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Implications of interactions between tyrosine kinase inhibitors and human solute carriers in cancer therapy

Rosie Johnston

December 2013

Discipline of Pharmacology,
School of Medical Sciences
A thesis submitted to the University of Sydney in fulfilment of the requirements for the degree of Master of Philosophy in Medicine
# Table of Contents

Statement of Authenticity vi
Acknowledgements vii
Abstract viii
List of Publications x
Abbreviations xi
List of Tables xiv
List of Figures xv

**Chapter 1 – Literature Review** 1

1.1 Human solute carriers 1

1.1.1 Transporters in the *SLCO* superfamily: OATPs 2

1.1.1.1 Nomenclature 2

1.1.1.2 Structure and transport mechanism 3

1.1.1.3 Tissue distribution 6

1.1.1.3.1 Modulated tissue distribution in cancer 9

1.1.1.4 Physiological role 11

1.1.1.5 Genetic polymorphisms and interindividual variations 13

1.1.1.6 Drug-drug interactions 15

1.1.1.7 Chemotherapeutic aspects 16

1.1.2 Transporters in the *SLC22* superfamily: OCTs, OCTNs and OATs 18

1.1.1.1 Nomenclature 18

1.1.1.2 Structure and transport mechanism 19

1.1.1.3 Tissue distribution 21
1.1.3.1 Modulated tissue distribution in cancer 21
1.1.4 Physiological role 23
1.1.5 Genetic polymorphisms and interindividual variations 24
1.1.6 Drug-drug interactions 25
1.1.7 Chemotherapeutic aspects 26

1.2 Tyrosine kinase inhibitors 28
  1.2.1 Role of tyrosine kinases 29
  1.2.2 Inhibitors of receptor tyrosine kinases 31
    1.2.2.1 Inhibitors of the HER family 32
    1.2.2.2 Multikinase inhibitors 36
  1.2.3 Inhibitors of nonreceptor tyrosine kinases 41
  1.2.4 Interactions of TKIs with SLCs 44
  1.2.5 Drug-drug pharmacokinetics interactions 47

1.3 Specific aims of the project 50

Chapter 2 – Materials and Methods 51

2.1 Materials 51
  2.1.1 TKI compounds 51
  2.1.2 Chemicals and reagents 51

2.2 Methods 53
  2.2.1 Expression of SLC transporters in HEK293 cells 53
  2.2.2 Transport studies 53
  2.2.3 IC₅₀ studies 55
  2.2.4 Kinetic studies 55

2.3 Data analysis and statistics 58
Chapter 3 – Results

3.1 Preliminary screening experiments

3.2 Determination of IC_{50} values for the inhibition of SLC-mediated uptake by TKIs

3.3 Kinetic study of the inhibitory effect of cediranib on OATP1A2-mediated estrone-3-sulfate uptake

   3.3.1 Michaelis-Menten graph of OATP1A2 activity
   3.3.2 Lineweaver-Burk plots of OATP1A2 inhibition by cediranib
   3.3.3 Dixon plots of OATP1A2 inhibition by cediranib

3.4 Kinetic study of the inhibitory effect of erlotinib on OATP2B1-mediated estrone-3-sulfate uptake

   3.4.1 Michaelis-Menten graph of OATP2B1 activity
   3.4.2 Lineweaver-Burk plots of OATP2B1 inhibition by erlotinib
   3.4.3 Dixon plots of OATP2B1 inhibition by erlotinib

Chapter 4 – Discussion

4.1 Evaluation of results from screening experiments

   4.1.1 Organic anion transporting polypeptides
   4.1.2 Organic anion transporters
   4.1.3 Organic cation transporters
   4.1.4 Organic zwitterion/cation transporters
4.2 In vitro-in vivo extrapolation of IC\textsubscript{50} and \( K_i \) values

4.2.1 Significance of findings

4.2.2 Appraisal of analytical techniques

4.3 Kinetic analysis of the inhibitory effect of cediranib and erlotinib on OATP1A2 and OATP2B1

4.3.1 Significance of findings

Chapter 5 – Conclusions and Directions for Future Work

5.1 Conclusion

5.2 Limitations and recommendations for future work

References
Statement of Authenticity

The work described in this thesis was performed between March 2012 and December 2013 in the Discipline of Pharmacology, School of Medical Sciences and Faculty of Pharmacy at the University of Sydney. I declare that this submission is my own work and the results have not been previously submitted for award of any other degree at the University of Sydney or any other educational institution. Unless otherwise specified in the text, all studies reported within this thesis were performed by the author.

Signed

Date 09/12/13
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First and foremost I must thank my supervisor Professor Michael Murray for his expertise and guidance throughout this research project. Michael’s involvement in my experiment planning and thesis writing was especially helpful. I also owe my gratitude to my co-supervisor, Dr Fanfan Zhou, for her excellent instruction in the lab and knowledge in the field of transporters. Without her help, I doubt I would have mastered the art of keeping cells happy so promptly! I am sincerely grateful to my lab group members Jian Zheng, Lizzie Li and Florence Cheung for their support during the past two years, both in terms of guiding me in the lab and being kind and generous friends. I am so glad to have met them through this project.

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Abstract

Members of the solute carrier (SLC) family of transporters govern the cellular influx of a multitude of endogenous compounds, xenobiotics and drugs. SLCs including OATP1A2 (*SLCO1A2*), OATP1B3 (*SLCO1B3*) and OCT1 (*SLC22A1*) participate in the disposition of several anticancer drugs, such as the tyrosine kinase inhibitor (TKI) imatinib. The regulation of most solute carrier transporters is modulated in cancer cells, hence individuals with altered expression of these proteins may have different antitumour efficacy with TKIs that are SLC substrates. Furthermore, some of these agents may elicit drug-drug interactions during therapy, so the accumulation of pharmacokinetic data concerning TKIs is of clinical interest.

In this project, the impact of TKIs on the uptake of model substrates in cells overexpressing SLC transporters was evaluated. Human embryonic kidney 293 (HEK293) cells were transfected with complementary DNA to express transport proteins, including organic anion transporting polypeptides (OATP1A2, OATP1B1, OATP1B3 and OATP2B1), organic anion transporters (OAT1-4), organic cation transporters (OCT1-3) and organic zwitterion/cation transporters (OCTN1-2). In the preliminary screening experiments, TKIs (10 µM) were tested for their capacity to inhibit the uptake of a specific transporter-mediated radiolabelled substrate into these cells. Half maximal inhibitory concentrations (IC$_{50}$) and inhibition constants ($K_i$) of potent inhibitors were determined.

Of the 13 clinically relevant TKIs tested, 11 effectively inhibited substrate uptake by some transporters. Two of these interactions had IC$_{50}$ and $K_i$ values in the nanomolar
range: the inhibition of OATP1A2- and OATP2B1-mediated estrone-3-sulfate uptake by cediranib and erlotinib, respectively.

Given that the predicted maximum unbound plasma concentration of cediranib is 6 nM, this drug has the potential to inhibit OATP1A2-dependent drug transport at clinically relevant concentrations, as the unbound IC\textsubscript{50} or \(K_i\) of cediranib is estimated to be approximately 1 nM.

Similarly, erlotinib-induced inhibition of OATP2B1 transport was regarded as clinically relevant since the IC\textsubscript{50} and \(K_i\) values were approximately 10-fold less than the maximum unbound plasma concentration achieved by this drug. Interactions of OATP1A2 and OATP1B3 with erlotinib were also found to be clinically significant. In particular the inhibition of OATP1B3 by erlotinib was suggested for further in vivo drug-drug interactions tests, because this transporter is known to transport several important anticancer drugs, including paclitaxel and the irinotecan metabolite SN-38, which may be used concomitantly with erlotinib in chemotherapy.

Implementing the R value extrapolation approach recommended by the International Transporter Consortium (ITC), model substrate uptake inhibition by gefitinib in OATP1A2- and OATP2B1-transfected HEK293 cells and neratinib in OATP1B3-HEK293 cells were additional transporter-TKI interactions found to be potentially implicated in in vivo drug-drug interactions. Furthermore, nilotinib and bosutinib were identified as inhibitors of OAT3 and OCT1, respectively, and are suggested for further clinical drug-drug interaction studies.
List of Publications


**Johnston, RA**, Zhou, F, Rawling, T, Murray, M. Selective inhibition of human solute carrier transporters by multikinase inhibitors. (Manuscript submitted to Drug Metabolism and Disposition.)

**Johnston, RA**, Murray, M. Implications of interactions between tyrosine kinase inhibitors and human solute carriers in cancer therapy: a systematic review. (Manuscript in preparation for Cancer Treatment Reviews.)
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the plasma concentration-time curve</td>
</tr>
<tr>
<td>BCRP</td>
<td>breast cancer resistance protein</td>
</tr>
<tr>
<td>BSEP</td>
<td>bile salt export pump</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cat</td>
<td>catalytic domain</td>
</tr>
<tr>
<td>CCK-8</td>
<td>cholecystokinin octapeptide</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
</tr>
<tr>
<td>c-Kit</td>
<td>stem cell factor receptor</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum plasma concentration</td>
</tr>
<tr>
<td>C&lt;sub&gt;max,sys&lt;/sub&gt;</td>
<td>maximum systemic plasma concentration</td>
</tr>
<tr>
<td>C&lt;sub&gt;max,portal&lt;/sub&gt;</td>
<td>maximum portal plasma concentration at the inlet to the liver</td>
</tr>
<tr>
<td>c-Met</td>
<td>hepatocyte growth factor receptor</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukaemia</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>DHEAS</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>F&lt;sub&gt;a&lt;/sub&gt;</td>
<td>fraction of drug absorbed</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>Flt-3</td>
<td>FMS-like tyrosine kinase 3</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;</td>
<td>unbound fraction of drug</td>
</tr>
</tbody>
</table>
GIST gastrointestinal stromal tumour
HCC hepatocellular carcinoma
HEK293 human embryonic kidney-293
HER human epidermal growth factor receptor
IC$_{50}$ half maximal inhibitory concentration
ITC International Transporter Consortium
IUR intracellular uptake and retention
$k_a$ absorption rate constant
$k_{cat}$ catalytic constant of transporter
$K_i$ dissociation constant of transporter-inhibitor complex
$K_m$ dissociation constant of transporter-substrate complex
$K_s$ dissociation constant of transporter-substrate complex
M mole per litre
M-1 $N'$-hydroxymethylsorafenib N-oxide
M-2 sorafenib N-oxide
M-3 $N'$-hydroxymethylsorafenib
M-4 $N'$-desmethylsorafenib
M-5 $N'$-desmethylsorafenib N-oxide
MATE multidrug and toxin extrusion protein
MFS major facilitator family
min minutes
MKI multikinase inhibitor
mmol millimole
MPP$^+$ 1-methyl-4-phenylpyridinium
MRP multidrug resistance protein
Myr N-terminal myristate
NCI-60 United States National Cancer Institute anticancer drug screen
nM nanomole per litre
NRTK nonreceptor tyrosine kinase
NSCLC non-small cell lung cancer
OAT organic anion transporter
OATP organic anion transporting polypeptide
OCT organic cation transporter
OCTN organic zwitterion/cation transporter
ORF  open reading frame
PAH  para-aminohippuric acid
PBS  phosphate-buffered saline
PDGF  platelet-derived growth factor
PDGFR  platelet-derived growth factor receptor
P-gp  P-glycoprotein
PIP_2  phosphatidylinositol 4,5-bisphosphate
pmol  picomole
Pro  proline
Q_h  hepatic blood flow rate
R_b  blood-to-plasma concentration ratio
RCC  renal cell carcinoma
Ret  rearranged during transfection
RTK  receptor tyrosine kinase
S  substrate
SH  Src homology domain
SLC  solute carrier
SN-38  7-ethyl-10-hydroxycamptothecin
SNP  single nucleotide polymorphism
TEA  tetraethylammonium
TI  transporter-inhibitor complex
TK  tyrosine kinase
TKI  tyrosine kinase inhibitor
TMD  transmembrane domain
TS  transporter-substrate complex
TSI  transporter-substrate-inhibitor complex
Tyr  tyrosine
μg  microgram
μL  microlitre
μM  micromole per litre
V  velocity of uptake reaction
VEGF  vascular endothelial growth factor
VEGFR  vascular endothelial growth factor receptor
V_{max}  maximal velocity of uptake reaction
List of Tables

Table 1.1 Substrates and tissue distribution of SLCs 6
Table 1.2 Interactions of TKIs with SLCs and other drugs 46
Table 2.1 Radiolabelled-to-unlabelled ratios of prototypical substrate used in uptake experiments 57
Table 3.1 Summary of IC$_{50}$ values for transporter-TKI interactions 79
Table 3.2 Kinetic parameters of the inhibition of OATP1A2- and OATP2B1-mediated estrone-3-sulfate uptake by cediranib and erlotinib 92
Table 4.1 Experimentally-determined IC$_{50}$ and $K_i$ values of TKI-mediated inhibition of transporter uptake, compared with clinically-derived unbound TKI $C_{\text{max,sys}}$ values 99
Table 4.2 Variables used for calculation of unbound $C_{\text{max, portal}}$ and R values 102
Table 4.3 Experimentally-determined IC$_{50}$ values from TKI-mediated inhibition of transporter uptake, compared with estimated unbound TKI $C_{\text{max,portal}}$ values 103
Table 4.4 Assorted R values for TKI-mediated inhibition of OATP substrate uptake 106
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Figure 1.1</strong></td>
<td>Predicted topological model of human OATP1B1</td>
<td>4</td>
</tr>
<tr>
<td><strong>Figure 1.2</strong></td>
<td>Diagram of major drug transporters in various tissues</td>
<td>7</td>
</tr>
<tr>
<td><strong>Figure 1.3</strong></td>
<td>Representation of the influence of <em>SLCO1B1</em> loss-of-function genetic variation on drug disposition</td>
<td>14</td>
</tr>
<tr>
<td><strong>Figure 1.4</strong></td>
<td>Selected anticancer drugs as substrates for organic anion transporting polypeptides</td>
<td>17</td>
</tr>
<tr>
<td><strong>Figure 1.5</strong></td>
<td>Predicted membrane topology of a structural model of rat OCT1</td>
<td>19</td>
</tr>
<tr>
<td><strong>Figure 1.6</strong></td>
<td>21 FDA approved TKIs and their targets</td>
<td>30</td>
</tr>
<tr>
<td><strong>Figure 1.7</strong></td>
<td>Overexpression of a normal receptor TK in cancer</td>
<td>31</td>
</tr>
<tr>
<td><strong>Figure 1.8</strong></td>
<td>Chemical structures of first generation HER inhibitors</td>
<td>33</td>
</tr>
<tr>
<td><strong>Figure 1.9</strong></td>
<td>Chemical structures of second generation HER inhibitors</td>
<td>34</td>
</tr>
<tr>
<td><strong>Figure 1.10</strong></td>
<td>Structures of the MKI sorafenib and five of its metabolites</td>
<td>37</td>
</tr>
<tr>
<td><strong>Figure 1.11</strong></td>
<td>Chemical structures of multikinase inhibitors</td>
<td>38</td>
</tr>
<tr>
<td><strong>Figure 1.12</strong></td>
<td>Schematic of the nonreceptor TK BCR-ABL in its inactive state</td>
<td>41</td>
</tr>
<tr>
<td><strong>Figure 1.11</strong></td>
<td>Chemical structures of nonreceptor tyrosine kinase inhibitors</td>
<td>42</td>
</tr>
<tr>
<td><strong>Figure 3.1</strong></td>
<td>Inhibitory effects of TKIs on SLC-mediated substrate uptake</td>
<td>60</td>
</tr>
<tr>
<td><strong>Figure 3.2</strong></td>
<td>Dose-response relationships for TKIs with respect to inhibition of estrone-3-sulfate uptake in HEK293-OATP1A2 cells</td>
<td>71</td>
</tr>
<tr>
<td><strong>Figure 3.3</strong></td>
<td>Dose-response relationships for erlotinib- and neratinib-mediated inhibition of CCK-8 uptake in HEK293-OATP1B3 cells</td>
<td>74</td>
</tr>
<tr>
<td><strong>Figure 3.4</strong></td>
<td>Dose-response relationships for TKIs with respect to inhibition of estrone-3-sulfate uptake in HEK293-OATP2B1 cells</td>
<td>74</td>
</tr>
</tbody>
</table>
Figure 3.5  Dose-response relationship for nilotinib-induced inhibition of estrone-3-sulfate uptake in HEK293-OAT3 cells  76
Figure 3.6  Dose-response relationship for bosutinib-influenced inhibition of MPP\(^+\) uptake in HEK293-OCT1 cells  77
Figure 3.7  Dose-response relationship for cediranib-induced inhibition of L-carnitine uptake in HEK293-OCTN2 cells  78
Figure 3.8  Michaelis-Menten family of plots for estrone-3-sulfate uptake in OATP1A2-transfected cells  80
Figure 3.9  Lineweaver-Burk plots for the inhibition of estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells by cediranib  81
Figure 3.10 Dixon plots for the inhibition of estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells by cediranib  84
Figure 3.11 Michaelis-Menten family of plots for estrone-3-sulfate uptake in OATP2B1-transfected cells  87
Figure 3.12 Lineweaver-Burk plots for the inhibition of estrone-3-sulfate uptake in OATP2B1-transfected HEK293 cells by erlotinib  89
Figure 3.13 Dixon plots for the inhibition of estrone-3-sulfate uptake in OATP2B1-transfected HEK293 cells by erlotinib  91
Figure 4.1  Schematic of the equilibria between transporter, substrate and inhibitor for linear mixed-type inhibition  111
Chapter 1

Literature Review

1.1 Human solute carriers

In recent years, members of the human solute carrier (SLC) classification of proteins have become recognised as major intermediaries in the uptake of pharmaceutical drugs into cells. The past view that therapeutic and intrinsic compounds are typically absorbed by diffusion via the lipid bilayer of the cell membrane has been superseded by the knowledge that transport proteins are the predominant means of cellular influx for most endogenous and exogenous molecules (1,2). Human organic anion and cation membrane transporters are categorised within two SLC gene families; SLCO (previously SLC21), consisting of the organic anion transporting polypeptides (OATPs), and SLC22, comprising the organic anion transporters (OATs), organic cation transporters (OCTs) and organic zwitterion/cation transporters (OCTNs). Some SLCs are expressed pervasively throughout the body, such as OCT3 and OCTN1-2 (3), while others are limited to particular tissues, for example OATP1B1 and OATP1B3 in hepatocytes (4), however all play significant roles in drug uptake, distribution and elimination (5). Given that members of both SLCO and SLC22 families are characterised by their broad substrate specificities (6), a multitude of drugs, xenobiotics and endogenous compounds can be transported by individual proteins. Consequently these uptake transporters may be principal determinants of drug disposition and response, and, salient to the focus of this thesis, sites at which drug-drug interactions can occur (7).
1.1.1 Transporters in the \textit{SLCO} superfamily: OATPs

OATP research has advanced considerably in the nearly 20 years since the first rat and human OATPs were cloned\(^\text{(8,9)}\), with emphasis on determining substrates and inhibitors of these transport proteins, in addition to clarifying the effect of interindividual variations and cancer on transporter expression and function\(^\text{(10,11)}\).

1.1.1.1 Nomenclature

The \textit{SLCO} family belongs to the major facilitator superfamily (MFS) of membrane transporters. OATPs are classified by their amino acid sequence where the guidelines stipulate that proteins with more than 40\% sequence identity belong to the same family (\textit{SLCO} or OATP), while proteins with greater than 60\% identity are part of the same subfamily (for example OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6)\(^\text{(12)}\). Each of these groups can have subfamilies, such as OATP1A, OATP1B, OATP1C etc., and the distinct OATPs within these subfamilies are assigned numbers based on when the proteins were identified. Alternatively, if there is already an orthologue known, they are given the same number\(^\text{(12)}\). The number of OATPs identified or predicted in protein databases now exceeds 300 from more than 40 species\(^\text{(2)}\), however there are only 11 OATPs known in humans, of which the four best characterised are expounded in this work: OATP1A2, OATP1B1, OATP1B3 and OATP2B1.
1.1.1.2 Structure and transport mechanism

OATPs are sodium-independent transport systems that are predicted to have 12 transmembrane regions, with a large extracellular loop between the 9th and 10th domains containing several conserved cysteine residues (13). Due to the challenges associated with crystallising membrane proteins (14), their structures have not thus far been determined experimentally. To this date, the Rhesus glycoprotein ammonium transporter (SLC42A3) is the only human SLC with an experimentally defined crystal structure (15). X-ray crystal structures have been solved for proteins in the bacterial SLC13 and SLC28 superfamilies (16,17), however no OATP homologues have been found in bacteria or yeast suggesting that OATPs are specific to the animal kingdom (2). Hence, alternative methods of protein structure elucidation are needed.

