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# *New Horizons in Predictive Drug Metabolism and Pharmacokinetics*

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# *Foreword*

It is a measure of the maturity of a branch of science as to how successfully it is able to predict the phenomena with which it deals. The development of a science proceeds in a series of steps; firstly, the observation and description of phenomena, then the development of an understanding of the origin of these phenomena and finally the emergence of a theoretical basis for the phenomena, which in turn provides a basis for *a priori* prediction. Experience across a wide range of sciences shows that this growth in the extent and depth of knowledge relies upon the interaction of experimental and theoretical approaches. The development of drug metabolism and pharmacokinetics, which depends upon progress in a group of chemical and biological sciences, illustrates this sequence very well.

The first experiments to elucidate the fate of foreign compounds in living organisms in the nineteenth century led to the description of the vast majority of the metabolic pathways for foreign compounds along with the recognition of the routes for their elimination. Although it was assumed that these pathways were mediated by enzymes, the basis for this did not emerge until the 1950s. Later, from the 1970s onwards, it became clear that these enzymes are not single entities but exist in families of closely related proteins. From the 1990s, the very rapid growth of knowledge in molecular genetics facilitated huge developments, providing a mechanistic basis to understand the myriad of genetic and environmental factors that had been observed to influence the disposition of foreign compounds.

Efforts to describe the physiological disposition of xenobiotics lagged somewhat behind. In the 1950s, it was shown that passage across membranes, the basis of absorption, distribution and excretion, was principally a passive process, in which unionized molecules passed through lipid-rich membranes, while ionized molecules did not. These models were refined by the inclusion of other physicochemical properties of drugs, notably lipid

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solubility, as well as both intra- and extracellular protein binding. However, it was evident that this framework did not describe the behaviour of all molecules and a fuller understanding emerged from the discovery of families of membrane transporter systems responsible for the import and export of xenobiotics from cells.

The mathematical modelling of drug disposition has a long history. The first attempts to describe blood levels of drugs led to compartmental models providing important insights into drug disposition. However, these models had no anatomical basis and could not be easily related to the chemical and biological aspects of drug metabolism mentioned above. The application of engineering principles refined these models, giving them more biological relevance in the form of pharmacokinetic–pharmacodynamic models linking drug disposition to drug effect.

Alongside progress in our ability to describe and understand biological systems, we have seen comparable advances in chemistry, particularly in computational chemistry. These allow the visualisation of the molecular structure and relevant physicochemical properties of both small molecule substrates, and the enzyme and transporter macromolecules with which they have key interactions. Insights into the molecular mechanisms of the function of these enzymes and transporters provide further foundations for predictive drug metabolism and disposition.

It is relatively rare to find that the fate of a drug in the body is dominated by a single mechanism; in general, the problem is describing the complex and subtle interactions between a very large number of processes, some passive, and many mediated by enzymes and transporters. The ability to handle very large datasets and the emergence of new paradigms in systems biology has transformed our ability to describe and predict biological systems, integrating reductionist and holistic approaches. The goal of achieving the *a priori* prediction of the metabolism and pharmacokinetics of a new drug thus becomes a feasible ambition.

This timely volume represents an authoritative summary of recent progress in relevant fields and the opportunities that exist for further developments from an international group of experts in predictive drug metabolism and pharmacokinetics, many of whom have made major contributions to the subject. I commend the initiative of the editor and publisher in bringing this volume together as well as the efforts of the contributors in producing a book that will be a point of reference for many scientists in academia, and the pharmaceutical and other industries where an understanding of the fate of drugs and xenobiotics is critical, as well as for regulators charged with making key decisions on the use of such compounds. This is a very rich volume that will provide the specialist and general reader alike with an immense amount of interest.

John Caldwell,
Emeritus Professor
*University of Liverpool, United Kingdom*

# *Preface*

Since the pioneering work and treatise by authors such as Tecwyn Williams, Dennis Parke, Bernard Brodie Milo Gibaldi, and other global leaders, a plethora of reviews and books have been published on absorption, distribution, metabolism, elimination and pharmacokinetics (ADME/PK). It may, therefore, seem foolhardy to think that a book on predictive ADME and PK could add substantially to the literature and assist in the shifting focus we are seeing in the pharmaceutical, chemical and life science industries towards predictive approaches. However, it is a testimony to a field that had its origins in the 19th century that it continues to evolve in sophistication, understanding and in its multi-disciplinary impact, and continues to be a critical component in the efficacy and safety of drugs, chemicals, and biologics. For those of us who have had the pleasure of working in this field, it has been a most rewarding experience and journey, since we have seen the continuing awareness and impact that our science has made to the safety and efficacy of new chemical entities. Perhaps there is no better example of this than the impact the introduction of high-throughput ADME screening has made in reducing ADME/PK related attrition in the pharmaceutical and biotechnology industries. However, despite this, a significant challenge still exists to improve the success rates of our discovery and development processes to allow patients faster access to safe and effective drugs. The high rate of attrition of promising drug candidates continues to be a major issue in meeting the medical needs of our patients and the future success of the pharmaceutical industry.

The current focus and urgency in the pharmaceutical industry is to shorten the time lines for all aspects of drug discovery and to improve the ability to filter out early potential ADME/PK, safety, and efficacy issues. We are therefore seeing increasing interest and focus on predictive approaches. These predictive approaches are completed in the early drug discovery phase and will

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gain increasing importance in the coming years in allowing early identification of potential ADME/PK issues.

This book presents a comprehensive treatise by leading experts on the current issues and challenges facing drug metabolism and PK (ADME/PK), and the role of predictive models in drug discovery and development in improving the success rate and safety assessment of pharmaceuticals. The hope of this book is that it will assist in the continuing paradigm shift of the incorporation of ADME/PK prediction and assessment into the drug discovery and development process, and in the overall paradigm of exposure assessment. The authors not only discuss the current state of the art methodologies, but perhaps more importantly focus on the future needs in ADME/PK that are likely to improve our prediction and optimization of ADME/PK, efficacy and human safety. The authors of the various chapters represent leading experts and investigators in the respective areas.

As mentioned previously, many books have been published over the past century in the field of drug metabolism and PK, these not only add to our continuing understanding of the complexity of the field within which we are privileged to work, but also add to the impact that our field has on improving human health and the environment in which we live. Any book of this nature is only as good as the expertise and vision of the contributing authors and I am deeply indebted to all of the experts who have contributed to making this book such a rewarding experience and a valuable addition to the field of ADME/PK.

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*Texas, USA*

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I personally want to thank all of the authors for providing their time and expertise in the completion of this book, and the Royal Society of Chemistry for giving me the opportunity to compile and edit this book on *New Horizons in Predictive Drug Metabolism and Pharmacokinetics* in the RSC Drug Discovery Series. A special thanks goes to Ayako Shinomiya Takei, who assisted me in the review and editing of the chapters. And finally, to the reader, I hope you will find this book of value to you in your quest for understanding the absorption, distribution, metabolism, elimination and pharmacokinetics of chemical entities, and as enjoyable to read as it was to compile.

This book is dedicated to my daughter Julia, my grandson Iain, and to the memory of my beloved Mary.

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CHAPTER 1

# *How Physicochemical Properties of Drugs Affect Their Metabolism and Clearance*

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## 1.1 Introduction

The physicochemical properties of compounds have been used for more than a century to predict or estimate pharmacokinetic processes. The most well known property is lipophilicity, often defined as the partition coefficient between octanol and water. This property is related to passive diffusion across cell membranes, solubility, interaction with receptors, metabolism and toxicity. To activate proteins, *e.g.* receptors and enzymes, the compound needs to bind to a binding pocket. Besides lipophilicity, physicochemical properties of importance for binding include molecular size, hydrogen bond acceptors/donors and charge. This chapter discusses the physicochemical properties of importance for drug metabolism. The primary organ for drug metabolism is the liver and to reach the liver the compound must cross cellular barriers. Absorption from the gastrointestinal tract (GIT) is therefore of critical importance for orally administered drugs, before distribution into and out of the liver can occur. We introduce the GIT in this chapter, and all

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of these processes are discussed in detail in other chapters. Thereafter we describe the enzymes responsible for drug metabolism in different tissues; the biology of these enzymes is further discussed in later chapters. Finally, the role of the enzymes and that of transporters in drug clearance is presented together with an analysis of the structural features of molecules of importance for binding to enzymes and transporters.

