

**DEVELOPMENT OF *IN SILICO* MODELS
FOR THE PREDICTION OF TOXICITY
INCORPORATING ADME INFORMATION**

PRZEMYSŁAW PIECHOTA

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Name of candidate: Przemyslaw Piechota

School: Pharmacy & Biomolecular Sciences

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(Candidate)

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Abstract

Drug discovery is a process that requires a significant investment in both time and resources. Although recent developments have reduced the number of drugs failing at the later stages of development due to poor pharmacokinetic and/or toxicokinetic profiles, late stage attrition of drug candidates remains a problem. Additionally, there is a need to reduce animal testing for toxicological risk assessment for ethical and financial reasons. *In silico* methods offer an alternative that can address these challenges.

A variety of computational approaches have been developed in the last two decades, these must be evaluated to ensure confidence in their use. The research presented in this thesis has assessed a range of existing tools for the prediction of toxicity and absorption, distribution, metabolism and elimination (ADME) parameters with an emphasis on absorption and xenobiotic metabolism. These two ADME properties largely determine bioavailability of a drug and, in turn, also influence toxicity. *In vitro* (Caco-2 cells and the parallel artificial membrane permeation assay) and *in silico* approaches, such as various druglikeness filters, can be used to estimate human intestinal absorption; a comparison between different methods was performed to identify relative strengths and weaknesses of the approaches. In terms of xenobiotic metabolism it is not only important to predict metabolites correctly, but it is also crucial to identify those compounds that can be biotransformed into species that can covalently bind to biomolecules. Structural alerts are routinely used to screen for such potential reactive metabolites. The balance between sensitivity and specificity of such reactive metabolite alerts has been discussed in the context of correctly predicting reactive metabolites of pharmaceuticals (using data available from DrugBank). Off-target toxicity, exemplified by human Ether-à-go-go-Related Gene (hERG) channel inhibition, was also explored. A number of novel structural alerts for hERG toxicity were developed based on groups of structurally similar compounds. Finally, the importance of predicting potential ecotoxicological effects of drugs was also considered. The utility of zebrafish embryos to distinguish between baseline and excess toxicity was investigated. In evaluating this selection of existing tools, improvements to the methods have been proposed where possible.

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Abbreviations

% F	Bioavailability
% FA	Fraction of drug absorbed
3Rs	Reduction, refinement and replacement
5HT4	5-Hydroxytryptamine
ABL	Aqueous Boundary Layer
ADME(T)	Absorption, Distribution, Metabolism, Excretion, (Toxicity)
ADR	Adverse Drug Reactions
AM1	Austin Model 1
AP	Applicability Domain
APIs	Active pharmaceutical ingredients
ATP	Adenosine triphosphate
Caco-2	Cells derived from colorectal carcinoma cells
CADD	Computer-Aided Drug Design
CAS RN	Chemical Abstracts Service Registration Number
CHO	Chinese Hamster Ovary cells
Cl(h)	(Hepatic) clearance
Cl_{int}	Intrinsic clearance
cLog P	Calculated partition coefficient
CNS	Central Nervous System
CoMFA	Comparative Molecular Field Analysis
CoMSiA	Molecular Similarity Indices in a Comparative Analysis
COS	Fibroblast-like cells
CSV	Comma Separated Values file
CYP450	Cytochrome P450
D	Dose of a drug
dG	Deoxyguanosine
DILI	Drug-Induced Liver Injury

Dmax	Maximum diameter
DOS	Diversity-Oriented Synthesis
DNA	Deoxyribonucleic Acid
E	Embryo
EC₅₀	Half maximal effective concentration
EFPIA	European Federation of Pharmaceutical Industries and Associations
E_{HOMO}	Energy of the Highest Occupied Molecular Orbital
ELE	Eleutheroembryo
E_{LUMO}	Energy of the Lowest Unoccupied Molecular Orbital
EQU	Equivocal
EU	European Union
f	Fraction of free drug
FATS	Fish Acute Toxicity Syndrome
FBS	Fragment-Based Screening
FDA	Food and Drug Administration
FET	Fish Embryo Test
FN	False Negative
FP	False Positive
FRB	Freely Rotated Bonds
GI	Gastro-Intestinal
GIT	Gastro-Intestinal Tract
GRIND	GRid-INdependent Descriptors
GSH	Glutathione
HAS	Human Serum Albumin
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HCS	High-Content Screening
HEK 293	Human Embryonic Kidney cells
Hep G2	Human hepatic carcinoma cell line
hERG	human Ether-à-go-go-Related Gene

HIA	Human intestinal absorption
HMG CoA	3-Hydroxy-3-MethylGlutaryl-COenzyme A
HOMO	Highest Occupied Molecular Orbital
HTD	High-Throughput Docking
HTS	High-Throughput Screening
IC₅₀	Half maximal inhibitory concentration
IGC₅₀	50% inhibitory growth concentration
I_k	Delayed rectifier current
IMI	Innovative Medicines Initiative
InChIKey	IUPAC International Chemical Identifier
IUPAC	International Union of Pure and Applied Chemistry
IVIVE	In Vitro-In Vivo Extrapolations
KCS	Potassium cyanide
kNN	k-nearest neighbours
K_{ow}	Octanol-water partition coefficient
LBVS	Ligand-Based Virtual Screening
LC₅₀	Concentration resulting in 50% lethality
LLE	Lipophilic Ligand Efficiency
Log D	Distribution coefficient
Log P	Partition coefficient
Log P_{apparent}	Permeability coefficient
Log S	Logarithm of aqueous solubility
LUMO	Lowest Unoccupied Molecular Orbital
M_{abs}	Total mass absorbed
MCTs	Monocarboxylate Transporters
MDCK	Madin-Darby Canine Kidney cell lines
MIST	Metabolites In Safety Testing
MLP	Multilayer Perceptron
MLR	Multiple Linear Regression
MOE	Molecular Operating Environment

MR	Molar Refractivity
MSE	Mean squared error
MW	Molecular weight
NA	Number of Atoms
NAPQI	N-acetyl-p-benzoquinone imine
NAS	New Active Substance
NBR	Number of Rotational Bonds
NME	New Molecular Entity
NMR	Nuclear Magnetic Resonance
NRC	National Research Council
NSAID	Non-Steroidal Anti-inflammatory Drug
OECD	Organization for Economic Cooperation and Development
PAMPA	Parallel artificial membrane permeability assay
PBPK	Physiologically-based pharmacokinetics
PLA	Plausible
PRO	Probable
PCA	Principal Component Analysis
pIC₅₀	Negative logarithm of IC ₅₀
pKa	Ionisation constant
PLS	Partial Least Squares
Q	Hepatic blood flow
Q²	Cross-validated correlation coefficient
QED	Quantitative Estimate of Drug-Likeness
(Q)SAR	(Quantitative) Structure-Activity Relationship
QSPR	Quantitative structure-Property Relationship
R	Correlation coefficient
R²	Coefficient of determination
R²_{adj}	Adjusted determination coefficient
RBD	Radioligand Binding Assay
REACH	Registration, Evaluation, Authorisation and restriction of Chemical substances

RF	Random Forest
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
SCA	Semicarbazide
Sdf	Structure-Data File
SLC	Solute carrier
SMARTS	SMiles ARbitrary Target Specification
SMILES	Simplified Molecular-Input Line-Entry System
SMOreg	Support vector machine for regression
SOM	Site Of Metabolism
SVM	Support Vector Machine
T_{1/2}	Half-life
T_e	Toxic ratio
TdP	Torsade de Pointes
TN	True Negative
TP	True Positive
TPSA	Total polar surface area
UDP	Uridine Diphosphate Glucose
UGT	UDP Glucuronosyl-Transferase
US	United States
UWL	Unstirred Water Layer
VS	Virtual Screening
vsurf	Volume and surface descriptors
zERG	Zebrafish orthologue of HERG
ZFET	Zebrafish embryo acute toxicity tests

1. Introduction

1.1. Drug discovery

Drug discovery is a process driven by the need for suitable pharmaceutical products that can be used, successfully, to treat a wide range of existing medical conditions (Hughes et al, 2011). A drug discovery project is a huge undertaking that is both time-consuming and requires substantial financial resources. The development of a novel drug can take up to 15 years - from the original concept to introducing the drug to the market. A systematic review of drug development costs showed that the figures may vary significantly but have been estimated to be in the range of USD\$92 million to USD\$883.2 before capitalisation (Morgan et al., 2011). However, drug development is a business that involves a high risk of failure. The probability of success for a drug development project may be as low as 7% (Lou & de Rond, 2006) although the rates vary. It is difficult to give accurate estimates of time and cost due to the number of variables involved and the applied methodology (Pronker et al., 2011), however the need for more rapid, cost-effective means of drug development is evident. This need has been recognised within the Innovative Medicines Initiative (IMI), a joint undertaking between the European Union and the association of pharmaceutical companies (EFPIA), which currently funds approximately 50 projects aimed at improving the drug discovery process. eTOX is one such project representing the combined effort of 25 participants from both industry (e.g. Novartis, Pfizer, Bayer) and academia (e.g. Liverpool John Moores University, University of Vienna, VU University Amsterdam) with the aim of speeding up the process of introducing safer and more effective medicines, by developing strategies and tools to predict safety and side-effects of drug candidates. The idea of the project grew from the realisation that the pharmaceutical industry is in possession of large amounts of unpublished data that were acquired during the drug development process. However, these data were not made available publicly due to their confidentiality. The main objectives of the eTOX project include: building a large database that would contain both public and private data, the implementation of methods that would allow access to this proprietary information, whilst protecting the intellectual property, and using the compiled database to develop or enhance toxicity prediction models (Briggs et al. 2012).

The aim of drug discovery is the development of new molecular entities (NME). NME refers to a drug that contains an active ingredient that has not been approved in any form by the United States Food and Drug Administration (US FDA). This term is very broad (in Europe the term new active substance (NAS) is used) and includes both new chemical entities (small molecules) and also new biological

entities (Paul et al., 2010). The drug discovery process can be divided into four distinct stages, which are characterised by different methodological approaches, each of which is discussed below.

I. Target discovery

The first stage of the drug discovery process involves target identification and validation. A broad spectrum of targets has been recognised such as: genes, proteins, RNA, disease biomarkers or elements of biological pathways that could be associated with a specific clinical condition (Yang, Adelstein & Kassis, 2012). Two different strategies are employed to target discovery, namely, systems approaches and molecular approaches. Systems approaches represent a more traditional way of identifying a target by using whole organisms to study the disease. Data are obtained from patients or *in vivo* animal models that focus on the relevant areas of physiology or pathology. The majority of drugs available on the market have been discovered through systems approaches and this methodology is still important in target discovery in the context of diseases for which specific phenotypes are detected at the whole organism level, e.g. obesity, hypertension, stroke, neurodegenerative conditions (Lindsay, 2003). However, recent advances in *omics* technology has led to a shift towards molecular approaches (e.g. in oncology) although phenotypic screening is still a major contributor in drug discovery when measured by number of new first-in-class small molecule drugs introduced to the market (Swinney & Anthony, 2009). In molecular approaches the emphasis is put on molecular mechanisms that are relevant to a specific pathological condition. In other words, molecular approaches are geared towards discovering targets at the cellular level through the study of molecular events. In this context, bioinformatics approaches are utilised to identify a target (Chen & Chen, 2008). These include data mining methods such as searching large databases of literature relevant to biomedical sciences (e.g. PubMed) and analysis of microarray data through supervised classification or unsupervised clustering. This can be enhanced by the analysis of data provided by proteomics and chemogenomics. Furthermore, target discovery can benefit from integrative approaches (Kim et al., 2007). A combination of different data mining approaches gives a more complete understanding of cellular mechanisms and, consequently, leads to selection of an appropriate target. Such a target should be characterised by a high druggability, i.e. it should be amenable to modulation when interacting with small molecules or proteins. The idea of a 'druggable genome' was introduced by Hopkins & Groom, (2002). They emphasised that a limited number of biological targets are available for drug discovery as not all genes are disease-modifying and some of them are not druggable, i.e. they are not capable of binding drug-like molecules (e.g. orally bioavailable compounds). In the case of the most common targets – proteins – one of the determinants of druggability is binding affinity at levels below 10 μ M (Keller, Pichota & Yin, 2006).

There are several different methods to assess or predict druggability; these include high-throughput approaches for identifying drug binding sites (Schmidtke & Barril, 2010), calculating druggability indices from data obtained in nuclear magnetic resonance (NMR) screening (Hajduk et al., 2005) or structure-based methods for assessing maximum binding affinity (Cheng et al. 2007). The properties of ideal druggable targets in drug discovery have been summarised by Gashaw et al., (2012).

II. Hit discovery

Once the target has been determined and validated the next step involves the identification of “hit” compounds. In the context of drug discovery, hits can be defined as small molecules (low molecular weight) that can display an adequate activity in relevant target assays (Bleicher et al., 2003). These assays can determine appropriate binding affinities (usually in micromolar range) to such therapeutic target classes as receptors, enzymes or ion channels. The identification of hits involves screening large libraries of compounds. These libraries may contain thousands or millions of compounds. High-throughput screening (HTS) of large libraries is a traditional approach to hit identification. However, the development and maintenance of large libraries is costly and despite the sheer size of the collection of compounds only a fraction of chemical space is represented. Libraries may also include compounds that are not druglike. Consequently, the potential to discover hits is limited and even if the hits are identified they may not be considered for further development due to unsuitable physico-chemical properties. Hence, HTS libraries may not be an optimal starting point for discovery of hit or lead compounds (Scott et al, 2012). In response to these challenges new methodologies were developed for the design of chemical libraries that addressed the balance of library size and molecular diversity. In the last two decades, two different strategies for library design have emerged: fragment-based screening (FBS) and diversity-oriented synthesis (DOS). Both FBS and DOS resulted from two different approaches to the concept of drug discovery. FBS libraries contain small molecular fragments that are screened for binding affinities to the target. Fragments that are found to bind target at micromolar concentrations are further utilised for identification of a lead compound in a processes of fragment evolution, linking, self-assembly and optimisation (Rees et. al, 2004). In comparison to HTS, this method offers a number of advantages: it is less resource-intensive, it identifies more efficient binders (although less potent), it may provide better understanding of binding interactions and it usually generates compounds with lead-like properties (Rees et al., 2004). Similarly to FBS, DOS libraries consist of hundreds to thousands of compounds. However, DOS libraries are composed of more druglike compounds with higher molecular weights or log P (partition coefficient) values. Such structurally diverse compounds are usually synthesised from common intermediates in a modular manner and then screened for lead-like molecules that

generally possess higher affinity for the target than do small fragments. DOS libraries are typically built without prior knowledge of activity of the members toward the biological targets. Both FBS and DOS libraries have been successfully used for drug discovery processes and each type of library can have certain advantages. For instance, the FBS approach has been characterised to provide a more structurally diverse library (Hajduk, 2011). However, this diversity may not necessarily be observed in actual FBS libraries due to the medicinal chemistry bias in selection of fragments. In consequence, FBS libraries tend to contain a large proportion of fragments based on aromatic rings, which leads to obtaining structurally flat leads as opposed to producing more 3-D-like scaffolds from the DOS approach, which could be more relevant in the biological context (Galloway & Spring, 2011).

A variety of screening strategies have been developed. HTS provides a means to test complete libraries either directly against a drug target or, increasingly, by using cell-based assays with application of high-content screening (HCS) (Fox et al., 2006). The advances in automation and robotics, miniaturisation of the microtiter plate wells, replacement of radioactive reagents with fluorescent labels and the development of new fluorescence methods resulted in HTS procedures that required smaller amounts of reagents and were more time-efficient (Phatak, Stephan, Cavasotto, 2009). Fragment screening, often accompanied by NMR used for structure determination, represents another strategy for hit discovery although, in contrast to HTS, it relies on the presence of the target crystal structure. Focused screens provide a cheaper alternative to HTS. This strategy involves screening compounds that are already known to bind to certain classes of targets, e.g. kinases. However, it may not be useful for discovery of novel pharmaceuticals. Lower throughput screening is also used, for instance, in physiological screens. In this case, the effects of selected potential drugs are studied at tissue level rather than by utilising cells or cellular components (Hughes et al., 2011).

The final stage of hit discovery is establishing a hit series. The screening generates a number of hits and their suitability for drug discovery has to be assessed. This is achieved by taking dose-response curves to establish half maximal inhibitory concentrations, which can be used to compare the potencies of the hits and also identify compounds that bind to the target in a reversible manner. This process can be supported by secondary *in vitro* bioassays to confirm that a specific hit not only interacts with an isolated target (e.g. a receptor) but can also induce functional change at the cellular or tissue level. The selected hits can be then clustered into groups based on structure-activity relationships (SARs). Compounds in each cluster usually have a common substructure that is responsible for the activity however, their potencies differ as a result of differences between chemical groups attached to the motif they share. At this stage, a further refinement process is

carried out. It involves the initial *in vitro* assessment of absorption, distribution, metabolism and elimination (ADME) properties and also gathering more information about potential selectivity issues. These processes allow for the definition of a number of hit series with each hit having activity in the range of 100 nM to 5 μ M (Hughes et al., 2011).

III. Hit to lead

The hit discovery process concentrates on identifying compounds with promising pharmacodynamic properties, i.e. the major role of the screening process is to search for molecular entities that can produce a desired biological activity. The hit to lead stage focuses on suitable pharmacokinetic properties of potential NMEs, whilst attempting to further increase the potency and selectivity of the hits. This stage encompasses consideration of the *in vivo* situation where the drug has to pass through biological barriers, e.g. permeability barriers such as the wall of the gastrointestinal tract or blood-brain barrier (Pardridge, 2001) before it can reach its target, i.e. it has to be bioavailable. The rates of xenobiotic metabolism (biotransformation) and excretion may significantly reduce bioavailability. Hence, ADME properties have to be carefully examined and modified where necessary in order to make a compound more bioavailable.

The output of the hit to lead stage comprises a series of leads. A lead can be defined as a ligand that has a suboptimal binding affinity towards a target. Characteristics of a lead, according to Oprea et al (2001), may include: i) the lack of complex molecular features so that the structure is more amenable to potential optimisation; ii) its presence in a clearly defined SAR series; iii) not being subject to a patent and iv) possessing good ADME properties. In the context of ADME, certain rules were defined to describe what constitutes a good lead molecule. These included Lipinski's "rule of five" defined for oral absorption or permeability (Lipinski et al., 2001) and Veber's rules for oral bioavailability (Veber et al., 2002). These druglikeness rules utilise simple molecular properties such as log P, molecular weight, the number of hydrogen bond donors and acceptors, polar surface area or the number of rotational bonds. The development of fragment based discovery led to the introduction of the concept of "lead-likeness". It was established that lead compounds usually have lower molecular complexity, are less lipophilic and less druglike (Oprea et al., 2001). Consequently, the rule of three was proposed to address the concept of lead-like 'hits' in fragment-based screening (Congreve et al., 2003).

A number of assays are used both during hit discovery and the hit to lead phase. Aqueous solubility and permeability across lipid membranes are key parameters that are measured. Permeability is often determined in *in vitro* studies using Caco-2 cells (representing the intestinal epithelium) or

Madin-Derby Canine Kidney (MDCK) cells transfected with the MDR1 gene to quantify the effect of efflux – a process which may limit gastro-intestinal uptake of molecules. Additionally, artificial membranes that mimic biological structures (for example PAMPA membranes) can be used for this purpose. Metabolism can be assessed using microsomal stability assays that measure compound clearance and may identify metabolites. Finally, cytotoxicity assays, including CYP450 inhibition and Hep G2 hepatotoxicity, may also be performed (Hughes et al., 2011).

IV. Lead optimisation

The final phase of drug discovery aims at obtaining a drug candidate that would be suitable for preclinical studies. This is achieved by further improvements in both pharmacodynamic and pharmacokinetic properties of a number of lead compounds so that only one or two structures belonging to different lead series are selected for further development. Optimisation of candidates is performed in terms of their potency, selectivity and ADME-Tox profile (encompassing oral absorption, bioavailability, metabolic clearance and off-target toxicity - such as cardiotoxicity related to hERG channel inhibition or adverse drug reactions resulting from the formation of reactive metabolites (Kalgutkar et al., 2005)). Various metrics have been introduced to assess the suitability of a lead. For instance, a number of ligand efficiency indices can be used to estimate the balance between modulation of physico-chemical properties and increasing target affinity during lead optimisation. This can include lipophilic ligand efficiency (LLE) which can be used to guide a medicinal chemist as a measure of lipophilicity and *in vitro* potency (Hopkins et al., 2014). Lipophilicity is an important property that affects both ADME and binding affinity. Reducing LLE has been shown to improve ADME properties and safety profiles as a result of lowered lipophilicity (Tarcsey, Nyiri & Keseru, 2013). Nevertheless, less than 10% of leads selected for development are eventually launched to the market (Peck, 2006). Bias towards the potency may be indicated as one of the reasons for drug candidates' attrition during the development stage. The bias may be a result of typically well-defined metrics during, such as binding affinity, while assessment of ADME properties seems to be a more complex task and more difficult to estimate. However, molecular properties that are important for binding to the target may be inversely correlated to ADME. Gleeson et al., (2011) showed that no strong correlation between *in vitro* potency and the therapeutic dose existed and that oral drugs seldom possess nanomolar activity. Moreover, off-target activities for many orally delivered drugs are considerable which is related to their promiscuity, i.e. binding to many targets despite drug discovery being focussed on a single target. In an attempt to quantify the role of ADME in lead optimisation strategies the concept of drug efficiency was introduced (Braggio et al., 2010). It was defined as a fraction of the dose that is

available at the site of action or, in other words, it is a ratio of the biophase concentration and the dose. This single parameter takes into account all factors (ADME properties) that can affect the concentration of a lead at the target site. The drug efficiency can be used to guide the medicinal chemist during the selection of drug candidates with the aim of increasing the biophase concentration and potentially reduce the attrition rate at the development stage.

The drug discovery process is complex and it is crucial that the output of this phase is represented by very carefully selected drug candidates for which the balance of both potency and ADME properties has been appropriately weighed. Such NMEs can then be subject to preclinical studies as investigational drugs and if approved they can enter clinical phases. Due to the high costs of clinical studies (Morgan et al., 2011) the necessity for high quality output from early drug discovery should be emphasised.

Research within this thesis is most applicable to stages III and IV of drug discovery i.e. hit-to-lead and lead optimisation processes. This thesis describes the development of *in silico* models that can be used to predict ADME-Tox properties of a range of molecules based solely on molecular structure, hence these models can be used to inform design and selection of the most promising drug candidates.

1.2. *In silico* methods in drug discovery

The process of drug discovery, as described above, uses various computational approaches at each stage. Such *in silico* methods not only allow the design of more potent drugs with better ADME profiles (computer-aided drug design, CADD) but also they do it in a less time-consuming manner and utilise less financial resources in comparison to *in vitro* and *in vivo* assays.

Virtual screening (VS) is a major application of CADD that has been utilised in the drug discovery process. VS can be applied to virtual libraries that usually contain $10^5 - 10^7$ compounds stored in a digital format. The aim of VS is to significantly reduce this initial number of potential candidates according to specific criteria, such as interaction with a biological target or desired physico-chemical properties. The output of VS could be one molecule, or it could be hundreds of compounds, depending on the purpose (Tanrikulu, Kruger & Proschak, 2013). VS can utilise either structure-based or ligand-based *in silico* methods (Sliwoski et al, 2013).

Structure-based methods rely on the knowledge of the 3D target structure. Ideally, the three-dimensional structure of a macromolecule should be based on experimental studies (X-ray

crystallography, NMR); if it is not readily available, homology models can be used as a substitute. The assumption of the structure-based approach is that, for a given molecule, more favourable interactions with a binding site on a biological molecule, e.g. a protein will result in more favourable biological activity. The structure-based methods are also referred to as high-throughput docking (HTD) methods. Firstly, HTD requires the potential binding sites and the druggability of the target to be predicted. Secondly, a suitable virtual chemical library is selected. Such a library may be subject to a filtering process, e.g. according to druglikeness rules. The remaining compounds are used in molecular docking studies, i.e. each molecule is placed into a binding site of a macromolecule and energetically favourable conformations of the protein and ligand pair are searched (e.g. by using molecular dynamics, genetic algorithms) which are in turn assessed by a scoring function. The scores represent the likelihood of favourable binding interactions and are used to rank the compounds of interest (Phatak, Stephan & Cavasotto, 2009). In essence, HTD can be regarded as a virtual equivalent of HTS and the increase in computational power, combined with more efficient calculations of ligand poses/protein flexibility led to a number of successful applications of HTD in drug discovery (McInnes, 2006).

Ligand-based virtual screening (LBVS) does not require the 3D structure of the target but instead, it utilises the existing research on ligands that are known to be active towards a specific target (Phatak, Stephan & Cavasotto, 2009). The assumption is made that similar molecules can exert similar biological activity. LBVS often utilises a variety of similarity searching methods such as 2D fingerprints, to filter compounds that are most similar to the active ligand(s). It can also use quantitative structure-activity relationship (QSAR) studies to determine physico-chemical properties important for binding of a particular ligand from which a predictive model can be generated. 3D pharmacophore screening can also be performed. A pharmacophore is a structural abstraction that describes steric and electronic features, such as hydrophobic regions, hydrogen bonding, number of aromatic rings etc, that are important for ligand – receptor interactions (Sliwoski, 2013). A pharmacophore model can be used to identify molecules that possess similar structural features that are also similarly distributed spatially.

In silico ADMET modelling offers another useful computational approach that is applied to drug discovery campaigns. QSAR, SAR and read-across methods are applied to ADMET data to prioritise hits and leads. Hits prioritisation involves obtaining virtual hits profiles that predict such characteristics as bioavailability, clearance, CYP450 metabolism, hERG or central nervous system (CNS) liabilities, presence of structural alerts for genotoxicity or reactive metabolite formation (Gleeson & Montanar, 2012). ADMET models are also useful in the process of lead optimisation,

especially in cases where pharmacokinetics and toxicity profiles are to be improved (Jorgensen, 2008). For instance, SAR information can be used to understand which structural changes could be introduced to obtain an optimal ADMET profile without sacrificing the potency of the lead. The incorporation of a variety of *in silico* filters, relating to ADME properties, in drug discovery has been indicated as one of the reasons for the recent reduction in clinical failures amongst drugs due to poor pharmacokinetics (Khanna, 2012).

1.3. Toxicity and its assessment

Toxicology is a scientific discipline that studies adverse effects of chemicals on living organisms. Depending on the methods used, three main approaches to toxicology can be distinguished: *in vivo*, *in vitro* and *in silico* approaches. *In vivo* toxicology is used to establish the relationship between a chemical agent and the toxic effects it elicits by testing on living animals. *In vitro* (literally translated to “within glass”) approaches apply a different methodology: cultured cells are exposed to potentially harmful substances and then the toxicity towards these cells (either of human or animal origin) is measured. *In vitro* testing is not limited to cells but can also involve assays on biological macromolecules. Finally, *in silico* toxicology uses computational techniques in an attempt to produce valid predictions so that the toxic effects of a chemical can be assessed without the necessity to conduct laboratory-based experiments.

In silico toxicology can be characterised as a multidisciplinary science, utilising the findings from areas such as chemistry, statistics, biological disciplines, computer studies and quantum mechanics. One of the central themes of computational toxicology is building predictive models using existing data. It is based on the assumption that compounds that are similar in terms of structural features may be associated with similar effects, e.g. possess a similar toxicity profiles. This is the basis for structure-activity relationship (SAR) analysis.

There are many benefits of applying computational techniques to toxicology. These include providing an alternative to animal testing or reducing the number of required experiments, thus saving both time and money which is especially important in the area of drug development. However, the methods also have broader applicability. There has been an increased interest in developing *in silico* tools to predict toxicity to address regulatory issues. For example, the EU legislative scheme REACH (Registration, Evaluation, Authorisation & Restriction of Chemicals) has been introduced to assess risks related to chemicals imported or manufactured in the EU in amounts larger than 1 tonne per year (REACH in Brief, 2007). REACH promotes alternatives to animal testing

by advocating the implementation of the 3Rs principles (reduction, refinement or replacement of animal use).

Toxicity of chemicals can be observed and studied at different levels in the context of pathological effects. Toxicity can manifest as cell death or tissue damage as the result of apoptosis or necrosis. In cases where the toxic effects are not lethal an alteration of the cell phenotype or function can be observed. For instance, small molecules may modulate the output of a specific signalling pathway which could in turn cause changes in hormone production and thus affect functioning of other cells. Immunological hypersensitivity is yet another type of toxicity. It is frequently related to the presence of hapten adducts which are formed when low molecular weight electrophilic compounds react with nucleophilic moieties on proteins, i.e. protein binding (Chipinda, Hettick, Siegel, 2011). Haptens can be responsible for downregulation of the immune system or can even induce auto-immune responses. Cancer is a pathological state that may be associated with genotoxic or non-genotoxic mechanisms (Liebler & Guengerich, 2005).

The assessment of toxicity of NMEs is an important challenge that is faced by drug discovery. Adverse effects of drugs can be classified into five different categories: on-target toxicity, hypersensitivity and immune response, off-target toxicity, toxicity related to bioactivation and finally, idiosyncratic reactions (Guengerich, 2011). On-target toxicity is related to the modulation of the primary target. In this case the drug binds to an appropriate target (providing the target has been well-chosen) but either the concentration is too high or the target is present in a tissue not intended for pharmacological intervention. The higher concentration may be a result of altered pharmacokinetics, e.g. due to liver or kidney disease or it may be related to pharmacodynamic interactions such as the increase in the number of target molecules (e.g. increased expression of specific receptors). An example of action at a non-target tissue is provided by statins for which the liver is the intended tissue - here they act to block cholesterol synthesis by the inhibition of HMG CoA reductase. However, statins can also bind to the same enzyme in muscles disrupting post-translational modifications of proteins, which can result in myopathy (Johnson et al., 2004). Hypersensitivity and immune response are associated with hapten formation via covalent protein binding. Four types of hypersensitivity reactions have been defined: immediate-hypersensitivity reactions (type I); antibody-dependent cytotoxic reactions (type II); immune complex disease (type III); and delayed-type hypersensitivity (type IV) (Bugelski, 2005). Off-target toxicity is related to the specificity of a drug. Although a drug is typically designed to bind to a specific target it can also interact with other targets. For instance, it may bind to other members of a protein family (e.g. kinases) or it can be involved in interactions with proteins that can bind structurally-diverse

compounds. The hERG channel represents an example of such a promiscuous target (Babcock & Li, 2013). The nonspecific binding of small molecules to the hERG channel can cause arrhythmias. The prediction of hERG channel inhibition is still a major challenge in pharmacological research. Another type of toxicity is associated with drug metabolism. Xenobiotics can be metabolised to reactive species, i.e. bioactivated. Reactive metabolites can either bind covalently to native proteins, modifying their biological functions, or they can act as haptens leading to an immune-mediated adverse drug effect. Acetaminophen is an example of drug that is metabolised to a hepatotoxic reactive species. The prediction of xenobiotic metabolism, with an emphasis on the identification of potentially reactive metabolites is another important challenge in drug-related research (Stepan et al, 2011). Idiosyncratic drug reactions are least understood and least amenable to prediction because they occur infrequently and animal models offer little predictivity. There are several theories postulated to explain the occurrence of idiosyncratic drug reactions: polymorphism of metabolising enzymes, hapten formation, inflammagen model, danger hypothesis and pharmacological intervention models (Guengerich et al, 2011), however predicting such toxicities is a significant challenge.

Within this thesis the areas of toxicity that have been investigated were toxicity due to inhibition of the hERG channel, formation of reactive metabolites and ecotoxicity.

1.4. ADME properties and their optimisation

The pharmaceutical industry is one of the areas where alternative approaches such as *in vitro-in vivo* extrapolations (IVIVE) and *in silico* modelling have gained much interest (Rostami-Hodjegan & Tucker, 2007). It has been recognised that desirable properties of a drug include not only its efficacy but also adequate pharmacokinetic characteristics. The toxicity of a drug is a function not only of its inherent pharmacological properties but also of its absorption, distribution, metabolism, and excretion (ADME) parameters. Poor ADME properties (for example, formation of toxic metabolites) have been linked to high drug attrition rates in late stages of drug development. Adverse drug reactions (ADRs) have led to the withdrawal of a number of drugs from the market due to high toxicity that was a result of bioactivation. Examples of such drugs include: alclofenac (antiinflammatory), tienilic acid (a diuretic) and amineptine (antidepressant) (Stepan et al., 2011). Hence, the pharmaceutical industry has implemented the assessment of ADME properties of drugs earlier in the drug design process (Kerns, 2008).

Within this thesis research has also been focussed on two ADME properties, namely absorption and metabolism, these are arguably the most important of the ADME properties and are discussed in more detail below. Distribution is also important as it describes the tendency of a compound to move out of blood and enter other tissues to reach a target. It is usually reported as a volume of distribution, i.e. the ratio of the amount of drug in body to the concentration of drug in blood. Distribution depends on the ability of compounds to cross biological compartments and on factors such as plasma protein binding – only unbound drug traverses biological membranes to reach a target. Excretion refers to removing xenobiotics from the body completely, predominantly via the renal route but also via bile, sweat or expired air. Renal excretion of unchanged drug, along with xenobiotic metabolism (mostly hepatic), are the two main factors that determine the elimination of a compound. The rate of elimination of a drug can be quantified by clearance (Cl; the volume of blood completely cleared of drug in a given time) or half-life ($t_{1/2}$; time for the concentration in the body to decrease by one half).

I. Absorption

The uptake of a xenobiotic depends on its route of exposure. A compound may enter the body via lungs, skin or can be absorbed via the gastrointestinal tract. Oral absorption is the most common method of delivery of drugs. Absorption can be measured as a percentage of the amount of compound take up via the gastrointestinal tract. A compound can cross the intestinal epithelium via a number of mechanisms, these include transcellular and paracellular transport. The paracellular mechanism, or passing between the cells, is a typical mechanism for small, hydrophilic molecules. Molecules with higher molecular weight and greater lipophilicity usually pass through the epithelial cells. The transcellular mechanism can be passive (a diffusion driven process) or it can be a carrier-mediated transport (facilitated or active) process that requires transporter proteins (Sugano et al., 2010). The logarithm of the aqueous solubility ($\log S$) and logarithm of the octanol:water partition coefficient ($\log P$) are two important properties that influence the uptake of a drug. ($\log D$ – partition coefficient corrected for ionisation may be used as an alternative to $\log P$). *In vitro* methods (e.g. Caco-2 or MDCK cells) and artificial membrane systems such as PAMPA are used to estimate absorption. Absorption alone does not determine the systemic availability of a compound (i.e. its bioavailability); this is in part determined by (avoidance of) first-pass metabolism and active efflux processes (Madden, 2010).

Various computational approaches have been used to predict absorption and bioavailability. These include QSAR methods, which typically utilise “medicinal-chemistry” types of descriptors (e.g. $\log P$, molecular weight, hydrogen bonding ability) or classification methods based on machine learning

algorithms. Rules that use certain thresholds for selected descriptors (log P, hydrogen bonds or total polar surface area) such as Lipinski's rules (Lipinski et al, 1997) or Veber's rules (Veber et al., 2002) offer simple but widely accepted methods used in drug discovery for early identification of compounds with potentially druglike characteristics. Further details on the processes of absorption and existing models will be given in Chapter 2, where existing models for absorption have been evaluated and modifications to improve prediction are proposed.

II. Metabolism

In the context of pharmaceuticals, metabolism can be described as a process of chemical modification of xenobiotics whereby they become more polar and, therefore, more readily partition into the aqueous urine or bile to be eventually removed from the body. This process can only occur if other ADME parameters such as absorption and distribution, also known as Phase 0, allow access to the site(s) of metabolism. Only then, can xenobiotics undergo biotransformation(s) (Phases I and II) into more soluble and usually less toxic chemical species that are more readily eliminated from the cells (Phase III). Phase I metabolism involves oxidation, hydroxylation, de-amination and de-alkylation reactions which are catalysed mostly by the cytochrome P450 superfamily of enzymes with CYP3A4, CYP2D6 and CYP2C9 isoforms being responsible for the majority of transformations of drugs (Madden, 2009). Phase II reactions yield hydrophilic conjugates, e.g. glucuronides via the action of glucuronyl transferases, and this step may either follow, or be independent of, phase I reactions. Although the majority of metabolites tend to be chemically inert, on some occasions drugs can be converted into species that exert non-desirable pharmacological activity or are chemically reactive. Reactive metabolites formed by bioactivation can bind covalently to biological macromolecules such as proteins leading to idiosyncratic drug toxicity also termed as type B ADRs (adverse drug reactions) (Kalgutkar et al., 2005). Thus, it is important to take into account ADME properties in order to build reliable models to predict activity/toxicity in the body. Otherwise, two problematic scenarios may occur: i) models are built using data for parent drugs only, without including any information on metabolites – in this case any metabolite-related toxicity will be missed; ii) models are based relating to inherent toxicity of molecules with no adjustment for absorption and distribution parameters. This can result in misleading models as apparent lack of activity/toxicity may in fact be the result of lack of ability to reach the target site.

Confidence in predictions for metabolism can be increased by the incorporation of *in vitro* data. However, *in vitro* predictions based on hepatocytes, human microsomes or recombinantly expressed microsomal systems may not give a complete picture. Moreover, the results of *in vitro* studies need to be validated in mammalian systems which can be both time-consuming and very costly (Eimon &

Rubinstein, 2009). Another important aspect is the compliance with recent animal testing policies. Further details on metabolism, methods to predict metabolism and potential refinements to existing methods are presented in Chapter 3.

1.5. QSAR models

Developing a QSAR model is a process that involves a number of steps. First of all, a high quality dataset comprising relevant (biological) activity data, associated with a given chemical structure, needs to be identified. Molecular descriptors for these chemicals can then be generated – those most relevant to the activity should be considered. Finally, a function describing the relationship between the activity and the selected descriptors is established and validated.

1.5.1. Data

A typical dataset used in QSAR modelling consists of a list of compounds with corresponding experimental measurements obtained for a specific endpoint. A variety of experimental values can be used in QSAR. For instance, data may be of biological origin and obtained from *in vitro* or *in vivo* studies. Usually, a biological activity constitutes a fixed response related to a concentration. A response that is halfway between the baseline and maximum effect is commonly used to quantify biological potency. For instance, the effect of an agonist on a receptor would be reported as an EC50 value (dose producing 50% of maximal effect) whilst IC50 values would be obtained for the inhibition of a receptor by an antagonist. ADME properties such as permeability in the form of fraction absorbed can also be used for QSAR modelling. It is important to appreciate the inherent variability of biological data as the large variation may influence the modelling process and can lead to unreliable models. The variability of data obtained from *in vivo* systems is usually higher than for activities measured in less complex *in vitro* systems (Dearden & Cronin, 2006).

1.5.2. Molecular descriptors

Molecular descriptors are obtained through the process of converting molecular structures into a set of continuous numerical or binary values describing specific molecular properties (Dudek, Arodz, Galvez, 2006). Two families of descriptors can be distinguished:

I. 2D QSAR descriptors

This family of descriptors is calculated without information relating to the 3D spatial arrangement of the molecule. 2D descriptors can be further classified as: i) constitutional descriptors – these describe simple properties related to the presence of elements that constitute a molecular

structure. Typical examples of such descriptors include the number of specific atoms, the number of single, double and triple bonds or the number of aromatic rings. ii) topological descriptors – these are the result of the application of graph theory to describe the way the atoms are connected. Atoms are represented by vertices and bonds are represented by edges. Wiener index, Balaban's J index (both calculated from the distance and adjacency matrices), Kier and Hall indices (consider the electrotopological state of the atoms within the molecule) (Hall & Kier, 1995) belong to this group of descriptors related to molecular connectivity. iii) electronic descriptors – these are related to electronic properties of molecules. Polarisability and hydrogen bonding are among such descriptors with the latter especially important in ligand-receptor interactions. This category also encompasses quantum mechanical descriptors such as the energy of the highest occupied molecular orbital (E_{HOMO}) and the energy of the lowest unoccupied molecular orbital (E_{LUMO}). These two descriptors are especially useful for studying chemical reactivity. iv) steric descriptors – these describe size and shape of a molecule. Molecular volume (a sum of the van der Waals volumes), molecular surface area or molar refractivity, as a measure of the size of a molecule, are commonly used in QSAR studies. v) hydrophobicity descriptors – this is an important group of descriptors that are widely used in drug design and discovery as they can be applied to modelling both pharmacodynamic (receptor binding) and pharmacokinetic properties (e.g. the uptake and distribution of a xenobiotic relying on partitioning through biological membranes). Partition coefficient ($\log P$), distribution coefficient ($\log D$) and aqueous solubility ($\log S$) are important hydrophobicity/hydrophilicity descriptors. vi) molecular fingerprints and fragment-based descriptors – these are very useful for screening purposes and can also be used in QSAR modelling. A molecular fingerprint is a string of bits representing the presence or lack of specific molecular features and as such it can be used to quantify the similarity between molecules. In cases where only a specific substructure is to be sought within a molecule SMARTS strings can offer an efficient method for the identification of such motifs (Willet, Barnard & Downs, 1998). Structural alerts, i.e. functional groups or substructures associated with a particular endpoint such as protein or DNA binding can be coded as SMARTS patterns that can be then used to screen compounds represented as SMILES strings.

II. 3D QSAR descriptors

These descriptors are more computationally expensive than 2D features. To obtain 3D descriptors the conformation of the structures has to be established either experimentally or by molecular mechanics calculations. Alignment-dependent and alignment-independent 3D descriptors exist. The former rely on aligning structures in space and therefore depends on knowledge of ligand-receptor complexes. Comparative molecular field analysis (CoMFA) is a common method. It samples

compounds, which are represented by their steric and electrostatic fields, in a three-dimensional lattice and then it analyses the data using the partial least square (PLS) technique to determine which parts of the molecule are important for the biological activity (Cramer, Patterson & Bunce, 1988). For alignment-independent 3D descriptors the superposition of structures is not a prerequisite as the calculations are not affected by rotational and translational movements of molecules in space. VolSurf and Grid-independent descriptors (GRIND) represent two common approaches (Dudek, Arodz & Galvez, 2006).

1.5.3. Statistical modelling

The relationship between the biological activity (toxicity) or property of a molecule and its chemical structure can be subject to statistical analysis: for instance, a linear regression can be obtained that shows a relationship between toxicity and a particular property of the chemicals, such as log P. In this case, the relationship is no longer qualitative analysis but also allows the measurement of the strength of the link between the structure and function, hence it is referred to as quantitative structure-activity relationship (QSAR) analysis (Cronin & Madden, 2010). Classification QSAR modelling represents another popular group of methods for obtaining toxicity predictions. In this case compounds are placed into a specific category (e.g. active or inactive) rather than a numerical value being determined as in the case of regression models.

Advances in artificial intelligence methods have increased the application of machine learning to QSAR modelling in an attempt to provide more accurate predictions. Machine learning provides sophisticated algorithms that can learn from data and then use this knowledge to build both linear and non-linear models. A variety of machine learning techniques are available. These include both supervised learning methods: multiple linear regression (MLR), partial least squares (PLS), decision trees, k-nearest neighbours (kNN), random forest (RF), multilayer perceptron (MLP) and support vector machines (SVM) and also unsupervised learning techniques such as: self-organising maps and principal component analysis (PCA). Supervised learning uses labelled data (e.g. inhibitor/non-inhibitor) to build a model that can predict a value or class for new data points (compounds). In other words, the training examples are provided with the output so that a function that describes the relationship between chemical descriptors and the target variable can be learnt. On the other hand, in unsupervised learning no labels are given to samples and an algorithm has to discover patterns in data without “the teacher”. The integration of machine learning to biological data modelling has been growing. For instance, in the context of ADME-tox property prediction, a 2.7-fold increase was observed in the number of articles published on this topic between 2001 and 2013 with SVM being the most popular and promising technique (Maltarollo et al, 2015).

Several methods for (Q)SAR development have been employed throughout this thesis and each is described in more detail within the relevant chapters.

1.5.4. QSAR validation

In order to be acceptable for regulatory purposes, QSAR or QSPR (quantitative structure-property relationship) models need to be scientifically valid. In 2004, the Organisation for Economic Co-operation and Development (OECD) introduced a set of guidelines known as the OECD principles for the validation of (Q)SARs (OECD, 2007). The following general rules were introduced to guide the development of QSAR models but are applicable to a broader range of *in silico* methods. A QSAR model should be associated with:

- i. A defined endpoint – to ensure that the modelled data were obtained using suitable experimental protocols and conditions. (Such an endpoint should also be of relevance to regulatory purposes.)
- ii. An unambiguous algorithm – methods used for building a QSAR model should be transparent, i.e. details should be known to the potential users to ensure the reproducibility of predictions. The lack of transparency has been recognised as an issue, particularly in relation to commercial software packages and this may be an obstacle for regulatory acceptance.
- iii. A defined domain of applicability – models are based on specific subsets of chemical space. Unreliable predictions may be obtained for compounds that do not belong to this subset of chemical space in terms of their molecular structure, physico-chemical properties or mechanism of action.
- iv. Appropriate measures of goodness-of-fit, robustness and predictivity – the quality of models should be assessed by both internal and external validation. Coefficient of determination (R^2), adjusted determination coefficient (R^2_{adj}), standard error of estimate (s), F-statistics and t-values were indicated to represent statistical parameters describing the goodness-of-fit for multilinear regression models. Explained variance of prediction (Q^2) and mean squared error (MSE) are amongst parameters that can be used to measure the predictive power of QSAR models. The use of external datasets is the most appropriate procedure to assess predictivity. However, in practical terms, the availability of external datasets may be limited. Therefore, it is common to use internal validation by following such

simulation procedures as: cross validation (leave-one-out and leave-many-out), bootstrapping, Y-scrambling or training/test set splitting.

- v. A mechanistic interpretation, if possible – the explanation of the QSAR in terms of chemistry or toxicology should be provided. However, such an interpretation is not always readily available. This may be a consequence of the fact that many models evolve as a result of refinement by the inclusion of more data and/or using different descriptors. Therefore, inferring mechanistic information from models at early stages of development may not be possible. Nevertheless, statistical models of good quality may still be used for regulatory purposes provided they meet the relevant criteria.

Despite guidelines being available for the development and validation of QSARs these rules are not always followed. Dearden, Cronin & Kaiser, (2009) provided a list of potential errors that may be made when building or using QSAR/QSPR models. Such errors include incorrect endpoint data, use of collinear and/or incomprehensible descriptors, use beyond the domain of applicability, unaccountable omission of data points, presence of duplicates within the dataset, data overfitting, lack, or misuse, of statistics or failure to correctly validate the model. Such errors may be a result of model developers or users not being fully aware of flaws or shortcomings in QSAR methods. Scior et al, (2009) suggested how to recognise such pitfalls and avoid them.

1.6. Zebrafish embryos as a screening tool

Additional research has been undertaken within this thesis to determine the applicability of zebrafish embryos as a means of screening compounds for activity/toxicity. Zebrafish embryos/larvae can be considered as a bridge between *in vitro* and *in vivo* studies as they provide experimental results in a broader physiological context and can be subject to high-throughput screening (Deo & MacRae, 2011). Zebrafish *in vivo* screens have been utilised in various stages of the drug discovery process such as target confirmation and lead discovery (Delvecchio, Tiefenbach & Krause, 2012).

There are many reasons why using zebrafish embryos is considered to be a useful solution to questions posed by toxicologists, for example: i) a great number of embryos can be obtained in short time intervals; ii) their maintenance is easy and relatively cheap; iii) zebrafish embryos can be cultured in microwell plates due to their small size and consequently only small amounts of tested compounds are required - this leads to high amenability to large-scale automated screens; iv) the zebrafish genome has been sequenced and it can be subject to genetic manipulations; v) the

embryos are transparent and phenotypic changes can be observed under the light microscope; vi) embryogenesis is rapid and most of the internal organs are fully developed 96 hours post fertilisation (Parng et al., 2002). This is also important in the regulatory context as it has been suggested that zebrafish larvae should be protected from 120 hours after fertilisation i.e. when they are capable of independent feeding (Strähle et al., 2011); vii) certain genes important in xenobiotic metabolism are very similar to those found in mammals, e.g. cytochromes CYP1A1, CYP2B6, CYP3A5 and also UDP glucuronosyl-transferase (UGT) 1A1 (Jones, 2010). This homology means that metabolic profiles comparable to those found in human can be identified. Additionally, zebrafish larvae were shown to be capable of both Phase I and Phase II metabolic reactions (Alderton et al., 2010). Such advantages may be very useful for drug toxicity screening as ADME properties can be studied (Sukardi et al., 2011). Therefore, zebrafish embryos are considered a potentially useful tool for safety assessment in mammalian toxicity. In addition, as part of the drug development process, an assessment of the environmental impact of the drug must be undertaken. As traditional ecotoxicity studies involve adult fish species, the use of zebrafish embryos may offer a refinement to this form of animal testing if they can be shown to respond to toxicants in a similar manner to adult fish. Chapter 6 of this thesis investigates the suitability of using zebrafish embryos in place of adult fish to determine environmental toxicity of compounds, including drugs.

1.7. The overall aims of the project

The overall aim of this thesis is to evaluate existing models for selected ADME-tox endpoints and develop improvements to these models where possible. These models should be of benefit not only to the drug discovery process within the pharmaceutical industry but have wider application to other areas where ADME-tox prediction is required (for example in the cosmetics, industrial chemical and pesticide industries).

1.7.1 Summary of aims and key findings for each chapter

A summary of the aims and key findings for each chapter of this thesis is presented below:

- Chapter 2 investigates the prediction of human intestinal absorption, in the context of bioavailability, using druglikeness filters as well as data from *in vitro* Caco-2 and PAMPA assays. The main aim was to compare the performance of *in vitro* (Caco-2 and PAMPA assays) and *in silico* methods for the prediction of HIA. Additional aims included the development of QSPRs in relation to HIA and bioavailability and the analysis of the role of

metabolism in the prediction of bioavailability. It was found that simple *in silico* druglikeness rules such as Lipinski's rule of five or a combination of medicinal chemist-type of descriptors such as log D and total polar surface area (TPSA) at certain cut-off values have comparable performance to existing *in vitro* methods (Caco-2 and PAMPA assays). This confirms the usefulness of simple rules in the early stages of the drug discovery. Nevertheless, such rules could not distinguish between high and low bioavailability. To account for the influence of xenobiotic metabolism on bioavailability, a QSPR model for the prediction of hepatic clearance was built, however, its predictions were not of sufficient accuracy to resolve this issue.

- The aim of Chapter 3 was to investigate the performance of three well-known, representative, software packages for the prediction of metabolism (Meteor, SMARTCyp, and MetaPrint2D-React). Performance was compared between two different datasets of drugs and the results showed that both Meteor and MetaPrint2d-React performed well. Further analysis of Meteor predictions was performed in order to address the tendency of this package to over-predict metabolites. This analysis showed that using permissive settings, but incorporating cut-offs for the number of metabolites investigated, can lead to good precision with much reduced computational effort and simplification of output. Interestingly, the combination of the empirically-based SMARTCyp program, for the prediction of SOM, with software for predicting metabolites (MetaPrint2D-React) also offers a pragmatic approach to directing the results of metabolite prediction to those metabolites that are most likely to be formed.
- Chapter 4 describes the development of structural alerts and other *in silico* models for predicting toxicity associated with hERG channel inhibition. Other aims included an evaluation of an existing regression model, using a larger dataset, and development of new QSAR models for hERG inhibition using a range of machine learning methods. It was found that the existing regression model could not provide accurate predictions for the larger hERG dataset. Due to a limited success with both global and local models for hERG inhibition another approach was used. Seven categories of compounds based on their 2-D similarity were analysed in an attempt to define structural features that could be important in hERG binding. These were coded as SMARTS patterns and offer the potential to be implemented as a screening tool for identification of putative hERG channel inhibitors.
- The main aim of Chapter 5 was to analyse the distribution of publically available structural alerts for reactive metabolites for a large dataset of drug molecules from the DrugBank database. Additionally, the importance of refining alerts was considered using the alert for a

carboxyl group as an example. The rationale was to consider sensitivity and specificity of structural alerts for reactive metabolites. The main finding was that the development of more specific structural alerts can reduce significantly the number of false positives in screening. The structural alert for carboxylic acids was refined and coded as a SMARTS pattern. Interestingly, the structural alerts were no more abundant in drugs that have been withdrawn from the market compared to drugs in use or experimental pharmaceuticals.

- In Chapter 6 the use of zebrafish embryos to model acute aquatic toxicity was investigated. The main aim was to build robust baseline QSAR models using zebrafish embryo toxicity data in order to investigate whether such models can distinguish between baseline and excess toxicity. Additionally, the Verhaar scheme was investigated, in the context of toxicity to zebrafish embryos, to determine whether improvements to the rules could be proposed for specific chemical classes. The Verhaar scheme is widely used for classification of compounds according to four modes of action and is one of the tools that can potentially be used in screening compounds in development for their potential impact on aquatic species. Although it was originally developed in the context of industrial chemicals there has been recent interest in assessing its suitability for application to pharmaceuticals. The environmental fate of active pharmaceutical ingredients (APIs) is of great importance, indeed pharmaceutical companies are required to produce an Environmental Risk Assessment when submitting a marketing authorisation for new pharmaceuticals. The main finding here was that zebrafish embryos can distinguish between compounds exhibiting baseline and excess toxicity. These results add weight to the evidence of the suitability of early developmental stages of zebrafish being used as an alternative to adult fish toxicity tests in the environmental assessment of new and existing chemicals (including drugs). Additionally, the analysis of aliphatic amines in the context of the Verhaar scheme, identified where improvements could be made to the scheme in terms of correctly classifying secondary and tertiary aliphatic amines.

2. Predicting Drug Absorption and Bioavailability

2.1. Introduction

Development of new drug candidates is a very time-consuming and costly undertaking. This process requires both pharmacodynamic and pharmacokinetic properties of molecules to be taken into account. Pharmacodynamics is related to the effect that a drug candidate could exert on a biological activity of interest, for instance, binding to a receptor. However, efficacy of a lead should be considered along with its ADME properties (absorption, distribution, metabolism and elimination). These properties describe the effects that the organism has on the drug molecule. Poor pharmacokinetics had previously been identified as the main reason for attrition in drug development (Waterbeemd & Gifford, 2003). The shift towards earlier assessment of ADME properties during drug discovery has contributed to the reduction in attrition rates from *circa* 40% in 1991 to *circa* 10% in 2000 (Wang & Hou, 2009). Advances in drug formulation also played an important role in this improvement (Basaravaj & Betageri, 2014). The development and incorporation of *in silico* methods for the prediction of ADME properties has become an important tool in assessing the potential pharmacokinetic profile of drug candidates in the early stages of drug discovery.