The implementation of comparative modelling based on crystal structures that have been solved for membrane transporters in the MFS has allowed the development of potential three-dimensional models for OATPs, including OATP1B1 (Figure 1.1) (18), OATP1B3 and OATP2B1 (19). Experiments utilising site-directed mutagenesis have been able to speculate the substrate binding sites of OATPs (20-22). Nevertheless, until it is possible to purify and crystallise an OATP, the mechanism by which OATPs transport substrates cannot be not fully understood.
Figure 1.1 Predicted topological model of human OATP1B1 with 12 transmembrane regions (18). Amino acids conserved in 77 out of 97 mammalian OATPs are indicated in black. Conserved cysteine residues are given in grey.

OATPs are believed to function as organic anion exchangers, as indicated by experiments with reduced glutathione, glutathione conjugates and bicarbonate acting as counterions in rat OATP-mediated uptake of endogenous substrates (23-25). The mechanism of transport appears to be electroneutral and operates by coupling the cellular uptake of substrates with the efflux of intracellular compounds (26,27). A low extracellular pH such as that found in the duodenum, compared with the lower part of the intestine, has been observed to enhance uptake by various OATPs including OATP2B1 (2). This transporter is localised at the apical membrane of human enterocytes, among other epithelia, and substrates of OATP2B1 such as pravastatin have been shown to be preferentially absorbed from the duodenum (28).
Furthermore, modulation of OATP-mediated transport of substrates by other endogenous substrates has also been demonstrated, such as in the stimulation of OATP2B1-mediated uptake of estrone-3-sulfate by prostaglandins A\textsubscript{1} and A\textsubscript{2} (29). It has been suggested that no general transport mechanism exists for OATPs and that they have more than one substrate binding site, which may or may not interact (2). The second point has been made evident by the observation of biphasic kinetic behaviour in OATP1B1-mediated uptake of estrone-3-sulfate, indicating that there are distinct high and low affinity components on OATP1B1 for this substrate (30,31).

In reviewing the current literature on OATPs, it is manifest that further research is required to explain their method of transport, as it may vary for different substrates in the same transporter. Likewise, depending on the transport mechanism, OATPs may or may not be able to concentrate a substrate within cells over the respective extracellular concentration, such as drugs in liver cells (2). While the knowledge on OATP transport mechanisms is incomplete, it is important to note that prototypical substrates and binding affinities of these substrates have been identified for the four key OATPs, including estrone-3-sulfate for OATP1A2, OATP1B1 and OATP2B1 (32,33), and cholecystokinin octapeptide (CCK-8) for OATP1B3 (34). Cellular uptake assays using these substrates, which possess relatively high affinities for their respective transporters, have allowed the straightforward in vitro identification of transporter inhibitors, which may be substrates competing for translocation across the cell membrane or inhibitors at the substrate binding site(s) (27). Numerous drug substrates and inhibitors of solute carriers have already been demonstrated (Table 1.1), which may alter the pharmacokinetics and toxicities of drugs utilising these transporters for uptake.
### 1.1.1.3 Tissue distribution

<table>
<thead>
<tr>
<th>Transporter (human gene symbol)</th>
<th>Endogenous substrates</th>
<th>Prototypical substrate</th>
<th>Anticancer drug substrates</th>
<th>Predominant tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1A2 (SLCO1A2)</td>
<td>Taurocholate, bile salts, organic anions and cations (2)</td>
<td>Estrone-3-sulfate (32)</td>
<td>Hydroxyurea, imatinib, methotrexate (39-41)</td>
<td>Brain, testis, kidney, intestine, liver, eye (2,4)</td>
</tr>
<tr>
<td>OATP1B1 (SLCO1B1)</td>
<td>Bile salts, organic anions (2)</td>
<td>Estrone-3-sulfate (33)</td>
<td>Hydroxyurea, methotrexate, paclitaxel, SN-38, sorafenib (39,42-45)</td>
<td>Liver (4)</td>
</tr>
<tr>
<td>OATP1B3 (SLCO1B3)</td>
<td>Bile salts, organic anions (2)</td>
<td>CCK-8 (34)</td>
<td>Docetaxel, hydroxyurea, imatinib, methotrexate, paclitaxel, SN-38 (39,40,46-49)</td>
<td>Liver (4)</td>
</tr>
<tr>
<td>OATP2B1 (SLCO2B1)</td>
<td>Estrone-3-sulfate, DHEAS (2)</td>
<td>Estrone-3-sulfate (33)</td>
<td>N/A</td>
<td>Liver, intestine, brain (2,27)</td>
</tr>
<tr>
<td>OAT1 (SLC22A6)</td>
<td>Medium-chain fatty acids, prostaglandins E₂ and F₂ (3)</td>
<td>p-aminohippuric acid (36)</td>
<td>Mesna, methotrexate (50,51)</td>
<td>Kidney, placenta, brain (3)</td>
</tr>
<tr>
<td>OAT2 (SLC22A7)</td>
<td>Glutamate, prostaglandins E₂ and F₂ (3)</td>
<td>Estrone-3-sulfate (3)</td>
<td>Docetaxel, fluorouracil, methotrexate, paclitaxel (52-54)</td>
<td>Liver, kidney (3)</td>
</tr>
<tr>
<td>OAT3 (SLC22A8)</td>
<td>cAMP, cortisol, prostaglandins E₂ and F₂ (3)</td>
<td>Estrone-3-sulfate (3)</td>
<td>Erlotinib, 6-mercaptopurine, methotrexate, topotecan (55-59)</td>
<td>Kidney, brain, skeletal muscle (3)</td>
</tr>
<tr>
<td>OAT4 (SLC22A11)</td>
<td>Estrone-3-sulfate, prostaglandins E₂ and F₂ (3)</td>
<td>Estrone-3-sulfate (3)</td>
<td>Mesna, methotrexate (50,60)</td>
<td>Kidney, placenta (3)</td>
</tr>
<tr>
<td>OCT1 (SLC22A1)</td>
<td>Putrescine, salolinol, agmatine (3)</td>
<td>MPP⁺ (37)</td>
<td>Cisplatin, imatinib, oxaliplatin, picoplatin (40,61-65)</td>
<td>Liver, intestine, kidney, lung, skeletal muscle, brain (3)</td>
</tr>
<tr>
<td>OCT2 (SLC22A2)</td>
<td>Acetylcholine, dopamine, epinephrine, histamine (3)</td>
<td>MPP⁺ (37)</td>
<td>Cisplatin, erlotinib, ifosfamide, oxaliplatin, picoplatin (55,61,65-67)</td>
<td>Kidney, intestine, lung, placenta, thymus, brain, inner ear (3)</td>
</tr>
<tr>
<td>OCT3 (SLC22A3)</td>
<td>Epinephrine, histamine (3)</td>
<td>MPP⁺ (38)</td>
<td>Oxaliplatin (61)</td>
<td>Broad (3,35)</td>
</tr>
<tr>
<td>OCTN1 (SLC22A4)</td>
<td>Acetylcholine, ergothionine, L-carnitine (3)</td>
<td>L-ergothionine (3)</td>
<td>Doxorubicin, mitoxantrone (68)</td>
<td>Broad (3,35)</td>
</tr>
<tr>
<td>OCTN2 (SLC22A5)</td>
<td>L-carnitine, choline (3)</td>
<td>L-carnitine (3)</td>
<td>Etoposide, imatinib (40,69)</td>
<td>Broad (3,35)</td>
</tr>
</tbody>
</table>
Table 1.1 Synopsis of some of the endogenous, prototypical and known anticancer drug substrates of the 13 solute carriers discussed in this text and the tissues in which these transporters are predominantly found (2,3,4,27,32-69). Abbreviation: N/A, not available.

Figure 1.2 Diagram of major drug transporters in various tissues (referenced from (27) with appropriate modifications). Essential drug transporters in the intestine (A), liver (B), kidney (C), and brain (D) are shown. Arrows illustrate the direction of substrate transport for each transporter. Circles coloured in blue represent uptake.
transporters in the SLCO and SLC22 superfamilies and are described in detail in this thesis. Circles coloured in red denote efflux transport proteins that are of importance but are not the topic of this thesis. *OATP1A2 is contrarily expressed in the distal tubules of the nephrons.*

OATPs are expressed in a variety of different tissues, in particular the intestine, liver, kidney and brain (Figure 1.2), which is indicative of their critical role in drug disposition (70). OATP1A2 has been detected at the apical (luminal) membranes of brain capillary endothelial cells (71), where it is believed to be part of the blood-brain barrier, and enterocytes (72), where it may mediate the uptake of exogenous compounds. Within the liver, OATP1A2 has limited expression at the basolateral (sinusoidal) membrane of hepatocytes (73), and is specifically localised to the apical membrane of cholangiocytes (74), where it may be involved in the reabsorption of drugs or xenobiotics excreted into the bile. This protein is also expressed at the luminal membrane of distal tubules of the nephrons, suggesting OATP1A2 may have an important role in the reabsorption of drugs that are filtered at the level of the renal proximal tubule (74). Additionally, it could be responsible for the secretion of exogenous compounds into urine (5).

Whereas most OATPs are prevalent in multiple tissues, OATP1B1 and OATP1B3 are predominantly, if not exclusively, localised to the sinusoidal membrane of liver cells, hence they are considered to be of particular importance for hepatic drug elimination (75). There is extensive research into the functional characterisation of polymorphisms in the genes encoding these proteins and drug-drug interactions involving substrates of these hepatic transporters, such that OATP1B1 and OATP1B3
are both recommended in the European Medicines Agency (EMA) guideline and the United States Food and Drug Administration (FDA) draft guidance for drug interaction studies (76,77).

While OATP2B1 is also found most abundantly on the basolateral membrane of liver tissue, it is expressed more ubiquitously throughout the body (78), for example at the apical membrane of brain capillary endothelial cells and the basal surface of the syncytiotrophoblast layer of placenta (71,79). OATP2B1 is additionally localised to the apical membrane of enterocytes (28), therefore it is possible that this transporter contributes to the absorption of its substrates from the intestinal lumen (80).

1.1.1.3.1 Modulated tissue distribution in cancer

Besides their expression in normal tissues, it is significant to note that several studies have shown altered tissue distribution of OATP proteins in some malignant tissues. For example, OATP1A2 has been detected in healthy colon tissue, yet this expression was markedly downregulated in colon cancer tissue (81). In the brain, OATP1A2 was localised to endothelial cells at the blood-brain and blood-tumour barriers, but not in glioma cells (71). Conversely in malignant breast cancer cells, such as the T47-D cell line derived from human breast ductal carcinoma and the hormone-dependent MCF-7 cell line, OATP1A2 expression was significantly upregulated compared with normal breast epithelial cells (82). OATP1A2 is a known transporter of the hormone precursor estrone-3-sulfate, which when exposed to estrogen-dependent breast cancer cells has been demonstrated to cause increased cellular proliferation in vitro (83,84). Modulation of OATP1A2-mediated estrone-3-sulfate uptake in these cells through
concomitant treatment with an inhibitor or competing substrate may result in suppressed cell proliferation (85). Hence, OATP1A2 could be pursued as a novel therapeutic target for breast cancer treatment, along with other SLCs which are highly expressed in breast cancer tissue and transport estrone-3-sulfate (see Table 1.1).

The liver-specific transporters OATP1B1 and OATP1B3 both appear to have decreased expression in hepatocellular carcinoma (HCC) (86,87), however interestingly these proteins are also expressed ectopically in several other cancer tissues (47). In contrast to OATP1A2, OATP1B1 levels were significantly enhanced in colon cancer tissue compared with normal colon tissue (81), whereas OATP1B3 is generally upregulated in a wide range of cancer types (11). Northern blot analysis has indicated the expression of OATP1B3 in gastric, colon, pancreatic and gallbladder cancer cell lines and cancer tissues (47,88,89), while in non-small cell lung cancer (NSCLC), OATP1B3 mRNA expression was considerably increased as opposed to the non-malignant surrounding tissue (90). OATP1B3 was also detected in cells of invasive ductal breast carcinoma, where its immunoreactivity was inversely correlated with tumour size and associated with a decreased risk of cancer recurrence and improved prognosis (91). The upregulation of these normally liver-specific proteins in cancer tissues may underlie their potential value as diagnostic and prognostic markers to improve cancer therapy. Additionally, OATP1B1 and OATP1B3 could serve as promising therapeutic targets given that they have been shown to transport several antineoplastic drugs, including the active irinotecan metabolite SN-38 (45,49), and paclitaxel (43,48). On the other hand, some anticancer drugs are inhibitors of these transporters, for example the tyrosine kinase inhibitor (TKI) lapatinib reduces OATP1B1-mediated uptake of the model substrate estradiol 17β-D-glucuronide (92).
OATP1B1 also shows high transport activity for estrone-3-sulfate and is upregulated in breast cancer cell lines (33,93), thus inhibition of OATP1B1-mediated uptake of this proliferation-inducing substrate into tumour cells may suggest a supplementary effect of treating breast cancer with lapatinib.

Broadly expressed OATP2B1 has exhibited upregulation in the colon adenocarcinoma cell line CX-1 and several breast cancer cells lines (33,93). In breast cancer patients, expression of OATP2B1 was demonstrated to be positively correlated with tumour grade (94). Conversely, recent clinical data have shown OATP2B1 expression to be decreased in malignant breast tumours compared with non-malignant tissue samples (95). With respect to the latter study, the authors considered that their findings were due to the high interpatient variability in the expression levels of OATPs as opposed to the effect of cancer. In terms of OATP2B1 downregulation in malignant tissues, expression of this transporter appeared to be decreased in osteocarcinoma tissues compared with non-malignant aneurysmal bone cysts (96). Similarly to OATP1A2, OATP2B1 was not detected in glioma cells, but was expressed at endothelium of the blood-brain and blood-tumour barriers in the brain (71).

1.1.1.4 Physiological role

OATPs transport a wide range of structurally unrelated compounds, such as endogenous bile salts, bilirubin glucuronides and steroid hormone metabolites (2), and xenobiotics, including some anticancer drugs (see Table 1.1), HMG-CoA reductase inhibitors (statins), and plant compounds (97,98). The majority of OATP substrates are anionic amphipathic molecules with a relatively high molecular weight (more than
350 Da) and a high degree of albumin binding under physiological conditions (12), though OATP1A2 has also been demonstrated to transport bulky type II organic cations such as \(N\)-methyl-quinine (99). The four OATPs of interest in this thesis share similar substrate specificities (100); in particular the high degree of amino acid similarity between OATP1B1 and OATP1B3 (80% sequence identity) gives rise to their substantially overlapping substrate spectrum (42,101). The cellular influx of the anticancer drugs methotrexate and hydroxyurea are mediated by OATP1A2, OATP1B1 and OATP1B3 (39,41,42,47), while estrone-3-sulfate is transported by OATP1A2, OATP1B1 and OATP2B1. Both OATP1A2 and OATP1B3 are involved in the uptake of the TKI imatinib (40), and all statins in clinical use are substrates of OATP1B1 and OATP1B3 (42,100,102). OATP2B1 also contributes to the disposition of statins (103), however despite its broad substrate selectivity, OATP2B1 is the only SLC discussed in this work that has not yet been shown to transport anticancer agents (104,105). Although OATP1B3 shares many substrates with OATP1B1 (5), it does selectively mediate the uptake of some compounds, for example the gastrointestinal peptide CCK-8 and the antihypertensive drug telmisartan (106,107).

OATPs form part of a network of proteins involved in the disposition of substrates that includes phase I and II metabolic enzymes, such as cytochrome P450 (CYP) enzymes and glucuronosyltransferases respectively, and efflux transporters for example P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) (see Figure 1.2) (105). The pharmacologic action of orally administered drugs is dependent on sufficient intestinal and hepatic absorption and distribution to sites of action, before their elimination by metabolic and excretory pathways (108). Regulation of OATPs at the transcriptional level, polymorphisms in the genes
encoding OATPs, and coadministration of inhibitors can lead to modulation of OATP activity (109), which may affect the bioavailability of drug substrates by hindering their absorption or facilitating their intracellular accumulation. The dynamic interplay between uptake and efflux transporters within epithelial cells is essential to our understanding of transporter-mediated drug disposition (108).

1.1.1.5 Genetic polymorphisms and interindividual variations

Extensive literature regarding the genetic variants of numerous drug transporters has been published (110-113), however, the impact of these variants on transport function and the scope of their contribution to interindividual variability of drug response requires further understanding. Recently, appropriate screening tools to investigate the functional consequence of large numbers of transporter single nucleotide polymorphisms (SNPs) in high-throughput assays have emerged (10,114).

The first systematic analysis of the membrane transporters OATP1B1, OATP1B3, and OATP2B1 on the interindividual variability of hepatic expression revealed that expression of OATP1B1 is significantly affected by SLCO1B1 variants (115). The genetic variations in this transporter appear to be major contributors to drug disposition; for example several studies have shown that individuals carrying a loss-of-function variant of OATP1B1 had increased area under the plasma concentration-time curve (AUC) of pravastatin (see Figure 1.3) (113,116,117).
Figure 1.3 Representation of the influence of SLCO1B1 loss-of-function genetic variation on drug disposition (113).

Expression of OATP1B3 or OATP2B1 is apparently not influenced by genetic variants, indicating that unidentified transcription factors, genetic variation in those genes, together with epigenetics such as DNA methylation or regulation by microRNA, may affect the interindividual variability of these transporters (115). Protein-altering variants of OATP1A2 potentially play a central role in interindividual variation in drug disposition and toxicity (41). Methotrexate and imatinib are two important anticancer drugs that have been determined as OATP1A2 substrates. Considering methotrexate therapy can result in severe toxicity in organs where OATP1A2 is expressed, including the kidney, liver, gastrointestinal tract and brain, clinical studies of OATP1A2 genetic variants are important to determine their
contribution to methotrexate therapeutic outcomes (41). It has recently been shown that polymorphisms in OATP1A2 and OATP1B3 are related to clearance of the imatinib (118,119), while a genetic variant of OATP2B1 has been associated with reduced bioavailability of the antihistamine fexofenadine (120).

1.1.1.6 Drug-drug interactions

Over the past decades, drug-drug interactions that inhibit intracellular drug metabolising enzymes, such as CYP enzymes, have been extensively studied, however knowledge of interactions inhibiting membrane transport proteins is still relatively limited (121). Many changes in the pharmacokinetics of drugs that cannot be explained by interactions at the metabolising enzyme level may be mediated by transporters (122). Some of the known inhibitors of the OATPs discussed in this thesis are summarised in Table 1.2 and include the antibiotic rifampicin and the grapefruit juice flavonoid naringin (123,124). Modulation of transport function by inhibitors may affect drug excretion pathways by suppressing OATPs involved in hepatobiliary elimination, in particular OATP1B1, OATP1B3 and OATP2B1 (125), thereby altering the plasma concentrations of a given drug substrate. In one study in healthy volunteers, it was shown that a single dose of rifampicin raised the AUC of coadministered atorvastatin by approximately seven-fold (126), probably by inhibiting OATP-mediated hepatic uptake of atorvastatin (100). The pharmacokinetics of drugs absorbed by OATPs from the intestine are potentially subject to interactions with other orally administered drugs and food; for example the immunosuppressant cyclosporine and green tea catechins have been demonstrated to inhibit the enteric transporters OATP1A2 and OATP2B1 (127-129). This may lead to a reduction in the
AUC or maximum systemic plasma concentration ($C_{\text{max,sys}}$) of oral drug substrates of these transporters. Interestingly, inhibition studies have shown that compounds can have stimulatory, inhibitory or no effect on OATP-mediated transport, depending on the model substrate used, therefore study design in transporter-mediated drug-drug interaction experiments is important (2).

