS[273]	http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/index.php
ProDom[274]	http://prodom.prabi.fr
ProSite[275]	http://prosite.expasy.org/
SCOP[19]	http://scop.mrc-lmb.cam.ac.uk/scop/
SFLD[276]	http://sfld.rbvi.ucsf.edu/
SMART[277]	http://smart.embl-heidelberg.de/
SUPERFAMILY[278]	http://supfam.cs.bris.ac.uk/SUPERFAMILY/
Protein Structures and Models	
ModBase[34]	http://www.salilab.org/modbase/
PDB[12]	http://www.pdb.org/
Protein Model Portal[195,279]	http://www.proteinmodelportal.org/
SwissModel Repository[280]	http://swissmodel.expasy.org/repository/
Miscellaneous	
DBALI[281]	http://www.salilab.org/dbali
GENECENSUS[282]	http://bioinfo.mbb.yale.edu/genome/
Alignment	
Sequence and structure based sequence alignment	
AlignMe[283]	http://www.bioinfo.mpg.de/AlignMe/
CLUSTALW[284]	http://www2.ebi.ac.uk/clustalw/
COMPASS[62]	ftp://iole.swmed.edu/pub/compass/
EXPRESSO[285]	http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi
FastA[286]	http://www.ebi.ac.uk/Tools/sss/fasta/
FFAS03[65]	http://ffas.burnham.org/
FUGUE[68]	http://www-cryst.bioc.cam.ac.uk/fugue
GENTHREADER[66,75]	http://bioinf.cs.ucl.ac.uk/psipred/
HHBlits/HHsearch[53]	http://toolkit.lmb.uni-muenchen.de/hhsuite
MAFFT[287]	http://mafft.cbrc.jp/alignment/software/
MUSCLE[288]	http://www.drive5.com/muscle
MUSTER[78]	http://zhanglab.ccmb.med.umich.edu/MUSTER
PROMALS3D[289]	http://prodata.swmed.edu/promals3d/promals3d.php
PSI-BLAST[39]	http://blast.ncbi.nlm.nih.gov/Blast.cgi
PSIPRED[290]	http://bioinf.cs.ucl.ac.uk/psipred/
SALIGN[291]	http://www.salilab.org/salign/
SAM-T08[74,77]	http://compbio.soe.ucsc.edu/HMM-apps/
Staccato[292]	http://bioinfo3d.cs.tau.ac.il/staccato/
T-Coffee[293,294]	http://www.tcoffee.org/
Structure	
CE[295]	http://source.rcsb.org/jfatcatserver/ceHome.jsp
GANGSTA+[296]	http://agknapp.chemie.fu-berlin.de/gplus/index.php
HHsearch[52]	ftp://toolkit.lmb.uni-muenchen.de/hhsearch/
Mammoth[297]	http://ub.cbm.uam.es/software/mammoth.php
Mammoth-mult[298]	http://ub.cbm.uam.es/software/mammothm.php
MASS[299]	http://bioinfo3d.cs.tau.ac.il/MASS/
MultiProt[300]	http://bioinfo3d.cs.tau.ac.il/MultiProt
MUSTANG[301]	http://www.csse.monash.edu.au/~karun/Site/mustang.html
PDBeFold[36]	http://www.ebi.ac.uk/msd-srv/ssm/
SALIGN[291]	http://www.salilab.org/salign/
TM-align[302]	http://zhanglab.ccmb.med.umich.edu/TM-align/

(*Continued*)

Table 1 (Continued)