2.1.1 Absorption

Absorption is related to the capability of a compound to permeate biological membranes. Orally administered drugs are absorbed from the gastro-intestinal tract (GIT). The intestine, due to its large surface area, is the main site of absorption, although the stomach can also contribute significantly, especially for acidic drugs. Human intestinal absorption (HIA) is experimentally determined as fraction absorbed (% FA) and is defined, according to Hou et al (2007a) as follows:

$$\% FA = M_{abs} / D \quad (Eq\ 2.1)$$

% FA = fraction of drug absorbed; M_{abs} = total mass absorbed; D = the given dose of drug

Drugs can cross intestinal epithelium either passively or by active transport. Active transport involves various transport proteins that, through energy-driven or facilitated processes, enable the movement of molecules across the membrane. This process is also termed carrier-mediated transport. It should be noted that this transport can be bi-directional, i.e. a compound can be pumped into the epithelium cell (influx) or it can be effluxed from cells into the intestinal lumen.

Two large families of transporters can be distinguished: the solute carrier family (SLC) and the ATP-binding cassette transporters, of which P-glycoprotein is a notable example because of its role in the efflux of drugs (Sugano et al, 2010) and the implications for drug resistance. Another important form of drug transport is via passive diffusion. This can occur either across the membrane of the epithelium cells (transcellular route) or via tight junctions (paracellular route). The paracellular route is known to be relevant for hydrophilic molecules with MW < 200 Da (Salama, Eddington, Fasano, (2006). The transcellular route is arguably the most important route for drug molecules but active transport and paracellular processes should be also considered when determining HIA. Efflux transporters can significantly reduce HIA so knowledge as to whether or not a drug is a substrate for such transporters is important for building predictive models. In the passive, transcellular mode of transport the molecules permeate the lipid membrane following the concentration gradient and it is dependent mostly on the fraction of the uncharged drug, which in turn is a function of the pKa of the drug and the local pH (pH varies in the different regions of the intestine, but is generally lower than the physiological pH of 7.4); this is known as pH-partition theory (Hogben et al, 1959). Hence, the lipophilicity of a drug, corrected for pH at the site of interest, can be considered as a crucial determinant of permeability across the lipid bilayer. Therefore, modelling intestinal absorption via the transcellular route will be heavily dependent on the partition coefficient (commonly represented as the logarithm of the octanol:water partition coefficient (log P)) corrected for pH; referred to as log D. Solubility, molecular weight, H-bond formation capability or polar surface area are among other molecular descriptors that can potentially correlate with HIA (Hou et al, 2006).

2.1.2. Prediction of HIA

Human intestinal absorption can be modelled via experiments on animals (*in vivo*) or using *in vitro* and *in silico* techniques. The utilisation of *in vitro* and especially *in silico* methods is advantageous in terms of reducing time and costs of drug discovery with the additional benefit of reduction of animal testing.

In vitro methods for prediction

Caco-2 cells derived from colorectal carcinoma cells or Madin-Darby Canine Kidney (MDCK) cell lines are commonly used as *in vitro* intestinal barrier models (Sugano et al, 2010). The parallel artificial membrane permeation assay (PAMPA) is another *in vitro* method, especially suitable for high-throughput screening (Akamatsu et al, 2009). PAMPA experiments are faster and cheaper but they can only model passive diffusion kinetics due to the lack of transport proteins that could simulate carrier-mediated transport. In contrast, Caco-2 cells express transport proteins and as such can more

comprehensively mimic *in vivo* conditions. Both these *in vitro* methods have been successfully used to model HIA (Artursson & Karlsson, 1991; Yee, 1997; Sugano et al, 2003).

In silico methods for prediction

In silico techniques include quantitative structure-property relationship (QSPR) modelling or rule-based filters. In QSPR, simple multi-linear regression (MLR) or more complex (but also less transparent) machine learning methods have been applied in order to classify compounds according to their potential permeability. The rule-based filters are, in practical terms, rules of thumb derived from the analysis of the type of molecular descriptors commonly employed by medicinal chemists. A variety of druglikeness filters have been defined with the Lipinski rule of five (Lipinski et al, 2001) and Veber's rules (Veber et al., 2002) being the most common. Other such filters were also devised (Rishton, 2003; Ghose, Viswanadhan, Wendoloski, 1999; Oprea et al., 2000a/b; Walters & Murcko, 2002). All these filters define thresholds and/or ranges of values for descriptors that have been selected as being determinants of HIA.

2.1.3. Bioavailability

Bioavailability (F) can be defined as the fraction of the administered dose of a drug that reaches the systemic circulation in an unchanged form. Bioavailability depends mostly on two factors: oral absorption and hepatic clearance (Berellini, Waters, Lombardo, 2012). These two variables have opposing effects on bioavailability and therefore, a high % HIA may not necessarily lead to high % F in cases where the rate of hepatic clearance is also high. First pass-metabolism occurring in the liver is one of the main contributors that determine human plasma clearance. Different models exist for describing hepatic elimination such as the "well-stirred" and the "parallel tubes" models (Liu & Pang, 2006). According to the well-stirred model, hepatic clearance can be described by equation 2.2.

$$Cl(h) = Q [(f \times Cl_{int}) / (Q + f \times Cl_{int})] \quad (Eq. 2.2)$$

Cl(h) = hepatic clearance; *Q* = hepatic blood flow; *f* = fraction of free drug; *Cl_{int}* = intrinsic clearance

According to equation 2.2 there are three factors that determine what happens to the drug following absorption from the GIT before it reaches the systemic circulation: (i) the rate of hepatic blood flow, (ii) the fraction of the drug that is not bound to plasma proteins and (iii) the capacity of hepatic enzymes to metabolise the drug (intrinsic clearance). Hence, in the context of intrinsic clearance, the capability to predict the nature and extent of xenobiotic metabolism for a compound could be useful for determining hepatic clearance, and in turn bioavailability.

The liver is host to many metabolising enzymes, with the CYP450 family of enzymes, notably CYP3A4, CYP2D6 and CYP2C9, being responsible for the Phase I metabolism (addition or exposure of a functional group) of the majority of drugs. Conjugation reactions of a parent drug, or its phase I metabolite, with a water soluble functionality, leads to Phase II metabolites that are more hydrophilic in comparison to the parent drug and as such are more readily excreted in urine. It should be noted that local metabolism occurring in the gut wall can also contribute to reduced bioavailability along with active efflux.

Application of druglikeness rules, such as Lipinski's rule of five or Veber's rules can provide an estimate of ADME properties and as such can be used for filtering out compounds that do not fulfil criteria for being a drug-like molecule. Predicting bioavailability is of high importance; not all compounds that are drug-like will be highly bioavailable due to the aforementioned factors such as xenobiotic metabolism.

The aims of the study described in this chapter are to: (i) compare the performance of *in vitro* (Caco-2 and PAMPA assays) and *in silico* methods for the prediction of HIA; (ii) develop QSPRs in relation to HIA and bioavailability and; (iii) analyse the role of metabolism in the prediction of bioavailability.

2.2. Methods

2.2.1. Datasets

Dataset A (HIA data):

This dataset comprised of Human Intestinal Absorption (HIA) data, expressed as % absorption, for 550 compounds. The data were obtained from Hou et al, (2007b) and were reported by the authors to be compounds that were known to be entirely absorbed by passive diffusion. For the analysis carried out here compounds with >30 % HIA were classified as "high" intestinal absorption and compounds with $\leq 30\%$ HIA were classified as low intestinal absorption (according to the criteria of Kansy et al (1998)). Hou et al, (2007b) used three main sources for their data: previous compilations of % HIA, reference books and bioavailability data. The structures and names of the compounds were available in an sdf file provided by the authors.

Dataset B (Caco-2 data):

This dataset comprised absorption values for 250 compounds, these were obtained using *Caco-2* cells and were recorded as permeability coefficients ($\log P_{\text{apparent}}$ (cm/s)). These were obtained from

Paixãoa, Gouveiaa, Morais, (2010) who collected the data from various published studies. To reduce interlaboratory variability the authors normalised the data with respect to one of the publications in their compilation i.e. the publication of Yazdanian et al., 1998.

Dataset C (PAMPA data):

This dataset comprised absorption values for 105 compounds, measured using the *PAMPA* assay and recorded as permeability coefficients ($\log P_{\text{apparent}}$ (cm/s); measurements were taken at pH values of 5.5 (56 compounds) and/or 7.4 (64 compounds). For 51 compounds $\log P_{\text{apparent}}$ was measured at both pH values. The data were taken from Verma, Hansch, & Selassie (2007) and Kerns et al., (2004), the measurements originated from two laboratories.

Dataset D (Bioavailability data):

This dataset was a subset of 80 compounds from Dataset A for which % bioavailability (% F) values were also available. For these compounds, hepatic clearance values (recorded as ml/min/kg) were obtained from Thummel et al, (2005).

Each dataset was stored as a spreadsheet (Microsoft Office 2013 Excel) which contained the following information: id number, SMILES string, chemical name, InChIKey and the relevant endpoint value (e.g. % HIA). SMILES strings were either generated by an automatic conversion (using OpenBabel, ver. 2.3.1) of an available sdf file (datasets A and D) or were obtained from Chemspider online chemical database (www.chemspider.com). InChIKeys were generated in OpenBabel.

Datasets A, B, C and D are presented in appendices I, II, III and IV.

2.2.2. Generation of molecular descriptors

The structures for the chemicals in each dataset were “washed” (i.e. salts removed) and neutralised in Chemaxon Standardizer (v. 14.11.24.0). A number of software packages were used to calculate molecular descriptors:

- MOE 2010.10 was used to generate 334 descriptors (both 2D and 3D – for details see appendix IX); structures were optimised using the AM1 Hamiltonian.
- ACDLabs (9.0) was used to calculate log D values at five different pHs (2, 5.5, 6.5, 7.4 and 10).

- MOSES.Descriptors Community Edition (Web Service by Molecular Networks) provided a total of 200 descriptors, including XlogP. The 3D structures were generated by an integrated CORINA library
- Vcclab (online tool) was used to obtain the logarithm of the aqueous solubility (log S; <http://www.vcclab.org/web/alogps/>)
- Joelib Descriptors (accessed via ChemMine Tools) was used to obtain molar refractivity (<http://chemmine.ucr.edu/>)

The descriptors required for the evaluation of druglikeness filters were mostly derived from a subset of descriptors generated using MOSES.Descriptors Community Edition. The exceptions being log D values (obtained from ACDLabs (9.0), log S (obtained from Vcclab) and molar refractivity (Joelib Descriptors).

2.2.3. Prediction of sites of metabolism and enzymes involved

Two online tools were used to obtain data concerning metabolism of the compounds present in dataset D:

- WhichCyp Web Service (ver. 1.2) (<http://drug.ku.dk/whichcyp/>) – predicts the P450 isoform that will be involved in metabolism. The input was provided in the form of an sdf file. The output contained predictions for the potential CYPs isoforms (1A2, 2C9, 2C19, 2D6 or 3A4) in a tabulated form (csv file).
- SMARTCyp Web Service (ver. 2.4.2) (<http://www.farma.ku.dk/smartcyp/>) – predicts sites of metabolism (SOM). The software considers only CYP3A4, CYP2D6 and CYP2C9 isoforms. The sdf file for dataset D constituted the input; the predictions of SOM for each compound were outputted to a csv file and contained atoms ranked by score. An atom with the lowest score had also the lowest rank and, in consequence, was most likely to be a site of metabolism.

2.2.4. Statistical analysis

Two software packages were used for the development of models relating to absorption and bioavailability:

- Minitab (v.17.1) was used for performing (multiple) linear regression analysis and calculating relevant statistics (R^2 and S). Two and three-dimensional plots were also produced using Minitab.
- Weka (v.3.6.11) was used for descriptor selection (using CfsSubsetEval evaluator and BestFirst or GreedyStepwise search methods) and applying classification methods such as

logistic regression (SimpleLogistic classifier) or support vector machine (SMO classifier) or MLR (LinearRegression). Molecular descriptors that were identified by Weka to be statistically significant were further assessed manually in terms of their relevance/interpretability and either selected or deselected for use in subsequent analyses. It should be noted that the process of descriptor selection in Weka took into account potential redundancy between descriptors resulting from their intercorrelation.

Analysis of the performance of *in silico* models (in terms of their sensitivity, specificity and accuracy) was performed using Microsoft Excel 2013. Here, sensitivity, specificity and accuracy are defined as follows:

$$\text{SENSITIVITY} = \text{TP}/(\text{TP} + \text{FN}) \quad (\text{Eq.3.3})$$

$$\text{SPECIFICITY} = \text{TN}/(\text{FP} + \text{TN}) \quad (\text{Eq.3.4})$$

$$\text{ACCURACY} = (\text{TP} + \text{TN})/(\text{P} + \text{N}) \quad (\text{Eq.3.5})$$

TP (true positive) = correctly predicted to be highly absorbed or drug-like; TN (true negative) = correctly predicted to be poorly absorbed or not drug-like; FP (false positive) = incorrectly predicted to be highly absorbed or drug-like; FN (false negative) = incorrectly predicted to be poorly absorbed or not drug-like; P = highly absorbed or drug-like compounds (experimental), N = poorly absorbed or not drug-like compounds (experimental).

2.3. Results and discussion

2.3.1. Dataset A: Applying druglikeness rules for prediction of HIA

2.3.1.1. Comparative analysis

Six different *in silico* filters, each containing a number of rules describing druglikeness, were applied (using an in-house procedure) to predict % HIA for the 550 compounds in dataset A. The following filters were utilised based on: Lipinski's (Lipinski et al, 2001), Veber's (Veber et al, 2002), Rishton's (Rishton, 2003), Ghose's (Ghose, Viswanadhan & Wendoloski, 1999), Oprea's (Oprea et al, 2000a/b) and Walters' (Walters & Murcko, 2002) rules. All of these filters utilise general physico-chemical properties to identify potential drug candidates and can be applied in drug development as broad screening tools. A range of filters was employed to determine which performed best alone, whether a combination of filters improved predictivity and if any improvements could be made to (use of)

these existing filters. Table 2.1 shows the molecular descriptors used by each of the filters, which account for the following properties of the molecules: (i) hydrophobicity (indicated by clog P); (ii) number of hydrogen bond donors or acceptors (HBD or HBA respectively); (iii) molecular rigidity (indicated by the number of freely rotatable bonds (NRB)); (iv) molecular weight (MW); (v) polarity (indicated by the topological polar surface area (TPSA)) and; (vi) the count of atoms/substructures. Note that the rules for each filter should be interpreted in the following way: if a rule is true then a compound is potentially drug-like. Usually, all criteria of a given filter have to be fulfilled to determine whether or not a compound is drug-like, although on some occasions certain rules can be broken, depending on the how the filter is applied. There are many overlaps between the different filters when it comes to the type of used descriptors. For instance, MW, log P and number of H-bond donors and acceptors are used by five of the six filters. On the other hand, TPSA or molar refractivity are less frequently used. Despite similar features being used in several filters, the threshold values (cut-off values or ranges of values) applied by the filters do differ. Hence a comparison was made as to the performance of the individual filters.

Table 2.1. Selected filters for druglikeness.

Filter	No of descriptors	Rules for drug likeness*
Lipinski	4	HBD \leq 5
		HBA \leq 10
		MW \leq 500
		cLogP \leq 5
Veber	2	NRB \leq 10
		TPSA \leq 140 Å ² or HBA+HBD \leq 12
Rishton	6	HBD < 5
		HBA(N+O) < 10
		MW < 500
		cLogP < 5
		NRB \leq 10
		TPSA \leq 140
Ghose	4	(A)logP range (-0.4 : 5.6)
		MW range (160 : 480)
		NA range (20 : 70)
		MR range (40 : 130)
Oprea	6	HBD \leq 5
		HBA(N+O) range (1 : 8)

		MW range (200 :450)
		clogP range (-2 : 4.5)
		NRB range (1 : 9)
		Nrings \leq 5
Walters	6	HBD \leq 5
		HBA(N+O) $< =$ 10
		MW range (200 : 500)
		logP range (-5 : 5)
		NRB \leq 8
		Formal charge range (-2 : 2)

*HBD = number of hydrogen bond donors; HBA = number of hydrogen bond acceptors; MW = molecular weight; clogP = calculated log P, NRB = number of rotational bonds; TPSA = total polar surface area; NA = number of atoms; MR = molar refractivity; Nrings = number of rings.

Figure 2.1 shows the results of applying the six filters in terms of their sensitivity, specificity and accuracy. As can be seen in Figure 2.1, filters based on Lipinski's rules and Veber's rules were found to have the greatest sensitivity (91% and 94%, respectively). True negative rates for these filters were considerably lower than for the other filters (63% and 67%, respectively). For this dataset the filters based on the rules of Walters and Oprea gave the least reliable predictions. Interestingly, the results obtained using the filter based on Veber's rules was the most successful in correctly classifying high and low absorption compounds despite the rules originally being defined to differentiate high from low bioavailability. Bioavailability is a composite parameter dependent on both absorption and metabolism. However, the results obtained here indicate that despite a more complex relationship between oral absorption and bioavailability, Veber's rules can be used to distinguish between high and low absorption.

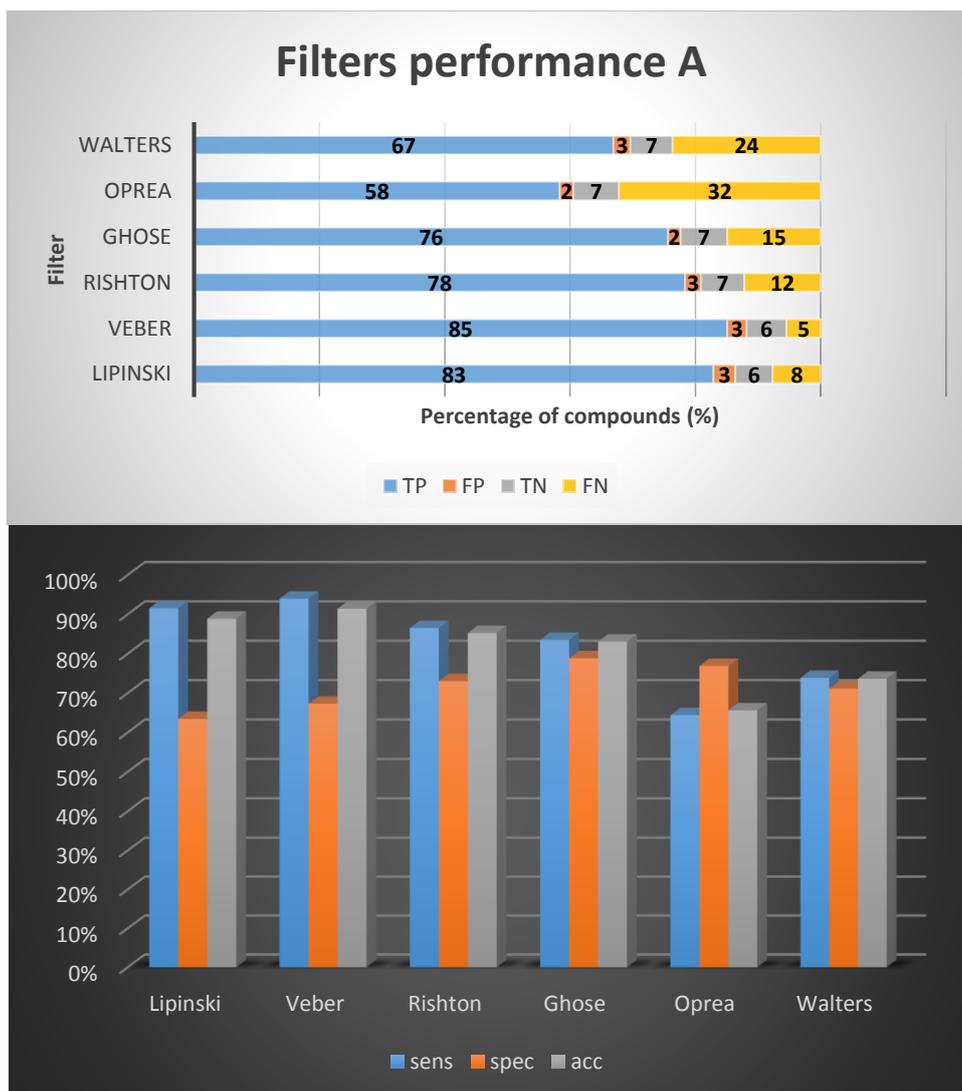


Figure 2.1. Performance of the six different filters for druglikeness in distinguishing high (>30 % HIA) from low (≤ 30 % HIA) absorption compounds

One of the key aims of this thesis is not only to investigate the performance of existing models, but also to determine if the performance of the models can be improved by a more flexible interpretation (or application) of the rules or by using a combination of different approaches. The idea of using the six druglikeness filters in combination was explored in an attempt to obtain a consensus classification for each compound. However, this approach did not improve the results (data not shown). The next step was to use a more flexible approach to applying each filter. The above six filters were again applied (individually) but in this case *one* violation of the rules for each filter was permitted without the compound being classified as poorly absorbed. Figure 2.2 shows the results of this more flexible application on the filters.

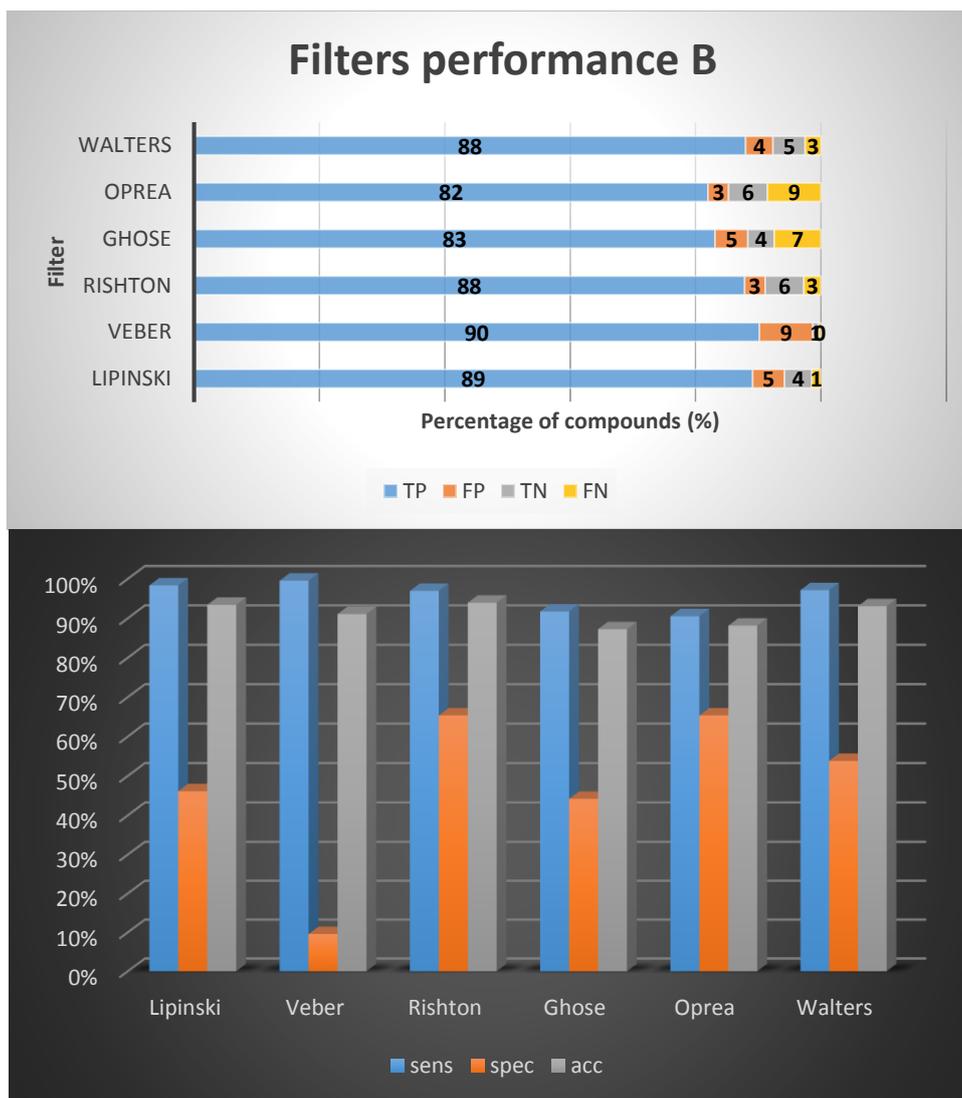


Figure 2.2. Performance of the six different filters for druglikeness in distinguishing high from low absorption compounds where one violation of the rules is permitted

The results show that the number of compounds that were highly absorbed but were not classified as such (false negatives) was reduced. For instance, only two compounds were classified by Veber's rules as false negatives. Oprea's and Walter's filters had significantly higher sensitivity values. In general, sensitivity was even further improved but at a cost of reduced specificity and precision. The increased number of false positives and a lower number of true negatives indicated that running these filters in a more tolerant way could result in a reduced capability to discriminate between two classes of absorption. It could be argued further whether it would be reasonable to run, for instance, Veber's filter in such a mode. The TPSA rule appears to be a more significant criterion for distinguishing between compounds with good or poor absorption; only two compounds with low absorption values had also a number of freely rotated bonds (FRB) > 10. In consequence, such a use of Veber's filter made it even more difficult to identify true negatives. On the other hand, Lipinski's

filter is known to predict poorly absorbed compounds when two or more rules are broken. It should also be noted that for an unbalanced dataset, which is the case here, a product of sensitivity and specificity (Sen x Spec) may be a more adequate measure of predictive power of a model (Newby, Freitas, Ghafourian, 2013). The balance between sensitivity and specificity should be considered in relation to the practical application of screening tools within drug discovery: i.e. if it is better not to screen out a potentially good drug candidate early in the process, then the reduction of false negatives would be the priority. On the other hand, low accuracy of predictions may render the whole filtering process as too unreliable and taking this into account it was decided to consider predictions where no rules were violated.

It is apparent from these results that of the six filters for druglikeness studied here, only Lipinski's rules and Veber's rules could provide good predictions for highly absorbed compounds. However, other filters gave a lower number of false positives and a greater number of compounds correctly classified as poorly absorbed. Hence, the different descriptors (and/or cut-off value for these descriptors) as applied in the other four filters were investigated in more detail to see if improvements could be made to *in silico* screens for absorption.

Selection criteria unique to the other filters were identified to be: molar refractivity and number of atoms (used in the Ghose's rules), number of rings (Oprea's rules) and formal charge (Walters' rules). The formal charge descriptor values were found to be the same for all compounds (value = 0), as this feature was clearly non-discriminatory it was discarded from further analysis. The molar refractivity descriptor is an indicator of general bulkiness of a molecule and it is related to the molar volume and London dispersion forces (Padron, Carrasco, Pellon, 2002). The number of atoms is also related to the size of molecules. The number of rings can be considered both as an indicator of the bulk of a molecule and also its hydrophobic properties. Two additional descriptors, that were not included in the original six filters but are nonetheless considered relevant in terms of drug absorption, were also considered. These were the logarithm of the aqueous solubility (log S) and the distribution coefficient (log D) at pH values of 5.5, 6.5 and 7.4 (representing different pH values found throughout the intestine).

It was noted that a level of redundancy exists between some of these additional descriptors e.g molar refractivity (MR) and MW were highly correlated ($r = 0.93$, $p = 0.000$). Additionally, MR showed poor discriminatory power when used as a sole descriptor to distinguish between high and low absorption (specificity = 27%) and was therefore not considered further. Similarly, the number of atoms was highly correlated with MW ($r = 0.86$, $p = 0.000$) and MR ($r = 0.93$, $p = 0.000$). Further

analysis showed that the number of atoms, the number of rings and log S also did not distinguish between high and low absorption compounds. However, log D showed some ability to discriminate between the classes. This supports the previous findings of Hou et al, (2007b) who identified log D (pH6.5) and TPSA as important features for modelling absorption using Support Vector Machine (SVM) analysis. Figure 2.3 shows the results of these analyses and demonstrates that of the nine descriptors investigated (molar refractivity, number of atoms, number of rings, log D (at pH 5.5, 6.5 and 7.4), log S, total polar surface area and molecular weight) log D and TPSA were most promising in terms of ability to differentiate high and low absorption compounds and hence were investigated further.

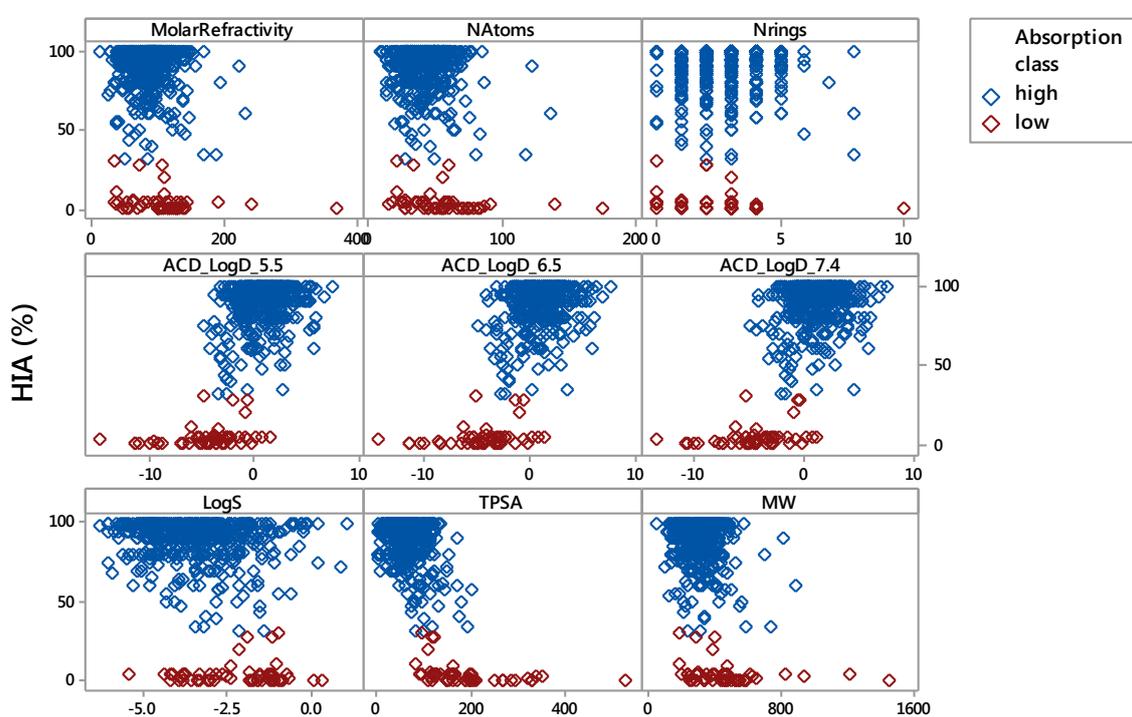


Figure 2.3. Scatterplots showing relationship between % HIA and molar refractivity, number of atoms, number of rings, log D (pHs 5.5, 6.5, 7.4), log S, TPSA and molecular weight.

2.3.1.2. Investigation of the influence of TPSA and log D

When the cut-off value for TPSA was taken to be the same as that used in Veber's and Rishton's rules ($TPSA \leq 140 \text{ \AA}^2$ for highly absorbed compounds) this descriptor alone could correctly classify 487 (out of 498) compounds as highly absorbed (sensitivity = 98%) which was better than Veber's or Rishton filters. However, there was no real improvement in prediction of poorly absorbed molecules (34 out of 52).

According to Hou et al., (2007b) log D values at pH 6.5 gave the best correlation with % HIA. They suggested an approximate threshold value for log D_{6.5} of -3.2 log units (i.e. compounds with log D_{6.5} above -3.2 are well-absorbed and below -3.2 log units are poorly absorbed). The result of using log D alone as a descriptor gave very similar results to using TPSA alone (sensitivity = 98% and specificity = 63%).

A combination of both TPSA and log D_{6.5} was considered to investigate whether the predictions for the low absorption class could be improved; the results are shown in Figure 2.4. The threshold values were kept at 140 Å² for TPSA and -3.2 log units for log D_{6.5}. Using this combination of TPSA and log D descriptors improved specificity (80%), whilst the true positive rate remained at a high level (96%). Applying a product of sensitivity and specificity as a statistical measure of performance revealed that a filter based on TPSA and log D (pH 6.5) descriptors may give the most promising results in terms of distinguishing between highly and poorly absorbed compounds (see Table 2.2). All compounds (except for ceftizoxime) that had TPSA > 140 Å² and log D_{6.5} ≤ -3.2 (i.e. 42 compounds in total) were correctly predicted to be poorly absorbed (as shown in Figure 2.4). Ceftizoxime is a beta-lactam and as such it may be absorbed by paracellular transport or be a substrate for the PepT1 transporter, as has been shown for ampicillin - another beta-lactam (Lafforque et al, 2008).

Table 2.2. Performance of selected filters for druglikeness as measured by a product of sensitivity and specificity. Note: Filters based on Lipinski's, Veber's, Rishton's, Ghose's, Oprea's and Walters' rules were used without allowing any rule violations.

Filter	sensitivity	specificity	sens X spec
Lipinski	92%	63%	58%
Veber	94%	67%	63%
Rishton	87%	73%	63%
Ghose	84%	79%	66%
Oprea	64%	77%	50%
Walters	74%	71%	53%
TPSA	98%	65%	64%
logD 6_5	98%	63%	62%
TPSA_logD 6_5	96%	81%	78%

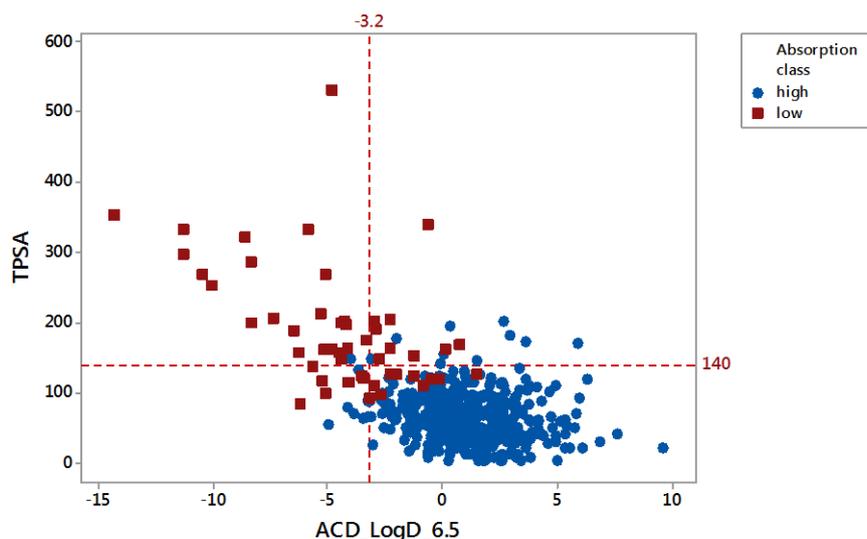


Figure 2.4. A scatterplot of $\log D_{6.5}$ and TPSA for 550 compounds. Reference lines represent the cut-off values used for $\log D$ and TPSA.

The results here indicate that the descriptors TPSA and $\log D_{6.5}$ may be useful for providing good predictions for high versus low absorption, however, these preliminary findings should be validated using an external dataset. Ideally, such a dataset should cover a wide range of values for both descriptors, contain a large number of diverse structures and have an appropriate number of both high and low absorption compounds. One of the problems in investigating HIA data is that it tends to be skewed towards high absorption compounds, making modelling more problematic, as discussed by Newby, Freitas, Ghafourian, (2013).

2.3.2. Relationship between *in vivo*, *in vitro* and *in silico* methods

2.3.2.1. Comparison of Caco-2 and % HIA data

Dataset B comprised measurements of permeability, assessed using Caco-2 cells, for 250 compounds. For 105 of these compounds HIA data were also available within Dataset A (taken from Hou et al (2007b)). This enabled investigation into the extent to which Caco-2 data were predictive of HIA data. Figure 2.5 illustrates the relationship between observed $\log P_{\text{apparent}}$ values from Caco-2 assays and % HIA.

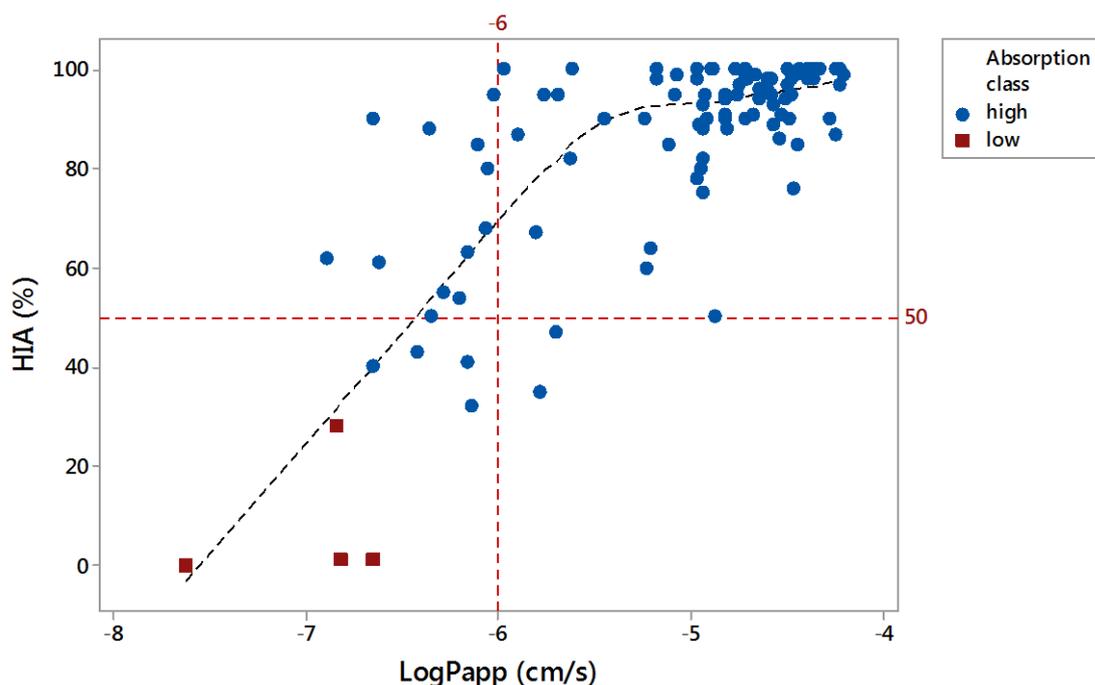


Figure 2.5. A scatterplot of % HIA versus $\text{Log } P_{\text{apparent}}$ (cm/s). $N = 105$. Compounds are classified as high absorption ($> 30\%$ HIA; blue circles) or low absorption ($\leq 30\%$ HIA; red squares). Reference lines for 50% HIA and $\text{log } P_{\text{apparent}} -6$ are also displayed.

The relationship between $\text{log } P_{\text{apparent}}$ and HIA has previously been reported to be sigmoidal in character (Artursson & Karlsson, 1991) although Hou et al (2007b) did not find a sigmoidal relationship in their analysis. The vast majority of the compounds in this study belonged to the category of highly absorbed molecules (HIA $> 30\%$), which complicated the analysis of classification; $\text{log } P_{\text{apparent}}$ spanned nearly 3.5 log units. Different threshold values for $\text{log } P_{\text{apparent}}$ have previously been suggested to be a criterion for good or poor absorption. Yee (1997) analysed 35 structurally diverse compounds with % HIA ranging from 0% to 100%. He found a good correlation between *in vivo* (oral) and *in vitro* (Caco-2) absorption and proposed a guideline for classifying compounds according to their absorption, i.e. compounds with poor oral absorption have $\text{log } P_{\text{apparent}} < -6$, moderately absorbed compounds have a $\text{log } P_{\text{apparent}}$ range of $-6 \leq \text{log } P_{\text{apparent}} \leq -5$ and high oral absorption compounds have $\text{log } P_{\text{apparent}} > -5$. In that study HIA values of $< 20\%$ were classed as 'low' absorption, values above 70% HIA were high absorption and values between 20 and 70 were considered as moderate. Other suggested threshold values, to differentiate between high and low absorption, include: $\text{log } P_{\text{apparent}} = -5.69$ (Gres et al, 1998), $\text{log } P_{\text{apparent}} = -6.15$ (Rubas et al, 1993), $\text{log } P_{\text{apparent}} = -4.69$ (Stewart et al, 1995) and $\text{log } P_{\text{apparent}} = -5.25$ (Hou et al, 2007b). Other authors (Ren & Lien, 2000) indicated that $\text{log } P_{\text{apparent}}$ values below -5.3 may not form the basis for reliable

predictions of human gastro-intestinal (GI) absorption although above this threshold the absorption is usually in a range of 50 – 100%. They also stated that experimental and inter-laboratory variability may be responsible for a poor correlation between Caco-2 permeability and GI absorption especially for compounds that are not very well absorbed.

It may be challenging to establish a Caco-2 permeability threshold value for this group of 105 compounds. One of the reasons is the previously mentioned lack of poorly absorbed chemicals. If compounds with < 30% HIA are classed as low absorption, only four chemicals would fall into this low absorption class. One way to overcome this issue was to redefine the classes. If the cut-off value for poorly absorbed compounds is set at 50% HIA an additional eight compounds fall into this newly defined 'low' absorption class. However, visual inspection of Figure 2.5 shows that for these additional compounds no clear trend is distinguishable between $\log P_{apparent}$ and % HIA.

Figure 2.5 shows a wide spread of HIA values for compounds with similar $\log P_{apparent}$ values, this serves as an example of reliability issues in using $\log P_{apparent}$ to predict % HIA for low absorption compounds. Despite these reliability issues, some general trends could be observed. Compounds with very high HIA tended to have a higher Caco-2 permeability. On the other hand, compounds with HIA close to 0% also had low $\log P_{apparent}$ values (refer to Figure 2.5). It was not possible to establish conclusively the nature of the relationship between HIA and Caco-2 for this dataset or to define threshold values to discriminate between high and low absorption. The plateau region in the top right corner of Figure 2.

Although Figure 2.5 suggests that the relationship may have a sigmoidal character, this cannot be verified as the bottom plateau cannot be determined due to the underrepresentation of poorly absorbed compounds in this dataset. This shape may be expected when a rate variable ($\log P_{apparent}$) is plotted against a fraction absorbed. In other words, highly absorbed compounds (approaching or equal to 100%) may be absorbed at different rates but will still achieve 100% absorption provided the rate is sufficiently rapid to allow for complete absorption.

The number of false negatives (defined as compounds with high $\log P_{apparent}$ but low % HIA) appearing in the top left corner of the Figure 2.5 suggests this particular Caco-2 model fails to reliably distinguish between compounds of high and low HIA. However, to examine this hypothesis fully, the dataset needs to be extended to consider a greater number of poorly absorbed compounds.

The potential for *in silico* rules to give more accurate predictions for the compounds from this dataset was investigated. As performed for the analysis of Dataset A, six druglikeness filters were applied to the 105 compounds from Dataset B that had both % HIA and Caco-2 data associated with them. Additionally, simple filters based on log D_{6.5} and TPSA (or combinations thereof) were used. Finally, two filters based on different log P_{apparent} threshold values were also utilised to enable a comparison between purely *in silico* methods and those based on a hybrid of both *in vitro* and *in silico* data. The results are presented in Table 2.3.

Table 2.3. Statistics for predictions of HIA class using selected filters. Filters I – VI are the same as applied for Dataset A. Filters VII and VIII are: TPSA ≤140; logD_{6.5} ≥-3.2 respectively and filter IX is a combination of both filters VII and VIII. The last two filters (X and XI) are based on *in vitro* Caco-2 permeability (log P_{apparent}) with the threshold values of -6 (Caco2_1) and -6.5 (Caco-2_2). TP = true positive; FP = false positive; TN = true negative; FN = false negative; sens = sensitivity; spec = specificity; acc = accuracy.

Id	Filter	TP	FP	TN	FN	Sens(%)	Spec(%)	Acc(%)	Sens x spec(%)
I	Lipinski	95	1	3	6	94.06	75.00	93.33	70.54
II	Veber	96	1	3	5	95.05	75.00	94.29	71.29
III	Rishton	91	1	3	10	90.10	75.00	89.52	67.57
IV	Ghose	87	0	4	14	86.14	100.00	86.67	86.14
V	Oprea	70	1	3	31	69.31	75.00	69.52	51.98
VI	Walters	78	1	3	23	77.23	75.00	77.14	57.92
VII	TPSA	97	1	3	4	96.04	75.00	95.24	72.03
VIII	logD _{6.5}	100	2	2	1	99.01	50.00	97.14	49.50
IX	TPSA_logD _{6.5}	96	1	3	5	95.05	75.00	94.29	71.29
X	Caco2_1	85	0	4	16	84.16	100.00	84.76	84.16
XI	Caco2_2	97	0	4	4	96.04	100.00	96.19	96.04

It is clear that some *in silico* methods show similar performance to filters based on Caco-2 permeability coefficients. This includes both Lipinski's and Veber's rules. Again, TPSA and logD_{6.5} descriptors appeared to be important, in a similar manner as that found for Dataset A. The rule based on TPSA, where compounds with TPSA > 140 Å² were considered to be poorly absorbed, could predict the same number of true positives as a threshold of -6.5 for Caco-2 permeability coefficient. TPSA was also able to predict 3 out of 4 compounds with low HIA values. The Caco-2 filter was capable of identifying all poorly absorbed values – but it should be noted that both thresholds were selected arbitrarily to obtain 100% specificity. The only true negative that was not identified by the

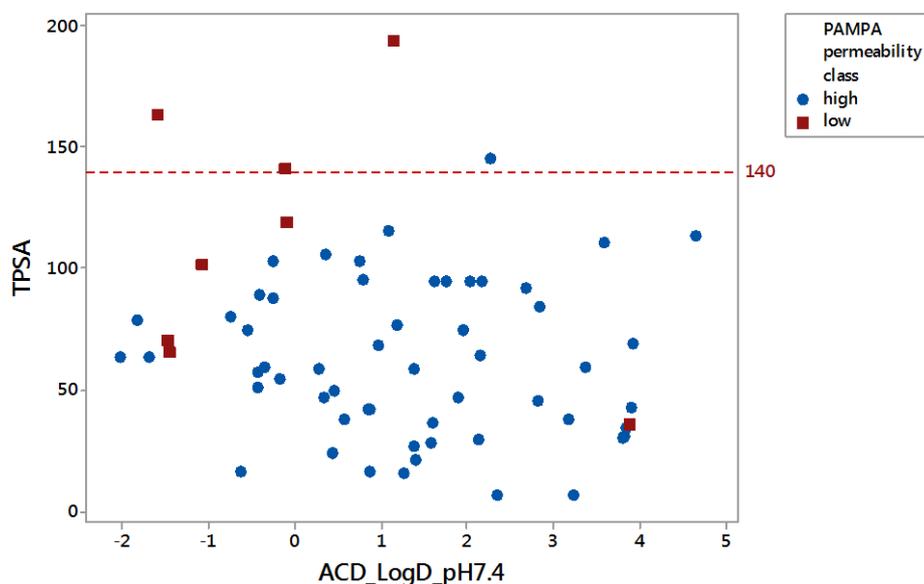
The scatterplot in Figure 2.6 shows no clear relationship between PAMPA log P_{app} and % HIA can be determined, in part due to the underrepresentation of compounds with low % HIA.

Avdeef (2005) suggested a threshold PAMPA log $P_{apparent}$ of -5.7 log units to distinguish low from high absorption compounds however, when that threshold was applied to this dataset eleven compounds were misclassified as being poorly absorbed. Two significant outliers were identified – practolol (log $P_{apparent}$ = -8) and clofibrate (log $P_{apparent}$ = -6.52) with HIA values of 95% and 96%, respectively.

The possibility of using log D and TPSA as molecular descriptors to model absorption in the PAMPA assay was also explored. Figure 2.7A shows the relationship between log $D_{7.4}$, TPSA and PAMPA permeability. TPSA alone did not seem to provide a good indication as to whether or not a compound would have a high or low PAMPA permeability. The majority of molecules with low PAMPA permeability values (Fig. 2.7A, red squares) had TPSA < 140 Å².

It was not possible to determine a threshold for log $D_{7.4}$ values that could differentiate between high and low permeability in the PAMPA assay. It should be noted that the two outliers – practolol and clofibrate – were correctly predicted to belong to highly absorbed compounds by TPSA and log $D_{6.5}$ during the analysis of the HIA dataset (dataset A).

(A)



(B)

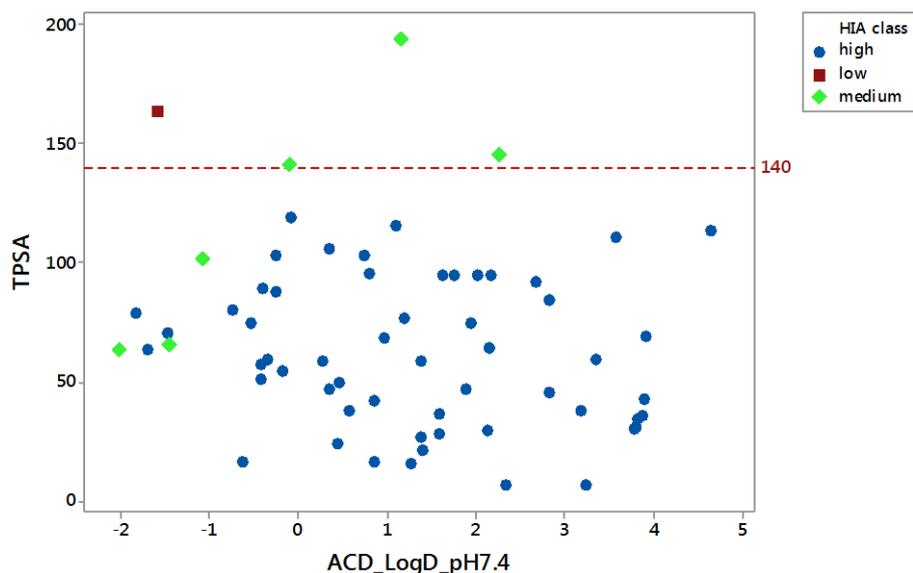


Figure 2.7. Scatterplot of TPSA vs $\log D$ (pH 7.4) for 64 compounds found both in PAMPA and HIA datasets. (A) Categories distinguished according to an arbitrary PAMPA $\log P_{\text{apparent}}$ threshold (where low < -6). (B) Categories according to HIA percentage thresholds (high >70%, 70% \leq medium >30%, low \leq 30%). It should be noted that an additional class of medium HIA was distinguished for comparison purposes.

Comparison of %HIA with PAMPA data obtained at pH5.5

56 compounds with PAMPA $\log P_{\text{apparent}}$ (pH = 5.5) were analysed. A similar general trend was observed as for the PAMPA pH 7.4 dataset, namely higher $\log P_{\text{apparent}}$ seemed to indicate a better oral absorption. There was only one compound with a poor oral absorption, however, four other compounds had the same $\log P_{\text{apparent}}$ (= -7), yet they were either moderately or highly absorbed (see Figure 2.8). Again, it was difficult to establish a threshold that would separate compounds into two classes of absorption for the reasons stated previously. For the purpose of the analysis, a threshold of PAMPA $\log P_{\text{apparent}} = -6$ was chosen where compounds were considered to have low absorption if $\log P_{\text{apparent}} < -6$.

Similarly to the pH 7.4 PAMPA dataset, a TPSA cut-off of 140 \AA^2 could not distinguish high and low PAMPA classes at the threshold of $\log P_{\text{apparent}} = -6$ (see Figure 2.9A). In this case applying the $\log D$ cut-off of -3.2 was also not able to separate high from low absorption compounds.

The analysis of both PAMPA datasets (pH 5.5 and pH 7.4) seemed to suggest that artificial membranes may be useful for identifying highly absorbed compounds; i.e. compounds that show good absorption via PAMPA have high % HIA. However, compounds that are poorly absorbed via PAMPA may be associated with either high or low % HIA. This result is not unexpected as PAMPA permeability relates only to passive transport; drugs that undergo passive transport and are highly

absorbed are well predicted. However, it has previously been suggested that many drugs could rely on a combination of active and passive transport mechanisms (Avdeef, 2005). These active transport mechanisms cannot be replicated in the PAMPA assay, hence actively transported drugs may be predicted to have low absorption although in reality % HIA is high.

Nevertheless, passive transport is an important way for drugs to enter the systemic circulation and PAMPA may be a valuable tool for estimating oral absorption via this process.

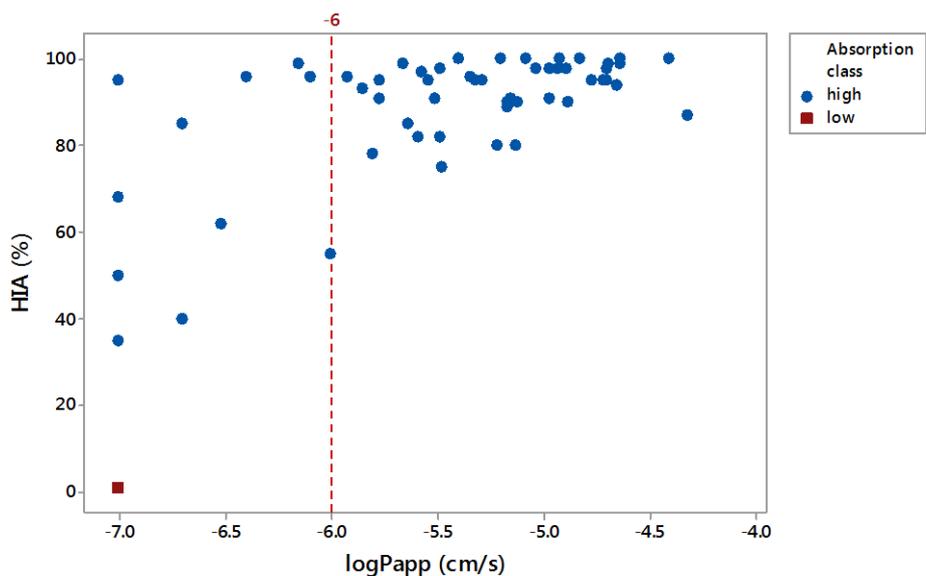
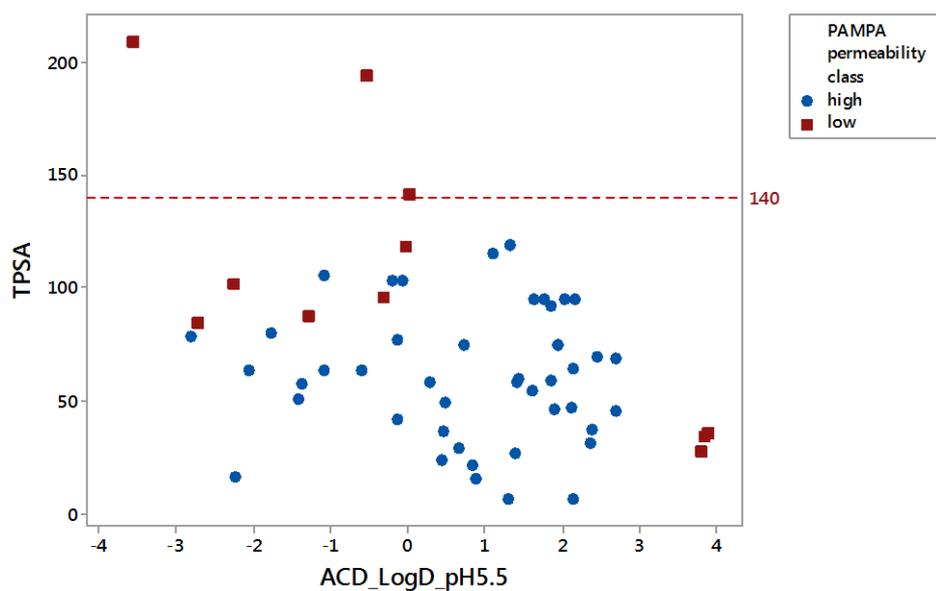


Figure 2.8. A scatterplot of HIA (%) vs PAMPA $\log P_{\text{apparent pH5.5}}$ (cm/s). The HIA classes (high and low) were colour coded (threshold = 30%).