1.1.1.7 Chemotherapeutic aspects

During tumour progression cells acquire an altered metabolism, which may be due to, or as a result of, an increased demand for energy and nutrients (130). The upregulation of some OATPs in tumour cells as a consequence of these changed needs may be of therapeutic value. For example modulating transporter activity can influence the biological processes required for tumour growth (130), particularly in hormone-related cancers (131), since OATPs mediate the uptake of steroid hormone precursors such as estrone-3-sulfate. A novel approach to drug design would be to modify pharmaceuticals so they become substrates of OATPs, which may help to increase their uptake into OATP-expressing cancer cells (11,132). Several anticancer drugs substrates of OATPs have already been identified, including such structurally diverse compounds as imatinib, methotrexate, paclitaxel, the potent irinotecan metabolite SN-38 and hydroxyurea (Figure 1.4) (39-49). In humanised transgenic mice, OATP1B1, OATP1B3, and possibly OATP1A2 have been shown to influence the pharmacokinetic behaviour of two of these substrates: the polar anion, methotrexate; and the uncharged, highly lipophilic compound, paclitaxel (133). It is therefore anticipated that many drugs with intermediate physicochemical properties in the broad spectrum of shared OATP substrates will be similarly affected (133).
Aside from mediating tissue distribution and elimination processes, these transport proteins provide intracellular drug concentrations necessary to reach a cytotoxic effect in cancer cells (105). This is inferred because there are anticancer drugs which accumulate in particular organs causing tissue-specific toxicity, but these adverse drug reactions can be modified by coadministration with chemical inhibitors of SLCs (134). Screening tumours for transporter expression prior to therapy with potential substrates could lead to SLC-targeted therapy with higher efficacy and decreased side effects (105). In considering SLC substrates or inhibitors for chemotherapy, it must also be conceded that suppressing these transporters therapeutically may interfere with normal physiological processes in the liver and impair the excretion of bilirubin, bile acids, drugs, and toxins (105).
1.1.2 Transporters in the SLC22 superfamily: OCTs, OCTNs and OATs

In the years since the first member of the SLC22 family was identified and characterised (OCT1) (135), substantial progress has been made in identifying substrates and inhibitors of these transport proteins, as well as in defining genetic variants with altered expression or function (10). The current focus is on the elucidation of their three-dimensional structure, to allow for the prediction of drug-related pathologies and the design of drugs targeted to individual SLCs (5).

1.1.2.1 Nomenclature

Members of the SLC22 family are predicted to adopt the structural fold associated with proteins in the MFS (136), in which they are assigned to subclass 2.A.1 (uniporters, symporters, antiporters) according to the Milton Saier transporter classification system (137,138). The SLC22 family comprises three subgroups of transporters with closely related primary structures: OCTs, OCTNs and OATs (3). Three OCTs (OCT1-3, SLC22A1-3), two OCTNs (OCTN1 and OCTN2, SLC22A4-5), and four of the OATs (OAT1-4, SLC22A6-8, 11) found in humans are researched in this thesis. Most of these proteins have a single rodent orthologue, but OAT4 is specific to humans (5).
1.1.2.2 Structure and transport mechanism

The SLC22 transporters all have a similar putative membrane topology consisting of 12 α-helical transmembrane domains (TMDs), a large extracellular loop between TMDs 1 and 2 and a large intracellular loop between TMDs 6 and 7 (see Figure 1.5) (3). The MFS fold has been associated with a wide array of substrates, which can be explained partly by its large pore and extensive conformational movements during transport (136). Hence, like the SLCO transporters, the majority of SLC22 family members are polyspecific for substrates. SLC transporters mediate the influx of nutrients and substances essential for cell survival, such as sugars, digested peptides, amino acids, nucleosides, and inorganic ions (139), though numerous drugs have been found that ‘hitchhike’ on these transport proteins (140), and several additional compounds can act as high or low affinity inhibitors (141,142).

Figure 1.5 Predicted membrane topology of a structural model of rat OCT1 (143).
Transporters within each subgroup tend to have similar transport mechanisms and substrate specificities (3). For example, all members of the OCT subgroup translocate organic cations, weak bases and some neutral compounds (141,144,145). The OCTs are facilitative diffusion systems that transport organic cations in both directions across the plasma membrane, where the impetus is supplied by the electrochemical gradient of the transported compounds (3,146). 1-methyl-4-phenylpyridinium (MPP+) is a model cation that is transported by OCT1-3 and exhibits high maximal uptake rates (37,38,141).

OCTN1 can act as a uniporter, such as in the transport of the zwitterionic antioxidant L-ergothioneine (147), while also mediating H+/organic cation antiport (141). OCTN2 is also involved in the uniport translocation of organic cations, and mediates Na+/L-carnitine co-transport (141). The relatively high binding affinity of OCTN1 and OCTN2 for L-ergothioneine and L-carnitine respectively (147,148), means that these compounds are useful as representative substrates in cellular uptake inhibition studies.

Members of the OAT subgroup of transporters are able to transport anions in either direction across cell membranes by exchanging extracellular against intracellular organic anions (3). The concentration of the intracellular counterpart anion, which for OAT1-4 is a divalent organic anion such as succinate or α-ketoglutarate, must be higher in the cytosol than outside the cell in order to stimulate the ‘uphill’ uptake of organic anions through OATs (149). This concentration gradient is maintained by sodium-coupled anion transporters localised to the same membrane as the respective OAT (149). Para-aminohippuric acid (PAH) is a prototypical substrate for OAT1 (36), while estrone-3-sulfate is a useful model substrate for OAT2-4 (3,150).
1.1.2.3 Tissue distribution

In the kidney, OAT1, OAT2, OAT3, OCT2, and OCT3 assist in the movement of organic anions from blood across the basolateral membrane into the proximal tubule cells (38,151-154). OAT4, OCTN1, and OCTN2 are expressed at the apical membrane of these cells (155-159), and may be involved in the reabsorption from, or the secretion of xenobiotics into, urine. OAT2 and OCT1 are responsible for the uptake of drugs into the liver at the sinusoidal membrane of hepatocytes, along with the previously discussed OATPs (160-162). In enterocytes, OCT3 was found to be localised to the luminal membrane (163), along with OCTN1 and OCTN2 (164), where it may mediate the uptake of orally administered drugs and other exogenous compounds from the intestine. OCT1 and OCT2 were found to be expressed at the basolateral membrane of enterocytes (141,165), which is indicative of their role in uptake and elimination. A pictorial representation of SLC22 localisation in these three major organs of drug disposition, as well as the brain, is shown in Figure 1.2.

1.1.2.3.1 Modulated tissue distribution in cancer

The variability in OCT1 expression is influenced by genetic and epigenetic factors including altered expression during diseases. OCT1 has been detected in several human cancer cell lines, suggesting that this transporter may be expressed in the corresponding tumours (64,166). OCT1 expression is a major determinant of the activity of oxaliplatin, a known OCT anticancer substrate, and the observed upregulation of this transporter in human colon cancer cell lines may serve as a prognostic factor in colorectal cancer (64). Furthermore, DNA methylation of the
SLC22A1 gene is associated with the downregulation of OCT1 in HCC (167), which is subsequently related to advanced tumour stages and a worse patient survival (168). Increasing evidence suggests that OCT1 activity influences therapeutic outcome in patients with chronic myeloid leukaemia (CML) receiving imatinib therapy (143,169). For example, most CML patients with low OCT1 activity were found to have a suboptimal response to treatment with imatinib (170). However, in this study OCT1 mRNA expression was not a strong predictor of clinical efficacy despite being correlated with OCT1 activity. This implies that OCT1 expression could merely be an indirect marker of imatinib transport or cellular accumulation and further study is required to interpret its clinical relevance (6).

Expression profiling studies in the NCI-60 panel of cell lines determined that OCT2 was distinctly upregulated in ovarian cancer cell lines, though tumour samples obtained from patients with ovarian cancer had low or absent OCT2 expression and no correlation between expression and clinical outcome was reported (67). Higher expression of OCT2 has been shown to increase cellular sensitivity to the anticancer drugs tetraplatin, and transplatin (67). OCT2 was found to be upregulated in colon cancer cell lines, (64) thus expression of this transporter may determine the efficacy of platinum-based therapy in colorectal tumours.

OCT3 has been suggested as an suitable candidate for individualised kidney tumour therapy, because the sensitivity of some human renal carcinoma (RCC) cell lines to several important chemotherapeutics is dependent on the expression of OCT3 (139,171). Real time PCR has shown a downregulation of the SLC22A3 gene encoding OCT3 in HCC compared to corresponding non-neoplastic tumour
surrounding tissue (168), however there was no significant difference in tumour characteristics due to OCT3 expression levels (168).

High levels of OCTN1 and OCTN2 mRNA expression have been reported in some human tumour-derived cell lines (157,158), though the relevance of this upregulation to tumour growth has not been determined. In OCTN2-transfected human embryonic kidney 293 (HEK293) cells, imatinib uptake was increased compared with cells expressing OCT1, indicating that OCTN2 expression or function may be a determinant of clinical efficacy in imatinib therapy (40).

An investigation of the NCI-60 panel revealed that OAT1, OAT2 and OAT3 mRNAs were not detected in any of the 60 cancer cell lines, though they were found to be expressed in kidney (OAT1-3) and liver (OAT2) mRNA samples (68), consistent with previous findings (152,153,160). Interestingly, elevated hepatocellular expression of OAT2 after castration in prostate cancer patients has been linked with an increased hepatic uptake of docetaxel and hence a decrease in systemic exposure of this OAT2 substrate (52), suggesting that dose escalation may benefit castrated patients.

1.1.2.4 Physiological role

SLC22 transporters function in a coordinated manner with each other, as well as other proteins including receptors, enzymes and efflux transporters, in various major biological and pharmacological processes. OCT1 regulates the first step in hepatic excretion of many cationic drugs and xenobiotics, by mediating their uptake across the sinusoidal membrane into the hepatocyte (3). OCT2 mediates the first step in renal
excretion of many organic drugs, for example, the antineoplastic drug oxaliplatin (3). OCT3 is involved in the uptake of organic cations into brain, heart, and liver and participates in their biliary excretion (3). OCTN1 is important for the absorption of ergothioneine in the small intestine and for its reabsorption in renal proximal tubules, while OCTN2 mediates the uptake of L-carnitine by enterocytes and its reabsorption in the kidney. OAT1 and OAT2 regulate the renal excretion of urate, and the reabsorption of endogenous and exogenous anions (3). In liver, OAT2 mediates the efflux of glutamate into the sinusoids and the uptake of various endogenous compounds, drugs and toxins into hepatocytes (3). In the kidney, OAT3 transports organic anions across the basolateral membrane of proximal tubules for renal secretion by apical transporters (151), such as OAT4. OAT4 also contributes to the reabsorption of filtered urate, prostaglandins, anionic drugs and xenobiotics (3). OAT4 is hypothesised to be important for the uptake of dehydroepiandrosterone (DHEAS) by the placenta in order to prevent intrauterine growth retardation caused by excess DHEAS (172).

1.1.2.5 Genetic polymorphisms and interindividual variations

Genetic variations in membrane transporters may contribute to the interindividual variability in the efficacy and safety of therapeutics, including cancer chemotherapy. Although uptake carriers have been extensively genetically characterised, relevant pharmacogenetic data are still very limited (10). Genetic variants of uptake transporters have predominantly been investigated for OATPs, however more recently a number of SNPs in the OCT (SLC22A1-3) genes were found to alter transport function and substrate selectivity in vitro (144). OCT1 has 18 SNPs that modify
amino acids; six have reduced transport activity and one has increased activity (173). The pharmacokinetics of imatinib and the antidiabetic drug metformin are significantly affected by OCT1 variants (174,175). OCT2 has ten genetic variants, all of which are functionally active (141), though variants of this transporter have also been shown to alter the transport kinetics of metformin (176), suggesting that OCT2 mutations may contribute to the interindividual variability in metformin disposition (177). Five SNPs have been identified in OCT3, three of which show reduced transport activity (178), while a novel susceptible locus in SLC22A3 encoding this transporter was reported to contribute to the risk of distal colon cancer in an Asian population (179). Mutations in the gene cluster that contains the OCTN1 and OCTN2 genes have been associated with autoimmune diseases (180,181). Debilitating mutations in the OCTN2 gene have been shown to cause systemic carnitine deficiency, which results from a lack of L-carnitine reabsorption in the kidney (182). In a retrospective study looking at patients treated with imatinib for gastrointestinal stromal tumour (GIST), polymorphisms were identified in the imatinib transporter genes SLC22A4 (OCTN1) and SLC22A5 (OCTN2) that improved time to progression (183). Genetic variants for the OATs are less well-characterised, though several have been found which affect transport function (151).

1.1.2.6 Drug-drug interactions

As with the OATPs, an important risk of pathology associated with members of the SLC22 family is that of adverse drug-drug interactions (5). Oxaliplatin was found to be a substrate of the OCTs, which are believed to play a role in its anticancer effects by facilitating oxaliplatin entry into tumour cells (64,184). The TKIs gefitinib,
erlotinib, nilotinib and sunitinib were shown to inhibit the uptake of metformin in cells overexpressing OCT proteins, and were hence evaluated for their drug-drug interaction potential with oxaliplatin (185). It was observed that the cellular uptake of platinum was reduced after oxaliplatin treatment in the presence of clinically relevant concentrations or erlotinib and nilotinib, suggesting that combination therapy involving oxaliplatin and these TKIs may not be optimal (185). Concomitant administration of the antigout drug probenecid and methotrexate was noted over 30 years ago to result in prolonged systemic circulation of methotrexate (186). This can today be explained by the inhibition of OAT3- and OAT1-mediated methotrexate transport by probenecid (187). Given that erlotinib has been determined as a substrate of OAT3 and OCT2 (55), cotreatment of this TKI with methotrexate or oxaliplatin may alter the pharmacokinetics of these drugs via transporter-mediated drug-drug interactions. Several other drug substrates and interactions of anticancer drugs with SLC22 proteins are listed in Tables 1.1 and 1.2.

1.1.2.7 Chemotherapeutic aspects

The modulated expression levels of SLC22 proteins in cancer have important consequences for an individual’s susceptibility to certain chemotherapy-induced side effects, drug-drug interactions, and treatment efficacy (6). Drug resistance, for instance, can be provoked by overexpression of efflux pumps or reduced expression of SLCs, whereas SLCs that are overexpressed in tumour cells may serve as chemosensitising targets (130). A novel treatment approach has recently been suggested which involves the chemically-induced enhancement of influx transporter expression in tumour cells. This results in the accumulation of anticancer substrates
within these sites, which may lead to an increased therapeutic window for chemotherapeutic agents (6,188).

The expression of OCT1 in CML cells has been correlated with the therapeutic effect of imatinib (62), however, only minor or no OCT1-mediated transport of imatinib has been demonstrated (40). A potential explanation for this inconsistency is that endogenous OCT1 substrates influence the therapeutic effect of imatinib that enters the cells by OCT1-independent pathways (3). The intracellular toxicity of some platinum derivatives besides oxaliplatin, such as cisplatin and picoplatin, is markedly increased in OCT1- and OCT2-transfected cells, suggesting that they are also substrates of these transporters. OCTN1 mediates the cellular uptake of the anticancer drugs mitoxantrone and doxorubicin, thereby conferring cellular sensitivity to these agents (68). A list of the known anticancer drug substrates of SLC22 transporters can be found in Table 1.1.
1.2 Tyrosine kinase inhibitors

Cancer is one of the most common causes of death globally and in Australia; in 2011 around 30% of all deaths registered in this country were cancer-related (189). Over the past decades, mechanistic studies on tumour cell proliferation have led to a superior understanding of the processes that trigger cancer growth, such as the activation of tyrosine kinases (TKs), which in turn stimulate signal transduction cascades within cells. In most cancers, one or more signal transduction pathways are activated or mutated resulting in cell proliferation. The development of specific targeting drugs, for example small molecule tyrosine kinase inhibitors which impede predefined TKs involved in carcinogenesis, has revolutionised cancer research since 1988 (190). Imatinib became the first TKI to be approved for cancer treatment in 2001, which blocks cell growth and induces apoptosis by inhibiting the BCR-ABL kinase (191). In addition to this enzyme, the main targets of currently approved TKIs include: epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and human epidermal growth factor receptor (HER), among other TKs and some TK-like kinases.

At present there are 21 orally available TKIs for the treatment of cancer (see Figure 1.6), 10 of which were approved by the FDA in the last two years (192), emphasising the clinical importance of these drugs. Notable examples of diseases that were previously only treated with chemotherapy, for which effective TKIs now exist, include CML, GIST and RCC (193). While TKIs are typically better tolerated than the alternative, chiefly non-specific cytotoxic chemotherapeutics (194), they are associated with a number of serious toxicities and adverse events in some patients.
There is a high degree of interindividual variability in the pharmacokinetics of these drugs, which may be related to differential expression or function of drug metabolising enzymes or transporters for which TKIs are substrates. Furthermore, TKIs have been implicated in several drug-drug interactions which may significantly influence drug safety and efficacy. Information on the clinical relevance of their interaction potential and inhibitory effects on proteins, especially influx transporters, is lacking.

### 1.2.1 Role of tyrosine kinases

Most disease states emanate from signal transduction pathways which have gone awry (195). Signal transduction is essential for proper cell function, as it regulates gene expression and ultimately protein synthesis (196). The majority of extracellular signals are transmitted into cells via the receptor-ligand signalling pathway, which drives a series of downstream effects targeting the nucleus, where important processes such as cancer cell proliferation, apoptosis, angiogenesis and metastasis are modulated. Tyrosine kinases involved in this signal transduction cascade are frequently altered or upregulated in malignant tumours, rendering them ideal targets for highly selective cancer treatment (196). Tyrosine kinases can be classified as receptor TKs (RTKs) and nonreceptor TKs (NRTKs). RTKs are transmembrane proteins with a ligand-binding extracellular domain and a catalytic intracellular kinase domain, while nonreceptor TKs lack transmembrane domains and are found in the cytosol, the nucleus, and the inner surface of the plasma membrane (197). Both are associated with many types of cancer including NSCLC, breast cancer and medullary thyroid cancer.
Figure 1.6 21 FDA approved TKIs and their targets (image cited from (195) supplemented with current information).
1.2.2 Inhibitors of receptor tyrosine kinases

RTKs consist of single transmembrane domains separating the intracellular kinase domains from extracellular domains. Ligand binding to the extracellular domain induces dimerisation of receptors, leading to activation of the kinase, usually by autophosphorylation on tyrosine residues (198). The precise reaction that TKs catalyse is the transfer of the γ-phosphate of adenosine triphosphate (ATP) to hydroxyl groups of tyrosine residues in signalling protein substrates (199), which initiates a cascade of cytoplasmic enzymatic reactions. RTKs can be dysregulated in cancer cells in several ways, for example they may be overexpressed (Figure 1.7), fused with partner proteins, or mutated.

![Diagram of receptor tyrosine kinase activation](image)

**Figure 1.7** Overexpression of a normal receptor TK (EGFR) in cancer, leading to activation of the TK. Small molecule TK inhibitors (labelled in red) usually act to block binding of ATP or substrate to the catalytic (Cat) domain of the TK (197).
Thus far, the ATP binding site in the tyrosine kinase domain has proven to be the most susceptible target for inhibitory drugs and all of the TKIs discussed in this thesis bind to the ATP binding clefts of the relevant targeted TKs. ATP-competitive inhibitors exhibit a remarkable degree of specificity given the general sequence conservation in the ATP binding pocket of protein kinases (199).