Name	*World Wide Web address*
Alignment modules in molecular graphics programs	
Discovery Studio	http://www.accelrys.com
PyMol	http://www.pymol.org/
Swiss-PDB Viewer[303]	http://spdbv.vital-it.ch/
UCSF Chimera[304]	http://www.cgl.ucsf.edu/chimera
Comparative Modeling, Threading and Refinement	
Web servers	
3d-jigsaw[94]	http://www.bmm.icnet.uk/servers/3djigsaw/
HHPred[305]	http://toolkit.genzentrum.lmu.de/hhpred
IntFold[306]	http://www.reading.ac.uk/bioinf/IntFOLD/
i-TASSER[307]	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
M4T[308]	http://manaslu.aecom.yu.edu/M4T/
ModWeb[291,309]	http://salilab.org/modweb/
Phyre2[310]	http://www.sbg.bio.ic.ac.uk/phyre2
RaptorX[311]	http://raptorx.uchicago.edu/
Robetta[81]	http://robetta.bakerlab.org/
SWISS-MODEL[95]	http://www.expasy.org/swissmod
Programs	
HHsuite[52]	ftp://toolkit.genzentrum.lmu.de/pub/HH-suite/
Modeller[35]	http://www.salilab.org/modeller/
MolIDE[312]	http://dunbrack.fccc.edu/molide/
Rosetta@home	http://boinc.bakerlab.org/rosetta/
RosettaCM[81]	https://www.rosettacommons.org/home
SCWRL[160]	http://dunbrack.fccc.edu/scwrl4/SCWRL4.php
Quality estimation	
ANOLEA[180]	http://melolab.org/anolea/index.html
ERRAT[313]	http://nihserver.mbi.ucla.edu/ERRAT/
ModEval	http://salilab.org/modeval/
ProQ2[184]	http://proq2.theophys.kth.se/
PROCHECK[187]	http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/
Prosa2003[177,178]	http://www.came.sbg.ac.at
QMEAN local[183]	http://www.openstructure.org/download/
SwissModel Workspace[314]	http://swissmodel.expasy.org/workspace/index.php?func=tools_structureassessment1
VERIFY3D[175]	http://www.doe-mbi.ucla.edu/Services/Verify_3D/
WHATCHECK[188]	http://www.cmbi.kun.nl/gv/whatcheck/
Methods evaluation	
CAMEO[195]	http://cameo3d.org/
CASP[315]	http://predictioncenter.llnl.gov

statistical potential (Pose & Rank),[190] protein-protein docking filtered by a SAXS profile (FoXSDock),[333,334] and merging SAXS profiles (SAXS Merge).[335,336]

Compared to protein structure prediction, the attempts at automation and integration of resources in the field of docking for virtual screening are still in their nascent stages. One of the successful efforts in this direction is ZINC,[317,318] a publicly available database of commercially available drug-like compounds, developed in the laboratory of Brian Shoichet. ZINC contains more than 21 million 'ready-to-dock' compounds organized in several subsets and allows the user to query the compounds by molecular properties and constitution. The Shoichet group also provides a DOCKBLASTER service[337] that enables end-users to dock the ZINC compounds against their target structures using DOCK.[247,338]

In the future, we will no doubt see efforts to improve the accuracy of comparative modeling and ligand docking. But perhaps as importantly, the two techniques will be integrated into a single protocol for more accurate and automated docking of ligands against sequences without known structures. As a result, the number and variety of applications of both comparative modeling and ligand docking will continue to increase.