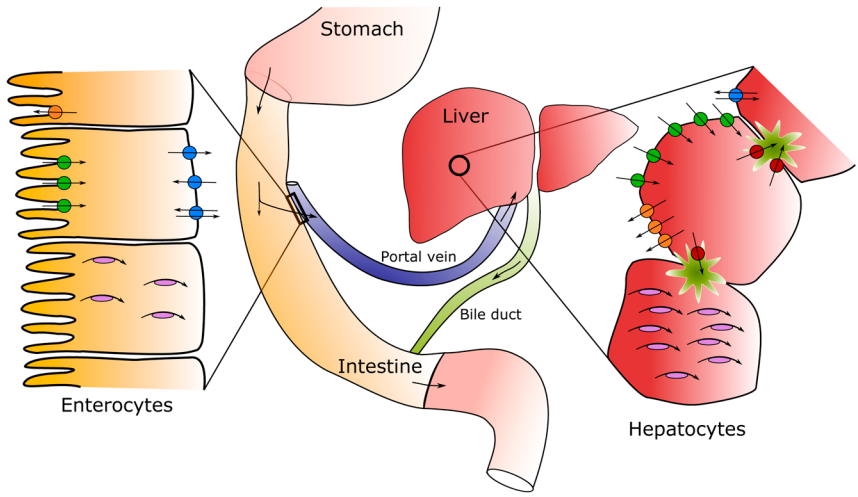
## 1.2 Intestinal Absorption, Liver Disposition and Enzyme Expression

Only free molecules can pass through cell barriers and, hence, only the unbound fraction of drugs can pass over the intestinal epithelium. Solubility governs the concentration reached in the intestinal fluid and is therefore a major driving force for the absorption. Hydrophilic and small molecules may be absorbed by diffusing through the paracellular route. This transport route has limited capacity as its total surface area is much smaller than that of the transcellular (membrane related) pathway. Furthermore, the tight junction reduces the pore size. In the small intestine compounds greater than 4 Å have limited permeability through this pathway whereas those greater than 15 Å are excluded from permeation.[1] To cross the cell membrane compounds have several options. The two most common pathways are passive diffusion through the lipoidal membrane or active transport mediated by transport proteins. The impact of these pathways is heavily debated. Kell and coworkers have challenged the theory that the majority of drugs use lipoidal passive diffusion to pass through cells.[2–4] Their hypothesis is that most of the transport across cells involves active processes and transport proteins. This debate has spurred research to determine to what extent the two pathways are involved in drug distribution.[5,6]

Once the compound has traversed the luminal membrane, it may either diffuse through the cytosol and cross the serosal membrane, or interact with enzymes, intracellular organelles (lysosomes, endoplasmic reticulum) or the cell nuclei. It has been proposed that there is a substrate overlap between cytochrome P450 3A4 (CYP3A4) and the efflux protein P-glycoprotein [P-gp; also known as multidrug resistance protein 1 (MDR1)]. Hence, these two different pathways may have synergistic effects in the clearance and detoxification of certain compounds.

When the compound has crossed the intestinal epithelium it reaches the portal vein from where the systemic circulation transports it to the main metabolic organ in the body—the liver. The compound can then reach the cytosol by either passive or active transport mechanisms across the basolateral membrane facing the bloodstream. The capacity of the liver as a detoxification organ is remarkable. Even compounds with high protein binding can be extracted to a large extent by the liver. This can be exemplified by atorvastatin, the cholesterol-lowering compound marketed as LIPITOR®. A high fraction of atorvastatin is absorbed from the intestine

but is also highly bound (98%) to proteins in the blood. Therefore, only 2% is available in an unbound free form that can permeate the cell membrane. In spite of this, the absolute bioavailability after oral administration is only 14%. This low number is a result of the cooperation between active influx transporters [mainly organic anion-transporting polypeptides (OATP) 1B1 and 1B3] in the basolateral membrane and CYP3A4 in the cytosol. In addition to these processes, atorvastatin is thereafter cleared from the hepatocytes through canalicular efflux by P-gp. The drug transporters and metabolic enzymes in the gut and liver that are crucial for the first-pass effect are shown in Figure 1.1. The metabolic capacity of the gut and liver are discussed in more detail below.




| | Uptake transporters | Efflux transporters | Bidirectional transporters | CYP enzymes |
|---|---|---|---|---|
| Enterocytes | Intestine<br>OATP2B1<br>OATP1A2<br>ASBT<br>MCT1<br>Blood<br>OCT1 | Intestine<br>MRP2<br>BCRP<br>P-gp<br>Blood<br>MRP3 | Blood<br>OSTα-OSTβ | CYP3A4<br>CYP3A5<br>CYP2J2<br>CYP2C9<br>CYP2C19<br>CYP2D6 |
| Hepatocytes | Blood<br>OATP1B1<br>OATP1B3<br>OATP2B1<br>NTCP<br>OCT1<br>OAT1<br>OAT7 | Blood<br>MRP3<br>MRP4<br>MRP6<br>Bile<br>MRP2<br>BCRP<br>P-gp<br>BSEP<br>MATE1 | Blood<br>OSTα-OSTβ | CYP1A2<br>CYP2A6<br>CYP2B6<br>CYP2C8<br>CYP2C9<br>CYP2C18<br>CYP2C19<br>CYP2D6<br>CYP2E1<br>CYP3A4<br>CYP3A5 |

**Figure 1.1** Overview of transporters and CYP enzymes of importance for drug absorption, liver distribution and hepatic elimination.

## 1.3 Metabolic Capacity of the Intestine and Liver

The intestine is the most important extrahepatic site of drug metabolism and its involvement in the first-pass metabolism of orally administered drugs makes it a major determinant of drug bioavailability. The most abundant CYP in the small intestine is CYP3A, constituting 50–82% of the intestinal CYP content.[7–10] However, compared with the liver, the total mass of CYP3A in the small intestine corresponds to only about 1% of the hepatic CYP3A levels.[9,11] Other CYPs expressed in the small intestine, as determined using immunoblotting or liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based protein quantification, are CYP2C9, CYP2C19, CYP2D6 and CYP2J2 (Table 1.1).[7,8] Their expression levels vary in the different regions of the intestine. CYP3A, CYP2C and CYP2D6 show highest expression in the proximal intestinal region and decreasing levels in the distal regions.[9,12] For CYP2J2, the expression is constant throughout the GIT.[13]

More CYPs are expressed in human liver and at higher expression levels than in the intestine. In 1994 Shimada *et al.* determined the expression levels of the major drug metabolizing CYPs in human liver using P450-spectra of total CYP content and SDS-PAGE and immunoblotting in 60 people (30 Caucasians and 30 Japanese).[15] Although the expression levels displayed both

**Table 1.1** Quantitative expression of CYPs in human intestine.

| CYP isoform | Amount in human intestinal microsomes | | | | |
|---|---|---|---|---|---|
| Total CYP | 61 (Immunoquantified) | | No data | No data | No data |
| CYP2C9 | 11 ± 0.5 | 15% of total CYP | 2.96 | 4.27 ± 0.97 | 0.32 ± 0.18 |
| CYP2C19 | 2.1 ± 0.1 | 2.9% of total CYP | | 2.79 ± 1.32 | 1.43 ± 0.25 |
| CYP2D6 | 0.7 ± 0.01 | 1% of total CYP | 2.25 | <LLOQ[a] | <LLOQ[a] |
| CYP2J2 | 1.0 ± 0.1 | 1.4% of total CYP | 2.92 | | |
| CYP3A4 | 58 ± 1.0 | 80% of total CYP | 26.3 | 18.7 ± 6.26 | 1.85 ± 0.36 |
| CYP3A5 | 16 ± 0.3 | | 1.44 | <LLOQ[a] | <LLOQ[a] |
| **Unit** | pmol $mg^{-1}$ protein | | fmol $\mu g^{-1}$ protein | pmol $mg^{-1}$ protein | pmol $mg^{-1}$ protein |
| **References** | 8 | | 14 | 7 | 7 |
| **Number of samples** | Pooled microsomes from 31 donors (11 for CYP3A5) | | Pooled microsomes from 8 donors | Pooled microsomes from 8 donors | Self-prepared microsomes from 3 donors |
| **Method of detection** | Western blot | | LC-MS/MS | LC-MS/MS | LC-MS/MS |

[a]LLOQ: lower limit of quantification.

interindividual and interethnic variations, the average expressions levels in comparison with total CYP content were: CYP3A (28.8%) > CYP2C (18.2%) > CYP1A2 (12.7%) > CYP2E1 (6.6%) > CYP2A6 (4.0%) > CYP2D6 (1.5%) > CYP2B6 (0.2%). Since then the methodological development of more sophisticated methods, *e.g.* different types of LC-MS/MS-based proteomics, has allowed quantification of CYPs.[7,14,16,17] Although the quality of the LC-MS/MS analyses may vary due to the level of method validation *etc.*, the results are consistent with those obtained by Shimada *et al.* that identified the CYP3A and CYP2C families as the most abundant hepatic CYPs (Table 1.2). All tissues have enzymatic activity to some extent; however, the gut and the liver are the two most important metabolic tissues. An overview of the metabolic profile of different tissues is provided in Table 1.3. It should be noted that not only the type of enzymes differs between tissues; the expression levels of these enzymes differ as well.