1.2.1 Inhibitors of the HER family

EGFR and HER2 belong to the HER family of RTKs, which is characterised by the binding of EGF-like or neuregulin ligands to the extracellular domain (200). The kinase sites of these enzymes are around 80% identical, however selective inhibitors have been generated that distinguish between EGFR and HER2 (201), including some TKIs. Overexpression of EGFR is a molecular hallmark of many human malignancies (195), particularly epithelial cancers such as metaplastic breast cancer (202); similarly, amplification of the gene encoding HER2 is associated with advanced disease and poor prognosis in around 30% of breast tumour cases (203). Mutations in the genes encoding EGFR and HER2, resulting in aberrant expression and signalling, have been correlated with cancer of the lung, oesophagus, head and neck, ovary and pancreas, among other tissues (204,205). Accordingly, the possibility of targeting HER family kinases for cancer therapy has been the focus of much research. The first generation of TKIs which target this family include gefitinib and erlotinib, while lapatinib, afatinib, neratinib and pelitinib are second generation inhibitors (206-209). Lapatinib is regarded as second generation because it is usually administered after treatment of breast cancer with the monoclonal antibody trastuzumab in cases where the disease has progressed (210).
TKIs of the 4-anilinoquinazoline class are rationally designed drugs to treat patients possessing tumours with defined genetic alterations of the HER TK domain. A number of groups independently reached the quinazoline scaffold, though Zeneca was the first to develop a clinically viable agent (gefitinib; Figure 1.8 (A)) (195). This was soon followed by erlotinib (Figure 1.8 (B)). Gefitinib is an orally active compound that selectively inhibits EGFR by blocking the ATP binding cleft in the intracellular kinase domain (211,212). Gefitinib is approved for the treatment of NSCLC. Erlotinib shares the 4-anilinoquinazoline framework with gefitinib, binds similarly to the ATP binding pocket of EGFR, and is also used to treat NSCLC (213). Furthermore, erlotinib is approved for the first-line treatment of metastatic pancreatic cancer (214).
Figure 1.9 Chemical structures of second generation HER inhibitors: (A) lapatinib, (B) afatinib, (C) neratinib and (D) pelitinib.
Lapatinib is the third TKI to be developed with the 4-aniloquinazoline core, as seen in Figure 1.9 (A). It is a dual EGFR/HER2 inhibitor which blocks the ATP binding cleft resulting in reduced autophosphorylation on EGFR or HER2 tyrosine residues (213). Lapatinib is approved for HER2-positive and hormone receptor-positive metastatic breast cancer therapy (215).

Despite the initial response to the first generation inhibitors gefitinib and erlotinib, the majority of patients will eventually develop resistance to these inhibitors and relapse after several months (216). Most cases of acquired resistance to gefitinib and erlotinib can be explained by specific secondary mutations in the EGFR kinase domain (217), therefore a new collection of TKIs have come into clinical development which target these altered TKs.

Afatinib is a second generation, irreversible inhibitor of EGFR and HER2 derived from the quinazoline chemical series, as shown in Figure 1.9 (B) (208). Irreversible binding occurs via a cysteine residue within the ATP binding site, which is conserved in EGFR and HER2 (218). Afatinib was approved by the FDA in July 2013 as the first-line treatment of patients with NSCLC, whose tumours have EGFR exon 19 deletions or exon 21 substitution mutations (219).

The first irreversible HER family kinase inhibitor to reach advanced clinical trials was neratinib (HKI-272; Figure 1.9 (C)) (209). Like afatinib, this compound is predicted to covalently modify a cysteine residue within the ATP binding pocket of EGFR and HER2 kinase domains (220). Neratinib has been evaluated in phase II trials for the treatment of NSCLC in patients who are resistant to gefitinib and erlotinib (209),
however there are no ongoing trials for this disease. It is currently in phase III trials for the treatment of HER2-positive breast cancer, in which it has been shown to overcome acquired resistance to trastuzumab (221). Neratinib has activity against the HER4 kinase in addition to EGFR/HER2 (222), hence its classification as a ‘pan-HER’ inhibitor.

Pelitinib (EKB-569) is a second generation, potent irreversible inhibitor of EGFR and a moderately less effective irreversible inhibitor of HER2 (223). Pelitinib is believed to suppress the downstream signalling instigated by these kinases by forming a covalent bond to a conserved cysteine residue located in their respective ATP binding sites (223). This compound is in phase I clinical trials for the treatment of gefitinib-resistant NSCLC patients (224).

1.2.2.2 Multikinase inhibitors

Multikinase inhibitors (MKIs) are compounds that inhibit multiple RTKs, for example PDGFRs, VEGFR1-3, and Raf kinases. MKIs have transformed anticancer therapy as they inhibit receptors involved in angiogenesis, the physiological process by which cells expand their vascular network, which is crucial for cancer cell survival (225). This type of targeted therapy has become the standard treatment for several tumour types, particularly solid tumours, such as metastatic RCC, NSCLC, GIST and colorectal cancer (226). One of the main angiogenic growth factors is VEGF, which binds to VEGFR1-3, causing ATP-mediated induction of the RTK. Activation of VEGFR mediates proliferation, vascular permeability, cell migration and cell
survival, leading to angiogenesis. Overexpression of VEGF and VEGFR has been reported for many clinically important solid cancers (227).

The PDGFRs, comprising PDGFR-α and PDGFR-β, are RTKs that are activated by PDGFs, and are involved in differentiation and cell proliferation (228). Alterations in the activities of these receptors are associated with a range of human cancers. Erroneous expression of both PDGF ligands and the PDGFRs is believed to influence the development of high-grade sarcomas and glioma (229).

\[ \text{Figure 1.10 Structures of the MKI sorafenib and five of its metabolites (230). Solid and dotted arrows indicate major and minor metabolic pathways respectively.} \]
The MKI sorafenib targets several RTKs including VEGFR1-3, PDGFR-β, FMS-like tyrosine kinase 3 (Flt-3, coded by the protooncogene known as FMS) and stem cell factor receptor (c-Kit), in addition to the serine/threonine kinases B-Raf and C-Raf (231). This drug is approved for the treatment of advanced RCC, HCC and differentiated thyroid cancer (230,232). Several sorafenib metabolites have been identified in the plasma of treated patients (233), five of which are shown in Figure 1.10. These compounds have also been found to suppress cell proliferation by inhibiting the ERK/MEK signalling cascade initiated by Raf kinase activation; in particular the M-4 metabolite N’-desmethylsorafenib decreased cell viability in the MDA-MB-231 cell line (230).
Figure 1.11 Chemical structures of multikinase inhibitors: (A) sunitinib, (B) vandetanib, (C) cediranib and (D) foretinib.

Figure 1.11 (A) shows sunitinib, an inhibitor of VEGFR2, PDGFRs α and β, Flt-3 and c-Kit (234). It is approved for the treatment of metastatic RCC, imatinib-resistant GIST and progressive and well-differentiated pancreatic neuroendocrine tumours (235). In several tumour models, sunitinib monotherapy appeared to have the cumulative antitumour activity associated with combined single-target inhibitors, supporting the hypothesis that the use of multitargeted agents may allow for improved overall efficacy (236).
The MKI vandetanib is the first effective therapeutic agent against metastatic medullary thyroid cancer (237,238). This compound is a member of the quinazoline class of TKIs, as illustrated in Figure 1.11 (B). Vandetanib simultaneously targets EGFR, which induces proliferation, as well as Ret kinase, an RTK for the glial cell line-derived neurotrophic factor, which mediates survival and migration of tumour cells, and VEGFR2 in the endothelial compartment (195). Hence, it addresses three aspects of cancer: proliferation, survival, and invasion of both the tumour cell and its growing vascular network (195).

Cediranib (AZD-2171) is another quinazoline derivative, as seen in Figure 1.11 (C), and is a highly selective potent inhibitor of VEGFR2, with moderate inhibition activity against VEGFRs 1 and 3, c-Kit and PDGFR-β (239). This compound has exhibited significant antitumour effect and acceptable toxicities in clinical trials of patients with advanced solid tumours (240,241), however its development in oncology has recently been halted owing to insufficient results in phase II trials for the treatment of NSCLC and HCC among other cancers (241). In 2012, cediranib was in phase III trials for the treatment of metastatic colorectal cancer in combination with an oxaliplatin-based regimen (FOLFOX or CAPOX), however the overall survival endpoint of this study was not met (242). Despite its discontinuation, there are ongoing studies investigating the antitumour activity of cediranib (243,244).

Foretinib (GSK1363089) is an inhibitor of the RTKs VEGFR2 and c-Met (the receptor for hepatocyte growth factor) (245,246). Antitumour activity of this compound has been observed in papillary RCC, head and neck cancer, and metastatic
gastric cancer. Foretinib is currently undergoing phase II trials for the treatment of each of these diseases (247-249).

1.2.3 Inhibitors of nonreceptor tyrosine kinases

The nonreceptor TKs are maintained in an inactive state by cellular inhibitor proteins and lipids and through intramolecular autoinhibition (Figure 1.12) (197).

**Figure 1.12** Schematic of the nonreceptor TK BCR-ABL in its inactive state (197). PIP$_2$, phosphatidylinositol 4,5-bisphosphate (an inhibitory membrane lipid); ATP, ATP binding domain; Cat, catalytic domain; Tyr, regulatory tyrosine residue; Pro, proline residue; SH, Src homology domain; Myr, N-terminal myristate (membrane association group).
NRTKs are critical components in the regulation of the immune system, for example in signalling by activated T and B cells (199). The prime example of a dysregulated NRTK in the hematologic cancers is BCR-ABL (199), which is invariably associated with CML (250). NRTKs are involved in signalling cascades triggered by RTKs and other cell surface receptors, which ultimately modulate cell proliferation and differentiation. They are activated by phosphorylation of tyrosine residues in their activation loop, which can occur via trans-autophosphorylation (i.e. between receptors) or phosphorylation by a different NRTK (199).

Figure 1.13 Chemical structures of nonreceptor tyrosine kinase inhibitors: (A) nilotinib and (B) bosutinib.
Along with imatinib, which is not discussed further in this thesis, nilotinib and bosutinib are TKIs approved for the treatment of CML. In action they target the BCR-ABL kinase which is involved in clonal expansion of leukemic cells by deregulation of cell proliferation (251).

Nilotinib (Figure 1.13 (A)) was rationally designed based on the concept that the potency and selectivity of imatinib could be improved by maintaining binding to the inactive conformation of the BCR-ABL kinase domain (252). Nilotinib is also active against other targets, including c-Kit and PDGFRs α and β (252). Aside from CML, nilotinib is being considered for the treatment of GIST (253).

Bosutinib, shown in Figure 1.13 (B), additionally inhibits another NRTK, Src kinase, overexpression of which is often linked to a migratory and invasive cancer cell phenotype (254). Upregulation of Src kinase has been detected in a number of cancer types, including prostate and colon (255,256). In addition to its use in CML patients, bosutinib is currently in phase I clinical trials for the treatment of various advanced solid tumours (257), and phase II trials for breast cancer therapy (258).

The 13 TKIs heretofore described are investigated for their interaction potential with SLCs in the present research.


1.2.4 Interactions of TKIs with SLCs

Despite the high lipophilicity of most TKIs, which is usually indicative of passive membrane permeation, most studies examining the interplay of TKIs with transporters in the \textit{SLCO} and \textit{SLC22} families have focused on substrate rather than inhibition interactions (185). Table 1.2 presents the already known substrate and/or inhibitor interactions of TKIs with SLCs.

OATP substrates are generally associated with having high molecular mass, low permeability and anionic, hydrophilic properties, for example statins (259). Regardless of this, several TKI substrates and inhibitors of OATPs have been determined. Erlotinib and lapatinib inhibit the transporters OATP2B1 and OATP1B1 respectively, at potentially clinically relevant concentrations (92,260). Currently, there are no data on the impact of either of these TKIs on the pharmacokinetics of OATP substrates in humans (261). Sorafenib was shown to be transported by OATP1B1 and OATP1B3 (44), and may be a substrate of the other hepatic \textit{SLCO} transporters, OATP1A2 and OATP2B1 (262). Gefitinib and nilotinib are also potential substrates of OATP1B1 and OATP1B3, while sunitinib and vandetanib may be substrates of OATP1B1 and OATP1B3 respectively (44). Conversely, uptake of nilotinib in OATP1A2-overexpressing cells showed no significant accumulation as compared with control cells (118).

A positive net charge and high lipophilicity were found to be key physicochemical properties required for OCT inhibition (263); characteristics which several TKIs possess (185). \textit{In vitro} experiments using transporter-overexpressing HEK293 cells
demonstrated that gefitinib is not a substrate of OCT1 and OCT2, however it does inhibit OCT1- and OCT2-mediated MPP\(^+\) uptake and OCT3-mediated metformin uptake (185,264). Erlotinib also inhibits OCT1 at potentially clinically relevant concentrations (185), and is a substrate of OCT2 (55). In contrast, lapatinib and sorafenib had little effects on the uptake of metformin by OCT1-3, while sunitinib was a potent inhibitor of all three OCTs (185). Recently, sorafenib has been determined as a substrate of OCT1 and downregulation of this transporter in HCC has been suggested to affect sorafenib uptake into tumour cells (262,265). Vandetanib was found to be a moderate inhibitor of OCT2-mediated MPP\(^+\) and metformin uptake (266). Nilotinib is not transported by OCT1 (63,267), though it does inhibit the uptake of tetraethylammonium (TEA) by this transporter (266), in addition to potently inhibiting OCT3-mediated metformin uptake in HEK293 cells (185).

Sorafenib and sunitinib are the only TKIs thus far to be investigated in substrate interaction studies involving organic zwitterion/cation transporters, and no significant uptake of either compound was observed in cells overexpressing OCTN1 or OCTN2 (234).

The binding of substrates to OATs appears to be driven by hydrophobicity (268), suggesting that highly lipophilic TKIs have the potential to be substrates or inhibitors of these proteins. Erlotinib was found to be a substrate of OAT3 (55), while lapatinib is a moderate inhibitor of this transporter (92). Contrastingly, lapatinib had little effects on OATs 1, 2 or 4 (92). Sorafenib and sunitinib are not substrates of OAT2 or OAT3 (234), and vandetanib is not an inhibitor of OAT1 or OAT3 (265).
<table>
<thead>
<tr>
<th>TKI</th>
<th>Known pharmacokinetic interactions</th>
<th>Transporter-TKI interactions</th>
<th>Transporter inhibitors (non-TKI)</th>
</tr>
</thead>
</table>
| Gefitinib    | Gefitinib ↑ metoprolol AUC (269)  
Itraconazole ↑ gefitinib AUC (269)  
Ketoconazole ↑ gefitinib AUC (270)  
Ranitidine ↓ gefitinib AUC (270)  
Sorafenib ↑ gefitinib AUC (269)  
Sorafenib ↑ metoprolol AUC (269)  | May be substrate of OATP1B1 and OATP1B3 (44)  
Inhibitor of OCT1-3 (185,264)  | Rifampicin inhibits OATP1B1 and OATP1B3 (123,272)  
Ranitidine inhibits OCT1-3 (273)  |
| Erlotinib    | Cigarette smoking ↓ erlotinib AUC (274)  
Ketoconazole ↑ erlotinib AUC (274)  
Phenytoin ↓ erlotinib AUC (275)  
Rifampicin ↓ erlotinib AUC (274)  | Substrate of OAT3 and OCT2 (55)  
Inhibitor of OATP2B1 (260)  
Inhibitor of OCT1-3 (185)  | Probenecid and novobiocin inhibit OAT3 (27)  
Ranitidine inhibits OCT1-3 (273)  
Naringin (grapefruit) and rifampicin inhibit OATP2B1 (123,124,272,277, 278)  |
| Lapatinib    | Carbamazepine ↓ lapatinib AUC (279)  
Grapefruit ↑ lapatinib AUC (280)  
Ketoconazole ↑ lapatinib AUC (279)  
Lapatinib ↑ SN-38 AUC (281)  
Paclitaxel ↑ lapatinib AUC (282)  | Inhibitor of OATP1B1 and OAT3 (92)  | Rifampicin inhibits OATP1B1 (272)  
Probenecid and novobiocin inhibit OAT3 (27)  |
| Afatinib     | Rifampicin ↓ afatinib AUC (219)  
Ritonavir ↑ afatinib AUC (219)  | N/A  | N/A  |
| Neratinib    | Ketoconazole ↑ neratinib exposure (283)  | N/A  | N/A  |
| Pelitinib    | N/A  | N/A  | N/A  |
| Sorafenib    | No effect of ketoconazole (284)  
Neomycin ↓ sorafenib AUC (232)  
Rifampicin ↓ sorafenib AUC (196)  
Sorafenib ↑ doxorubicin AUC (285)  
Sorafenib ↑ gefitinib AUC (271)  
Sorafenib ↑ SN-38 AUC (285)  | Substrate of OATP1B1 and OATP1B3 (44)  
May be substrate of OATP1A2 and OATP2B1 (262)  
Substrate of OCT1 (265)  | Rifampicin inhibits OATP1B1, OATP1B3, OATP1A2 and OATP2B1 (123,272)  
Naringin (grapefruit) inhibits OATP1A2 and OATP2B1 (124,277,278,286)  
Quinidine inhibits OCT1 (27)  |
| Sunitinib    | Ketoconazole ↑ sunitinib AUC (287)  
Rifampicin ↓ sunitinib AUC (288)  | May be substrate of OATP1B1 (44)  
Inhibitor of OCT1-3 (185)  | Rifampicin inhibits OATP1B1 (272)  
Ranitidine inhibits OCT1-3 (273)  |
| Vandetanib   | Itraconazole ↑ vandetanib AUC (289)  
Rifampicin ↓ vandetanib AUC (290)  | May be substrate of OATP1B3 (44)  
Inhibitor of OCT2 (266)  | Rifampicin inhibits OATP1B3 (272)  
Ranitidine inhibits OCT2 (273)  |

TKI: Targeted kinase inhibitors; OCT: Organic cation transporter; OAT: Organic anion transporter; OATP: Organic anion transporting polypeptide; OCT1, OCT2, OCT3: Organic cation transporters; OAT1, OAT3: Organic anion transporters; OATP1A2, OATP2B1, OATP1B1: Organic anion transporting polypeptide 1A2, 2B1, 1B1.
Table 1.2 Pharmacokinetic consequences of TKIs coadministered with drugs/food and known interactions of TKIs and other compounds with SLCs. Abbreviations: N/A, not available; IUR, intracellular uptake and retention.

<table>
<thead>
<tr>
<th>TKI</th>
<th>Known pharmacokinetic interactions</th>
<th>Transporter-TKI interactions</th>
<th>Transporter inhibitors (non-TKI)</th>
</tr>
</thead>
</table>
| Cediranib| • Cediranib ↑ temozolomide AUC (291)  
             • Ketoconazole ↑ cediranib AUC (292)  
             • Rifampicin ↓ cediranib AUC (292)                           | N/A                          | N/A                                    |
| Foretinib| N/A                                                                                                                                            | N/A                          | N/A                                    |
| Nilotinib| • Grapefruit ↑ nilotinib AUC (293)  
             • Imatinib ↑ nilotinib IUR (294)  
             • Ketoconazole ↑ nilotinib AUC (295)  
             • Rifampicin ↓ nilotinib AUC (295)                           | • May be substrate of OATP1B1 and OATP1B3 (44)   
                          • Inhibitor of OCT1-3 (185,267)                      | • Rifampicin inhibits OATP1B1 and OATP1B3(272) 
                          • Ranitidine inhibits OCT1-3 (273)               |
| Bosutinib| • Bosutinib ↑ digoxin AUC (296)  
             • Ketoconazole ↑ bosutinib AUC (297)  
             • Lansoprazole ↓ bosutinib AUC (296)  
             • Rifampicin ↓ bosutinib exposure (296)       | N/A                          | N/A                                    |

No interactions of afatinib, neratinib, pelitinib, sorafenib metabolites, cediranib or bosutinib with SLC transporters are reported in the literature, however it has been determined that bosutinib is not an OCT1 substrate in unpublished work (298).