(A)



(B)

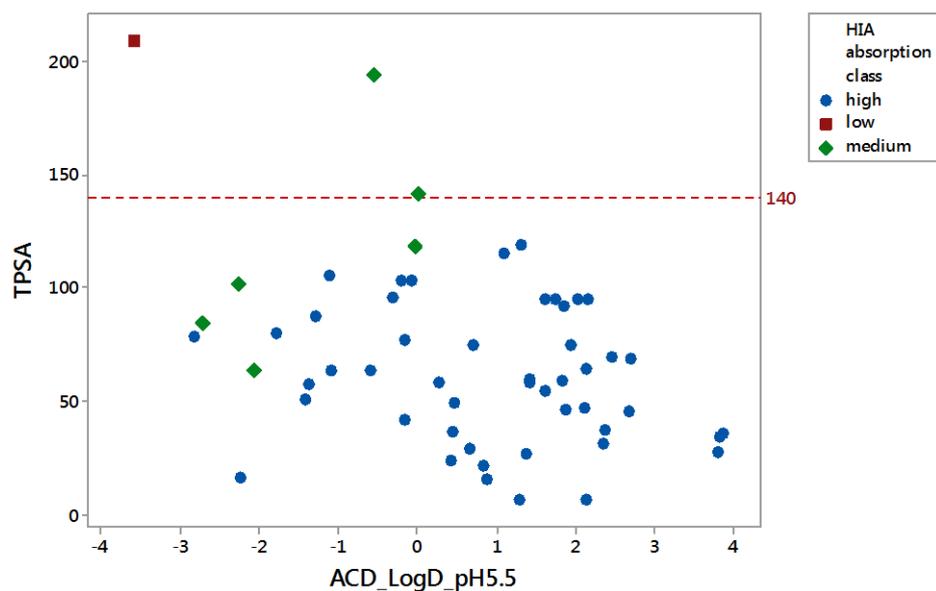


Figure 2.9. Scatterplots of TPSA versus logD (pH 5.5) for 56 compounds found both in Datasets A and C.

(A) Categories according to arbitrary PAMPA log $P_{apparent}$ threshold (low < -6). (B) Categories according to HIA percentage thresholds (high >70%, 70% ≤ medium >30%, low ≤30%). It should be noted that the medium HIA class was distinguished for comparison purposes.

As PAMPA log P_{app} values were measured at two discrete pH values (for the majority of the compounds in Dataset C) the question arose as to how the pH used in the PAMPA experiments could influence the predictions of absorption. As the pH of the GIT varies, spanning a range of pHs from 2 to 8, using PAMPA log $P_{apparent}$ values taken at only one pH may be not sufficient to reliably predict oral absorption. It has previously been suggested that log of a sum of PAMPA effective permeability values, taken at three different pHs (5.0, 6.2 and 7.4) could be useful for discriminating high and low absorption compounds in a “binning strategy” (Avdeef, 2005).

To investigate this further, the difference between log $P_{apparent}$ (at pH 7.4) and log $P_{apparent}$ (at pH 5.5) for each compound containing both PAMPA permeability values was analysed; 51 compounds in total (all having medium to high HIA). For 11 of these compounds the difference was > 0.5 log units. In some cases, the correct predictions depended on pH-conditions under which the measurements were taken. For instance, seven compounds were predicted to have low oral absorption based on a threshold of PAMPA log $P_{apparent}$ = -6 at a pH of 5.5. However, for three of these compounds the corresponding log $P_{apparent}$ at pH 7.4 correctly predicted high HIA. Five misclassified cases were present when the predictions were made on PAMPA permeability values taken at pH 7.4 and, in fact, one of these compounds was correctly predicted when a PAMPA log $P_{apparent}$ measured at pH 5.5 was used (see Table 2.4) .

Table 2.4. Misclassified compounds from dataset C.

Name	HIA (%)	HIA class	log P _{apparent} pH5.5	PAMPA 5.5 class	log P _{apparent} pH7.4	PAMPA pH7.4 class	LogD pH5.5	LogD pH6.5	LogD pH7.4	XlogP	TPSA
<i>Sulpiride</i>	40	med	-6.7	low	-7	low	-2.24	-1.83	-1.07	0.62	101.73
<i>Acebutolol</i>	85	high	-6.7	low	-5.48	high	-1.27	-0.94	-0.24	1.68	87.66
<i>Clofibrate</i>	96	high	-6.4	low	-6.52	low	3.88	3.88	3.88	3.32	35.53
<i>Sulfasalazine</i>	62	med	-6.52	low	-7	low	0.02	-0.09	-0.10	4.51	141.31
<i>Progesterone</i>	96	high	-6.1	low	-5.4	high	3.83	3.83	3.83	3.47	34.14
<i>Labetalol</i>	95	high	-7	low	-5.35	high	-0.30	0.11	0.80	2.52	95.58
<i>Erythromycin</i>	35	med	-7	low	-7	low	-0.54	0.35	1.16	1.23	193.91
<i>Bumetanide</i>	96	high	-5.34	high	-6.52	low	1.32	0.44	-0.08	2.59	118.72

The PAMPA classes were based on a log $P_{apparent}$ threshold = -6. The HIA classes were: high >70%, 70% ≤ medium >30%, low ≤30%. TPSA, XlogP and logD values predicted for three different pH conditions are also present. Green colour indicates compounds that were correctly predicted by PAMPA log $P_{apparent}$ (at pH 7.4 or 5.5) to have a high % HIA.

Selected compounds present in Table 2.4 were further analysed in terms of their chemical properties. Bumetanide was correctly predicted to have a high oral absorption by log $P_{apparent}$ at a pH of 5.5. This compound could be categorised as an organic acid and as such it can be present in a solution in an ionised or unionised form, depending on the pH. Analysis of the major microspecies (Chemicalize, Chemaxon) showed that the unionised form (COOH) was prevalent at pH 4 but as pH increased the deprotonated species (COO⁻) became more prevalent. These changes were reflected in both log D values and PAMPA permeability. The neutral form was expected to be more lipophilic (greater log D value at pH 5.5) and, therefore, more easily cross the PAMPA membrane. It shows that a pH gradient needs to be considered when carrying out the PAMPA experiments so that permeability coefficients are established for microspecies relevant to the GIT pH range. This also supports the previous report that recommended using a combination of permeability coefficients as a binning strategy (Avdeef, 2005). In addition, it should be mentioned that bumetanide can be actively transported by proteins belonging to the class of monocarboxylate transporters (MCTs) (Morris & Felmler, 2008). This could account for the low value of PAMPA log $P_{apparent}$ despite high bumetanide HIA (96%). Acebutolol and labetalol (beta-adrenergic antagonists) were correctly predicted by PAMPA log $P_{apparent}$ 7.4 to be highly absorbed. Acebutolol is a base and a charged form (with a protonated nitrogen) would be the prevalent species under GIT conditions where pH values range from 1.7 in the stomach through ≈6.5 in jejunum and ileum to 8 in the colon (Avdeef, 2001). Labetalol is an example of a zwitterionic ampholyte and at GIT pH it is present as a mixture of a protonated and zwitterionic form. It is difficult to precisely calculate log D for zwitterions. The contributions of such species to overall absorption could be negligible in comparison to the non-charged form however such a contribution may be noticeable depending on the relative fraction

present among other microspecies (Mazak & Noszal, 2012). Nevertheless, log D accounts for a mixture of species present at a given pH and for both acebutolol and labetalol the log $D_{7.4}$ increased by about one log unit when compared to log $D_{5.5}$. An even more prominent increase was observed for PAMPA log P_{apparent} . Again, taking into account only one pH may not be sufficient to predict HIA. Interestingly, progesterone had different permeability at pHs 5.5 and 7.4 despite the fact that it had the same log D value, which suggested it was present in the unionised state. In this case, log D, log P and TPSA would indicate that progesterone is a highly absorbed compound while PAMPA experimental differences could lead to less reliable predictions.

It should be noted that when it comes more lipophilic compounds PAMPA permeability may depend on log D in a bilinear fashion (Akamatsu et al, 2009). This is related to the presence of the unstirred water layer (UWL) on both sides of the artificial membrane, which in turn may affect the rate and extent of the absorption. Retention of compounds in the membrane could be also a factor influencing oral absorption, i.e. compounds of medium to high lipophilicity (log $D > 2$) could partition into a membrane but a lower fraction would then permeate into the cell. These factors are also relevant to HIA. For instance, the aqueous boundary layer (ABL) present in the intestine can affect the absorption significantly and the ABL effect should be identified and then its influence accounted for when building an appropriate QSPR (Sugano, 2010). Factors such as the ABL effect are beyond the scope of this study and will not be discussed further.

2.3.2.3. Comparison of HIA, Caco-2 and PAMPA

47 compounds that were common to Datasets A, B and C i.e. those for which HIA, Caco-2 and PAMPA (pH = 7.4) data were all available were further investigated and used for comparative analysis. Figure 2.10 shows the general trend that as log P_{apparent} as measured in both PAMPA and Caco-2 assays increases, % HIA also increases although outliers may introduce distortion to this image. However, as previously discussed, data for compounds with lower HIA values are needed to fully explore these trends and establish reliable thresholds as none of the 47 common compounds studied here had a HIA value of < 30%.

Despite the obvious difficulties in modelling such data, an overall trend can be seen - both PAMPA and Caco-2 are useful in identifying compounds that are highly absorbed. Compounds identified by the assays as poorly absorbed may in fact demonstrate high % HIA because of processes such as active transport which are not modelled by PAMPA and only modelled to a limited extent by Caco-2.

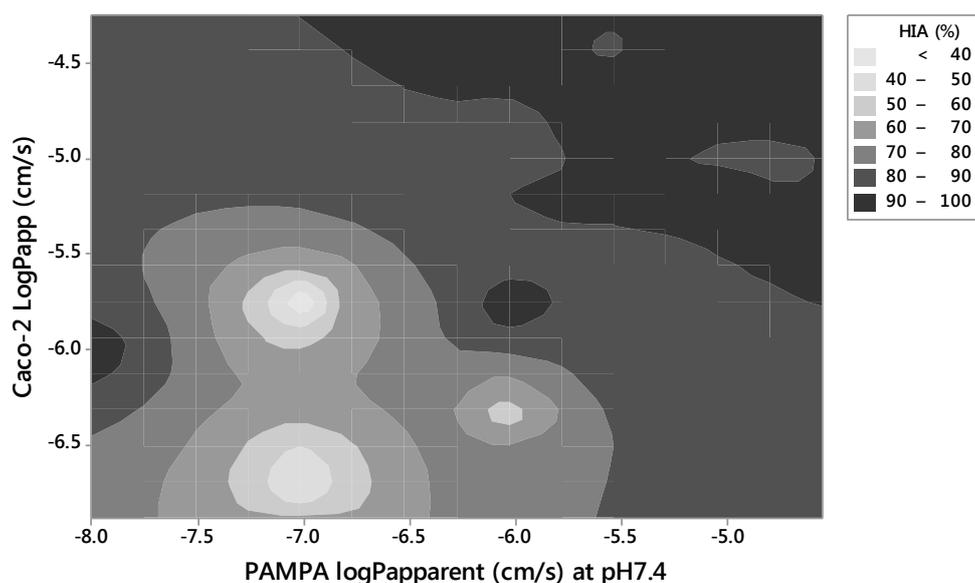


Figure 2.10. A contour plot of HIA (%), Caco-2 and PAMPA permeability.

The purpose of this study was to determine whether simple rules such as druglikeness filters or combinations of “medicinal chemist”-type descriptors could classify compounds in terms of their oral absorption (high or low) and investigate the usefulness of *in vitro* screening. Whilst Caco-2 and PAMPA assays may provide useful screens, they still have the disadvantage of requiring experimental measurements. Reliable *in silico* tools are more beneficial as screens because they can be performed on virtual datasets. The results of the above analysis indicate that the *in silico* methods used performed reasonably well in predicting the correct class for HIA (high or low); results were comparable with, or in some cases superior to, results obtained from *in vitro* methods (Caco-2 and PAMPA).

2.3.3. Metabolic contribution to bioavailability

For an orally administered drug to be useful, it is desirable that not only is absorption suitable but also that the drug is not metabolised too extensively by first pass metabolism. Bioavailability is a composite parameter, based on both absorption and avoidance of first pass metabolism. The final part of this study was to investigate the influence of metabolism on overall bioavailability. For this analysis a subset of 80 compounds from Dataset A was used, for which hepatic clearance values were available from Thummel et al. (2005).

2.3.3.1. Effect of clearance on bioavailability

The relationship between human oral bioavailability and % HIA taking into account the relative hepatic clearance rate for the compounds is depicted in Figure 2.11. Bioavailability and % HIA data are skewed towards highly bioavailable/highly absorbed compounds. However, the hepatic clearance classes contained a similar number of compounds (high = 34 and low = 46). The relationship between bioavailability and oral absorption is demonstrably influenced by the rate of clearance. Compounds with high absorption and low clearance show high bioavailability, however for compounds with extensive clearance bioavailability is reduced.

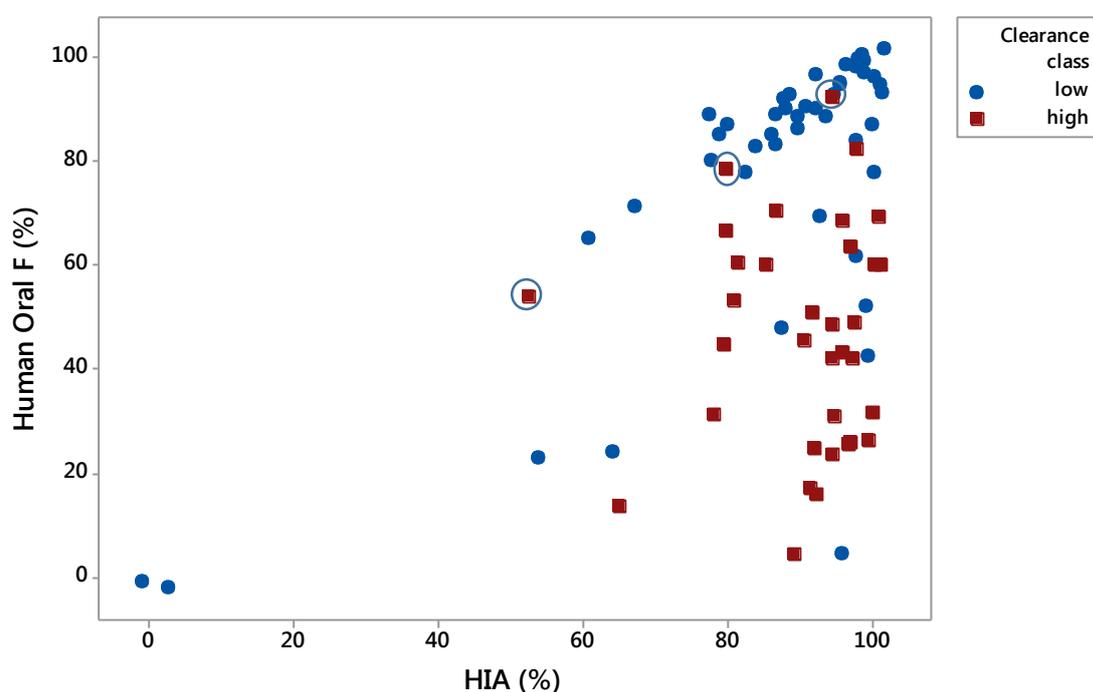


Figure 2.11. A scatterplot of bioavailability (% F) versus % HIA. Each compound was classified according to its clearance: high clearance ≥ 7.5 ml/min/kg and low clearance is < 7.5 ml/min/kg. $N = 80$. For the description of three compounds (high clearance class) circled in blue refer to the text below.

When only compounds with low clearance values, i.e. < 7.5 ml/min/kg were analysed, a high correlation coefficient ($r = 0.7$, $p < 0.001$) was obtained, which indicated that there was a strong positive relationship between % F and % HIA. This indicates that, for compounds which are not subject to extensive first-pass effect, bioavailability depends mostly on the extent of intestinal absorption. On the other hand, no correlation between % F and % HIA was observed for compounds with high clearance; although a few exceptions to this rule were noted. The position of three compounds, belonging to the high clearance class (Figure 2.11, blue circles), shows that their

bioavailability correlated with their HIA. The clearance values for these compounds were either on the borderline of the threshold of 7.5 ml/min/kg, or relatively close to this cut-off, for instance 9.9 (ml/min/kg) for nitrofurantoin. Also, nine compounds with low clearance values were found to have large differences (> 20%) between % F and % HIA. Four of these compounds had very low clearance values (< 1.3 ml/min/kg); clofibrate was of particular interest being an extreme example where HIA was high (96%) and bioavailability was low (5%), however this large discrepancy could not be accounted for by clearance as this was also low.

Of special interest were compounds with significant differences between % HIA and % F. As reported by Hou et al (2007b) this difference may indicate a considerable effect of metabolism. Despite the mentioned outliers, the majority of compounds (75% of the compounds for which the difference between % HIA and % F was > 20%) belonged to the high clearance class. It is evident that the first-pass effect contributed significantly to the reduced bioavailability for the studied compounds. Whilst this result is predictable, given the nature of the bioavailability parameter, it was useful to demonstrate that it applied to the data studied here so that the possibility of modelling bioavailability by consideration of both absorption and clearance could be investigated.

2.3.3.2. Prediction of metabolic clearance

In this section of work two *in silico* methods were employed in an attempt to predict hepatic metabolism, so that the effect of metabolism could be accounted for in overall prediction of bioavailability.

Prediction of CYP isoform / SOM

Firstly, all 80 compounds from dataset D were grouped according to which cytochrome they were most likely to bind to; this was carried out using the WhichCyp software. Only three enzymes were considered: CYP3A4, CYP2D6 and CYP2C9. These enzymes are the most important in terms of drug development as over 90% of drugs are metabolised by these three enzymes. These are the three enzymes for which the SMARTCyp software could predict the probability of xenobiotic metabolism occurring at a particular atom (site of metabolism; SOM). SMARTCyp calculates ranking scores by considering such variables as energy of activation or accessibility. The top ranking atoms (highest chances of being a SOM) are those with the lowest scores. Due to this interpretation it was speculated that compounds with lower scores (for the top ranking atom) would have higher clearance. 14 compounds were predicted to bind to 3A4 and there was a high negative correlation between clearance and scores (Pearson coefficient = -0.75, p = 0.02). A similar analysis for

compounds predicted to be metabolised by CYP2D6 was also performed (n = 33). However, this time a poor correlation (positive) between clearance values and ranking scores was observed. The same analysis was also performed for CYP2C9, which resulted in obtaining a moderate negative correlation ($R = -0.55$, $p = 0.052$). It was apparent that this approach could not lead to identification of compounds with high clearance.

There are several reasons why no reasonable correlation could be obtained. In many cases more than one cytochrome may be involved in the metabolism of a given drug, so a given score for one enzyme may not be representative of the complete metabolic profile. Additionally, only metabolism related to the CYP family of enzymes was considered; these are involved in Phase I metabolism. While cytochromes are known to be heavily involved in xenobiotic metabolism other enzymes can also play a role, for instance those that facilitate the formation of conjugates in phase II metabolism.

Development of a QSAR model for clearance

A QSAR approach was also used to predict hepatic clearance. Li et al, (2009) built a successful quantitative structure-hepatic clearance model for a dataset of 50 compounds. This regression model was solely based on molecular descriptors (obtained using the TSAR software package, ver. 3.3). H-bond acceptor ability, energies of the highest occupied and lowest unoccupied molecular orbitals (E_{HOMO} and E_{LUMO}) and torsional energy were identified as important features.

This section reports an attempt to build a similar model for the 80 compounds in this study using descriptors calculated by MOE. Unfortunately, this attempt did not prove successful. All models that were built using MLR or support vector machine for regression (SMOreg), did not compare favourably with the model of Li et al. All R^2 values obtained here were in the region of 50 % (specific values were dependent on the Weka feature selection and classification method) whereas Li et al obtained a value of 85% for their training set. In the study here, features that were found to be correlated with clearance included LUMO energy (AM1 ELUMO) and various surface area/volume (vsurf) and adjacency and distance matrix descriptors (for example petitjean), however, none of the models were considered adequate to predict clearance

The reasons for the failure to obtain a model of similar predictive performance to that of Li et al are possibly due to the differences between the datasets (only 18 compounds were common to both studies) and the differences in software packages used for calculation of the molecular features. It should be noted that MOE descriptors used here were of a similar type to those from TSAR (used by Li et al) and the calculations were based on the same structure optimisation method (Hamiltonian,

AM1). Nevertheless, although the LUMO energy was found to be important in both models, HOMO was not present in the model developed here. This suggests that building a robust model capable of predicting hepatic clearance may be a challenging task and the applicability domain of such models needs careful consideration.

Other efforts were made to establish *in silico* rules that could account for the differences between HIA and bioavailability. Compounds were classified according to the difference (Δ) between % F and % HIA ($\Delta > 20\%$ indicated metabolism has a significant influence on F; $\Delta < 20\%$ indicates metabolism has little influence on F). The logistic regression model obtained included, similar to the clearance QSAR, features related to surface, volume and shape of molecules (seven vsurf MOE descriptors) or molecular density. The model correctly classified 72.5% of compounds. However, *kappa* statistics (0.45) did not indicate strong predictive capability.

2.3.3.3. Other considerations

Other reasons for the difference between % F and % HIA were investigated. Metabolic capacity is an important factor that influences clearance, which in turn affects bioavailability. However, clearance can also be affected by plasma protein binding and the kinetics of this process (Baker & Parton, 2007). The degree to which this binding occurs is different for acids and bases, with acids showing relatively higher binding than bases to human serum albumin (HSA) (Ghafourian & Amin, 2013). α -1 acid glycoprotein is more important in binding neutral and basic drugs. The ability to enter hepatocytes, and therefore be available for metabolism, is another factor to consider. The difference between plasma pH (approximately 7.4) and the pH in the GIT can affect log D of a compound and therefore alter the rate at which it crosses the hepatocyte membrane (assuming a passive transport); this will affect rate and extent of metabolism.

The role of medicinal chemistry-type descriptors for prediction of bioavailability was also considered. As previously shown, molecular features such as TPSA or log D could be considered very useful for the prediction of druglikeness of compounds and implicitly HIA. Using simple threshold values can provide rapid (not computationally intensive) and effective ways to screen for compounds that could be potential drug candidates. This might be especially important when exploring a vast chemical space. When it comes to prediction of human oral bioavailability these simple criteria may still hold valid. In this dataset of 80 compounds, seven drugs had a bioavailability of $< 20\%$; two of these compounds had very low absorption (1% and 0%) and this was predicted by both TPSA and log D descriptors. Five compounds had either high % HIA (over 90%) or medium % HIA (67%). All of these compounds, except for clofibrate, had high clearance and TPSA and log D values within acceptable

boundaries. In two cases a greater value of log D at pH 7.4 might have contributed to better passive permeability across hepatocyte membranes (potentially higher clearance). On the other hand, such an increase in lipophilicity could also facilitate binding to plasma proteins and in turn affect the metabolic rate (only unbound fraction can enter hepatocytes and be subject to metabolism). However, for three other compounds log D values were not affected by pH yet, these three drugs had poor % F to % FA ratios (the highest was 21%).

It is evident that prediction of bioavailability is a complex task; estimation of first-pass metabolic effects is crucial to making accurate predictions. However, prediction of metabolism includes predicting which CYP450 isoform(s) are involved, which are the potential SOM on the xenobiotic and the kinetics of the reaction (dictating intrinsic clearance). One of the ways to approach this issue could be the identification of structural features that are associated with high clearance/fast metabolism. However, the CYP450 system is capable of binding a very broad range of structurally diverse compounds. Such a lack of structural specificity may constitute a challenge for determination of structural information important for high or low clearance. Building a large database of compounds with known hepatic/intrinsic clearance values and then performing a similarity search for an unknown compound could be useful.

In conclusion, the *in silico* methods studied here showed reasonable predictive performance in determining which compounds belonged to the high or low class for HIA. The results were comparable with (and in some cases better than) existing *in vitro* methods (i.e. Caco-2 and PAMPA assays) but the *in silico* methods have the advantage of being much faster and more cost-effective. *In silico* screens can be performed on virtual datasets early in the drug discovery process.

Attempts to build models to predict bioavailability were less successful. It was not possible to build a model that could identify those compounds where % HIA was high but % F was low (which was assumed to be related to metabolic effects). The simple rules for druglikeness (such as a combination of TPSA and log D) although useful for the prediction of HIA, could not distinguish high and low bioavailability.

Whilst the prediction of rate and extent of metabolism is of key importance to predicting bioavailability, prediction of potential metabolites is also an important issue that will be investigated in Chapter 3.

3. Pragmatic Approaches to Using Computational Methods to Predict Metabolites

The results of this analysis have been published in *Journal of Chemical Information and Modeling* (Piechota et al., 2013).¹

3.1. Introduction

Prediction of xenobiotic metabolism is a research priority not only in drug development but also in many other areas including cosmetic and food safety assessment and environmental studies. The prediction of metabolism is important in the context of drugs' efficacy. For example certain pharmaceutical compounds require conversion from an inactive parent to an active metabolite. However, the predominant reason for predicting metabolism is concern for consumer or environmental safety as xenobiotics may be biotransformed to compounds that cause adverse effects. Poor absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of drug candidates have resulted in high drug attrition rates in late stages of drug development and withdrawal of drugs from the market due to adverse drug reactions (ADRs), resulting in great financial and societal cost. Reactive metabolites formed by bioactivation may bind covalently to biological macromolecules such as proteins leading to idiosyncratic drug toxicity, otherwise known as type B ADRs (Kalgutkar et al., 2005). Hence, the pharmaceutical industry has implemented the assessment of ADMET properties earlier in the drug development process which has already resulted in significantly reduced attrition rates (Kerns & Li, 2008; Kruhlak et al., 2012). Other relevant factors for the prediction of metabolism include identifying which enzymes may be involved in the metabolism of specific drugs. For instance, one of the problems of an aging population is polypharmacy and the potential for drug-drug interactions where the presence of one drug affects the metabolism, and hence the *in vivo* concentration, of another. Drug-food or drug-herbal interactions can similarly alter *in vivo* concentrations and knowledge of metabolic routes can help predict such interactions. Therefore, there is a great deal of interest in predicting potential metabolites and routes of metabolism for xenobiotics. This has resulted in a plethora of software and techniques available to predict metabolism. Different methods provide alternative solutions to

¹ Adapted with permission from: Piechota P, Cronin MT, Hewitt M, Madden JC. Pragmatic approaches to using computational methods to predict xenobiotic metabolism. *J Chem Inf Model*. 2013 Jun 24; 53(6):1282-93. doi: 10.1021/ci400050v. Copyright © 2013 American Chemical Society.

the problem and each is associated with advantages and disadvantages. Some methods are highly computationally expensive, others may result in vast numbers of potential metabolites being produced, where the true metabolites become obscured by too many data or other packages where fewer of the true metabolites are predicted. Kirchmair et al., (2012) provide an excellent review of the computational approaches to predicting metabolites and sites of metabolism. Table 3.1 lists some of the methods available. There are many other methods available particularly with respect to quantum chemical and docking approaches to predict likely sites of metabolism.

Table 3.1. *In Silico* Methods for the Prediction of Metabolites and Sites of Metabolism for Xenobiotics (adapted from Kirchmair et al., 2012).

Prediction of metabolites	Summary of method	Website or key citation
META	Uses a dictionary of biotransformations to predict the structure of likely metabolites. Analyses metabolite stability.	Klopman, Dimayuga & Talafous, (1994)
Metabolexpert	Uses rule-based knowledge to predict likely metabolites in humans, animals or plants.	http://www.compudrug.com/?q=node/36
Metabolizer, (ChemAxon, Budapest)	Includes libraries of biotransformation - enumerates all possible metabolites, predicts major metabolites, estimates metabolic stability and supports species-specific predictions of likely metabolites.	http://www.chemaxon.com/products/online-tryouts/metabolizer/
Metaprint2D-React	Predicts structures of metabolites based on data-mining and statistical analysis – refer to Metaprint2D below	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react
MetaSite	Considers enzyme-substrate recognition and predicts metabolic transformations for cytochrome-mediated reactions in phase I metabolism. Provides the structure of metabolites and provides a ranking derived from estimated likelihood of metabolic reaction at a given position.	http://www.moldiscovery.com/soft_metasite.php
Meteor	Uses expert knowledge rules for metabolism to predict metabolites which	https://www.lhasalimited.org/meteor/

	are presented in metabolic trees	
OECD QSAR Toolbox	Contains both rat liver and skin metabolism simulators in addition to database of known biotransformations	https://www.qsartoolbox.org
SyGMa	Predicts structures and probability of metabolites based on rules derived from Accelrys Metabolite Database	Ridder & Wagener, (2008)
TIMES (Tissue METabolism Simulator)	A heuristic algorithm to generate plausible metabolic maps from a comprehensive library of biotransformations and abiotic reactions	http://oasis-lmc.org/?section=software&swid=4
<hr/>		
Prediction of Sites of Metabolism	Summary of method	Website or key citation
ADMET Predictor – Metabolite Module	Derives likelihood of metabolic reactions occurring at specific atom positions; identifies substrates for five CYP isoforms	http://www.simulations-plus.com/Products.aspx?PID=13&MID=15
Metaprint2D	Predicts sites of phase I xenobiotic metabolism in dog, human and / or rat through data-mining and statistical analysis of known metabolic transformations reported in literature.	http://www-metaprint2d.ch.cam.ac.uk/metaprint2d
MetaSite	See above - also predicts metabolites	http://www.moldiscovery.com/soft_metasite.php
SMARTCYP	Uses pre-calculated density functional theory activation energies with topological accessibility descriptors to sites liable to cytochrome P450 metabolism.	http://www.farma.ku.dk/smartcyp/index.php
StarDrop	Combines quantum chemical analysis and ligand-based model of CYP substrates to estimate potential sites of metabolism	http://www.optibrium.com/stardrop/stardrop-p450-models.php
RS-WebPredictor	Uses topological and quantum chemical atom-specific predictors to identify sites of metabolism. Regioselectivity models	http://reccr.chem.rpi.edu/Software/RS-WebPredictor/

	for substrates have been developed for nine key CYP isozymes	
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Kirchmair et al., (2012) also listed many methods for the prediction of enzyme interactions, such as binding affinity, inhibition and induction of cytochromes incorporating docking studies and binding energy calculations. Such methods tend to be computationally expensive and were not considered in the present study which focusses only on software to predict metabolites or sites of metabolism.

In general, metabolism results in chemical modification of xenobiotics so that they become more polar and, therefore, more readily excreted via urine or bile. Phase I metabolism involves oxidation, hydroxylation, de-amination and de-alkylation reactions which are catalysed by the cytochrome P450 superfamily of enzymes with CYP3A4, CYP2D6 and CYP2C9 isoforms being responsible for the majority of transformations for drugs. Phase II reactions yield hydrophilic conjugates, for example glucuronides may be formed via the action of glucuronyl transferases. Phase II metabolism may follow or be independent of phase I reactions. Hydrolysis and reduction reactions are also significant in terms of metabolism. Metabolism is a highly complex process involving a multitude of biotransformations that may occur consecutively, concurrently or in competition with each other. Genetic and environmental factors can also affect the nature and abundance of metabolites making prediction a difficult task. Nevertheless, software packages for prediction of metabolism are widely used, particularly by the pharmaceutical industry, and especially during the screening phase of the drug development process. Predictions made by the software may aid the development of drugs with improved pharmacokinetic profiles and highlight potential metabolite induced toxicity (Sun & Scott, 2010).

In 2002, the Metabolites in Safety Testing (MIST) committee defined how to approach metabolic data when assessing the toxicity of drug candidates (Baillie et al., 2002); these guidelines were subsequently modified (Smith & Obach, 2005). Because it is essential that the products of metabolic pathways are considered for their potential toxicity, *in silico* methods to predict metabolism are becoming increasingly important. Such methods need to be used with confidence, hence performance and best practice in using the methods needs to be assessed.

To assess any software used for metabolism prediction, specific criteria should be established so that the design of a particular package is taken into account. Due to differences between programs it is not always possible to apply the same criteria to each program when assessing performance. Some software (e.g. SMARTCYP Web Service) only provides sites of metabolism (SOM) rather than metabolite structures. However, predicting SOM is not equivalent to identifying the correct

biotransformation that would take place at the particular atom. Prediction of SOM has been successful with studies indicating up to 90% correct identification of the three most likely SOM. However, prediction of the absolute likelihood of the formation of a certain metabolite is less accurate (Kirchmair et al., 2012).

Where predicted metabolite structures are provided in the output of an algorithm, direct comparison between different algorithms still may not be feasible. For example, some software may only predict the outcome of Phase I, and not Phase II, metabolism or models may only consider specific cytochrome P450 enzymes. Other issues affecting direct comparison of software packages include selection of user-defined constraints such as species, probability level for a particular metabolite being formed etc. Statistical analysis of predictions may provide a general indication of algorithm performance, but comparisons between packages must be assessed with caution. The underlying design and limitations of the software should be taken into consideration. Another important consideration is the training set on which the software was developed; this information may or may not be available. Output for a given package may reflect overall performance in identifying metabolites but not give a true indication of predictive power of the model when faced with unknown compounds. Therefore, a combination of the quantitative and qualitative assessment of performance may be more informative. For example, investigating not only statistical values for percentage metabolites correctly predicted, but also considering which metabolites are not predicted e.g. if there are specific metabolic routes that are not identified in the software; this gives scope for further improvements of algorithms. Clearly there are too many software routines of diverse functionality and output to evaluate in a fully consistent manner.

The aim of the present study was to investigate the performance of three well-known, representative, software packages for the prediction of metabolism. Performance was compared between two different datasets. The first dataset (DS1) comprised 28 non-steroidal anti-inflammatory drugs (NSAIDs) plus paracetamol, representing a homogenous chemical space. The second dataset (DS2) comprised the first 30 drugs listed in the Top 200 Drugs for 2010 by Sales in the United States, excluding drugs containing more than one active ingredient or peptides (Drugs.com, 2010). DS2 represented a more diverse chemical space than DS1 with a wider range of potential metabolic biotransformations.

Investigation into the influence of modifying user-defined constraints was also carried out, for appropriate packages, to enable recommendations to be made as to the most pragmatic software settings for a given query.

The three software packages selected from those listed in Table 3.1 were:

(I) Meteor

This is an industry-standard, knowledge-based expert system developed by the not-for-profit organisation Lhasa Limited, Leeds, England. Meteor (currently marketed by Lhasa Limited as Meteor Nexus) predicts metabolites from the structure of the parent compound, based on an extensive set of biotransformation rules which have been extracted from the literature or informed by confidential data from the pharmaceutical industry. It uses two types of reasoning: absolute reasoning describes the probability of a biotransformation taking place (in terms of probable, plausible, equivocal, doubted and improbable) and relative reasoning that allows further ordering of all possible metabolic outcomes in cases when there are competing biotransformations at a given SOM (Marchant, Briggs & Long, 2008).

(II) Metaprint2D / Metaprint2D-React

Metaprint2D is a freely available algorithm that predicts the likelihood of a metabolic reaction occurring at a given position in the molecule, using a circular fingerprints technique. Circular fingerprints are used to represent the chemical environment of an atom. They are generated by using concentric layers that radiate out from a central atom and then store information about the number and types of atoms in each layer. The first layer contains only a central atom, the second layer includes the direct neighbours of the central atom and the consecutive layer contains atoms that are directly bonded to the atoms in the second layer. Circular fingerprints used by MetaPrint2D can be based on up to six layers. To predict sites of metabolism, MetaPrint2D utilises data found in the Symyx[®] Metabolite database, which is a collection of 80,000 xenobiotic transformations (predominantly for pharmaceuticals) collated from the literature. The predictions are obtained by generating fingerprints for the query compound and then comparing these using a similarity search against two databases of fingerprints. The first of the two databases contains fingerprints calculated for all the substrate atoms present in transformations found in the Symyx[®] Metabolite database, whilst the second database contains fingerprints for only those atoms from the Symyx[®] database that are known to be sites of metabolism (reaction centres). The results of the similarity search for each atom of the query compound are reported as the occurrence ratio which measures how often a similar chemical environment was found at a reaction centre relative to all occurrences of similar environment in the Symyx[®] Metabolite database. Metaprint2D-React

is an extension of Metaprint2D that can provide structures of potential metabolites associated with reaction at the identified positions (Carlsson et al., 2010; Adams, 2010).

(III) SMARTCyp

This software determines the sites in a molecule liable to metabolism using the 2D structure of the compound and calculations of energies required for oxidation. These are matched against pre-calculated activation energies for fragments represented as SMARTS patterns (Rydberg et al., 2010). SMARTCyp uses two descriptors to predict a site of metabolism: reactivity (E) and accessibility (A). The reactivity descriptor is based on transition state energies calculated for fragments using density functional theory. The calculations are grouped by fragments and then average activation energies are obtained and defined as SMARTS rules for each group. The accessibility is described as the distance of the atom from the centre of the molecule. Both reactivity and accessibility descriptors are used to obtain a score for each atom; an atom with the lowest score is ranked as the most likely site of metabolism (Rydberg et al., 2010). Currently, CYP3A4, CYP2C9 and CYP2D6 are the only models available in SMARTCyp.

3.2. Methods

3.2.1. Datasets

Dataset 1 (DS1) and dataset 2 (DS2) were chosen to represent homogenous and diverse drugs respectively, in terms of action, structure and metabolic transformation. DS1 comprised 28 commonly used NSAIDs and paracetamol; DS2 comprised 30 drugs taken from the Top 200 drugs in 2010 by sales in the United States. The top 30 drugs in the list (excluding peptides) were considered; if a drug comprised more than one active ingredient each of them were included providing they were subject to CYP450 metabolism.

3.2.2. Collecting and storing metabolism data

A thorough literature search was performed to obtain the known *in vivo* metabolites, in humans, for the 59 drugs considered in this study. Sources of information (see Appendix V) included original research papers, reviews and online databases such as: Martindale: The Complete Drug Reference

and Clarke's Analysis of Drugs and Poisons. As some of the predictive software investigated here (such as SMARTCyp) was not designed to predict Phase II metabolites, data collection focused on Phase I metabolism only.

The complete list of known metabolites found in the literature are available as supplementary information (see Appendices VI and VII).

The structures of all parent drugs and metabolites were drawn in MarvinSketch, version 5.0.3 (Chemaxon) and stored in mol format. Information concerning the specific CYP450 isoform(s) involved in biotransformations for DS2 was also recorded and is also available as supplementary data.

In total, 78 known metabolites for DS1 and 101 known metabolites for DS2 were obtained from the literature.

Table 3.2 lists the common names for the drugs used in this study along with their Chemical Abstracts Service Registration Number (CAS RN), International Union of Pure and Applied Chemistry (IUPAC) name and the number of phase I metabolites retrieved from the literature for each drug. The Supporting Information Available also gives the SMILES strings for the parent drugs and their known phase I metabolites.

Table 3.2. Common names, CAS registry numbers, IUPAC names and number of phase I metabolites obtained from the literature for drugs used in datasets 1 and 2 in this study.

Dataset 1			
Common Name	CAS RN	IUPAC Name	No. of metabolites
Alclofenac	22131-79-9	[4-(Allyloxy)-3-chlorophenyl]acetic acid	3
Aspirin	50-78-2	2-Acetoxybenzoic acid	3
Azapropazone	13539-59-8	5-(Dimethylamino)-9-methyl-2-propyl-1H-pyrazolo[1,2-a][1,2,4]benzotriazine-1,3(2H)-dione	2
Bromfenac	91714-94-2	[2-Amino-3-(4-bromobenzoyl)phenyl]acetic acid	3
Carpofen	52263-47-5	2-(6-Chloro-9H-carbazol-2-yl)propanoic acid	3
Diclofenac	15307-86-5	{2-[(2,6-Dichlorophenyl)amino]phenyl}acetic acid	6
Diflunisal	22494-42-4	2',4'-Difluoro-4-hydroxy-3-biphenylcarboxylic acid	1
Fenbufen	36330-85-5	4-(4-Biphenyl)-4-oxobutanoic acid	4
Fenclofenac	34645-84-6	[2-(2,4-Dichlorophenoxy)phenyl]acetic acid	1

Fenoprofen	31879-05-7	2-(3-Phenoxyphenyl)propanoic acid	1
Feprazone	30748-29-9	4-(3-Methyl-2-buten-1-yl)-1,2-diphenyl-3,5-pyrazolidinedione	1
Flufenamic acid	530-78-9	2-[[3-(Trifluoromethyl)phenyl]amino]benzoic acid	3
Flurbiprofen	5104-49-4	2-(2-Fluoro-4-biphenyl)propanoic acid	3
Ibuprofen	15687-27-1	2-(4-Isobutylphenyl)propanoic acid	4
Indomethacin	53-86-1	[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]acetic acid	3
Ketoprofen	22071-15-4	2-(3-Benzoylphenyl)propanoic acid	3
Ketorolac	66635-83-4	5-Benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid	1
Mefenamic acid	61-68-7	2-[(2,3-Dimethylphenyl)amino]benzoic acid	2
Nabumetone	42924-53-8	4-(6-Methoxy-2-naphthyl)-2-butanone	5
Naproxen	22204-53-1	(2S)-2-(6-Methoxy-2-naphthyl)propanoic acid	1
Paracetamol	103-90-2	N-(4-Hydroxyphenyl)acetamide	3
Phenylbutazone	50-33-9	4-Butyl-1,2-diphenyl-3,5-pyrazolidinedione	3
Piroxicam	36322-90-4	4-Hydroxy-2-methyl-N-(2-pyridinyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide	1
Pirprofen	31793-07-4	2-[3-Chloro-4-(2,5-dihydro-1H-pyrrol-1-yl)phenyl]propanoic acid	5
Sulindac	38194-50-2	{(1Z)-5-Fluoro-2-methyl-1-[4-(methylsulfinyl)benzylidene]-1H-inden-3-yl}acetic acid	2
Suprofen	40828-46-4	2-[4-(2-Thienylcarbonyl)phenyl]propanoic acid	5
Tiaprofenic acid	33005-95-7	2-(5-Benzoyl-2-thienyl)propanoic acid	2
Tolmetin	26171-23-3	[1-Methyl-5-(4-methylbenzoyl)-1H-pyrrol-2-yl]acetic acid	2
Zomepirac	33369-31-2	[5-(4-Chlorobenzoyl)-1,4-dimethyl-1H-pyrrol-2-yl]acetic acid	2

Dataset 2

Aripiprazole	129722-12-9	7-[4-[4-(2,3-Dichlorophenyl)-1-piperazinyl]butoxy]-3,4-dihydro-2(1H)-quinolinone	5
Atorvastatin	110862-48-1	(3R,5R)-7-[2-(4-Fluorophenyl)-5-isopropyl-3-phenyl-4-(phenylcarbamoyl)-1H-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid	5
Buprenorphine	52485-79-7	(5 α ,6 β ,14 β ,18R)-17-(Cyclopropylmethyl)-18-[(2S)-2-hydroxy-3,3-dimethyl-2-butanyl]-6-methoxy-18,19-dihydro-4,5-epoxy-6,14-ethenomorphinan-3-ol	1

Celecoxib	169590-42-5	4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide	2
Clopidogrel	90055-48-4	Methyl (2S)-(2-chlorophenyl)(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate	3
Donepezil	120011-70-3	Methyl (2S)-(2-chlorophenyl)(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)acetate	4
Duloxetine	116539-58-3	(3S)-N-Methyl-3-(1-naphthyloxy)-3-(2-thienyl)-1-propanamine	11
Efavirenz	154635-17-3	(4S)-6-Chloro-4-(cyclopropylethynyl)-4-(trifluoromethyl)-1,4-dihydro-2H-3,1-benzoxazin-2-one	3
Emtricitabine	143491-57-0	4-Amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H)-pyrimidinone	1
Escitalopram	59729-33-8	(1S)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile	5
Esomeprazole	73590-58-6	6-Methoxy-2-[(S)-[4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole	5
Ezetimibe	163222-33-1	(3R,4S)-1-(4-Fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl)-2-azetidinone	1
Fenofibrate	49562-28-9	Isopropyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate	2
Fluticasone propionate	80474-14-2	(6 α ,11 β ,16 α ,17 α)-6,9-Difluoro-17-[[[(fluoromethyl)sulfanyl]carbonyl]-11-hydroxy-16-methyl-3-oxoandrost-1,4-dien-17-yl] propionate	1
Levofloxacin	100986-85-4	(3S)-9-Fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	2
Methylphenidate	113-45-1	Methyl phenyl(2-piperidinyl)acetate	6
Modafinil	68693-11-8	2-[(Diphenylmethyl)sulfinyl]acetamide	2
Montelukast	158966-92-8	{1-[[{(1R)-1-{3-[(E)-2-(7-Chloro-2-quinolinyl)vinyl]phenyl}-3-[2-(2-hydroxy-2-propanyl)phenyl]propyl}sulfonyl)methyl]cyclopropyl}acetic acid	5
Naloxone	465-65-6	(5 α)-17-Allyl-3,14-dihydroxy-4,5-epoxymorphinan-6-one	2
Olanzapine	132539-06-1	(5 α)-17-Allyl-3,14-dihydroxy-4,5-epoxymorphinan-6-one	6
Oxycodone	76-42-6	(5 α)-14-Hydroxy-3-methoxy-17-methyl-4,5-epoxymorphinan-6-one	3

Pioglitazone	111025-46-8	5-{4-[2-(5-Ethyl-2-pyridinyl)ethoxy]benzyl}-1,3-thiazolidine-2,4-dione	6
Pregabalin	148553-50-8	(3S)-3-(Aminomethyl)-5-methylhexanoic acid	1
Quetiapine	111974-69-7	2-{2-[4-(Dibenzo[b,f][1,4]thiazepin-11-yl)-1-piperazinyl]ethoxy}ethanol	5
Rosuvastatin	287714-41-4	(3R,5S,6E)-7-{4-(4-Fluorophenyl)-6-isopropyl-2-[methyl(methylsulfonyl)amino]-5-pyrimidinyl}-3,5-dihydroxy-6-heptenoic acid	2
Salmeterol	89365-50-4	2-(Hydroxymethyl)-4-(1-hydroxy-2-[[6-(4-phenylbutoxy)hexyl]amino]ethyl)phenol	2
Sildenafil	139755-83-2	5-{2-Ethoxy-5-[(4-methyl-1-piperazinyl)sulfonyl]phenyl}-1-methyl-3-propyl-1,4-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one	4
Sitagliptin	486460-32-6	(3R)-3-Amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl]-4-(2,4,5-trifluorophenyl)-1-butanone	1
Valsartan	137862-53-4	N-Pentanoyl-N-[[2'-(2H-tetrazol-5-yl)-4-biphenyl]methyl]-L-valine	1
Venlafaxine	93413-69-5	1-[2-(Dimethylamino)-1-(4-methoxyphenyl)ethyl]cyclohexanol	4

3.2.3. Use of software

(I) Meteor

Meteor, version 13, Lhasa Limited, Leeds, England was used to predict metabolites for both datasets. The parent structures were submitted as mol format and the resulting metabolites were stored as Meteor generic files (mtr). As stated previously, Meteor employs two types of reasoning - absolute and relative.

With regard to absolute reasoning, three levels were selected in this analysis: probable, plausible and equivocal (doubted and improbable were not considered). For each of these three levels of absolute reasoning, three levels of relative reasoning were considered: levels 1, 2 and 3.

Table 3.3 shows the nine combinations of reasoning employed and the abbreviations for these that will be used throughout this paper.

Table 3.3. Combinations of reasoning used in Meteor and abbreviations utilised in this paper

Absolute Reasoning	Relative Reasoning 1	Relative Reasoning 2	Relative Reasoning 3
Probable	PRO1	PRO2	PRO3
Plausible	PLA1	PLA2	PLA3
Equivocal	EQU1	EQU2	EQU3

All the other parameters selected from the option “*Processing Constraints*” were kept constant for all combinations of reasoning. Processing constraints were: phase option = phase I only; species = human; enzyme = not specified; max number of metabolites = 400; max number of steps in a pathway = 4.

The presence of known metabolites for DS1 and DS2 was identified by means of the Meteor functionality that allows direct searching for structures in a metabolic tree (mol format). All the true positives (i.e. known metabolites from the literature that were correctly predicted by the software) along with their positions in the Meteor metabolic tree were recorded and saved in a Microsoft Excel spreadsheet. In the first part of the analysis described here, the percentage of known metabolites that were correctly predicted within each metabolic tree (of up to 400 metabolites) for each compound in DS1 and DS2 was calculated using reasoning level EQU3.

Further analysis was subsequently undertaken to establish the sensitivity for each of the nine reasoning settings given in Table 3.2 by taking into account the number of metabolites correctly predicted at different cut-off points. The cut-off points were 5, 10, 15, 20, 25, 30, 35, 40 and 400 and refer to the number of predicted metabolites appearing sequentially in the metabolic tree that were subsequently checked against structures of known metabolites. For example, using a cut-off value of 20 means that only the first 20 metabolites appearing in the Meteor tree of predicted metabolites were considered; a cut-off value of 400 means that all 400 predicted metabolites were checked against known metabolites to determine if the known metabolite had been predicted. The purpose of this was to investigate the most pragmatic use of the Meteor software. Whilst it is possible to generate 400 metabolites, investigation into the structure of each of these (as may be performed for subsequent estimation of metabolite toxicity) is a very time-consuming process. It was considered useful to determine whether or not it was possible to identify the majority of known metabolites within the first 5, 10 or 20 etc. metabolites that appear in the highest positions of the metabolic tree(s), to increase the efficiency of using the program.

(II) MetaPrint2D-React

The structures of the parent drugs were entered as SMILES strings into the MetaPrint2D-React web-service software at the following URL: <http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react/>. Fingerprint matching was set to a default value and the human model was selected. The resulting predictions for metabolite structures were compared to known metabolites.

(III) Combination of SMARTCyp and MetaPrint2D-React

A subset of metabolites from DS2 that are known to be products of CYP3A4 or CYP2D6 metabolism were identified from the literature. 39 of the metabolites from DS2 are known to be products of CYP3A4 and 11 of the metabolites are known to be products of CYP2D6.

For each parent drug contained in this subset of data the following steps were followed. The parent structure was submitted as a SMILES string to the SMARTCyp Web Service version 2.0; URL: <http://www.farma.ku.dk/smartcyp/>. SMARTCyp was used to identify the top five ranking atoms (in terms of most likely site of metabolism) for both CYP3A4 and CYP2D6. The parent structure of the drug was then submitted as a SMILES string into the MetaPrint2D-React program. (URL: <http://www-metaprint2d.ch.cam.ac.uk/metaprint2d-react/>); fingerprint matching was set to the default value and the human model was selected. The output was analysed in terms of biotransformations occurring at the atoms corresponding to the SOM previously predicted as being in the top five ranked sites by SMARTCyp. The metabolites predicted from biotransformations at these sites were compared to known metabolites. The procedure was repeated using only the top three ranked SOM from SMARTCyp. This process is outlined in Figure 3.1.

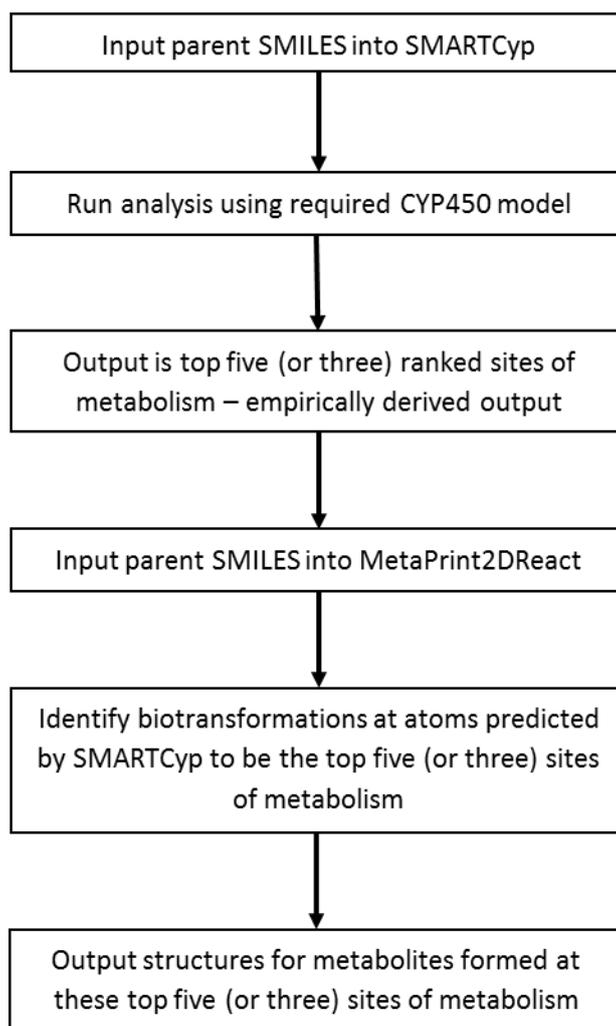


Figure 3.1. Flow diagram showing the method to predict metabolites using a combination of SMARTCyp (models for CYP3A4 and CYP2D6) and MetaPrint2D-React.