Although the abundance of CYPs is of major interest it does not provide the full picture of the importance of the specific CYP enzymes for drug metabolism. One striking example showing discrepancy between expression levels and importance is the CYP2D6 enzyme. This enzyme is only expressed in low levels in human liver (1.5–2% of the total CYP content). However, it is one of the major drug-metabolizing enzymes and metabolizes up to 25% of clinically used drugs.[31–34] Another example is CYP1A2. It constitutes approximately 18% of the human hepatic CYP content,[15] but its relative importance in drug metabolism is only 3–9% (Table 1.4).[32–34]

## 1.4 Pharmacogenomics

In addition to interindividual variation in expression levels, many drug-metabolizing enzymes, and especially some of the CYPs, are highly polymorphic. Approximately 40% of CYP-dependent phase I metabolism is performed by polymorphic CYPs, including CYP2D6, CYP2C9, CYP2C19 and CYP2B6.[36] For CYP2D6 more than 100 different alleles and suballeles have been identified. These include alleles where the entire *CYP2D6* gene is deleted, alleles with duplicated or multiduplicated *CYP2D6* genes, and alleles containing single-nucleotide polymorphisms (SNPs).[36] Such gene variants may of course have a major impact on the pharmacokinetics and may result in adverse effects of drugs that are CYP2D6 substrates (*cf.*[37,38]). A classic example of this is the prodrug codeine, which is activated by CYP2D6 into the active drug morphine. For people who are poor CYP2D6 metabolizers, *i.e.* their *CYP2D6* genes are deleted or contain mutations leading to non-functional enzymes, codeine does not give the desired analgesic effect.[39] On the contrary, for individuals with alleles with duplicated or multiduplicated *CYP2D6* genes, *i.e.* ultra-rapid metabolizers, codeine is activated rapidly, which can lead to codeine toxicity and central nervous system depression.[40] There are also a few cases of infant mortality where ultra-rapid metabolizer mothers treated with codeine transferred fatally high morphine concentrations to their breastfed infants.[41–43]

**Table 1.2** Quantitative expression of CYPs in human liver.

| CYP isoform | Amount in human liver | | | | | |
|---|---|---|---|---|---|---|
| Total CYP determined spectrally | 0.344 ± 0.167 | 411 | 255 ± 17 | 534 | No data | No data |
| CYP1A2 | 0.042 ± 0.023 | 17.7 ± 0.6 | | 45 | 12.8 ± 0.17 | 19.0 |
| CYP2A6 | 0.014 ± 0.013 | 49.2 ± 1.7 | | 68 | | 61.1 |
| CYP2B6 | 0.001 ± 0.002 | 6.86 ± 0.44 | | 39 | 9.59 ± 0.38 | 29.3 |
| CYP2D6 | 0.005 ± 0.004 | 11.5 ± 0.3 | | 10 | 9.34 ± 0.15 | 38.6 |
| CYP2C | 0.060 ± 0.027 | | | | | |
| CYP2C8 | | 29.3 ± 0.6 | 11.5 ± 2.9 | 64 | 26.9 ± 0.54 | 55.8 |
| CYP2C9 | | 80.2 ± 1.4 | 88.5 ± 8.7 | 96 | 37.3 ± 2.50 | 93.0 |
| CYP2C18 | | | | | | 2.82 |
| CYP2C19 | | 3.64 ± 0.22 | 17.8 ± 3.3 | 19 | 2.18 ± 0.18 | 15.6 |
| CYP2E1 | 0.022 ± 0.012 | 51.3 ± 0.9 | | 49 | 65.3 ± 1.52 | 103 |
| CYP2J2 | | | | | | 4.95 |
| CYP3A (3A4/3A5) | 0.096 ± 0.100 | | | | | |
| CYP3A4 | | 64.0 ± 1.9 | | 108 | 32.6 ± 0.38 | 109 |
| CYP3A5 | | 3.54 ± 0.28 | | 1 | 1.96 ± 0.05 | 7.24 |
| CYP3A7 | | | | | | 11.4 |
| CYP4A11 | | | | | | 16.5 |
| CYP51A1 | | | | | | 4.89 |
| **Unit** | nmol $mg^{-1}$ protein | pmol $mg^{-1}$ protein | pmol $mg^{-1}$ protein | pmol $mg^{-1}$ protein | pmol $mg^{-1}$ protein | fmol $\mu g^{-1}$ protein |
| **References** | 15 | 16 | 18 | 19 | 7 | 14 |
| **Number of liver specimens** | 60 | 10 | 17 | No data | 25 | 50 |
| **Method of detection** | Immunochemical | LC-MS/MS | Immunochemical | Immunochemical | LC-MS/MS | LC-MS/MS |

**Table 1.3** Overview of CYPs (family 1–3) expressed in hepatic and extrahepatic tissues.

| CYP isoform | Gastrointestinal tract | Liver | Brain[b] | Kidney | Heart | Skin[e] | Respiratory tract[e] |
|---|---|---|---|---|---|---|---|
| CYP1A1 | | | X | | X | X | X |
| CYP1A2 | | X | X | | X[d] | | X |
| CYP1B1 | | | X | | X | X | X |
| CYP2A6 | | X | | | | X | X |
| CYP2A13 | | | | | | | X |
| CYP2B6 | | X | X | X | X[d] | X | X |
| CYP2C8 | | X | | X[c] | X | | X |
| CYP2C9 | X | X | | X[c] | X | X | |
| CYP2C18 | | X | | | | X | X |
| CYP2C19 | X | X | X | | | X | |
| CYP2D6 | X | X | X | | X | X | X |
| CYP2E1 | | X | X | | X[d] | X | X |
| CYP2F1 | | | | | | | X |
| CYP2J2 | X | | | X[c] | X | X | X |
| CYP2S1 | | | | | | X | X |
| CYP2R1 | | | | | | X | |
| CYP2U1 | | | X | | | X | |
| CYP2W1 | | | | | | X | |
| CYP3A | | | | | | X | |
| CYP3A4 | X | X | X | X[c] | X | | X |
| CYP3A5 | X | X | X | X | | | X |
| CYP3A7[a] | | X | | | | | |
| CYP3A43 | | | X | | | | |
| **References** | 7,8,14 | 7,14–16,18–20 | 21–25 | 26 | 27 | 28 and 29 | 30 |

[a]Major CYP3A enzyme expressed in fetal liver.
[b]Expression levels vary greatly between different brain regions and cell types.
[c]Data regarding human kidney expression are conflicting.
[d]Not seen in healthy human heart.
[e]mRNA and/or protein expression.

For other CYPs, *e.g.* CYP2C9, CYP2C19 and CYP2B6, many variant alleles and suballeles have been described, some of which have significant clinical impact. The most well known clinical CYP2C examples are the *CYP2C9* polymorphisms involved in warfarin metabolism[44,45] and *CYP2C19* polymorphisms associated with clopidogrel activation.[46,47] Both of these were highlighted in 2011 as important pharmacogenomics biomarkers.[37] The warfarin (COUMADIN®) and the clopidogrel (PLAVIX®) Food and Drug Administration (FDA) drug labels have been updated to contain recommendations for initial doses based on *e.g. CYP2C9* genotype[48] and a warning about diminished effectiveness in CYP2C19 poor metabolizers,[49] respectively. A complete and updated overview of CYP and CYP oxidoreductase (POR) polymorphisms can be found on the home page of The Human Cytochrome P450 (CYP) Allele Nomenclature Committee (http://www.cypalleles.ki.se).