1.2.5 Drug-drug pharmacokinetic interactions

Cancer patients are often polymedicated, elderly and treated by different physicians who might not be in regular contact with each other, hence the risk of adverse drug reactions in this population is high (299). Drug-drug interactions can affect plasma drug monitoring, which is an important factor to optimise antitumour efficacy and to minimise drug toxicity towards normal tissues (300). Anticancer drugs also have a
narrow therapeutic range, so it is important to prevent drug interactions or detect them early (299). All TKIs approved for the treatment of cancer are administered orally, which is highly convenient for the patient compared with conventional chemotherapeutics that are usually administered intravenously (301). However, oral delivery often results in less control over the toxicity profiles of TKIs and increased opportunities for drug-drug interactions. Table 1.2 highlights some of the important known interactions of TKIs with coadministered drugs, or food, such as grapefruit juice, which alter the exposure (AUC) or intracellular uptake (IUR) of either the TKIs or the drugs they are taken with. Extreme variations have been demonstrated in gefitinib and lapatinib AUCs which may contribute to the fluctuation in their anticancer pharmacodynamic effect (301). As shown in this table, several TKIs which are known or postulated to be substrates of OATP1B1 and OATP1B3 also have interactions with rifampicin, a known substrate and inhibitor of these transporters (123), where the outcome is decreased systemic exposure to the TKI. Since transporter inhibition is usually associated with an increase in AUC and reduced cellular uptake, it is probable that rifampicin is interacting with TKIs at a different site, for example CYP3A4, the hepatic drug metabolising enzyme which rifampicin has been demonstrated to strongly induce (123).

While most patients tolerate TKIs, resistance to these compounds frequently develops and adverse reactions are evident in a subset of individuals. TKIs are primarily used at a fixed dose, which may result in suboptimal treatment or excessive toxicity, considering the wide interindividual variability in the pharmacokinetics of these therapies (301). The most common adverse events associated with TKI treatment include cutaneous toxicity (rash), hypertension, thyroid dysfunction, diarrhoea,
hepatotoxicity, cardiotoxicity and nephrotoxicity (194,301,302). Whether these toxicities arise from TKI-drug interactions at CYP enzymes or transporters is not determined. The severity of several cutaneous side effects, for example hand-foot skin reaction, is suggested to be dose-dependent (303), which supports the possible need for dose individualisation of TKIs (301).

Disparity exists between the toxicity profiles of TKIs because of the different kinases they target (303). Clinical experience has revealed that inhibition of VEGFR induces several side effects including hypertension and renal and cardiac toxicity (304). Additionally, inhibitors of HER2 are associated with cardiac dysfunction, while inhibition of EGFR has been correlated with significant dermatologic and liver toxicity (194).

The observed variability in TKI efficacy and toxicity may also be due to genetic polymorphisms, age, gender, ethnicity, disease state, and organ function, among other factors (305). Approximately 20 to 30% of adverse reactions to drugs are assumed to be caused by interactions between drugs (306). Accordingly, the current EMA guideline and FDA draft guidance for drug interaction studies call for mandatory pharmacokinetic studies in clinical trials of new drugs (76,77). Using in vitro-in vivo extrapolation methods such as those suggested for SLCs by the International Transporter Consortium (ITC), many interactions can be discovered or predicted (27).
1.3 Specific aims of the project

Excluding inhibition studies of the organic cation transporters (185), little research has been conducted to assess TKIs for their capacity to inhibit other human solute carriers in the *SLCO* and *SLC22* families. Considering the broad range of pharmaceutical compounds that have been established as substrates of these transporters, and the likelihood of several anticancer and palliative drugs being concomitantly administered, there is the potential for adverse effects arising from transporter-mediated drug-drug interactions. Moreover, modulation of drug uptake, for example by drug-drug interactions occurring at hepatic influx transporters, may also affect their intracellular metabolism by CYP enzymes (307). This project was designed to investigate the inhibitory capabilities of 13 different TKIs and five metabolites of the TKI sorafenib on 13 members of the SLC superfamily of transporters. The transporters evaluated include OATP1A2, OATP1B1, OATP1B3, OATP2B1, OAT1-4, OCT1-3 and OCTNs 1 and 2. *In vitro-in vivo* extrapolation methods will be utilised to quantitatively assess the drug-drug interaction potential of TKIs with these important solute carriers.
Chapter 2
Materials and Methods

2.1 Materials

2.1.1 TKI compounds

Sorafenib and five metabolites of sorafenib, including $N'$-hydroxymethylsorafenib $N$-oxide (M-1), sorafenib $N$-oxide (M-2), $N'$-hydroxymethylsorafenib (M-3), $N'$-desmethylsorafenib (M-4), $N'$-desmethylsorafenib $N$-oxide (M-5) were synthesised by Dr Tristan Rawling according to literature procedures (308–311). Reagents for the synthesis of sorafenib and its metabolites were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

Gefitinib, lapatinib, nilotinib, sunitinib, bosutinib, afatinib, cediranib, erlotinib, pelitinib, neratinib and foretinib were purchased from Selleck Chemicals (Houston, Texas, USA).

2.1.2 Chemicals and reagents

$[^3]$H-estrone-3-sulfate (57.3 Ci/mmol), $[^3]$H-CCK-8 (97.5 Ci/mmol), and $[^3]$H-MPP$^+$ (82.1 Ci/mmol) were purchased from PerkinElmer (Melbourne, Victoria, Australia). $[^3]$H-PAH (60 Ci/mmol), $[^3]$H-L-ergothioneine (1.7 Ci/mmol), and $[^{14}]$C-L-carnitine (56 mCi/mmol) were purchased from BioScientific Pty. Ltd. (Gymea, New South
Wales, Australia). HEK293 cells were obtained from the American Type Culture Collection (ATCC) and were frozen at passage number 4. Unless otherwise stated, all cell culture media and reagents were obtained from Invitrogen (Mount Waverley, Victoria, Australia).

The plasmids incorporated with human OATP1A2 (reference sequence: NM_005075.1), OATP1B1 (reference sequence: AB026257.1), OATP1B3 (reference sequence: NM_019844), and OATP2B1 (reference sequence: NM_007256) complementary DNAs (cDNAs) were obtained from United BioResearch Pte Ltd. (Dural, New South Wales, Australia). Plasmids cloned with the open reading frames (ORFs) of human OAT1 (reference sequence: NM_004790.4), OAT2 (reference sequence: NM_006672.2), OAT3 (reference sequence: NM_004254.2), OCT1 (reference sequence: NM_003057.2), OCT2 (reference sequence: NM_003058.2), and OCT3 (reference sequence: NM_021977.2) were purchased from Australian Biosearch (Balcatta, Western Australia, Australia). The plasmids of human OAT4 were cloned in house as described in the literature (312). The plasmids containing the full-length human OCTN1 (reference sequence: NM_003059) and OCTN2 (reference sequence: NM_003060) cDNAs were obtained from Gene-Ethics (Asia) Pte Ltd. (The Franklin, Singapore).
2.2 Methods

2.2.1 Expression of SLC transporters in HEK293 cells

HEK293 cells were maintained at 37°C in an atmosphere of 95% air and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS). All cells were sub-cultured twice a week and passage numbers 9 to 37 were used throughout the study. Two days prior to performing the transport experiments, cells were seeded in 48-well human fibronectin-coated plates at a density of 250,000 cells/well. One day after seeding, cells were transfected with 0.32 μg plasmid cDNA/well using Lipofectamine 2000 Reagent (Invitrogen) or DNAfectin 2100 (Applied Biological Materials Inc., Richmond, British Columbia, Canada) following the manufacturer’s instructions. 24 hours after transfection, transport activities were measured.

2.2.2 Transport studies

Uptake of a mix of radiolabeled and unlabelled prototypical substrate of each SLC (see Table 2.1), was initiated at room temperature in phosphate-buffered saline (PBS), pH 7, containing 5 mM glucose. Total substrate concentrations and time used in the study were compatible with the previously determined time- and concentration-dependent studies of these transporters: 300 nM [³H]-estrone-3-sulfate for OAT3, OAT4, OATP1A2, OATP1B1, and OATP2B1 (31,312-315); 2 μM [³H]-estrone-3-sulfate for OAT2 at pH 5.5 (53); 5 μM [³H]-PAH for OAT1 (316); 2 nM [³H]-CCK-8 for OATP1B3 (34); 100 nM [³H]-MPP⁺ for OCT1, OCT2, and OCT3 (317); 5 μM
**[3]H-L-ergothioneine for OCTN1 (147); and 5 μM [14C]-L-carnitine for OCTN2 (159), respectively. Preliminary experiments indicated that initial rates of transporter-mediated substrate uptake in HEK293 cells were linear over at least 8 minutes, which was the time selected for subsequent experiments. The uptake mixture, consisting of PBS solution, prototypical substrate and inhibitor (10 μM TKI or TKI metabolite dissolved in DMSO) was added to confluent cells upon removing media from the wells. The uptake was terminated by rapidly washing the cells in ice-cold PBS. The cells were then solubilised in 0.2 M NaOH and neutralised with 0.2 M HCl before aliquoting for liquid scintillation counting. The intracellular concentrations of model substrate were analysed with an Ultima Gold scintillation cocktail (PerkinElmer) using a PerkinElmer Tri-Carb 2810TR liquid scintillation analyzer. Uptake count was standardised to the amount of protein in each well (0.32 μg). In all experiments, untransfected cells were included on each plate to correct for passive permeability of the model substrate. The actual inhibition of uptake function of the transporter was calculated by subtracting the passive uptake counts in untransfected cells from the total uptake counts in the SLC-expressing cells according to the following equation:*

\[
\text{inhibition (\%)} = \left(1 - \frac{\text{uptake (SLC+inhibitor)} - \text{uptake (untransfected)}}{\text{uptake (SLC-inhibitor)} - \text{uptake (untransfected)}}\right) \times 100 \quad [1]
\]

All experiments were performed on three separate occasions and each data group was conducted with triplicate wells (OATP1A2, OATP1B1, OATP1B3, OATP2B1, OAT1, OAT3, OAT4, OCTN1, OCTN2) or duplicate wells (OCT1-3, OAT2). Data are presented as the mean ± standard deviation. TKIs were classified as inhibitors and were further investigated if they reduced uptake of the prototypical substrate by more than 50%. This criterion for further inhibition analysis was selected in order to
account for potential error in transporter expression and efficiency of substrate uptake by cells within each well, for the purpose of avoiding false positives.

2.2.3 IC₅₀ studies

The inhibitory potency of each compound was evaluated by determining their half maximal inhibitory concentration (IC₅₀), i.e. the concentration of TKI required to reduce transporter-mediated uptake of prototypical substrate by 50%. Uptake measurements were performed by adding increasing concentrations of a particular TKI to the uptake buffer solution containing the relevant radiolabeled substrate at the concentrations indicated in Table 2.1. Different concentration ranges for each transporter-TKI interaction were chosen on the basis of the estimated 50% inhibitory concentration in the preliminary screening experiments. Experiments were repeated three times with triplicate wells for each data group on each occasion. Data are presented as the mean ± standard error.

2.2.4 Kinetic studies

For kinetic analysis, OATP1A2-transfected HEK293 cells were prepared for uptake studies as indicated in Section 2.2.2, however in place of a fixed concentration of estrone-3-sulfate in the uptake buffer solution, concentrations of 0.5 μM, 1 μM, 5 μM, 10 μM, 15 μM and 30 μM were added to separate wells in duplicate (see Table 2.1). These concentrations were chosen in order to include the dissociation constant for the transporter-substrate complex (Kₘ) for OATP1A2 and estrone-3-sulfate, which is approximately 15 μM. For each different concentration of estrone-3-sulfate, six
concentrations of cediranib were added to the uptake solution in duplicate; 0 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM. The reactions were conducted as outlined in Section 2.2.2 and the uptake of estrone-3-sulfate was determined using liquid scintillation counting. Data are presented as the mean ± standard error.

In the kinetic analysis of OATP2B1-mediated transport of estrone-3-sulfate, HEK293 were prepared for uptake as outlined previously, however five different concentrations of estrone-3-sulfate (0.5 μM, 1 μM, 2 μM, 5 μM and 10 μM) were added to separate wells in duplicate (see Table 2.1). These concentrations were selected in order to include the dissociation constant for the transporter-substrate complex ($K_m$) for OATP2B1 and estrone-3-sulfate, which is approximately 5 μM. For each different concentration of estrone-3-sulfate, six concentrations of erlotinib were added to the uptake solution in duplicate; 0 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM. The reactions were conducted as outlined in Section 2.2.2 and the uptake of estrone-3-sulfate was determined using liquid scintillation counting. Results are given as the mean ± standard error.

Substrate uptake rates were expressed as pmol/min/μg protein, using the following equations:

\[ \text{specific activity of substrate} = \frac{\text{counts per minute (untransfected cells)}}{\text{pmol substrate}} \]  \[2\]

\[ \text{specific activity of transporter} = \frac{\text{counts per minute (SLC-expressing cells)}}{\text{specific activity of substrate} \cdot \text{min} \cdot \text{μg protein}} \]  \[3\]
where pmol substrate is calculated from the substrate concentration (0.5 – 30 μM) used in 120 μL uptake solution/well, min refers to the reaction time in minutes (8 minutes), and μg protein is equal to 0.32 μg transporter cDNA/well.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Transporter</th>
<th>Prototypical substrate</th>
<th>Concentration ratio radioabelled:unlabelled (nM)</th>
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<tbody>
<tr>
<td>Transport and IC₅₀ studies</td>
<td>OATP1A2, OATP1B1, OATP2B1, OAT3</td>
<td>estrone-3-sulfate</td>
<td>10:290</td>
</tr>
<tr>
<td></td>
<td>OAT2</td>
<td>estrone-3-sulfate</td>
<td>20:1980</td>
</tr>
<tr>
<td></td>
<td>OAT4</td>
<td>estrone-3-sulfate</td>
<td>20:280</td>
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<tr>
<td></td>
<td>OATP1B3</td>
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<tr>
<td></td>
<td>OCT1</td>
<td>MPP⁺</td>
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<td>OCT2, OCT3</td>
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<td>L-ergothioneine</td>
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<tr>
<td></td>
<td>OCTN2</td>
<td>L-carnitine</td>
<td>1000:4000</td>
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<tr>
<td>Kinetic studies</td>
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<tr>
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<td></td>
<td></td>
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<td>10:29990 (30 μM)</td>
</tr>
</tbody>
</table>

Table 2.1 Radiolabelled-to-unlabelled ratios of prototypical substrate used in uptake in preliminary screening experiments, IC₅₀ determinations and kinetic tests.
2.3 Data analysis and statistics

2.3.1 Statistics

The statistical significance of differences between treatments in the preliminary screening experiments was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test (p<0.05). The software used was Microsoft Excel and GraphPad Prism version 5.01 (GraphPad Inc., La Jolla, California, USA).

2.3.2 \(IC_{50}\) determination

\(IC_{50}\) values for each positive transporter-TKI inhibitory interaction were calculated by nonlinear regression models in GraphPad Prism. Nonlinear goodness-of-fit values (\(R^2\)) for were also determined using this software.

2.3.3 Kinetic analysis

Kinetic parameters were calculated by applying the Michaelis-Menten equation to nonlinear regression analysis of the rate of transporter activity (V) and substrate concentration ([S]) in GraphPad Prism. Inhibitory data were further analysed using Lineweaver-Burk plots in conjunction with Dixon plots to determine the mode of TKI-mediated inhibition (318,319). Goodness-of-fit values (\(R^2\)) for Lineweaver-Burk and Dixon replots were computed in GraphPad Prism.
Chapter 3

Results

3.1 Preliminary screening experiments

With regard to elucidating the role of SLC proteins in TKI disposition, and the potential for drug-TKI interactions at transporter sites, 13 clinically important TKIs and five metabolites of the TKI sorafenib were tested for their capacity to inhibit the transport of specific SLC-mediated radiolabelled substrates into HEK293 cells. HEK293 cells were transfected with transporter cDNA to overexpress 13 different SLCs, including OATP1A2, OATP1B1, OATP1B3, OATP2B1, OAT1, OAT2, OAT3, OAT4, OCT1, OCT2, OCT3, OCTN1 and OCTN2. These SLCs constitute more than 50% of the functionally characterised members of the human SLCO and SLC22 families and are responsible for the uptake of important anticancer drugs such as imatinib, paclitaxel and cisplatin (40, 43, 61).

The SLC-selective prototypical substrates used in these experiments were estrone-3-sulfate for OATP1A2, OATP1B1, OATP2B1, OAT2, OAT3 and OAT4, CCK-8 for OATP1B3, PAH for OAT1, MPP+ for OCT1, OCT2 and OCT3, L-ergothioneine for OCTN1 and L-carnitine for OCTN2. The uptake of these substrates was quantified using liquid scintillation counting as mentioned earlier in Section 2.2.2. Results are given as the mean ± standard deviation. TKIs were considered to be significant inhibitors and were further investigated if they reduced uptake of the prototypical substrate by more than 50%.
(A) OATP1A2

[3H]-estrone-3-sulfate uptake (percent of control activity)

TKI

(B) OATP1B1

[3H]-estrone-3-sulfate uptake (percent of control activity)

TKI
**Graphs (C) and (D)**

**Graph (C):**
- Title: OATP1B3
- Y-axis: [3H]-CCK-8 uptake (percent of control activity)
- X-axis: TKI
- Bar graph showing the uptake of [3H]-CCK-8 under different TKIs.

**Graph (D):**
- Title: OATP2B1
- Y-axis: [3H]-estrone-3-sulfate uptake (percent of control activity)
- X-axis: TKI
- Bar graph showing the uptake of [3H]-estrone-3-sulfate under different TKIs.
**OCT3**

$[^3]H$-MPP$^+$ uptake (percent of control activity)

TKI

Control, Sorafenib, M-1, M-2, M-3, M-4, M-5, Gefitinib, Lapatinib, Nilotinib, Sunifranib, Bosutinib, Vandetanib, Afinitor, Cediranib, Erlotinib, Palitinib, Neralitinib, Foretinib

---

**OCTN1**

$[^3]H$-L-ergothioneine uptake (percent of control activity)

TKI

Control, Sorafenib, M-1, M-2, M-3, M-4, M-5, Gefitinib, Lapatinib, Nilotinib, Sunifranib, Bosutinib, Vandetanib, Afinitor, Cediranib, Erlotinib, Palitinib, Neralitinib, Foretinib

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*Significance levels: * p < 0.05, ** p < 0.01, *** p < 0.001
Figure 3.1 Inhibitory effects of TKIs on (A) OATP1A2-mediated estrone-3-sulfate uptake, (B) OATP1B1-mediated estrone-3-sulfate uptake, (C) OATP1B3-mediated CCK-8 uptake, (D) OATP2B1-mediated estrone-3-sulfate uptake, (E) OAT1-mediated PAH uptake, (F) OAT2-mediated estrone-3-sulfate uptake, (G) OAT3-mediated estrone-3-sulfate uptake, (H) OAT4-mediated estrone-3-sulfate uptake, (I) OCT1-mediated MPP\(^+\) uptake, (J) OCT2-mediated MPP\(^+\) uptake, (K) OCT3-mediated MPP\(^+\) uptake, (L) OCTN1-mediated l-ergothioneine uptake, (M) OCTN2-mediated l-carnitine uptake in HEK293 cells (n=3) in vitro. 13 TKIs and five metabolites of sorafenib were tested at a concentration of 10 μM. Statistically different from control: *p<0.05, **p<0.01, ***p<0.001.
As shown in Figure 3.1 (A), cediranib emerged as a highly potent selective inhibitor of OATP1A2-mediated estrone-3-sulfate uptake in HEK293 cells. The transporter activity was essentially abolished in the presence of 10 μM cediranib, while gefitinib, nilotinib, sunitinib, vandetanib, afatinib, erlotinib, pelitinib, neratinib and foretinib also showed more than 50% inhibition of estrone-3-sulfate uptake. Sorafenib and its five metabolites (M-1 to M-5), lapatinib and bosutinib did not show any inhibitory effect. Given that 10 μM is generally too high a concentration of TKI to achieve in vivo (the relevant therapeutic TKI $C_{max,sys}$ values vary between 0.08 μM to 9.90 μM; refs. 222,245,320-333), these positive interactions were repeated using a lower range of TKI concentrations to determine the half maximal inhibitory concentrations (IC$_{50}$).