3.2.4. Statistical analysis

The performance of Meteor and MetaPrint2D-React was assessed in terms of the number of correctly predicted metabolites (sensitivity) and was calculated according to the following formula:

$$\text{Sensitivity (\%)} = ([TP] / \text{TOTAL}_{in\ vivo}) * 100\%$$

Precision in the context used here was calculated as:

$$\text{Precision (\%)} = [TP] / ([TP] + [FP]) * 100\%$$

$\text{TOTAL}_{in\ vivo}$ = total number of known metabolites observed (from *in vivo* literature data)

TP = No. of correct predictions (predicted metabolites that are also observed *in vivo*)

FP = No. of incorrect predictions (predicted metabolites that are not observed *in vivo*)

3.3. Results and discussion

3.3.1. General performance of the algorithms

Two datasets were used to analyse performance of different software in predicting metabolites and sites of metabolism and to investigate pragmatic approaches to using the software. Dataset 1 (DS1) was a more chemically homogenous set of 28 NSAIDs and paracetamol for which a total of 78 known metabolites had been identified from a literature search. Dataset 2 (DS2) was a more diverse set of 30 drugs for which 101 metabolites had been identified in the literature. Figure 3.2 shows an example of results for aripiprazole (and its five known metabolites) for illustrative purposes, along with the predictions from Meteor and MetaPrint2D. Note that M1, M4 and M5 were not predicted by Meteor when the probable (PRO) absolute reasoning engine was used.

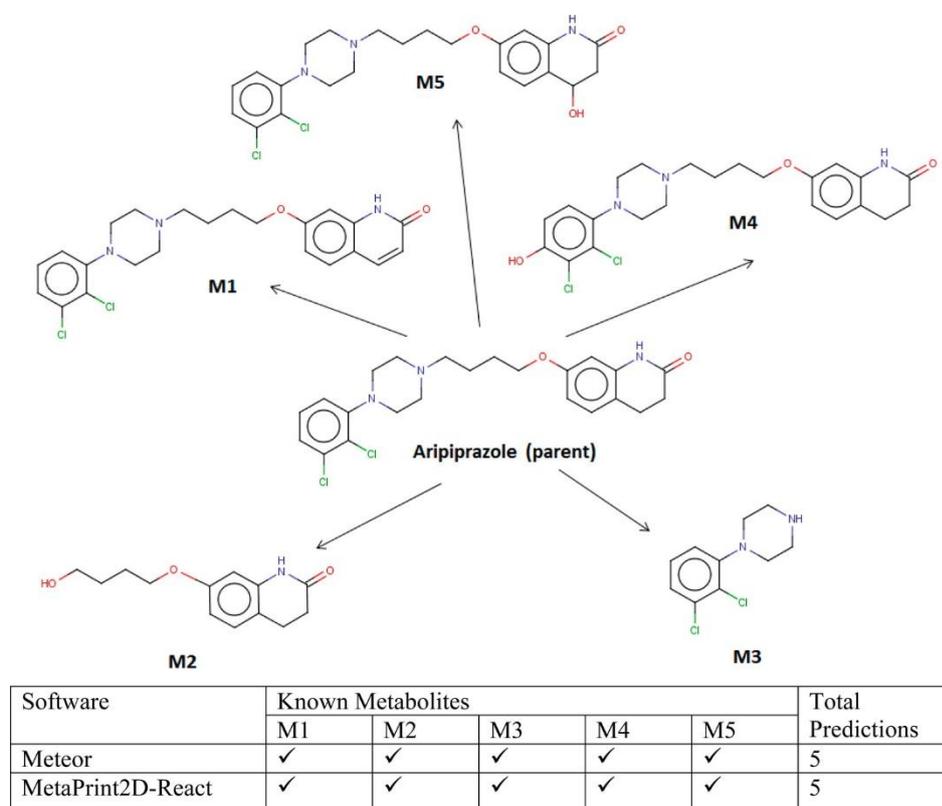


Figure 3.2. Known metabolites of aripiprazole and the predictions obtained by Meteor and MetaPrint2D-React.

Table 3.4 shows the overall performance of the software packages in predicting the known metabolites (as obtained from the literature) for DS1 and DS2.

Table 3.4. Performance of Meteor and Metaprint2D-React in Predicting Metabolites of datasets 1 and 2.

Software	No. metabolites correctly predicted for DS1	No. metabolites correctly predicted for DS2
Meteor (setting EQU3)	57 (73%)	86 (85%)
Metaprint2D-React	62 (80%)	90 (89%)

Note that this analysis was carried out in order to obtain a general indication of performance and compare the number of correct predictions that are obtained when using two different datasets (one homogenous, one diverse). The results are not a true measure of overall predictivity of the software used as it is likely that the drugs analysed are present to a greater or lesser extent in the information used to develop the individual software packages.

Meteor and Metaprint2D-React both show good performance in predicting metabolites for datasets DS1 and DS2. What is interesting to note is that in all cases predictions were marginally better for the diverse dataset (DS2) than for the more homogenous dataset (DS1). This suggests that areas of chemical space more predominant in one set of compounds may not be as well represented in the training sets for the software than other areas. For example, none of the software packages predicted the metabolites of the NSAID bromfenac. The metabolites of bromfenac are dependent on the formation of a cyclic amide metabolite which was not predicted by the software tested. Similarly, two metabolites of nabumetone and one metabolite of suprofen were not predicted by any of the software indicating a possible knowledge gap in metabolite prediction. Such information can be used to direct future improvements in software development. Aside from these examples, for DS1 72 of the metabolites (i.e. 92%) were predicted by at least one of the software packages (or combination of packages).

Cases where specific metabolites were not found by any of the packages studied also occurred for DS2. They include: three metabolites of clopidogrel, two metabolites of pioglitazone and one metabolite of sitagliptin which gave a total of 6 out of 101. Thus, 96 metabolites (95%) were predicted by at least one of the packages used in the study.

3.3.2. Pragmatic approach to using Meteor

Table 3.5 shows the results of the predictions for the Meteor software where nine different reasoning levels were investigated for the prediction of metabolites of DS1. The cut-off values in row 1 show the number of metabolites that were checked against known metabolites e.g. a cut-off value

of five indicates the first five metabolites only in the metabolic tree *for each compound* were checked; for DS1 78 metabolites had been identified from the literature.

Table 3.5. Predictions from Meteor for dataset 1 using nine settings for reasoning (as defined in Table 3.3) and nine cut-off values.

Cut-off values	5		10		15		20		25		30		35		40		400			
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%		
PRO1	6	8	7	9	7	9	7	9	7	9	7	9	7	9	7	9	7	9	7	9
PRO2	7	9	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10
PRO3	7	9	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10	8	10
PLA1	33	42	39	50	39	50	39	50	39	50	39	50	39	50	39	50	39	50	39	50
PLA2	37	47	45	58	46	59	46	59	46	59	46	59	46	59	46	59	46	59	46	59
PLA3	37	47	45	58	46	59	46	59	46	59	46	59	46	59	46	59	46	59	46	59
EQU1	37	47	46	59	47	60	48	62	48	62	48	62	48	62	48	62	48	62	48	62
EQU2	41	53	49	63	52	67	54	69	55	71	55	71	55	71	55	71	55	71	55	71
EQU3	41	53	48	62	51	65	54	69	56	72	57	73	57	73	57	73	57	73	57	73

N = number of correctly predicted metabolites; % = percentage of correctly predicted metabolites.

Trends in the data shown in Table 3.5 can be more clearly visualised graphically as shown in Figure 3.3

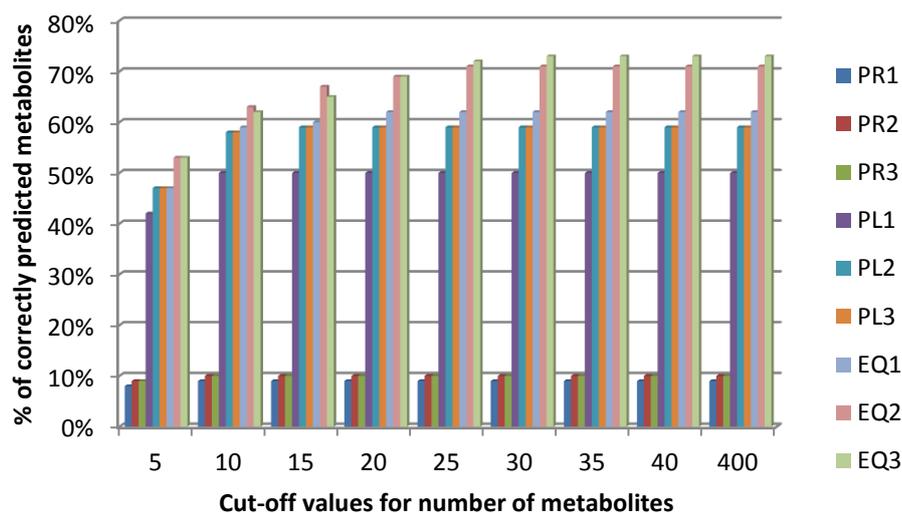


Figure 3.3. Meteor predictions for DS1 showing the variation in number of metabolites correctly predicted as a function of cut-off values and reasoning levels selected.

Table 3.6 shows the results of the Meteor predictions for DS2 using nine reasoning settings and nine cut-off values; for DS2 101 metabolites had been identified from the literature.

Table 3.6. Predictions from Meteor for dataset 2 using nine settings for reasoning (as defined in Table 3.3) and nine cut-off values.

Cut-off values	5		10		15		20		25		30		35		40		400		
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	
PRO1	27	27	28	28	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29
PRO2	27	27	30	30	32	32	32	32	32	32	32	32	32	32	32	32	32	32	32
PRO3	27	27	30	30	32	32	32	32	32	32	32	32	32	32	32	32	32	32	32
PLA1	37	37	52	51	57	56	60	59	60	59	61	60	61	60	62	61	62	61	61
PLA2	37	37	58	57	67	66	69	68	71	70	73	72	74	73	74	73	75	74	74
PLA3	37	37	57	56	68	67	70	69	71	70	74	73	75	74	75	74	76	75	75
EQU1	39	39	58	57	64	63	67	66	68	67	69	68	69	68	70	69	70	69	69
EQU2	42	42	57	56	71	70	74	73	76	75	77	76	79	78	80	79	84	83	83
EQU3	42	42	56	55	67	66	73	72	78	77	80	79	81	80	82	81	86	85	85

N = number of correctly predicted metabolites; % = a percentage of correctly predicted metabolites.

Similarly to dataset 1 the results for the analysis can be visualised graphically as shown in Figure 3.4.

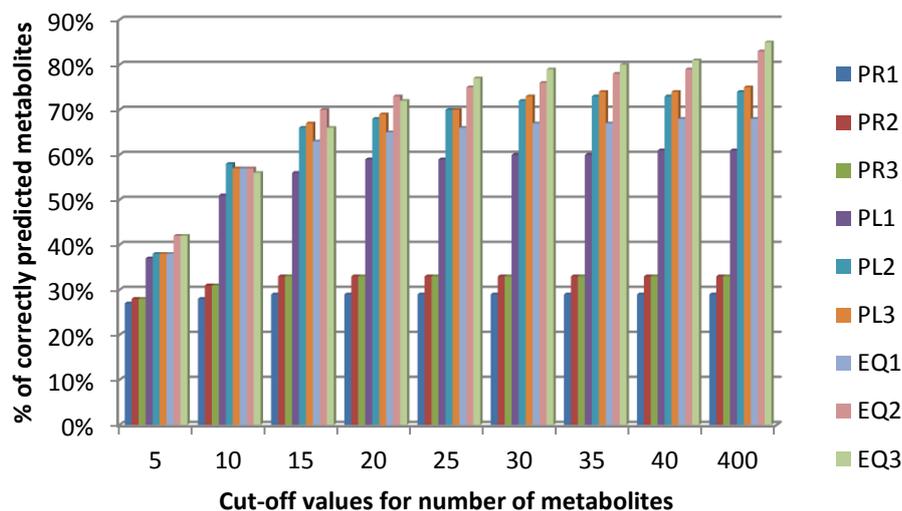


Figure 3.4. Meteor predictions for DS2 showing the variation in number of metabolites correctly predicted as a function of cut-off values and reasoning levels selected.

The above results show clear trends for both the homogenous and diverse datasets. Setting absolute reasoning to probable is restrictive and many known metabolites are missed; only 10% and 32% of known metabolites are correctly predicted for DS1 and DS2, respectively. When altering the absolute reasoning level to plausible many more metabolites are found. However, using the absolute reasoning level of equivocal, results in the highest number of metabolites being found for DS1, for all levels of relative reasoning (1-3). For DS2 the combination of absolute reasoning of equivocal and relative reasoning 2 and 3 almost always gave the highest number of known metabolites.

One well-known drawback of using the least stringent reasoning settings (e.g. EQU2 or EQU3) is that these are associated with a high number of potential metabolites being generated. For example, the study of Tjollyn et al., (2011) which involved investigation of the predictive power of Meteor, showed that although the program is sensitive (i.e. performs well in correctly predicting known metabolites) it also has tendency to over-predict biotransformations (for example, simple hydroxylations) giving the program low specificity. Hence the true metabolites may become “lost” in the plethora of possible metabolites. However, the analysis carried out here indicates that using cut-off values is a pragmatic solution to this problem. For DS1 using EQU3, but only investigating the structures of the top 30 metabolites gave the same results (in terms of number of metabolites correctly predicted) as investigating the top 35, 40 or 400 metabolites. Similarly, for DS2 using a cut-off value of 30 returns 79% of true metabolites, with 400 metabolites needing to be investigated to obtain 85% of true metabolites.

It is evident that the sensitivity of Meteor can be controlled by selecting different absolute and relative reasoning levels. Due to the design of the program it is expected that changing the absolute reasoning from probable to plausible and then to equivocal is associated with an increase in sensitivity in each case. Similarly, more true positives can be identified using higher levels of the relative reasoning. However, this increased sensitivity is paired with a great reduction in precision. For instance,esomeprazole, a drug present in DS2, is known to have five metabolites *in vivo*. Three of these metabolites were correctly identified using reasoning PRO1, which produced a total of five predicted metabolites, hence precision can be calculated as 3/5. For all five metabolites to be correctly predicted required a reasoning setting of PLA1, which produced 77 predictions (precision = 5/77). This example illustrates how excellent sensitivity can be obtained but at the cost of a large number of false positives. On the other hand, it also indicates that by limiting the total number of predicted metabolites it would be possible to increase precision. Thus, instead of applying high filtering levels, and so reducing the variety of possible biotransformations, analysis of a subset of the output (as was performed here using cut-off values) may be more pragmatic. Cut-off points can be

selected according to the level of sensitivity required by the user. The results here indicate that in terms of drug development a more pragmatic approach to predicting metabolites using Meteor may be to use EQU3 but to limit the number of predicted metabolites investigated to the top 25 or 30. This would offer significant savings in time and effort in predicting likely metabolites that can then be processed as necessary for toxicity assessment.

It should be stressed that the known metabolites, collated from the literature for both datasets, contained not only major metabolites, but also those that were present in plasma or urine in trace amounts. Advances in analytical methods allow identification of less abundant metabolites so that the total number of metabolites can be relatively high. For almost 50% of the drugs in DS2, four or more metabolites have been identified *in vivo* (e.g. 11 known metabolites have been identified for duloxetine). Thresholds for identification of metabolites *in vivo* may vary from 5% to 10% (relative to the administered drug dose) according to different regulatory guidelines (OECD, 2008; FDA. U.S. Food and Drug Administration, 2008; European Medicines Agency, 2009). The software studied here was assessed for its ability to predict metabolites that had been identified *in vivo* irrespective of their relative abundance i.e. it was tested without any quantitative assumptions. Meteor assesses probabilities of metabolite formation according to its absolute and relative reasoning engines rules; it does not provide explicit information concerning the relative abundance of these metabolites in plasma or urine. Additionally, the method that Meteor uses to categorise metabolites in a tree may not always reflect an actual *in vivo* abundance.

It should be noted that the precision values were calculated relative to cut-off points. For instance, using the PRO1 setting gave 11 predicted metabolites for donepezil and three of these (at positions 1, 2 and 3) were true positives; as the correct predictions were within the threshold of the cut-off point of 5 the precision was calculated as 3 out of 5 (not 3 out of 11) this gives a more favourable value for prediction. However, on some occasions, if the total number of predictions was lower than 5 and true positives were found, the value for precision appears lower. Limiting the number of metabolites in the output allows more permissive settings to be used (such as EQU2 or EQU3) where even hundreds of predictions can be provided. As most true positives concentrate within the first 15 or 20 predicted metabolites for the compounds considered here, it is reasonable to include equivocal reasoning as it improves sensitivity. For instance, when Meteor is used at EQU3 with the number of metabolites set at default value of 400, the sensitivity is 85.2% whilst the precision equals 3.4%. If a cut-off point of 25 is selected using the same reasoning engine, the sensitivity drops to 78.2% but the precision increases threefold to 10.7%. Although the obtained precision values are not very high in absolute terms, their significant increase with only a slight drop in sensitivity can still be valuable

when computational time is considered. For example where identified metabolites are subsequently assessed for toxicity (e.g. using Derek Nexus (Lhasa Limited), or other predictive software); the significant reduction in metabolite numbers is beneficial not only in the time to generate the metabolite information but also in their subsequent processing. Figures 3.5 and 3.6 show the improvement in precision where lower cut-off values are used.

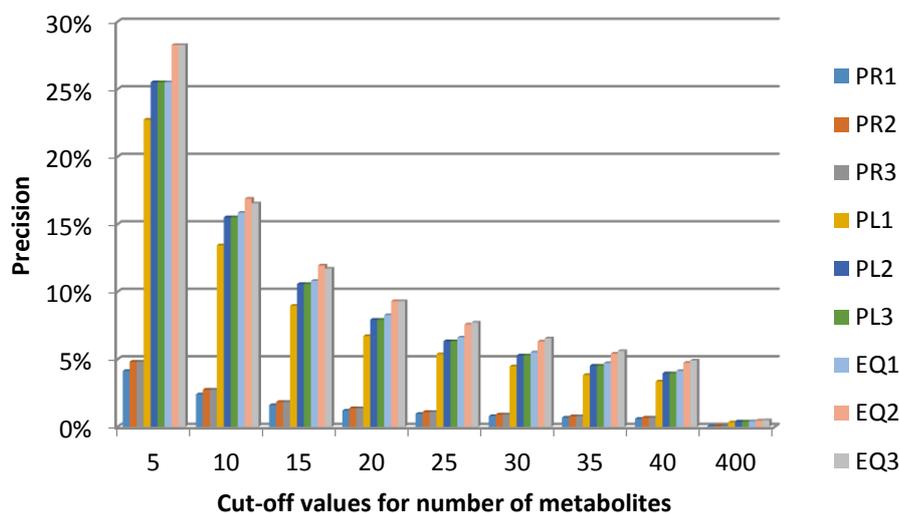


Figure 3.5. Percentage precision for DS1 as a function of cut-off values.

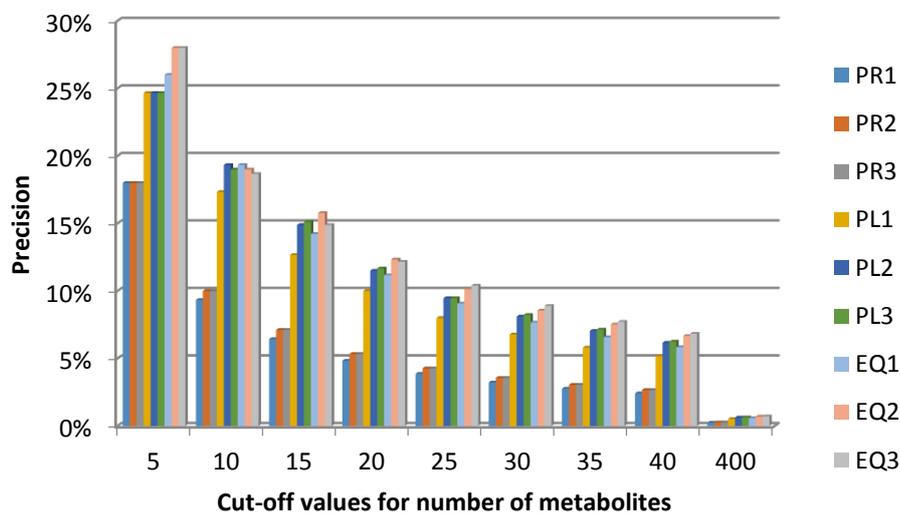


Figure 3.6. Percentage precision for DS2 as a function of cut-off values.

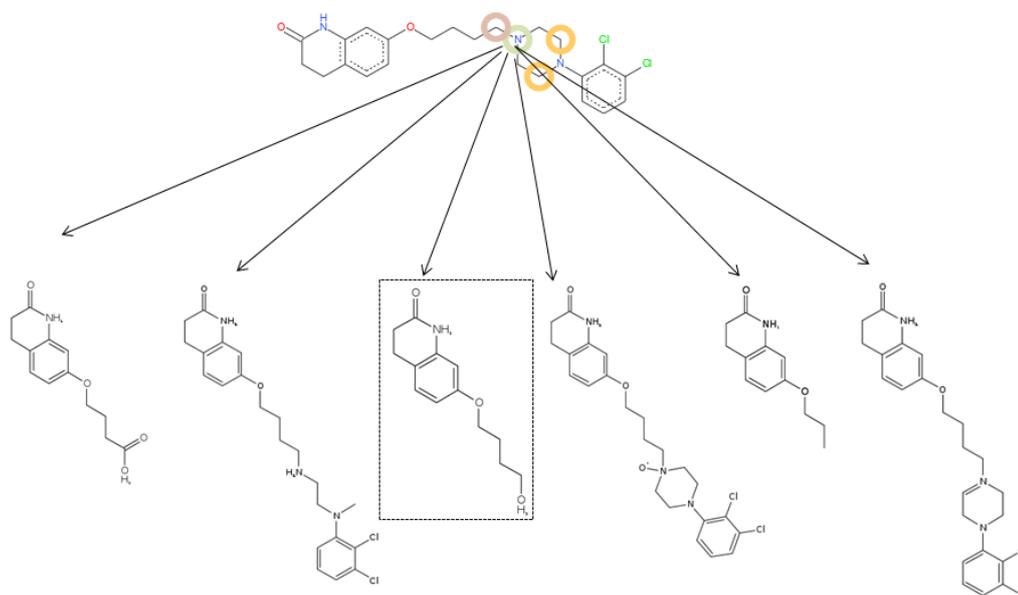
The results from this study show that, pragmatically, cut-off values in the region of 20 – 30 give a reasonable balance between sensitivity and specificity. The use of low filter settings with relatively low cut-off values is further supported by the fact that Meteor absolute reasoning categories are not directly correlated with the abundance of metabolites observed *in vivo* (Tjollyn et al., 2011). Meteor does not indicate which prediction is ‘more important’ therefore the selection of settings to enhance predictive power and adjustment of threshold points to levels that best suit the user is warranted.

3.3.3. Combining SMARTCyp and MetaPrint2D-React

SMARTCyp and MetaPrint2D can both be used to indicate SOM (site of metabolism) however, in this study SMARTCyp (not MetaPrint2D) was combined with MetaPrint2DReact for the prediction of metabolites. The rationale behind selecting a combination of these two approaches is that the SMARTCyp method, in contrast to MetaPrint2D, does not depend on historical reaction data. SMARTCyp utilises pre-calculated energies for a number of sub-fragments and as such can be described as an empirical approach. One of the benefits of such a method is that it could be applied with greater confidence to predict SOM for molecules that do not share substantial structural similarity with compounds found in historical databases. Therefore, such predictions would be less biased (towards already existing data) providing that the array of sub-fragments is large enough to cover a variety of structures. On the other hand, SMARTCyp does not provide structures of metabolites, which can be required by some users. Hence, MetaPrint2D-React was used to fill this gap. It should be noted that although accurate prediction of SOM presents a greater challenge than providing putative structures of the metabolites associated with a reaction at a given site, producing definitive structures is advantageous for further processing, e.g. predicting toxicity of identified metabolites. A combination of SMARTCyp and MetaPrint2D-React could be implemented to produce a metabolic tree, i.e. MetaPrint2D-React could be used to obtain structures of metabolites for SOM predicted by SMARTCyp. An example of such a tree for Aripiprazole is presented in Figure 3.7.

Investigation of the literature had revealed that 39 of the primary metabolites for 17 drugs from DS2 were known to be a product of CYP3A4 metabolism. Similarly, CYP2D6 was responsible for the formation of 11 of the primary metabolites for six of the drugs in DS2. SMARTCyp was used to determine the top three and top five sites of metabolism for these drugs. These drugs were then entered into MetaPrint2D-React and metabolites corresponding to the SOM identified using SMARTCyp were determined, according to the scheme shown in Figure 3.1, using the models for CYP3A4 and CYP2D6.

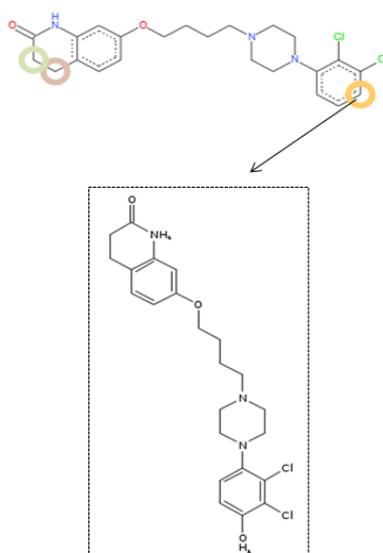
SMARTCyp 3A4 model



MetaPrint2D-React structures predictions

(A)

SMARTCyp 2D6 model



MetaPrint2D-React structures predictions

(B)

Figure 3.7 (A) A simulation of a metabolic tree using a combination of SMARTCyp CYP3A4 and (B) CYP2D6 models and MetaPrint2D-React. The circles (present here in SMARTCyp output screenshots) indicate the top ranked SOM (colour code: yellow = rank 1, brown = rank 2, green = rank 3). One SOM for each model was selected to obtain possible structures from MetaPrint2D-React. The metabolites (shown in the boxes with dotted lines) are those that correspond to known *in vivo* metabolites. Please note that the parent structure with circled SOM as well as the structures of metabolites are screenshots of the original outputs from SMARTCyp and MetaPrint2D-React, respectively.

For aripiprazole and quetiapine, both CYP3A4 and CYP2D6 were involved in metabolism; in these cases the corresponding models were explored in SMARTCyp. Analysis of the predictions obtained using this combination of methods indicates that the CYP2D6 model performed better than the CYP3A4 model. The CYP2D6 model predicted 91% of metabolites correctly when the top five ranking SOM were considered and predicted 73% of metabolites when only the top three SOM were taken into account. For CYP3A4 the model predicted 56% of metabolites correctly when the top five SOM were selected and 44% correctly when the top three SOM were considered as shown in Figure 3.8.

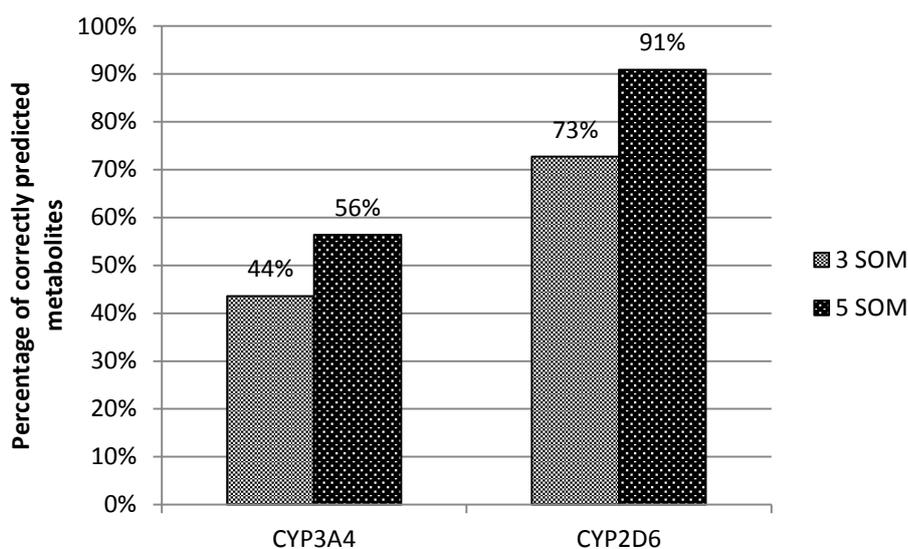


Figure 3.8. The percentage of known metabolites that were correctly predicted using a combination of SMARTCyp and MetaPrint2D-React using three (3 SOM) or five (5 SOM) sites of metabolism associated with CYP3A4 and CYP2D6. Note: The number of known metabolites in DS2 that were identified to be products of cytochrome CYP3A4 and CYP2D6 were 39 and 11 respectively.

It should be noted that these percentages were calculated by taking into account all of the experimentally observed metabolites. Rydberg et al., (2010) used a different approach wherein they calculated the percentage of compounds for which SMARTCyp was able to correctly predict at least one metabolic site among the top-ranked atoms. They found that for a dataset of 394 compounds, 81% of these had a correct SOM among the top three ranked SOM. Applying the method of Rydberg et al. (2010) to the dataset of 17 drugs, known to produce 39 metabolites via CYP3A4, resulted in correct predictions for 76% of the compounds i.e. for 13 of the 17 parent drugs SMARTCyp identified a correct SOM within the top three ranked SOM. In a similar analysis Rydberg and Olsen (2012) showed that for a dataset of 45 compounds, known to be metabolised by CYP2D6, 91% of the structures were found to have a metabolic site among the top-three atoms. In the dataset studied here, six drugs are known to be metabolised by CYP2D6 and of these the metabolites associated

with the top three ranked SOM were correctly identified in 83% of cases. Although smaller datasets were analysed here than those studied by Rydberg et al. (2010) and Rydberg and Olsen (2012) the results support the usefulness of the SMARTCyp tool.

SMARTCyp's use of pre-calculated energies for sub-fragments allows very fast processing in the prediction of SOM which is of great value when applied to large scale screening in drug discovery. Furthermore, the program has recently been enriched by including three additional cytochrome models: CYP2C9, CYP2C19 and CYP1A2 so that predictions can be made for five major cytochromes involved in drug metabolism. An evaluation of the latest three models (Liu et al., 2012) confirmed that the accuracy of SMARTCyp in terms of identifying metabolic hot spots is comparable or even better than that of the state-of-the-art software based on 3D structure-based methods such as Metasite and StarDrop. The benefits of using SMARTCyp in conjunction with other programs to predict metabolism, as a filter to identify most likely SOM, and therefore reduce number of false positives is another pragmatic approach that warrants further research. Development of a method that allows discrimination between different models is in progress in collaboration with Lhasa Ltd (Lhasa Limited, 2012); this directs a user in terms of identification of major contributors to metabolism for a query compound.

In conclusion, within this study the overall performance of selected computational methods (Meteor, SMARTCyp, MetaPrint2D and MetaPrint2-React) in predicting metabolites or sites of metabolism has been assessed. The results indicate relative performance and highlight areas where improvements may be made in the software (or in use of the software); the results do not indicate true predictive performance as in many cases the compounds studied here may have been included within the training set for the programs.

The results show that both Meteor and MetaPrint2D-React performed well in predicting metabolites for both homogenous (DS1) and heterogenous (DS2) datasets. Of particular interest is that all packages performed better in predicting metabolites of DS2 rather than DS1. This suggests that there may be specific areas of chemical space that are less populated within the data used to train the models. This study has identified structural types for which predictions could be improved if metabolite data for these compounds (such as bromfenac) were incorporated.

The combination of the empirically-based SMARTCyp program, for the prediction of SOM, with software for predicting metabolites also offers a pragmatic approach to directing the results of metabolite prediction to those metabolites that are most likely to be formed.

A significant part of the research undertaken here was to determine a pragmatic approach to using the Meteor software. One problem identified with this package is its tendency to over-predict metabolites. The approach used here, i.e. using permissive settings such as EQU3, but incorporating cut-offs for the number of metabolites investigated shows that good precision can be attained with much reduced computational effort and simplification of output. As the importance of metabolites and their potential to elicit toxicity is becoming increasingly recognised, tools that can aid the rational prediction of such metabolites, such as the methods proposed here, will be of benefit.

The endpoints considered in this thesis thus far (absorption/bioavailability and metabolism) relate to only one aspect of drug development i.e. investigation of internal exposure of drug candidates. Another important aspect to consider is the potential toxicity elicited by drug candidates, hence the subsequent chapters relate to prediction of toxicity. Clearly prediction of toxicity is a very broad topic, therefore only a few specific examples, of particular relevance to drug design, have been selected for investigation.

4. Development of Models to Predict hERG Channel Inhibition

4.1. Introduction

An important challenge in drug design is to predict potential off-target adverse reactions. Efficacy cannot be the only criterion for a lead compound; toxic effects must also be considered, including those that are not related to the original activity. The hERG (human Ether-à-go-go-Related Gene) potassium channel is known to be involved in off-target adverse reactions related to cardiotoxicity. Inhibition of the hERG channel has been linked to life-threatening arrhythmias (Sanquinetti, Tristani-Firouzi, 2006). The potassium channel is responsible for producing the delayed rectifier current (I_K). This outward current is responsible for the process of membrane repolarisation. Efflux of potassium ions (positive current) causes the loss of positive charge from the cell which allows the membrane to return to the resting state (around -80mV). A decreased function of the hERG channel can manifest as QT interval prolongation (observable on electrocardiograms) which is the result of reduced repolarisation. Lengthening of the QT interval is a risk factor for the occurrence of Torsade de Pointes (TdP), a polymorphic ventricular tachycardia. Different drug classes such as antiarrhythmics, microbial agents or antihistamines have been shown to bind to, and inhibit, the hERG channel. Consequently, many previously approved drugs have either been withdrawn from the market (e.g. cisapride or grepafloxacin) or their use has been limited (e.g. sertindole, pimozone). This has led to obligatory drug screening for hERG1 inhibition by both the United States Food and Drug Administration and the European Medicines Agency (Durgardi, 2014).

4.1.1. Data sources

Many data used in *in silico* modelling are obtained from *in vitro* experiments. The voltage clamp electrophysiological assay is a technique commonly applied to mammalian cells (chinese hamster ovary (CHO) cells, fibroblast-like cells (COS) or human embryonic kidney (HEK) 293 cells) transfected with a gene coding for the hERG channel (Aronov, 2005). Non-mammalian cells such as *Xenopus laevis* oocytes are also used to study hERG inhibition. However, using these cells may lead to underestimation of IC₅₀ (half maximal inhibitory concentration) as a result of reduced accessibility of the hERG channel due to the highly lipophilic environment in *Xenopus laevis* oocytes (Netzer et al, 2002). Therefore, data used for modelling hERG channel inhibition should originate preferably from

mammalian cell lines or, if pIC50 values from non-mammalian sources are used, these should be modelled separately. Other *in vitro* methods include the rubidium efflux assay, radioligand binding assays (RBD) and fluorescence-based assays (Polak, Wisniowska & Brandys, 2008). In recent years, advances in cell preparation techniques and automation of patch clamp assays has enabled higher throughput hERG screening (Danker & Moller, 2014). *In vivo* tests are usually carried out on rodents and, less frequently, on dogs.

The advances in experimental *in vitro* methods have contributed to the accumulation of larger datasets of hERG inhibition values. Doddareddy et al., (2010) published a hERG dataset for 2644 compounds from more than 250 publications. However, this collection may require further curation due to issues such as the presence of duplicates. Other large datasets are also available (Wang et al. (2012) reports 806 compounds; Broccatelli et al. (2012) reports 1173 compounds). These collections were also mostly compiled from scientific publications or publically available databases such as WOMBAT-PK (<http://www.sunsetmolecular.com>), ChEMBL (<https://www.ebi.ac.uk/chembl/>) and Tox-Portal (<http://www.tox-portal.net/index.html>).

4.1.2. QSAR approaches to modelling hERG inhibition

HERG channel inhibition has been modelled by various regression or classification techniques. Regression models aim to predict absolute values of activity/inhibition. Multiple linear regression (MLR) is one of the traditional methods used in developing quantitative structure-activity relationships (QSARs). MLR was used by Aptula & Cronin, (2004) to model hERG inhibition for a set of 19 compounds. The model obtained showed that molecular size and lipophilicity may influence hERG inhibition. Keseru, (2003) developed a stepwise linear regression analysis model which included five descriptors: calculated logarithm of the octanol:water partition coefficient (cLogP), molar refractivity, partial negative surface area and two Volsurf-type descriptors (training set, n = 55). Coi et al, (2006) used a set of CODESSA descriptors to perform regression analysis. Another regression model was built by Yoshida & Niwa, (2006; n = 104). They utilised easily interpretable descriptors (calculated using the Molecular Operating Environment (MOE) software) such as log P, topological polar surface area (TPSA), diameter and sum of the van der Waals surface area of atoms. The correlation coefficients (R^2) for the different models generated ranged from 0.71 to 0.94.

Classification methods attempt to predict a class (for instance, active/inactive) for data points rather than indicate an absolute activity value. Many classification models have been developed for hERG inhibition. In 2002, Roche et al., used a variety of machine learning techniques such as: self-organising maps, principal component analysis (PCA), partial least squares (PLS) and supervised

neural networks (training set n = 244; validation set n = 72). Their best model (neural network) had an accuracy of 71% and 93% for blockers and non-blockers, respectively. Other approaches used include applying genetic programming to fragment-based descriptors (Bains, Basman & White, 2004) and using genetic algorithm classifiers based on molecular similarity using topomers (Nisius & Goller, 2009). Atom-type descriptors, calculated for a dataset of 977 compounds, enabled a good classifier to be developed using support vector machine (SVM) (accuracy = 94%, test set n=66) (Jia & Sun, 2008). Molecular fingerprint-based descriptors were successfully used for modelling large datasets. For instance, Doddareddy et al. (2010) constructed SVM and linear discriminant analysis models using extended-connectivity fingerprints (training set n = 2389). One of their SVM models achieved an accuracy of 88% for the test set of 255 compounds. Wang et al., (2012) utilised molecular fingerprints to obtain a binary hERG classification model using a naïve Bayesian classifier and recursive partitioning (training set n = 620). They obtained 85% accuracy for their test set (n = 120), in addition to 89.4% accuracy for an external test set from WOMBAT-PK (n = 66) and 87.3% accuracy for another external test set derived from the PubChem database (n = 1953).

In addition to the ligand-based approaches there are also binding studies such as molecular docking. However, as the crystal structure of hERG is not available these techniques have to rely on homology models. Unfortunately, the sequence similarity of the hERG channel and available templates is low which limits the potential to provide accurate predictions (Wang, et al., 2013).

4.1.3. hERG channel structure

The hERG channel is a membrane protein, many of which have proven to be challenging subjects for crystallography, mainly due to issues related to their solubility, purity and stability (Carpenter et al, 2008). Although a crystal structure of the hERG channel has not yet been established, some studies on mammalian Shaker (Kv1.1,) or bacterial (KcsA, TthK and KvAPThe hERG) potassium channels have provided considerable structural information about the hERG channel. The hERG channel comprises four identical subunits (homotetramer). Each of the subunits is composed of six-transmembrane segments (S1-S6) that are helices. Domains S1-S4 form a voltage sensing functional subunit while S5 and S6 helices (linked by a selectivity filter and a smaller helix) constitute the pore domain (Sanguinetti & Tristani-Firouzi, 2006) (see Figure 4.1A). The promiscuity of hERG binding may be related to two factors. Firstly, it possesses a large central cavity in the pore domain where drug binding occurs. Drugs enter the pore domain from the intracellular side and bind to the cavity, hence the channel has to be in an open (or inactivated) conformation so that a drug can have access to the cavity (see Figure 4.1.B).

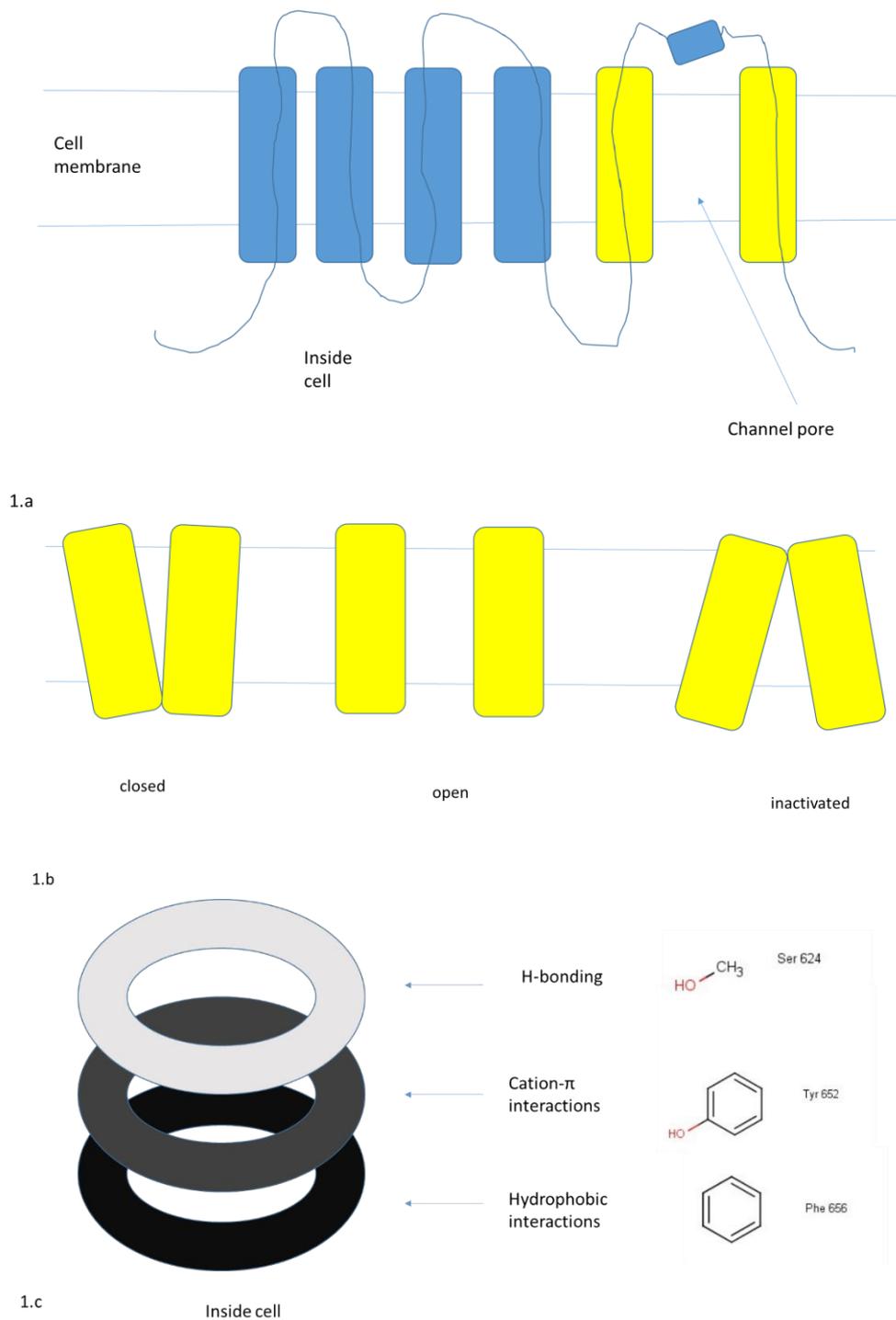


Figure 4.1. a) schematic representation of the hERG channel. Blue colour represents S1-S4 alpha-helices while yellow is for the S-5 and S-6 domains (also alpha-helices); b) shows the three conformations of the hERG channel. c) shows a schematic representation of regions of the pore that are important for drug binding

Another factor is the presence of specific amino acids in the pore region. Mutagenesis studies have identified a number of amino acids that may play an important role in stabilisation of drug binding to the hERG channel. The potentially important residues are: Phe 656 (hydrophobic interactions), Tyr

652 (cation- π interactions) or Thr 623 and Ser 624 (which may be involved in hydrogen bonding). The concentric rings that are formed by the side chains of these specific amino acids may constitute a characteristic topology of the channel pore and partially explain why structurally diverse compounds can bind to the channel (see Figure 4.1C).

4.1.4. hERG pharmacophore models

A number of pharmacophore models for hERG inhibition have also been developed. Ekins et al, (2002) proposed a five-point pharmacophore model developed using a set of 15 compounds. This model included four hydrophobic features and one positive ionisable centre. A similar model was developed by Cavalli et al, (2002). The CoMFA analysis of 31 hERG channel blockers led to identification of four features: three aromatic moieties (hydrophobic) and a tertiary amine that possesses a nitrogen atom that is protonated at physiological conditions (pH7.4). Du et al, (2004) developed a pharmacophore (for 34 Class III antiarrhythmic agents) that consisted of a positive ionisable feature, two aromatic rings and a hydrophobic moiety. The pharmacophore model developed by Aronov & Goldman (2004) indicated the presence of a hydrogen bond acceptor, separated from a protonated nitrogen and an aromatic ring. A pharmacophore model for neutral hERG blockers ($n = 194$), i.e. those that do not contain a basic nitrogen, has also been reported (Aronov, 2006). This six-point model included three hydrogen bond acceptors and three hydrophobes/aromatic moieties. Tan et al. (2012) have suggested that a single rigid pharmacophore model may not be sufficient due to the flexible nature of the homotetramer. They developed an ensemble 3D-QSAR model that could account for a number of interaction modes. Despite differences between pharmacophore models, four elements appear to be important: i) between two and four hydrophobic/aromatic features that take part in hydrophobic and/or π - π stacking interactions with Phe 656 and Tyr 652 residues in the hERG binding pocket. ii) a basic centre (protonated nitrogen) that can be involved in π -stacking interactions with Tyr 652. iii) hydrogen bond acceptors - in case of neutral molecules and iv) ligand flexibility (Wang et al., 2013).

The aim of this study was to develop models for the prediction of hERG inhibition for a dataset of 244 compounds with known pIC50 values. In particular, the research included:

- i. An evaluation of an existing regression model (Aptula & Cronin, 2004) using a larger dataset. This was to determine whether simple medicinal-chemistry type descriptors, such as log D or molecular diameter, could be used to predict hERG inhibition for a larger and more chemically-diverse dataset.

- ii. Development of new QSAR models for hERG inhibition using different approaches such as, multiple linear regression and binary classification, using a range of machine learning methods (logistic regression, naïve Bayes, k-nearest neighbour, support vector machine and random forest).
- iii. Development of structural alerts for hERG blockers using categories of compounds grouped according to 2-D similarity. Such alerts can be coded as SMARTS patterns enabling rapid screening of large datasets in drug discovery.

4.2 Methods

4.2.1. Dataset

The dataset used in this analysis was taken from (Nisius & Goller, 2009) and is subsequently referred to as H_244. The authors compiled their dataset from scientific publications (32 references in total). The following information was available from their supporting material:

- i) A table containing 681 hERG patch-clamp measurements on mammalian cell lines (HEK, CHO, COS) expressed as pIC50 values. Duplicate compounds with corresponding pIC50 values were present.
- ii) An sdf file containing structures of 275 compounds. Additionally, this file also included information about pIC50 classes to which the 275 compounds belonged; the classes were used by the authors to distinguish between active and inactive: compounds with $pIC50 \leq 5$ were considered as inactive, compounds with $pIC50 > 6$ were considered as active, and compounds with $5 < pIC50 \leq 6$ were considered as medium active.

The dataset was subjected to a “cleaning” process. First of all, in cases where there were multiple pIC50 values for a compound the mean values were calculated. This procedure was performed only when the difference between the highest and lowest values was not > 1.5 log units. This threshold value was chosen arbitrarily to account for such factors as experimental errors. On occasions where one of the multiple measurements was a clear outlier such a value was removed and the mean pIC50 value was calculated from the remaining measurements.

Overall, after applying the above procedures, 244 compounds were present in the final dataset (H_244) (see also Appendix VIII); these were stored in a Microsoft Office 2013 Excel file along with the following information: identification number, SMILES string, chemical name, InChIKey, an

endpoint value (pIC50) and toxicity class (active, medium-active, inactive). SMILES strings and InChIKeys were generated by an automatic conversion (using OpenBabel, ver. 2.3.1) of the available sdf file.

The H_244 dataset was also stored in the following formats: sdf, SMILES (in OpenBabel, ver. 2.3.1) and xyz (using Chemaxon Marvin View, ver. 14.11.23) for prospective processing by software packages.

4.2.2. Generation of molecular descriptors

A number of software packages were used to calculate molecular descriptors:

- Molecular Operating Environment (MOE; version 2010.10, Chemical Computing Group) software was used to generate 334 descriptors (both 2 and 3- dimensional); structures were optimised using the AM1 Hamiltonian.
- ACDLabs (version 9.0, Advanced Chemistry Development) software was used to calculate log D values at five different pHs (2, 5.5, 6.5, 7.4 and 10).
- HYBOT software (Molecular Properties Optimisation Project) was used to generate descriptors related to H-bond thermodynamics
- MOPAC2012 (Molecular Orbital Package) software (applying AM1) was used to obtain the maximum diameter (Dmax) descriptor. The maximum diameter values had to be extracted from the MOPAC output and to facilitate this process a perl script was written to automatically identify the maximum value present in the matrix of interatomic distances (from the MOPAC results table). Another perl script was also written to separate the previously obtained batch xyz file into separate files to fulfil MOPAC input requirements. Calculations were performed for one compound at a time.

A total of 458 descriptors was obtained from the software packages and recorded in the form of a spreadsheet (Microsoft Excel 2013). The data were subject to further processing to enable subsequent analyses in the statistical software packages (Minitab and Weka). Columns containing empty cells (missing descriptor calculations), or strings / descriptors for which a limited range of values was present were removed. Consequently, the number of descriptors constituting the dataset was reduced to 365. The full dataset of compound names, identifiers and descriptor values are given in Appendix IX.

4.2.3. Similarity search

2-D similarity searching was performed using the Tanimoto distance similarity method in the ToxMatch software (v. 1.07). Scripts in R and perl were written to extract automatically groups of similar compounds according to pre-determined cut-off values (these were selected as similarity scores of 0.6, 0.7, 0.8 and 0.9).

4.2.4. Field points analysis

Five compounds that were determined during this investigation to belong to category I (refer to section 4.3.2; Table 4.2) were analysed using Activity Miner present in the Torch software (ver. 10.3, Cresset) to determine the influence of 3-D features on their molecular fields. SMILES strings and pIC50 values for the five compounds were provided in spreadsheet format (csv).

4.2.5. Statistical analysis

Two software packages were used for the development of hERG inhibition models:

- Minitab (ver. 17.0) was used to perform MLR analysis and to build QSAR models for hERG inhibition.
- Weka (ver. 3.6) machine learning and data mining software was used to obtain classification modes for the H_244 dataset. Logistic regression, naïve Bayes, k-nearest neighbour (IBk classifier), support vector classifier (SMO – sequential minimal optimization) and random forest were among the algorithms applied. The input for Weka was provided in a csv format and it contained pIC50 values, toxicity classes and previously calculated descriptors. 10-fold cross-validation was used to test the obtained models. The process of cross-validation was performed automatically by Weka. The dataset was divided into 10 parts (folds) and then each fold was used as test set (exactly once) whilst the rest of the data (nine folds) was used to train a particular model. Finally, the performance of the models was averaged and the estimate of accuracy of each classifier was reported.

4.2.6. Structural alerts development

- Structural features and fragments associated with hERG channel inhibition were captured using SMARTS (SMiles ARbitrary Target Specification) strings. SMARTSEditor (ver. 0.9.0.) was used to develop SMARTS (Schomburg, Wetzer, Rarey, 2013) and SMARTSviewer (ver. 0.9.0.) was utilised for visualisation of SMARTS (Schomburg et al, 2010). Both these software

packages were developed by the Center for Bioinformatics Hamburg (www.zbh.uni-hamburg.de).

- OpenBabel (ver.2.3.1.) was used to identify the fragments described by the SMARTS strings in the H_244 dataset.

4.3. Results and discussion

4.3.1. Global models for predicting hERG inhibition

Different strategies can be utilised to build models for hERG inhibition. The dataset in this study comprised hERG channel inhibition values (pIC50) so it was possible to use regression techniques in order to establish any potential relationship between pIC50 values and the calculated descriptors. hERG inhibition values can also be categorised into three different classes with respect to their biological activity. Classes associated with (in)activity have previously been reported by Nisius & Geller, 2009) who allocated compounds to classes based on the following criteria: $pIC50 \leq 5$ = inactive; $5 < pIC50 \leq 6$ = medium active and; $pIC50 > 6$ = active. Presence of nominal activity class (e.g. active/inactive) can be used to apply machine learning techniques, for instance, those that can perform binary classification.

4.3.1.1. Evaluation of the model of Aptula and Cronin (2004) using $\log D$ and D_{max} descriptors to predict hERG inhibition

Initially, an attempt was made to build a simple model based on $\log D$ (pH = 7.4) and D_{max} . These descriptors were used previously to model successfully a set of 19 compounds (Aptula & Cronin, 2004). The dataset studied here (H_244) is much larger and more chemically-diverse and therefore provided the opportunity to test whether the existing model was broadly applicable to a wide range of compounds. 17 of the 19 compounds from the Aptula and Cronin dataset were also present in H_244. Figure 4.2 shows the predictions for the H_244 dataset based on Aptula and Cronin's model (Eq. 4.1).