**Table 1.4** Relative contribution of the CYP isoforms to hepatic drug metabolism.

| CYP isoform | Relative contribution to hepatic drug metabolism (%) | | | |
|---|---|---|---|---|
| CYP3A (3A4/3A5) | 51 | 46 | 53 | 30.2 |
| CYP2D6 | 24 | 12 | 25 | 20 |
| CYP2C | 19 | — | 18 | — |
| CYP2C8 | — | — | — | 4.7 |
| CYP2C9 | — | 16 | — | 12.8 |
| CYP2C19 | — | 12 | — | 6.8 |
| CYP1A | — | 9 | — | — |
| CYP1A2 | 5 | — | 3 | 8.9 |
| CYP2E1 | 1 | 2 | — | 3 |
| CYP2A6 | — | — | — | 3.4 |
| CYP2B6 | — | 2 | — | 7.2 |
| CYP2J2 | — | — | — | 3 |
| **References** | 33 | 32 | 34 | 35 |
| **Number of drugs studied** | All prescribed drugs | 200 drugs (of which ~100 cleared by CYP-mediated metabolism) | 315 drugs (of which 175 cleared by CYP-mediated metabolism) | 248 drugs cleared by CYP-mediated metabolism |

## 1.5 Molecular Features of Importance for Transporter Interactions: Substrates *Versus* Inhibitors

Many substrates and inhibitors have been identified for transporters that are of importance for drug distribution into and out of cells. For a selection of these, see Table 1.5. While a substrate of the transporter can also be an inhibitor of the transport protein and block transport of other compounds, compounds that have been identified as inhibitors may not be transported. The latter is related to the inactivation of the transport protein by binding to sites other than the one crucial for mediating transport. Interaction with the transport-mediating site allows the drug compound (or its metabolite) to traverse the lipophilic membrane. Hence, the molecular requirements of the different transporters have been studied to better understand what physicochemical properties of a compound will result in them being actively transported by a particular transport protein. While metabolism is a chemical reaction that turns a substrate into a product that is chemically different, the substrates of transport proteins remain the same; no chemical reaction occurs. However, the terminology of transporters and experimental procedures to study transport have been inspired by those in the metabolism field. So, for example, the Michaelis–Menten equation is often used to describe the efficiency of transporters to flux compounds across the membrane.

The structural requirements for transport by influx and efflux transport proteins have been heavily studied. The majority of studies have been

**Table 1.5** Transporters of clinical relevance for drug clearance, expressed in the gut and liver.[a]

| Transporters | Gut | Liver | Selected substrates | Selected inhibitors |
|---|---|---|---|---|
| **Influx** | | | | |
| ASBT (SLC10A2) | X | | Bile salts | Cyclosporin A |
| MCT1 (SLC16A1) | X | | Nateglinide | Nateglinide |
| NTCP (SLC10A1) | | X | Bile salts | Bumetanide, chlorpropamide, cyclosporin A, furosemide, ketoconazole, progesterone |
| OAT2 (SLC22A7) | | X | Methotrexate, tetracycline, theophylline | Cefamandole, cefoperazone, cefotaxime, cephaloridine, cephalothin, cilastatin, clarithromycin, erythromycin, ganciclovir, minocycline, oxytetracycline, pravastatin, probenecid |
| OATP1A2 (SLCO1A2) | X | | Enalapril, fexofenadine, pravastatin, rifampicin | Dexamethasone, erythromycin, ketoconazole, lovastatin, naloxone, nelfinavir, quinidine, rifampicin, ritonavir, saquinavir, verapamil |
| OATP1B1 (SLCO1B1) | | X | Atorvastatin, benzylpenicillin, cerivastatin, irinotecan, methotrexate, pitavastatin, pravastatin, rifampicin, simvastatin | Cyclosporin A, indinavir, lovastatin, nelfinavir, pioglitazone, pravastatin, quinidine, rapamycin, ritonavir, rosiglitazone, saquinavir, troglitazone |
| OATP1B3 (SLCO1B3) | | X | Digoxin, methotrexate, pioglitazone, pitavastatin, rifampicin | Rifampicin |
| OATP2B1 (SLCO2B1) | X | X | Benzylpenicillin, glibenclamide, ibuprofen, fexofenadine, pravastatin, rifampicin, tolbutamide | Tangeretin, rifamycin |
| OCT1 (SLC22A1) | X | X | Acyclovir, cimetidine, cisplatin, ganciclovir | Amiloride, chlorpromazine, clonidine, desipramine, disopyramide, metformin, midazolam, prazosin, progesterone, quinidine, ranitidine, verapamil |
| OCT3 (SLC22A3) | X | | Carboplatin, cimetidine, cisplatin | Clonidine, desipramine, imipramine, prazosin, progesterone |
| OCTN2 (SLC22A5) | X | | Cimetidine, valproic acid | Aldosterone, amphetamine, ampicillin, cefadroxil, cefdinir, cefepime, cefixime, cefluprenam, cefoselis, cefsulodin, ceftazidime, cephalexin, cephalothin, clonidine, cyclacillin, desipramine, furosemide, lomefloxacin, norfloxacin, benzylpenicillin, probenecid, verapamil |
| PEPT1 (SLC15A1) | X | | Benzylpenicillin, cefadroxil, cefixime, ceftibuten, enalapril, faropenem, lisinopril, temocapril, valacyclovir | Amoxicillin, ampicillin, captopril, cefadroxil, cefluprenam, cefotaxime, cefpirome, cefsulodin, ceftazidime, ceftriaxone, cefuroxime, cephadroxil, cephalexin, cephaloridine, cloxacillin, cyclacillin, dicloxacillin, glycylsarcosine, L-dopa, metampicillin, moxalactam |

(*continued*)

**Table 1.5** (*continued*)

| Transporters | Gut | Liver | Selected substrates | Selected inhibitors |
|---|---|---|---|---|
| **Efflux** | | | | |
| BCRP (ABCG2) | X | X | Cerivastatin, daunorubicin, glibenclamide, lamivudine, methotrexate, mitoxantrone, prazosin, pravastatin, tamoxifen, topotecan | Cyclosporin A, doxorubicin, nelfinavir, novobiocin, omeprazole, pantoprazole, ritonavir, saquinavir, silybin, silymarin, verapamil |
| BSEP (ABCB11) | | X | Daunorubicin, doxorubicin, vincristine | Chlorpromazine, cimetidine, clofazimine, cyclosporin A, glibenclamide, ketoconazole, paclitaxel, progesterone, quinidine, reserpine, tamoxifen, troglitazone, valinomycin, verapamil, vinblastine |
| P-gp, MDR1 (ABCB1) | X | X | Acetaminophen, acetylsalicylic acid, albendazole, aldosterone, atenolol, carbamazepine, chlorpromazine, ciprofloxacin, clozapine, cyclosporin A, daunorubicin, diazepam, digoxin, dipyridamole, docetaxel, emetine, fluconazole, flumazenil, fluoxetine, haloperidol, hydrocortisone, ibuprofen, imatinib, ivermectin, ketamine, loperamide, losartan, naloxone, neostigmine, nitrazepam, olanzapine, paclitaxel, quinidine, risperidone, scopolamine, sumatriptan, valinomycin, verapamil, vinblastine | Amiodarone, amitriptyline, astemizole, atorvastatin, bromocriptine, buspirone, candesartan, captopril, cimetidine, clarithromycin, clofazimine, clotrimazole, desipramine, desloratadine, dexamethasone, diclofenac, erythromycin, felodipine, fentanyl, glibenclamide, indinavir, itraconazole, ketoconazole, lidocaine, lopinavir, loratadine, lovastatin, methadone, metoprolol, miconazole, morphine, nelfinavir, nicardipine, nifedipine, norverapamil, omeprazole, pantoprazole, ranitidine, reserpine, ritonavir, saquinavir, simvastatin, sirolimus, spironolactone, tamoxifen, terfenadine, verapamil, vincristine |
| MRP2 (ABCC2) | X | X | Cerivastatin, etoposide, indinavir, methotrexate, pravastatin, ritonavir, saquinavir, vinblastine, vincristine | Benzbromarone, cyclosporin A, daunorubicin, furosemide, lovastatin acid, probenecid, quinidine, reserpine, sulfinpyrazone, verapamil |
| MRP3 (ABCC3) | X | X | Etoposide, glibenclamide, glutathione, methotrexate | Benzbromarone, doxorubicin, indomethacin, probenecid, verapamil, vincristine |
| MRP4 (ABCC4) | | X | Adefovir, methotrexate | Benzbromarone, celecoxib, diclofenac, dipyridamole, ibuprofen, indomethacin, indoprofen, ketoprofen, probenecid, rofecoxib, sildenafil, verapamil |
| MRP6 (ABCC6) | | X | Cisplatin, daunorubicin, doxorubicin, etoposide, teniposide | Benzbromarone, indomethacin, probenecid, sulfinpyrazone |

[a]Data on clinically relevant transporters were taken from ref. 70–73. Representative examples of substrates and inhibitors for each of the transport proteins were extracted from the database established by Prof. Sugiyama (http://togodb.dbcls.jp/tpsearch/). Substrates also being identified as inhibitors are not listed. Note that inhibitors listed may be substrates but to date only data on inhibition are available in the open literature.

directed towards investigation of transport protein inhibition. The reason for this is mainly methodological issues associated with substrate assays. While analyses of molecular features of substrates require determination of the intracellular concentration of a large number of compounds, inhibition assays rely on screening a large number of compounds for their inhibition of the transport of one substrate. Hence, analytical demands for the latter are reduced and a higher throughput mode is possible. The most important transport proteins for clearance are discussed below.