Figure 3.1 (B) showed that no TKIs exerted major decreases in OATP1B1-mediated estrone-3-sulfate uptake in HEK293 cells, although small decreases were produced by the sorafenib metabolites M-1 and M-2.

Erlotinib and neratinib emerged as effective inhibitors of CCK-8 uptake by OATP1B3-transfected HEK293 cells, as indicated by Figure 3.1 (C). Significant inhibitory interactions of sorafenib, a known substrate of this SLC, and gefitinib, nilotinib and vandetanib, which are potential substrates of OATP1B3 (44), were not apparent; hence these TKIs may utilise an alternative binding site from CCK-8. The interactions of OATP1B3 with erlotinib and neratinib were analysed further using a lower range of TKI concentrations to establish IC$_{50}$ values.

Gefitinib, nilotinib, afatinib, pelitinib and neratinib showed strong inhibitory effects on OATP2B1-mediated estone-3-sulfate uptake, as shown in Figure 3.1 (D); erlotinib
in particular emerged as a highly potent selective inhibitor of this transporter. The transporter activity was reduced by almost 100% in the presence of 10 μM erlotinib, while gefitinib, nilotinib, afatinib, pelitinib and neratinib also elicited more than 50% inhibition of estrone-3-sulfate uptake. Erlotinib-induced inhibition of OATP2B1 has been demonstrated previously (260), though in that study the authors used 20 μM erlotinib and further testing on this interaction was limited to obtaining an IC\textsubscript{50} value (0.55 μM). Sorafenib and its five metabolites, lapatinib, sunitinib, bosutinib, Vandetanib, cediranib and foretinib did not show any inhibitory effect. Since 10 μM is essentially unattainable during therapy \textit{in vivo}, the positive interactions were repeated using a lower range of TKI concentrations to determine IC\textsubscript{50} values.

Figures 3.1 (E) and (F) demonstrate no inhibitory action of TKIs on OAT1-mediated PAH or OAT2-mediated transport of estrone-3-sulfate uptake in HEK293 cells, respectively.

Nilotinib was found to be a potent inhibitor of OAT3-mediated estrone-3-sulfate uptake in HEK293 cells, as indicated by Figure 3.1 (G). Although not shown here, lapatinib has been previously identified as an inhibitor of OAT3 (92), however the concentration of lapatinib used in that study was 30 μM compared with 10 μM in this project, and even at this high concentration lapatinib only reduced estrone-3-sulfate uptake by 60%. Thus, it appears likely that lapatinib will not inhibit OAT3-mediated estrone-3-sulfate uptake at clinically relevant concentrations. Erlotinib has also been shown to interact with OAT3 as a substrate rather than an inhibitor (55), though this potential interaction was not indicated in Figure 3.1 (G). It is possible that erlotinib
enters cells via OAT3 using a different binding site to estrone-3-sulfate. No TKIs other than nilotinib inhibited OAT3 sufficiently for further IC50 analysis.

Figure 3.1 (H) revealed no inhibitory effect of TKIs on OAT4-mediated estrone-3-sulfate uptake in HEK293 cells.

Bosutinib emerged as a potent inhibitor of OCT1-mediated transport in HEK293 cells, as indicated by Figure 3.1 (I), reducing the uptake of MPP+ by more than 75%. Gefitinib, nilotinib, sunitinib and erlotinib have been found previously to inhibit OCT1-, OCT2- and OCT3-mediated uptake of metformin (185). However, in the present study, only gefitinib and cediranib inhibited MPP+ uptake by nearly half (44% and 46%, respectively). As the criterion for further inhibition analysis was set at 50% reduction in model substrate uptake, an IC50 value was determined for bosutinib alone.

Figures 3.1 (J), (K) and (L) demonstrate no inhibitory action of TKIs on OCT2- and OCT3-mediated MPP+ or OCTN1-mediated transport of L-ergothioneine uptake in HEK293 cells. Vandetanib has been shown to inhibit OCT2 transport of MPP+ and metformin (266), however the IC50 values that were calculated (73.4 μM and 8.8 μM respectively) are not clinically relevant. Erlotinib has also been shown to interact with OCT2 as a substrate (55), though this interaction was not observed in Figure 3.1 (J). As with OAT3, erlotinib may have a different binding site to MPP+.

Cediranib was a moderate inhibitor of OCTN2, reducing uptake of L-carnitine in HEK293 cells by more than 50%, as indicated by Figure 3.1 (M). No other TKIs
appeared to have any effect on transporter uptake, hence further analysis to determine IC₅₀ values was only undertaken for cediranib.

Overall, 11 TKIs were found to inhibit the activity of particular transporters by more than 50%, comprising 21 significant inhibitory interactions. IC₅₀ tests were performed for each of these interactions.
3.2 Determination of IC<sub>50</sub> values for the inhibition of SLC-mediated transport by TKIs

From the preliminary screening tests it was determined that model substrate uptake by OATP1A2, OATP1B3, OATP2B1, OAT3, OCT1 and OCTN2 was inhibited by 11 different TKIs. The corresponding IC<sub>50</sub> values for these transporter-TKI inhibition interactions were calculated by fitting the data for increasing concentrations of TKI to a sigmoid dose-response regression curve using GraphPad Prism (Figures 3.2-7).

All experiments were performed in triplicate and repeated on three independent occasions. Uptake of a fixed concentration of model substrate was measured in HEK293 cells overexpressing human transporter cDNA with the addition of six to eight increasing concentrations of TKI. Different concentration ranges for each transporter-TKI interaction were chosen on the basis of the estimated 50% inhibitory concentration in the preliminary screening experiments. The percentage of transporter activity remaining was plotted against each concentration of the inhibitor. Results are displayed as the mean ± standard error.

(A) IC<sub>50</sub> = 1.17 μM
(B) IC<sub>50</sub> = 34.96 nM

\[
\text{[3H]-estrone-3-sulfate uptake (percent of control activity)}
\]

\[
\log(\text{afatinib, μM})
\]

\[
\log(\text{cediranib, μM})
\]
(C) IC_{50} = 1.10 \mu M

(D) IC_{50} = 3.55 \mu M

(E) IC_{50} = 0.88 \mu M

(F) IC_{50} = 0.39 \mu M

(G) IC_{50} = 1.46 \mu M

(H) IC_{50} = 3.77 \mu M
Figure 3.2 Dose-response relationships for (A) afatinib, (B) cediranib, (C) erlotinib, (D) foretinib, (E) gefitinib, (F) neratinib, (G) nilotinib, (H) pelitinib, (I) sunitinib and (J) vandetanib with respect to inhibition of $[^{3}H]$-estrone-3-sulfate uptake in HEK293-OATP1A2 cells. Six to eight different TKI concentrations were used.

Cediranib emerged as the most potent TKI inhibitor of OATP1A2-mediated estrone-3-sulfate uptake, with an IC$_{50}$ value of 35 nM. IC$_{50}$ values derived for gefitinib and neratinib were also submicromolar, being 0.88 μM and 0.39 μM respectively, while afatinib, erlotinib and nilotinib inhibited OATP1A2 transport with IC$_{50}$ values around 1 μM. The interactions of sunitinib and vandetanib with OATP1A2 resulted in IC$_{50}$ values of 2.03 μM and 2.45 μM respectively, however given the very low $C_{\text{max,sys}}$ that sunitinib can achieve in vivo (329), it is unlikely this result is clinically relevant. IC$_{50}$ values for foretinib and pelitinib were more than 100-fold the IC$_{50}$ for cediranib. Cediranib was identified as a candidate for further kinetic testing to determine the mode of inhibition (Section 3.3).
Figure 3.3 Dose-response relationships for (A) erlotinib- and (B) neratinib-mediated inhibition of $[^3]$H-CCK-8 uptake in HEK293-OATP1B3 cells. Six to seven different TKI concentrations were used.

The IC$_{50}$ value for neratinib-mediated inhibition of OATP1B3 substrate uptake was submicromolar (0.65 μM), while erlotinib inhibited this transporter slightly less effectively, with an IC$_{50}$ of 1.19 μM. Both interactions are likely significant in vivo as erlotinib has a particularly high unbound $C_{\text{max,sys}}$ (323,324).
Figure 3.4 Dose-response relationships for (A) afatinib, (B) erlotinib, (C) gefitinib, (D) neratinib, (E) nilotinib and (F) pelitinib with respect to inhibition of [³H]-estrone 3-sulfate uptake in HEK293-OATP2B1 cells. Six to seven different TKI concentrations were used.
Erlotinib emerged as the most potent TKI inhibitor of OATP2B1-mediated estrone-3-sulfate uptake, with an IC$_{50}$ value of 32 nM. This is 17-fold lower than the previously reported IC$_{50}$ for this interaction (0.55 μM; ref. 260), though in that report, the concentration range used for erlotinib IC$_{50}$ determination varied between 20 nM and 400 μM. The present experiment used 0 nM to 100 nM which delivers a much more accurate estimate of IC$_{50}$, since the range of percentage inhibitions are approximately symmetrical around the 50% inhibition point. The IC$_{50}$ derived for gefitinib was also submicromolar (0.65 μM), while afatinib, neratinib, nilotinib and pelitinib inhibited OATP1A2 transport with IC$_{50}$ values around 2-3 μM. Erlotinib was identified as a candidate for further kinetic evaluation to determine the mode of inhibition (Section 3.4).

![Graph](image.png)

**Figure 3.5** Dose-response relationship for nilotinib-induced inhibition of $[^3H]$-estrone-3-sulfate uptake in HEK293-OAT3 cells. Seven different nilotinib concentrations were used.
Nilotinib was demonstrated to be a highly potent inhibitor of OAT3-mediated estrone-3-sulfate uptake with an IC$_{50}$ of 0.41 μM.

![Graph showing dose-response relationship for bosutinib-influenced inhibition of [H]-1-MPP$^+$ uptake in HEK293-OCT1 cells. Seven different bosutinib concentrations were used.](image)

**Figure 3.6** Dose-response relationship for bosutinib-influenced inhibition of [H]-1-MPP$^+$ uptake in HEK293-OCT1 cells. Seven different bosutinib concentrations were used.

Bosutinib was shown to be a moderately effective inhibitor of OCT1-mediated MPP$^+$ uptake with an IC$_{50}$ of 2.06 μM. Further analysis comparing this value with the $C_{\text{max,sys}}$ achieved by bosutinib is required to ascertain whether this is a clinically relevant interaction.
Figure 3.7 Dose-response relationship for cediranib-induced inhibition of $[^{14}C]$-L-carnitine uptake in HEK293-OCTN2 cells. Seven different cediranib concentrations were used.

Cediranib was a modest inhibitor of OCTN2-mediated L-carnitine uptake with an IC$_{50}$ of 2.49 μM. Further exploration comparing this value with the $C_{\text{max,sys}}$ for cediranib is needed to assess whether this result is clinically significant.

Inhibitors were considered to be selective if their IC$_{50}$ values were at least 10-fold lower than those of other transporters (260). Hence, cediranib was identified as a selective inhibitor of OATP1A2, as the IC$_{50}$ for this interaction was more than 70-fold lower than the IC$_{50}$ derived from cediranib-mediated inhibition of OCTN2. Consistent with previous findings (260), erlotinib was established as a specific inhibitor of OATP2B1.
<table>
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<tr>
<th>Transporter</th>
<th>TKI</th>
<th>IC$_{50}$ (μM)</th>
<th>R$^2$</th>
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</thead>
<tbody>
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<td>Cediranib</td>
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<td>Pelitinib</td>
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<td>0.97</td>
</tr>
<tr>
<td>OAT3</td>
<td>Nilotinib</td>
<td>0.41</td>
<td>0.99</td>
</tr>
<tr>
<td>OCT1</td>
<td>Bosutinib</td>
<td>2.06</td>
<td>0.99</td>
</tr>
<tr>
<td>OCTN2</td>
<td>Cediranib</td>
<td>2.49</td>
<td>0.99</td>
</tr>
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Table 3.1 Summary of IC$_{50}$ values for transporter-TKI interactions and corresponding goodness-of-fit values (R$^2$) for the nonlinear regression curves in Figures 3.2-7 calculated using GraphPad Prism software.
3.3 Kinetic study of the inhibitory effect of cediranib on OATP1A2-mediated estrone-3-sulfate uptake

To further investigate the mode of OATP1A2 inhibition by cediranib, kinetic experiments were carried out using six inhibitor concentrations and six substrate concentrations. The results were analysed using graphical methods, as discussed earlier in Section 2.3.3.

3.3.1 Michaelis-Menten graph of OATP1A2 activity at a range of cediranib concentrations

![Graph showing Michaelis-Menten family of plots for $[^3]H$-estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells. Experiments were performed with 0.32 μg transporter protein/well and inhibitor (cediranib) concentrations of 0 nM (●), 10 nM (■), 25 nM (▲), 50 nM (▼), 75 nM (♦) and 100 nM (○), with 0.5 – 30 μM of estrone-3-sulfate [S]. V refers to the velocity of substrate uptake.]

Figure 3.8 Michaelis-Menten family of plots for $[^3]H$-estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells. Experiments were performed with 0.32 μg transporter protein/well and inhibitor (cediranib) concentrations of 0 nM (●), 10 nM (■), 25 nM (▲), 50 nM (▼), 75 nM (♦) and 100 nM (○), with 0.5 – 30 μM of estrone-3-sulfate [S]. V refers to the velocity of substrate uptake.
Figure 3.8 demonstrates the concentration-dependent inhibition of OATP1A2-mediated estrone-3-sulfate uptake in HEK293 cells by cediranib. Michaelis-Menten plots provide useful parameters to describe the affinity of a transporter for its substrate and the rate of substrate uptake, which can be used in subsequent analysis for the determination of inhibition constants ($K_i$) from IC$_{50}$ values. $K_s$ (or $K_m$) pertains to the dissociation constant of the transporter-substrate complex, while $V_{max}$ refers to the limiting maximal velocity that would be observed when all of the transporter is present as the transporter-substrate complex. The value for $V_{max}$ was obtained from the Michaelis-Menten plot and the value for $K_s$ was determined from the Lineweaver-Burk plot in the absence of inhibitor (cediranib = 0 nM). This analysis was conducted in OATP1A2-transfected HEK293 cells (n=3) in vitro ($V_{max} = 12,400$ pmol estrone-3-sulfate/min/µg protein, $K_s = 5.3$ µM).

3.3.2 Lineweaver-Burk plots of the inhibition of OATP1A2-mediated estrone-3-sulfate uptake by cediranib

(A)
Figure 3.9 (A) Lineweaver-Burk plot for the inhibition of [$^3$H]-estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells by cediranib. The transporter activity was determined at six concentrations of cediranib, including 0 nM (●), 10 nM (■), 25 nM (▲), 50 nM (▼), 75 nM (♦) and 100 nM (○), and six concentrations of estrone-3-sulfate [S]; 0.5 μM, 1 μM, 5 μM, 10 μM, 15 μM and 30 μM. (B) Primary replot of inhibitor concentrations versus Lineweaver-Burk plot slope data.

The mode of substrate inhibition by cediranib can be inferred using graphical methods such as double reciprocal Lineweaver-Burk graphs (Figure 3.9) and Dixon plots (Figure 3.10). The intersection point of a family of plots presented on a double reciprocal graph, such as that in Figure 3.9 (A), is characteristic of inhibition behaviour. In this case, the family of plots intersect above the 1/[S]-axis and to the left of the 1/V-axis, suggesting that cediranib may inhibit OATP1A2-mediated uptake of
estrone-3-sulfate in a mixed fashion (334). Whereas the family of reciprocal plots for a competitive inhibitor intersect on the 1/V-axis, and a non-competitive inhibitor produces plots which converge on the 1/[S]-axis, mixed-type inhibition is a combination of both of these modes (334).

By graphing the slopes of each Lineweaver-Burk plot against the inhibitor concentrations, as in the Figure 3.9 (B) replot, the equilibrium dissociation constant for the transporter-inhibitor complex (K_i) can be determined (334). K_i is a favourable inhibition parameter for in vitro-in vivo extrapolation compared with IC_{50}, as it is a value independent of substrate concentration (335), while IC_{50} values change according to substrate concentration. From analysis of Figure 3.9 (B), the point of intersection with the x-axis is equal to -38 nM (R^2 = 0.94), hence the K_i for the interaction between OATP1A2 and cediranib was calculated to be 38 nM. This indicates that cediranib is a potent mixed-type inhibitor and may exhibit a greater affinity for OATP1A2 than the substrate, estrone-3-sulfate (K_s = 5.3 μM).

The K_i value determined is highly comparable with the corresponding IC_{50} for this inhibition (35 nM). Considering the K_i is only 3 nM different from the IC_{50} calculated previously, it is likely that this is a simple mixed inhibition system (335).
Dixon plots, which present the reciprocal velocity \((1/V)\) of the reaction against inhibitor concentration \([I]\), and replots of the slopes of Dixon plot lines versus reciprocal substrate concentration \((1/[S])\), were implemented to further clarify the mode of inhibition.

3.3.3 Dixon plots of the inhibition of OATP1A2-mediated estrone-3-sulfate uptake by cediranib
Figure 3.10 (A) Dixon plot for the inhibition of $[^3H]$-estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells by cediranib (0-100 nM). Estrone-3-sulfate concentrations were 0.5 μM (●), 1 μM (■), 5 μM (▲), 10 μM (▼), 15 μM (♦) and 30 μM (○). (B) Dixon plot slope replot versus reciprocal substrate (estrone-3-sulfate) concentration.

The appearance of the Dixon plot intersection in Figure 3.10 (A) confirms the mixed-type inhibition mode identified in previous analyses, as the family of plots intersect above the $x$-axis. Dixon plots for partial and most mixed-type inhibition systems are curved, however when the transporter-substrate-inhibitor complex (TSI) is not functional, Dixon plots tend to be linear ($\beta=0$, where $\beta$ is the factor by which the rate of substrate uptake is altered by the inhibitor). The linearity of the relationship in
Figure 3.10 (B) indicates that \( \beta \) is 0 in this system, while the \( \alpha \) factor, which corresponds to the change in the dissociation constant of the transporter-substrate complex \( (K_s) \) when cediranib occupies OATP1A2, can be calculated from the \( x \)-intercept of this replot. When \( \alpha \) is greater than 1, the family of Dixon plots intersect above the horizontal axis, and the plot resembles that obtained for a competitive inhibitor, as is the case in Figure 3.10 (A). The \( \alpha \) value (from \(-1/\alpha K_s\) at the \( x \)-intercept in part (B) of Figure 3.10 was determined to be 3.5 (\( R^2 = 0.99 \)). Therefore, the mode of cediranib-induced inhibition of OATP1A2-mediated estrone-3-sulfate uptake is best considered as linear mixed-type with a pronounced non-competitive component (334).
3.4 Kinetic study of the inhibitory effect of erlotinib on OATP2B1-mediated estrone-3-sulfate uptake

To further investigate the mode of OATP2B1 inhibition by erlotinib, kinetic experiments were carried out using six inhibitor concentrations and five substrate concentrations. The results were analysed using graphical methods, as discussed earlier in Section 2.3.3.

3.4.1 Michaelis-Menten graph of OATP2B1 activity at a range of erlotinib concentrations

![Michaelis-Menten graph of OATP2B1 activity](image)

**Figure 3.11** Michaelis-Menten family of plots for $[^3]$H-estrone-3-sulfate uptake in OATP2B1-transfected HEK293 cells. Experiments were performed with 0.32 μg transporter protein/well and inhibitor (erlotinib) concentrations of 0 nM (●), 10 nM (■), 25 nM (▲), 50 nM (▼), 75 nM (♦) and 100 nM (○), with 0.5 – 10 μM of estrone-3-sulfate [S]. V refers to the velocity of substrate uptake.
Figure 3.11 indicates that as erlotinib concentration increases, the rate of estrone-3-sulfate uptake by OATP2B1-transfected HEK293 cells is slightly reduced. The value for $V_{\text{max}}$ was obtained from the Michaelis-Menten plot and the value for $K_s$ was determined from the Lineweaver-Burk plot in the absence of inhibitor (erlotinib = 0 nM). This analysis was conducted in OATP2B1-transfected HEK293 cells (n=3) in vitro ($V_{\text{max}} = 6,672$ pmol estrone-3-sulfate/min/μg protein, $K_s = 1.6$ μM).
3.4.2 Lineweaver-Burk plots of the inhibition of OATP2B1-mediated estrone-3-sulfate uptake by erlotinib

(A)

(B)
**Figure 3.12** (A) Lineweaver-Burk plot for the inhibition of $[^3H]$-estrone-3-sulfate uptake in OATP2B1-transfected HEK293 cells by erlotinib. The transporter activity was determined at six concentrations of erlotinib, including 0 nM (●), 10 nM (■), 25 nM (▲), 50 nM (▼), 75 nM (♦) and 100 nM (○), and five concentrations of estrone-3-sulfate [S]; 0.5 μM, 1 μM, 2 μM, 5 μM, and 10 μM. (B) Primary replot of inhibitor concentrations versus Lineweaver-Burk plot slope data.