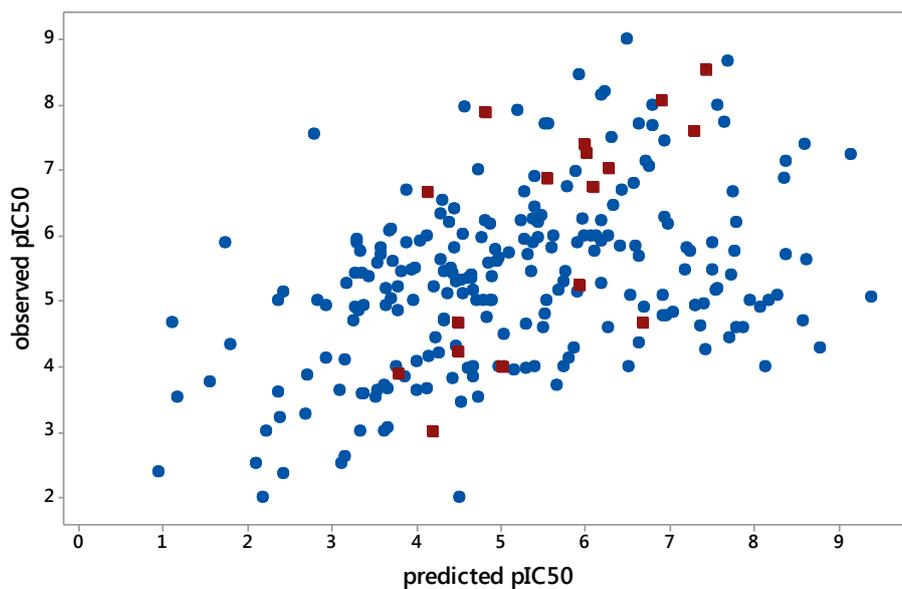


Figure 4.2. A plot of observed pIC_{50} values versus those predicted using Aptula and Cronin's model. Data points that were present in both H_244 and Aptula and Cronin's dataset are depicted as red squares. Eq.4.1. was used to obtain predictions.

$$pIC_{50} = 0.58 \log D + 0.3 D_{max} - 0.36 \quad (\text{Eq. 4.1})$$

$$n = 19, R^2 = 0.87, S = 0.73$$

The poor correlation between predicted and observed values in this study indicates that $\log D$ and D_{max} descriptors are not sufficient to model activity within the H_244 dataset. One reason for using these descriptors is their transparency, hence interpretability of the model; such descriptors are more easily rationalised. $\log D$ describes hydrophobic properties (in a holistic way) at a specified value of pH, for instance at physiological pH, and as such it can be useful for the prediction of a compound's fate in the organism, including whether it can reach the target and potentially bind to it. As hydrophobic interactions play an important role in ligand-binding to the hERG channel pocket (e.g. π -stacking interactions) it would be expected that hydrophobicity descriptors would be useful in modelling. D_{max} could also be potentially important in determining whether a compound can fit into the binding pocket and then be positioned relative to crucial side chains of amino acids so that the binding is strong enough to reduce activity of the hERG channel. However, the good correlation obtained by Aptula's model ($R^2 = 0.87$) was not replicated in this larger H_244 dataset; this may be due to many of these compounds falling outside of the applicability domain of the original model and it could also be a result of using different versions of software (e.g. MOPAC2012 was used in this study to calculate the maximum diameter descriptor). Other issues could also be important such as the complexity of the interactions at the hERG channel. Ligands can bind to various conformations of

the hERG channel and some of these states (inactivated) can even promote high affinity binding (Perrin et al, 2008). A broad spectrum of compounds can potentially inhibit the hERG channel and this indicates a lack of binding specificity, hence finite predictions of binding affinity are difficult.

4.3.1.2. Testing maximum diameter and log D rules for H_244

In addition to the regression model, Aptula & Cronin (2004) identified broad rules regarding maximum diameter and log D in the context of hERG inhibition. The authors proposed that: i) a log D > 3.5 could be an indicator of strong hERG channel binding and ii) compounds with maximum diameter (D_{max}) > 18 Å (not applicable to antipsychotics) and log D_{7.4} > 2.5 may prolong the QT interval. Figures 4.3A and 4.3B show the distribution of active, medium-active and inactive compounds in relation to these cut-off values for D_{max} and log D. For this analysis compounds from H_244 that were also present in the original Aptula and Cronin dataset were omitted.

It is clear from the below plots that the second of the above rules cannot be extrapolated to the dataset studied here as many inactive and medium-active compounds had log D > 3.5. Similarly, using the criterion of maximum diameter > 18 did not seem to discriminate between active and inactive compounds. However, utilising the second rule that combined both maximum diameter and log D, resulted in identification of 19 compounds that were mostly active or medium active (data not shown). Five compounds were inactive with the lowest pIC₅₀ value being around 4 log units. On the other hand, less than a quarter of most active blockers (pIC₅₀ > 6) were not identified using this rule, which puts in question its practical application.

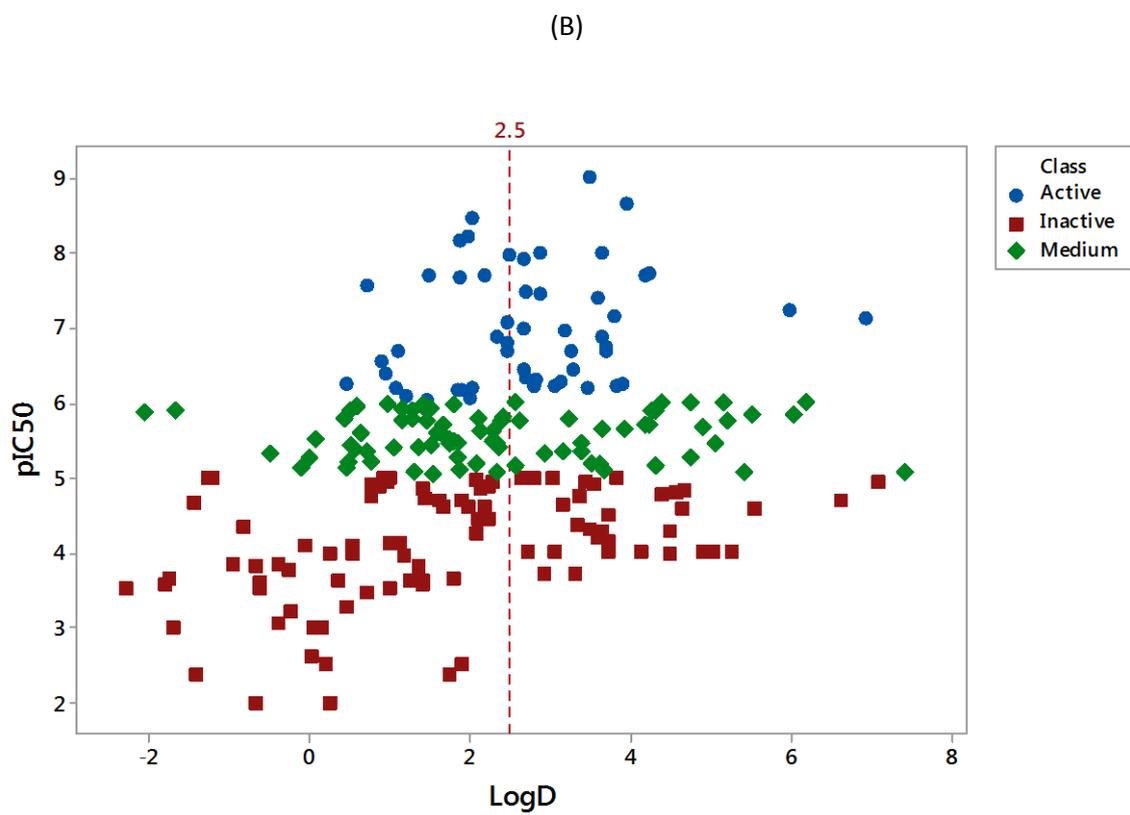
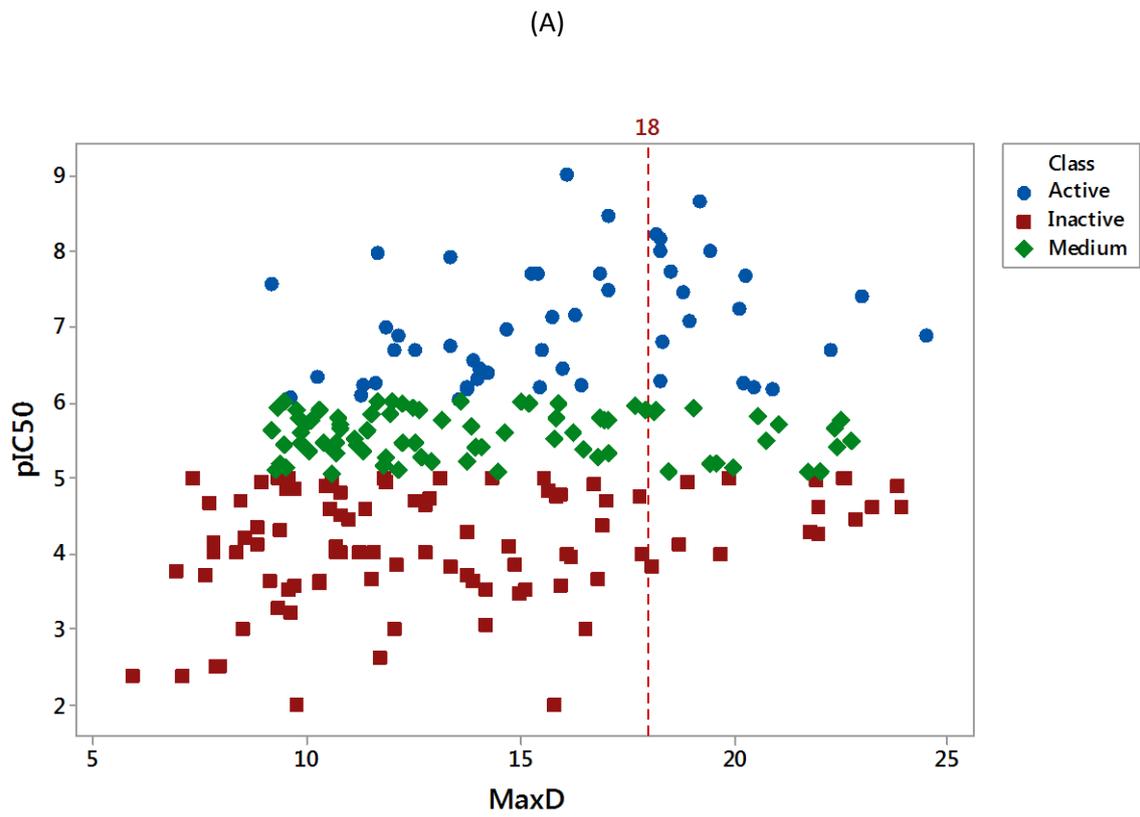


Figure 4.3. (A) a scatterplot of maximum diameter vs pIC50 values. (B) a scatterplot of log D vs pIC50 values.

4.3.1.3. Global regression model

As modelling the hERG inhibition using log P and Dmax descriptors did not yield a good correlation an attempt was made to identify the most relevant descriptors, for modelling H₂₄₄ data, from a large pool of descriptors that had been calculated previously (i.e. 364 descriptors generated as detailed in section 4.2.2). Initially, all descriptors were employed in an attempt to obtain a linear regression model for the 244 compounds. It was difficult to obtain a model with a correlation coefficient greater than 0.5 without an unrealistically high number of terms in the equation. Although Weka software eliminates collinear descriptors it was possible that some descriptors that were more meaningful in terms of interpretability were removed and more “obscure” ones were included in the model. An additional issue is that linear regression methods may not be the best choice for modelling datasets where number of instances is lower than the number of descriptors. Therefore, it was decided to select only a small subset of descriptors (from those that were calculated by ACD) that were expected to be most relevant in the context of binding ligands to the hERG S5 and S6 subunits. Selected descriptors were related to hydrophobicity (log P), H-bond formation capability (potentially important in stabilising interactions between a ligand and side chains of amino acids within the binding pocket) and constitutional descriptors, for instance the number of aromatic rings (potentially important π -stacking interactions with aromatic side chains of amino acids). From this subset, three descriptors were automatically selected by Weka as most relevant for further processing. Selecting these descriptors resulted in the following global model being generated using MLR (as shown in Eq. 4.2).

$$pIC50 = 0.21 \log P + 0.43 \text{ No_arom} + 0.23 \text{ No_rings_6} + 3.15 \quad (\text{Eq. 4.2})$$

$$n = 244, R^2 = 0.52, S = 1.14$$

Where No_{arom} = number of aromatic rings; No_{rings_6} = number of six-membered rings

The above equation indicates limited correlation between the selected descriptors and hERG inhibition, however as a good correlation was not obtained using the MLR approach alternative methods were investigated.

4.3.1.4. Binary classification models

The possibility of building models to enable binary classification of active versus inactive compounds was investigated. Compounds from the H₂₄₄ dataset, as previously mentioned, had been classified as active, inactive or medium-active. This classification (as shown in Figure 4.4) was simplified for

machine learning techniques purposes so that only two classes were present: inactive and active. Compounds in the “medium-active” category were placed into the “active” category. This resulted in 143 compounds now being classed as “active” and 101 compounds being classed as “inactive”.

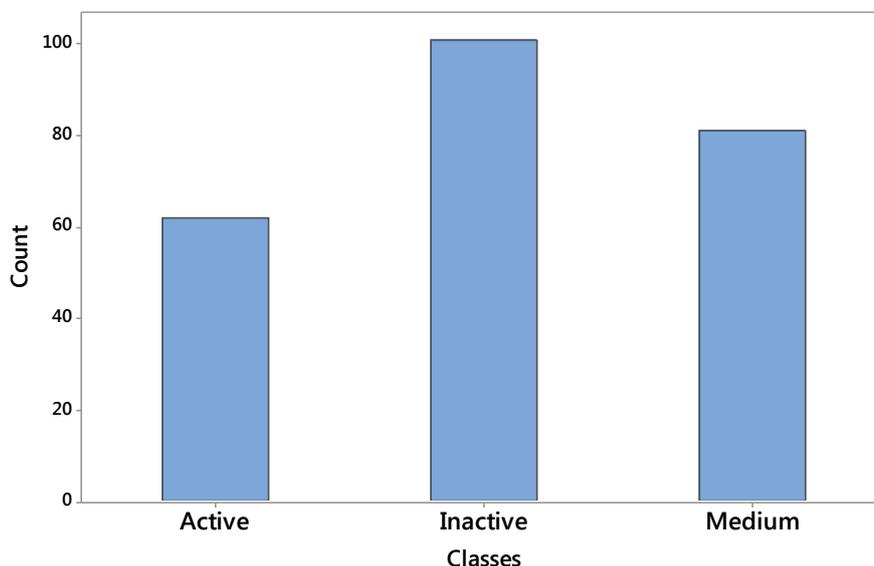


Figure 4.4. Three classes of compounds from the hERG_244 dataset according to their potency to block the hERG channel. No. actives = 62; no. inactives = 101; no. medium-actives = 81.

A number of classification techniques were then applied to attempt to distinguish between the active and inactive classes. The results of these analyses are shown in Table 5.1. All models were tested using 10-fold cross-validation. Note that within the table the following calculations have been applied:

$$\text{Recall} = \text{TP}/(\text{TP}+\text{FN}) \quad (\text{Eq. 4.3})$$

$$\text{Precision} = \text{TP}/(\text{TP}+\text{FP}) \quad (\text{Eq. 4.4})$$

$$\text{FP Rate} = \text{FP}/(\text{FP} + \text{TN}) \quad (\text{Eq. 4.5})$$

$$\text{F-Measure} = 2(\text{precision}*\text{recall})/(\text{Precision}+\text{Recall}) \quad (\text{Eq. 4.6})$$

Where TP = true positive; FP = false positive, TN = true negative, FN = false negative.

Kappa statistics (a scalar value) are used to indicate the agreement of predicted class with the true class or in other words it assesses the accuracy of the model (Othman & Yau, 2007). The Receiver Operating Characteristic (ROC) curve is used as another performance measure for binary classification. ROC curve is a two-dimensional graph where true positives are a function of false positives. Kappa and ROC curves take into account randomness in estimating accuracy and both these measures have been shown to be closely related concepts (Ben-David, 2008). Kappa and ROC area are expressed as values in the range between 0 and 1. There is no standardised interpretation of kappa and ROC area, however, higher values (approaching 1) are an indication of greater accuracy of a model. On the other hand, a kappa value of 0 and ROC area value of 0.5 suggest that the classification accuracy is comparable to that obtained by chance.

Table 4.1. A summary of the statistics obtained using a selection of hERG binary classification models. The statistical approaches are described in the text.

Classifier	Correctly Classified Instances	Incorrectly Classified Instances	Kappa statistic	Precision	FP Rate	F-Measure	ROC Area
Logistic Regression	61.1%	38.9%	0.16	0.60	0.46	0.59	0.65
Naïve Bayes	71.3%	28.7%	0.38	0.72	0.35	0.70	0.75
k-nearest neighbour (1Bk)	67.2%	32.8%	0.32	0.67	0.35	0.67	0.67
SMO	68.6%	31.2%	0.37	0.70	0.31	0.69	0.73
Random forest	78.3%	21.7%	0.53	0.80	0.28	0.77	0.84

The results presented in Table 4.1 show that the random forest model performed best out of all models tested in terms of accuracy as it was able to correctly classify 78% of instances, with a moderate kappa statistic and relatively high ROC area value. For the analyses presented here, only the performance of the random forest classifier could be considered as acceptable, the other machine learning methods applied to the dataset H_244 showed worse performance with logistic regression the least successful of the methods applied.

4.3.2. Groups based on 2-D similarity

The ToxMatch software (v 1.07) was used to perform a 2-D similarity search on dataset H_244, using the Tanimoto distance similarity method. This analysis revealed that some compounds could be classified into groups on the basis of their structural similarities. Various cut-off values for similarity

for grouping were initially trialled; the value of 0.6 was selected empirically as this enabled categories of suitable chemical diversity to be formed. Seven categories containing five or more chemicals were identified (as shown in Table 4.2). 64 out of 244 compounds were classified into one of the categories. Subsequently, each of these groups was subjected to further modelling.

Table 4.2. The seven categories containing similar compounds identified from the H₂₄₄ dataset using 2-D similarity searching

Category	Id.	Name	pIC50	Class
I	11	2-Amino-N-pyrimidin-4-ylacetamide-1	6.02	Active
	12	2-Amino-N-pyrimidin-4-ylacetamide-2	6.55	Active
	13	2-Amino-N-pyrimidin-4-ylacetamide-3	5.78	Medium
	14	2-Amino-N-pyrimidin-4-ylacetamide-4	5.38	Medium
	15	2-Amino-N-pyrimidin-4-ylacetamide-5	6.19	Active
II	25	AF_3013_(NM-394)	3	Inactive
	95	Ciprofloxacin	3.02	Inactive
	138	Gatifloxacin	3.89	Inactive
	140	Grepafloxacin	4.23	Inactive
	155	Levofloxacin	3.06	Inactive
	158	Lomefloxacin	2.62	Inactive
	182	Moxifloxacin	3.98	Inactive
	214	Prulifloxacin	3.46	Inactive
	228	Sparfloxacin	4.67	Inactive
III	29	Aminomethyl-tetrahydronaphthalene-ketopiperazine_1	5.09	Medium
	30	Aminomethyl-tetrahydronaphthalene-ketopiperazine_2a	5.48	Medium
	31	Aminomethyl-tetrahydronaphthalene-ketopiperazine_2b	5.77	Medium
	32	Aminomethyl-tetrahydronaphthalene-ketopiperazine_2c	5.48	Medium
	33	Aminomethyl-tetrahydronaphthalene-ketopiperazine_2d	5.92	Medium
	34	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4a	5.7	Medium
	35	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4b	5.4	Medium
	36	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4e	4.96	Inactive
	37	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4f	4.89	Inactive
	38	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4g	4.62	Inactive
	39	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4h	4.6	Inactive

	40	Aminomethyl-tetrahydronaphthalene-ketopiperazine_4i	4.6	Inactive
IV	51	Benperidol	8.47	Active
	117	Domperidone	6.79	Active
	121	Droperidol	7.49	Active
	200	Oxatomide	6.45	Active
	207	Pimozide	7.59	Active
V	57	BMCL_03_13_1829-1835_1	7.06	Active
	58	BMCL_03_13_1829-1835_10	8.21	Active
	60	BMCL_03_13_1829-1835_14	6.69	Active
	61	BMCL_03_13_1829-1835_15	7.96	Active
	62	BMCL_03_13_1829-1835_16	4.59	Inactive
	63	BMCL_03_13_1829-1835_17	5.83	Medium
	64	BMCL_03_13_1829-1835_18	5.34	Medium
	65	BMCL_03_13_1829-1835_19	5.71	Medium
	66	BMCL_03_13_1829-1835_2	8	Active
	67	BMCL_03_13_1829-1835_20	4.8	Inactive
	68	BMCL_03_13_1829-1835_21	5.66	Medium
	69	BMCL_03_13_1829-1835_22	5.46	Medium
	70	BMCL_03_13_1829-1835_23	4	Inactive
	71	BMCL_03_13_1829-1835_3	8.15	Active
	72	BMCL_03_13_1829-1835_4	6.24	Active
	73	BMCL_03_13_1829-1835_5	4.12	Inactive
	74	BMCL_03_13_1829-1835_7	6.88	Active
	75	BMCL_03_13_1829-1835_8	7.44	Active
	76	BMCL_03_13_1829-1835_9	4	Inactive
	80	BMCL131829-16	4.59	Inactive
	81	BMCL131829-17	5.83	Medium
	224	Sertindole	8.07	Active
VI	98	Clarithromycin	4.26	Inactive
	129	Erythromycin	3.95	Inactive
	130	Erythromycylamine	3.52	Inactive
	150	Josamycin	3.99	Inactive

	185	N-Demethylethromycin	3.83	Inactive
	221	Roxithromycin	4.44	Inactive
VII	99	Clebopride	6.21	Active
	173	Metoclopramide	5.27	Medium
	181	Mosapride	5.32	Medium
	213	Prucalopride	5.31	Medium
	218	Renzapride	5.7	Medium

4.3.2.1. Relationship between 2-D similarity and pIC50

Each of the categories was investigated in the context of the relationship between their similarity and their pIC50 values. This was to identify whether or not a decreased similarity to a reference molecule (the most potent hERG blocker in each group) was correlated with a decrease in pIC50 value. The rationale being that compounds less similar to the reference compound, should have lower activities.

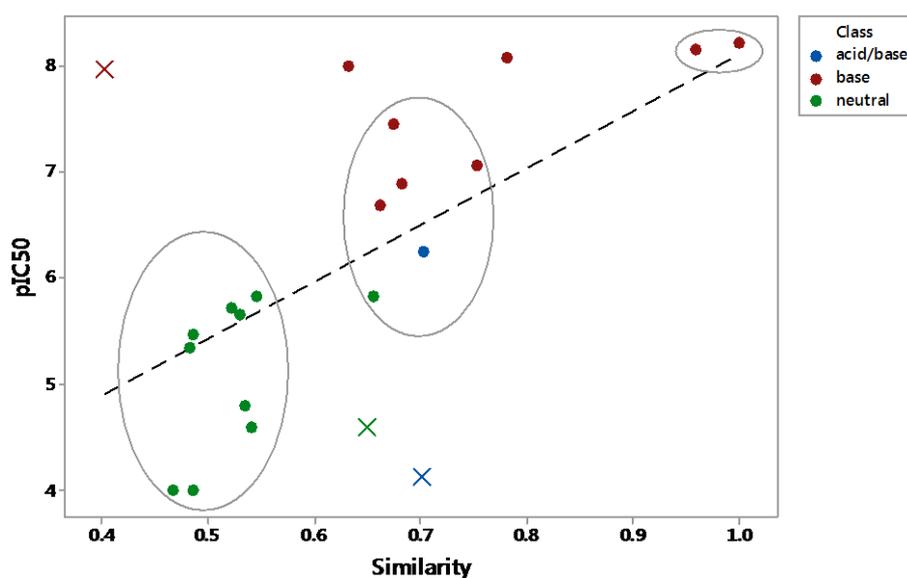


Figure 4.5. A scatterplot of 2-D similarity values versus hERG pIC50 values for compounds belonging to category V. Compounds inside grey ellipses form clusters of similar compounds; outliers are depicted as crosses. Note that similarity values are relative to the most active compound in the group.

However, no such qualitative trends were observed for most categories; the data were randomly scattered when pIC50 values were plotted against similarity to the most potent compound. The only exception was observed for compounds from category V which appeared to have greater hERG channel inhibition potency with increasing 2-D similarity values (refer to Figure 4.5), where the dotted regression line indicates a potential linear relationship between similarity and pIC50. Moreover, three clusters of similar compounds were identified. These clusters were then analysed in terms of whether the compounds present in them were acidic, basic or neutral. The smallest cluster contained two bases that both had high pIC50 values. The cluster of compounds with the similarity value around 0.7 was composed of was a mixture of bases, neutrals and a compound that contained both acidic and basic groups. Finally, only neutral substances were found in the cluster of least similar compounds. Interestingly, all basic compounds had an increased potency to inhibit the hERG channel in comparison to neutral compounds. This trend could also be observed within a cluster that contained all classes of compounds; all bases had also higher pIC50 values. Three outliers, each belonging to a different class, were identified. The difference in pIC50 values between the most potent, basic outlier (compound no.61; red cross) and compounds no. 80 (neutral; green cross) and 73 (containing both acidic and basic groups; blue cross) was approximately 3.5 log units. It is also apparent that the presence of an acidic group reduces the potency of hERG channel binding despite the presence of a basic group. This analysis demonstrates the important role of the protonated nitrogen in hERG binding. 2-D similarity, although useful for identifying categories, was of rather limited application to SAR analysis for hERG toxicity. The promiscuity of interactions between the hERG channel and potential ligands may even further complicate the analysis. Hence, it was decided to place more emphasis on the investigation of the chemistry of the compounds in each group and to try to establish a correlation between the molecular descriptors and the pIC50 values.

4.3.2.2. Local multi-linear regression models for individual categories

To build QSAR models for the individual categories identified in section 4.3.2, it was decided to use a small subset of descriptors selected from the large number of descriptors previously calculated. The criteria for selecting these descriptors were their interpretability and relevance to binding to the hERG channel. The descriptors included: maximum diameter (Dmax), Log D (at pH 7.4), MW, topological polar surface area, counts of H bond donors and acceptors, number of aromatic rings and number of rotatable bonds. These descriptors could be categorised as being medicinal chemistry-type and were similar to those used in attempts to develop a global model for the complete H_244 dataset, including the descriptors used in the model of Aptula and Cronin (2004).

The models for the individual categories (where it was possible to generate a model) are presented in Table 4.3.

Table 4.3. A summary of MLR models for the seven categories of compounds.

Category	Model	R ²	R ² (adj)	S	R ² (pred)	pIC50 range
I (n=5)	No model could be developed					5.38-6.55
II (n=9)	No model could be developed					2.62-4.67
III (n=12)	pIC50 = 10.28- 0.2296 MOP_DMAX	43.56%	37.92%	0.38	29.75%	4.6-5.92
IV (n=5)	No model could be developed					6.45-8.47
V (n=22)	pIC50 = 3.364 + 0.1891 MOP_DMAX	34.38%	31.10%	1.20	20.22%	4.0-8.21
VI (n=6)	pIC50 = 2.294 + 0.0905 MOP_DMAX	87.70%	84.63%	0.13	73.73%	3.52-4.44
VII (n=5)	No model could be developed					5.27-6.21

It is clear that it was not possible to build good models based on the manually preselected features. In four cases it was not possible to obtain any linear regressions as the terms would not fulfil the condition of $\alpha < 0.05$. Maximum diameter was the only feature that was included in cases where models were obtained. The coefficients of correlation were rather poor as less than 50% of variability in pIC50 values was explained by the independent variable (Dmax) - except for the model for category VI. Interestingly, no model was obtained for category II compounds although five out of nine compounds were also drugs were found in the training set of Aptula and Cronin (2004). Moreover, running regression analysis for the overlapping (common to H_244 and Aptula and Cronin datasets) compounds from category II enabled a high R² to be obtained although log D was the only descriptor present in the equation.

The model for compounds from category VI suggested that pIC50 values correlate positively with a maximum diameter of the drug. A predicted R² of 74% (determined using 10-fold cross-validation) indicated that the model had reasonably good predictive power, however the limited number of compounds present in each category made quantitative analysis problematic.

4.3.2.3. Qualitative analysis of seven categories of compounds

As little success was achieved with developing quantitative models for hERG channel inhibition for each category of compounds, the next step was to investigate key chemical features common to the compounds within each category. Analysis of the size and hydrophobic properties of compounds

within each category was also undertaken. The main focus was to identify, qualitatively, factors that may play a role differentiating binding affinity for the hERG channel.

Category I.

Category I comprised five compounds as shown in Figure 4.6.

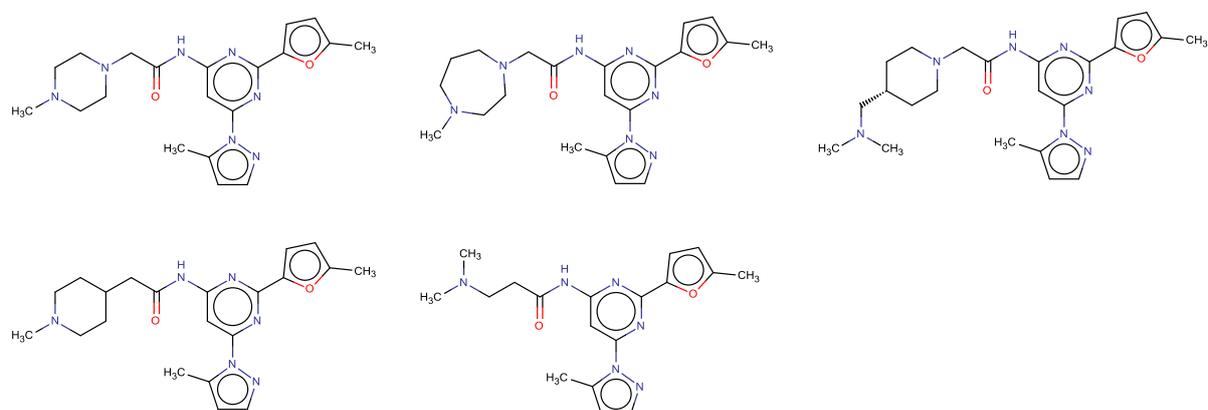
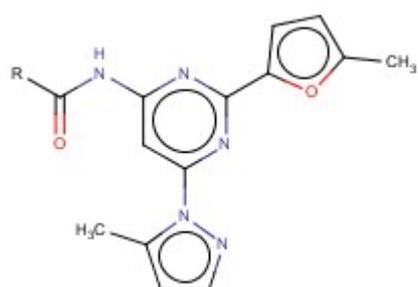


Figure 4.6. Compounds that belong to category I. The compounds are (left to right): 2-amino-N-pyrimidin-4-ylacetamide-1, 2-amino-N-pyrimidin-4-ylacetamide-2, 2-amino-N-pyrimidin-4-ylacetamide-3, 2-amino-N-pyrimidin-4-ylacetamide-4, 2-amino-N-pyrimidin-4-ylacetamide-5.

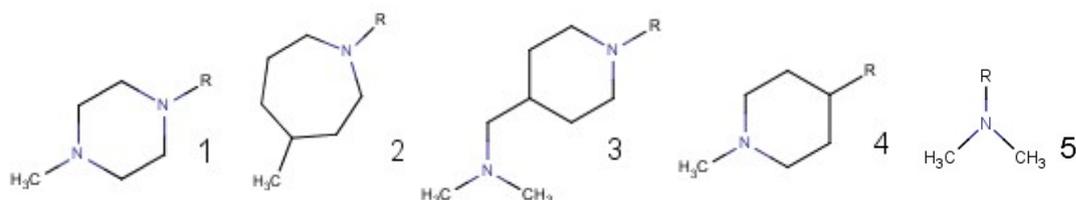
This group contains a series of analogous compounds. All of them contain a pyrimidine ring attached to furan (containing a methyl group), pyrazole (also with a methyl group present) and acetamide (see Figure 4.7A). The differences between molecules are a consequence of various substitutions on the acetamide. These substituents include: heterocyclic amines (piperidine, piperazine, homopiperazine) or aliphatic tertiary amines (see Figure 4.7B).

Of the five compounds in category I, three were active and two were medium active. It was assumed that the common substructure was important for binding these molecules to the hERG channel. The presence of rings could play a role in stabilising the molecule inside a pocket by hydrophobic or stacking interactions with side chains of Phe or Tyr. The unique subfragments seemed to contribute to changes in activity. It should be noted that all of them contained nitrogen atom(s) that had potential to contribute a positive charge and as such was capable of interacting with π electrons of Phe or Tyr. The activities range spanned over one log unit although all the five molecules were very similar in terms of their 2D structures. This was an interesting case of studying potential activity cliffs. The biggest difference in pIC_{50} values for compounds in this category was between a compound containing the substructure 2 ($\text{pIC}_{50} = 6.55$) and a molecule with the fragment 4 ($\text{pIC}_{50} =$

5.38). Visual inspection shows the more active compound had one more nitrogen atom and an additional C-C bond inside a ring (7-membered ring) compared to the less active compound.



(A)



(B)

Figure 4.7 (A) The molecular skeleton common to all compounds from category 1. (B) Substructures unique to each molecule from category 1. The substructures are numbered for identification purposes.

To identify which of these differences contributed more to the increase in activity another comparison was made between fragments 1 and 4 (Figure 4.7B). This time the difference between the activities was lower (0.64 log units) and structural differences were similar as between fragments 2 and 4 with the exception of the presence of six-membered ring and a methyl group attached to nitrogen. This could suggest that the number of atoms in the ring may influence the activity. If only 2D structures were considered, the sequence of the mentioned three molecules (increasing pIC_{50}) would be: compound or fragment 4 (6-membered ring, one N) < compound 1 (6-membered ring, two Ns) < compound 2 (7-membered ring, 2Ns). However, it would be difficult to draw any possible conclusions about potential structural alerts. It seems that these substructures contribute to the activity yet they cannot be considered without the main skeleton. For instance, comparing fragments 1 and 2 (H in fragment 2 is replaced by C from acetyl group) they are almost identical (2-D similarity of around 0.93). That would mean that they should have similar activity, which is not the case (half log unit difference). It could be hypothesised that a 7-membered ring, having more ring strain than 6-membered cyclic systems, may in turn change the conformation of

the whole molecule (if rotatable bonds are present). On the other hand, the difference of half log units may also be a result of experimental variability so that effectively, for compounds containing fragments 1 and 2 there would be little difference in activities. Although, as was already mentioned, it would be difficult to develop a clear structural alert it may be reasonable to indicate that a presence of nitrogen atom(s) with a positive charge may play an important role in binding to the hERG channel; this is in agreement with previous studies on hERG channel binding.

As investigation of structures at the 2-D level was of limited success, further work was carried out that considered the influence of the 3-D features of the molecules in the context of molecular fields. The Torch software package allows alignment of molecules against a reference molecule and then calculates disparity values in a pairwise manner. Disparity is obtained by the following formula:

$$\text{Disparity} \approx \Delta\text{Activity}/(1-\text{Similarity}) \quad (\text{Eq. 4.7})$$

It follows that high disparity values indicate potential activity cliffs, in other words, high disparity indicates molecules which are similar (in terms of 3-D molecular fields) but have significant differences in activity. Similarity can be calculated for 2D-structures (fingerprints) or for molecular fields. Figure 4.8 shows a visualisation of fields for two pairs of molecules. The first pair (Figure 4.8A) has approximately 50% field similarity which is significantly different from the value obtained when considering 2-D similarity. In this context, the changes in the activity could be potentially related to the changes in molecular field. On the other hand, molecules with relatively different subfragments: i.e. compounds 1 and 5 have higher field similarity (0.77) in comparison to values obtained from 2-D method (both compounds have similar pIC50 values). This shows how potential SAR information could be lost if steric and electronic conditions were not taken into account and only 2D structures were examined. Here, the highest disparity value (3.0) was obtained for molecules with subfragment 2 and 4 which may indicate that introducing another heteroatom and much greater ring strain does lead to a more favourable conformation when it comes to binding to the hERG pocket.

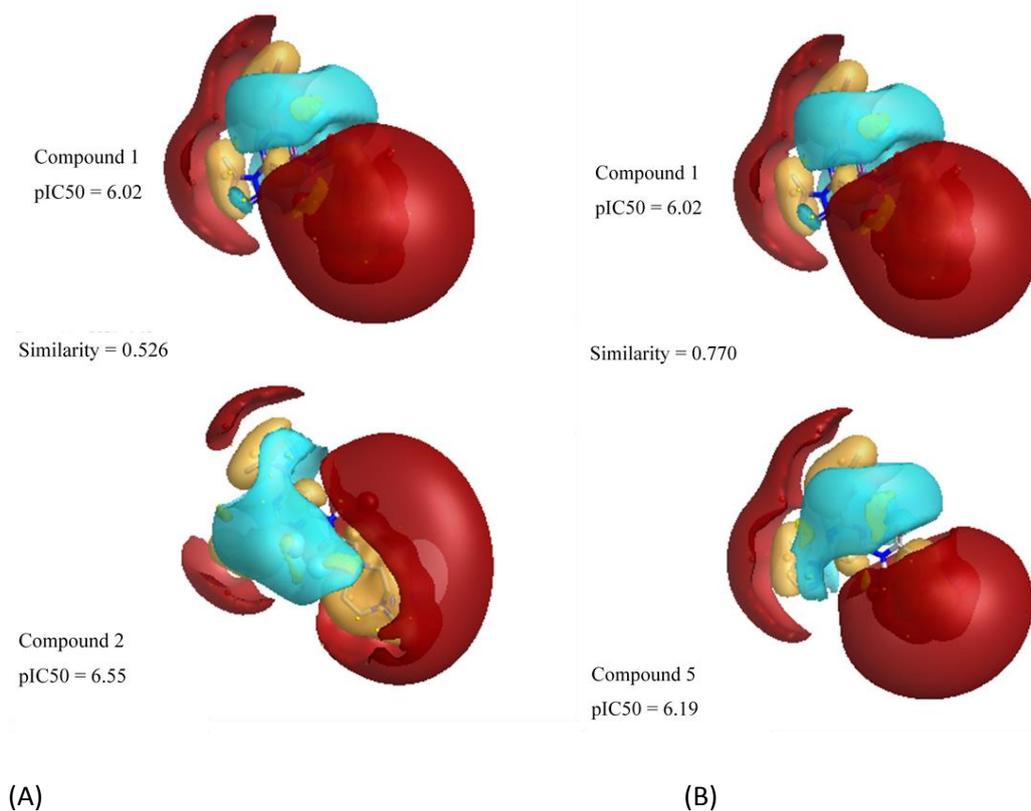


Figure 4.8. Comparison of molecular fields for selected two pairs of molecules from category 1. (A) compounds 1 and 2; (B) compounds 1 and 5.

Category II.

Nine compounds were placed into category II, the structures of which are shown in Figure 4.9. The compounds are all antimicrobial agents and chemically they are classified as fluoroquinolones. All of the nine compounds were categorised as inactive with pIC₅₀ values ranging from 2.62 (lomefloxacin) to 4.67 (sparfloxacin). Although the compounds from category II are classed as inactive according to their pIC₅₀ values many fluoroquinolones are known to possess potential to cause arrhythmias. Sparfloxacin and grepafloxacin have already been removed from the market due to cardiotoxicity; others, such as: ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin have also been indicated as being associated with TdP (Minotti, 2010). Although incidents of TdP caused by fluoroquinolones are rare and depend on other factors such as sex, age, co-administration of drugs or xenobiotic metabolism, they can still result in serious consequences, including sudden death. The case of fluoroquinolones also illustrates that the conventional classification of chemicals according to their hERG blockage potency may be, on occasions, misleading and some “borderline” pIC₅₀ values can also be responsible for substantial cardiotoxicity through inhibition of the hERG channel.

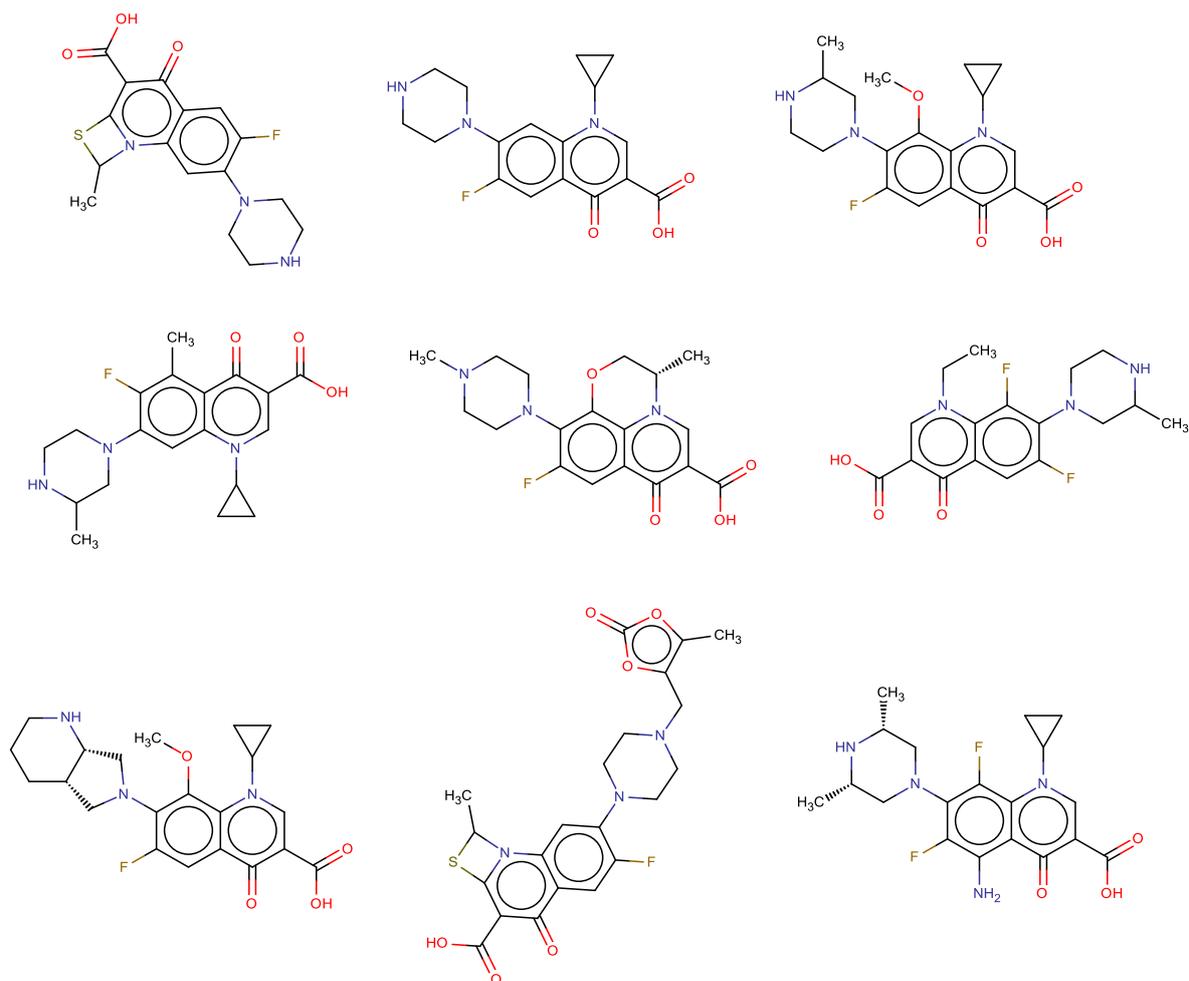


Figure 4.9. Category II compounds: (left to right) AF_3013_(NM-394), Ciprofloxacin, Gatifloxacin, Grepafloxacin, Levofloxacin, Lomefloxacin, Moxifloxacin, Prulifloxacin, Sparfloxacin.

It is also important to realise that the adverse effects depend not only on absolute inhibition values but they also should be considered in relation to therapeutic plasma concentrations of a drug. For instance, sparfloxacin had a ratio of $IC_{50}/[plasma] = 10$ whilst the same ratio for ciprofloxacin was ten times greater (Kang et al, 2001). Nevertheless, there are great differences in absolute pIC_{50} values for fluoroquinolones from group II. This may indicate that the fluoroquinolone skeleton alone is not sufficient to explain these differences and that focus should be rather put on substituents.

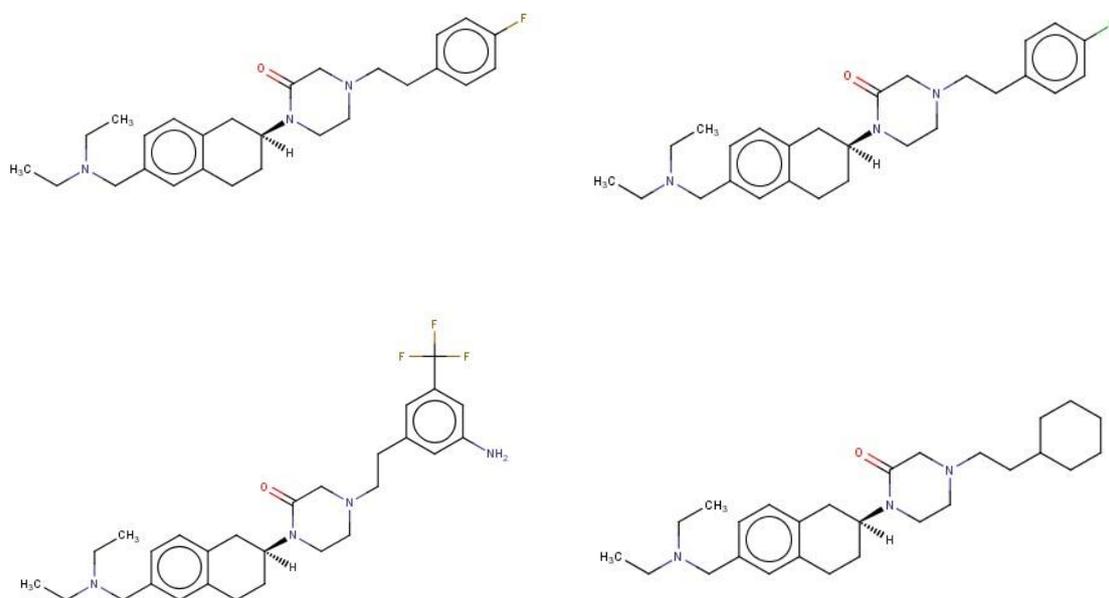
Substituent analysis may reveal some SAR trends. For instance, ciprofloxacin, grepafloxacin and sparfloxacin differ by a substituent at position 5 (a hydrogen, a methyl and aminogroup, respectively) but they also have a different number of methyl groups on the piperazine ring (zero, one and two respectively). The toxicity exerted to hERG could possibly depend on an atom/group attached at position 5 (hydrogen < methyl < amino). The increasing number of methyl groups on the piperazine group may have a lower significance for blockage of the hERG channel. The least potent

compound has a hydrogen at position 5 and additionally differs from sparfloxacin (most potent) by one less methyl group on the piperazine ring and the lack of the propyl group attached to N1 (only methyl group present). The importance of the position 5 of the fluoroquinolone skeleton was previously indicated. For the compounds from category II position 5 could be a candidate for forming a potential structural alert where the presence of methyl or amino group would be an indicator of increased hERG inhibition.

The presence of hydrogen was associated with QTc prolongation relatively lower in comparison to the presence of other groups (< 2ms for ciprofloxacin, 3 ms for gatifloxacin, and 5-6 ms for moxifloxacin and levofloxacin). In contrast, QTc prolongation time for grepafloxacin (methyl group at position 5) was 11 ms whilst 15 ms was the prolongation of the QTc interval for sparfloxacin containing NH₂ group (Rubinstein & Camm, 2002).

Category III.

The twelve compounds in category III are a group of analogues of MCH-R1 (melanin-concentrating hormone receptor 1) antagonists. Structurally, the molecules shared four common moieties: tetralin, ketopiperazine, a tertiary amine linked to tetralin via a methyl group and finally, a hydrophobic residue N-linked to ketopiperazine (see Figure 4.10).



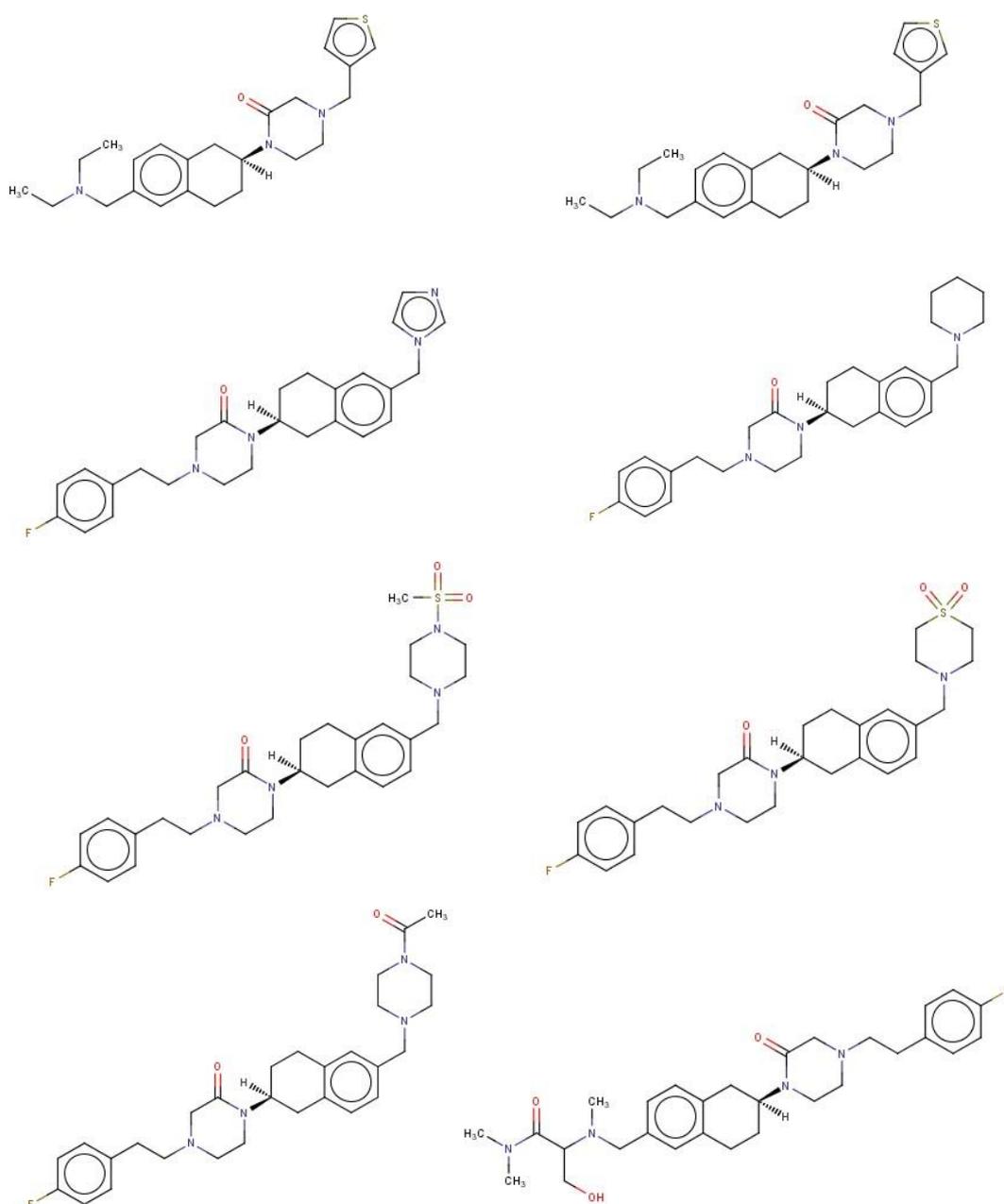


Figure 4.10. Compounds from category III.

Meyers et al (2007) analysed the role of different substituents for hERG binding for these compounds. They indicated that a hydrophobic residue does not need to be aromatic and hence the binding of this part of a molecule is governed by hydrophobic interactions with Phe 565. They also concluded that adding a polar group in the proximity of the tertiary amine residue decreased the inhibition of the hERG channel. It should be noted that the analogues contained two “cation centres” in the form of protonated nitrogen atoms (on tertiary amine residue and ketopiperazine)

and as such they could be involved in cation- π interactions with Tyr 652. However, considering the disruptive role of incorporating polar groups, hydrophobic interactions may be important for stabilisation of binding of this terminus of a molecule in addition to cation- π interactions.

Category IV.

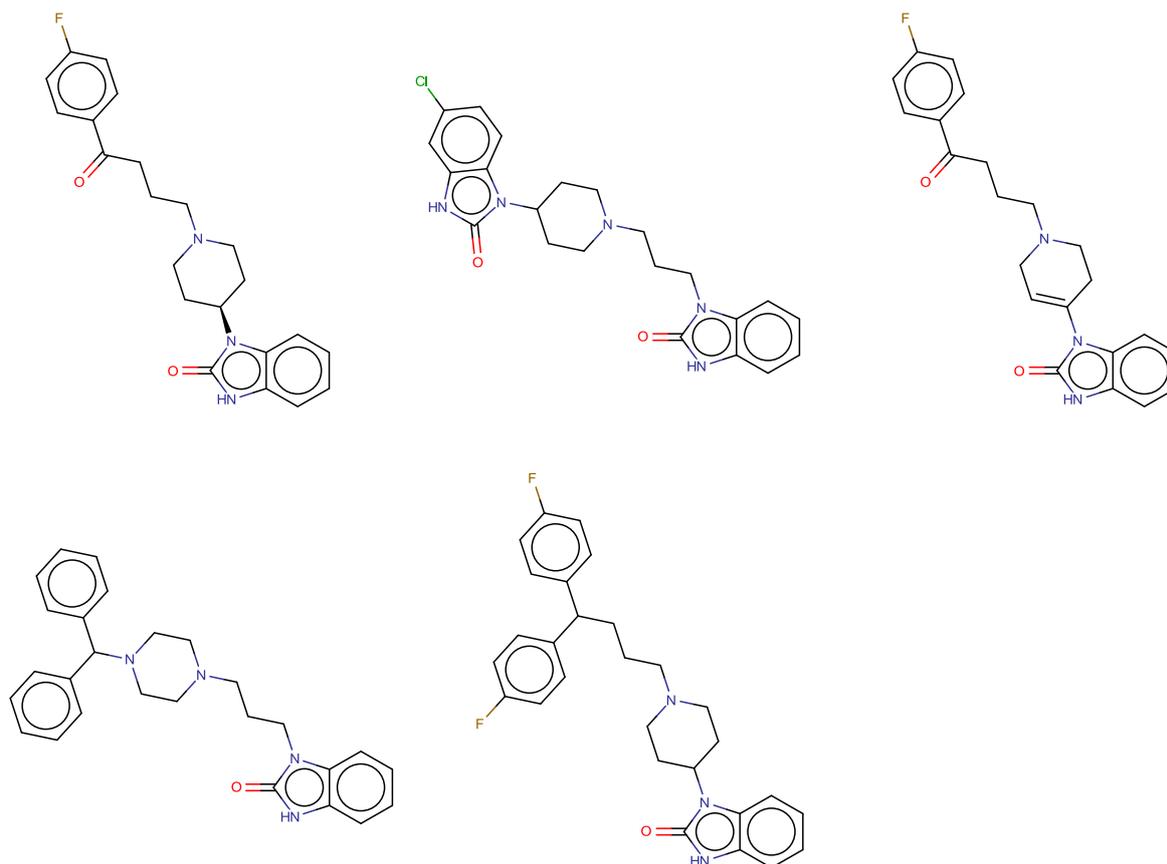


Figure 4.11. Category IV compounds.