## 1.5.1 Efflux Proteins

### *1.5.1.1 P-gp Substrate Recognition Pattern*

One of the most studied transport proteins is the efflux protein P-gp since it is important for drug distribution to several tissues, including the gut and liver. Drug–drug interactions (DDIs) have also been identified that are mediated by P-gp. Among the most well known are those that occur between digoxin and the P-gp inhibitors amiodarone, cyclosporin A, quinine and verapamil.[50] Seelig and coworkers were pioneers in the study of the recognition pattern of P-gp (*cf.*[51,52]). Based on studies of ~100 compounds, they suggested that a special spatial separation of electron donor groups is required for compounds to be transported by P-gp. Their work was followed by a number of structure–activity relationship (SAR) studies in which P-gp substrates are predicted on the basis of chemical information calculated from the molecular structure. The SAR models are typically classification models used to distinguish compounds that are substrates from those that are not transported by the P-gp. One classification model used the sum of atomic electrotopological states (MolES), a descriptor of molecular bulkiness, to predict substrates.[53] Compounds with a MolES >110 are regarded as substrates for P-gp whereas a MolES <49 indicates non-substrates. For compounds with a value between 49 and 110 other descriptors are needed to identify whether they would be substrates.[53] A similar study using a classification approach established the rule of four.[54] This rule states the following: compounds with (N + O) ≥ 8, molecular weight > 400 and acid p$K_a$ > 4 are likely to be P-gp substrates. Compounds with (N + O) ≤ 4, molecular weight < 400 and base p$K_a$ < 8 are likely to be non-substrates. Both of these SAR studies identified that P-gp transports larger molecules. Furthermore, it seems that compounds with many hydrogen bonds, and to some extent negative charges, are transportable by P-gp. The non-substrates have fewer hydrogen bond acceptors and are neutral, or at least not highly positively charged. The importance of N and O demonstrated by this study confirms the work by Seelig and colleagues. Finally, P-gp substrates are amphipathic and lipophilic.[55] It has been suggested that the substrate binding pocket sits inside the cellular membrane and needs to be accessed by distribution into the lipid bilayer.[56–58] Based on this, the lipophilic and amphiphilic nature of the substrates is to be expected.

### 1.5.1.2 *Inhibition of P-gp, BCRP, MRPs, BSEP: Specificity and Overlap*

While it is important to understand molecular features that result in substances being substrates to efflux proteins, it is also of interest to look at which molecular features lead to inhibition of transport. Inhibition may result in severe DDIs. Inhibitors may be competitive (they bind to the same binding site as the substrate) or non-competitive (they bind to another site on the transport protein and thereby block the transport). Therefore, a substrate may inhibit the transport of another substrate, and an inhibitor is not necessarily transported by the protein. Artursson and colleagues have explored large compound series to identity inhibitors of the transport proteins most important for drug disposition. They identified specific molecular requirements of the different transporters and the extent to which the molecular requirements for inhibition of these transporters overlap. For example, the ABC transporters P-gp, breast cancer resistance protein (BCRP), multidrug resistance-associated protein 2 (MRP2) and bile salt export pump (BSEP), all of which are expressed in the canalicular membrane of the hepatocyte, have a significant overlap of inhibitors, *i.e.* the same compound may block several of these transporters at the same time. The impact on drug clearance, for instance from hepatocytes to bile, may therefore be greatly affected. Such inhibition may also result in reduced enterohepatic recycling of endogenous substances such as bile acids and bilirubin, which can result in, among others, fatal cholestasis.[59] In a study of 122 compounds, all tested for their inhibition of P-gp, BCRP and MRP2, molecular features of specific inhibitors (interacting with only one of the transporters) and of those that interacted with all three transporters were identified.[60] The inhibitors of P-gp were lipophilic, non-polar and had higher structure connectivity. BCRP inhibitors were also more lipophilic than non-inhibitors and the number of aromatic rings correlated positively with inhibition. Inhibitors of MRP2 had similar properties; lipophilicity and unsaturated bonds (double bonds) positively correlated with inhibition, as did shape. Thus, inhibitors of P-gp, BCRP and MRP2 are all lipophilic and aromatic, but to different degrees. The specific inhibitors of P-gp are less aromatic than those of MRP2 and BCRP, and the BCRP inhibitors generally have more aromatic nitrogens than the P-gp inhibitors. P-gp inhibitors are the most lipophilic ($\log D_{\mathrm{pH7.4}}$ of 2.3) followed by BCRP ($\log D_{\mathrm{pH7.4}}$ of 1.9) and MRP2 ($\log D_{\mathrm{pH7.4}}$ of 1.2). By contrast, multi-specific inhibitors, *i.e.* compounds that inhibit all three proteins, are 100- to 1000-fold more lipophilic ($\log D_{\mathrm{pH7.4}}$ of 4.5).[60]

Another study investigated 250 compounds for their inhibition of BSEP. Of the 86 inhibitors identified, 58% were neutral at physiological pH, 36% were negatively charged and only 6% were positively charged. By contrast, BSEP substrates are typically monovalent, negatively charged bile acids. BSEP inhibition is also favored by lipophilicity, hydrophobicity and number of halogens. Reciprocally, hydrophilicity and hydrogen bond acceptors negatively correlate with inhibition.[61]

## 1.5.2 Uptake Transporters

### *1.5.2.1 Inhibition of OATP1B1, OATP1B3, OATP2B1: Specificity and Overlap*

There are a number of studies on the inhibition of OATP uptake transporters, particularly OATP1B1 (which is the most important hepatic OATP). Two studies by Karlgren *et al.* investigated the inhibition of OATP1B1 by 146 compounds and the inhibition of OATP1B1, OATP1B3 and OATP2B1 by 225 compounds.[62,63] In both studies, a significantly larger proportion of the inhibitors were negatively charged compounds compared with the non-inhibitors. This is not surprising given that OATPs are known to primarily transport anionic drugs. Furthermore, these studies showed that compared with the non-inhibitors, the OATP inhibitors had a significantly higher lipophilicity (mean NNLogP of 3.6–4.0 *vs.* 2.3–2.7), larger molecular weight (mean weight of 481–514 *vs.* 325–336 g $mol^{-1}$) and a larger polar surface area (PSA; mean PSA of 115–142 *vs.* 66–74 $Å^2$).[62,63] OATP1B1 inhibitors also displayed a lower mean square distance index (MSD), a topological distance descriptor normalized for size.[63] Inhibitors of OATP1B3—but not of OATP1B1 and OATP2B1—had more hydrogen bond donors than the non-inhibitors, whereas the OATP2B1 inhibitors were less dependent on polarity than those of OATP1B1 and OATP1B3.[62] These findings were confirmed by an *in vitro* study of 2000 compounds on OATP1B1 and OATP1B3.[64] It was also found that a low number of aromatic bonds (<7) correlated positively with OATP1B1 inhibition but negatively with OATP1B3 inhibition, whereas a log*D* value of >7.5 and 3–4 hydrogen bond donors correlated positively with OATP1B3 inhibition. Interestingly, due to the high number of compounds investigated, they could also identify substructures that favored inhibition of a specific transporter or favored inhibition of both OATP1B transporters.