The double reciprocal graph in Figure 3.12 (A) shows the family of plots intersecting above the $1/[S]$-axis and to the left of the $1/V$-axis, suggesting that erlotinib may inhibit OATP2B1-mediated uptake of estrone-3-sulfate in a mixed fashion (334).

From Figure 3.12 (B), the $x$-intercept value is equal to -41 nM ($R^2 = 0.99$), which corresponds to the $K_i$ for the interaction between OATP2B1 and erlotinib (334). This indicates that erlotinib is a potent mixed-type inhibitor and may exhibit a greater affinity for OATP2B1 than the substrate, estrone-3-sulfate ($K_s = 1.6 \mu M$).

The $K_i$ value calculated is very similar to the corresponding IC$_{50}$ for this inhibition (32 nM). Considering the $K_i$ is only 11 nM different from the IC$_{50}$ determined previously, it is possible that this is a simple mixed inhibition system (335).
3.4.3 Dixon plots of the inhibition of OATP2B1-mediated estrone-3-sulfate uptake by erlotinib

(A)

(B)
Figure 3.13 (A) Dixon plot for the inhibition of $[^3H]$-estrone-3-sulfate uptake in OATP2B1-transfected HEK293 cells by erlotinib (0-100 nM). Estrone-3-sulfate concentrations were 0.5 μM (●), 1 μM (■), 2 μM (▲), 5 μM (▼), 10 μM (○). (B) Dixon plot slope replot versus reciprocal substrate (estrone-3-sulfate) concentration.

The intersection point of the family of Dixon plots in Figure 3.13 (A) confirms the mixed-type inhibition mode postulated from previous analyses, as the plots meet above the x-axis. The Dixon replot in Figure 3.13 (B) is linear, so β is equal to 0. The α factor, corresponding to the change in $K_s$ when erlotinib occupies OATP2B1, was determined from the x-intercept of the replot in Figure 3.13 (B) to be 1.5 ($R^2 = 0.99$). Analogous to the nature of OATP1A2 inhibition by cediranib, the mode of erlotinib-induced inhibition of OATP2B1-mediated estrone-3-sulfate uptake can be regarded as linear mixed-type with a pronounced non-competitive component (334).

<table>
<thead>
<tr>
<th></th>
<th>OATP1A2-mediated estrone-3-sulfate uptake inhibition by cediranib</th>
<th>OATP2B1-mediated estrone-3-sulfate uptake inhibition by erlotinib</th>
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</thead>
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<tr>
<td>$K_s$</td>
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<td>1.6 μM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>12,400 pmol substrate/min/μg protein</td>
<td>6,672 pmol substrate/min/μg protein</td>
</tr>
<tr>
<td>$K_i$</td>
<td>38 nM</td>
<td>41 nM</td>
</tr>
<tr>
<td>α</td>
<td>3.5</td>
<td>1.5</td>
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<tr>
<td>β</td>
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<td>0</td>
</tr>
</tbody>
</table>

Table 3.2 Kinetic parameters of the inhibition of OATP1A2 and OATP2B1-mediated estrone-3-sulfate uptake by cediranib and erlotinib. These values were calculated from replots of the inhibition data using GraphPad Prism software.
Chapter 4

Discussion

4.1 Evaluation of results from screening experiments

Cell-based assay systems using recombinant transporters that are transiently expressed in cell lines such as HEK293 are useful tools for characterising drug-transporter interactions \textit{in vitro}. The principal qualities which have made HEK293 cells a popular choice to study membrane proteins include their simple reproduction and maintenance, responsiveness to transfection using a wide variety of methods, high efficiency of transfection and protein production, and dependable translation and processing of proteins, among other attributes (336). HEK293 cell monolayers were therefore employed to assess the potential of TKIs to inhibit substrate uptake by individual recombinant transporters from the \textit{SLCO} and \textit{SLC22A} gene families.

4.1.1 Organic anion transporting polypeptides

Ten potentially clinically significant inhibitors of OATP1A2 were identified from preliminary screening results, including afatinib, cediranib, erlotinib, foretinib, gefitinib, neratinib, nilotinib, pelitinib, sunitinib and vandetanib. At a concentration of 10 $\mu$M, these TKIs inhibited estrone-3-sulfate uptake in HEK293 cells overexpressing OATP1A2 by more than 50%; cediranib essentially inhibited uptake completely.
No inhibitors of OATP1B1-mediated uptake of estrone-3-sulfate were identified. This contradicts the literature where lapatinib has been demonstrated to be an inhibitor of OATP1B1 (92), though in that study the authors used estradiol 17β-D-glucuronide as a prototypical substrate and Chinese hamster ovary (CHO) cells heterologously expressing human OATP1B1, which may explain the discrepancies.

OATP1B1 has been shown to have biphasic kinetics for estrone-3-sulfate uptake, indicating the presence of two distinct components of substrate transport (33). This has been exemplified in the influence of the lipid-lowering drug gemfibrozil on OATP1B1-mediated transport of estrone-3-sulfate, where inhibition of substrate uptake by gemfibrozil was apparent only at low substrate concentration (10 nM) and not at high (3 μM) (31). It was concluded that gemfibrozil solely interferes with the high affinity component of OATP1B1, suggesting that estrone-3-sulfate may not be an ideal model substrate to use for OATP1B1 interaction studies. Since the concentration of estrone-3-sulfate used in this experiment was relatively high (300 nM), it is possible that lapatinib is exclusively an inhibitor of the high affinity component on OATP1B1 for estrone-3-sulfate, otherwise it may only interfere with the binding site for estradiol 17β-D-glucuronide.

Sorafenib has recently been identified as a substrate of OATP1B1 (44), though its interaction with this transporter was not evident in the present findings. Furthermore, gefitinib, nilotinib and sunitinib have been proposed as potential substrates for OATP1B1 (44), which was also not supported by the current data. It is possible these TKIs utilise a different binding site from estrone-3-sulfate for OATP1B1-mediated uptake.
In summary, use of estrone-3-sulfate as the model substrate for OATP1B1 uptake is a possible confounding factor for predictions of the interaction potential of this transporter. Estradiol 17β-D-glucuronide or another well-characterised OATP1B1 substrate such as pitavastatin could be used in order to cover the different binding sites of OATP1B1 (31,34).

Two OATP1B3 inhibitors of potential clinical relevance were identified from initial screening uptake tests: erlotinib and neratinib. At a concentration of 10 μM, these TKIs inhibited CCK-8 uptake in OATP1B3-HEK293 cells by more than 50%.

Inhibitors of OATP2B1-mediated estrone-3-sulfate transport identified from screening results, included afatinib, erlotinib, gefitinib, neratinib, nilotinib and pelitinib. At a concentration of 10 μM, these TKIs reduced estrone-3-sulfate uptake in HEK293 cells overexpressing OATP2B1 by more than 50%, with erlotinib emerging as a potent inhibitor (only 2% uptake of estrone-3-sulfate). Different interacting sites for estrone-3-sulfate have also been described for OATP2B1. The transport of this substrate follows biphasic uptake kinetics with a high-affinity component ($K_m$ value of 1.8 μM) and a low-affinity component ($K_m$ value of 1.4 mM) (337). The $K_s$ value of 1.6 μM calculated from Section 3.4 can be considered as $K_m$ in this situation, owing to the fact that the transporter-substrate complex is not functional (Dixon plots are linear; $\beta=0$), hence the turnover number, or $k_{cat}$ of the transporter is very low and $K_m$ approaches $K_s$ (338). This $K_s$ indicates that erlotinib probably inhibits the transport of this substrate by OATP2B1 at its high-affinity binding site.
4.1.2 Organic anion transporters

No inhibitors of OAT1, OAT2 or OAT4-mediated model substrate uptake were identified, however, nilotinib (10 μM) was observed to strongly inhibit estrone-3-sulfate transport by OAT3-HEK293 cells (only 19% uptake of estrone-3-sulfate).

4.1.3 Organic cation transporters

No inhibition of OCT2 or OCT3-mediated MPP\(^+\) uptake was observed in the preliminary screening tests, however, bosutinib (10 μM) was seen to substantially reduce substrate uptake by OCT1-HEK293 cells (only 24% uptake of MPP\(^+\)).

4.1.4 Organic zwitterion/cation transporters

Cediranib emerged as a potential inhibitor of OCTN2, decreasing the uptake of L-carnitine by more than 50% in OCTN2-HEK293 cells. OCTN1 did not appear to be inhibited by any TKIs.
4.2 *In vitro-in vivo* extrapolation of IC$_{50}$ and $K_i$ values

TKIs that reduced the uptake of SLC-mediated prototypical substrate by more than 50% were further investigated for their drug-drug interaction potential by determining their IC$_{50}$ values. IC$_{50}$ values are useful criteria as they can be applied in a number of *in vitro-in vivo* extrapolation algorithms, such as R value estimation (27), to predict the likely clinical significance of these inhibitory interactions. The R value refers to the change in systemic exposure of a transporter substrate in the presence of an inhibitor. An R value of 2 indicates a 2-fold increase in substrate exposure. In such forecasting methods, the steady-state $C_{\text{max,sys}}$ achieved by repeated administration of the maximum tolerated dose of TKI, is usually used to compare with the IC$_{50}$ value.

Another parameter than can be implemented in these calculations is the $K_i$, which is the dissociation constant for the transporter-inhibitor complex (TI). This value is determined from kinetic experiments analysed using Michaelis-Menten, Lineweaver-Burk and Dixon plots (see Sections 3.3 and 3.4). $K_i$ values can be predicted from IC$_{50}$ values and vice versa using Michaelis-Menten constants ($V_{\text{max}}$ and $K_m$), which is useful because IC$_{50}$ values are the most commonly used assessment of inhibitory interactions (335).

Among the 13 TKIs tested and the five metabolites of sorafenib, 11 TKIs including afatinib, bosutinib, cediranib, erlotinib, foretinib, gefitinib, neratinib, nilotinib, pelitinib, sunitinib and vandetanib were assessed for IC$_{50}$ determination, while the most potent inhibitors cediranib and erlotinib were subjected to kinetic analysis.
Recently, the ITC has proposed specific criteria for using *in vitro* data to predict potential *in vivo* drug-drug interactions occurring at SLCs important in drug disposition, in particular the OATPs, OATs and OCTs (27). Table 4.1 lists the transporter-TKI interactions that exceeded 50% inhibition of model substrate uptake in the preliminary screening tests and displays the IC$_{50}$ values obtained, along with the $K_i$ values from kinetic experiments.

$C_{\text{max,sys}}$ values for TKIs, along with the fraction of unbound TKI in blood (i.e. the percentage of drug that is not bound to plasma proteins), were obtained from the literature (222,245,320-331) and compared with IC$_{50}$ or $K_i$. If the IC$_{50}$ is less than 10-fold the unbound $C_{\text{max,sys}}$ in cells expressing OAT or OCT proteins (i.e. $C_{\text{max,sys}}$/IC$_{50}$ ≥ 0.1), a clinical study to directly assess drug-drug interaction potential could be warranted (27).
<table>
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<th>Transporter</th>
<th>TKI</th>
<th>IC$_{50}$ (μM)</th>
<th>$K_i$ (μM)</th>
<th>Unbound $C_{\text{max,sys}}$ (μM)</th>
<th>References</th>
<th>Clinical relevance: Unbound $C_{\text{max,sys}}$/IC$_{50}$ ≥ 0.1 (value)</th>
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<td>0.006</td>
<td>(321,322)</td>
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$^a$ Information regarding the unbound fraction of TKI in plasma is not reported, hence total $C_{\text{max,sys}}$ is given.

* With respect to the estimated maximum concentration of unbound TKI at the inlet of the liver (185,339,340), these transporter-TKI inhibition interactions are also considered clinically significant (see Table 4.3).

*Table 4.1* Experimentally-determined IC$_{50}$ and $K_i$ values of TKI-mediated inhibition of transporter uptake, compared with clinically-derived unbound TKI $C_{\text{max,sys}}$ values.
As shown, nilotinib inhibited OAT3-mediated transport of estrone-3-sulfate sufficiently (unbound $C_{\text{max,sys}}/IC_{50} = 0.21$) to be considered for subsequent clinical analysis with a sensitive substrate such as methotrexate (51,341). Concomitant use of nilotinib with substrates of OAT3 may elicit significant drug-drug interactions resulting in altered exposure or cellular uptake of the inhibited substrate.

The procedure for OATPs is slightly more complex. If the above criterion is met ($C_{\text{max,sys}}/IC_{50} \geq 0.1$), it is advised that an in vitro-in vivo extrapolation method is employed, such as the R value approach. The R value method uses the maximal unbound drug concentration at the inlet of the liver ($C_{\text{max,portal}}$) rather than the unbound $C_{\text{max,sys}}$ which refers to the maximum concentration of inhibitor in systemic circulation. Generally orally-administered drug concentrations are highest at the inlet of the liver, where the blood flow from the hepatic artery and portal vein meet (185,340), therefore use of the unbound $C_{\text{max,portal}}$ value can avoid false negative predictions of drug-drug interactions at transporters in the liver.

Given that the majority of inhibitory interactions identified in this research involve hepatic transporters (OATP1A2, OATP1B3, OATP2B1, OCT1 and OCTN2), it seemed prudent to include the $C_{\text{max,portal}}$ value for TKIs in the analysis of these particular SLCs. Hence, the unbound $C_{\text{max,sys}}/IC_{50} \geq 0.1$ measure of significance was adapted to consider unbound TKI in portal plasma in order to identify other potentially clinically relevant interactions for further in vitro-in vivo extrapolation (Table 4.2).
The clinical maximum concentration of unbound TKI in portal plasma at the inlet of the liver (unbound $C_{max,portal}$) was calculated using the following equation (339):

$$\text{Unbound } C_{max,portal} = f_u \cdot C_{max,sys} + \frac{(f_u/R_b) \cdot \text{Dose} \cdot F_a \cdot k_a}{Q_h}$$  \[4\]

where $f_u$ is the unbound fraction of TKI in plasma, $R_b$ is the blood-to-plasma concentration ratio, dose is the maximum oral dose given, $F_a$ is the maximum oral bioavailability of TKI, $k_a$ is the absorption rate constant, and $Q_h$ is hepatic blood flow rate (1,500 mL per min; ref. 27). Unless otherwise specified, $F_a$ and $R_b$ were assumed to be 1 in the present study, while two values of $k_a$ were evaluated; 0.1 per min, which is the theoretical maximum value for $k_a$ taking gastric emptying into account (339), and 0.03 per min, suggested by the ITC (27).

The $f_u$ values for foretinib, neratinib and pelitinib in systemic plasma are not available in the literature, however to err on the conservative side in the estimation of potential in vivo drug-drug-interactions effected by these TKIs, the unbound fraction 0.01 (1%, i.e. TKI is 99% bound to plasma proteins) can be used (77).
<table>
<thead>
<tr>
<th>TKI</th>
<th>$C_{\text{max,portal}}$ (μM)</th>
<th>Fraction absorbed ($F_a$)</th>
<th>Dose (mg)</th>
<th>Fraction unbound ($f_u$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afatinib</td>
<td>2.45</td>
<td>1</td>
<td>55</td>
<td>0.05</td>
<td>(27,77,219,320)</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>18.6</td>
<td>1</td>
<td>500</td>
<td>0.04</td>
<td>(27,77,296,331)</td>
</tr>
<tr>
<td>Cediranib</td>
<td>1.54</td>
<td>1</td>
<td>30</td>
<td>0.03</td>
<td>(27,77,239,321,322)</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>10.6</td>
<td>0.6-1</td>
<td>150</td>
<td>0.07</td>
<td>(27,185,276,323,324,342-344)</td>
</tr>
<tr>
<td>Foretinib</td>
<td>7.74</td>
<td>1</td>
<td>240</td>
<td>0.01</td>
<td>(27,77,245)</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>8.58</td>
<td>0.59</td>
<td>225</td>
<td>0.09</td>
<td>(27,185,212,261,324,325)</td>
</tr>
<tr>
<td>Neratinib</td>
<td>11.6</td>
<td>1</td>
<td>320</td>
<td>0.01</td>
<td>(27,77,222)</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>13.6</td>
<td>0.31</td>
<td>800</td>
<td>0.02</td>
<td>(27,77,185,326,327,345,346)</td>
</tr>
<tr>
<td>Pelitinib</td>
<td>3.29</td>
<td>1</td>
<td>75</td>
<td>0.01</td>
<td>(27,77,328)</td>
</tr>
<tr>
<td>Sunitinib</td>
<td>0.85</td>
<td>1</td>
<td>50</td>
<td>0.05</td>
<td>(27,185,324,329)</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>13.2</td>
<td>1</td>
<td>300</td>
<td>0.065</td>
<td>(27,77,330)</td>
</tr>
</tbody>
</table>

\[^{a}\] $R_b$ used = 0.76 (212)

Table 4.2 Variables used for the calculation of unbound $C_{\text{max,portal}}$ and $R$ values.

Results that were not significant from initial analysis using $C_{\text{max,sys}}$ values (unbound $C_{\text{max,sys}}/IC_{50} << 0.1$), included the interactions between OATP1A2 and foretinib, pelitinib and sunitinib, OATP2B1 and pelitinib, and OCTN2 and cediranib. Despite application of the default parameters ($F_a = 1$, $k_a = 0.1$ per min) when calculating their maximum unbound portal plasma concentrations using the variables in Table 4.2, the $IC_{50}$ values for these TKIs exceeded the estimated unbound $C_{\text{max,portal}}$ values by at least 10-fold and are therefore not considered clinically relevant. Hence, these interactions were excluded from Table 4.3.
<table>
<thead>
<tr>
<th>Transporter</th>
<th>TKI</th>
<th>IC₅₀ (µM)</th>
<th>Unbound Cₘₐₓ,portal (µM)</th>
<th>Unbound Cₘₐₓ,portal/IC₅₀ ≥ 0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Afatinib</td>
<td>1.17</td>
<td>0.123&lt;sup&gt;c&lt;/sup&gt; – 0.387&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.11 – 0.33</td>
</tr>
<tr>
<td></td>
<td>Gefitinib&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88</td>
<td>0.772</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Neratinib</td>
<td>0.39</td>
<td>0.116&lt;sup&gt;c&lt;/sup&gt; – 0.385&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.30 – 0.99</td>
</tr>
<tr>
<td></td>
<td>Nilotinib&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.46</td>
<td>0.273</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Vandetanib</td>
<td>2.45</td>
<td>0.857&lt;sup&gt;c&lt;/sup&gt; – 2.772&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.35 – 1.13</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Neratinib</td>
<td>0.65</td>
<td>0.116&lt;sup&gt;c&lt;/sup&gt; – 0.385&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.18 – 0.59</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Afatinib</td>
<td>2.08</td>
<td>0.387&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Neratinib</td>
<td>2.68</td>
<td>0.385&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Nilotinib&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.67</td>
<td>0.273</td>
<td>1.10</td>
</tr>
<tr>
<td>OCT1</td>
<td>Bosutinib</td>
<td>2.06</td>
<td>0.745&lt;sup&gt;c&lt;/sup&gt; – 2.447&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.36 – 1.19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fₐ used = 0.59 (261); R₉ used = 0.76 (212)

<sup>b</sup> Fₐ used = 0.31 (345)

<sup>c</sup> Unbound Cₘₐₓ,portal determined using the parameter kₐ = 0.03 per min.