All compounds from this category (Figure 4.11) were very active with the lowest pIC₅₀ value of 6.45. Benperidol, droperidol (butyrophenones) and pimozide (diphenylbutylpiperidine) are antipsychotic drugs and dopamine antagonists. Domperidone is an antiemetic and also a dopamine antagonist. Oxatimide is a histamine H₁ antagonist and as such can be categorised as an anti-allergic agent. All molecules contain a bicyclic benzimidazole residue (with a carbonyl group at position 2). They also contain a six-membered N-heterocyclic ring, an alkyl linker and hydrophobic residues such as phenyl rings. N-heterocyclic ring contains a basic nitrogen that is protonated at physiological pH. Despite

sharing common structural features the range of pIC50 values spanned two log units. Three antipsychotics were most potent hERG inhibitors.

Category V.

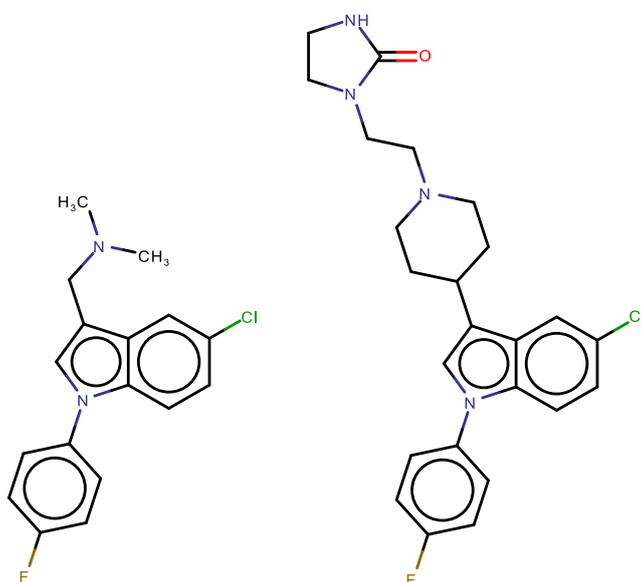


Figure 4.12. Two examples of category V compounds.

Compounds that belong to this category (Figure 4.12) include sertindole (antipsychotic drug) and its 21 analogues. Pearlstein et al (2003) used these compounds (among others) to study hERG channel inhibition utilising CoMSiA and homology modelling. All these compounds contain a basic scaffold consisting of indole with an attached phenyl group and a large proportion of the analogues also contain a piperidyl substituent. This skeleton possesses a great deal of structural rigidity which to some extent simplifies the analysis of the analogues. As sertindole is a potent hERG channel inhibitor (the drug has already been withdrawn from the market due to cardiotoxicity) the analogues were expected to exhibit similar properties and bind to the channel pocket. Indeed, the majority of the compounds were either active (ten) or medium-active (six compounds). The remaining six compounds were inactive and the lowest pIC50 value was four.

The pIC50 values spanned over four log units hence it was interesting to analyse some features of 2-D structures that may influence the inhibition of the hERG channel. The five most potent inhibitors from this group had pIC50 values around 8. Two analogues were only slightly stronger inhibitors than sertindole although structurally they were very similar – apart from a missing chlorine substituent on the indole ring and additionally in one chemical (no. 58) with the highest pIC50 value

the oxygen on the imidazolidine end was substituted by sulphur. Introducing a double bond to a piperidyl group of the sertindole did not seem to change inhibition values (no. 66). The only compound that was structurally least similar to sertindole (no. 61) had neither the imidazolidine nor piperidyl rings. The piperidyl ring was replaced by a tertiary amine. Although such a structural change reduced the diameter and molecular weight by nearly one third, the properties of this molecule in terms of binding to the hERG channel remained nearly the same as those of the original drug. However, it should be noted that nitrogen from the amine group is protonated under physiological conditions and it may be an equivalent of the charged nitrogen from piperidyl group. As previously mentioned, such protonated nitrogen may form interactions with π -electrons of Tyr 652. To further investigate this matter, other compounds were also analysed. Figure 4.13 illustrates the fact that all compounds from category V form two distinctive groups when it comes to their size (diameter). The chemicals with a greater diameter are associated with greater pIC50 values. On the other hand, the analogues that are “truncated” versions of sertindole tend to have a reduced affinity towards the hERG channel. However, three compounds do not follow these rules. Two compounds represented by red triangles in Figure 4.13 were the only ones from the group of smaller analogues that had a protonated nitrogen either present in a piperidyl ring (bottom triangle) or represented by tertiary amine (top triangle). On the other hand, a compound represented by a red square had a low pIC50 value although it possessed a protonated nitrogen. This could be explained by the presence of negative charge introduced by a carboxyl group. This might suggest that the hydrophobic part of the molecule may be important for interacting with Phe 656 and hindrance of this hydrophobic area by a negative charge (in this case) could prevent such hydrophobic attraction between the side chains of phenylanilines of the hERG channel and a molecule. This could be further supported by the fact that replacing hydroxyl group with a methoxy substituent increased the pIC50 values by nearly 3.5 log units (compare compounds no. 73 and 75). However, it should be noted that when a carboxyl group was attached to the phenyl substituent via a methyl link (compound no. 72) the activity was much higher (pIC50 = 6.24). This may suggest that adding another C-C bond allowed more access to the hydrophobic phenyl ring. Nevertheless, it is clear that a presence of both a positive charge (protonated nitrogen) and an accessible hydrophobic group (in this case, a phenyl ring) might be important structural features that would indicate a potential hERG binder channel binder. The spatial arrangement of crucial groups seems to play a role as well and a certain degree of spatial separation may be required to accommodate for the distances between crucial side chains of amino acids.

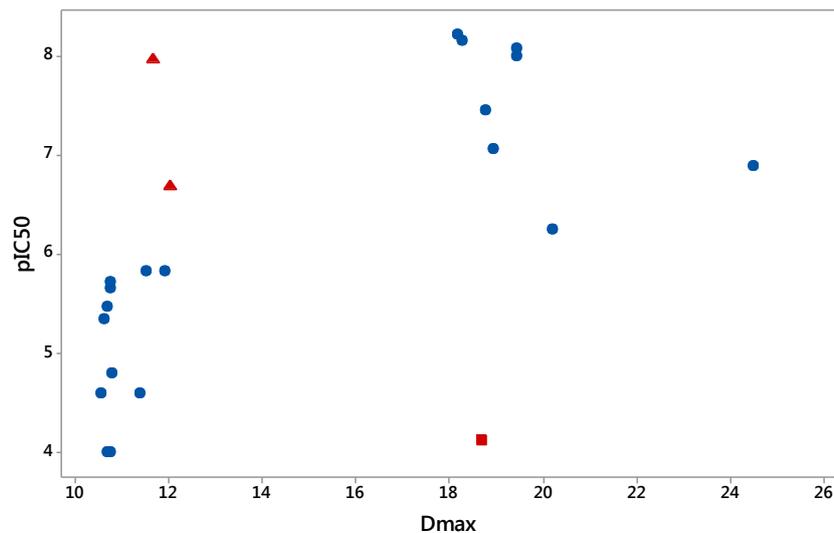


Figure 4.13. Scatterplot of pIC_{50} vs maximum diameter for compounds belonging to category V. Red colour indicates unusual observations and the shape of the red data points refers to whether a compound is in the group with larger diameter (triangle) or is a “truncated” analogue (square).

Category VI.

Category VI comprised six compounds (Figure 4.14) all of which are macrolide antibiotics (or, in the case of N-demethylerythromycin) an active metabolite of a macrolide antibiotic). Structurally, they share a macrocyclic lactone ring to which deoxy sugar moieties can be attached (such as cladinose or desosamine in erythromacin) or other side chains such as N-oxime as in the case of roxithromycin. Similarly to the antimicrobial agents from category II, the pIC_{50} values for macrolides were below the cut-off value of 5 log units, nevertheless they are known to cause hERG channel blockage (Volberg et al, 2002).

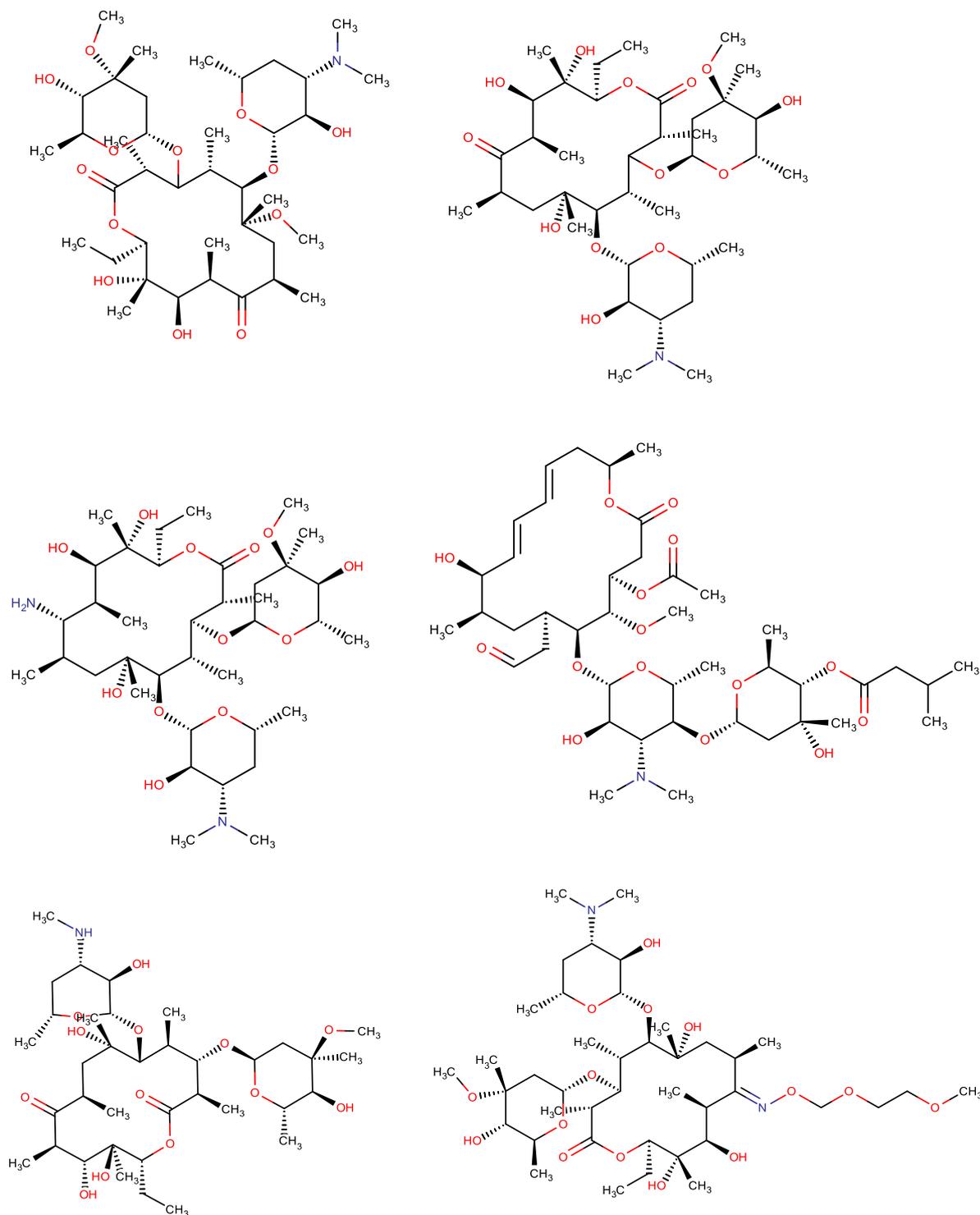


Figure 4.14. Compounds from category VI. (left to right) Clarithromycin, Erythromycin, Erythromyclamine, Josamycin, N-Demethylerythromycin, Roxithromycin.

All macrolides from category VI contain amines which can be protonated in physiological conditions. Again, the protonated nitrogen from the amine group could be important for taking part in cation- π interactions with Tyr 652 hydroxyphenyl group. The rather large size and specific shape of these

molecules confirms the hERG channel binding pocket can accommodate a variety chemical entities. The lactone ring may enable hydrophobic interactions with Phe 656 residues. However, the relatively large number of polar groups may decrease the hydrophobicity of this region and, thus, it should reduce the strength of the hydrophobic interactions. The analysis of log D values (pH = 7.4) showed that compounds with the lowest pIC50 values (no. 130 and 185) also had considerably lower log D values. Interestingly, the N-demethylated form of erythromycin (active metabolite) showed insignificant reduction of the potency to block the hERG channel although the log D values differed by approximately two log units. This may suggest that the change in lipophilicity was not prohibitive neither for transmembrane transport nor access to the channel pocket. However, it may be noticed that the metabolism site on erythromycin was the amine group and thus possibly involved in cation- π interactions. Furthermore, the removal of the methyl group may potentially positively influence the strength of the cation- π bond by removing steric hindrance and increasing charge density. Nevertheless, the structural changes due to metabolism do not occur in that part of the molecule that possibly took part in hydrophobic interactions. Hence, considering differences in log D values may be misleading if the analysis of the corresponding structural changes is not present and it does not take into account pharmacophorically important regions of the molecules.

Category VII.

The five compounds from category VII (Figure 4.15) are drugs with gastroprokinetic and/or antiemetic properties. Clebopride and metoclopramide are dopamine antagonists while mosapride, prucalopride and renzapride are serotonin receptors (5HT4) agonists. Chemically, all these molecules are either substituted benzamides or (in case of prucalopride) carboxamides. All these drugs were either active or medium active towards the hERG channel. All have a nitrogen that can be protonated in physiological conditions (this N was always found on a substituent attached to an amide nitrogen). Again, the positive charge had the potential to interact with tyrosine side chains in the hERG channel pocket. Benzamide and carboxamide (with benzofuran) cores of the molecules are planar structures and as such they could be considered as approximately constant in terms of conformational geometries. Thus, the differences in potency to block the hERG channel could result from differences in substituents that have more flexibility to adopt a greater number of conformations. For instance, clebopride was about one log unit more active than metoclopramide. They both share an identical benzamide structure. However, the clebopride molecule has both a larger diameter and greater log D (by two log units). This could suggest that the presence of a phenyl group (as piperidyl group is assumed to be involved in cation- π interactions through a protonated nitrogen) may be important for stacking interactions. On the other hand, mosapride has pIC50

values very close to metoclopramide even though it is structurally (at least in 2-D) more similar to clebopride. It could be hypothesised that introducing oxygen into a piperidyl group in mosapride may have reduced the strength of cation- π bond. Again, it seems that although certain chemical features can indicate the potential to bind to the hERG channel, very often small structural changes can have a great influence on binding. For instance, Durdagi et al, (2014) analysed a number of cisapride (another prokinetic drug – withdrawn from the market due to hERG toxicity) analogs. They concluded that the truncation of the flexible linker between N-heterocyclic ring and the phenyl group reduced the blockage of the hERG channel. Shortening alkyl chains influenced torsional energy, reduced van der Waals interactions with the protein (hydrophobic residues) and also affected binding of the aromatic groups of the drug with Tyr 652 and Phe 656.

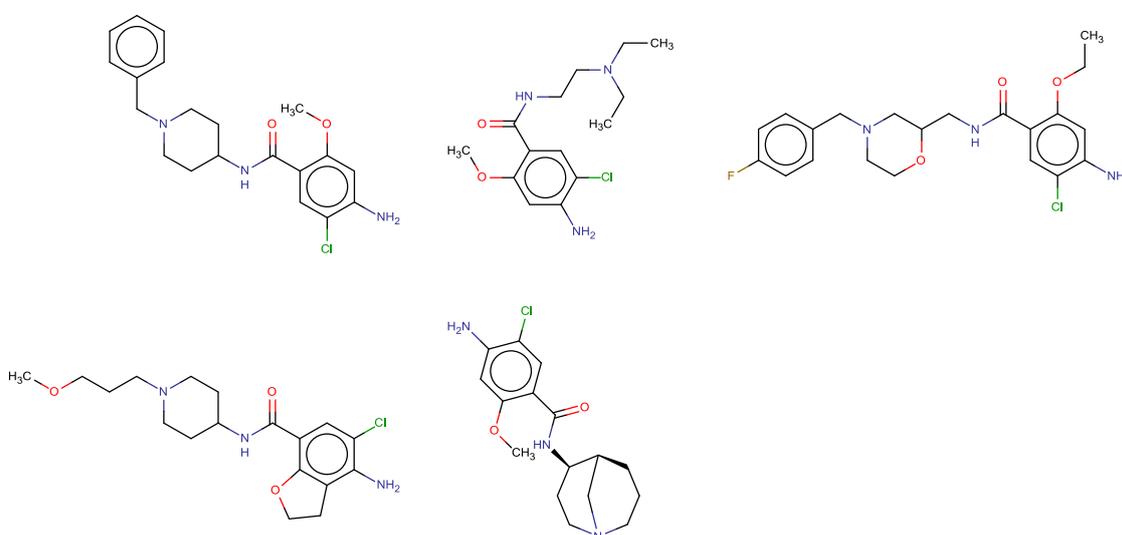


Figure 4.15. Structures of compounds from category VII.

4.3.3. Development of structural alerts for hERG toxicity

During the investigation of the categories based on 2D similarity it appeared that specific structural fragments were potentially associated with hERG channel binding. These fragments formed the basis for the development of structural alerts; these were coded into SMARTS patterns where possible.

Structural alerts for hERG toxicity have been recently reported by Liu et al, (2014) who utilised extended-connectivity fingerprints for their Bayesian classification model. They identified 20 fingerprint features which contributed most positively to their model and were present in hERG inhibitors. Half of the fingerprints used by Liu et al originated from sertindole analogues, enabling comparison to the compounds from category V in this study.

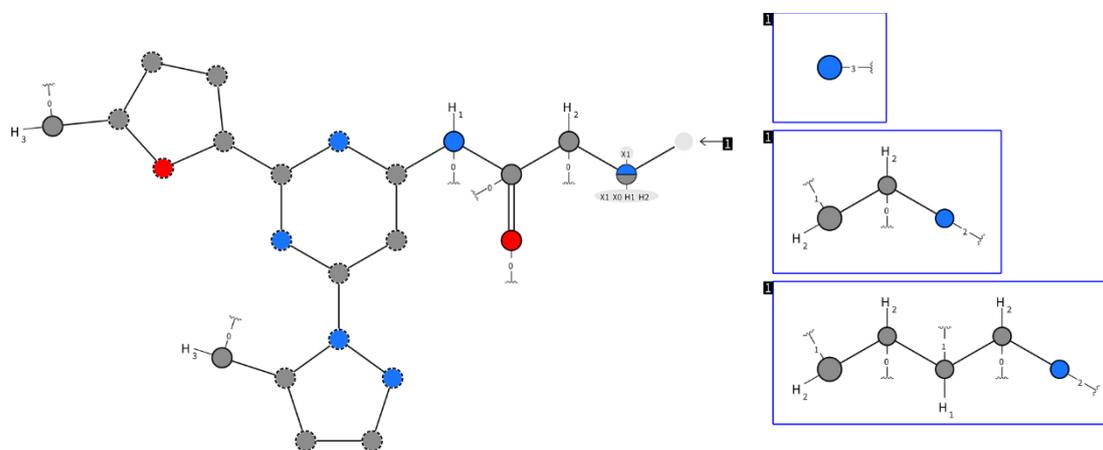
The proposed structural alerts for each of the seven categories are summarised in Figures 4.16 - 4.21. It should be noted that some categories were formed solely by compounds with pIC50 < 5 (categories II and VI). Nonetheless, structural alerts were still developed as some compounds from these categories were known to cause blockage of the hERG channel. The alerts were visualised using SMARTSviewer (ver. 0.9.0.) (Schomburg et al, 2010).

Category I

The structural alert developed for category I was based on the common skeleton for each molecule (as shown in Figure 4.7A). The SMARTS pattern for this alert is:

```
[CX4H2]([CX3](=[OX1])[NX3H1]c1cc(nc(n1)c2ccc(o2)[CX4H3])n3nccc3[CX4H3])[CX4H1,CX4H2,NX3][*
$([NX3]),*$([CX4H2][CX4H2][NX3]),$([CX4H2][CX4H2][CX4H1][CX4H2][NX3])]
```

The differences between pIC50 values of the subfragments from Figure 4.7B were insufficient to allow further consideration of these subfragments. A log unit range of potencies could be considered, at least partially, as a result of experimental error that could range from 0.5 to 1 log unit. However, the nitrogen atom present in each of the five subfragments was included in the structural alert. At physiological pH (7.4) these nitrogen atoms would be protonated and as such they would potentially stabilise interactions with the hERG channel.



Picture created by the SMARTSviewer (www.smartviewer.de).
Copyright: ZfH Center for Bioinformatics Hamburg.

Figure 4.16. Structural alert for category I compounds.

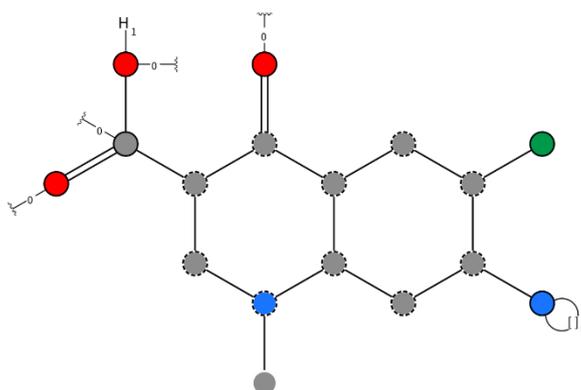
Category II

Fluoroquinolones can prolong the heart's QT interval. Consequently, despite all compounds from category II being classified as inactive a structural alert was developed. The main skeleton of fluoroquinolones was included. The previously discussed potency differences potentially resulting

from a different substituent on position 5 (ciprofloxacin, grepafloxacin and sparfloxacin) were not taken into account. This means that the least potent ciprofloxacin is also captured by the SMARTS pattern. Matsuo et al, (2013) showed that ciprofloxacin can change the electrocardiographic characteristic when used at supra-therapeutic dosage (10mg/kg rather than 3mg/kg) to prevent bacterial resistance. Moreover, ciprofloxacin was also shown to inhibit other cardiac channels (Na⁺, Ca²⁺). The piperazine ring was not included in the alert even though it was present in eight compounds. Possibly, the basic nitrogen, present in piperazine ring and in the N heterocyclic subfragment of moxifloxacin, may play a role in hERG binding although this was not reflected in the final SMARTS. This was an arbitrary choice in order to develop an alert that is less specific so that it can match a broader category of compounds. However, this was compensated by adding a nitrogen at the 7-position. The presence of a fluorine atom may be arguable, however, it was present in all nine compounds.

SMARTS:

```
c21c(cc(c(c1)[NR1])F)c(=[OX1])c([CX3](=[OX1])[OX2H1])cn2[#6]
```



Picture created by the SMARTSviewer [www.smartsview.de].
Copyright: ZBH Center for Bioinformatics Hamburg.

Figure 4.17. Structural alert for category II compounds.

Category III

All common substructures for compounds from category III were included in the structural alerts for this group. The following four moieties were included: tetralin, ketopiperazine, a tertiary amine linked to tetralin via a methyl group and finally, a hydrophobic residue N-linked to ketopiperazine. The hydrophobic residue was coded as a carbon atom (aliphatic or aromatic).

This category was formed by a mixture of active and inactive compounds according to the pIC50 based classification. However, the pIC50 values for the inactive compounds were close to the cut-off

value of 5 log units. Again, such differences could be within the range of an experimental error, which supports the argument that a structural alert could be a better indicator of activity than pIC50 values alone.

SMARTS:

```
c12cc([CX4H2][#7]([#6])[#6])ccc1[C@@X4H1]([NX3]3[CX3](=[OX1])[CX4H2][NX3]([CX4H2][#6])
)[CX4H2][CX4H2]3)[CX4H2][CX4H2]2
```

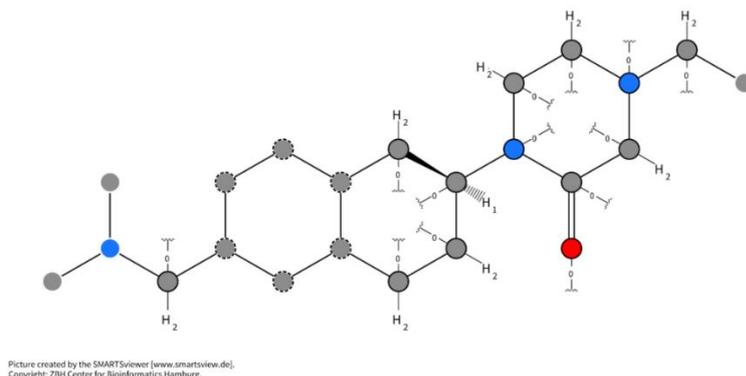


Figure 4.18. Structural alert for category III compounds.

Category IV

All five compounds were very potent hERG blockers. The structural alert was based on benzimidazole moiety. However, N-heterocyclic residue linked to benzimidazole via an aliphatic linkage could also be potentially included in the SMARTS, especially in the context of including basic nitrogen.

SMARTS:

```
c12ccccc1n([CX4,CX3])c(=[OX1])[nH]2
```

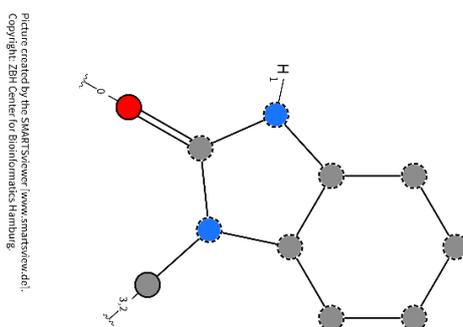


Figure 4.19. Structural alert for category IV compounds.

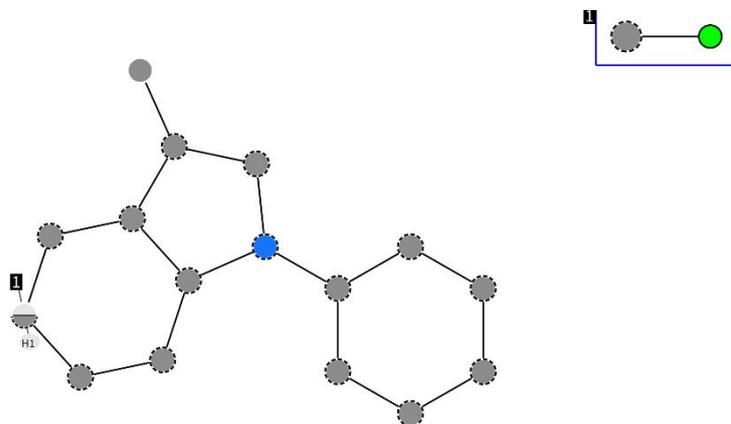
Category V

The structural alert was based on the indole and the phenyl ring with an optional fluorine. No piperidine or tertiary amine was included. Therefore, the structural alert was based on the truncated version of sertindole. As previously discussed, the analogues with lower maximum diameter tended to be weaker inhibitors although some of them were medium-active or even highly active when they contained a basic nitrogen in the form of cyclic or acyclic tertiary amine (compounds no. 60 and 61). However, it was decided to use a common skeleton in order to capture all of the analogues. Potentially, it could be useful to develop two different alerts that distinguish between sertindole analogues that are strong hERG inhibitors and those with less binding affinity. Nevertheless, when the pIC50 values range for category 5 was taken into account, the proposed alert may still be sufficient for broad screening of chemical space.

The developed alert was not identical to those suggested by Liu et al although one of their fragments (G13) included an indole without a phenyl ring.

SMARTS:

```
c21c(cn(c1cc[cH1,$(c[Cl])])c2)c3ccccc3)[#6]
```



Picture created by the SMARTSviewer [www.smartsview.de].
Copyright: ZBH Center for Bioinformatics Hamburg.

Figure 4.20. Structural alert for category V compounds.

Category VI

All six compounds were classified as inactive. A lactone ring (a cyclic ester) was potentially indicated to play part in binding to the hERG channel. However, no structural alert was developed. The size of the ring (14 to 16 C) and differences between substituents made the process of developing a straightforward alert quite challenging. It should be noted that lactones were previously associated with toxicity endpoints such as genotoxic carcinogenicity (Kazius, McGuire & Bursi, 2005) or skin

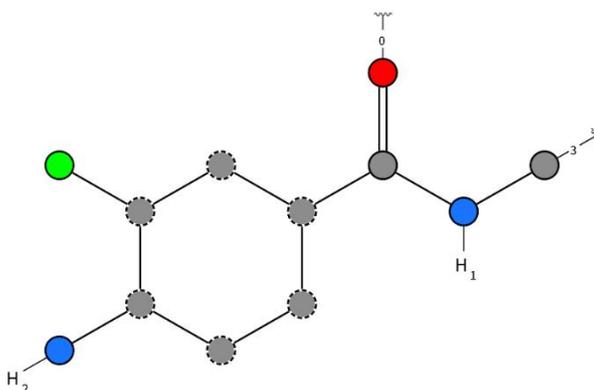
sensitisation (Enoch, Madden & Cronin, 2008). However, it could be assumed that in the case of the antibiotics from this category the size of the macrocyclic ring was much more important from a hERG binding perspective than any involved reactivity.

Category VII

Benzamide was a common substructure for all the compounds from category 7 and as such it was used to develop a structural alert for this group. Again, the role of other substructures, was not specified in SMARTS even though some of them, especially those that contain basic nitrogen, may be important for hERG binding.

SMARTS:

```
[NH2]c1c([Cl])cc(C(=[OX1])[NH][CX4])cc1
```



Picture created by the SMARTSviewer (www.smartsview.de).
Copyright: ZBH Center for Bioinformatics Hamburg.

Figure 4.21. Structural alert for category VII compounds.

To ensure that the SMARTS captured all the compounds in the categories the developed alerts were tested against the H_244 dataset. The re-screening process was successful. Further validation on an external dataset would be required and is intended to be included in future work.

One of the challenges during development of structural alerts is to make a decision about how specific the rule should be. This dilemma was present when writing SMARTS for the described categories. For instance, in some cases the basic nitrogen was not included in the SMARTS pattern as this would require writing a rule so specific to compounds from a given category that its use for screening purposes would be questionable. The only exception was made for the structural alert for category I that was more amenable to the inclusion of the ionisable nitrogen. On the other hand, according to various pharmacophore models, a protonated nitrogen seemed to be an important

feature and as such it should be included in SMARTS (if applicable). Possibly, a better solution could be provided by coding structural fragments that represent good features contributing to a model such as those present in study by Liu et al, or Wang et al., (2012) – another naïve Bayesian classifier. Additional work could be done on developing SMARTS for strong hERG inhibitors. Tobita, Nishikawa & Nagashima, (2005) proposed features for strong hERG blockers and these included non-aromatic nitrogen, hydrophobic fragments and also a ring connected through a nitrogen atom (see Figure 4.22). Such rules could be used for general screening of chemical space.

Developing structural alerts for inhibition of the hERG channel is a difficult task due to the promiscuity of this channel. However, this approach offers some advantages such as being less prone to experimental errors and less dependent on pIC50 threshold values. In this work, 1 μ M (pIC50 = 6) was used as this was used to distinguish between actives and inactive in other studies (Nisius & Goller, (2009), Keseru, (2003). However, other thresholds have also been used. For instance, Aronov & Goldman, (2004) used 40 μ M cut-off which corresponds to pIC50 of 4.4.

Another possibility for future work could involve combining structural alerts for hERG channel inhibitors with a set of rules based on molecular descriptors such as log P, TPSA or molar refractivity (Buyck et al., 2003). This may provide a useful filter to be used by medicinal chemists in terms of screening for hERG blockers.

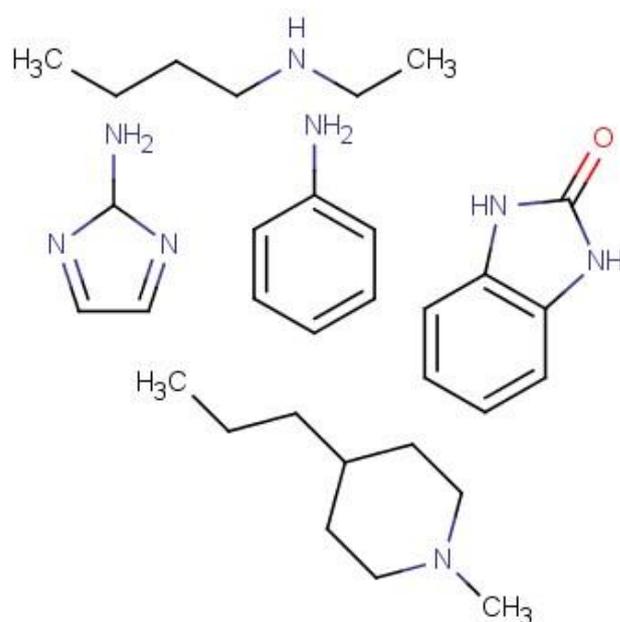


Figure 4.22. Structural features for strong hERG inhibitors. Adapted from Tobita, Nishikawa & Nagashima, (2005).

Development of predictive models for hERG channel inhibition is challenging. In the work carried out here, a previously reported model, that used hydrophobicity and molecular size descriptors, was evaluated to determine if it had broad applicability. Aptula and Cronin's model did not provide accurate predictions for the much larger H_244 dataset studies here. This shows that models developed for smaller datasets have a more limited applicability domain. In this work, both global and local regression models developed were not of sufficient quality to offer improvement to existing models. It could be argued that the regression methods may not be the optimal choice for hERG modelling possibly due to the promiscuity of the hERG channel that can bind structurally diverse compounds. However, binary classification modelling was more successful with the best performing random forest classifier that achieved a recall of 78%.

Due to limited success with modelling hERG binding a new approach was used in an attempt to overcome poor accuracy of hERG models. Seven categories of compounds were identified through a 2-D similarity search. Each group was discussed in terms of potential structural features that could play a role in hERG binding. This analysis resulted in the development of structural alerts for 6 out of 7 categories formed from the H_244 dataset. The author is not aware of any SMARTS rules for hERG described in scientific literature and therefore, the developed SMARTS patterns may offer a potential to be implemented as a screening tool for identification of putative hERG blockers. Future work could include a further development of structural alerts both in terms of their number and quality. Incorporation of such rules into decision trees that utilise medicinal chemistry-type descriptors would constitute a further extension of this work.

Some analysis of structural alerts in the context of screening will be provided in the next chapter where the specificity of alerts related to reactive metabolism will be discussed, particularly with respect to the balance between the specificity and sensitivity.

5. Structural Alerts for Reactive Metabolites

5.1. Introduction

The formation of reactive metabolites in the process of bioactivation has been known to be responsible for adverse drug effects, especially idiosyncratic toxicities (Kalgutkar et al., 2005; Stepan et al., 2011). The prediction of bioactivation is not a trivial task as it depends not only on the structure of the compound but also on factors that are characteristic to an individual - such as genetic makeup, age or health. Moreover, the formation of reactive metabolites does not always lead to toxic effects due to detoxification processes, such as reaction with glutathione that can bind electrophilic species.

Reactive metabolites are either electrophiles, which are electron-deficient species, or free radicals that contain unpaired electrons. Electrophiles can be further categorised as soft electrophiles (low positive charge density) and hard electrophiles (high positive charge density). Soft electrophiles tend to react with soft nucleophiles (e.g. quinone methides can bind covalently to lysine or histidine residues on proteins) while hard electrophiles generally bind to hard nucleophiles (e.g. benzyl carbonium ions can react with bases in DNA) (Stachulski et al., 2012). Reactive species can bind covalently to proteins and disrupt their biological function, e.g. inhibiting a specific enzyme or receptor activity or modulating a signalling pathway. Furthermore, a reactive metabolite bound to a protein becomes a hapten and as such it forms an antigen and can potentially elicit an immune response. Reactive metabolites can also induce oxidative stress. Finally, reactive species can form DNA adducts (Byrns et al., 2006) that can introduce mutations which could further lead to carcinogenic effects. Any of the described scenarios can result in cell death in either a controlled or uncontrolled manner (apoptosis and necrosis). Reactive metabolites are formed from a structurally heterogeneous spectrum of chemicals that includes a diverse group of small molecule drugs. Moreover, there exists numerous potential targets for reactive metabolites. Hence, the association between reactive metabolites formation and toxicity is not always clear (with the exception of genotoxicity (Stachulski et al., 2012)). This means that the development of a screening tool that could determine potential toxicity, via reactive metabolite formation, is an extremely challenging task. It has been recognised that early and reliable detection and identification of reactive metabolites can be a bottleneck in drug discovery and integrated approaches have been proposed (Stepan et al., 2011; Reese et al., 2011; Thompson et al., 2012; Pelkonen et al., 2014).

A number of *in vitro* methods are employed to detect reactive metabolites; for instance, small-molecular trapping agents. Among the trapping agents glutathione (GSH) is used to detect soft electrophiles while hard electrophiles are detected using potassium cyanide (KCS) or semicarbazide (SCA). Other trapping agents may include peptides and oligonucleotides, such as deoxyguanosine (dG), the latter being utilised for detection of species that target DNA. Ideally, these tests for adduct formation should be performed in combination with tests for biomarkers of toxicity (Pelkonen et al., 2014).

In silico methods used for prediction of reactive metabolites include screening compounds against a set of structural alerts. Such a preliminary screening process represents a strategy of avoidance, i.e. not incorporating certain functional groups into a drug structure. The development of structural alerts for reactive metabolites is based on the identification of molecular fragments/functional groups that are known to be subject to bioactivation. However, structural alerts also have limitations. First of all, they are knowledge-based, which means that they only represent what is currently known and available in the published literature. Secondly, the binary categorisation of structural alerts being present or absent within a molecule can be ambiguous (Stachulski et al., 2012) as it may not be entirely clear what constitutes an alert. Recently, it has been argued that structural alerts may be applied too stringently so reducing the chemical diversity required in drug discovery (Liu, Yu & Wallqvist, 2015). These authors also argued that a structural alert should be only developed if it occurs more significantly in compounds that exert toxicity rather than in those chemicals that are negative for the toxicity.

The main aim of this chapter was to analyse the distribution of publically available structural alerts for reactive metabolites for a large dataset of drug molecules (DrugBank). Additionally, the importance of refining alerts has been considered using the alert for a carboxyl group as an example.

5.2 Methods

5.2.1. Datasets

Structures of 6858 drug molecules were downloaded from DrugBank (<http://www.drugbank.ca>) in sdf format. The DrugBank dataset included the following types of drugs: (i) approved (n = 1543), (ii) experimental (n = 5023), (iii) nutraceutical (n = 84), (iv) illicit (n = 169), (v) withdrawn (n = 149) and (vi) investigational (n = 522).

Some overlaps between categories were present. For instance, a drug could be approved in one country and withdrawn in another. The total of all groups was, therefore, greater than the number of compounds (n = 7490).

In addition to structural information, the sdf file also contained a number of calculated medicinal chemistry-type descriptors, for instance, log P, PSA (polar surface area), or NRB (the number of rotatable bonds) and also a number of rules violations for selected druglikeness filters such as Lipinski Rule of Five or Veber's rules. Not all descriptors were available for all compounds

Duplicates (including stereoisomers) were removed from the dataset by comparing InChIKeys. All structures were subject to a standardisation procedure performed in OpenBabel (v. 2.3.1), i.e. explicit hydrogens were added, dative bonds were converted, solvents were removed and salts were stripped (using the method of removing all but the largest contiguous fragment).

5.2.2. Structural alerts

The 35 structural alerts selected for reactive metabolites were taken from an online chemical database - oCHEM (<https://ochem.eu>). oCHEM allows a user to screen compounds against all alerts within the software or to select subgroups of alerts against which to screen. In this study the alerts associated with idiosyncratic toxicity (Reactive Metabolite formation) were selected. The alerts were present in the form of SMARTS which were developed from the work of Kalgutkar & Soglia (2005). The alerts are listed in Table 5.1.

Table 5.1. Structural alerts for detection of indirectly acting electrophiles (reactive metabolites).

ID	ALERT	SMARTS
1	Anilines_anilides	<chem>c1ccccc1[NX3]([#1,CX4!R,\$(c1ccccc1,\$([CX3]=[OX1])[#6]),\$([CX3]=[OX1])[OX2][#6]),\$([SX4]=[OX1])(=[OX1])[#1,#6])][#1,CX4!R,\$(c1ccccc1,\$([CX3]=[OX1])[#6]),\$([CX3]=[OX1])[OX2][#6]),\$([SX4]=[OX1])(=[OX1])[#1,#6])]</chem>
2	Hydrazine_hydrazides	<chem>[#6,#1][NX3H1][NX3H1][#6,#1]</chem>
3	Nitrobenzenes	<chem>a[\$([NX3]=[OX1])=[OX1]),\$([NX3+]=[OX1])[O-]]</chem>
4	Dibenzazepines	<chem>N([CX4])([CX4])C1=Nc2ccccc2[NX3H1]c3c1ccccc3</chem>
5	Benzylamines	<chem>[#1,CX4!R][NX3]([#1,CX4!R])[CH2]c1ccccc1</chem>
6	Propargyl_amines	<chem>[#1,CX4,\$(c1ccccc1)][CX2][CX2][NX3]([#1,CX4,\$(c1ccccc1))][#1,CX4,\$(c1ccccc1)]</chem>
7	Cyclopropyl_amines	<chem>[CX4]1[CX4][CX4]1[NX3]([#1,CX4,\$(c1ccccc1))][#1,CX4,\$(c1ccccc1)]</chem>
8	N-Substituted-4-aryl-1,2,3,6-tetrahydropyridines	<chem>[NX3]1([CX4!R])[CX4][CX3]=[CX3](c2c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c2([#1,#6,F,Cl,Br,I]))[CX4][CX4]1</chem>
9	N-Substituted-4-arylpiperidin-4-ol	<chem>[NX3]1([CX4!R])[CX4][CX4][CX4]([OH1])(c2c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c([#1,#6,F,Cl,Br,I])c2([#1,#6,F,Cl,Br,I]))[CX4][CX4]1</chem>
10	Formamides	<chem>[CX3H1])(=[OX1])[NX3H1][#6]</chem>

11	Hydantoin_(glycolylurea)	[NX3H1][CX3](=[OX1])[CX4]([#1,#6])([#1,#6])[NX3H1][CX3]1(=[OX1])
12	Thioamides	[#1,#6][CX3](=[SX1])[NX3]([#1,#6])[#1,#6]
13	Thioureas	[#1,#6][NX3]([#1,#6])[CX3](=[SX1])[#7]
14	Sylfonylureas	[#6][Sv6X4](=[OX1])(=[OX1])[NX3H1][CX3](=[OX1])[NX3H1][#6]
15	Thiols	[SX2!H0][#1,#6&!\$([CX3]([SH1])=[OX1,SX1,NX2])]
16	Disulfides	[#6,#1][SX2][SX2][#6,#1]
17	Ortho-_or_parahydroquinones	[\$(c1([OH1])c([OH1])cccc1),\$(c1([OH1])ccc([OH1])cc1)]
18	Ortho-_or_paraquinones	[\$([#6]1(=[OX1])-,:[#6]=,:[#6]-,:[#6](=[OX1])-,:[#6]=,:[#6]1),\$([#6]1(=[OX1])-,:[#6](=[OX1])-,:[#6]=,:[#6]-,:[#6]=,:[#6]1)]
19	Ortho-_or_paraalkylphenols	[\$(c1([OH1])c([CX4]([#1,#6])[#1,#6])cccc1),\$(c1([OH1])ccc([CX4]([#1,#6])[#1,#6])cc1)]
20	Quinone_methide	[\$([OX1]=[#6X3]1-,:[#6X3]=,:[#6X3]-,:[#6X3]-,:[#6X3]-,:[#6X3]1=[CX3]([#1,#6])[#1,#6]),\$([OX1]=[#6]1-,:[#6]=,:[#6]-,:[#6](=[CX3]([#1,#6])[#1,#6])-,:[#6]=,:[#6]1)]
21	Benzo-dioxolanes	c1ccc([OX2]2)c([OX2][CX4H1]2[OH1])c1
22	3-Methylene_indoles	c1ccc([nX3]2([#1,#6]))c(c([CH2][#1,#6])c2)c1
23	Furans	c1ccc[oX2]1
24	Thiophenes	c1ccc[sX2]1
25	Thiazoles_and_2-amino_derivatives	[\$(c1([#1,CX4!R])c([#1,CX4!R])[nX2]c([#1,CX4!R])[sX2]1),\$(c1([#1,CX4!R])c([#1,CX4!R])[nX2]c([NX3]([#1,CX4!R])[#1,CX4!R])[sX2]1)]
26	Thiazolidinediones	[SX2]1[CX3](=[OX1])[NX3H1][CX3](=[OX1])[CX4]1[#6]
27	Arenes	NOT [#6!#1] AND c1ccccc1
28	Bromoarenes	NOT [#6!#1!Br] AND c1ccccc1Br
29	2-_or_4-Halopyridines	[\$(n1c([Cl,Br,I,F])cccc1),\$(n1cccc([Cl,Br,I,F])cc1)]
30	Heterocycles_with_C-2_LG	nc[\$([OX2][Sv6X4](=[OX1])(=[OX1])[OH]),\$([OX2][Sv6X4](=[OX1])(=[OX1])[OX2][CH3]),\$([OX2][Sv6X4](=[OX1])(=[OX1])[CH3]),\$([OX2][Sv6X4](=[OX1])(=[OX1])[CF3]),\$([OX2][Sv6X4](=[OX1])(=[OX1])c1ccc([CH3])cc1),\$([I,Br,Cl])]
31	Alkynes	[#6][CX2]#[CX2][#1,#6]
32	Michael_acceptors	[\$([CX3]=[CX3](\$([NX3](=[OX1])=[OX1]),\$([NX3+](=[OX1])[O-]))),\$([CX3](=[OX1])[OX2][#1,#6]),\$([Sv6X4](=[OX1])(=[OX1])[OH]),\$([CX3](=[OX1])[#1,F,Cl,Br,I]),\$([CX2]#[NX1]),\$([CX4]([F,Cl])([F,Cl])[F,Cl]),\$([CH2][OH]),\$([CH2](\$([NX3](=[OX1])=[OX1]),\$([NX3+](=[OX1])[O-]))),\$([CH2][F,Cl])),\$([CX3]=[CX3][CX3](=[OX1])[#1,#6])]
33	Alkylhalides	[\$([CX4]([F,Cl,Br,I])([H,!F!Cl!Br!I])([H,!F!Cl!Br!I])[H,!F!Cl!Br!I]),\$([CX4]([#6])([F,Cl,Br,I])([F,Cl,Br,I])[H,!F!Cl!Br!I]]
34	Carboxylic_acids	[#6][CX3](=[OX1])[OH1]
35	Fluoroethyl_ethers_and_amines	[\$([F][CX4](\$([CH2]),\$([CH]),\$(C[OH1]))[OX2][#1,#6]),\$([F][CX4](\$([CH2]),\$([CH]),\$(C[OH1]))[NX3][#6])]

5.2.3. Detection of alerts

The presence of structural alerts for all compounds from the DrugBank dataset was evaluated using OpenBabel. Structures in the sdf file were screened against each of the 34 SMARTS patterns. For a given structural alert, when a hit was identified the given structure from the sdf file was converted

to SMILES format which was then appended to a text file. The results of the screening (34 runs in total) were saved in a csv file for further analysis.

5.3. Results and discussion

5.3.1. The distribution of alerts

Out of the 35 structural alerts for idiosyncratic toxicity, related to reactive metabolite formation, available in oCHEM the vast majority (i.e. 32) were matched against at least one compound from the DrugBank dataset. The remaining alerts for which no matches were found were: disulfides, benzo-dioxolanes and bromoarenes. The distribution of the individual alerts is shown in Figures 5.1A.

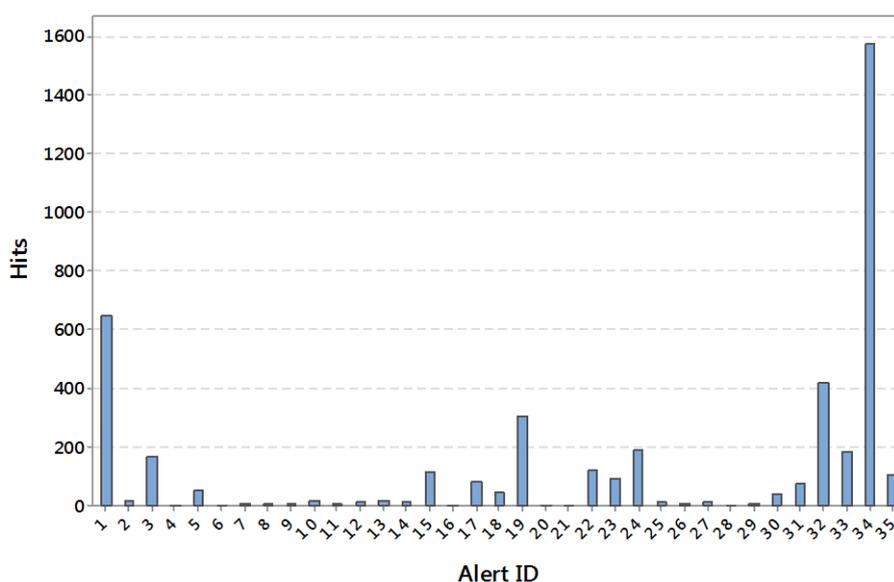


Figure 5.1 A. The histogram of reactive metabolism (RM) alerts found in the DrugBank dataset.

The total number of hits was 4342. Carboxylic acids (alert 34) and anilines/anilides (alert 1) were the most frequently matched alert (1587 and 645, respectively). Alkyl phenols (ortho and para) as well as Michael acceptors were also relatively frequent hits. For 52% of compounds from the DrugBank dataset no alerts were matched.

The high number of structural alerts for carboxylic acids in the dataset was relatively surprising. Although the $-\text{COOH}$ moiety is known to be crucial for the mechanism of action of some drugs, for instance non-steroidal anti-inflammatory drugs (NSAIDs), retinoids, cannabinoids, fibrate hypolipidaemic agents, diuretics, anticonvulsants, antitumour agents and antibiotics (Regan et al,

2010), it is also an acidic functional group that can influence an ionisation state of a drug, which in turn may affect solubility and lipophilicity, i.e. properties that contribute to the ADME profile. Acidic drugs are most likely to be completely ionised in pH values greater than 7 (Charifson & Walters, 2014). In the DrugBank dataset, the carboxylic group was present in 23% of all drugs considered (17% of approved drugs and 15% of withdrawn drugs).

It is possible for a compound to hit multiple alerts. As can be seen in Figure 5.1B, the total number of identified alerts for any compound was never > 4 and in a majority of cases one or two alerts were found (see Figure 5B).

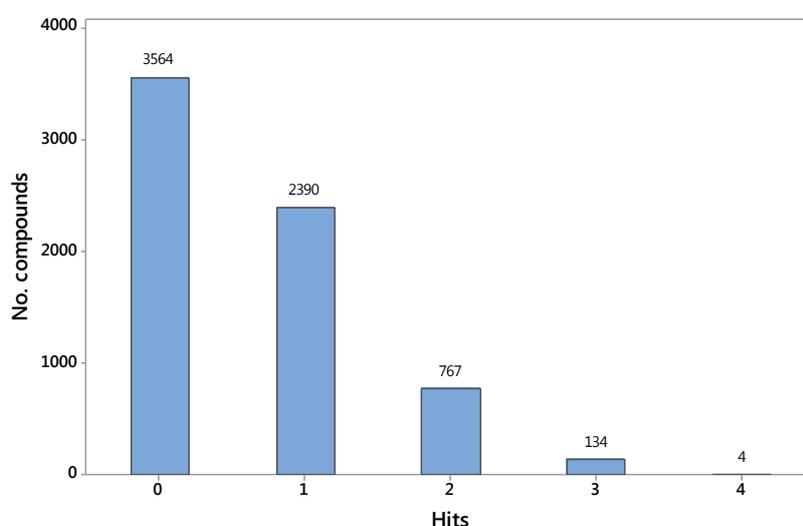


Figure 5.1B. The counts of compounds for each of the hits categories. The categories were formed on the basis of total number of hits for each compounds. 'Hit' is defined here as a presence of a structural alert.

One question that was considered was whether there was any correlation between the number of identified alerts and the class of a drug. The assumption was that the higher number of detected alerts may be associated with higher chances of idiosyncratic toxicity, simply because of the greater number of sites in a molecule that could potentially lead to the formation of reactive metabolites. None of the four compounds with the highest occurrence of alerts were found in the withdrawn class; one of them was an approved drug (fluticasone furoate), the remaining three molecules were experimental. Four compounds containing three structural alerts were found in the withdrawn or withdrawn/approved class: cefadroxil, fluprednidene acetate, bentiromide and bunamiodyl. Nevertheless, approved and experimental drugs constituted a much larger proportion of compounds containing three structural alerts. Out of 130 compounds with three alerts there were 48 drugs from the 'approved' category and 77 from the 'experimental' drug category. However, the absolute values could not give the correct picture, as the number of compounds in each class was different. Hence,

values relative to the number of compounds in each drug group were obtained. Figure 5.2 presents the frequencies of occurrence of structural alerts for the three different categories of drugs (approved, experimental and withdrawn).