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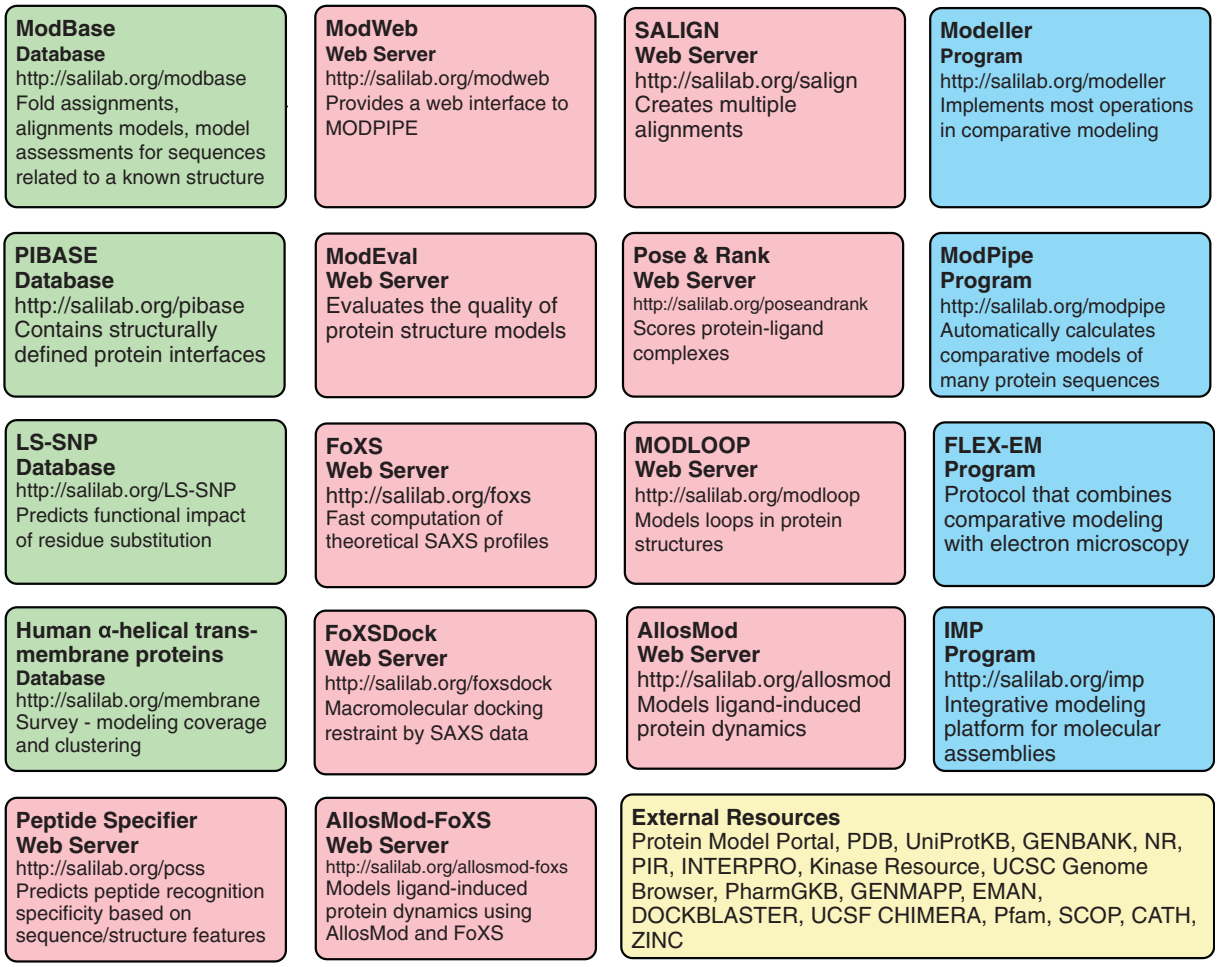

Figure 5 An integrated set of resources for comparative modeling.[34] Various databases and programs required for comparative modeling and docking are usually scattered over the internet, and require manual intervention or a good deal of expertise to be useful. Automation and integration of these resources are efficient ways to put these resources in the hands of experts and non-specialists alike. We have outlined a comprehensive interconnected set of resources for comparative modeling and hope to integrate it with a similar effort in the area of ligand docking made by the Shoichet group.[317,318]

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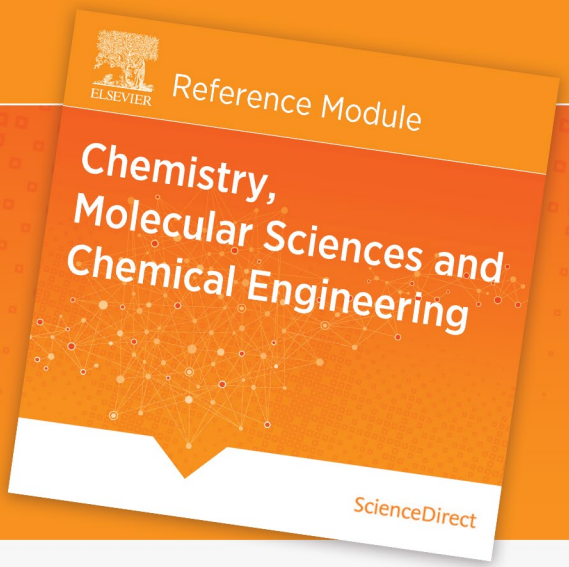

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