The three OATP transporters share many inhibitors. Two examples are atazanavir and ritonavir, which are considered general OATP inhibitors.[62] In one study of 91 identified inhibitors, 42 were common for OATP1B1 and OATP1B3. Of these 42 inhibitors, 16 did not inhibit OATP2B1. By contrast, only 9 of the inhibitors were identified as inhibitors of OATP1B1 and OATP2B1 but not OATP1B3. Only one compound, nefazodone, interacted with both OATP1B3 and OATP2B1 but did not inhibit OATP1B1.

Many of the compounds identified as inhibitors of the OATP transporters are also inhibitors or substrates of other transporters or metabolizing enzymes. For example, the FDA and/or the European Medicines Agency (EMA) list that 67 of the 225 compounds included in the studies above are substrates, inhibitors or inducers of CYP enzymes. Of these 67 compounds, 21 compounds were also identified as inhibitors of one or more OATP transporters.[62] The largest overlap was for OATPs and CYP2C8, followed by OATPs and CYP3A4. Previously it was suggested that there was a substrate overlap between OATP1B1 and the efflux transporter MRP2.[65] However, an

investigation of common inhibitors of OATP1B1 and MRP2 found no such corresponding overlap of inhibitors.[63]

### *1.5.2.2 Inhibition of OCT1*

Organic cation transporter 1 (OCT1) is the major cationic uptake transporter in the liver. An investigation of 191 compounds identified 62 as inhibitors of OCT1.[66] These inhibitors tended to be positively charged (66%) or neutral (32%) at physiological pH. They were more lipophilic (mean ClogP of 3.50 *vs.* 1.43), had a lower PSA (mean PSA of 42.9 *vs.* 95.5), and a lower number of both hydrogen bond donors (1.07 *vs.* 2.66) and acceptors (3.38 *vs.* 5.09) than the non-inhibitors.[66] These results agree with a previous study of OCT1 inhibition that used a more homogeneous dataset ($n = 30$).[67] The results also support previous observations that a positive charge is important for interactions with the OCT1 transporter.[68,69]

## 1.6 Molecular Features of Importance for Enzyme Interactions: Substrates *Versus* Inhibitors

Table 1.6 presents a representative sample of the many substrates and inhibitors of the enzymes responsible for drug metabolism. The liver is the organ with the highest metabolic capacity (Table 1.4). The enzymes of highest importance for drug metabolism in this tissue are CYP3A4, CYP2C9, CYP2C19 and CYP2D6. Below we discuss the molecular features of the substrates and inhibitors of these four enzymes. We focus on CYP3A4 as this enzyme is of the greatest importance for drug metabolism and therefore the most studied.

### 1.6.1 CYP3A4

The binding pocket of CYP3A4 is quite large. Pharmacophore modeling has been used to reveal the molecular requirements of compounds that bind and activate the enzyme.[74] Information extracted from 38 compounds and the software Catalyst showed that the large binding pocket required interaction with a hydrophobic fragment and hydrogen bond interactions through a hydrogen bond donor and a hydrogen bond acceptor. These different features require a particular spatial distribution in the molecule to interact with the binding pocket. The pharmacophore was later regenerated in a study by Norinder, who also identified another pharmacophore with similar accuracy.[75] Norinder included more hydrophobic interaction points (three hydrophobic fragments) and only one hydrogen bond acceptor to achieve the same quality of pharmacophore as the previous one. This shows the complexity in identifying the molecular features that characterize substrates. A more complex approach, also using pharmacophore modeling, identifies structures vulnerable to metabolism and the reactive site. This methodology calculates the fingerprint of both the enzyme and the substrate. The calculations are

**Table 1.6** Substrates and inhibitors of CYP enzymes of importance for drug clearance in the gut and liver.[a]

| CYP isoform | Substrates | Inhibitors |
|---|---|---|
| **CYP1A2** | Amitriptyline, caffeine, clomipramine, clozapine, cyclobenzaprine, duloxetine, estradiol, fluvoxamine, haloperidol, mexiletine, nabumetone, naproxen, olanzapine, ondansetron, phenacetin, propranolol, riluzole, ropivacaine, tacrine, theophylline, tizanidine, triamterene, verapamil, (*R*)-warfarin, zileuton, zolmitriptan | Amiodarone, ciprofloxacin, cimetidine, efavirenz, fluoroquinolones, furafylline, interferon, methoxsalen, mibefradil, ticlopidine |
| **CYP2B6** | Artemisinin, bupropion, cyclophosphamide, efavirenz, ifosfamide, ketamine, meperidine, methadone, nevirapine, propafol, selegiline, sorafenib | Clopidogrel, thiotepa, ticlopidine, voriconazole |
| **CYP2C8** | Amodiaquine, cerivastatin, paclitaxel, repaglinide, sorafenib, torsemide | Gemfibrozil, glitazones, montelukast, quercetin, trimethoprim |
| **CYP2C9** | Amitriptyline, celecoxib, diclofenac, fluoxetine, fluvastatin, glimepiride, glipizide, glyburide, ibuprofen, irbesartan, lornoxicam, losartan, meloxicam, *S*-naproxen, nateglinide, phenytoin-4-OH2, piroxicam, rosiglitazone, suprofen, tamoxifen, tolbutamide, torsemide, valproic acid, *S*-warfarin, zakirlukast | Amiodarone, efavirenz, fenofibrate, fluconazole, fluvoxamine, isoniazid, lovastatin, metronidazole, paroxetine, phenylbutazone, probenicid, sertraline, sulfamethoxazole, sulfaphenazole, teniposide, voriconazole |
| **CYP2C19** | Amitriptyline, carisoprodol, citalopram, chloramphenicol, clomipramine, clopidogrel, cyclophosphamide, diazepam, esomeprazole, hexobarbital, indomethacin, labetalol, lansoprazole, *S*-mephenytoin, *R*-mephobarbital, moclobemide, nelfinavir, nilutamide, omeprazole, pantoprazole, phenobarbitone, primidone, progesterone, proguanil, propranolol, teniposide, *R*-warfarin, voriconazole | Cimetidine, esomeprazole, felbamate, fluoxetine, fluvoxamine, isoniazid, ketoconazole, modafinil, oxcarbazepine, probenecid, ticlopidine, topiramate |
| **CYP2D6** | Alprenolol, amphetamine, amitriptyline, aripiprazole, atomoxetine, bufuralol, carvedilol, chlorpheniramine, chlorpromazine, clonidine, codeine, clomipramine, debrisoquine, desipramine, dexfenfluramine, dextromethorphan, donepezil, duloxetine, flecainide, fluvoxamine, fluoxetine, haloperidol, imipramine, lidocaine, metoclopramide, *S*-metoprolol, methoxyamphetamine, mexiletine, minaprine, nebivolol, nortriptyline, ondansetron, oxycodone, paroxetine, perhexiline, perphenazine, phenacetin, phenformin, promethazine, propafenone, propranolol, risperidone, sparteine, tamoxifen, thioridazine, timolol, tramadol, venlafaxine, zuclopenthixol | Amiodarone, bupropion, cinacalcet, cimetidine, celecoxib, citalopram, clemastine, cocaine, diphenhydramine, doxepin, doxorubicin, escitalopram, halofantrine, hydroxyzine, levomepromazine, methadone, metoclopramide, mibefradil, midodrine, moclobemide, quinidine, ranitidine, ritonavir, sertraline, terbinafine, ticlopidine, tripelennamine |

(*continued*)

**Table 1.6** (*continued*)

| CYP isoform | Substrates | Inhibitors |
|---|---|---|
| **CYP2E1** | Acetaminophen, aniline, benzene, chlorzoxazone, enflurane, ethanol, *N*,*N*-dimethylformamide, halothane, isoflurane, methoxyflurane, sevoflurane, theophylline | Diethyl-dithiocarbamate, disulfiram |
| **CYP3A4/3A5/3A7** | Alfentanil, alprazolam, amlodipine, aprepitant, aripiprazole, astemizole, atorvastatin, boceprevir, buspirone, carbamazepine, cafergot, caffeine → TMU, cerivastatin, clarithromycin, chlorpheniramine, cilostazol, cisapride, cocaine, codeine-*N*-demethylation, cyclosporine, dapsone, dexamethasone, dextromethorphan, diazepam, diltiazem, docetaxel, domperidone, eplerenone, erythromycin, estradiol, felodipine, fentanyl, finasteride, gleevec, haloperidol, hydrocortisone, indinavir, irinotecan, lercanidipine, lidocaine, lovastatin, methadone, midazolam, nateglinide, nelfinavir, nevirapine, nifedipine, nisoldipine, nitrendipine, ondansetron, pimozide, progesterone, propranolol, quetiapine, quinidine, quinine, risperidone, ritonavir, romidepsin, salmeterol, saquinavir, sildenafil, simvastatin, sirolimus, sorafenib, tacrolimus, tamoxifen, taxol, telaprevir, telithromycin, terfenadine, testosterone, torisel, trazodone, triazolam, vemurafenib, verapamil, vincristine, zaleplon, ziprasidone, zolpidem | Amiodarone, chloramphenicol, cimetidine, ciprofloxacin, delavirdine, diethyl-dithiocarbamate, fluconazole, fluvoxamine, gestodene, imatinib, itraconazole, ketoconazole, nefazodone, mibefradil, mifepristone, norfloxacin, norfluoxetine, suboxone, voriconazole |