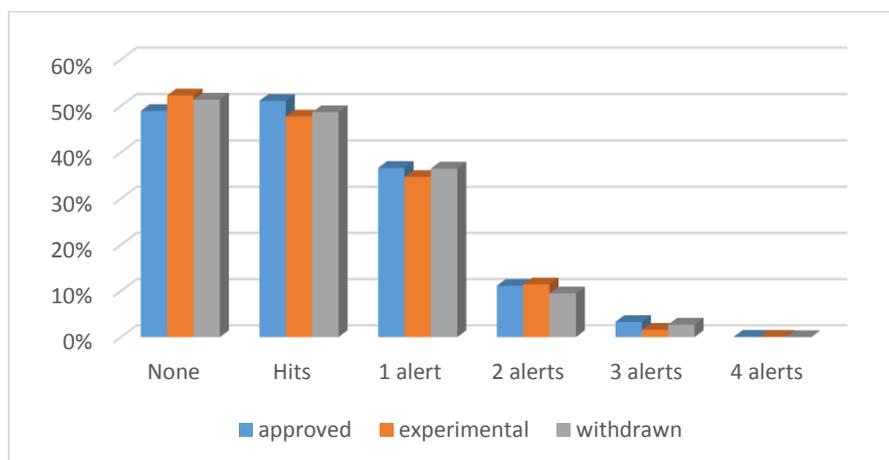


Figure 5.2. Distribution of numbers of alerts identified for three categories of compounds (approved, experimental, and withdrawn) from DrugBank dataset.

There is no significant difference in the number of alerts identified for each of the three categories of drugs (approved, withdrawn or experimental). Approximately 50% of compounds from each of these three classes contained no structural alerts. The fraction of compounds with one, two, three or four structural alerts was also similar across all three categories. Hence, it can be concluded that there was no correlation between the number of hits and the class to which the drug belonged, i.e. a higher number of hits was not associated with the 'withdrawn' class. It should be noted that idiosyncratic toxicity is only one of the many reasons due to which drugs may be removed from the market. Other reasons may be off-target toxicity such as hERG-channel inhibition. Similarly, some compounds may be approved and not withdrawn and yet they are known to be associated with adverse drug reactions. A famous example of such a drug is acetaminophen that can form a reactive metabolite (NAPQI - quinone-imine intermediate, which can lead to liver necrosis (Mitchell et al, 1973). Nevertheless, it is interesting that the distribution of alerts was similar for approved, withdrawn and experimental drugs. Additionally, the presence of relatively large number of hits for approved drugs was also an indication that identifying structural alerts can have a limited use as a predictive tool for determination of toxicity. Kalgutkar & Didiuk (2009) suggested that *in silico* methods, including the toxicophore approach, are not extensively applied to prediction of bioactivation in the context of drug discovery, because of limited validation studies.

5.3.2. Structural alerts – an issue of sensitivity and specificity

The rather poor specificity of the structural alerts in terms of prediction of toxicity may be related to the way in which the alerts are defined. The alerts describe structural features that can potentially be bioactivated, however, they do not contain any information on whether or not the process of bioactivation will actually occur. Other sites of metabolism (SOM) on a molecule may be favoured by a specific metabolising enzyme and in consequence, either no reactive metabolites are formed, or if they are formed they are only present as minor species at concentrations that do not reach the thresholds for toxicity. Furthermore, even the presence of reactive metabolites at higher concentrations may not necessarily result in adverse effects, due to elimination mechanisms such as glutathione (GSH) detoxification system that is capable of binding reactive species. All these factors have to be considered when assessing toxic effects. As discussed in section 5.3.1 the alert for a carboxyl group was found to be the most frequent alert for the DrugBank dataset. This moiety is known to be involved in the formation of reactive acyl glucuronide by conjugation of a parent drug with glucuronic acid (refer to Figure 5.3).

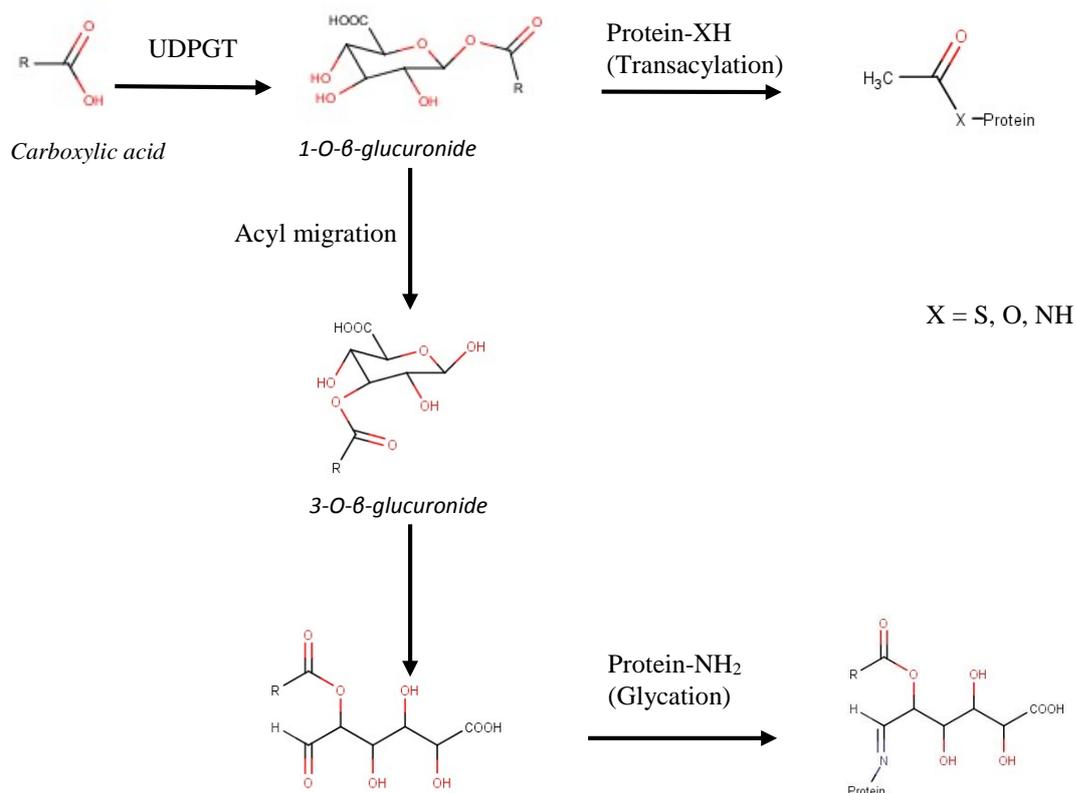


Figure 5.3. Mechanim of acyl glucuronide-mediated protein adduction. UDPGT is UDP glucuronosyltransferase.

The electrophilic acyl glucuronides can form protein adducts through transacylation or glycation reactions and as such they can lead to haptensisation of proteins, and in turn to evoke immunological responses. Such covalent protein binding has been shown to occur *in vitro*. However, *in vivo* toxicity of acyl glucuronides may depend on pharmacokinetic properties for instance, rapid elimination ensure that toxic concentrations are not reached. The intrinsic reactivity of acyl glucuronides has been shown to be dependent on the structure of a parent drug. Wang et al, (2004) indicated the following order of reactivity - acetic acid > propionic acid > benzoic acid as a result of differences in electronic and steric properties. Apart from structural features, physico-chemical properties may influence elimination. Moreover, the presence of a carboxylic acid group does not guarantee that glucuronidation will be the major metabolic process. For instance, the glucuronidation fractions for zomepirac and lumiracoxib (both NSAIDs) are 90% and 2.5%, respectively. Although 17% (263/1521) of all approved drugs contained a COOH group only a fraction of these will be implicated in idiosyncratic toxicity despite the fact that the formation of acyl glucuronides is the most important metabolism route for carboxylic acids (Skonberg et al., 2008). It is clear that the path from the presence of a structural alert to the occurrence of toxicity is not simple even without considering individual differences, such as age, health, specific genes expression level, etc..

The example of the carboxylic acid group exemplifies a typical challenge related to utilisation of structural alerts that is the possibility of a large number of false positives. This is a recurring theme for software packages that use structural information to define predictive rules. An example of such an *in silico* tool is Meteor Nexus, which was previously shown to have a tendency to “overpredict” potential metabolites (Piechota et al, 2013).

How can such a specificity issue be addressed, in relation to structural alerts? One thing that could be considered is to refine a structural alert so that it can be used as a more restrictive filter by taking into account additional structure-activity information. For instance, regarding the carboxylic acid group – instead of using a SMARTS pattern for a very general form of a carboxylic acid group (*R-COOH*), some SAR information could be incorporated to redefine the alert. As mentioned, acyl glucuronides reactivity is related to the aglycone structure. In practical terms, one SMARTS rule could be transformed into a number of more detailed rules. In the case of a carboxylic acid, a number of NSAIDs drugs withdrawn from the market contained aryl-alkyl acid (Kalgutkar et al., 2005) and this could form the basis for writing a refined version of the SMARTS pattern for the alert. This idea was explored for the carboxylic acid alert. The general SMARTS pattern was rewritten so that it could match aryl-alkyl acid, where the alkyl chain contained up to three carbon atoms and any

aryl group was present. This pattern could also be written in pseudoSMARTS as aryl-alkyl(C or CC or CCC)-COOH (see Figure 5.4.).

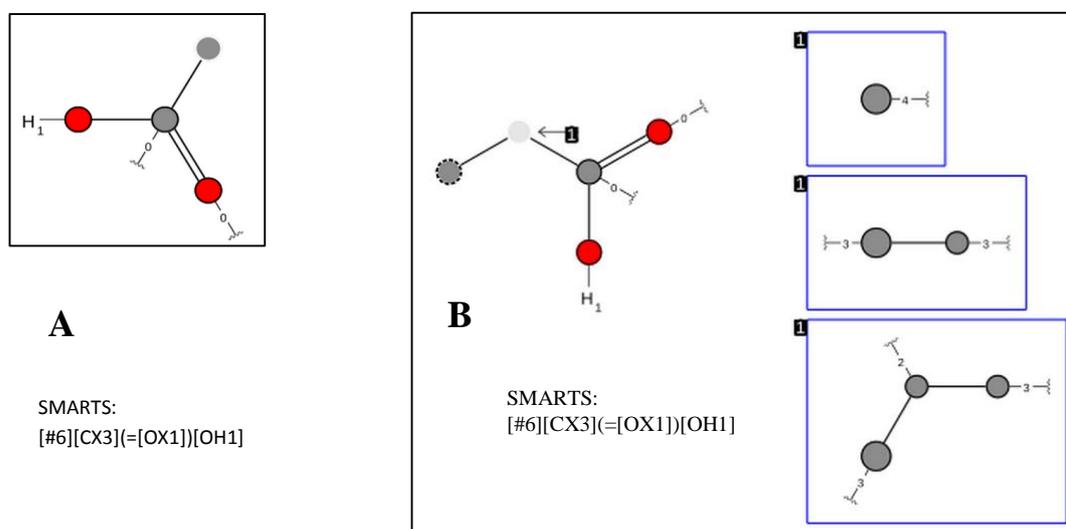


Figure 5.4. The original oCHEM structural alert for carboxylic acids (A) and its refined version (B).

The re-written SMARTS for carboxylic acids was used to test the full DrugBank dataset. The number of hits was reduced to 99 drugs. However, the new SMARTS rule missed a few drugs that contain carboxylic group and are known to cause idiosyncratic toxicities, such as valproic acid or amineptine. However, these drugs exert their toxicity through a different mechanism. They do not form acyl glucuronides but instead they can be oxidised by CYP450 to form reactive α,β -unsaturated carbonyl species, which can cause hepatotoxicity through inhibition of mitochondrial β -oxidation (Kalgutkar et al, 2005). Therefore, a refinement of SMARTS rules could be based on the mechanism of toxic action. This approach may be more useful than simply identifying a functional group as it carries more information relevant to drug discovery. Depending on the mechanism of action, the potential toxicity of a lead compound could be assessed by selecting a specific assay relevant to the structural alert and thus, possibly reduce the amount of experimental work required. However, it could also be argued that the more specific SMARTS rules can decrease the sensitivity and therefore, a cost/benefit scenario has to be considered when developing structural alerts. Alerts/toxicophores can only be as good as the results and the extent of *in vivo/in vitro* studies they are based on. In some cases, arbitrary decisions have to be included when defining the rule. For instance, a cut-off of up to three C atoms in the alkyl group was arbitrarily chosen for the refined SMARTS pattern for carboxylic aglycons.

Another factor to be considered when refining alerts is the amount of SAR information that can be translated into SMARTS rules. Benet et al, (1993) reported that for a series of NSAIDs the lower the number of substituents on the α -carbon (to the carboxyl group) was greater the capacity for covalent protein binding. The question arises as to whether such mitigating factors should be included directly into an alert or if separate rules should be written and their influence on toxicity indicated by a classification scheme. This could be developed in a similar manner to the relative reasoning engine developed within the software Meteor or Derek Nexus.

In conclusion, structural alerts for reactive metabolites are relatively common in drug molecules. These structural alerts were no more abundant in drugs that have been withdrawn from the market compared to drugs in use or experimental drugs. Although structural alerts may be useful for general screening purposes other factors, such as dosage or pharmacokinetic properties, must also be considered in a holistic approach to drug development. The development of more specific structural alerts would be beneficial in reducing the potential for false positives in screening. This has been demonstrated here, in the refinement of the carboxylic acid alert and indicates the future potential of this approach in drug development. Identification of additional alerts and validation of all alerts are also important but were beyond the scope of this thesis

6. Assessing the Suitability of Zebrafish Embryos for Predicting Acute Aquatic Toxicity

6.1. Introduction

Acute toxicity testing on fish is a requirement for hazard and risk assessment of chemicals (OECD, 1992a). It is also mandatory for obtaining toxicological data for the purpose of registering new chemicals. However, the REACH regulation (OECD/REACH, 2006) and the 3Rs philosophy to reduce, replace and refine animal tests, where possible, has led to greater interest in alternatives to animal testing for these purposes. Fish embryos are of interest as a potential alternative to testing on juvenile or adult fish and have already been introduced as an alternative in certain ecotoxicological scenarios. For instance, Germany introduced legislation in 2005 regarding mandatory testing of sewage effluent on fish embryos (Braunbeck & Lammer, 2006).

In terms of drug development there are also regulations concerning the assessment of the potential impact of drugs on environmental species (CDER, 1998; EMA, 2006; WHO, 2011). Active pharmaceutical ingredients (APIs) are usually released into surface waters via the sewage systems. Although the concentrations are low, the APIs are reported to remain in the environment for months or even years (Monteiro & Boxall, 2009). As drugs are designed with high safety margins they usually have low acute toxicity in mammals; this is also to some extent applicable to aquatic species. It has been estimated that acute toxicity to fish at levels below 1mg/L characterises less than 6% of all pharmaceuticals (Berninger & Brooks, 2009). However, environmentally persistent APIs can cause chronic effects, where longer duration of exposure is observed (Brooks et al., 2012). Despite low occurrence of acute toxicity in fish caused by APIs it is still important to predict such effects. The Verhaar scheme is a well-established predictive model used to categorise industrial chemicals by their mode of acute toxicity to aquatic organisms. Although historically, the Verhaar scheme has not been used to assess APIs, there has been a recent upsurge in interest in application of the scheme to pharmaceuticals (Vestel et al., 2015).

Currently, environmental assessment is generally performed towards the end of the drug development process. More rapid screens that could be used earlier in the process would be beneficial and the methods could also be applied to priority setting for assessing the environmental impact for existing drugs for which little data are available. Zebrafish embryos may provide a

solution to these testing requirements, however, there exists a need for assessment and validation of the suitability of fish embryos for predicting acute aquatic toxicity.

6.1.1. Use of Zebrafish embryos in toxicity testing

Zebrafish embryos/eleutheroembryos have been recognised as a potential replacement for fish acute toxicity testing. Lammer et al., (2009) have shown that fish embryo toxicity (FET) tests are equivalent to tests conducted on fully developed fish. Moreover, embryos can be also used to measure genotoxicity or possibly replace fish chronic toxicity test. The Gene-DarT project (gene expression Zebrafish embryo test) has attempted to establish gene markers that correlate with chronic toxicity (Scholz et al., 2008). Cardiotoxicity is another area where zebrafish embryo tests have been applied. *Danio rerio* expresses a hERG ortholog (zERG) that shares high similarity in the binding pocket region with the human version of the gene. It was shown that zebrafish embryos could be used to study hERG inhibition (Mittelstadt et al., 2008) or to observe QT prolongation effects (Langheinrich, Vacun & Wagner, 2003). Hepatotoxicity, for instance drug-induced liver injury (DILI), may be modelled in zebrafish early developmental stages such as the embryonic or larval stages (Jones et al, 2009; He et al., 2013). The transparency of the organism enables visual assessment of phenotypic characteristic for this particular endpoint. Zebrafish embryos have also been proposed for high-throughput screens for identification of small molecules that target common phenotypes associated with cancer (Terriente & Pujades, 2013).

Zebrafish embryonic development includes embryo and eleutheroembryo stages. The embryonated egg is enclosed in an acellular structure called chorion. The enzymatic breakage of inner chorion layer leads to a rupture of the chorion envelope, which is observed as the process of hatching. This occurs around 72h after fertilisation. The eleutheroembryo formed (sac fry) is still an autotroph and feeds on the yolk that is present in yolk sac. The difference between hatched and unhatched embryonic stages should be taken into account in the context of replacement of acute fish toxicity tests with fish embryo tests (FET). The chorion forms a permeability barrier and therefore the eleutheroembryo, lacking the chorion, would be more sensitive to tests (Embry et al., 2010). The question of the effectiveness of the chorion barrier is still open. Lipophilic substances have been shown to pass through chorion and accumulate in yolk (Wiegand et al., 2000). This accumulation is possibly due to the presence of the very lipophilic environment (phospholipids, triglycerides) of the yolk. This fact may also explain why older embryonic stages, which continue to consume yolk, may become more sensitive to chemicals. On the other hand, a set of highly lipophilic pesticides were shown to be more toxic to adult fish and larvae than to the embryo (Lammer et al., 2009; Wendler, 2006), which indicates that the chorion can prevent, at least to some extent, the intake of lipophilic

substances. In an attempt to avoid false negatives when using the non-protected stages of the embryo rather than fully developed organisms, the use of dechoriation procedures at 24 h post-fertilisation has been proposed (Henn & Braunbeck, 2011). Nevertheless, the work of Lammer et al., (2009) showed a very good correlation between the toxicity to the embryo and the toxicity to the eleutheroembryo.

The standard apical endpoints for assessing lethality to zebrafish embryos (LC50) include: i) coagulation of the embryo, ii) lack of somite formation, iii) non-detachment of the tail bud from the yolk sac and iv) lack of heart-beat. Exposure times typically used for toxicity assessment are 48h- 96h (OECD, 1992). A recent validation study that considered these endpoints and exposure times showed that zebrafish embryo acute toxicity tests (ZFET) have good inter- and intra- laboratory reproducibility. These authors also concluded that taking into consideration the good predictivity of the ZFET in relation to acute fish toxicity (Belanger et al, 2013) the ZFET could be considered as a valid alternative method of toxicity testing (Busquet et al. 2014).

6.1.2. Modes of acute aquatic toxicity

Acute aquatic toxicity can be characterised by two modes, namely general and specific toxicity. General toxicity, or narcosis, is a process that can lead to anaesthesia or stupor in organisms living in the aquatic environment. Narcosis is a non-specific and reversible mechanism that leads to the disruption of the functioning of the biological membrane which is caused by the accumulation of organic compounds in the lipid bilayer. Several categories of narcosis can be distinguished: non-polar, polar, ester and amine narcosis (Ren, 2002). Narcosis can be modelled solely with log P – a variable that describes the hydrophobicity of a compound. Using this descriptor it is possible to obtain a baseline toxicity model that will describe compounds exhibiting minimum toxicity, i.e. non-polar narcotics. Specific (excess) toxicity is closely related to the reactivity of chemicals. In this case, reactive compounds possess such electronic and steric properties that allow them to interact with specific biological entities (e.g. receptors) or to bind covalently to proteins, in both cases causing disruption of the cell function. Similar to narcosis, different mechanisms are involved in reactive toxicity: electrophilic interactions, oxidative phosphorylation uncoupling, central nervous system seizure or acetylcholinesterase inhibition (Gunatilleka & Poole, 1999). Compounds that act via such mechanisms are usually associated with toxicity higher than baseline. For instance, the excess toxicity of electrophiles is related to the fact that, in a biological context, they are capable of reacting with nucleophilic groups found in proteins. Such groups are present in nitrogen and sulphur containing side chains of amino acids and include: thiol (cysteine), sulphur atom (methionine), and

primary amino moiety (lysine), (Schwöbel et al, 2011). Typical reactions include: aliphatic and aromatic nucleophilic substitution, Michael addition or Schiff base formation (Enoch et al, 2011).

6.1.3 QSAR modelling of narcosis

Many QSAR models exist for prediction of non-polar and polar narcosis. *Pimephales promelas* (fathead minnow), *P. reticulata* (rainbow trout), *Danio rerio* (zebrafish), *Daphnia magna* (water flea) or *Tetrahymena pyriformis* (protozoa) are among common aquatic organisms used for testing purposes. The majority of reported QSAR models use regression techniques such as (multi-)linear regression or Partial Least Squares analysis; log P has been shown to be the most important descriptor. For instance, Verhaar, Mulder & Hermens, (1995) obtained high quality models for non-polar ($n = 58$, $R^2 = 0.94$, $Q^2 = 0.93$) and polar narcosis ($n = 86$, $R^2 = 0.90$, $Q^2 = 0.90$) for fathead minnow. Another study (von der Ohe et al, 2005) obtained a baseline toxicity model for *Daphnia magna* using a set of 36 compounds ($R^2 = 0.90$, $Q^2 = 0.94$); they also built a regression model for polar narcosis for a group of 33 chemicals these had been classed as Verhaar class 2 (see section 6.1.4), however anilines were excluded. Ellison et al, (2008) obtained a regression model for non-polar narcosis (*Tetrahymena pyriformis*) using a training set of 87 classic non-polar narcotics (alcohols, ketones) with $R = 0.97$. Recently, Zhang et al, (2013) reported regression models for non-polar and polar narcosis for *Tetrahymena pyriformis* and *Daphnia magna*. They also analysed interspecies correlation of toxicity values and concluded that there exists a poor relationship between the toxicity to *T. pyriformis* and the toxicity to *D. magna* with significant differences observed for anilines and their derivatives.

QSAR studies have also been carried out on data obtained from zebrafish embryos. Van Leeuwen, Adema, Hermens, (1990) showed excellent correlation of the toxicity to zebrafish embryo and the hydrophobicity descriptor (log P) both for inert compounds (chlorobenzenes, $n = 6$) and polar narcotics (anilines, $n = 6$). Kammann, Vobash & Wosniok, (2006) studied the effect of brominated phenols and indoles. They concluded that bromophenols are not associated with excess toxicity in zebrafish embryos but rather act as non-polar narcotics. Ding et al, (2013) identified that hydrophobicity (indicated by a calculated value for log P), the energy of the lowest unoccupied molecular orbital (E_{LUMO}) and shape descriptors (e.g. molecular shape factor) correlated with the toxicity of triazole fungicides ($n = 17$) to zebrafish embryos.

6.1.4. The Verhaar scheme

The well-established Verhaar scheme (Verhaar, Leeuwen & Hermens, 1992) offers a way of categorising chemicals according to different mechanisms of aquatic toxicity. The Verhaar scheme originated from previous works of McKim et al., (1987) and Bradbury et al., (1990) who studied the

physiological response of immobilised rainbow trout exposed to a range of industrial chemicals that were known to be present in the aquatic environment. The analysis of the data allowed the characterisation of six distinct fish acute toxicity syndromes (FATS): non-polar narcosis, polar narcosis, uncoupling of oxidative phosphorylation, acetylcholinesterase inhibition, central nervous system seizure, and respiratory membrane irritation. Veith & Broderius, (1990) showed that two types of narcosis could be distinguished, namely, narcosis I syndrome (baseline) and narcosis II syndrome. Their conclusion was based on an observation that chemicals such as anilines and phenols did not produce a strictly additive toxicity when combined with octanol (a reference baseline chemical). This indicated that phenols, anilines and their derivatives, exert greater toxicity than predicted by the baseline model, i.e. they act via a different mechanism of action. Hermens, (1989) analysed a number of aquatic toxicity models for various groups of compounds and concluded that whilst octanol-water partition coefficient (K_{ow}) is the most important descriptor for modelling aquatic toxicity of less reactive compounds, it needs to be combined with descriptors related to electronic and steric features in order to model more reactive chemicals. Verhaar et al., (1992) further developed the work by Hermens and defined four classes of compounds on the basis of physicochemical properties (e.g., K_{ow} or MW ranges) and by consideration of structural characteristics for each class. The following four classes of compounds were distinguished:

- i. Non-polar narcotics or inert chemicals – this includes compounds that are not reactive and also do not exert any specific mode of action, for instance by acting on molecular receptors. Toxicity of non-polar narcotics can be modelled by taking into account only their hydrophobicity. These inert compounds determine baseline toxicity (e.g. aliphatic alcohols, ketones).
- ii. Polar narcotics or less inert chemicals – this class encompasses compounds that are not reactive but exert higher than baseline toxicity. The term “polar” refers to a relatively common characteristic of such compounds, namely they possess functional groups that can act as hydrogen bond donors (e.g. anilines, phenols).
- iii. Reactive chemicals - a very diverse category of compounds which are reactive and can covalently bind to side chains of amino acids (e.g. α,β -unsaturated carbonyl compounds, imines) .
- iv. Specifically acting chemicals – this group comprises compounds that bind non-covalently to biological receptors (e.g. phosphate esters acting as acetylcholinesterase inhibitors).

Verhaar et al., (1992) rationalised their scheme by the analysis of calculated toxic ratios for a dataset of 166 compounds. The toxic ratio (TR) was defined as a ratio between the baseline LC_{50} , and the

experimental LC₅₀. The analysis of log TR distributions showed that polar narcotics had, on average, nearly 1 log unit higher toxic ratios in comparison to the baseline model. Similarly, the mean log TR values for compounds from classes 3 and 4 were almost 2 log units higher. It was also found that non-polar narcotics and polar narcotics formed two distinct classes while no significant difference for classes 3 and 4 chemicals was observed, which in turn suggested that classes iii and iv are not composed of homogenous data. Verhaar et al., (2000) successfully validated their aquatic toxicity classification scheme on a dataset of 176 compounds. In addition, they also reclassified some groups of compounds (e.g. phthalate esters were reassigned from class iii to class i. It should be noted that the Verhaar scheme was developed in the context of organic compounds of industrial origin and as such it may not be directly applicable to pharmaceuticals despite the aforementioned interest in the environmental fate of APIs.

The Verhaar classes have been implemented in the Toxtree software (Toxtree 2.6.0, <http://toxtree.sourceforge.net>). This open source application uses a decision tree approach to estimate toxic hazard. The method organises the query compounds into five categories; four of them represent the aforementioned types of aquatic toxicity whilst the fifth category is reserved for compounds that could not be classified into any of the Verhaar classes. The initial implementation of the Verhaar scheme rules was updated following the work of Enoch et al, (2008) who evaluated the performance of this method and suggested a number of improvements to avoid misclassification.

The aim of this chapter was to build robust baseline QSAR models using zebrafish embryo toxicity data, in order to investigate whether such models can distinguish between baseline and excess toxicity. The results should add to the discussion whether the early developmental stages of this species can be used as an alternative to fish toxicity tests in environmental assessment of new and existing chemicals (including drugs). Additionally, the Verhaar scheme was investigated, in the context of toxicity to zebrafish embryos, to determine whether improvements to the rules could be proposed for specific chemical classes i.e. aliphatic amines.

6.2. Methods

6.2.1. Datasets

Two datasets were used for the analysis of acute toxicity to zebrafish embryo: DS1 (training set) and DS2 (test set).

DS1 (training set):

This dataset was extracted from Lammer et al., (2009). The authors collected fish embryo (E) and leutheroembryo (ELE) toxicity data from various sources. 203 toxicity values (EC50, expressed in $\mu\text{g/L}$) were present for a wide range of compounds. Zebrafish (*Danio rerio*) was the main species considered. Others included: fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*) and *Clarias gariepinus*. Experiment duration times varied from 24h to 120h. The effects measured included lethal and sublethal apical endpoints, mostly developmental anomalies.

In order to build QSAR models a subset of the data was selected. It included only zebrafish species, both E and ELE (i.e. hatched but not yet free feeding embryos) developmental stages. The whole range of exposure times (24h – 120h) was present. Highly volatile compounds (e.g. acetone) and metals or their salts (e.g. cadmium or cadmium chloride) were excluded. Measurements obtained for compounds with high volatility (high vapour pressure at room temperature) may be a subject to large experimental differences. Many software packages do not calculate molecular descriptors for inorganic substances due to the lack of defined molecular graphs for such compounds (Toropova et al., 2010). Similarly, substances with ambiguous names (e.g. high solubility alkyl sulphate) were also removed as it was not possible to generate SMILES strings for such compounds. EC50 values were converted to μM units and then $\log(1/\text{EC50})$ values were obtained. When multiple toxicity data were present for a compound, mean values were calculated. The final dataset consisted of 117 compounds.

DS2 (test set):

The dataset was derived from Belanger et al., (2013). This publication contained fish embryo (zebrafish, fathead minnow, medaka and *Clarias gariepinus*) toxicity values (LC50 ($\mu\text{g/L}$)) extracted from scientific literature for 228 compounds. All compounds that were duplicates of structures present in DS1 were excluded. Additionally, where compounds could not be identified by their CAS numbers (13 compounds in total) these were removed from the analysis. With regard to selecting species, experimental variables, removing certain classes of compounds and calculating mean toxicity values, the same criteria were used as for DS1. This resulted in 76 compounds remaining for investigation. Those compounds that were assigned to Verhaar classes 1 and 2 and had $\log P$ values within the range of those from the models obtained for the Lammer dataset were used to assess the utility of the regression equations developed using the Lammer data (DS1). The remaining compounds were used to investigate excess toxicity.

For both DS1 and DS2, the structures in SMILES format were obtained from ChemSpider (<http://www.chemspider.com/>) by manual search using CAS numbers. Finally, both datasets were stored in Microsoft Excel format for further analysis (see also Appendix X).

6.2.2. Calculating molecular descriptors

All compounds (from both DS1 and DS2; 193 in total) were 'washed' (i.e. inorganic counterions removed) and energy minimised by MOE (Molecular Operating Environment) software (ver. 2011.10, Chemical Computing Group).

- KOWWIN version 1.67a (a program that can estimate the log octanol-water partition coefficient of chemicals using an atom/fragment contribution method) was used to extract experimental log P values providing these were available; otherwise values that were predicted using KOWWIN were used. Overall, experimental log P values were present for 141 compounds out of 193 substances from both training and test datasets.

6.2.3. Classifying compounds according to the Verhaar scheme

The Verhaar scheme (modified) as implemented in Toxtree 2.6.0 was used for grouping compounds into categories for aquatic toxicity. Subsequently, these groups were refined manually by subdividing these Verhaar classes into smaller groups that were considered potentially relevant to aquatic toxicity (such as primary amines or phenols and anilines).

6.2.4. Statistical analysis

- Minitab (version 17.1) was used to perform statistical analysis and to generate plots. Linear regression equations and their associated statistical parameters e.g. the standard error (S), the square of the correlation coefficient (R^2) and R^2 adjusted for degrees of freedom were recorded; outliers were removed, where applicable, as discussed below.

6.3. QSAR models for Verhaar classes

6.3.1. Preliminary analysis

The initial step in the analysis of the relationship between the hydrophobicity descriptor (log P) and toxicity to zebrafish embryos was to inspect visually the distribution of Verhaar classes for all compounds in DS1. No attempt was made to build a global model in the true sense of this term due

to the fact that not all the compounds in DS1 exhibited the same mode of toxic action. It would not be appropriate to include aliphatic alcohols (known non-polar narcotics) and aromatic alcohols (known polar narcotics) in the same model. Instead, all the compounds from DS1 were classified according to the Verhaar scheme (Table 6.1) and then plotted as colour-coded groups (Figure 6.1).

Table 6.1. The results of Verhaar classification for DS1.

Verhaar class	No. of compounds (Toxtree)
1 (non-polar narcotics)	27
2 (polar narcotics)	32
3 (reactive)	7
4 (specifically-acting)	5
5 (non-classified)	46

Initially, plotting all Verhaar classes (Figure 6.1) shows that the non-polar narcotics (class 1) define a level of baseline toxicity, as expected. Similarly, a regression line for class 2 compounds, shows that polar narcotics have higher toxicity, in comparison to the less inert, polar narcotics. Class 2 compounds show correlation with log P but fall on a separate line. Compounds that belong to classes 3, 4 are scattered reflecting a lack of correlation with log P; this again is as expected as these compounds act via diverse modes of action (e.g. specific receptor interactions or covalent binding to proteins).

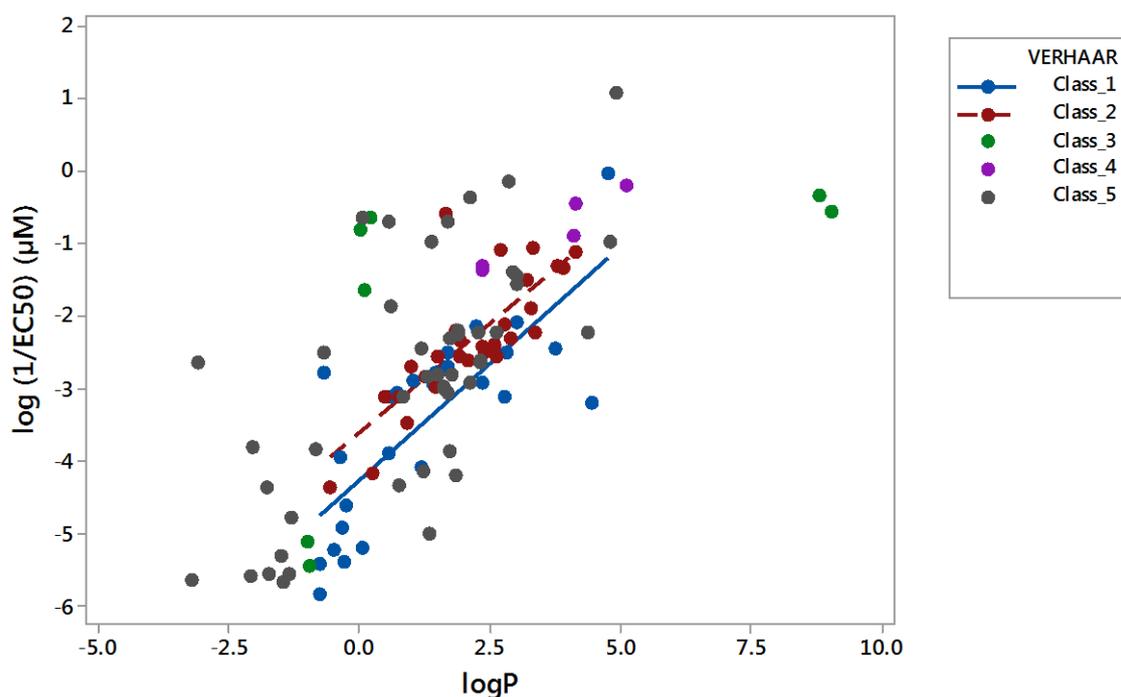


Figure 6.1. Scatterplot of toxicity vs log P for all Verhaar classes for compounds from DS1 ($n = 117$). Regression lines shown are for compounds from class 1 (blue line) and class 2 (dashed red line); refer to text.

Table 6.1 shows that for a significant number of compounds (39%), no decision could be made as to class allocation, therefore they are found in class 5. Class 5 compounds do not show any particular trend; however, many compounds from this category show toxicity lower than baseline which may indicate experimental errors in determining their toxicity. It is possible that some of these compounds should in fact be classified as non-polar narcotics and this possibility will be considered later in this chapter.

6.3.2. Verhaar class 1 compounds

27 compounds from Verhaar class 1 were used to obtain a baseline toxicity model. The initial regression analysis is presented in Figure 6.2.

The determination coefficient for the Verhaar class 1 model was not very high when toxicity was plotted against log P ($R^2_{adj} = 60.7\%$; $s = 0.83$). However, visual inspection of the scatterplot indicated the presence of at least three “obvious” outliers: methoxyacetic acid, tributylamine and triclosan (an aromatic ether). These compounds had large residuals: 1.9, -1.8 and 1.2, respectively. If the maximum residual permissible was set to ± 1 , and only those compounds that were found within these boundaries were included in the model, methanol could also be considered an outlier (residual

= -1.1). It was decided not to remove methanol from this model, as it is a classic example of a non-polar narcotic. The reasons for the presence of the outliers were investigated. First of all, the toxicity and log P values were investigated. For methoxyacetic acid and tributylamine only estimated log P values could be used and toxicity values originated from a single measurement, respectively. These factors could influence the quality of the data points for modelling however, they are not conclusive; in the case of triclosan, two relatively close experimental values for toxicity were present along with a measured log P but this compound also appears to be an outlier. None of the outliers are considerably volatile (low vapour pressures) a factor known to influence bioavailability. It may be the case that methoxyacetic acid has been misclassified. In Figure 6.2 it appears to be a polar narcotic, which can be supported by the presence of a polar, carboxylic group. Tributylamine and triclosan are relatively insoluble, potentially reducing their bioavailability, in addition their log P values are high (4.46 and 4.76, respectively), hence their toxicity may not show a linear dependence on log P. The toxicity of triclosan is underpredicted whilst tributylamine is over-predicted. Tributylamine is a pharmaceutical and may exert greater toxicity than that predicted by baseline narcosis. In order to develop a more robust baseline QSAR model these three outliers were removed due to the significant leverage effect they exert. This resulted in the following regression equation (Eq. 6.1):

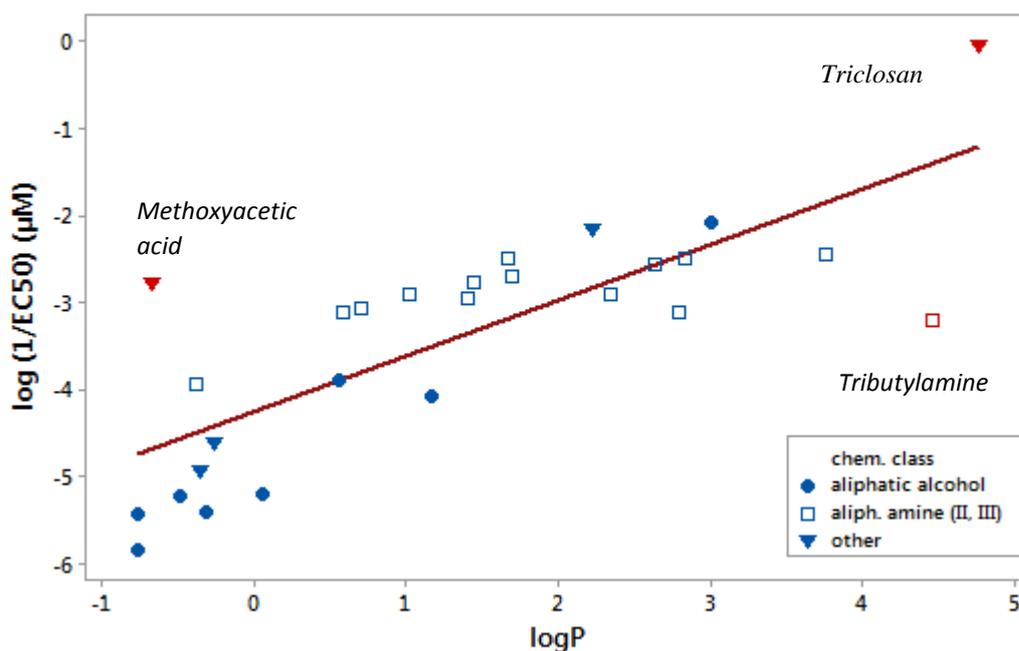


Figure 6.2. Regression model for compounds from Verhaar class 1 ($n = 27$).

$$\text{Log}(1/\text{EC50}) = -4.44 + 0.77 \text{ Log } P \quad (\text{Eq. 6.1})$$

$$N = 24, S = 0.61, R^2 = 75.1\%, R^2_{\text{adj}} = 74.0\%$$

The removal of the three outliers significantly increased the correlation coefficient as well as reduced the standard error of the previous model (previously $R^2_{\text{adj}} = 60.7\%$; $s = 0.83$).

6.3.3. Verhaar class 2 compounds

Similarly, an attempt was made to build a model for Verhaar class 2 compounds. The previous analysis of all compounds in DS1 (refer to figure 6.1) showed that compounds from Verhaar class 2 showed greater toxicity in comparison to class 1 compounds.

In this case visual inspection did not indicate significant outliers. Closer analysis of the residuals (based on Eq. 6.2) also did not reveal any outliers. The largest residuals were found to be 0.96 for 3,4-dichloroaniline and -0.61 for isopropylamine; however, removing these data points would not be justified considering the previous assumptions about the residuals range. Therefore, all 32 compounds were used to build the final model for Verhaar class 2 (Figure 6.3, Eq. 6.2). The statistics (both R^2 and S) of the model for Verhaar class 2 compounds indicated a good relationship between $\text{Log } P$ and toxicity.

$$\log(1/\text{EC50}) = -3.73 + 0.63 \text{ Log } P \quad (\text{Eq. 6.2})$$

$$N = 32, S = 0.36, R^2 = 80.2\%, R^2_{\text{adj}} = 79.6\%$$

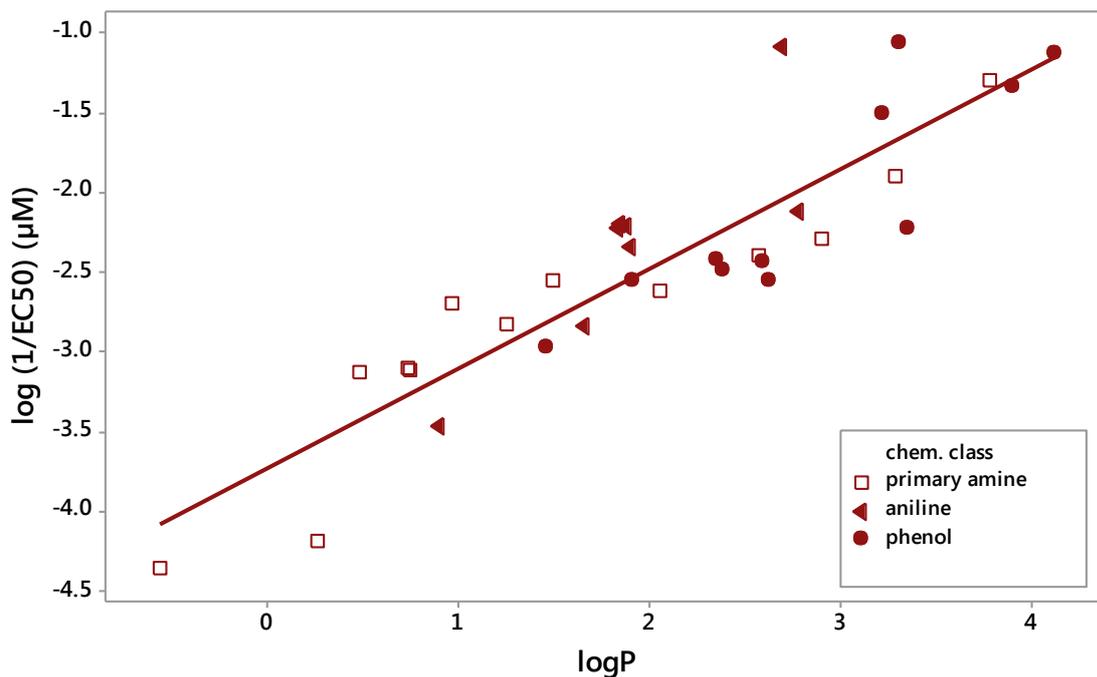


Figure 6.3. Regression model for Verhaar class 2 compounds ($n = 32$) where different chemical groups have been identified.

6.4. Refinement of models

The two models built so far for Verhaar classes 1 and 2 showed that zebrafish embryos could be used to model the relationship between the hydrophobicity descriptor and toxicity. However, the question whether they could be used to distinguish between non-polar and polar narcosis may be more difficult to answer. The preliminary analysis showed that zebrafish embryos can distinguish between non-polar and polar narcotics (or at least between Verhaar class 1 and class 2 compounds). On the other hand, a closer examination of these two Verhaar classes (Figure 6.4) revealed that for compounds with log P values within a range of about 0.5 to 2.0 log units the distinction between the non-polar and polar narcotics was less clear. An additional issue may be related to the fact that a lot of compounds from class 1 had toxicities lower than that predicted by the baseline model (Eq. 6.1, Figure 6.4) and this included classic non-polar chemicals such as aliphatic alcohols. This may indicate that the baseline was not defined correctly which in turn may render the discussion about different types of toxicity irrelevant.

So far, the models were solely based on the Verhaar classification without a detailed analysis of compounds that belonged to each particular class. However, these models may need further refinement in order to provide more robust regression equations, if possible. One of the ways to approach this issue is to divide the Verhaar classes 1 and 2 into smaller groups of compounds. A similar approach is utilised by ECOSAR software (the US EPA). This program is used to estimate the aquatic toxicity of industrial chemicals. It distinguishes between three major classes of compounds: i) neutral organics, ii) organics with excess toxicity and iii) surfactant organic chemicals. These general categories are further subdivided into distinct chemical classes for which individual, more accurate, SAR models have been built. For instance, neutral organics class includes such subclasses as: alcohols, aliphatic hydrocarbons, alkyl halides, aromatic hydrocarbons, arylhalides, cyanates, disulfides, ethers, ketones, and sulphides (EPA, 2012).

Figure 6.4 shows this attempt to group the chemicals from Verhaar classes 1 and 2 into smaller chemical categories. Grouping compounds was based on the possible mechanism of action. Plotting Verhaar class 1 and 2 subcategories on the same graph was intended to provide some insight into the question about poor segregation of non-polar and polar narcotics within the mentioned log P range. The distribution of compounds in both Verhaar classes is summarised in Table 6.2. The most numerous group of compounds were aliphatic amines as they comprised nearly half of the chemicals. The primary aliphatic amines comprised 40% of all compounds from class 2 while secondary and tertiary aliphatic amines constituted 54% of chemicals belonging to class 1. The rest of the compounds from class 1 were mostly aliphatic alcohols. The group "other" contained otherwise unclassified compounds and was composed of an ether, a ketone and an acid and as such this group is excluded from further analysis. In addition to aliphatic amines, Verhaar class 2 also included anilines and phenols. The subgroups defined here are relatively homogenous with the exception of "other" class. The presence of a large number of amines in both Verhaar classes affords the opportunity to build a separate model(s) for this group. The Toxtree implementation of Verhaar scheme classifies amines as either belonging to Verhaar class 1 (secondary, tertiary) or to Verhaar class 2 (primary). Nonetheless, it is worth exploring the idea of separate model(s) for amines to see whether zebrafish embryos could distinguish this type of narcosis. Another reason to consider amines separately is to determine whether this would lead to an improved model for baseline toxicity.

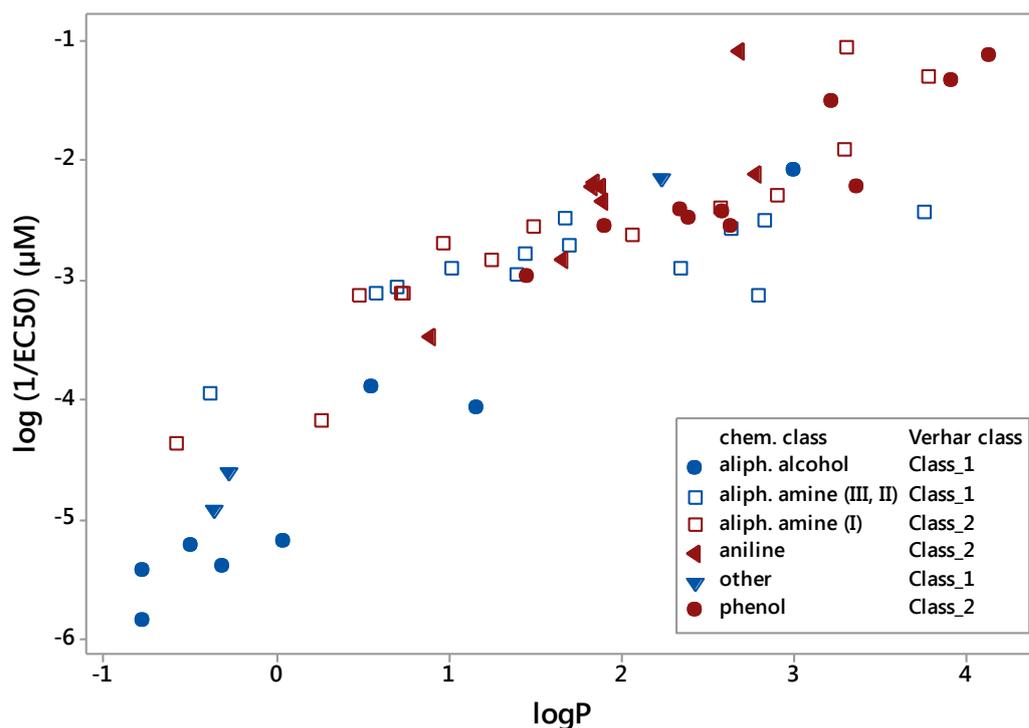


Figure 6.4. Chemical subcategories for Verhaar classes 1 and 2. Groups are depicted by different shapes and classes differentiated by a colour scheme (blue = class 1, red = class 2). Note: primary aliphatic amines are in Verhaar class 2 while secondary and tertiary aliphatic amines are in Verhaar class 1. All amines are represented as empty squares.

Table 6.2. The distribution of compounds for different chemical subgroups from Verhaar classes 1 and 2 for DSI.

Chemical group	Verhaar class 1	Verhaar class 2
Anilines	0	8
Phenols	0	11
Primary aliphatic amines	0	13
Secondary and tertiary aliphatic amines	13	0
Aliphatic alcohols	8	0
Other	3	0
Total	24	32

6.4.1. Refined baseline model

The first step to improve the baseline model was to exclude aliphatic amines from Verhaar class 1 compounds. The refined baseline model was based on eight aliphatic alcohols and three compounds from “other” category. Standard error (S) was significantly decreased while a coefficient of

determination increased to 92%; the largest residual had a value of 0.46. The refined baseline model is described by Eq. 6.3 (see also Figure 6.5).

$$\text{Log}(1/\text{EC50}) = -4.80 + 0.98 \text{Log } P \quad (\text{Eq. 6.3})$$

$$N = 11, S = 0.37, R^2 = 92.7\%, R^2_{\text{adj}} = 91.9\%$$

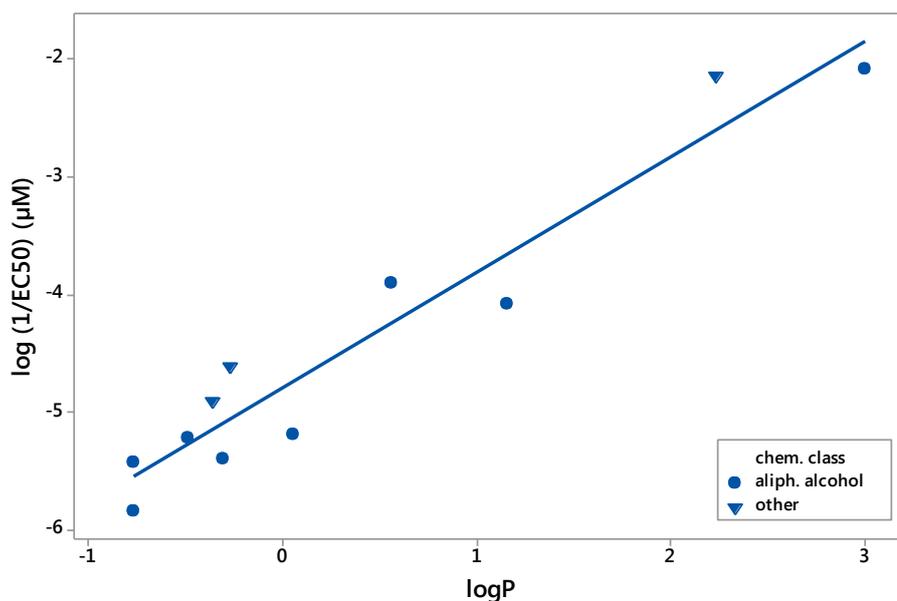


Figure 6.5. A refined baseline toxicity model for zebrafish embryos. The compounds included are aliphatic alcohols and three compounds from “other” subgroup ($n = 11$).

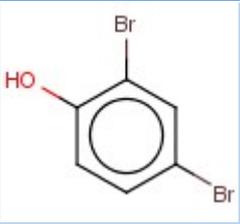
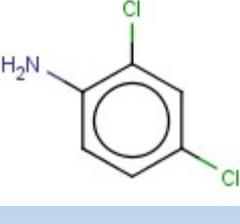
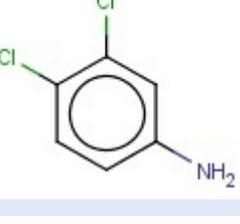
6.4.2. Refinement of model for polar narcotics (phenols and anilines)

This model was based on compounds from Verhaar class 2. The possibility of improving the model was explored by removing aliphatic primary amines so that only anilines and phenols were subject to modelling.

After removing the aliphatic primary amines from Verhaar class 2, the initial model (regression equation not shown) for phenols and anilines had $R^2_{\text{adj}} = 64.8\%$ so it was reduced in relation to R^2 obtained for Eq. 6.2. Phenols and anilines are usually associated with the polar narcosis mechanism and, therefore, a better model was expected in this case. One of the ways to improve the model was to identify statistical outliers. The analysis of residuals plot showed that 3,4-dichloroaniline was the compound most significantly under-predicted whilst 2,6-dibromophenol was the most over-predicted (see Table 6.3). However, the residual values were still within the previously considered range of ± 1.0 , therefore, removal of these compounds would not be justified.

A further step in an attempt to refine the polar narcotics model was to perform the analysis of chemical structures. This revealed the presence of two pairs of homologous compounds and within each pair relatively high differences between toxicity values were present despite log P being similar. 3,4-dichloroaniline and 2,4-dichloroaniline had toxicities that differed by more than 1 log unit. Similarly, 2,4-dibromophenol is fivefold (μM units) more toxic than 2,6-dibromophenol (Table 6.3). The chemical differences between the compounds in each of these two pairs (relative halogen positions) could not sufficiently explain such large differences between toxicity values. Most likely, experimental errors may account for these discrepancies.

Table 6.3. Four halogenated compounds from the model for polar narcotics. The residuals values were based on the model for phenols and anilines (data not shown).