<sup>d</sup> Unbound Cₘₐₓ,portal determined using the parameter kₐ = 0.1 per min.

**Table 4.3** Experimentally-determined IC₅₀ values from TKI-mediated inhibition of transporter uptake, compared with estimated unbound TKI Cₘₐₓ,portal values.

As shown in Table 4.3, some of the SLC-TKI interactions, such as the inhibition of OATP2B1 by afatinib and neratinib, were only regarded as significant when utilising the higher limit of kₐ = 0.1 per min. All other interactions, however, were important at the lower criterion of kₐ = 0.03 per min, while the interactions between OATP1A2 and the TKIs gefitinib and nilotinib, and OATP2B1 and nilotinib resulted in unbound Cₘₐₓ,portal/IC₅₀ values greater than 0.1 using the minimum parameters known for these
TKIs. For example, the absolute bioavailability ($F_a$) for gefitinib is 0.59 (59%) and the blood-to-plasma concentration ratio for this drug is 0.76 (212,261). The absolute bioavailability of nilotinib is not known, however it is estimated to be low (approximately 0.31 or 31%; ref. 345) owing to the evidence that systemic exposure to nilotinib is increased by 82% when the drug is given with a high-fat meal compared with the fasted state (324,326). So as not to overestimate the effect of nilotinib on OATP1A2- and OATP2B1-mediated estrone-3-sulfate uptake, the lower limit of 0.31 was used for $F_a$.

Inhibition of OCT1-mediated uptake of MPP$^+$ by bosutinib produced unbound $C_{max,portal}/IC_{50}$ values of 0.36 and 1.19, with $k_a$ set to 0.03 per min and 0.1 per min respectively. With regard to the guidelines suggested for OCTs by the ITC (27), since the $IC_{50}$ is less than 10 times the estimated unbound $C_{max}$, a clinical drug-drug interaction study is recommended using a probe substrate that is likely to be co-administered with bosutinib in a therapeutic setting, such as cisplatin or imatinib (347,348).

Table 4.3 summarises all OATP-TKI interactions for which the $IC_{50}$ values are less than 10-fold the estimated maximum unbound portal plasma concentrations, hence R-extrapolation is recommended (27). Additionally, positive results from Table 4.1, where the criterion of unbound $C_{max,sys}/IC_{50} \geq 0.1$ was applied, also require further in vitro-in vivo evaluation by the R value paradigm. In particular, $IC_{50}$ and $K_i$ values for erlotinib-induced inhibition of OATP2B1 transport generated unbound $C_{max,sys}/IC_{50}$ or $K_i$ values of 13 and 10 respectively; at least 2 orders of magnitude higher than the threshold value of 0.1. Consequently the concentration of erlotinib necessary to
significantly inhibit OATP2B1-mediated uptake of estrone-3-sulfate is 10 times lower than the maximum unbound concentration of erlotinib in the systemic circulation. Other interactions were not quite as pronounced, though sufficient to be considered for R value estimation, including OATP1A2 inhibition by the TKIs cediranib and erlotinib, OATP1B3 inhibition by erlotinib, and OATP2B1 inhibition by gefitinib.

The R value can be calculated from the following equations (27):

\[
R = 1 + \frac{f_u \cdot C_{\text{max,portal}}}{IC_{50}} \quad [5]
\]

\[
R = 1 + \frac{f_u \cdot C_{\text{max,portal}}}{K_i} \quad [6]
\]

The criterion of R>2, indicating that the AUC or \(C_{\text{max,sys}}\) of an OATP substrate is predicted to increase more than 2-fold in the presence of an inhibitor, is applied to all OATPs, although the FDA recommends that the limit be set at R>1.25 for OATP1B1 and OATP1B3 (77). Hence, in this evaluation, an R value greater than 2 indicates that a clinical drug-drug interaction study is advised for OATP1A2 and OATP2B1 inhibitors, while an R value of 1.25 or more suggests potential clinical relevance for OATP1B3 inhibitors. Using these parameters and equations 5 and 6, in addition to combining different values for the bioavailability \((F_a = \text{reported value if known, or 1})\) and absorption rate constant \((k_a = 0.03 \text{ per min or 0.1 per min}; \text{refs. 27,349})\), R values for TKI-mediated inhibition of model substrate uptake in three OATPs were calculated for use in comparisons with clinical data.
Table 4.4 Assorted R values for TKI-mediated inhibition of OATP substrate uptake calculated using equations 3 and 4.

4.2.1 Significance of findings

Six novel inhibitory interactions of OATPs with TKIs could be predicted from Table 4.4 (in bold). In addition, the interaction of OATP2B1 with erlotinib was elaborated upon to determine the R value for this inhibition. As shown in the above table, the latter interaction produced an average R value of 37.5, suggesting that the exposure of OATP12B1 substrates can be expected to increase 37.5-fold in the presence of erlotinib. Erlotinib has an absolute bioavailability between 60% and 100%, with an
estimated average of approximately 76% in cancer patients (350). Even using the lowest possible criteria in the R-extrapolation for this interaction \( (F_a = 0.6, k_a = 0.03 \text{ per min}) \), produced an R value of 24 or 18, applying the IC\(_{50}\) and \( K_i \) values respectively. Therefore the exposure of OATP2B1 substrates is predicted to be enhanced at least 18-fold in the presence of erlotinib.

The potential for drug-drug interactions is considerable, such that erlotinib is recommended for further clinical drug-drug interactions studies with drug substrates of OATP2B1. No anticancer drug substrates for this transporter have been reported to date, though co-treatment of erlotinib with statins is not infrequent (351). While commonly used to treat hypercholesterolemia, increasing evidence suggests that statins exert pleiotropic effects, independent of cholesterol reduction, including antiproliferative and antimetastatic properties (352). Concurrent treatment of the HCC cell line HepG2 with erlotinib has been shown to augment the antiproliferative effects of fluvastatin (353). Several statins, including atorvastatin, fluvastatin, pravastatin and pitavastatin, are OATP2B1 substrates (31,103,125,349). Accordingly, there is potential for erlotinib to affect the efficacy and toxicity of statins in therapy, which may be related to interference with OATP2B1-mediated transport of substrates, such as fluvastatin.

The second potent inhibitor of OATP2B1, gefitinib, was determined to have an R value greater than 2, hence the presence of this drug can be predicted to increase the AUC or \( C_{\text{max,sys}} \) of OATP2B1 substrates by at least 2-fold. Along with erlotinib, gefitinib can be added to the growing compendium of OATP2B1 interacting compounds.
Erlotinib was identified as a potentially significant inhibitor of OATP1B3. Neratinib is also an inhibitor of OATP1B3 of possible clinical relevance, however it is noted that the pharmacokinetic parameters used in this assessment were estimated and not measured. OATP1B3 is known to transport several anticancer drugs, including docetaxel, hydroxyurea, imatinib, methotrexate, paclitaxel, and the potent irinotecan metabolite, SN-38 (39,40,46-49). Erlotinib has been administered concomitantly with docetaxel, paclitaxel and irinotecan, though significant pharmacokinetic interactions have not been identified in vivo (354-356). Both erlotinib and neratinib are recommended for further clinical drug-drug interaction studies with sensitive OATP1B3 substrates, for example statins (27).

The novel OATP1A2 inhibitors include cediranib, erlotinib and gefitinib (mean R values of 3.07, 2.22 and 3.42 respectively), and possibly vandetanib (mean R value of 1.74). The absolute bioavailability of cediranib in human is unknown, though in rat it is around 60% (239). This value was used in the R value approximations in Table 4.4. The average R value calculated for vandetanib-induced inhibition of OATP1A2 estrone-3-sulfate transport was less than 2. However applying the uppermost boundaries for the R equation parameters ($F_a = 1$, $k_a = 0.1$ per min) produced an R value of 2.13, indicating this interaction has potential clinical significance and further drug-drug interactions studies are needed.

Imatinib, methotrexate and hydroxyurea are anticancer drugs that are transported by OATP1A2 (39-41). There is potential for interactions between cediranib, erlotinib, gefitinib or vandetanib and these anticancer drugs, in addition to the many others drug substrates identified so far for OATP1A2 including numerous statins and
antihypertensive drugs (357). Inhibition of VEGFR by cediranib and vandetanib can lead to vasoconstriction, causing hypertension (226). Consequently, it is highly likely that antihypertensive drugs will be paired with cediranib and vandetanib in a therapeutic setting (358). The exposure and cellular uptake of OATP1A2 substrates, such as the antihypertensive drugs atenolol and celiprolol, may be altered by concomitant treatment with these VEGFR inhibitors.

4.2.2 Appraisal of analytical techniques

The use of estimated unbound hepatic inlet concentrations ($C_{\text{max,portal}}$) of inhibitors has proven to be a reliable tool for the in vivo prediction of reversible inhibitor concentrations that are likely to elicit drug-drug interactions in CYP enzymes (359). In terms of transporters, it has been demonstrated that use of the $C_{\text{max,portal}}$ variable in the calculation of R values for hepatic OATP interactions has been successful in the classification of compounds into interacting and non-interacting drugs, which was in good agreement with reported clinical data (360). Nonetheless, predicting the magnitude of these interactions based on R values was less definitive. For more quantitative estimations, the contribution of additional factors, such as drug metabolising enzymes and efflux transporters needs to be considered (360). Additionally, calibration of the R values calculated in Table 4.4 using compounds with known clinical inhibition patterns in OATPs, such as statins, is required in order to fully comprehend the drug-drug interaction potential of these TKIs.
4.3 Kinetic analysis of the inhibitory effect of cediranib and erlotinib on OATP1A2 and OATP2B1

In the analysis of results from the kinetic experiments described in Section 3.3, the $K_i$ value for the cediranib-induced inhibition of estrone-3-sulfate uptake in OATP1A2-transfected HEK293 cells was calculated to be 38 nM. The corresponding $K_s$ value for this interaction was found to be 5.3 μM. As the $K_i$ is indicative of the binding affinity of the transporter for an inhibitor, and $K_s$ is interpreted as the binding affinity between the substrate and transporter, it can be deduced that cediranib has approximately 140-times greater affinity for OATP1A2 compared with the prototypical substrate estrone-3-sulfate.

In Section 3.4, the $K_i$ value for the erlotinib-effected inhibition of estrone-3-sulfate transport in OATP2B1-transfected HEK293 cells was calculated to be 41 nM. The related $K_s$ value for estrone-3-sulfate uptake was measured as 1.6 μM. From these parameters, it can be interpreted that OATP2B1 has almost 36-times greater affinity for erlotinib than for estrone-3-sulfate. As mentioned previously, it would appear that erlotinib inhibits the high affinity binding site on OATP2B1 for estrone-3-sulfate by a mixture of partial competitive and pure non-competitive inhibition modes (see Sections 3.4 and 4.1) (334). Erlotinib was identified as a selective inhibitor of OATP2B1, because the IC$_{50}$ for this interaction was more than 10-fold lower than the IC$_{50}$ values for the other OATPs (260).

The kinetic analyses of these inhibitory interactions indicated that the mode of inhibition was linear mixed-type with a pronounced non-competitive component in
both cases (334). In this system, the β factor is equal to 0 and the transporter-substrate-inhibitor complex (TSI) between OATP-estrone-3-sulfate-TKI is not functional. In non-competitive inhibition, the inhibitor binds to both unbound (T) and substrate-bound transporter (TS); likewise in mixed-type inhibition, the inhibitor can bind to T and TS, but also may overlap with the substrate-binding site, as seen in competitive inhibition. The equilibria describing this system are shown in Figure 4.1.

\[
\begin{align*}
T + S & \xrightleftharpoons[K_i]{K_s} TS \xrightarrow{k_p} T + S \\
+ & \\
I & I \\
K_i & aK_i \\
TI + S & \xrightleftharpoons[aK_i]{aK_i} TSI
\end{align*}
\]

*Figure 4.1 Schematic of the equilibria between OATP (T), substrate (S) and inhibitor (I) for linear mixed-type inhibition adapted from (334) with minor modifications. In this case, S = estrone-3-sulfate and I = cediranib or erlotinib (TKI).*

As long as the inhibitor is present, some of the transporter will always be in the non-productive TSI form, even at infinitely high substrate concentration. Furthermore, at any concentration of inhibitor, a portion of the transporter that is available for combination with estrone-3-sulfate will exist in the lower affinity protein-inhibitor complex form (TI). At an infinitely high concentration of inhibitor, all of the protein can be directed to the TI and TSI forms. Given that TSI is non-productive, the velocity of the uptake reaction can be driven to zero by increasing the inhibitor concentration. This explanation is consistent with the double reciprocal Lineweaver-
Burk plots presented in Figures 3.9 (A) and 3.12 (A), where increasing concentration of inhibitor results in increasing slopes and 1/V-axis intercepts.

4.3.1 Significance of findings

Cediranib is a highly potent and selective VEGF signalling inhibitor with activity against all three VEGFRs and has demonstrated encouraging antitumour activity in early clinical studies across a broad range of tumours, both as monotherapy and in combination with certain chemotherapy regimens (239,361). Although the clinical development of cediranib has been discontinued due to negative results in the treatment of HCC, NSCLC and bowel cancer (241), studies are ongoing in the investigation of its therapeutic effect on glioblastoma (243,244). VEGFR inhibitors such as cediranib and vandetanib have been associated with renal toxicity (304), which could be related to their inhibition of OATP1A2, given that this transporter is expressed at the luminal side of distal tubules of the nephron and may be involved in the secretion of toxins into urine (5).

Cediranib is administered as a once-daily 30 mg dose which reaches an average $C_{\text{max,sys}}$ of approximately 0.2 μM (292,321,322,364-366). The maximal unbound plasma concentration of cediranib was determined to be 6 nM (323), indicating that this TKI has an unbound fraction of approximately 3% in blood. The absolute bioavailability in human is unknown for this drug, therefore in the previous R value calculation (Section 4.2.1), this variable was set to 1 (100% bioavailable). 60% bioavailability was also used in this calculation based on bioavailability in rat.
Erlotinib is an orally active, selective inhibitor of EGFR and has been approved for the therapy of advanced NSCLC and first-line treatment of metastatic pancreatic cancer in combination with gemcitabine (214). Augmented tumour growth inhibition has been reported when erlotinib is coadministered with paclitaxel and cisplatin (362), while concomitant treatment of this TKI with irinotecan in human colorectal cancer xenograft models was more effective than erlotinib alone (363). Both paclitaxel and the potent irinotecan metabolite SN-38 are known to be substrates of OATP1B3 (48,49), which in this study was found to be significantly inhibited by erlotinib, hence it is possible that erlotinib alters the pharmacokinetics of these drugs by modulating their transmembrane transport.

Erlotinib is given as a once-daily dose of 150 mg for the treatment of NSCLC or 100 mg for pancreatic cancer, though for the in vivo-in vitro extrapolation for erlotinib-induced interactions, the maximum tolerated dose (150 mg) was used to calculate $C_{\text{max,portal}}$. The maximum unbound concentration of erlotinib in systemic plasma is 0.424 μM (185), and its absolute bioavailability ranges from 60% to 100% depending on the fed or fasted state of patients (342). Erlotinib is highly bound to albumin and $\alpha_1$-acid glycoprotein in the blood resulting in a rather low unbound fraction of drug in plasma (7%).

In the present study, the IC$_{50}$ values for cediranib and erlotinib, against OATP1A2- and OATP2B1-mediated uptake of estrone-3-sulfate, were calculated to be 35 nM and 32 nM respectively. Applying the parameters detailed in Table 3.2, the following
equation for mixed-type inhibition can be rearranged to determine $K_i$ from $IC_{50}$ for comparison with the experimentally-derived values (335):

$$IC_{50} = \frac{[S] + K_s}{[S] + \frac{K_s}{\alpha K_i} + \frac{K_s}{K_i}}$$  \hspace{1cm} [7]$$

Using equation 7, where the $K_s$ and $\alpha$ value were 5.3 $\mu$M and 3.5 respectively for the interaction between OATP1A2 and cediranib, while for OATP2B1 and erlotinib, the values for $K_s$ and $\alpha$ were 1.6 $\mu$M and 1.5, the theoretical in vivo $K_i$ values were determined as 34 nM (cediranib) and 28 nM (erlotinib). These values are comparable with the experimentally determined $K_i$ values of 38 nM and 41 nM correspondingly. Considering the unbound $C_{\text{max,portal}}$ is predicted to be 46 nM for cediranib and 724 nM for erlotinib, these TKIs are able to inhibit OATP1A2 and OATP2B1 at concentrations that may be observed after oral doses of the drugs.
Chapter 5

Conclusions and Directions for Future Work

115
5.1 Conclusion

The present research identified six novel inhibitors of OATPs and one novel inhibitor of each of OAT3 and OCT1 at clinically relevant concentrations. These interactions included: the inhibition of OATP1A2-mediated estrone-3-sulfate uptake by cediranib, erlotinib and gefitinib; the inhibition of OATP1B3-mediated CCK-8 uptake by erlotinib and neratinib; the inhibition of OATP2B1-mediated estrone-3-sulfate uptake by erlotinib and gefitinib; the inhibition of OAT3-mediated estrone-3-sulfate uptake by nilotinib; and the inhibition of OCT1-mediated MPP⁺ uptake by bosutinib. All are recommended for further clinical drug-drug interaction studies with SLC-specific drug substrates.

Using the in vitro-in vivo R value extrapolation approach recommended by the ITC, the inhibition of OATP2B1-mediated uptake by erlotinib was found to be particularly potent, with an R value of at least 18, suggesting that the systemic exposure to OATP2B1 substrates might be increased substantially in the presence of erlotinib. This has serious implications for the in vivo use of erlotinib as it may change the pharmacokinetics of OATP2B1 substrates, including some statins.

Kinetic testing was implemented to determine the mode of inhibition for the highly potent interactions of OATP1A2 with cediranib and OATP2B1 with erlotinib, which was linear-mixed in both cases. Cediranib has approximately 140-times greater affinity for OATP1A2 compared with the prototypical substrate estrone-3-sulfate, therefore it could be used as a selective inhibitor in clinical drug-drug interaction studies for this transporter. Erlotinib has almost 36-times greater affinity for
OATP2B1 than estrone-3-sulfate and is postulated to bind at the high affinity binding site for this substrate on OATP2B1 in a mixed-type fashion.

SLCs contribute to the disposition of a broad spectrum of endogenous and exogenous compounds. Thus, inhibition of these transporters by TKIs may alter the pharmacokinetics and toxicities of concurrently administered drugs, including numerous anticancer drugs. Besides their expression in normal tissues, it is significant to note that several studies have shown altered tissue distribution of SLCs in some malignant tissues, hence substrate uptake into tumour cells could be modulated by the TKIs that were found to be inhibitory in the present study.
5.2 Limitations and recommendations for future work

While several TKIs were found to be inhibitors of SLCs, whether they were also substrates was not assessed. Further uptake screening experiments using radiolabelled TKIs would be useful. Moreover, several TKIs are established in the literature as SLC substrates, but were not found to interact with the relevant transporters in this thesis. Therefore, standardised assays using a variety of substrates at different concentrations and pH would add to the present findings and reduce interlaboratory variability.

Many challenges remain in mimicking the *in vivo* situation using *in vitro* experiments. Single-transfected cell uptake assays are limited in their ability to simulate the dynamic interplay between endogenous enzymes and transporters, and the possible compensatory increase in activities of one or more other transporters when the function of a transporter is suppressed. To assist the interpretation of the present data, polarised cells that stably express multiple transporters would be useful. For example, cells transfected with a recombinant uptake transporter in the basolateral membrane and an efflux transporter in the apical membrane, could be informative. Ideally, low passage cell lines expressing the full complement of drug transporters present in a given tissue or cell should be used, such as in primary cell-based assays. By adopting improved methods of evaluating transporter activity, and accounting for the contribution of drug metabolising enzymes and albumin to TKI disposition, enhanced quantification of potential *in vivo* drug-drug interactions will be possible to predict.

The possibility of TKIs acting as allosteric stimulators of SLC uptake activity was outside the scope of this project but could be evaluated in future studies.
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