[a]Data on CYP substrates and inhibitors were taken from the cytochrome P450 drug interaction table established by Prof. Flockhart. Flockhart DA. Drug Interactions: Cytochrome P450 Drug Interaction Table. Indiana University School of Medicine (2007). http://medicine.iupui.edu/clinpharm/ddis/clinical-table/, accessed 2014-12-09. http://togodb.dbcls.jp/tpsearch/.

based on GRID methodology, *i.e.* the enzyme fingerprints are calculated by the GRID flexible molecular interaction fields and the substrate fingerprints are obtained through GRID probe pharmacophore recognition. The latter calculates hydrophobicity, hydrogen bond donors and acceptors, and charge to obtain a fingerprint of each atom in the molecule. These descriptors are then assessed for their capacity to interact with the reactive heme atom of the enzyme. This is performed through assessment of how accessible they are for interactions with the heme. This fingerprint method has resulted in the software MetaSite for prediction of vulnerable sites for CYP metabolism of CYP3A4, CYP2C9 and CYP2D6 (see Section 1.7.1).[76–78]

Another method to predict the site of metabolism (SOM) in a drug molecule was developed at the University of Copenhagen. Their approach uses quantum chemical calculations with the density functional method B3LYP to estimate the activation energy required for different atoms to become the SOM. The group has also tested less time-consuming calculations by making use of the semi-empirical AM1 method.[79] Using the two methods together, the following descriptors were calculated for the substrate and the radical obtained after dehydration: the Mulliken charges on the carbon and hydrogen atoms involved in the reaction; the spin of the carbon atom in the radical; the energies of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO); and the energy difference between these two orbitals. The coefficients for the hydrogen 1s and carbon 2p atomic orbitals in the HOMO and LUMO were also calculated. Their analyses of computational models of different complexity revealed that simpler and less computationally demanding methods could be used to identify SOM. This spurred many articles (*cf.*[80–82]) as well as the development of the software SMARTCyp, further discussed in Section 1.7.2.

Inhibitors of CYP3A4 have been studied extensively. Datasets in earlier studies often had fewer than 30 compounds and, based on these, typically linear SAR models were developed. These studies identified lipophilicity as an important descriptor for achieving inhibition.[75,83,84] Later studies used datasets with several hundreds of compounds. The aim of these was to find molecular motifs of importance for CYP3A4 inhibition or to develop global computational models for predicting the risk that a new compound might be a CYP3A4 inhibitor. AstraZeneca studied 463 compounds for their CYP3A4 inhibition and the response data were analyzed by either partial least squares or regression tree methodology.[85] The modeling used molecular descriptors based on atoms and fragments as input. The greater the aromaticity and lipophilicity, the more potently the compound inhibited CYP3A4. Furthermore, neutral compounds and bases inhibited CYP3A4 whereas negatively charged compounds bound to other isozymes. Another study of 741 compounds developed a classification model to distinguish inhibitors from non-inhibitors. The model was then validated with a test set of 186 compounds. The recognition rate of the model was relatively high and 73% of the compounds were correctly identified as either inhibitors or non-inhibitors. The final model was based on constitutional, electrostatic

and geometric descriptors. Examples of these are molecular weight, flexibility (number of flexible bonds, rigid bonds and rings), charge, lipophilicity and van der Waals surface area. This model identified that inhibitors are larger than non-inhibitors and extracted a cut-off value of 354 g $mol^{-1}$. Inhibitors are also more hydrophobic and have fewer chargeable groups, the latter being <5% of the molecular composition for inhibitors. Another molecular property that discriminates inhibitors from non-inhibitors is the number of nitrogens; a compound with more than two nitrogens does not inhibit CYP3A4.[86] A study of 1756 compounds on the inhibition of CYP3A4 concluded that the models for prediction of CYP3A4 inhibition must be based on algorithms that can handle the complexity of enzymatic inhibition and the resulting non-linear data.[87]

### 1.6.2 CYP2C9, CYP2C19 and CYP2D6

Computational analyses of pharmacophore modeling, protein conformation analyses and multivariate data analyses have identified that substrates to CYP2C9 are hydrophobic (up to two functions), and include at least one hydrogen bond donor and one hydrogen bond acceptor.[88–91] Substrates of CYP2C9 are favored by being negatively charged but metabolism by CYP2C19 enzyme is not. Substrates of CYP2D6 often contain overlapping hydrophobic features, a hydrogen bond donor function well separated from the hydrophobic features and negative molecular electrostatic potential.[92,93] The hydrophobic domain near the oxidation site interacts with a large, flat and lipophilic region of the CYP2D6 that contains residues Leu121, Leu213, Ala305, Val370 and Thr309. The hydrogen bond donor group can have two different spatial locations from the hydrophobic region. Substrates with the nitrogen atom positioned 10 Å from the oxidation center interact with CYP2D6 through hydrogen bonds between the nitrogen and Glu216 and Gln117. Other substrates have the nitrogen atom positioned 5 or 7 Å away from the oxidation center and the nitrogen interacts only with the Asp301 residue of CYP2D6.[93]

Gleeson and colleagues studied a dataset of 457 compounds to predict CYP2C9 inhibitors.[85] Molecular features that correlated with CYP2C9 inhibition were lipophilicity, aromaticity and non-ionizability. The enzyme was also found to be inhibited by negatively charged compounds, a finding also confirmed by Manga *et al.*[94] Pharmacophore models based on three different datasets ($n$ = 9, 29 and 13, respectively) each generated a different pharmacophores that inhibited CYP2C9. The pharmacophores differed from each other spatially and in other important molecular features.[95] All three pharmacophore models included one hydrophobic pocket and one hydrogen bond acceptor but they differed in number of hydrophobic pockets (1–2), hydrogen bond acceptors (1–2) and whether they had hydrogen bond donors. There is a large overlap in the physicochemical properties of inhibitors of CYP2C9 and CYP2C19. For example, Gleeson *et al.*, who used a dataset of 369 compounds, found that CYP2C19 is also inhibited by lipophilic and aromatic compounds.[85] However, CYP2C19 has a preference for neutral compounds.

The overlap between these two enzymes is understandable as they share 95.7% homology—only 43 of their 490 amino acids differ from each other.[96] Studies of CYP2C19 have also revealed that stronger inhibitors are more lipophilic at the N-3 position. The binding affinity of the inhibitors also increases with the degree of steric bulk. This is a result of the general entropic effect associated with solvation where the increased order of the bulk water for larger compounds favors binding of such molecules to the enzyme.

In contrast to the CYP3A4, CYP2C9 and CYP2C19 inhibitors, the role of lipophilicity for inhibition of CYP2D6 is less clear. Using a dataset of 170 compounds, Gleeson *et al.* identified that inhibitors of this enzyme are aromatic structures with weak basic functions, but not lipophilic *per se*.[85] Groot *et al.* reviewed different models for the prediction of CYP2D6 inhibitors (and substrates).[92] Based on 3500 compounds, they extracted the following rules for inhibitors: (i) inhibitors are weak bases (92% of the compounds with CYP2D6 $IC_{50}$ < 1 mM were weak bases); and (ii) decreasing polarity, as measured by the total PSA (TPSA), increases CYP2D6 inhibition (*e.g.* 73% of the weak bases had CYP2D6 $IC_{50}$ < 10 mM when the TPSA was <50 Å$^2$). In comparison, this number was 37% when TPSA was >100 Å$^2$. In contrast to the study by Gleeson *et al.* Groot and coworkers found that lipophilicity was positively related to inhibition. The majority (73%) of the weak bases with a calculated log*P* of 3–5 had CYP2D6 $IC_{50}$ < 10 mM, whereas this number was 45% for the weak bases with a log*P* of 1–3.