Compound name	Structure	Log(1/EC50)(μM)	Log P (experimental)	Comments
2,4-Dibromophenol		-1.50	3.22	residual = 0.18
2,6-Dibromophenol		-2.22	3.36	overpredicted (residual = -0.63) potential outlier
2,4-Dichloroaniline		-2.12	2.78	residual = -0.16
3,4-Dichloroaniline		-1.09	2.69	underpredicted (residual = 0.93) potential outlier

An attempt to build a new model after removing the two compounds with highest residuals (3,4-dichloroaniline and 2,6-dibromophenol) resulted in a regression equation (for phenols and anilines) with improved statistics. The determination coefficient was very close to that obtained for initial

Verhaar class 2 that included primary aliphatic amines (Eq. 6.2) while standard error was reduced even further. Despite this improvement it was eventually decided not to remove these two compounds from the QSAR model for phenols and anilines, mainly due to being consistent with the chosen residuals range. Nevertheless, it was suspected that 3,4-dichloroaniline was an outlier in both Eq. 6.2 and a model for phenols and anilines.

Another question arose whether the first step of the refinement process for polar narcosis model, i.e., removing primary aliphatic amines from Verhaar class 2, was justified? It seems that the answer should be negative in this case. First of all, a high $R^2_{adj} \approx 80\%$ achieved for Verhaar class 2 model (Eq.6.2) was obtained for a larger number of compounds and the quality of predictions based on QSAR models was shown to correlate positively with the dataset size (Gedeck, Rohde, Bartels, 2006). Secondly, the intermediate model for phenols and anilines had to be improved by a more detailed analysis of potential chemical outliers. This indicates that the model represented by Eq.6.2 is more robust and – to apply Occam’s razor - the described refinement steps are not required. Therefore, it could be concluded that for zebrafish embryos primary aliphatic amines should be modelled along with anilines and phenols as polar narcotics.

Reliable assignment of compounds into their respective classes within the Verhaar scheme is important as each class is associated with different modes of action and this, in turn, may be reflected in toxicity values. The obtained QSAR model for Verhaar class 2 (Eq.6.2) did not benefit from removing a group of primary aliphatic amines. On the other hand, the model for non-polar narcotics was improved by removing aliphatic amines (both secondary and tertiary) although at an expense of significant reduction in the size of the modelled data (by more than 50%) and also a possibility of model overfitting. This could potentially compromise the robustness of the baseline model and this will be addressed later during the validation of the models using an external test set.

6.4.3 Aliphatic amines models

The previous analysis showed that primary amines modelled well together with other compounds from Verhaar class 2, i.e. phenols and anilines. However, secondary and tertiary aliphatic amines were removed from Verhaar class 1 to improve the baseline model. This may indicate that secondary and tertiary aliphatic amines exert excess toxicity and as such they could potentially be modelled with other aliphatic amines (primary). If this is the case, tertiary and secondary amines could be potentially categorised as Verhaar class 2 compounds, i.e. polar narcotics. This may add to a discussion about the usage of such tools as Verhaar scheme for regulatory and risk assessment purposes in the context of the REACH legislation (Enoch et al., 2008).

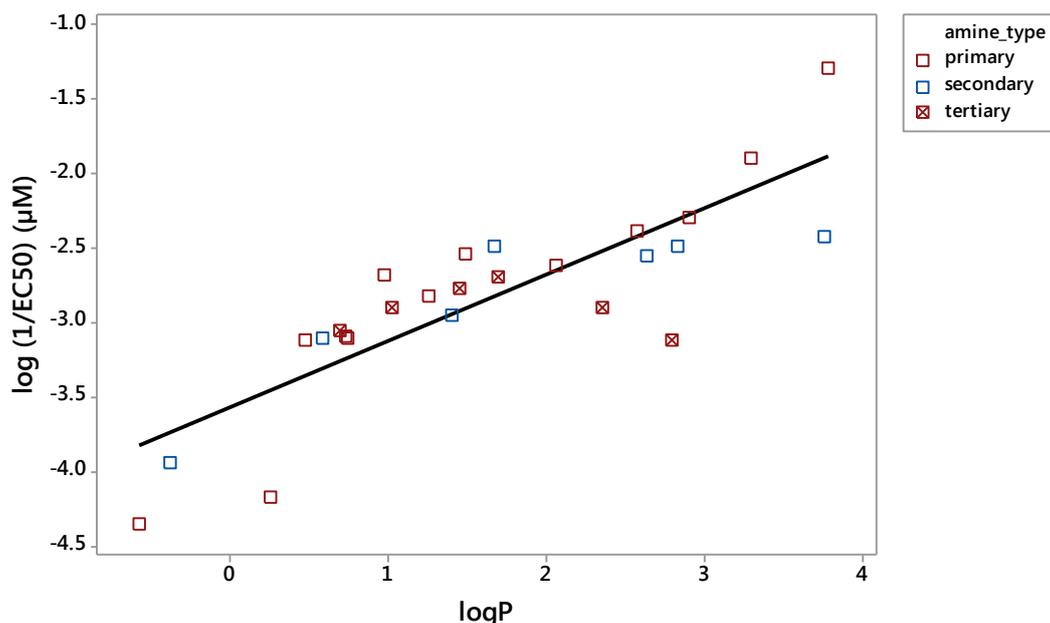


Figure 6.6 Regression model for primary, secondary and tertiary aliphatic amines ($n = 26$).

Aliphatic amines have been successfully modelled as a separate category of compounds in the context of acute aquatic toxicity to fish. Newsome et al., (1991) developed a QSAR model ($R^2 = 84\%$, $S = 0.56$) for a dataset of 41 aliphatic amines using solely log P. This model was validated for a larger dataset of aliphatic amines ($n = 61$) and a very similar model was obtained in terms of goodness-of-fit (Newsome, Johnson & Nabholz, 1993). Later studies indicated that amine narcosis might be a separate mechanism of toxic action (Schultz, Jaworska & Hunter, 1995b). Sinks, Carver & Schultz, (1998) studied 22 aliphatic amines in *Tetrahymena pyriformis* and they found that difference in intercept between the previous model for aliphatic alcohols (non-polar narcosis) and their regression equation was 0.6 log units, which again indicated that amines might exert excess toxicity. Their model for IGC50 (50% inhibitory growth concentration) used log P to explain the differences in toxicity and although it was developed for primary aliphatic amines the authors found it applicable to prediction of toxicity for other aliphatic amines in their dataset (four compounds). Further evidence that indicated the enhanced toxic effect of aliphatic amines in aquatic organisms was provided by Dimitrov et al., (2003). They also identified hydrophobicity (log Kow) to be the only significant variable that could explain the changes in LC50 values.

A model was developed for all 26 aliphatic amines present in DS1 (Figure 6.6, Eq. 6.4). The goodness-of-fit was worse than in case of models for non-polar and polar narcosis. The lower R^2_{adj} (68%) could be a result of the presence of possible outliers with large residuals. Isopropylamine and

tripropylamine were potential candidates with the largest absolute residuals (-0.8 and -0.7, respectively). Interestingly, isopropylamine was also indicated to be a potential outlier in the model for Verhaar class 2 compounds (Eq. 6.2). The removal of these outliers would result in $R^2_{adj} = 77\%$. Nevertheless, no compounds were removed from the global aliphatic amines model as they fulfilled the residual range criteria (± 1).

The developed models for subclasses of aliphatic amines (Eq. 6.5, Eq. 6.6 and Eq. 6.7 – see Table 6.4) were of different quality with primary aliphatic amines showing the best correlation between log P and toxicity whilst a very poor model (Eq. 6.7) was obtained for tertiary aliphatic amines. The challenges of modelling aliphatic tertiary amines using lipophilicity was previously reported (Newsome et al., 1991). For tertiary amines from DS1 that had log P values higher than 2.0 it was observed that with an increase in hydrophobicity the toxicity was reduced. The analysis of water solubility (log S) showed that for aliphatic amines, with solubility below 0.5, the toxicity decreased as solubility decreased (Figure 6.7). The same pattern was not observed for primary and secondary aliphatic amines. Primary and secondary aliphatic amines have structures that are similar to phospholipids present in the biological membranes and as such their partitioning may be governed by energetically favourable interactions with the membrane (Sinks, Carver & Schultz, 1998) - the positively charged nitrogen can interact with phosphate polar head group and the lipophilic alkyl chains are involved in hydrophobic interactions with the phospholipid tails. The interaction of protonated nitrogen with phosphate is less favourable in case of tertiary aliphatic amines (Austin, Davis & Mannes, 1998). In this context it could be reasonable to reconsider the classification of tertiary amines as non-polar narcotics. Possibly, they could be classified as exerting baseline toxicity only if their log P or log S values are above certain cut-offs. Otherwise, tertiary aliphatic amines could be classified as polar narcotics.

An attempt to include seven secondary aliphatic amines along with other Verhaar class 2 chemicals (primary aliphatic amines, phenols and anilines) reduced the determination coefficient (compare Eq. 6.2 and Eq. 6.10) however, the intercept difference was minimal, which could indicate that secondary amines could be classified as Verhaar class 2 compounds.

Consequently, the global aliphatic amines model was not further developed. It was previously suggested that measurements of partition coefficient based on 1-octanol system may not completely reflect actual partitioning taking place in the biological membranes where protonated amine species may be inserted. This could explain the excess of toxicity exerted by aliphatic amines (Dimitrov et al., 2003). Additionally no specific FATS (fish acute toxicity syndrome) was identified for

amine narcosis although such FATS are known to exist for non-polar and polar narcotics (lethargy and hyperactivity respectively) (Netzeva, Pavan & Worth, 2007).

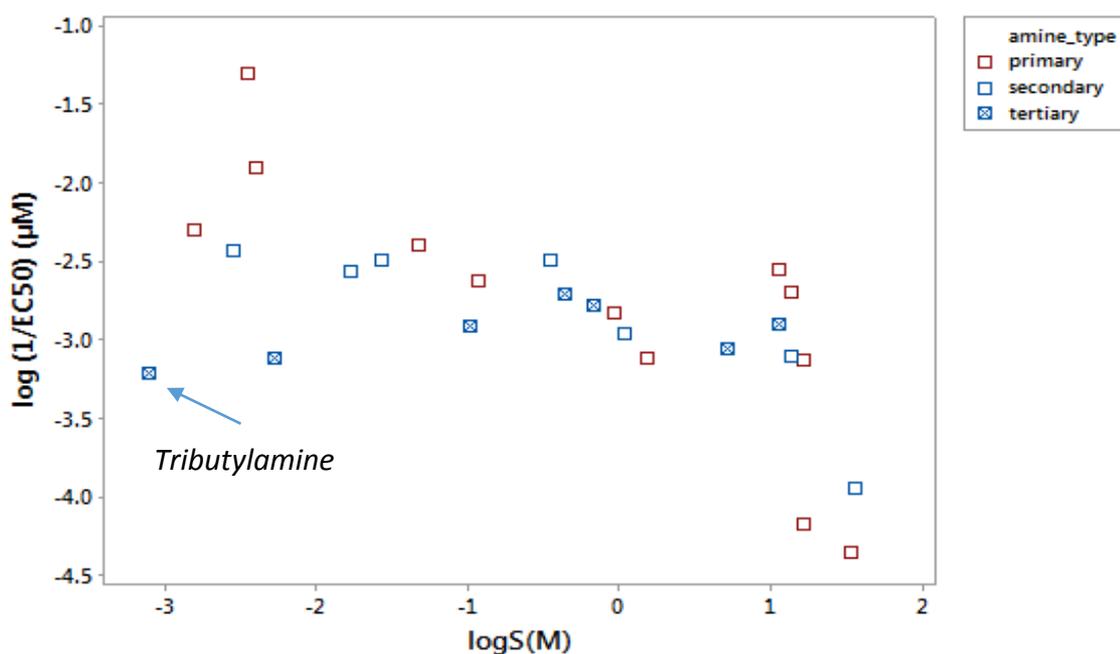


Figure 6.7. A plot showing the relationship between $\log S$ (calculated) and $\log(1/EC50)$ for primary, secondary and tertiary aliphatic amines. Note that tributylamine (an outlier removed from the baseline model) was included

Table 6.4. Models for aliphatic amines from Verhaar classes 1 and 2.

Equation Id.	Group	Equation	N	R ² (%)	R ² _{adj} (%)	Q ² (%)	S
Eq.6.4	IA, IIA, IIIA	$\log(1/EC50) = -3.57 + 0.45 \log P$	26	69.6	68.3	62.0	0.36
Eq.6.5	IA	$\log(1/EC50) = -3.72 + 0.59 \log P$	13	86.3	85.0	80.6	0.32
Eq.6.6	IIA	$\log(1/EC50) = -3.47 + 0.34 \log P$	7	78.8	74.6	40.6	0.28
Eq.6.7	IIIA	$\log(1/EC50) = -2.85 - 0.04 \log P$	6	3.70	0.00	0.00	0.17
Eq.6.8	IIA, IIIA	$\log(1/EC50) = -3.34 + 0.26 \log P$	13	55.2	51.1	25.3	0.28
Eq.6.9	IA, IIA	$\log(1/EC50) = -3.62 + 0.49 \log P$	20	78.9	77.7	71.8	0.34
Eq.6.10	IA, IIA, Anil., Phen.	$\log(1/EC50) = -3.66 + 0.57 \log P$	39	76	75.3	73.0	0.38

IA = primary aliphatic amines, IIA = secondary aliphatic amines, IIIA = tertiary aliphatic amines, Anil. = anilines, Phen. = phenols.

The described refinement process showed that for Verhaar class 2 compounds the original model that included anilines, phenols and primary amines was of good quality and it indicated that these groups of compounds are correctly classified as less inert compounds. Removing secondary and tertiary aliphatic amines led to an improved baseline model although the smaller number of compounds used to build a model and uneven distribution of log P values could decrease the confidence in terms of the predictive power of this model. Secondary and tertiary aliphatic amines with log P smaller than 2.0 log units may be classified less adequately and they could potentially be classified as polar narcotics or Verhaar class 2 (see Figure 6.8).

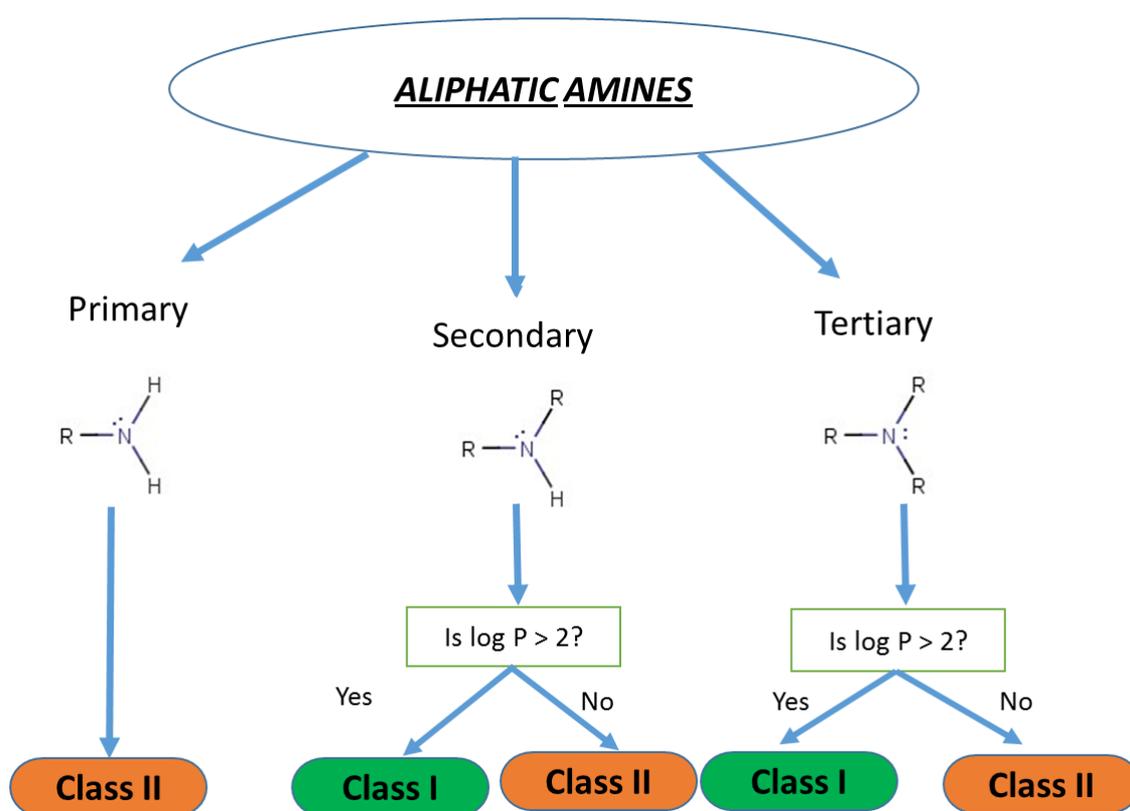


Figure 6.8. A schematic representation of a suggested rule for Verhaar reclassification of aliphatic amines.

6.5. Other Verhaar classes

Other Verhaar classes were not suitable for regression analysis as the compounds present in them were expected to exert toxic effects by a variety of mechanisms, hence it would be difficult to obtain a linear model based on a single hydrophobicity descriptor. However, these classes were still utilised to provide further analysis of the aim of this chapter – that is, whether zebrafish embryos can

distinguish between baseline and excess toxicity. The compounds from Verhaar classes 3, 4 and 5 were compared against the refined baseline model (Eq. 6.3) (Figure 6.9).

It is clear that the majority of compounds from Verhaar classes 3, 4 and 5 have toxicity greater than predicted by the developed baseline. It should be noted that the range of log P values for Verhaar classes 3, 4 and 5 is considerably greater than in baseline models; this indicates that the baseline models should not be used for the prediction of toxicity for these compounds. This is particularly important in the cases of extreme values for partition coefficient. These compounds could be either very insoluble (not bioavailable) or so hydrophilic that their interactions with the biological membrane would be thermodynamically unfavourable.

Interestingly, for a number of compounds from Verhaar class 5 the actual toxicities were lower than was predicted according to the baseline models. This should not occur by the definition of the baseline toxicity although some margin of error should be taken into account. These compounds are shown in Table 6.5 and it appears that they are diverse in terms of chemical structure.

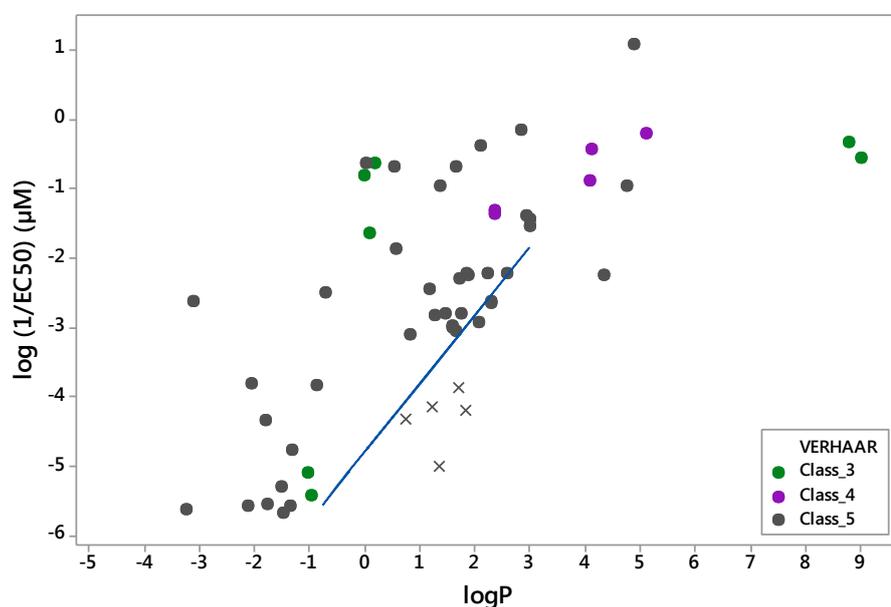
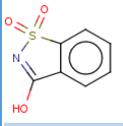
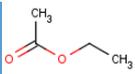
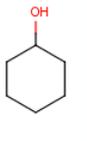
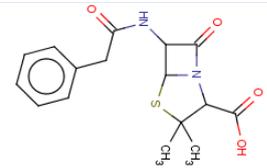
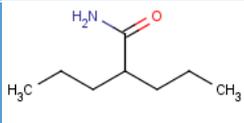


Figure 6.9. A scatterplot of log P toxicity to zebrafish embryos for Verhaar classes 3, 4 and 5. The added regression line corresponds to a refined baseline model (Eq. 6.3). Compounds from class 5 (for which toxicity is over-predicted) are depicted as crosses – these are presented in Table 6.5

Table 6.5. Selected compounds from Verhaar class 5. All of these had lower actual toxicity values than predicted by the developed baseline models.

Compound name	Structure	Comments
Saccharin		N,S heterocyclic, a common sweetener
Ethyl acetate		ester; can be hydrolysed <i>in vivo</i>
Cyclohexanol		cyclic alcohol
Penicillin		pharmaceutical
Valpromide		pharmaceutical; possible metabolism to valproic acid

Some of the compounds identified are heterocyclic compounds while others are structurally less complex, for instance ethyl acetate (ester). Interestingly, a high quality separate model for ester narcosis was previously obtained (Jaworska, Hunter, Schultz, 1995). However, although esters seemed to exert excess toxicity in fish, this was not observed for ciliates (e.g. *Tetrahymena pyriformis*). This was rationalised by indicating higher activity of esterases in fish. Hydrolysis of ester by these enzymes was hypothesised to lead to changes in concentration gradient between fish and water, which in turn would increase the uptake of a chemical by shifting the thermodynamic equilibrium. On the other hand, no specific FATS were identified for monoesters. Additionally, the presence of additivity of octanol and monoesters indicated that esters may act as non-polar narcotics. Ethyl acetate present in the DS1 is a monoester and although it could not be classified according to Verhaar rules (class 5) its low toxicity value (below a baseline) could indicate that it is a non-polar narcotic.

Two pharmaceuticals (penicillin and valpromide) were among compounds with low toxicity that were found in Verhaar class 5. Both these compounds can be a subject to xenobiotic metabolism. In humans, valpromide is rapidly metabolised to valproic acid (a compound present in baseline models) while penicillin is biotransformed to either 6-aminopenicillanic acid or penicilloic acid. Zebrafish

embryo expresses various CYP isoforms and is capable of oxidative and conjugative xenobiotic metabolism (Jones et al., 2010). However, it is not known to what extent (if at all) the biotransformations occur in the case of valpromide and penicillin in zebrafish embryos.

Visual inspection of Figure 6.9 reveals that a fraction (20 compounds) of Verhaar class 5 chemicals had baseline level toxicity values. Some examples include: cyclohexylamine or piperidines (heterocyclic amines). This might suggest that such categories of compounds could be reclassified as non-polar narcotics. On the other hand, it may be the case that the slope of the regression line is too high and such compounds exert excess toxicity. Nevertheless, it seems that some of the Verhaar class 5 compounds could be incorporated into non-polar or polar models and therefore, reclassified as Verhaar class 1 or 2. However, chemical diversity of these chemicals should be carefully examined before drawing conclusions about creating new toxicophores.

6.6. Validation of baseline non polar narcosis and polar narcosis models

The quality of selected narcosis models for zebrafish embryos was tested using compounds from DS2. The test set presented more heterogeneous chemical space than DS1. All compounds from DS2 were grouped according to the Verhaar scheme as implemented in Toxtree. For 43% of compounds from DS2 the mode of aquatic toxicity was not identified (see Figure 6.10).



Figure 6.10. Distribution of compounds for DS1 and DS2 according to Verhaar classes. The number of chemicals in each class is presented in absolute values.

To ensure the correct usage of the models an attempt was made to define the applicability domain, i.e. to determine a space for which the model can produce reliable predictions (Ellison et al., 2011). Such a domain can be described in terms of x-space of the model that consists of independent

variables and also in y-space that constitutes a predictions space (Jaworska, Nikolova-Jeliazkova & Aldenberg, 2005). One of the methods to define x-space is to determine descriptor ranges. All of the obtained models for DS1 modelled toxicity using only a single variable, log P. Hence, the range of log P values was described for each of the tested models (Eq. 6.1, 6.2 and 6.3) as well as the prediction domain (see Table 6.6).

Table 6.6. Ranges of independent (log P) and dependent (toxicity) variables for selected subsets of DS1 or DS2. Red colour indicates minimum and maximum values that are outside of the domain.

Model/subset	log P			Toxicity (log (1/EC50) (µM))		
	min.	max.	span	min.	max.	span
Eq.6.1	-0.77	3.76	4.53	-5.84	-2.08	3.76
Eq.6.2	-0.57	4.13	4.70	-4.36	-1.06	3.30
Eq.6.3	-0.77	3.00	3.77	-5.84	-2.08	3.76
test set (Verhaar class 1)	-0.49	4.60	5.09	-4.47	0.70	5.16
test set (Verhaar class 2)	0.60	5.76	5.16	-2.79	-0.23	2.56

Apart from determining the range of utilised physicochemical descriptors another important step involves defining structural domain. For instance, Ellison et al., (2008), defined structural domain for non-polar narcosis for *Tetrahymena pyriformis* by identifying structural fragments associated with excess toxicity. The structural domains of the three tested models inherently originated from the Verhaar rules. It is important to appreciate that the test set compounds should belong to the training set structural domain to enhance reliability of predictions. The presence of some structural features may determine the mode of action and constitute the differences in toxicity between compounds with similar values of physicochemical descriptors, such as log P.

6.6.1. Performance of baseline models

The initial step was to observe (visual inspection of Figure 6.10) whether the refined baseline model (Eq. 6.3) could distinguish between baseline and excess toxicity for compounds from DS2. The rationale was that no compounds should have toxicity lower than the baseline, hence the diversity of the test set would not greatly influence the classification of a compound exerting an excess toxicity considering that the baseline model (Eq. 6.3) was developed from non-polar narcotics.

The observation of the scatterplot present in Figure 6.11 showed that at least within (or close to) a range of Log P values in which the baseline models were developed only one compound (cyclohexane/Verhaar class 1) had a toxicity value much lower than predicted by Eq. 6.3. Interestingly, two chemicals from Verhaar class 1 were significantly underpredicted. Nevertheless, it

was clear that the majority of compounds (not present in Verhaar class 1) had toxicity greater than baseline.

Further analysis was focused on the test compounds that were classified as belonging to class 1 of the Verhaar scheme. The applicability domains of Eq. 6.1 and 6.3 were used as a filter for 17 Verhaar class 1 compounds from DS2. In terms of x-space, the log P range was greater for non-polar narcotics from DS2, and therefore, the number of compounds available for testing was reduced by four and eight chemicals for models 6.1 and 6.3, respectively. The consideration of the y-space brought a further reduction in the number of compounds to be tested so that eventually the predictions were obtained for eight substances for model 6.1 and six chemicals for model 6.3. This was a very strict filtering process as it did not allow any extrapolations to be made for the test set. Applying the appropriate log P range seemed to be fully justifiable as the relationship between independent and dependent variables may lose its linear character, which can be exemplified by the case of the tertiary aliphatic amines from DS1. However, limiting y-space may lead to a reduction in the capability to identify outliers as some of these are inherently excluded from testing. This shows that the definition of the applicability domain may depend on the purposes of modelling.

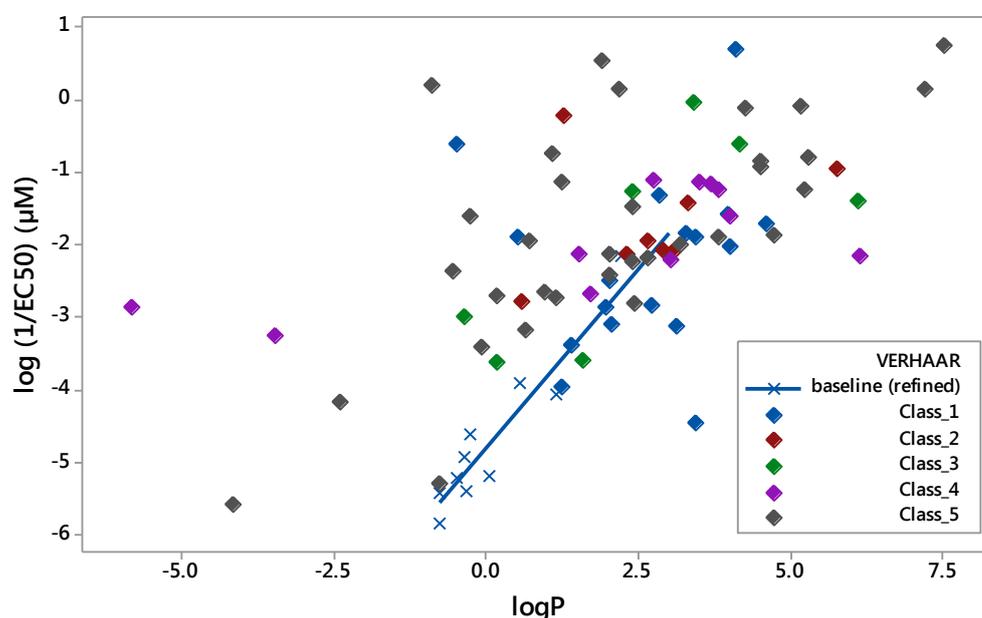


Figure 6.11. A scatterplot of toxicity to zebrafish embryos versus log P for DS2 (test set). The added regression line represents Eq.6.3 (The refined baseline model). The colour scheme for Verhaar classes is identical as for compounds from DS1. The chemicals depicted as blue crosses belong to baseline model from DS1 (Eq. 6.3).

In terms of the structural domain, some compounds such as aromatic hydrocarbons (e.g. toluene) were not used to build the baseline models. However, they were not excluded from testing as it was decided to use a broader definition of structural domain for less inert chemicals that was equivalent to Verhaar class 1 structural domain. Dataset 2 did not contain any aliphatic amines in the class 1 or class 2 compounds therefore, no information was provided to add to the discussion about classification of aliphatic amines. Nevertheless, both baseline models (Eq. 6.1 and Eq. 6.3) were tested to see whether the refinement process led to obtaining a better model for non-polar narcotics.

Predictions were classified as correct when predicted values did not deviate from experimental values by more than 0.5 toxicity units (Ellison et al., 2008). The performance of both baseline models is summarised in Table 6.7.

Table 6.7. Performance of the non-polar (Eq. 6.1 and Eq. 6.2) and polar (Eq. 6.2) narcosis models. The tested compounds belonged to DS2.

Narcosis type	Model	No. of tested compounds	Correct predictions (%)
<i>Non-polar</i>	<i>Eq.6.1</i>	8	75
	<i>Eq.6.3</i>	6	83
<i>Polar</i>	<i>Eq.6.2</i>	7	71

The majority of compounds from Verhaar class 1 were predicted to be non-polar narcotics. Model 6.1 could not correctly predict two compounds: 2,3-dimethyl-1,3-butadiene and cyclohexane. These chemicals were not included in model 6.3 as their log P values were not inside the applicability domain. Additionally, the refined model overpredicted toluene. All three compounds, which were not correctly predicted, were either aliphatic or aromatic hydrocarbons and as such did not contain polar moieties that are required for solvation in water; this is also reflected in their low log P values. Moreover, cyclohexane is immiscible in water. Volatility of toluene and dimethylbutadiene may also affect measurements (overestimation of concentration). All these compounds could be treated as outliers in the developed QSAR baseline models. A similar deviation from a positive correlation between hydrophobicity descriptor and toxicity was observed when discussing tertiary aliphatic amines at higher log P values (> 2). However, from the classification point of view, these chemicals were correctly classified as inert substances (Verhaar class 1) and, therefore, their acute toxic effects would be assessed appropriately. However, from the regression model perspective, the applicability domain should be extended by additional rules, either related to structural information or regarding other physicochemical properties, such as vapour pressure or water solubility. Such rules, for

instance only compounds with MW < 600 Da can be a subject to Verhaar classification, were indeed included in the original Verhaar scheme (Verhaar, Leeuwen & Hermens, 1992).

The refined baseline model (Eq. 6.3) provided more correct predictions than the initial baseline model (Eq. 6.1). However, to have a better understanding of the performance of the models some additional aspects needed to be considered. First of all, only a limited number of test compounds was available and it was even further reduced by the defined applicability domain. Structural domain differences between the training set and test set were also present (e.g. hydrocarbons were only present in DS2). The distribution of chemical groups within Verhaar class 1 differed with only a single aliphatic alcohol and, more prominently, no aliphatic amines were present in the test set. In this context the good performance of model 6.1 might support the classification of secondary and tertiary amines as inert compounds and as such they could be used to build a baseline narcosis model.

6.6.2. Testing the polar narcosis model

Similar analysis as for class 1 was carried out with regard to test compounds classified as Verhaar class 2. Initially, this subset contained 8 chemicals however, one compound (nonylphenol) was later removed due to the log P being outside of the applicability domain for polar narcosis model (Eq. 6.2). In terms of the chemical classes, the final test set for polar narcosis was composed of four phenols, one aniline and two nitropyridines. The model provided correct predictions for five out of seven compounds (see Table 6.7). 3-nitropyridine (residue = -0.57) and 2-chloro-5-nitropyridine (residue = -2.7) were the two compounds for which the toxicity values were underpredicted. The training set did not contain pyridines and therefore, this class of compounds did not define the structural domain. However, these compounds were categorised as Verhaar class 2 although their toxicity values were higher than for classical polar compounds. In *Tetrahymena pyriformis*, nitropyridines were previously shown to have high toxic ratio (T_e) value that discriminated between polar narcosis and reactivity related to covalent binding (Seward, Cronin & Schultz, 2000). It is possible that nitropyridines should not be allocated to Verhaar class 2 and, consequently they should not be modelled with polar narcotics. Instead, nitropyridines could be classified as Verhaar class 3 compounds.

Possible future work on this subject could include investigation of the usefulness of zebrafish embryo for prediction of the ADME properties. For instance, it would be interesting to compare the uptake of compounds by the zebrafish embryos to human intestinal absorption. Berghmans et al, (2008) investigated nine drugs and observed that the amount of drug that penetrated the zebrafish was related to the hydrophobicity of the drug (cLogP) and compounds with high cLogP values (> 3.8)

had a better ability to penetrate the zebrafish larvae. The author of the thesis identified six of these compounds in the Hou et al., (2007b) HIA dataset (see Chapter 2 of the thesis) to compare how the fish penetration was related to HIA. It appeared that 50% of these drugs were high absorption despite their poor uptake by zebrafish embryos. More data are needed to derive any rules about the relationship of zebrafish embryo and HIA.

In summary, zebrafish embryos appear to distinguish between baseline and excess toxicity. The developed regression models for non-polar narcosis (Eq. 6.1 and Eq. 6.3) and polar narcosis (Eq. 6.2) were validated using an external dataset. The applicability domains of the models were determined by the ranges of independent and dependent variables. The models could predict the toxicity for the majority of test compounds that belonged to the applicability domain. However, the validation process could benefit from the availability of a greater number of compounds. The analysis of aliphatic amines and the context of Verhaar scheme suggested that secondary and tertiary aliphatic amines with log P smaller than 2.0 log units could potentially be reclassified as Verhaar class 2. Interestingly, a number of Verhaar class 5 compounds appeared to exert baseline type of toxicity which might suggest that further rules could be introduced. Additional rules may also reduce the number of compounds for which, in terms of Verhaar scheme, no decisions can be made.

7. Discussion

The area of *in silico* toxicology has developed rapidly over the past few decades. There are many factors that have driven the advances in non-testing methods (Raunio, 2013). The reduction refinement and replacement philosophy (the 3Rs framework) has led to an appreciation of the need to approach animal testing, and the search for alternatives, in a different way. Furthermore, legislation and regulation have also played an important role. For example, the REACH regulation promotes the use of non-testing methods and provides guidelines on the application of computational methods, such as QSARs or read-across, in risk assessment (ECHA, 2008). In addition the 7th amendment to the EU Cosmetics Directive effectively bans the use of animals for studying toxicity of cosmetic ingredients. The US National Research Council has proposed a strategy for the assessment of environmental toxicity (of industrial chemicals and pesticides) using *in vitro* and *in silico* approaches to identify human toxicity pathways. This strategy involves the incorporation of systems biology and “omics” methods (NRC, 2007). The pharmaceutical industry has also been a significant contributor to the development of alternative methods by progressing the development of *in silico* methods for drug discovery and assessment of ADME-tox profiles. The IMI project eTOX is an example of a collaborative effort of pharmaceutical companies to develop novel approaches to assessing drug-related toxicity. Despite the progress in computational approaches for the prediction of toxicity, these methods still find limited application in the area of regulatory toxicology. For instance, there is no “list” of *in silico* methods that have been approved formally for use complying with REACH requirements. The limitations of applying *in silico* methods within REACH are in part due to the lack of experimental data available for model development and also the lack of experience of using such models for regulatory purposes (Benfenati et al., 2013). In the context of pharmaceuticals, neither the US FDA nor the European Medicines Agency require *in silico* data to be submitted (Valerio, 2009).

The wider acceptance of computational models for risk assessment could be achieved through the development of high quality, validated models for clearly defined endpoints (OECD, 2007). In the context of risk assessment such models should also be transparent, i.e. have a clear chemical and biological interpretation. It is also important to critically evaluate existing *in silico* tools to determine their suitability for purpose, both in terms of overall predictivity and their domain of applicability. Assessment and evaluation of existing software for ADME-tox prediction can enhance the understanding of these tools and provide guidelines of how they can be applied, most effectively, in practice. This is particularly important for non-expert users. This was one of the themes that was explored within this thesis.

For example, in Chapter 3, selected software for metabolism prediction was evaluated. It was noted that, especially in the case of the Meteor software, there was the tendency for overprediction of metabolites. Although Meteor provides a number of settings that may be selected by the user (absolute and relative reasoning engines; number of metabolites in the output etc) it is not obvious which settings are the most appropriate to select in order to identify correct metabolites without producing a large number of false positives. Within this work it was demonstrated that using relatively permissive reasoning engine settings (equivocal) and reducing the number of metabolites to be predicted improved the precision of the results. Menikarachchi et al., (2013) suggested that a certain number of false positives does not need to be disadvantageous providing that these can be filtered out in an efficient way. In the case of Meteor, this software may be used in combination with Derek Nexus (Lhasa Limited, Leeds) to determine the toxicological liability of the metabolites predicted by Meteor. It may be argued that this combination represents a safer option in terms of toxicity prediction i.e. toxicity of both parent drug and metabolites can be predicted. On the other hand, obtaining Meteor predictions is computationally expensive and time consuming; this may present a significant limitation when applying this combination approach to screening a large library of compounds, as may be necessary in a pharmaceutical industry setting. Other freely available software that was investigated here (SMARTCyp and MetaPrint2D-React) also proved to be very useful tools for prediction of xenobiotic metabolism. Another outcome of the studies within this thesis was the proposal to combine SMARTCyp and MetaPrint2D-React, whereby SMARTCyp could be used to predict potential sites of metabolism (SOM) and MetaPrint2D-React could then provide the structures of the metabolites generated at the given SOM. Although MetaPrint2D can also provide SOM it relies solely on a historical database and hence, it may fail to correctly predict metabolite structures for compounds representing a different chemical space, as may be the case in specific drug development programs. SMARTCyp is based on quantum mechanical calculations and therefore it is less dependent on historical data. (This advantage was realised by Lhasa Limited, during the time in which these PhD studies were undertaken, and SMARTCyp has now been incorporated into Meteor Nexus). The assessment of Meteor and MetaPrint2D-React also showed that despite good overall performance, some metabolites were not correctly predicted, for example in the case of bromfenac. This illustrates that each *in silico* tool is associated with certain limitations. For bromfenac a particular biotransformation was not adequately represented within the expert system (Meteor) nor was it present in the historical database utilised by the data mining approach (MetaPrint2D-React). This is not the only limitation of concern. For instance, for SMARTCyp, potential limitations relate to the number of fragments for which the energy calculations have been performed and also by the number of CYP450 models (3A4, 2D6, etc.) that are currently available. In

each case, the limitations have to be recognised by the users. This also means understanding the applicability domain of a model and having realistic interpretation of the output. Again, in the case of the *in silico* tools for xenobiotic metabolism it should be appreciated that the predictions are purely qualitative as they do not estimate the relative amounts of each metabolite likely to be formed. These limitations add to the lack of confidence in decisions concerning the safety of a chemical. This is especially important in a regulatory context where computational methods are not widely accepted and carry relatively little weight in risk assessment (Modi et al., 2011).

Xenobiotic metabolism is a key component of ADME-Tox profiles. However, it cannot be considered in isolation from absorption. If a compound is poorly absorbed it will not enter the systemic circulation and therefore, it will not reach the CYP450 system in the liver where the majority of metabolism occurs. Hence, the prediction of human intestinal absorption was another important pharmacokinetic aspect that was considered (Chapter 2). *In silico* approaches may offer a fast and cost-effective method for assessing the absorption of compounds especially at the early stages of drug discovery when performing virtual screens on large number of compounds. Druglikeness filters, such as Lipinski's rule of five or Veber's rules, are of special interest due to their simplicity relating to the use of transparent, and therefore more easily-interpretable, medicinal-chemist type descriptors. The accuracy of the predictions obtained from applying druglikeness filters were comparable (and in some cases better) than those that resulted from *in vitro* approaches to predicting absorption (Caco-2 and PAMPA). Interestingly, a combination of TPSA ($\leq 140 \text{ \AA}^2$) and $\log D_{6.5}$ (Hou et al., 2007b) gave the most promising results in terms of distinguishing between high and low human intestinal absorption. This indicates that in the case of pharmaceuticals the distribution coefficient ($\log D$), especially at pH values similar to those encountered in the GIT, may be a better predictor of oral drug absorption than $\log P$ as $\log D$ takes into account the ionisation of a compound. However, druglikeness filters should be used with caution even though they are widely applied in the pharmaceutical industry. One of the factors to consider is the binary nature of the predictions obtained by application of druglikeness rules. This means that "hard" cut-offs may be applied in too rigorous a manner and result in some useful compounds being discarded during screening. In response to this potential pitfall, more sophisticated druglikeness measures have been introduced recently, such as quantitative estimate of druglikeness (QED) (Bickerton et al., 2012). QED combines eight characteristics (medicinal chemist-type descriptors) into a single metric that describes how similar a compound is to known orally administered drugs. It would be interesting to examine whether the QED could successfully be applied to the absorption dataset studied herein. Another issue identified with the generic "druglikeness" rules is the poor distinction between human intestinal absorption and bioavailability (in humans), for example Veber's rules were developed for

oral bioavailability in rats. Nonetheless, a druglike molecule should be expected to be absorbed, at least to some extent. Finally, druglikeness rules cannot provide a quantitative prediction for absorption or bioavailability which might be useful, especially at later stages of drug discovery, e.g. during the optimisation process. This is an area where *in vitro* techniques may be more advantageous.

Within the work conducted here, application of rules such as the combination of TPSA and log $D_{6.5}$ was not useful for the prediction of bioavailability. This may be expected due to the metabolic clearance as these properties do not adequately account for the contribution of first pass hepatic clearance. An attempt to build a model that could predict hepatic clearance was not successful; hepatic clearance is a complex process and therefore is difficult to model. Similarly, *in vitro* techniques (liver microsomes, human hepatocytes) have been shown to underpredict *in vivo* metabolic clearance (Chiba, Ishii & Sugiyama, 2009). The extent to which a compound will be metabolised was recently shown to be related to *in vitro* permeability rate measurements and also to *in silico* permeability rate predictions. However, knowledge of metabolic rate is often limited (Kirchmair et al., 2015) making it difficult to develop models to predict rate and extent of metabolism and the contribution of these to bioavailability.

During analysis of oral absorption and bioavailability all the datasets (except that for hepatic clearance) were significantly skewed towards high absorption/bioavailability values. More evenly distributed datasets would be required to increase confidence in the models developed.

Xenobiotic metabolism is also linked to toxicity; as a result of bioactivation, reactive metabolites can be formed. These tend to be electrophilic species that can readily attack nucleophilic centres on biomolecules. This process may lead to a disruption of biological functions and may lead to toxic effects such as type B adverse drug reactions. Elements of electrophilic chemistry, relevant to protein binding, have been captured and described in terms of electrophilic reaction domains (Enoch et al., 2011). The structural alerts associated with protein binding have been incorporated in a number of software packages, such as the OECD (Q)SAR Toolbox or Toxtree or they can be accessed via web services such as the ToxAlerts platform (Sushko et al., 2012).

Structural alerts offer a promising tool for screening purposes, for instance in drug discovery. Work carried out here included analysis of the DrugBank dataset in relation to the distribution of structural alerts for reactive metabolism. Interestingly, nearly half of the compounds from DrugBank contained at least one structural alert. There were no significant differences in distribution of structural alerts among different drug classes (approved, experimental, and withdrawn). There was also no correlation between the number of identified alerts and the class of the drug. This shows that the presence of a structural alert is not sufficient to predict the adverse outcome. A more holistic

approach is required that takes into account such factors as: dosage, route of clearance or the existence of alternative sites on a molecule that are more liable to metabolism. Pharmacokinetic properties, such as absorption need to be considered as well. A compound that is poorly absorbed will not be subject to extensive xenobiotic metabolism as it will not reach the liver in large quantities. This is where the aforementioned rule-based filters such as Lipinski's rule of five can be utilised to obtain more complete profiles for screened compounds. Despite the complexity of the problem some non-expert users may not appreciate that structural alerts cannot be the only basis of decision-making about the future of a compound. There may be a temptation to take advantage of the binary nature of the result i.e. if an alert is present - reject the compound, otherwise take it forward in development.

The structural alerts within oCHEM were initially used without refinement, leading to a high number of compounds being associated with "hits". As it is possible many of these "hits" could relate to false positives in terms of toxicity prediction, refinement of the structural alerts was investigated. This was exemplified by re-writing the SMARTS pattern for the carboxylic acid alert in order to reduce the number of false positives. However, this approach may also carry a risk of missing potentially reactive structures. The balance between sensitivity and specificity requires careful consideration of mitigating factors and may be challenging to validate due to the lack of data.

Toxicity related to poor pharmacokinetic properties compounds is only one of the challenges for the pharmaceutical industry. Off-target toxicity represents another challenging area in drug discovery and development. Within this thesis, aspects of off-target toxicity related to hERG channel inhibition, were also investigated. A previously reported QSAR model for hERG toxicity (Aptula & Cronin, 2004) was evaluated. This model offered a transparent interpretation due to using simple medicinal chemistry-type descriptors related to hydrophobicity (log D) or molecular size (maximum diameter). Despite this advantage, the model could not provide accurate predictions for the larger dataset (H_244) studied here. A limited applicability domain of the model developed by Aptula & Cronin, (2004) was indicated to be an important factor that influenced the predictions. Building global linear regression and classification models did not offer improvements to existing models. The best model (78% correct predictions) was obtained using a random forest classifier. Local models, based on 2D-similarity, were also developed for seven defined categories of compounds identified within the dataset. The multiple linear regression models obtained for the categories investigated were not satisfactory, except for compounds from category VI. The limited success with global modelling can be explained by the fact that the hERG channel can bind very structurally diverse compounds. However, better models were expected for local categories. The results suggest that multiple linear regression methods are not suitable for hERG modelling. On the other hand, good regression models

were obtained by Yoshida & Niwa, (2006) although for a smaller dataset. The quality of models can be affected by interlaboratory variability. The differences in IC_{50} values obtained from different laboratories can be greater than 10-fold (Ekins, 2007). Additionally, the inhibition values may originate from different cell lines increasing variability in results; this was noted to be the case for the dataset studied here.

Due to the difficulties in building quantitative predictive models a more qualitative approach was taken. The alerts developed for six (out of the seven) defined categories could be utilised as a potential screening tool. However, these alerts require further validation. One of the limitations of the structural alerts for hERG may be their specificity for the group of compounds on which they were based. This is the opposite situation to that encountered during the analysis of structural alerts for carboxylic acid in the context of reactive metabolites. Whilst the structural alert for carboxylic acids was refined to be more specific, the alerts for hERG would benefit from greater generalisation so that they capture more compounds. However, it should be appreciated that the hERG channel inhibition is a result of weak interactions between a drug and a channel pocket rather than being related to specific reactivity. As a result it may be more beneficial to develop SMARTS for fragments based on pharmacophore models and then combine them with filters based on physico-chemical properties, similar to those developed by Aptula & Cronin, (2004). This could be captured in the form of a decision tree. Such fragments could be more useful for screening purposes as they could capture broader chemical space, but they would have to be considered in conjunction with a filter based, for instance, on molecular size that prohibits larger molecules entering the channel pocket. This could narrow down the results of the screen and give a more pragmatic approach to prediction of hERG-related off-target toxicity.

The trend to reduce animal testing stimulates the development of new approaches. One such approach in the context of aquatic toxicity is using fish embryos, rather than adult organisms. It has been established that the zebrafish embryo toxicity (ZFET) test is a valid alternative method of determining aquatic toxicity (Belanger et al., 2013; Busquet et al., 2014). Research undertaken here investigated whether zebrafish embryos could distinguish between baseline and excess toxicity. The analysis was performed by utilisation of the Verhaar scheme. The implementation of the Verhaar scheme was also of interest in terms of its performance. Whilst the scheme has been well established there is a need to update and/or add new rules. This statement is based on the fact that within both the training and test sets analysed here many compounds could not be placed into one of the Verhaar categories (i.e. the software place many compounds into category V). Moreover, some of these compounds exerted baseline toxicity. Zebrafish embryos were shown to distinguish between non-polar and polar narcosis. The analysis of amines showed that secondary and tertiary

aliphatic amines, with log P smaller than 2.0 log units, may potentially be classified as polar narcotics. However, more data points are required for this statement to be conclusive.

The applicability domain of the QSAR models developed for the prediction of aquatic toxicity was also considered. This is a specific recommendation of the OECD guidelines for building valid QSAR models and as such should be considered during model development and testing. The importance of applicability domain may not be fully appreciated by naive users of toxicity prediction software and therefore can have significant consequences for the reliability of predictions obtained.

Zebrafish embryos can also be useful for the assessment of ecotoxicity of pharmaceuticals that constitute a class of environmental pollutants. Active pharmaceutical ingredients (APIs) that originate from drug production for both medicinal and veterinary use can reach drinking water through various pathways (Santos et al., 2013). Moreover, APIs can be subject to biotransformations with the possibility of formation of reactive metabolites. The significance of the biotransformation of the parent and the environmental risks of the metabolites have not yet been fully investigated (Kummerer, 2010). The regulatory bodies have recognised the issue of APIs and in consequence, the environmental risks and impact of medicinal products are required to be evaluated and assessed (European Directive 2001/83/EC). Zebrafish embryos have been successfully used in the context of toxicity testing of APIs. A recent study showed that pharmaceutical pollutants can have detrimental effects on zebrafish embryos development (Pruvot et al., 2012). In another study, zebrafish embryos were utilised to assess the toxicity of 15 veterinary pharmaceuticals and their metabolites (Carlsson et al., 2013). As zebrafish embryos can distinguish between baseline and excess toxicity they appear to be a promising organism for studying the effects of reactive metabolites.

The work undertaken within this thesis covered several aspects of ADME-tox prediction. Emphasis was placed on topics related to drug discovery and development with a particular focus on prediction of pharmacokinetic properties such as absorption and metabolism. A discussion of screening tools such as structural alerts was also presented. Off-target toxicity and the use of alternative fish developmental stages for the assessment of aquatic toxicity have been explored. A number of existing software packages have been evaluated and recommendations made as to how the packages, or the methods of their application, may be improved.

Future work

The studies conducted for this thesis have explored various aspects of computational toxicology ranging from modelling ecotoxicity to prediction of ADME properties and off-target effects of drugs. A variety of tools were evaluated, different modelling techniques were applied and improvements to existing methods suggested where appropriate. It has been appreciated that *in silico* approaches to prediction of toxicity may be useful, although they are not free from limitations. One of the most important limitations is related to the availability of high quality, diverse, datasets that can be used to train models. It is obvious that the quality of a model depends on the quality of data used for its construction; size and diversity are also of importance. Nevertheless, large, well-curated and balanced datasets are rather difficult to find in the public domain. The pharmaceutical industry may be privileged in this respect as they own large collections of data. This cornucopia has been explored by the eTOX project for building models without compromising the confidentiality of the data. However, this can present another issue related to the applicability domain of such models. It relates to the question whether a model built, for instance, on a dataset of leadlike/druglike molecules can also give reliable predictions for toxicity of compounds belonging to alternative regions of chemical space, such as that applicable to cosmetic ingredients, industrial chemicals etc. Detailed analysis and accurate definition of the applicability domain of models is a key area for future development. In particular how can the structural similarity between compounds be defined and what are the consequences in terms of the similarity of training and test sets.

Structural alerts comprise another interesting area. For example the work on structural alerts for hERG inhibition, as carried out in this thesis, might serve as a starting point for future work in this area. Data mining approaches that allow the discovery of chemical fragments related to specific endpoints in a more automated manner may enable results to be obtained more rapidly in future. This may be more relevant to such an endpoint as hERG channel inhibition where there is no definitive mechanistic interpretation of the channel inhibition due to the nature of the drug-channel interactions of such a diverse range of drugs. Such an approach could also be applied to the identification of potentially reactive metabolites which could add a new perspective to the existing alerts, especially in the context of their specificity.

Finally, further work could also be carried out in relation to the prediction of ADME properties possibly in combination with physiologically-based pharmacokinetic modelling (PBPK). The prediction of pharmacokinetic profiles requires knowledge of some basic physico-chemical properties such as solubility or permeability. The accurate and reliable prediction of medicinal

chemistry-type descriptors is a *sine qua non* for simulation of pharmacokinetics and, therefore, a crucial area for future investigation in terms of improving the existing models.

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Appendices

The list of appendices includes:

1. Appendix I. Dataset A (HIA data) used in Chapter 2.
2. Appendix II. Dataset B (Caco-2 data) used in Chapter 2.
3. Appendix III. Dataset C (PAMPA data) used in Chapter 2.
4. Appendix IV. Dataset D (bioavailability and hepatic clearance data) used in Chapter 2.
5. Appendix V. List of references that were used to obtain *in vivo* metabolites for the 59 drugs considered in Chapter 3.
6. Appendix VI. List of known metabolites for 29 NSAIDs discussed in Chapter 3.
7. Appendix VII. List of known metabolites for 30 top selling drugs discussed in Chapter 3.
8. Appendix VIII. H_244 dataset used in Chapter 4.
9. Appendix IX. Descriptors used for modelling compounds from H_244 dataset (Chapter 4).
10. Appendix X. DS1 (training) and DS2 (test) discussed in Chapter 6.

The data listed in each appendix can be found in an enclosed CD at the back of this thesis.