## 1.7 Software

A number of different methods and software are available for the prediction of metabolism. These enable predictions of metabolic sites, metabolic reactions and products, mechanisms, and enzyme dynamics. More traditional structure–activity approaches are also used and models have been developed to predict CYP inducers and inhibitors. An extensive list of software can be found in the article published by Kirchmair and coworkers.[97] Three commonly used pieces of software that make use of a combination of different *in silico* approaches are MetaSite, SMARTCyp and StarDrop. These are briefly described below.

### 1.7.1 MetaSite

MetaSite (Molecular Discovery, Italy) predicts phase I metabolism mediated by CYP enzymes and flavin-containing monooxygenases. It predicts the binding between substrates and enzymes (a thermodynamic factor) and the chemical transformation (a kinetic factor). The predictions are obtained by a combination of molecular interaction fields that analyze ligand and enzyme properties, together with quantum mechanics and knowledge-based components that relate to the kinetics of metabolism (*i.e.* the reactivity). The software enables identification of molecular sites vulnerable to metabolism

so that medicinal chemists can redesign structures that are rapidly cleared through metabolism. To further improve predictions of metabolism patterns and reactivity, Molecular Discovery has formed a human CYP consortium with some pharmaceutical companies. The overarching goal of this consortium is to produce high-quality experimental data to improve predictions for metabolic rate, site and reaction pathways, and the likelihood of a compound being a substrate/inhibitor for a specific enzyme. Information about the program can be found on the MetaSite webpage (http://www.moldiscovery.com/software/metasite).

### 1.7.2 SMARTCyp and StarDrop

The software SMARTCyp originates from the University of Copenhagen. It includes a fragment-based database for which the density functional theory activation energy has been calculated. This database is then used to match structural fragments of drug molecules to estimate CYP3A4-, CYP2D6- and CYP2C9-mediated transformation. Data from 211 transitions were used to develop the fragment-based energy rules. To rank the SOM in the molecules, an accessibility descriptor is used.

Figure 1.2 shows clopidogrel, an antithrombosis drug discussed in the pharmacogenomics Section (1.4). SMARTCyp provides probability estimations of a particular atom being the site of enzymatic activation. For clopidogrel, which is composed of 18 heteroatoms, the atoms are numbered 1 to 18. The estimation is based on three factors related to the molecular structure: activation energy, accessibility and solvent-accessible surface area. The

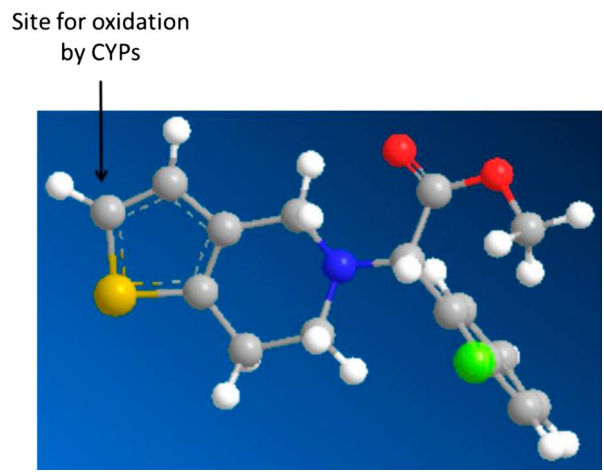



**Figure 1.2** Clopidogrel's main site for enzymatic degradation. SMARTCyp predicts the probability of this atom being the site of metabolism to be among the top 33% (with CYP2D6 the highest probability; and for CYP2C9 and CYP3A4 ranked as atoms 5 and 6, respectively, out of 18). Predictions are based on the methodology described by Rydberg and coworkers.[80]

activation energy is the approximate energy required for the reaction of the catalytic site of the enzyme (*i.e.* CYP3A4, CYP2C9 or CYP2D6) to occur at this atom. Accessibility is a measure of the distance of the particular atom from the center of the molecule and always has a number between 0.5 (atom is positioned in the center) and 1.0 (atom is positioned at the far end of the molecule). The solvent-accessible surface area is the total surface area of that particular atom exposed to *e.g.* water and thereby accessible for interaction with the enzyme. This surface area is predicted from 2D molecular topology descriptors. CYP3A4 and CYP3A5 are the most important enzymes for hepatic metabolism of clopidrogel.[98] Clopidogrel is a prodrug and requires enzymatic activation *in vivo*, the atom of importance for this is the carbon next to the sulfur in the thiophene group (Figure 1.2).[99] It should be noted that *in vivo* about 85% of clopidogrel is inactivated by hydrolysis of the ester group, *i.e.* this ester is necessary to obtain active inhibition of platelet aggregation. This type of reaction is not predictable by the SMARTCyp software.

Similar to SMARTCyp, the software Stardrop (Optibrium, UK) predicts reaction sites. It makes use of quantum mechanics calculations to predict metabolic sites and their vulnerability to different CYPs. This software has also been suggested as a useful tool in the redesign of enzymatically liable molecules. The accuracies of the predictions of SMARTCyp and StarDrop are similar.[80]

## 1.8 Conclusion

This chapter has reviewed the physicochemical properties of compounds that determine their interaction with transport proteins and the enzymes involved in drug clearance. Lipophilicity is an important physicochemical property resulting in interaction and, in particular, inhibition of both transport proteins and enzymes. Other specific features of the substrates and inhibitors are summarized below.

- Substrates to OATP1B1, OATP2B1 and OATP1B3 are negatively charged. Inhibitors of these transport proteins are lipophilic (~log$P$ 4), large (~500 g mol$^{-1}$) and polar ~120 Å$^2$, although OATP2B1 inhibitors are less dependent on polarity than those of OATP1B1 and OATP1B3. Inhibitors of OATP1B3 have a larger number of hydrogen bond donors (3–4). A low number of aromatic bonds (<7) increases the risk of OATP1B1 inhibition, but reduces the risk of OATP1B3 inhibition.
- Substrates to OCT1 are cationic and, hence, many of the inhibitors also carry a positive charge. Furthermore, inhibitors are lipophilic (log$P$ of 3.50) and less polar (PSA of 43), with a lower number of hydrogen bond donors (1) and acceptors (~3) than the non-inhibitors.
- P-gp substrates require specific hydrogen bond interactions and compounds with (N + O) ≥ 8, molecular weight > 400 g mol$^{-1}$ and acid p$K_a$ > 4 are likely to be P-gp substrates. Inhibitors of P-gp and other efflux proteins (BCRP and MRP2) are lipophilic and aromatic. P-gp is

the most lipophilic ($\log D_{pH7.4}$ of 2.3) followed by BCRP ($\log D_{pH7.4}$ 1.9) and MRP2 ($\log D_{pH7.4}$ of 1.2). Multi-specific inhibitors are 100- to 1000-fold more lipophilic ($\log D_{pH7.4}$ of 4.5). Specific inhibitors of P-gp are less aromatic than those of MRP2 and BCRP. In addition, BCRP inhibitors have a greater number of aromatic nitrogens than P-gp inhibitors.

- Studies of CYP substrates have identified the importance of an acidic function, hydrogen bond donors and hydrogen bond acceptors for CYP2C9, a weak basic function (*i.e.* cationic charge), and aromatic ring features for CYP2D6 and hydrophobic features for CYP3A4.
- Inhibition of CYP2C9, CYP2C19, CYP2D6 and CYP3A4 is highly related to the lipophilicity and aromaticity of the drug—inhibition increases with lipophilicity for all four enzymes. Inhibitors of CYP2C9 and CYP2C19 have common features; however, CYP2C9 prefers negatively charged compounds whereas CYP2C19 is inhibited by neutral compounds. Inhibition of CYP3A4, which is the most studied enzyme, is favored by aromatic structures, larger molecules (molecular weight > 354 g $mol^{-1}$) and compounds with a low number of nitrogens (<2).

The literature on substrates and inhibitors of the transporters and CYP enzymes suggests that different computational approaches are required to arrive at reliable and robust predictions of interactions. Increasing the prediction accuracy of SOM and the development of quantitative models, for the prediction of *e.g.* $IC_{50}$ values, will require elucidation of the pathway (competitive/non-competitive inhibition and binding site of the enzyme for substrates) based on large, high-quality datasets. In addition, the interplay between transporters and enzymes needs further attention to understand how drug-like compounds access the intracellular enzymes.

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